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Modern Trends in Human Leukemia III

Newest Results in Clinical and Biological Research

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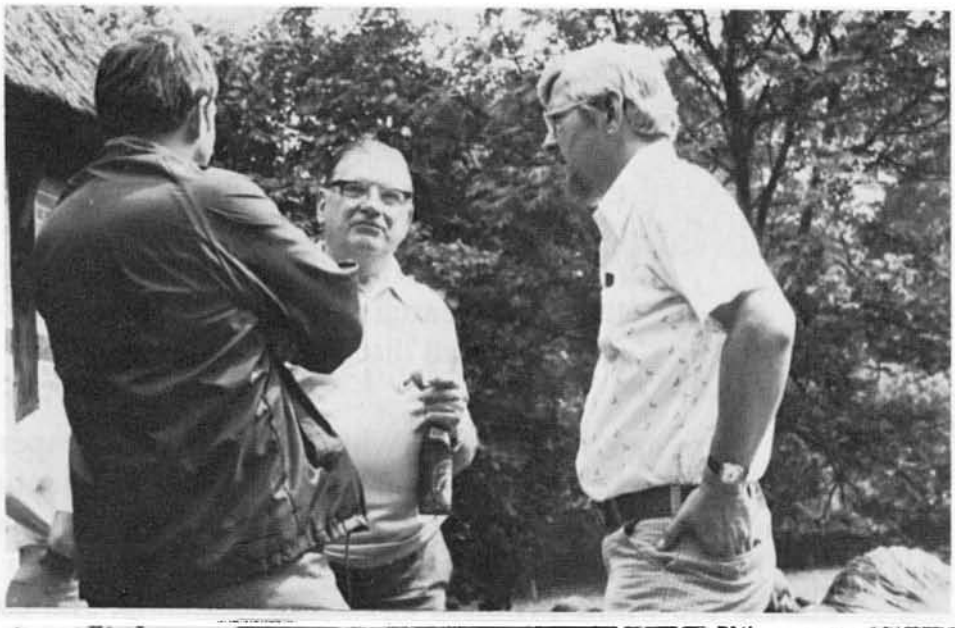
Preface

„Es ist ja ganz einerlei, wer es gefunden hat, die Hauptsache ist, daß es gefunden worden ist."
This was said by Hermann Wilbrand, director of the department of opthalmology at the university hospital Hamburg-Eppendorf (1919–1923), when others claimed priority for the long searched for cortical visuell center, which he had discovered.

Five years ago we tried in Wilsede an unusual experiment, bringing together for three long days and nights scientists and medical doctors to learn like students about each others work. The hope was that the participants in the workshop would discuss the whole problem of human leukemia, and cooperative programmes among the different specialized research groups and medical centers would be stimulated. Now five years later we feel that the experiment was a success. Cooperative research programmes around the world were started and most of us understand more and more the Frederic Stohlman question about the pratical application of our research and its benefit for the patient.

The chairman have done a tremendous job organizing an up to date scientific programme for the third Wilsede meeting. I would like to thank all of them for a programme which includes all the important results and future aspects of human leukemia.

Personal and scientific discussion in Wilsede, June 1978



Highly qualified scientists did not only prepare themselves for a talk, but also for posters to give us a chance for more personal discussions about the clinical and research fields we are interested in. I hope that these possibilities of individual discussions will help understanding the different fields important for human leukemia, as an example for human cancer.

The special Wilsede atmosphere probably will have again a good influence on our discussions and perhaps you will take home some of this spirit and remember it sometimes.

Than we should be grateful to the Verein Naturschutzpark e.V. and especially to Alfred Toepfer and his associates for their idealistic efforts to save this little piece of nature for man.

Rolf Neth

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Memorial Tribute to Dr. Frederick Stohlman

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I would like to express sincere thanks on behalf of the Stohlman children and myself to Dr. Rolf Neth for arranging for these memorial lectures and for the privilege of speaking to you briefly about the late Fred Stohlman. As most of you know, Fred and his lovely wife, Bernadette, died tragically on September 8, 1974 when the explosion of a terrorist bomb plunged their plane into the Ionian Sea. This senseless act of violence deprived 4 children of their beloved parents and ruthlessly terminated, at the age of 45, the career of a brilliant investigator and physician. Much has since been published concerning Fred Stohlman's outstanding achievements and I will not dwell on these accomplishments. Rather I would like to recall to you, his friends, associates and fellow scientists, some of the human and endearing traits of this unique individual.

Fred was very young when he graduated from Georgetown Medical School, in fact he was only 21 when I first met him as an intern on the 3rd Medical Service (Tufts) at Boston City Hospital. As the staff photograph shows (Fig. 1) he did indeed look very boyish and youthful. Following his medical training and 9 years at the National Institute of Health where he worked closely with George Brecher, Fred returned to Boston in 1962 and became Chief of medicine and research at St. Elizabeth's Hospital and professor of medicine at Tufts Medical School. There he built up a staff and trained hematology fellows many who were to become world famous. Some of his staff members are shown in the accompanying photograph (Fig. 2). Fred's involvement and concern for his fellow workers and associates was proverbial; his intense interest in discussion of research problems is demonstrated in this photograph (Fig. 3).

Among his numerous activities Fred probably enjoyed participation in meetings and symposiums most of all. His expertise as a chairman and his ability in editing proceedings of conferences was universally recognized. He organized meetings which attracted outstanding scientists from all over the world and Fred developed warm and lasting friendships on an international basis. Dr. Stohlman was not only a distinguished scientist and physician, he was also a warm and congenial human being who loved good food, good drink and pleasant companionship. He probably satisfied a life time ambition during an international meeting when he made an impromptu performance as conductor of the Munich Orchestra as shown in this photograph (Fig. 4).

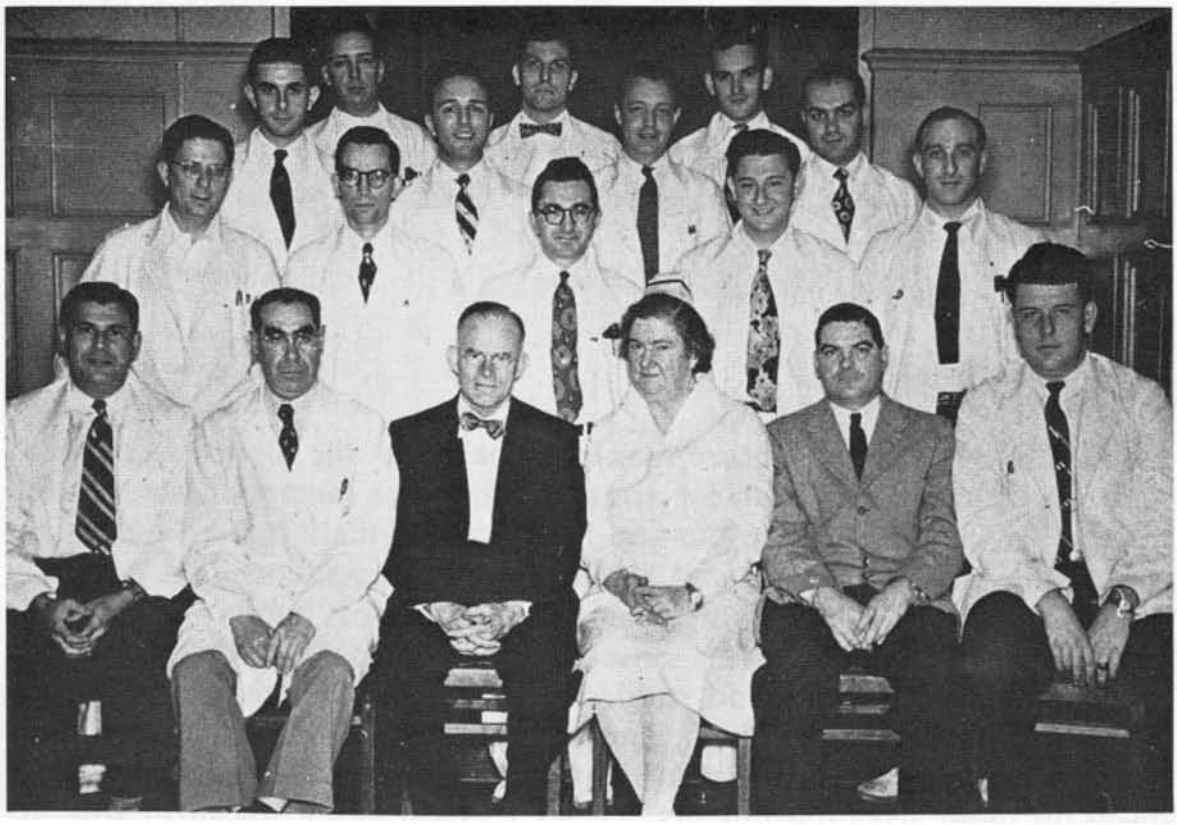

Fig. 1

Fig. 2

A few months before his death, in June 1974, Fred came to Wilsede. His contributions on that occasion were memorable and he endeared himself to a host of new friends. The following picture (Fig. 5) depicts typical scenes of that first meeting of the now famous conferences on Modern Trends in Leukemia Research. These photographs epitomize the spirited intensity and the charm of the Wilsede atmosphere. The last picture (Fig. 6) shows Fred Stohlman as I, and I am sure all of you, would like to remember him; smiling, happy and of course, at a meeting. It is particularly appropriate that these lectures, by Donald Pinkel who has done so much for leukemia in childhood and Bob Gallo, as outstanding in the field of viral leukemo-

Fig. 4

Fig. 5

Fig. 6

genesis, are dedicated to his memory. I am sure that somewhere in Heaven Fred Stohlman's spirit is looking down on this gathering and his memory will inspire all of us to a continuing dedication to the high scientific goals he attained during his all too brief career.

Cellular and Virological Studies Directed to the Pathogenesis of the Human Myelogenous Leukemias

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It is a particular honor for me to give the first Frederick Stohlman memorial lecture because of my personal respect and friendship with Fred. His courage, honesty, and stimulation of good science in the hematological field will be remembered by all who knew him. It is also very fitting to have these lectures in Wilsede where special friends were made at the first meetings and where association and friendship with Fred Stohlman became much closer for many of us.

Introduction

There have been enormous advances in the therapy of lymphocytic malignancies as exemplified by the treatment and apparent cures of some childhood acute lymphoblastic leukemias (see D. Pinkel elsewhere in this book). The myelogenous leukemias remain much more difficult to treat, and for this reason and because they also provide an interesting hematopoietic system for the study of differentiation our interests for the past several years has focused on the origin and pathogenesis of this disease group.

The origin of the myelogenous leukemias appears to involve proliferation of a stem cell with various degrees of commitment to differentiation and arrests in differentiation at various cellular levels. These diseases appear to be monoclonal (see Fialkow and also Rowley elsewhere in this book) when they are clinically manifest. However, it is not known if the initiation of the disease is multiclonal or monoclonal. Their appearance as monoclonal when presented to clinicians might be because of a select growth advantage of one clone as often seen in tissue culture. We do not yet know the mechanism(s) initiating the abnormality of growth characteristic of these diseases, but certain factors have clearly been shown in select cases to induce or play some role in leukemic development. Studies in mice, chickens, and humans implicate genetic factors which may be involved at multiple levels. Thus, some congenital diseases with chromosomal abnormalities and certain families have shown an unusual incidence of leukemia (see report by R. Miller elsewhere in this book). One family of unusual interest has recently been described by F. Gunz and his colleagues in Australia. Thirteen members of the family developed some form of myeloproliferative disease, clearly implicating genetic factor(s). Yet, some members developed the disease within a relatively short time of each other even

though the age of each varied [17]. This certainly suggests an environmental factor may have been operative as well as genetic factor(s). There was no known unusual exposure of any member of the family to chemicals or radiation.

What about environmental factors? Radiation can induce leukemias in animals, and it has been clearly associated with myelogenous leukemia in humans under unusual circumstances. Benzene also has been associated with leukemia in very rare instances, and some other chemicals with such rarity that their leukemogenic potential in man is undefined.

Epidemiological studies indicate that leukemia is not increased in people living at higher altitudes with greater exposure to radiation, nor is leukemia generally higher in industrial areas than in rural regions [24]. These observations together with the facts that 1. the incidence of childhood leukemia has apparently stayed about the same since industrialization and in fact has declined in recent years, 2. the incidence peaks in young children, 3. the incidence is greater in whites than in black people, and 4. association with chemicals and radiation is very rare (see R. Miller elsewhere in this book) all suggest to me that the leukemias may be predominantly biological diseases and that all biological factors must be thoroughly explored.

Retroviruses and Retrovirus Related Information: Reasons for Intensive Exploration of Human Tissues

We have felt that a search for retrovirus (RNA tumor virus, oncornavirus) information or related information in human cells was mandated by several considerations. 1. As discussed above epidemiological considerations are a stimulus for consideration of biological factors. Although these same broad studies also do not lend strong support to a virus causation of the disease in a conventional manner, I think they are quite consistent with a role for viral information if one considers: a) long latency, b) a second or third factor in addition to appropriate viral information may be a requisite, c) retroviruses can be vertically transmitted either in the germ line as an endogenous cellular element or by congenital infection (see R. Weiss elsewhere in this book). These factors would obscure epidemiological approaches. 2. Several retroviruses can produce leukemia in a variety of animals in laboratory experiments. 3. Retroviruses can sometimes transform cells and not be seen again as discrete virus particles in in vitro experiments (see P. Duesberg elsewhere in this book). Moreover, some data suggests this may also be true with some naturally occurring leukemias of animals. For instance, in a significant number of cats with leukemia (perhaps approaching 50%), feline leukemia virus (FeLV) has not been found (see O. Jarrett elsewhere in this book). In many of these cats antibodies to FeLV has also not been identified, and recent data from our laboratory in collaboration with W. Hardy and M. Essex indicate that FeLV proviral nucleic acid sequences may also not be readily detectable (Koshy, Wong-Staal, Gallo, Hardy, and Essex, in preparation). Yet there is evidence that FeLV may still cause the disease in

these so called "virus negative" cats. This is based on some epidemiological results (M. Essex, personal communication) and on the finding of feline oncornavirus membrane antigen (FOCMA) in the leukemic cells. This protein is believed to be specifically coded for or induced by FeLV and by feline sarcoma virus (FeSV) (see M. Essex elsewhere in this book). 4. Bone marrow transplants studies have indicated that in some exceptional cases normal bone marrow donor cells may be transformed to leukemic cells when given to a leukemic individual [43]. 5. We have recently been able to show that some of the primate type-C retroviruses can transform human B-lymphocytes and may also interfere with differentiation of myelopoietic cells. 6. The most compelling reason to think of retroviruses in the etiology of leukemias of humans is the results of animal models. It appears now that in every instance where we know the cause of *naturally* occurring leukemia in a *sizable* fraction of a leukemic animal population it involves a retrovirus. This now includes chickens (see R. Weiss and O. Jarrett, A. Burney elsewhere in this book), some wild type mice [16], cows (see O. Jarrett in this book), cats (see Jarrett and also Essex) and gibbon apes (see next section). 7. Since retroviruses can recombine with cellular genes and since some of them can affect cell differentiation (see later section of this report and also reports by M. Dexter, by N. Teich, by M. Moore, and by T. Graff in this book), it is possible and perhaps likely that sometimes these viruses contain cell derived genes involved with growth and/or differentiation. If so then it should be important to use retroviruses as probes in human leukemia to see if such genetic information is altered during leukemogenesis whether or not the disease is due to a virus. For this we would choose a primate retrovirus.

Primate Type-C Retroviruses

There were no isolates of any primate retrovirus before this decade. Now there are many, and they are from diverse species. I will focus on two groups since they were the earliest isolates and the only ones which have been shown to have pathological effects on cells. We have been particularly interested in the members of the infectious type-C virus group isolated once from a woolly monkey and called simian sarcoma virus (SiSV), simian sarcoma associated virus (SiSAV) complex and some isolated several times from gibbon apes and collectively called gibbon ape leukemia virus (GaLV). SiSV (SiSAV) and the various GaLV are very closely related viruses, and the evidence suggests their ancestral origin was probably a rodent virus which entered these primates by interspecies infection [27,47]. It is of particular interest that the virus entered two primates which are only distantly related. We have especially focused our attention on this virus group because the gibbon is the species closest to man for which a retrovirus has been isolated, because it is the species closest to man for which we have an animal model of leukemia and notably one which we know something of the etiology, and because we think viruses like this have been in humans. It

Table 1. Infectious primate type-C retroviruses: The Woolly Monkey (Simian) Sarcoma Virus (SSV-SSAV) – Gibbon ape Leukemia Virus (GaLV) Group[a]

Ancestral Origin	Vectors	Transmission	Biological Effects: Tumorigenicity in vivo natural	Biological Effects: Tumorigenicity in vivo experimental	Biological Effects: Transformation in vitro	Members	
Rodents	unknown (? man)	Horizontal to woolly monkey, gibbon apes, ? man. Vertical only by infection parent to progeny. Infections clearly evident gibbon to gibbon. Source of infection of one woolly monkey is unknown	Fibrosarcoma in a woolly monkey (SSV) CML and ALL by GaLV	Fibrosarcoma and fibromas in marmosets and cerebral tumors by SSV. CML and ALL by GaLV	SSV transforms fibroblasts. It's helper virus (SSAV) as well as GaLV can transform human blood B-lympho-blasts. (see text)	SSV (SSAV)	isolated once from a pet woolly monkey. Very related isolates from human cells reported by 5 laboratories
						$GaLV_{Thai}$	From leukemic animals in a colony in Thailand inoculated with human malaria blood
						$GaLV_{SF}$	From leukemic animals in San Franzisco Zoo
						$GaLV_{Br}$	From brains of 3 animals 2 of which were inoculated with extracts of human brains
						$GaLV_{H}$	From a spontaneous acute lymphoblastic leukemia of an animal free roaming with a group of gibbons on Hall's Island, Bermuda

[a] References to original reports (except for recent results) can be obtained from the review by Gallo and Todaro "Oncogenic RNA Viruses". In: Seminars in Oncology. Yarbro, J.W., Bornstein, R.S., Mastrangelo, M.J. (eds.), pp. 81–95. New York: Grune & Stratton, Inc. 1976

appears sufficiently clear that this virus entered gibbons directly or via an unknown vector from a rodent, and it is certain that the virus is transmitted by infection among gibbons in captivity. The immediate vector to the one woolly monkey is unknown. A summary of these viruses is given in Table 1.

Shortly after the isolation of viruses of the SiSV-GaLV group [9,21,22, 39,42,46], baboon endogenous type-C viruses (BaEV) were isolated from different tissues and different species of baboons [20,40]. These viruses exhibit all the characteristics of endogenous viruses, i.e., transmitted in the germ line of baboon cells, and had no demonstrable effect in vivo or in vitro on mammalian cells. Apparently BaEV entered an ancestor of domestic cats several millions of years ago, infecting the germ line, and now maintained as an endogenous virus of cats known as RD114 (reviewed by Todaro in reference [41]). There is also evidence from several laboratories that a related virus is sometimes identified in humans (see below).

Table 2. Endogenous Type-C Virus of Baboons (BaEV)[a]

Ancestral Origin	Transmission Vertical	Interspecies Infection	Biological Effects	Members
Babon	in germ line: baboon to baboon	1. to a feline ancestor of domestic cat; and now vertically transmitted in germ line of cat as a variant of BaEV known as RD114 2. ? to humans 3 reports of related virus from human tissue and several reports of related antigens and nucleic acid sequences (see text)	none reported to date –	Papio cynocephalus Papio anubis Papio papio Papio hamadryas

[a] References to original reports (except for recent results) can be obtained from either the text or the review by Gallo, R.C. and Todaro, G.J.: Oncogenic RNA Viruses. In: Seminars in Oncology. Yarbro, J.W., Bornstein, R.S., Mastrangelo, M.J. (eds.), pp. 81–95. New York: Grune & Stratton, Inc. 1976

Selected Results with Human Cells

Numerous reports suggest that some fresh uncultured human cells contain intracytoplasmic virus like particles (discussed in references [10] and [37]), but since these are not isolated as infectious virus and have not been shown to exhibit biological activity it is not certain thay they represent defective or abortive type-C viruses or are cellular artifacts. However, the presence of high molecular weight RNA with some sequence homology to RNA from

some viruses and, in some samples of human leukemic cells, a DNA polymerase with properties resembling reverse transcriptase (RT), associated with these "particles", emphasize their viral-like properties. In some cases these polymerases were shown to be immunologically specifically related to RT from SiSV-GaLV group (reviewed in references [10,11] and [12]), but in most cases immunological relatedness to primate viruses was not found and extensive comparisons (by immunological tests) to other animal viruses was usually not performed because of limited amount of enzyme. These results were a stimulus to consider the possibility that humans may harbor viruses related to this group.

Our objective was to purify sufficient enzyme to enable us to make peptide maps of the RT and compare this to RT from these viruses. This has not been achieved, and there has been little progress in our laboratory with this problem since the initial reports. On the other hand Chandra and colleagues [1,2] have reported the purification of this enzyme (related to SiSV-GaLV RT) from the spleen of a child with a preleukemic disease, myelofibrosis, which eventually turned into AML. Similar enzymes have been found in orbital chloromas from leukemic people living near Ankara, a disease appearing as a cluster (see R. Miller and also P. Chandra elsewhere in this book).

On rare occasions whole type-C virus has been reported isolated from human cells. These reports, reviewed in references [11] and [13], have come from our laboratory (HL23V), from Panem and Kirsten (HEL-12), from Nooter and Bentvelzen and their colleagues (SKA-21), from Gabelman and Waxman, and recently from H. Kaplan and colleagues. In each case the viruses are related to the SiSV-GaLV group, and at least in three cases (HL23V, SKA-21, and HEL-12), a second virus related to BaEV has been detected. This peculiar combination remains unexplained. Because of their similarity to existing primate viruses it is possible that these isolates are all laboratory contaminants. Regarding HL23V we offer the following results against contamination: 1. Reproducible isolation from separate clinical specimens; 2. previous evidence reported in references [30] and [7] for RT related to RT of SiSV-GaLV in the uncultured blood cells; 3. previous evidence for cytoplasmic RNA sequences related to SiSV and BaEV in the fresh uncultured blood cells of this patient [33]; 4. previous evidence for DNA proviral sequences related to BaEV [33,48], although SiSV proviral sequences could not be detected [33,48]. We did not subsequently find evidence for a humoral antibody response to either the SiSV or BaEV component of HL23V in the sera of this patient, but recently we have obtained confirmation of the presence of the BaEV provirus in the DNA of the uncultured blood cells from this patient. As described elsewhere in detail in this book [49], we have used the technique of DNA digestion with a restriction endonuclease followed by separation of DNA fragments by agarose gel electrophoresis, transfer of the DNA fragments to nitrocellulose filters by the Southern blotting technique, hybridization of I^{125}-labeled 35S viral RNA to this DNA, and examination for virus specific bands after development over X-ray films. This approach allows for more sensitive molec-

ular hybridization because much of the irrelevant DNA is excluded (the labeled viral RNA probe is in excess) and for qualitative assessment because the positive bands can be visualized and compared to bands of virus infected cells. As shown elsewhere in this book [49] DNA from fresh uncultured blood cells from patient HL-23 and from another AML patient labeled HL-49 contain several clearly visible bands after digestion with the endonuclease HIND III which hybridize to I^{125}-35S RNA from BaEV (M7). For control purposes DNA from normal human leukocytes and from human cells (A204) deliberately infected by BaEV are also shown. No viral specific fragments with BaEV as a probe were found in the normal cell DNA. The only band seen is from ribosomal DNA which was detected with labeled rRNA which was used as a control because sometimes rRNA can contaminate viral RNA. Multiple viral specific bands are, or course, found in the positive control, BaEV infected A204 cells. It is interesting that some of the proviral bands found in the DNA from the leukemic cells of patients HL-23 and HL-49 are not found in the BaEV infected A204 cell positive control. This suggests that the integration sites may be different or that the viral fragments detected in HL-23 and HL-49 are related but not identical to the M7 strain of BaEV. Despite the evidence for some cytoplasmic RNA sequences related to SiSV (SiSAV) in HL23 fresh blood cells [33] no novel proviral bands of SiSV (SiSAV) were found. M. Reitz in our laboratory had previously shown that DNA from the kidney and spleen of patient HL-23 contained more SiSV (SiSAV) hybridizable sequences than did DNA from normal cells or DNA from the leukemic blood cells of patient HL-23. It is possible that only a small percent of the cells contained the SiSV (SiSAV) proviral sequences, and failure to detect them is due to insufficient sensitivity. Such cells may have been concentrated in the kidney and spleen. Unfortunately, DNA or tissue from these organs is no longer available so they cannot be examined by the restriction endonuclease blotting technique. In summary, we have evidence now that the BaEV component of HL23V was present in the *primary uncultured blood cells* of patient HL-23, but inconclusive data for the SiSV (SiSAV) component.

The important question regarding these or similar viruses is to ask whether they could be important causes of human leukemia. In an effort to obtain a preliminary answer to this question we surveyed DNA from many human tissues for the presence of integrated novel viral sequences by the standard techniques of molecular hybridization. We used molecular probes (both labeled RNA and labeled cDNA) from many animal viruses, including SiSV and BaEV. We did not find significant differences between normal and leukemic cells with probes from the majority of viruses, and the level of hybridization were insignificant. An example of this is the negative data obtained with a rat type-C endogenous virus and with GaLV illustrated in Fig. 1 (parts B and C). The data is a summary of results of many samples presented as a distribution frequency, i.e., it represents the percent of human DNA samples (ordinate) which hybridize to a certain maximum extent (abscissa). Note that the vast majority of cases (normal and leukemic hybridize very little to rat virus nucleic acid probes and the distri-

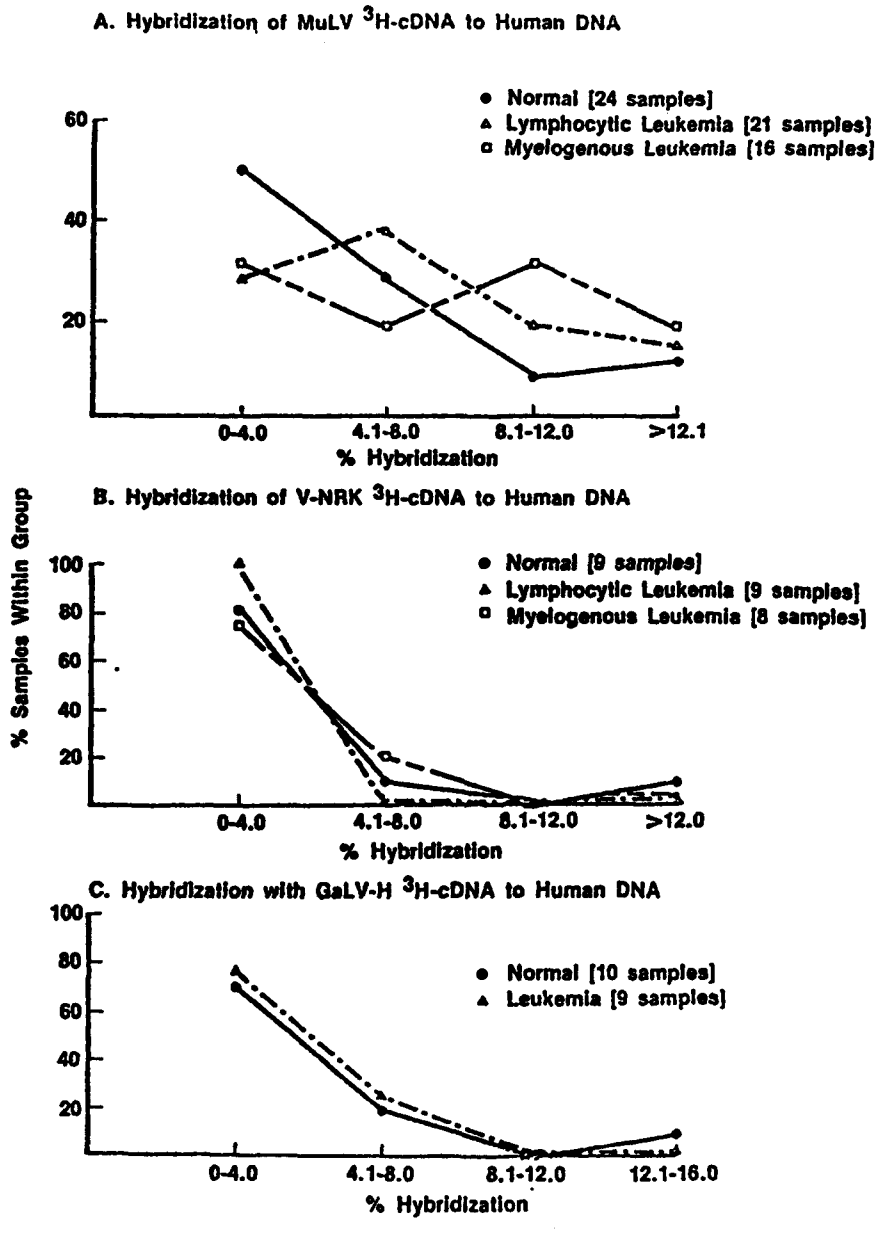

Fig. 1. Survey of human DNA from normal blood and from people with various types of leukemia for murine leukemia virus (MuLV) (Rauscher strain) related sequences (panel A), rat endogenous virus (V-NRK) related sequences (panel B), and gibbon ape leukemia virus (GaLV) related sequences (panel C).

The probes are ^{3}H-cDNA prepared from endogenous reverse transcriptase reactions of the respective viruses. Data are given as the frequency and extent of hybridization found in the various DNA samples. As shown all samples hybridized less than 8% of cDNA probes from V-NRK and GaLV, and there are no differences between normal and leukemic samples. These hybridization results are not significantly above background levels. Therefore, within the limits of sensitivity of the assay such sequences could not be detected in humans. In contrast, some human DNA samples did hybridize some of the MuLV probe to levels which appear to be significant. These results do not discriminate between MuLV sequences themselves or sequences related to MuLV. This survey was carried out by N. Miller and M. Reitz in our group.

bution of samples is virtually identical with DNA from normal and leukemic cells. In contrast, using BaEV probes we find a small number of leukemic DNA samples which hybridize significantly more of the probe (data not shown). We have not conducted an extensive survey for BaEV related sequences using the more sensitive approach of restriction endonuclease-Southern blotting, but we have applied it to a few very select cases and verified the higher hybridization obtained in the molecular hybridization survey. These results were mentioned above and presented with AML

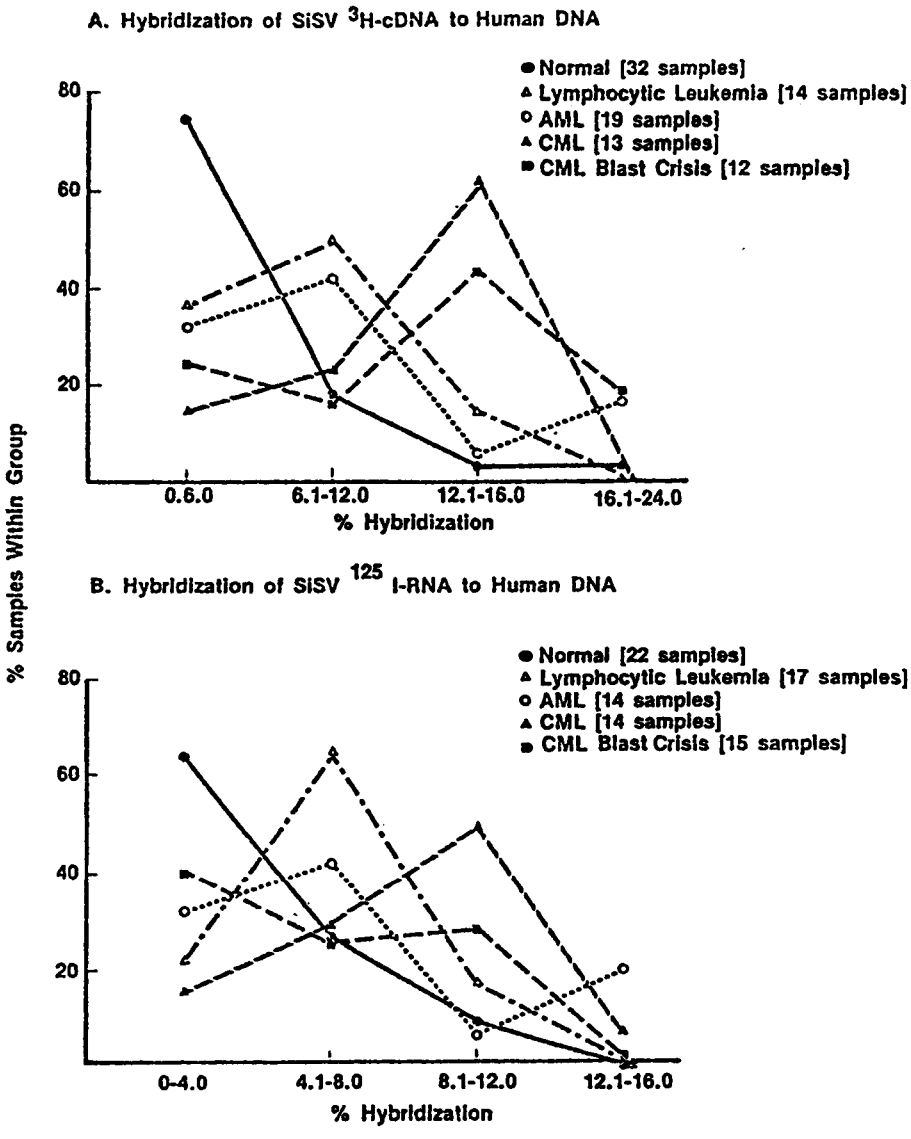

Fig. 2. Survey of human DNA from normal blood and from people with various types of leukemia for simian sarcoma virus (woolly monkey) (SiSV) related sequences.
In the upper panel (A) the probe used was ^{3}H-cDNA prepared from endogenous reverse transcriptase reactions. In the lower panel (B) the probe was I^{125} labeled 70S RNA. In both cases the vast majority of DNA samples from normal tissues hybridized less than 8% and all but a few less than 16%. In contrast, a significant percent of leukemic samples hybridized more than 12% and several more than 16%. Again, these results do not discriminate between SiSV sequences and SiSV related sequences. This survey was carried out by M. Reitz and N. Miller in our group.

patients HL-49 and HL-23 elsewhere in this book [49]. If the survey results are a true indication of the presence of these sequences then we can safely rule out BaEV or a closely related virus as commonly associated with human leukemia and therefore as a *common* cause of human leukemia. However, it may be important to keep in mind that the results could be underestimations, e.g., if only fragments of provirus are present and/or only a small number of cells in a population contain viral sequences, the sequences might easily be undetected, but possibly sufficient for leukemic transformation.

We also surveyed DNA purified from many human tissues for SiSV related sequences, and as illustrated in Fig. 2 several leukemic DNA samples hybridized more nucleic acid probes of SiSV (grown in marmoset cells) than did DNA from normal tissues. This is more clearly evident when one compares the distribution of hybridization of viral probes to various human DNA samples between rat virus (Fig. 1) and SiSV (Fig. 2). However, one DNA sample from a normal tissue (one of six human placentas tested) also showed unusually high hybridization to SiSV probes, so clearly higher hybridization is not unique to certain leukemic cells. To verify these higher hybridization results and to obtain greater sensitivity, some of these samples were also analyzed by restriction endonuclease digestion–Southern blotting, and hybridization of the specific fragments to SiSV 35S RNA. As described elsewhere in this book [49], the positive results with the one placenta derived from a normal person (termed NP3) obtained in the standard molecular hybridization survey was confirmed as was one leukemic sample. In these cases it is difficult to escape the conclusion of infection by an SiSV related virus. The presence in placenta is particularly interesting since the finding suggests the possibility of congenital virus transmission, a mechanism apparently involved in natural virus transmission seen in chickens [44], cats [19], cattle [26], and gibbon apes [23]. The data are also consistent with the isolation of an SiSV related virus from a human embryo by Panem and Kirsten [32].

As noted earlier SiSV and GaLV are not endogenous to primates but present in them only after infection. Although their recent history (? vectors) is unknown their ancestral origin is believed to be rodents. An unexpected observation, that came out of the search of human DNA for inserted viral sequences related to SiSV and GaLV was the finding of an SiSV related sequence in *all human DNA*. This was detected after digestion of the DNA with BAM I, with HIND III, and with Xpa restriction endonucleases (see paper by F. Wong-Staal et al. elsewhere in this book). This sequence was specific to this virus group since no endogenous viral sequence (i.e. sequences in DNA from normal uninfected tissues from various cell samples) were detected with probes from other viruses. We found this sequence using either SiSV (SiSAV) or SiSAV, and the results were obtained with viruses grown in non-human cells. Therefore, the results cannot be due to a trivial explanation such as presence of human cellular sequences with the viral probe. Moreover, it appears that this is not an evolutionarily conserved sequence since it was not found in the DNA we

have tested from other normal uninfected primates. We, therefore, tentatively interpret these results to indicate that this group of viruses has been in the human population or an ancestor of humans.

The Possibility of an Immune Response to Type-C Viruses in Humans

One of the most important questions relevant to type-C viruses and humans is whether a serological response against them is detectable. Results presented previously [25,38] and elsewhere in this book by R. Kurth and also by H. Snyder and by N. Hogg and their colleagues deal with this complex and as yet unsettled issue. We recently explored a different approach. Stimulated by the findings of I. Witz and his colleagues [45], P. Jacquemin, C. Saxinger, and I examined human blood cells for surface immunoglobulins. Both IgG and IgM were found, and appear to be chiefly associated with non-lymphocytic cells, a finding in agreement with Metzgar et al. [29] and Cotropia et al. [5]. We discovered that among the IgG were some which react with high specificity and at low concentrations with purified reverse transcriptases (RT) from select mammalian leukemia type-C viruses. In AML we find the IgG generally reacts with RT from SiSV, and to our surprise the reaction can be specific enough to distinguish this RT from the RT of the other members of the SiSV-GaLV group. In normal people (bone marrow) we find about 20% positive for IgG chiefly reactive with RT from one of the GaLV isolates, namely $GaLV_{SF}$. In CML, we find the remission and chronic phase patients to be like normal (negative or reactive with RT from $GaLV_{SF}$), while in almost every CML in blast crisis the IgG is chiefly reactive with RT from FeLV and rodent type-C viruses [18,36]. Unlike the sporadic detection of apparent inserted viral nucleic acid sequences the finding of IgG with specific RT enzymatic neutralizing activity is common. Because of this and because we have not as yet developed an assay independent of RT activity neutralization,[1] it is premature to conclude that this IgG represents an immune response to a viral protein. It is possible that the IgG is directed against determinants of a protein coincidentally very close to the amino acid sequences of the catalytic sites of certain RTs. The answer to this question should come from the isolation and characterization of the putative cell surface antigen.

Growth and Differentiation of Human Leukemic Myeloid Cells and Effects of Primate Type-C Viruses

We have reported that in the presence of conditioned media (CM) from certain human embryo cell strains, human myelogenous leukemic cells can often be induced to grow in liquid suspension tissue culture [8,14]. In many instances the cells terminally differentiate [14,15]. Sometimes they retain marker chromosomes, and this observation combined with the fact that this CM generally does not induce growth of normal myeloid cells led us to

conclude that the maturation arrest of many human myelogenous leukemias is not always irreversible, in agreement with L. Sachs [35] and M. Moore [31] and their colleagues. The differences in response of normal myeloid cells and leukemic cells also suggested to us that membrane receptors for regulation may be modified in leukemia. An interesting by product of this research was the establishment of the first human cell line with distinct myeloid characteristics [3]. This line, called HL-60, was shown to consist mostly of promyelocytes [31] which can be terminally differentiated when DMSO or other compounds active in the Friend murine erythroleukemia system are added to the culture [3].

Effect of Primate Type-C Viruses on Normal Fresh Human Blood and Bone Marrow Cells

To our knowledge there are surprisingly no reported studies of the effects of primate type-C viruses on fresh human blood cells. We have recently completed a preliminary study of the effects of various type-C viruses, including the primate viruses described here on human blood and bone marrow cells. We find that SiSV (SiSV), SiSAV alone, GaLV, HL23V (SiSV component), but not BaEV induce growth (Fig. 3) of B-lymphocytes (Fig. 4). In about one third of cases studied, the cells become immortalized, and despite apparent normal karyotype, they are tumorigenic in nude mice (Fig. 5). These cells are Ig and EBNA positive and do not have myeloid or T-cell characteristics [28]. The observation is different from that of "spontaneous" transformation of EBV positive human B-lymphocytes because the induction of growth is faster, the frequency of establishment of cell lines in our hands much greater, and the cells are tumorigenic despite apparent normal chromosomal makeup. The addition of BaEV or FeLV (despite some virus replication in a few cases) did not produce this effect. Finally, recent results indicate that the same phenomenon can be induced with EBV negative cord blood but at a lower frequency. We conclude that by indirect or direct means and by mechanisms not yet understood, some primate type-C viruses can be involved in the in vitro transformation of human B-lymphocytes.

Working Hypothesis

A currently interpretable and simple model for leukemogenesis is that the leukemogenic event, whatever the cause, leads to auto-production of a growth promoting molecule which prevent binding of normal regulatory molecules. This model is similar to one proposed by Todaro and colleagues in the genesis of sarcomas [6]. The alternative is that receptors for regulators are themselves modified. These ideas should become testable in the very near future.

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[1] Since this talk precipitation of RT has been obtained with these IgG.

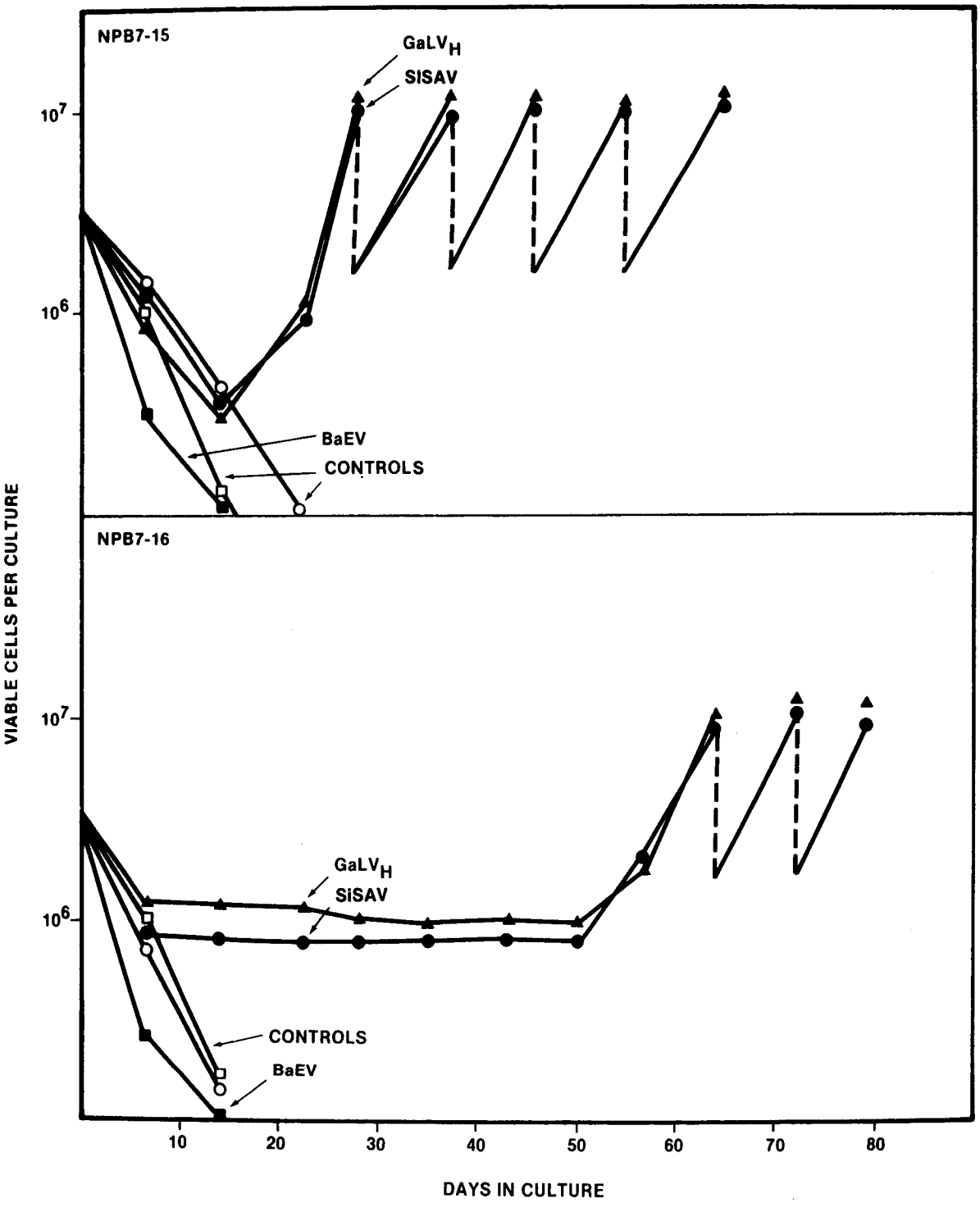

Fig. 3. Induction of growth of B-lymphocytes from normal human blood by primate type-C retroviruses (GaLV$_H$ and SiSAV).
Human blood leukocytes were cultured with or without the Hall's Island strain of GaLV or with the simian sarcoma associated virus (helper virus) (SiSAV). In some cases (e.g., sample NPB 7–15 shown in the figure) induction of growth was rapid. In other cases (e.g., sample NPB 7–16 shown in the figure lower panel) growth induction occurred after 50 days. Spontaneous growth (no virus) occurred in only 1 to 2% of samples but in more than 40% of cells treated with virus if a co-cultivation method was used (P. Markham, F. Ruscetti, Z. Salahuddin, R. Gallagher, and R. Gallo, in press).

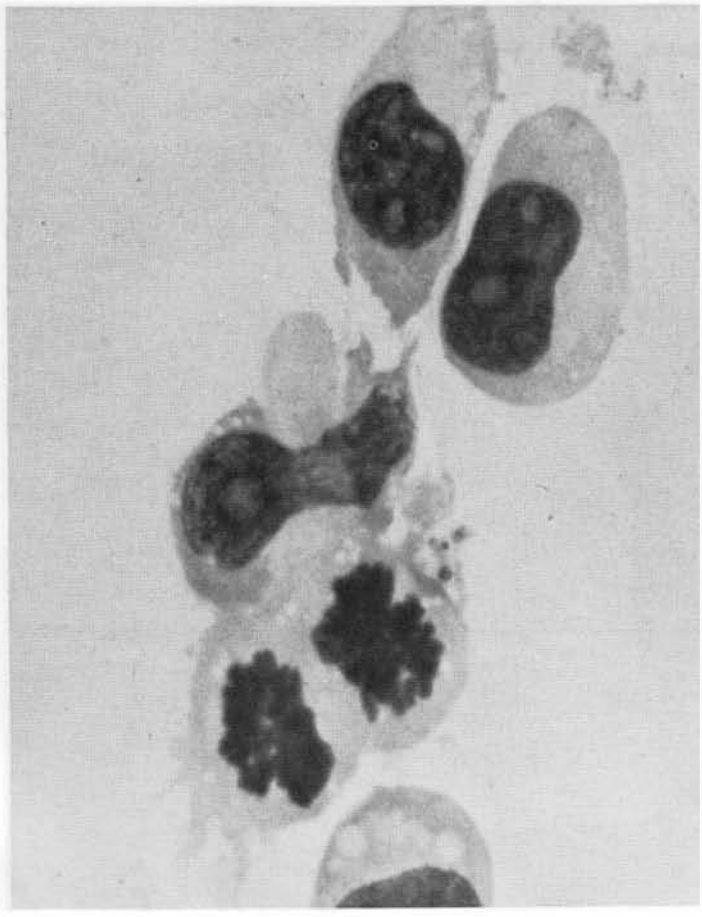

Fig. 4. Morphology of "immortalized" cell line induced from normal human blood after infection by Ganot lV_H.
Cells are lymphoblasts which are IgG and EBV positive
(Magnification 1000×).

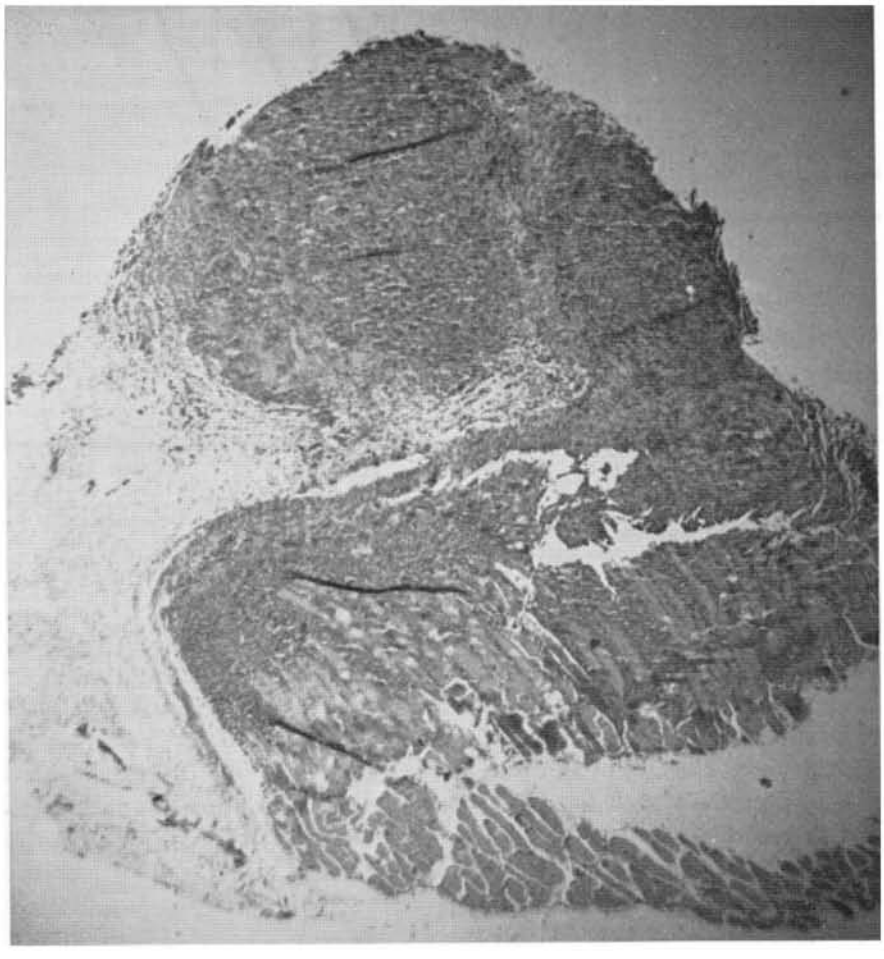

Fig. 5

Summary and Conclusion

1. Work over the past years and especially results of the past few years indicate that type-C viral or viral related genetic information exists in humans.
2. We do not know how this information entered humans or whether it causes disease, but it is of interest that the probes from the viruses used to detect this information are from the very same viruses which we find can affect growth and differentiation of some human hematopoietic cells.
3. The status of actual virus isolates from humans, though encouraging because of similarities of isolates from five different laboratories, remain very perplexing and so far have not been especially informative to human leukemogenesis.
4. In the near future we hope to clone in bacteria the viral related sequences detected in human DNA in order to more precisely determine their chemical and biological properties. The HL-60 system may also afford an opportunity to purify receptors for CSF. When CSF and other, perhaps more important, regulatory factors are purified, we would like to determine if they bind differently to leukemic and normal cells.

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Fig. 5. B-Cell lymphoma of nude mouse induced by injection of mouse with cells induced to grow by $GaLV_H$.
Cells are human as determined by karyotype analysis. (From: P. Markham, F. Ruscetti, Z. Salahuddin, R. Gallagher, and R. Gallo, in press).

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Treatment of Childhood Acute Lymphocytic Leukemia *

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Thank you for the honor of sharing in this memorial to Frederick Stohlman. The work I will report today represents the efforts of many physicians and scientists who have tried to understand and control childhood leukemia.

In North America and Europe acute lymphocytic leukemia (ALL) represents approximately 80 percent of childhood leukemia and 30 percent of childhood cancer. The disease usually occurs without warning in the well child who has been well cared for. It is characterized by fever, pallor, fatigue, malaise, bone pain, bleeding and enlarged viscera and lymph nodes. Without effective treatment the child soon dies of hemorrhage, infection or tumor encroachment. The diagnosis is made by examination of aspirated bone marrow.

In 1948 it was demonstrated that antifolate compounds produced clinical and hematological remissions in some children with ALL [10]. However, the remissions were only partial – cessation of treatment was followed by relapse in a few weeks, and temporary – relapse usually occurred within a few months despite continued administration of the drug. Subsequently, corticosteroids, mercaptopurine, vincristine and occasionally cyclophosphamide were demonstrated to induce remissions of a similar nature [11]. By 1961 it was possible to prolong the lives of children with ALL for a year or more but mortality remained near 100 percent. The major obstacles to cure were: drug resistance, initial and acquired; inadequate distribution of drugs to the leptomeninges resulting in primary meningeal relapse; treatment-related hematosuppression, immunosuppression and epithelial damage; and a pessimism about curing leukemia that imprisoned the wills of many physicians [15, 18].

The "total therapy" plan of treating ALL, initiated in 1962, embodied several innovative features: Combination chemotherapy for induction of remission and continuation treatment; reduction of leukemia cell mass to subclinical levels and restoration of hematopoiesis prior to antimetabolite

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therapy; meningeal irradiation early in remission to prevent meningeal relapse; cessation of chemotherapy after 2–3 years of continuous complete remission; and most important, a purpose to cure rather than palliate leukemia (Table 1) [15].

Early pilot studies suggested that the plan was feasible and useful [15]. Approximately $^{9}/_{10}$ths of the children experienced complete remission, hematological remissions were four times the usual length, and $^{1}/_{6}$th of the children remained in complete remission after treatment was stopped. However, the low doses of meningeal irradiation utilized were not effective in preventing meningeal relapse. In a later study the meningeal irradiation was increased and limited to the cranium and upper cervical area, and intrathecal methotrexate was administered during the irradiation period [2]. When followed by five-drug combination chemotherapy for 2½ to 3 years this treatment program resulted in a low frequency of meningeal relapse and ½ of the children are now surviving free of leukemia and off treatment for many years [19]. A comparative study proved that moderately high doses of preventive craniospinal meningeal irradiation reduced the risk of initial meningeal relapse 15fold and again led to one-half of the children surviving free of leukemia when they subsequently received three years of multiple drug chemotherapy [3]. At present meningeal irradiation is the only method demonstrated by long-term comparative study to prevent meningeal relapse both during chemotherapy and after its cessation [3,8,14,19,21].

Most of the children who survive continuously free of leukemia for five years and off treatment for two years are apparently cured. In Fig. 1 the initial continuous complete remission duration of 76 children entering complete remission in 1967 to 1970 are plotted on a semilogarithmic graph. All of the children received 2400 rads of cranial irradiation with simultaneous intrathecal methotrexate or 2400 rads of craniospinal irradiation early during complete remission. Subsequently they received multiple drug chemotherapy for 2½ to 3 years or until relapse or death during remission. As indicated, the complete remission duration curve forms a plateau after 4 to 5 years. All children represented in the plateau have been in complete remission for 8 to 10½ years and have been off treatment for 5 to 8 years. Except for the one child who relapsed after 5½ years of complete

Table 1. Plan of total therapy of acute lymphocytic leukemia 1962–75

Remission induction 4–6 weeks
To convert "late" leukemia into "early leukemia"
Prednisone + vincristine ± daunorubicin ± asparaginase
Preventive meningeal therapy 2–4 weeks
To eradicate arachnoid leukemia early in remission
cranial irradiation + intrathecal methotrexate or craniospinal irradiation
Continuation chemotherapy 2–3 years
To eliminate residual systemic leukemia
methotrexate + mercaptopurine + cyclophosphamide ± prednisone ± vincristine ± arabinosyl cytosine

remission all children in remission at 5 years remain so. This suggests that these children are biologically different from the children in the descending portion of the curve and that this difference represents biological cure of leukemia.

Since a plateau of continuous complete remission has been achieved the height of this plateau is now the criterion of success of curative treatment of ALL. Any new treatment or modification of treatment must be assessed with respect to this criterion. Since a plateau cannot be predicted or extrapolated statistically it is necessary to delay judgement about the curative value of treatment until actual experience demonstrates it.

The quality of survival for most children with ALL is satisfactory (Fig. 2, 3). Within a few weeks after initiation of treatment most children can return to normal activities such as school attendance and athletics [15,19,21]. Many children have 1–2 weeks of fever and somnolence approximately 6 weeks after cranial irradiation. All the children have various degrees of hematosuppression and immunosuppression, many exhibit inhibition of skeletal growth, and some demonstrate mucosal or skin disorders, elevation of hepatic enzymes in the serum, and macrocytic anemia. The children need to be monitored carefully to avoid excessive toxicity and to control infection. Trimethoprim and sulfamethoxazole is effective in preventing *Pneumocystis carinii* pneumonia in children at high risk [12].

After termination of chemotherapy an immunological rebound may occur with lymphocytosis of the bone marrow and rise in immunoglobulin levels [6]. Hematopoiesis recovers and elevated enzymes return to normal. Often growth and weight gain are accelerated and the children have increased energy and vitality [21]. Neuropsychological studies indicate that preschool children may experience impairment of short memory and

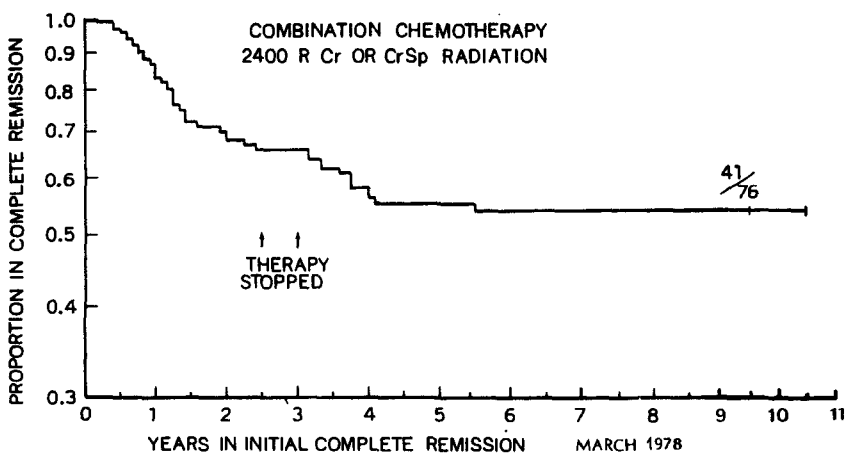

Fig. 1. This semilogarithmic graph describes the initial continuous complete remission duration of children who began receiving total therapy including preventive meningeal irradiation in 1967 to 1970. Treatment was stopped in all patients remaining in continuous complete remission after 2½ to 3 years. None of the children experienced initial meningeal relapse after cessation of therapy and only one child developed relapse after five years of complete remission. The level of this plateau of continuous complete remission is now the measure of curative value of treatment. It must be established by actual experience for each treatment plan or its modification

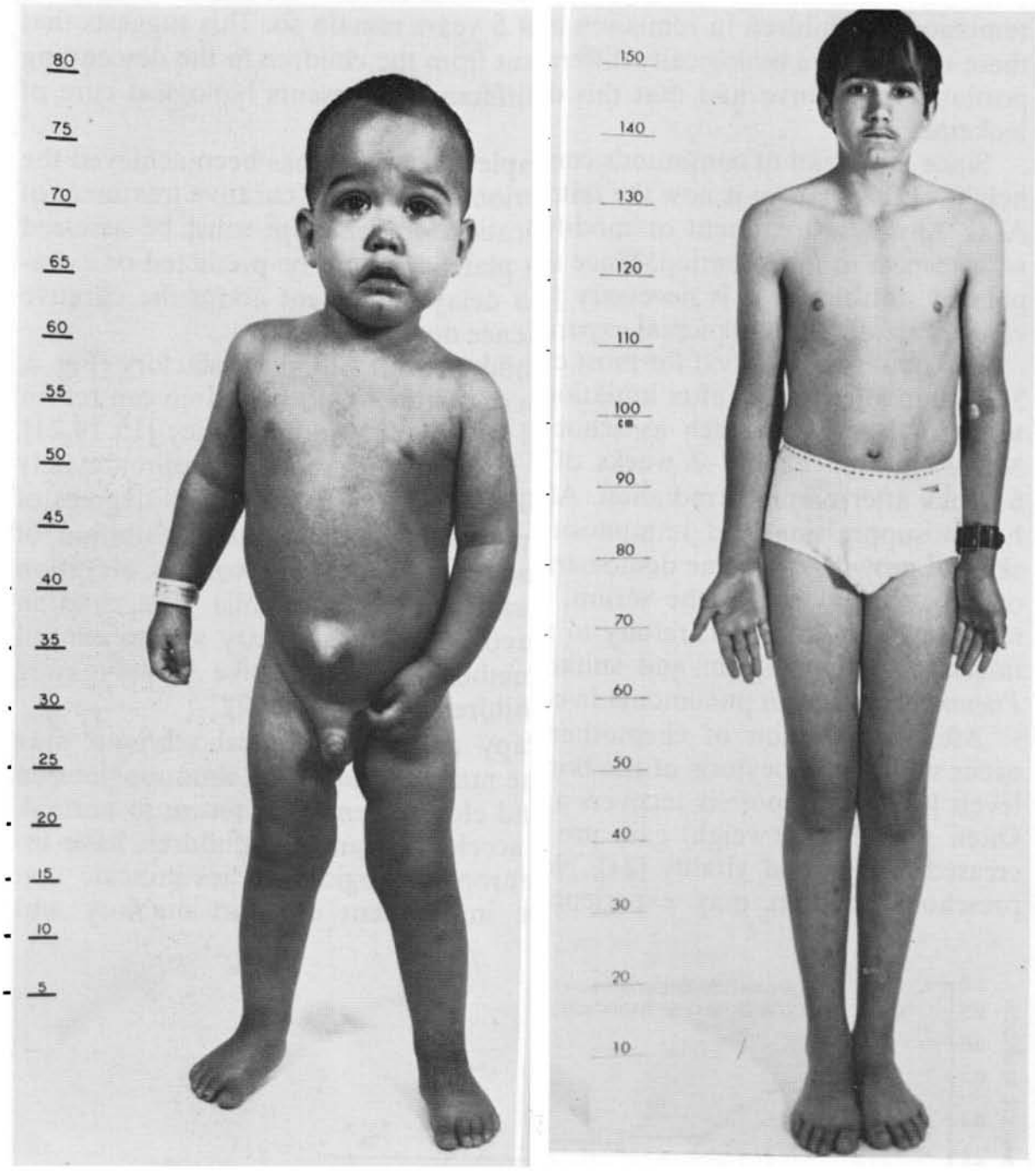

Fig. 2. Patient J.E. was admitted with ALL in August 1964 at age 1½ years. He survives continuously free of leukemia for 14 years and off therapy 11 years while enjoying normal growth, development and function

mathematical ability [9]. Early detection of these defects is important to allow remedial measures and to minimize school difficulties.

Reports of abnormal computerized cranial tomography, of paraventricular calcifications and of serious functional neurological defects have caused concern [13, 17]. The evidence suggests that clinical meningeal leukemia, high doses of parenteral methotrexate following cranial irradiation and intercurrent infectious encephalitis may be responsible for many of

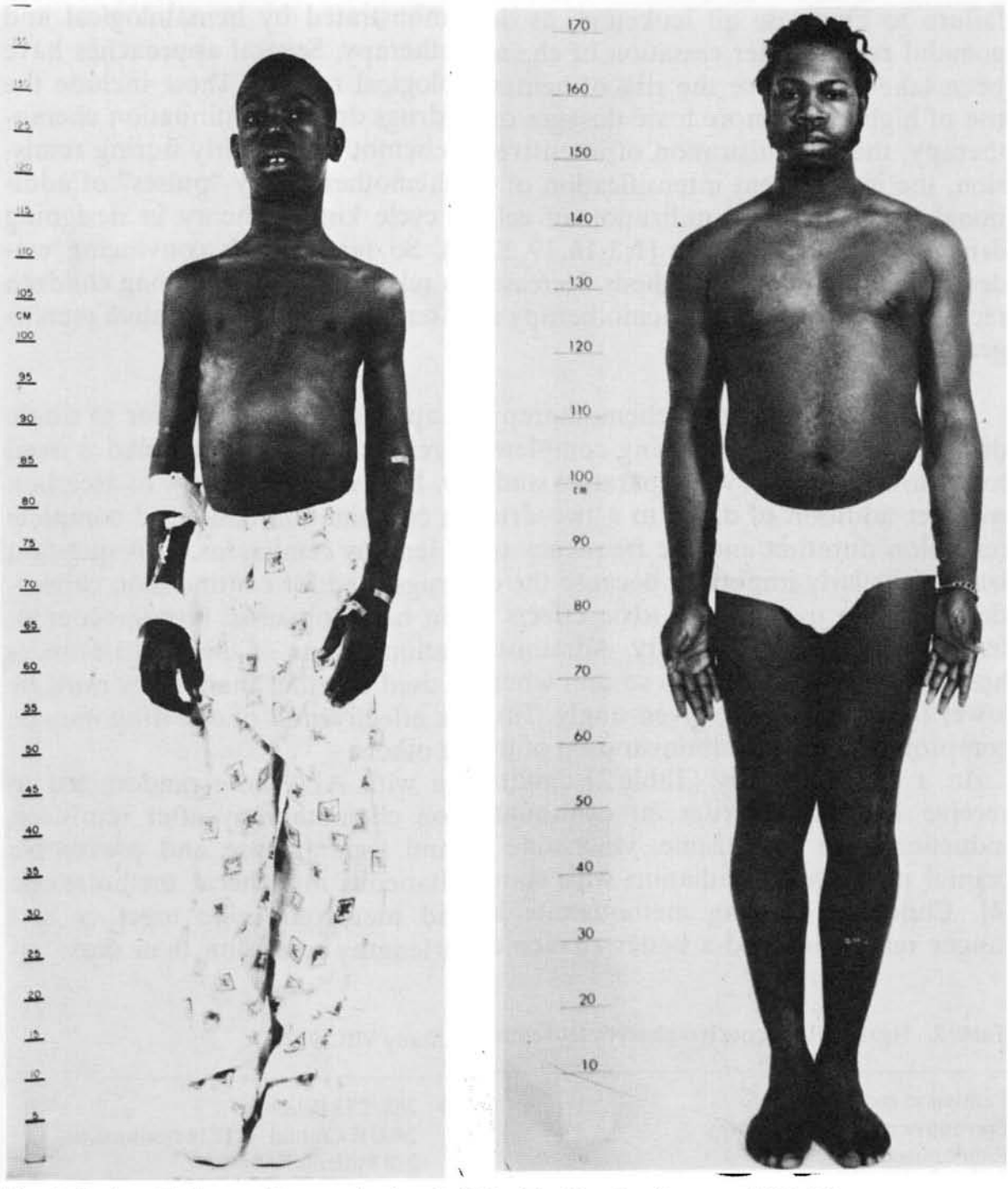

Fig. 3. Patient F.G., age 13 years, had an initial white blood cell count of 225,000 per cu mm. He has developed from adolescent to adult while continuously free of leukemia for 10 years. He has not received treatment for eight years

these problems. Also to be considered, however, are other neurotoxic drugs such as vincristine, asparaginase and prednisone, and the most common cause of cerebral atrophy in young children, protein-calorie malnutrition, a frequent concomitant of leukemia and its treatment.

The two most important reasons why children still die of ALL are drug resistance, as manifest by hematological relapse during chemotherapy, and

failure to eradicate all leukemia, as demonstrated by hematological and gonadal relapse after cessation of chemotherapy. Several approaches have been taken to reduce the risk of hematological relapse. These include the use of higher and more toxic dosages of drugs during continuation chemotherapy, the administration of intensive chemotherapy early during remission, the intermittent intensification of chemotherapy by "pulses" of additional drugs, and the utilization of cell cycle kinetic theory in designing drug treatment schedules [1,3,16,19,20]. So far we lack convincing evidence that any of these methods decreases relapse frequency among children receiving multiple agent chemotherapy after appropriate preventive meningeal treatment.

Although combination chemotherapy appeared to be superior to single drug treatment for continuing complete remission, there remained a need to demonstrate this by comparative study. It was also necessary to ascertain whether addition of drugs to a two-drug combination enhanced complete remission duration and the frequency of lengthy remissions. This question was particularly important because the drugs used for continuation chemotherapy have overlapping toxic effects on hematopoiesis, immunocompetence and epithelial integrity. Administration of one of the drugs reduces host tolerance to the others so that when used together their doses must be lower than if they were given singly. Thus, effectiveness of one drug may be compromised by the administration of the others.

In a 1972–75 study (Table 2) children with ALL were randomized to receive 1, 2, 3 or 4 drugs for continuation chemotherapy after remission induction with prednisone, vincristine and asparaginase and preventive cranial meningeal irradiation with simultaneous intrathecal methotrexate [4]. Children receiving methotrexate and mercaptopurine together had longer remissions and a better chance of lengthy remission than those re-

Table 2. Treatment of acute lymphocytic leukemia. Study VIII 1972–75

Remission induction	268/283 Patients			
Preventive meningeal therapy	2400 R Cranial + IT methotrexate			
Randomized	228 Patients			
	M	MMp	MMpC	MMpCA
No. Patients	20	68	70	70
Relapses	15	24	32	28
Died in complete remission	1	0	4	5
Continuous complete remission	4	44	34	37
Off therapy in continuous complete remission	4	37	26	28

In this comparative study remission was induced with prednisone, vincristine and asparaginase and followed by preventive cranial meningeal irradiation with simultaneous intrathecal methotrexate. Patients were assigned at random to one of four regimens: methotrexate alone (M), methotrexate and mercaptopurine (MMp), methotrexate, mercaptopurine and cyclophosphamide (MMpC), or methotrexate, mercaptopurine, cyclophosphamide and arabinosyl cytosine (MMpCA). The results indicate superior efficacy for the MMp combination.

ceiving methotrexate alone. On the other hand, patients receiving cyclophosphamide or cyclophosphamide and arabinosyl cytosine in addition to methotrexate and mercaptopurine tended to have shorter remissions and fewer lengthy remissions than those in the two-drug group. These results indicate that addition of simultaneous cyclophosphamide or cyclophosphamide and arabinosyl cytosine did not improve the efficacy of the methotrexate and mercaptopurine combination. Whether a cyclic or sequential schedule of two two-drug combinations might prove superior needs to be determined.

The morbidity and mortality of the one, three and four-drug regimens were greater than those of the two-drug combination (Table 3). Children on methotrexate alone received two to three times higher doses of this drug than those receiving the combinations. Nine out of 20 suffered leukoencephalopathy during initial complete remission while none of the other 218 children developed evidence of this complication during initial remission. In the three- and four-drug groups immunosuppression was more pronounced and was accompanied by higher risk of varicella-Zoster infection and *Pneumocystis carinii* pneumonia, more frequent hospitalizations and deaths during complete remission. Thus the most efficacious treatment regimen also had the least morbidity.

The most significant opportunity for improving the treatment of ALL in the past five years has been its biological and clinical classification by immunological cell surface markers (Table 4) [5,7]. This allows species identification of the leukemia cells, the first step toward developing specific cytocidal or cytostatic therapy. This may also provide further specific biological and chemical correlates of sensitivity and resistance of ALL cells to current drugs and may lead to new concepts of control of ALL. For example, the relatively good prognosis of common type ALL could be related to increased glucocorticoid receptors on the common type leukemic lymphoblasts [22]. Other speculations for the good prognosis of common type ALL include: its origin in the bone marrow where drug diffusion is probably superior than in the visceral masses characteristic of thymic cell and B-cell ALL; its low mitotic rate and less DNA synthesis, which might reduce the risk of mutation to drug resistance; its lower number of leu-

Table 3. Morbidity during initial complete remission. Study VIII

	M	MMp	MMpC	MMpCA
No. Patients	20	68	70	70
Cases of Leukoencephalopathy	9	0	0	0
Cases of Pneumocystis pneumonia	0	1	7	19
Cases of Varicella-Zoster	0	8	16	17
Total No. Hospitalizations	13	25	49	72

See table 2 legend for explanation of abbreviations. The two-drug regimen was accompanied by the least morbidity during initial complete remission. This suggests that efficacy and morbidity of a chemotherapy regimen may be unrelated.

kemia cells and therefore greater susceptibility to chemical eradication and less possibility of a drug resistant variant; the better stimulation of normal lymphocytes by common type lymphoblasts in mixed leucocyte cultures and therefore greater susceptibility to a theoretical immune control. Another speculation is that common type ALL is a developmental disorder of lymphocytes. Children normally experience rapid lymphocytic proliferation from age two to six years, the most frequent age for common type ALL. Is it possible that factors controlling lymphocytic proliferation after age six years contribute to control of common type ALL? Does leukemia therapy simply repress common type lymphoblast replication until normal controls take over as the child becomes older?

Table 4. Immunologic classification, childhood lymphocytic leukemia

	Common	Null	T cell	B cell
Frequency	60%	15%	22%	3%
Sex, Age	M = F, 2–6 yrs.	?	M, > 8 yrs.	?
Origin	Marrow	?	Thymus	Alimentary
Mitotic Rate	Low	?	High	High
Initial white blood cell count	Low	High	High	Low or High
Remission	Long	Less	Less	Least
Surface immunoglobulin	—	—	—	+
Erythrocyte rosettes	—	—	+	—
Thymic antigens	—	—	+	—
Common ALL antigen	+	—	—	+
Ia antigen	+	±	—	+

This tentative classification of ALL is based on immunological cell surface markers of the leukemic lymphoblasts. For valid determination of species, leukemic lymphoblasts of the bone marrow need to be studied prior to chemotherapy. The table was prepared by Dr. Luis Borella.

Summary

ALL in children cannot be considered incurable. Approximately one-half of children receiving modern therapy survive free of leukemia 5–10 years after cessation of treatment and at little or no risk of relapse.

The value of any treatment program must be measured by the proportion of children surviving free of leukemia off therapy and at little or no risk of relapse, that is, the proportion that is apparently cured. This cannot be projected or extrapolated from preliminary data.

The classification of ALL into biological species by immunological markers may lead to the development of more specific and effective treatment as well as to better understanding of its origin and nature.

Most important, it must be emphasized that the majority of children in the world do not benefit from advances in treatment of ALL because of their complexity, hazards, expense and inaccessibility. Therapeutic research needs to be directed away from more complex, expensive technology such as bone marrow transplantation and sophisticated radiotherapy. Effort

should be concentrated on understanding the fundamental biology of children's ALL and on its practical application for specific, effective, simple, safe and cheap treatment. In this way we can assure that all children in the world will benefit from our science and we can best fulfill our obligations as scientists and physicians.

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Epidemiology of Leukemia

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Demography

In studying etiology it is important to learn not only who is most susceptible but also who is least susceptible to certain forms of leukemia. Chronic lymphocytic leukemia (CLL) has a peculiarly low frequency in Chinese and Japanese, and is not induced by ionizing radiation [21]. CLL comprises only 1.5% of adult leukemias in Chinese and Japanese as compared with 30% in Europeans [10], and the frequency does not rise with migration to Hawaii [9]. These observations separate CLL etiologically from other forms of leukemia and indicate the extent to which inherent susceptibility may vary.

Acute lymphocytic leukemia (ALL) in childhood has a peak frequency at about 4 years in white children, but not in Blacks. The peak emerged in Great Britain in the 1920's, in the U.S. in the 1940's and in Japan in the 1960's [24], but not yet in the People's Republic of China [25]. Thus ALL appears to be related to "industrialization" or development of the nation, but Blacks seem not to be susceptible. In the United States the peak rose progressively until the late 1950's, when a decline set in before the era of chemotherapy (Fig. 1). Since 1972 the decline has steepened as new treatments have taken hold. Studies of cell surface markers indicate that the peak is due to ALL of the non-B non-T cell type [29].

Environmental Agents

Ionizing radiation: X-ray was the first environmental agent implicated in leukemogenesis. Case reports in the 1920's were followed by retrospective studies in the 1940's and a prospective study of atomic-bomb survivors in Japan since the 1950's [21]. Myelogenous leukemia, either acute or chronic, is the predominant type induced by radiation, but ALL was occasionally induced, usually when the age at exposure was under 20 years. An increased frequency of CML persisted until 1965, but the rates for acute leukemia continue to be elevated [4, 16]. Recently leukemia has been observed as a complication of radiotherapy for Wilms' tumor [28], indicating the need to seek a lower dose that would cure the original cancer without causing leukemia.

Emphasis is now being placed on the leukemogenic potential of low-dose radiation in the general population. The question is more likely to be resolved by understanding the biologic mechanisms involved than by argu-

ments about threshold and linearity of dose-response – at levels below which epidemiologic studies are impractical because of the large sample sizes required.

Beginning in 1956 Dr. Alice Stewart published data which seemed to indicate that each form of childhood cancer could be induced by small diagnostic x-ray exposures of the mother during pregnancy. The fullest presentation of her findings appeared in 1975 [3]. One by one she had dealt with the criticisms of her original study. The largest remaining puzzle was the constancy of the increase in relative risk (1.5-fold), regardless of the form of cancer, be it leukemia, lymphoma, Wilms' tumor, cerebral tumors, neuroblastoma or all other childhood cancer [21]. This finding seemed biologically implausible [21]. Recent studies have failed to duplicate her findings except possibly for the increased risk of leukemia [8,15,17]. A lingering doubt thus remains about the interpretation of her results.

Chemicals: Among chemicals known or suspected to be leukemogenic are benzene in persons occupationally exposed [1,31], and alkylating agents used for cancer chemotherapy [5]. Maternal exposure during pregnancy could conceivably induce leukemia in the offspring transplacentally. This possibility seems enhanced by a Swedish report that after occupational benzene exposure during pregnancy, an increased frequency of sister chromatid exchanges was observed in both mother and child [12] – a finding which needs to be confirmed elsewhere.

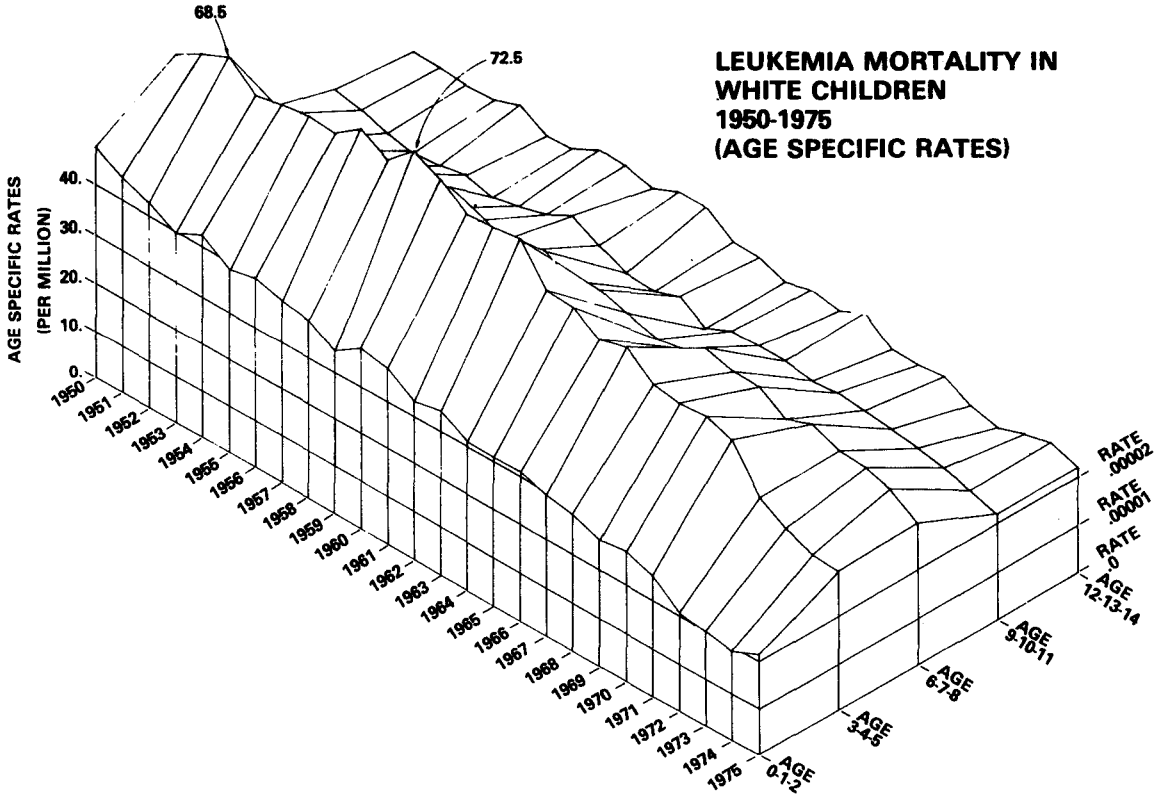

Fig. 1. Annual leukemia mortality rates for U.S. white children, 1950–1975, by 3-year age-intervals

In the treatment of multiple myeloma with melphalan, the predominant subtype of leukemia induced has been acute monomyelogenous (AMML) [19].

Leukemia Clusters: In the mid-1960's leukemia clusters were taken by virologists as evidence for horizontal transmission of the disease. Creative statisticians were stimulated to develop dispassionate procedures to determine if clustering of such rare events in time and space was attributable to chance. When applied to leukemia, these methods showed no striking excess of clusters suggestive of an infectious mode of transmission [6]. Individual clusters may nonetheless be environmentally induced, as by ionizing radiation in Hiroshima and Nagasaki [16], and by benzene in Italian shoemakers [31]. Clusters are more likely to be meaningful if they are of a particular subtype as in Ankara, Turkey, where AMML accounted for 40% of childhood leukemia [7] as contrasted with 4% in Boston [11].

Host Susceptibility

Inborn Chromosomal Abnormalities: It is now well known that leukemia, principally ALL, occurs excessively in Down's syndrome, but it is not well known that the childhood peak occurs three years earlier than in the general population [22]. An increased risk of leukemia might also exist in other autosomal trisomies, but may be less apparent because of the short lifespan. The risk of leukemia is markedly elevated in two recessively transmitted chromosomal fragility disorders, Bloom's syndrome [13] and Fanconi's anemia [26]. In Fanconi's anemia the cell type has almost invariably been AMML. It should be noted that this rare form of leukemia is the predominant form that occurs not only in this syndrome, but also after multiple myeloma treated with melphalan, and as a cluster in Ankara.

In ataxia-telangiectasia (AT) there is both chromosomal fragility and immunodeficiency, and a predisposition especially to lymphoma, but also to ALL [14]. Each of the foregoing constitutional disorders, as well as exposure to ionizing radiation or benzene, has as a feature in common chromosomal abnormality before the onset of leukemia [23]. With the development of banding techniques for the examination of chromosomes, leukemia in the general population is increasingly being associated with particular chromosomal aberrations (Rowley, this volume). The piecing together of clinical and epidemiological rarities has thus led to a more broadly applicable understanding of cytogenetics of leukemia in the general population. It now becomes a challenge to explain the exceptions in which no connection is yet known between leukemia and chromosomal abnormality. Among inborn diseases with a predisposition to leukemia but as yet without characteristic chromosomal abnormalities are Poland syndrome [32], Rubinstein-Taybi syndrome [18] and multiple neurofibromatosis, in which childhood leukemia is of the non-lymphocytic type [2].

A DNA Repair Defect in Familial AML?

The discovery of DNA repair defects in xeroderma pigmentosum and AT, two disorders with acute sensitivity to radiant energy, led us to seek such defects in disorders with sensitivity to a delayed effect of radiation; namely, neoplasia. An extension of this reasoning led us to studies of persons with multiple primary cancers or familial cancer of types that could be radiogenic, but in these cases were not. In one instance a boy with hereditary retinoblastoma and multicentric osteosarcoma of the limbs, not due to therapy, showed diminished survival of skin fibroblasts in culture after x-irradiation. Another such case is under study.

Study was also made of a family in which four siblings and three maternal relatives had acute myelogenous leukemia, and two other maternal relatives had malignant reticuloendotheliosis [30]. The occurrence of AML in the most recent sibling affected seemed to be predicted by increased transformation of skin fibroblasts in culture by SV40 seven years before the onset of leukemia [20]. This response was similar to that seen in Fanconi's anemia (FA) or in heterozygotes for the disease, but no stigmata of FA was observed in the family. The available skin fibroblasts, from two of the affected siblings and the mother, showed diminished cell survival, but those from healthy twin brothers and the father did not [27]. The cells are now being studied for DNA repair defects. These observations illustrate once again the importance of an interaction among epidemiology, clinical observations and laboratory research.

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Chromosome Abnormalities in Leukemia

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A. Introduction

The renewed interest in the study of chromosome abnormalities in hematologic malignancies, particularly in the leukemias, is the result of technical improvements which permit the precise identification of each human chromosome, and of parts of chromosomes as well. The information obtained raises a number of questions regarding the validity of older notions, such as the variability of the chromosome pattern (karyotype) in acute leukemia, or the rarity of associations of specific chromosome abnormalities with particular types of leukemia. One of the surprising observations of the last few years has been the frequent occurrence of consistent translocations in a variety of hematologic malignancies. The challenging questions at present are how and why nonrandom changes, particularly consistent translocations, occur.

B. Methods

An analysis of chromosome patterns in malignancy must be based on a study of the karyotype of the tumor tissue itself. In the case of leukemia, the specimen is usually a bone marrow aspirate that is processed immediately or is cultured for a short time [29]. Cells in metaphase from a 24-hour culture of peripheral blood will have a karyotype similar to that of cells obtained from the bone marrow. The chromosome analysis may be performed by means of one of several pretreatments prior to staining with Giemsa [34], or the slides can be stained with quinacrine mustard for fluorescence, as previously described [3,29]. The chromosomes are identified according to the Paris Nomenclature [22], and the karyotypes are expressed as recommended under this system.

C. Chronic Myelogenous Leukemia

I. Chronic Phase

Nowell and Hungerford [20] reported the first consistent chromosome abnormality in a human cancer; they observed an unusually small G-group chromosome, called the Philadelphia (Ph^1) chromosome, in leukemic cells from patients with chronic myelogenous leukemia (CML). Bone marrow cells from approximately 85% of patients who have clinically typical CML

contain the Ph^1 chromosome (Ph^1+) [38]. Chromosomes obtained from PHA-stimulated lymphocytes of patients with Ph^1+ CML usually are normal.

Chromosome banding techniques were first used in the cytogenetic study of leukemia for identification of the Ph^1 chromosome. Caspersson et al. [2] and O'Riordan et al. [21] reported independently that the Ph^1 chromosome was a No. 22q –. The question of the nature of this chromosome was answered in 1973, when Rowley [24] reported that it represents a translocation, rather than a deletion as many investigators had previously assumed. The first report in 1973 presented data on nine Ph^1 patients, in all of whom there was additional dully fluorescing chromosome material at the end of the long arm of one No. 9 (9q+). The amount and staining characteristics of this material were similar to those of the distal portion of the long arm of No. 22. The abnormality in CML is, therefore, an apparently balanced reciprocal translocation, t (9;22) (q34;q11). Measurements of the DNA content of the affected pairs (9 and 22) have shown that the amount of DNA added to No. 9 is equal to that missing from the Ph^1 [14]; thus there is no detectable loss of DNA in this chromosome rearrangement.

The karyotypes of 569 Ph^1+ patients with CML have been examined with banding techniques by a number of investigators, and the 9; 22 translocation has been identified in 529 cases (94%) (reviewed in Rowley [27]). Unusual or complex translocations were identified in 40 patients, in 17 of whom the translocation involved No. 22 and one of several other chromosomes. In two patients, the translocated material could not be detected and was presumed to be missing. Twenty-one cases have also been reported in which the rearrangement involved three or more chromosomes; in all of these cases, with one exception [13], two of the chromosomes were Nos. 9 and 22 with breaks in the usual bands.

The great specificity of the translocation involving Nos. 9 and 22 remains an enigma. At present, patients with a variant translocation appear not to differ clinically from those with the usual Ph^1 [32].

II. Acute Phase

When patients with CML enter the terminal acute phase, about 20% appear to retain the 46, Ph^1+ cell line unchanged, whereas other chromosome abnormalities are superimposed on the Ph^1+ cell line in 80% of patients [27,28]. In a number of cases, the change in the karyotype preceded the clinical signs of blast crisis by 2–4 months.

Bone marrow chromosomes from 178 patients with Ph^1+ CML, who were in the acute phase, have been analyzed with banding techniques [27,28].

Thirty-five showed no change in their karyotype, whereas 143 patients had additional chromosome abnormalities. The most frequent gains or structural rearrangements of particular chromosomes observed in 136 patients who underwent relatively complete analyses are summarized in Table 1. These changes frequently occur in combination to produce modal numbers of 47 to 52.

Table 1. The most frequent chromosome changes determined with banding in 136 Ph[1]-positive patients in the acute phase of CML

Type of chromosome change	Number of chromosome						
	8	9	10	17	19	21	Ph[1]
	Number of patients						
Gain	53	8	11	8	24	11	48
Loss				5	1		2
Rearrangement[a]	3	4	3	36[b]	2	1	4[c]
Total	56	12	14	49	27	12	54

[a] Includes translocations, deletions, inversions, and ring chromosomes.
[b] Thirty of these were said to be an isochromosome for the long arm, i (17q)
[c] All of these were said to be a dicentric Ph[1].

The single most common change in the acute phase of CML is the addition of a second Ph[1] chromosome. Prior to the use of banding, the most commonly observed abnormality was an additional C-group chromosome; of 64 patients whose cells contained additional C's, 53 had an additional 8.

The i(17)q, which was observed in 30 patients, appears to be the second most common structural rearrangement, after the 9;22 translocation. It was the only abnormality in addition to (9;22) in 16 cases, whereas in 14 it was associated with an extra C, identified as No. 8 in every patient. Fifty-one other structural rearrangements, such as balanced reciprocal translocations, deletions, and unidentified additions to chromosomes, were identified in combination with i(17q) and the dicentric Ph[1]. In 13 cases, a second balanced reciprocal translocation (separate from the 9;22 translocation) was the only change noted in the acute phase as compared with the karyotype in the chronic phase. With one exception, the additional F noted in 25 cases was a No. 19; it was never seen as the only new abnormality in the acute phase of CML.

III. Identity of Ph[1]-positive Cells

The identity of the cells that contain the Ph[1] chromosome has recently become a topic of considerable interest. This problem has at least two facets; one concerns the nature of the blast cells in the acute phase of CML and the other, the proper classification of patients with Ph[1]+ acute leukemia.

In regard to the first aspect, Boggs [1] noted that the blast cells in some patients in the acute phase of CML appeared to be lymphoid rather than myeloid, and that some patients in the acute phase achieved a remission with vincristine and prednisone, which were usually effective primarily in lymphoid leukemias. Several laboratories are currently examining the surface markers of cells from patients in the acute phase of CML; unfortunately, the cytogenetic analyses are frequently not done with banding techniques, and often the karyotype is obtained only from the initial sample. Since Whang-Peng et al. [39] have identified the Ph[1] chromosome in two of four Ph[1]+ ALL patients as a 21q –, banding is essential.

In regard to the second question, of 13 patients [26] with Ph^1+ ALL who had a 22q – chromosome identified with banding, six had a translocation of 22q to 9q34; two others had variant translocations, one to 14q and one to 21q. The presence of a translocation was not determined for the other five. Ten of the 13 patients were studied a second time; two of these had no remission and continued to have an abnormal karyotype. The remaining eight patients achieved a remission and had a normal karyotype in cells from the bone marrow or from unstimulated peripheral blood. It remains to be determined whether it is logical, or correct, to classify all Ph^1+ leukemias as CML, or whether we are dealing with two different diseases.

D. Acute Nonlymphocytic Leukemia (ANLL)

I. Nonrandom Patterns

Little information is available regarding the chromosome pattern determined with banding in acute lymphocytic leukemia; therefore this section includes the data available for ANLL only. Cells from approximately 216 patients with ANLL have been analyzed with banding; 113 patients (51%) had a chromosome abnormality, which was identified precisely in 100 [26,36]. The chromosome gains, losses, and rearrangements are summarized in Fig. 1. There is evidence that some portion of the apparent chromosome variability is related to evolution of the karyotype in ANLL. In an attempt to distinguish primary from secondary events, we have indicated the aberrations noted in 90 patients who had minimal changes, i.e., modal chromosome numbers of 45–47, in the shaded area of the figure. Although a gain of No. 8 and a loss of No. 7 are the most frequent changes in either case, other aberrations, such as a gain of Nos. 1, 6, or 7, are seen only in patients with higher modal numbers.

In some patients, it is possible to follow the development of other chromosome changes in the course of serial analyses of bone marrow samples. In a series of 90 patients with ANLL [35], 17 showed a change in their karyotype as the disease progressed. In 11 patients, this involved the gain of a chromosome, which was a No. 8 in nine cases. Thus, an additional No. 8 is a common occurrence both in the evolution of ANLL and in CML in the acute phase.

Two structural rearrangements are sufficiently important to merit special mention. The first occurs in acute myeloblastic leukemia (AML) and is seen in about 10% of all patients with aneuploidy. Prior to banding, it was described as $-C,+D,+E,-G$ (37); Rowley [25] showed that this is a translocation, presumably reciprocal, involving Nos. 8 and 21, t(8;21)(q22;q22). This translocation is unique in that its presence is frequently associated with the loss of a sex chromosome, an X in females (33%) and the Y in males (59%); such loss is otherwise a rare occurrence.

The other consistent rearrangement has been identified only recently (30) as a 15;17 translocation, t(15;17)(q25;q22), in acute promyelocytic

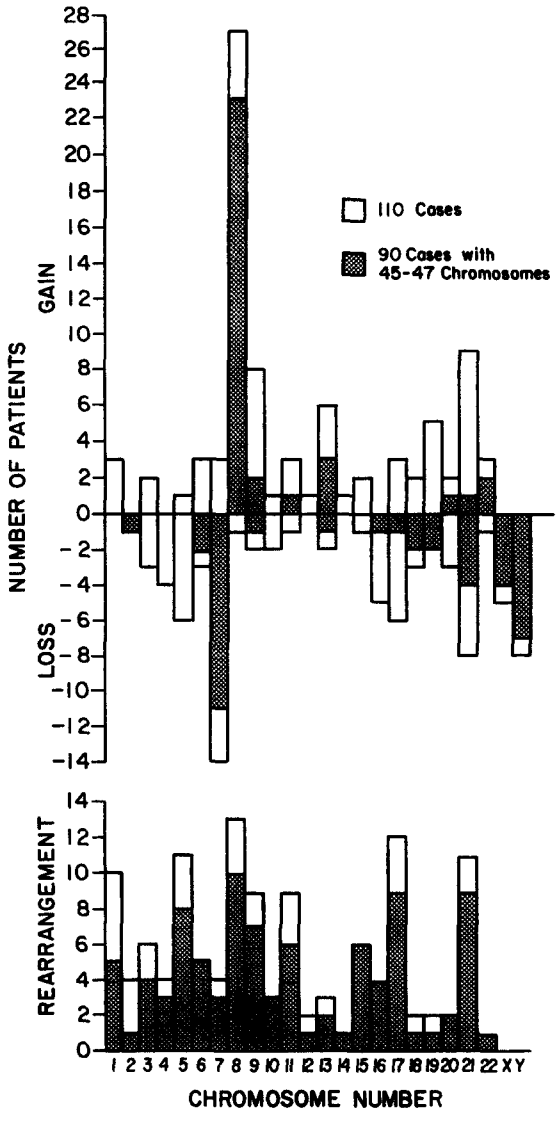

Fig. 1. Diagram of chromosome changes seen in 110 patients with ANLL; 45 patients were studied in my laboratory. The chnages in 90 patients with modal chromosome numbers of 45–47 are indicated in the shaded portion.

leukemia (APL). Our first two patients with APL were found to have a deleted 17q [10]. Metaphase chromosomes from a third patient had clearer bands, and a structural rearrangement involving No. 15 as well as No. 17 was noted. Nine of 17 patients with APL included in data from the Workshop on Chromosomes in Leukemia had a 15; 17 translocation [7].

II. Clinical Significance of Chromosome Abnormalities

About 50% of patients with ANLL are found to have a normal karyotype even with the use of banding techniques. Sakurai and Sandberg [31] were the first to note (prior to banding) that the presence, in the initial bone sample, of even one cell with a normal karyotype was associated with a substantially better prognosis. In our first series of 50 patients studied with banding [11], particularly among those with acute myeloblastic leukemia (AML) who had a normal karyotype, 85% achieved a complete remission (median survival 18 months), compared with 25% of those with only abnormal cells (median survival 2.5 months). The difference in survival for patients with acute myelomonocytic leukemia (AMMoL) was not signif-

icantly related to the karyotype. In an enlarged series of 90 patients, we noted the same relationships [12]. These observations have also been confirmed by Nilsson et al. [19] and by data correlated by the Workshop on Chromosomes in Leukemia [7].

The significance of these findings is not clear. It may be that leukemic cells with a normal karyotype have not yet evolved to the same state of malignancy, and that patients with normal cells therefore have a better prognosis. Alternatively, it may be that the mechanism associated with leukemogenesis in cells with a normal karyotype is different and does not require chromosome changes for the malignant transformation. These patients may, therefore, represent a different etiologic category in which the cells could be more readily reversible or more sensitive to chemotherapy. In any event, hematologists might consider whether chemotherapeutic protocols should be specifically tailored to patients with normal karyotypes, whereas a different protocol may be appropriate for patients who have only chromosomally abnormal cells.

E. The Production of Consistent Translocations

The mechanism for the production of specific, consistent reciprocal translocations is unknown. Possibly, specific translocations are the result of cell selection. In such a model, chromosome breaks and rearrangements occur continuously at a low frequency. Many of these rearrangements do not lead to changes in cell metabolism, and the cells therefore do not proliferate preferentially; other rearrangements may be lethal to the cells. Still others provide the cell with a proliferative advantage, and cells with these changes not only persist, but eventually become the predominant cell type. In such a model, the chromosome change is the fundamental, initial event that leads to the neoplastic nature of the cell.

Other possible explanations depend on either [1] chromosome proximity, since translocations may occur more frequently when two chromosomes are close together, or [2] regions of homologous DNA that might pair preferentially and then be involved in rearrangements. The fact that many of the affected chromosomes, e.g., Nos. 1, 9, 13, 14, 15, 21, and 22, are involved in nucleolar organization supports these proposals.

On the other hand, proximity of homologous DNA sequences should lead to an increased frequenced of these rearrangements in patients with constitutional abnormalities, but this has not been observed. It is possible that either or both of these mechanisms are subject to selection; a translocation might occur because the chromosomes are close together, but only certain specific rearrangements may have a proliferative advantage which results in leukemia and thus leads to their detection.

Another genetic mechanism that may account for consistent chromosome changes is related to transposable elements, called controlling elements in maize [6, 15] and insertion sequences in bacteria [4]. Transposable elements have been detected in every organism in which the genetic structure is known

with reasonable precision. In maize, for example, there are at least three distinct controlling elements, each with its own characteristics and with different chromosome locations that influence the production of anthocyanin pigment in each kernel of an ear of corn [6,15]. Similar genetic systems that modify the action of host genes may be present in mammalian cells. If so, these transposable elements may play a role in malignant transformation. The following features of transposable elements are relevant to the "how" and "why" of consistent translocations: 1. Change in location within the DNA, 2. the transferring of adjacent DNA in this change, and 3. the alteration of the normal mechanism for genetic regulation, depending on the site and orientation of the inserted sequences. These properties, plus a selective system for removal of changes that do not have a proliferative advantage in hematologic cells, are just those required to explain consistent translocations occurring as somatic mutations.

F. The Role of Nonrandom Changes

There is good cytological [8] and biochemical [5] evidence that, in an individual patient with chronic myelogenous leukemia or Burkitt lymphoma, the tumor cells have a clonal origin. In CML, initially only a single cell has the 9;22 translocation, and when the patient comes to the physician, frequently all cells in division contain the Ph^1 chromosome. It is necessary to examine the kinds of genetic mechanisms that can provide the cell containing the 9;22 translocation with this proliferative advantage.

Two points that should be emphasized are the genetic heterogeneity of the human population and the variety of cells involved in malignancy. There is convincing evidence from animal experiments that the genetic constitution of an inbred strain of rats or mice plays a critical role in the frequency and type of malignancies that develop [23,33]. We are much more aware now than formerly of certain genes in man that predispose to cancer, such as the genes for Bloom syndrome, Fanconi anemia, and ataxia-telangiectasia [9]. We are completely ignorant of the number of gene loci in man which, in some way, control resistance or susceptibility to a particular malignancy.

The second factor affecting the karyotypic pattern relates to the different cells that are at risk of becoming malignant, and the varying states of maturation of these cells. There is good evidence that the same chromosomes may be affected in a variety of tumors; No. 8 is a good example [18]. On the other hand, some chromosomes seem to be involved in neoplasia involving a particular tissue; the involvement of No. 14 in lymphoid malignancies is an example.

When one considers the number of nonrandom changes that are seen in a single malignancy such as ANLL, it is clear that not just one gene, but rather a class of genes is involved. Our knowledge of the human gene map [17] has developed concurrently with our understanding of chromosome changes in leukemia. It is now possible to try to correlate the affected chro-

mosomes with the genes that they carry. Clearly, these efforts must be very tentative, since relatively few genes have been mapped, and since some of the chromosomes that are most frequently abnormal have few genetic markers.

Preliminary data suggest that chromosomes which carry genes related to nucleic acid biosynthesis may frequently be abnormal in hematologic malignancies. Moreover, specific chromosome regions associated with these genes may also be involved. Thus, the most frequent abnormalities of No. 17 result either in an isochromosome for the long arm or in a translocation with No. 15 in which the break in No. 17 is in band 17q22. This region of No. 17 contains genes for thymidine kinase, galactokinase, and a site that is particularly vulnerable to AD-12-induced breakage [16]. Furthermore, induction of host cell thymidine kinase and a high frequency of breaks in 17q22 are early functions of this virus, as is the synthesis of a tumor antigen which may play a role in the control of DNA synthesis.

Thus it is possible that nonrandom chromosome aberrations, when they occur, change the level of some enzymes related to nucleic acid metabolism, either through a change in location or through duplication of gene loci. Nonrandom chromosome changes, particularly consistent, specific translocations, now seem clearly to be an important component in the proliferative advantage gained by the mutant cell in neoplasia. The challenge is to decipher the meaning of these changes.

G. Summary

The consistent occurrence of nonrandom chromosome changes in human malignancies suggests that they are not trivial epiphenomena. Whereas we do not understand their significance at present, one possible role which they may fulfill is to provide the chromosomally aberrant cells with a proliferative advantage as the result of alteration in the number or location of genes related to nucleic acid biosynthesis. The proliferative advantage provided by various chromosome aberrations is likely to differ in patients with different genetic constitutions.

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Use of Glucose-6-Phosphate Dehydrogenase Markers to Study Human Myeloproliferative Disorders

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In accordance with inactivation of one X-chromosome in each somatic cell, females heterozygous at the X-chromosome linked glucose-6-phosphate dehydrogenase (G-6-PD) locus for the usual B gene (Gd^B) and a common variant allele such as Gd^A have two populations of cells – one producing type B G-6-PD and the other, type A. Thus, normal tissues from a Gd^B/Gd^A heterozygote manifest both B *and* A isoenzymes (a double-enzyme phenotype), but a tumor with a single cell (clonal) origin shows B *or* A G-6-PD (a single-enzyme phenotype). The same rationale allows delineation of stem-cell relationships. If a tumor arises in a multipotent stem cell of type A, all descendants of that stem cell will type as A.

In this communication G-6-PD studies are reviewed that indicate that chronic myelocytic leukemia and related disorders have clonal origin at the time of study and involve stem cells pluripotent for granulocytes, erythrocytes, platelets and monocytes/macrophages. The questions of whether marrow fibroblasts or blood lymphocytes arise from the leukemia progenitor and of whether there are any residual normal stem cells are emphasized.

I. Chronic Myelocytic Leukemia (CML)

A. Clonal Origin in Pluripotent Marrow Stem Cells

Thus far, 12 women with Philadelphia-chromosome (Ph^1)-positive CML and heterozygous at the G-6-PD locus have been studied. Both B and A isoenzymes were found in normal tissues, but only one type of G-6-PD was seen in the CML granulocytic cells (8 patients typed as B and 4 as A) [8,9]. The fact that single-enzyme phenotypes occur in CML granulocytic cells, whereas granulocytes from G-6-PD heterozygotes without hematopoietic diseases have double-enzyme phenotypes [6], strongly favors a clonal origin of CML. This postulate is also supported by studies with other isoenzyme and chromosomal markers (references given in [9]). However, the conclusion that CML has a clonal origin applies only to the stage of the disease at the time of study. Conceivably, at a very early phase many cells may be affected, but by the time CML is evident one clone has evolved. The fact that at the time of diagnosis all CML cells are of clonal origin virtually ex-

cludes any hypothesis of pathogenesis based on continuous recruitment of hitherto normal cells.

In G-6-PD heterozygotes with CML, single-enzyme phenotypes are found in erythrocytes, platelets and blood monocytes/macrophages as well as in granulocytes [8]. Thus, the disease involves a multipotent marrow stem cell, a conclusion supported by studies with other markers (references given in [7]). In contrast to the blood cells, cultured marrow fibroblasts from 3 patients with CML displayed both B and A enzymes, indicating that at least in these cases, these cells do not arise from the CML clone. Similar conclusions were reached using Ph^1 as a marker [5, 11, 14]. One of the patients we studied had myelofibrosis [8]. The facts that marrow fibroblasts grown from this patient lacked Ph^1 and had a normal double-enzyme phenotype suggest that the myelofibrosis is not part of the CML clonal proliferation and is probably a secondary phenomenon.

B. Do Blood Lymphocytes Arise from the CML Stem Cell?

To investigate the origin of different lymphocyte populations we studied three G-6-PD heterozygotes with CML. The CML myelocytic cells in each patient showed a single type: B.

Simple preparative methods all failed to separate lymphocytes from granulocyte precursors and other immature forms. Thus we adopted complex multistaged preparative protocols. These methods and the procedures used to identify T or B lymphocytes are described in detail elsewhere [10]. The results provide evidence for at least two and possibly three lymphocyte populations.

1. T-lymphocytes which do not Arise from CML Stem Cells
In each of the three patients there is a population of E-rosette forming lymphocytes which has a normal double-enzyme phenotype in marked contrast to the single-enzyme phenotypes found in the CML clones. These T cells were most easily demonstrated when the patient was in complete clinical remission.

2. Non-T lymphocytes which Arise from CML Stem Cells
A population of non-E-rosette forming lymphocytes was identified which had the same single-enzyme phenotype as did the CML clone. This population was demonstrated when the patient was in relapse or in clinical remission. These cells had complement receptors and many of them manifested B-lymphocyte characteristics such as cell-surface and intracytoplasmic Ig, and Ig synthesis.

3. T-lymphocytes which may Arise from CML Stem Cells
Preliminary studies suggest that there may be a population of lymphocytes which has a single-enzyme G-6-PD phenotype and T-cell characteristics including lack of cell-surface Ig and formation of E-rosettes. In contrast to the non-clonal T-cells which were most easily demonstrated in clinical remission, these "clonal" "T"-cells have thus far been demonstrated only when the disease is in relapse. However, conclusions based on these find-

ings must be guarded. For example, since the disease was active at the time of study, the difficulties of isolating and characterizing lymphocyte populations were increased. In addition, it cannot be concluded firmly that this clonal subpopulation is analogous to normal T-lymphocytes since it may consist of B-lymphocytes or undifferentiated cells of the leukemic clone which have acquired T-cell markers during evolution of leukemia.

The likely demonstration in CML of clonal lymphocytes suggests that there is a common hematopoietic stem cell for some lymphocytes as well as for myelogenous cells and it is this stem cell which is involved in the leukemia. These data may explain why in some cases of blast crisis, the cells have characteristics which resemble those found in the common type of acute lymphoblastic leukemia (e.g., see Chapters by Boggs and Greaves, this volume). The demonstration of E-rosetting cells from patients with CML in remission that do not arise from the leukemia stem cell may reflect persistence of restricted stem cells committed to differentiate only into T-lymphocytes.

Mitogen-stimulated mitoses within the lymphocytes having single-enzyme phenotypes and thereby presumably arising from the CML stem cells generally lacked Ph^1. One possibility is that the cells in metaphase are not representative of the vast majority of enzyme-producing cells, but a more intriguing possibility is that cells which are clonally derived only acquire Ph^1 at a later stage in leukemogenesis (see below).

C. Are there any Residual Normal Stem Cells in CML?

The fact that during remission in CML, the single-enzyme G-6-PD phenotypes persist provides no evidence for residual normal stem cells [8]. However, it was possible that a minor isoenzyme component had been missed if it had contributed less than 5% of the total G-6-PD activity. To study this problem at a more sensitive level, we analyzed granulocytic colonies grown in semi-solid medium. Such colonies from normal G-6-PD heterozygotes have single-enzyme phenotypes and arise from single cells. Thus, analysis of a single colony is equivalent to study of the one progenitor cell from which it was derived. Of almost 1000 granulocytic colonies studied, one colony had a G-6-PD phenotype different from that observed in the CML blood clone [9]. These data provide no evidence for residual, normal granulocyte colony-forming cells (CFU-C) in patients with CML, a situation which contrasts with that found in polycythemia vera (see below).

On the other hand, some studies using Ph^1 as a marker do suggest persistence of normal stem cells. For example, the presence of some normal stem cells in CML was suggested by the observation of Ph^1-negative granulocytic colonies in 3 of 5 patients in one study [3]. (However, other investigators have found such colonies to be uniformly Ph^1-positive [2,15,17]). Further evidence favoring persistence of some Ph^1-negative cells in CML derives from the appearance of such cells in patients treated with cycle-active intensive therapy [4]. How are these chromosome observations suggesting persistence of normal stem cells in CML reconciled with the failure

using G-6-PD as a marker to detect granulocytic colonies arising from non-CML progenitors? One possibility is that only some patients have Ph^1-negative CFU-C, but not the ones we studied. Alternatively, there may be some Ph^1-negative CFU-C in all patients. If this hypothesis is correct, then our failure using G-6-PD to find evidence of stem cells that do not derive from the CML clone suggests that cells which are clonally derived acquire Ph^1 at a later stage in leukemogenesis. According to this hypothesis, CML would be a multi-staged disease. As indicated above, the possibility that some clonally derived lymphocytes lack Ph^1 would be in accord with this hypothesis.

II. Polycythemia Vera and Myeloid Metaplasia with Myelofibrosis

A. Clonal Origin in Pluripotent Marrow Stem Cells

Studies of two G-6-PD heterozygotes with polycythemia vera [1] and two with agnogenic myeloid metaplasia with myelofibrosis [12, 13] indicate that at least in the patients investigated, the disorders involve multipotent hematopoietic stem cells and suggest that at the time of study, the diseases have a clonal origin. According to some theories of pathogenesis, polycythemia vera and agnogenic myeloid metaplasia result from proliferation of normal stem cells in response to unknown myeloproliferative stimuli. The G-6-PD data do not support these hypotheses and are more compatible with neoplastic origin.

B. Are there any Normal Stem Cells in Polycythemia Vera?

As described in detail elsewhere in this volume (see Chapter by Adamson), in contrast to CML, analyses of granulocytic and erythroid colonies from patients with polycythemia vera indicate that there are stem cells which do not arise from the polycythemia vera clone detected in the blood and therefore are presumably residual normal stem cells [16]. However, these cells are demonstrable in vitro only in the presence of erythrocyte or granulocyte stimulating factors. Thus, although there are normal stem cells in patients with polycythemia vera, their expression is suppressed in vivo. This contrast between polycythemia vera and CML suggests a basic difference in the regulatory abnormalities in the two disorders although they may involve the same or a similar multipotent hematopoietic stem cell.

C. Myelofibrosis in Agnogenic Myeloid Metaplasia

The factors underlying the marrow fibrosis, the predominant clinical feature in this disease, are unknown. Many workers feel that it is part of the same process as that which affects the myeloid cells. Our results suggest that this hypothesis, which predicts finding the same single-enzyme phenotype in the marrow fibroblasts as the one observed in the blood cells, is not correct.

In the G-6-PD heterozygote with agnogenic myeloid metaplasia who had equal amounts of B and A isoenzymes in cultured skin fibroblasts and only type A in blood cells, both B and A isoenzymes in equal proportions were found in cultured marrow fibroblasts [12]. This patient also had a distinctive chromosome abnormality (47, XX, +8) in the blood cells which was not detected in a single marrow fibroblast. These G-6-PD and cytogenetic findings strongly suggest that the marrow fibrosis in this patient was not part of the basic process which led to clonal proliferation of hematopoietic stem cells and that the myelofibrosis was a secondary abnormality. Similar conclusions were reached from chromosome studies of a patient with *acute* myelofibrosis [18].

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Histopathology of Bone Marrow in Human Chronic Leukemias

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Chronic myelogenous leukemias have arisen a considerable new interest, since lymphoblastic crises recently turned out to complicate and terminate a primary myeloproliferative disease (Beard et al., 1976; Janossy et al., 1977; Rosenthal et al., 1977). Ways of stem cell differentiation (Metcalf, 1973, 1974, 1977; Boggs, 1974; Moore, 1976; Greenberg, 1976, 1978) as well as biological markers of leukemic cells (Fialkow et al., 1977, 1978a, b) give sufficient proof for the suggestion that hematopoietic stem cells have been primarily involved in this disease.

The morphology of leukemias is based on histopathology, cytochemistry and electron microscopy, whereas gross pathology is of lesser importance for their determination. There is a large variety of subgroups from chronic leukemia – CML – which may be classified comprehensivly only by the histopathology of biopsies from bone marrow cores. Thus it is not clearly known which pathogenetic relationships are existing between the numerous subgroups, as erythro- and megakaryocytic leukemias for instance, and how they are related to each other and to CML. Moreover the pathways are not sufficiently understood by which CML and its subgroups or other myeloproliferative diseases, as Polycythemia vera, terminate into final stages of myelofibrosis and myeloid metaplasia (Gralnick et al., 1971; Ward and Block, 1971; Buyssens and Bourgeois, 1977).

A more detailed morphology of chronic myelogenous diseases ought to bring up new insights to these basic questions, provided histopathology is supported by electron microscopy (Thiele et al., 1977a, b, c), chromosome analysis (Sandberg and Hossfeld, 1970; Rowley, 1976, 1978) and completed with results of enzyme markers (McCaffrey et al., 1975; Gallo, 1975) and membrane phenotypes (Greaves, 1975) and compared with clinical findings.

The acute leukemias, however, can be characterized according to their cellular composition by methods of membrane (Janossy et al., 1977; Catovsky and Galton, 1977; Catovsky et al., 1978; Gordon and Hubbard, 1978) and enzyme markers (Hoffbrand et al., 1977; Mertelsmann et al., 1978) alone with more reliability than morphological methods are able to do. Even the enzyme cytochemistry (Leder, 1975; Bennett and Reed, 1975; Bennett et al., 1976; Löffler, 1978; Schmalzl et al., 1977) electron microscopy (Bessis, 1973, , 1975) and chromosome analysis (Golomb et al., 1976; Alimena et al., 1977) are only of a supporting value to characterize acute leukemias but do not offer a final diagnostic clue.

For these reasons this study will be restricted to the histopathology of biopsies from bone marrow cores in chronic myelogenous leukemias and other myeloproliferative diseases and is supported by findings from chromosome analysis and by former results of electron microscopy as well as clinical findings in these same patients.

Material and Methods

Results of this study are based on core biopsies of bone marrow from the anterior iliac crest by the method of Burkhardt (1966a, b) and Georgii and Thiele (1976) of the posterior iliac crest by the method of Jamshidi and Swaim (1971). Resin embedding was done using methacrylate, semi-thin sections of 3 μ were stained with the usual procedures of hematopathology as elsewhere reported (Georgii and Thiele, 1976; Vykoupil et al., 1976). Electron microscopy by thin section and freeze-fracture techniques was formerly described (Georgii and Thiele, 1976) as have been methods of chromosome analysis in short term cultures from bone marrow cells which were obtained from the puncture sites after withdrawing the core of the biopsy (Krmpotic et al., 1968; Golomb et al., 1976). All biopsies were done before any therapy and selected from routine samplings out of a pool of 7,000 patients, among which, 718 cases with chronic leukemias or myeloproliferative diseases and 190 with acute leukemias were found.

Results

The own material shows a prevalence of chronic to acute diseases in a relation of 3 : 1, which is another reason to restrict this study to chronic myeloproliferative disorders. These disorders can be classified into 9 subtypes including the Polycythemia vera and unclassifiable entities (Table 1).

Table 1. Distribution of chronic myeloproliferative disorders in a total of 718 patients found in 7,000 sequential biopsies of bone marrow: There is a conspicuous group of myeloses with mixed cellularity, so called chronic megakaryocytic granulocytic myelosis – CMGM

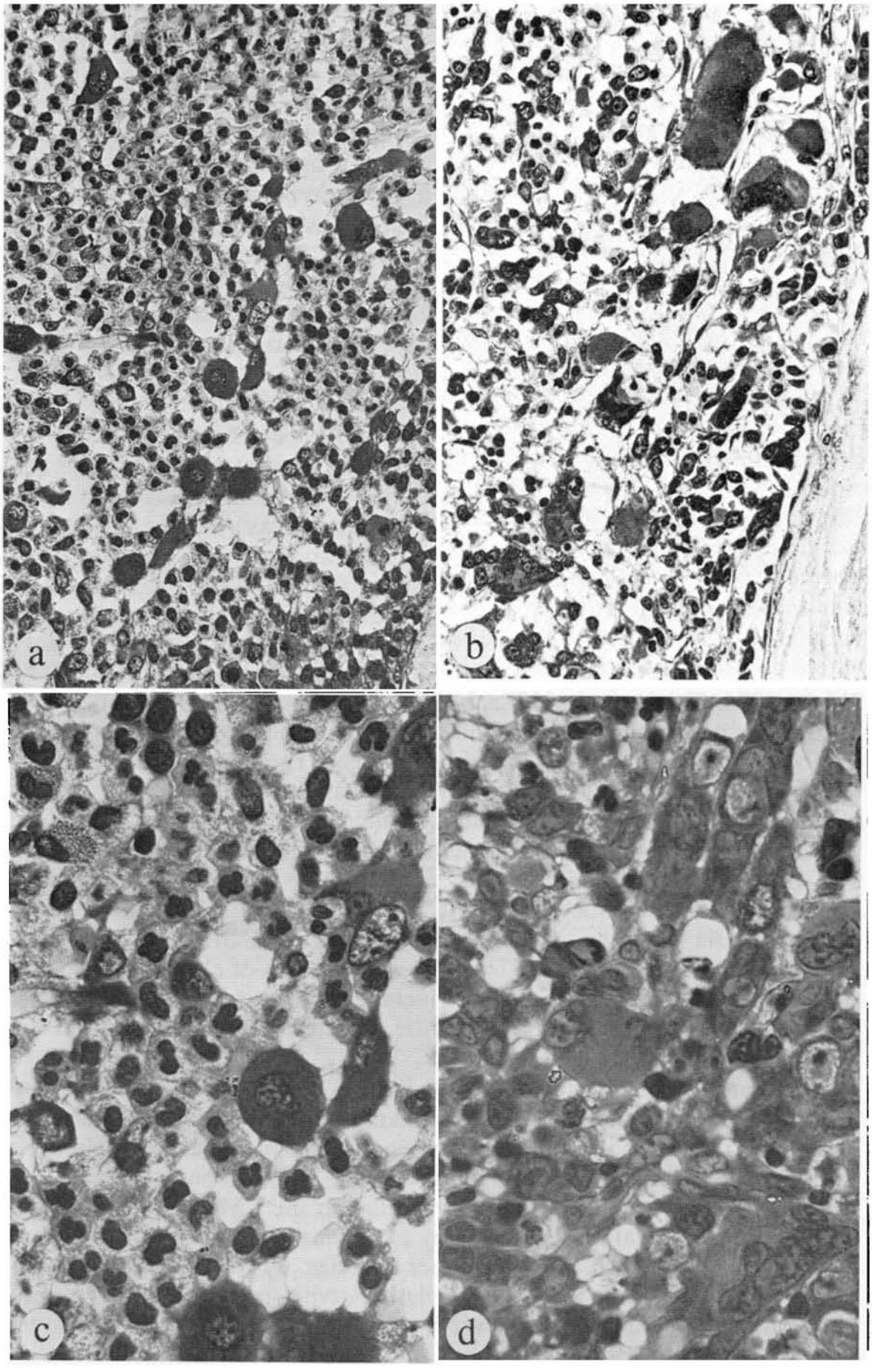
Chronic leukemias		n	%
Chronic granulocytic leukemia – CGL –		133	19
Smouldering leukemia		5	1
Mixed myeloses	– CMGM Ia. II –	187	26
Myelofibroses	– CMGM IIIa. IV –	238	33
Primary myelofibroses		14	2
Thrombocythemia		11	2
Myelo-monocytic leukemia		3	0
Polycythemia vera		65	9
Unclassified		62	9
Total		718	100

Among chronic myelogenous leukemias – CML – the chronic granulocytic leukemias – CGL – are those with a neoplastic proliferation of granulopoiesis. This atypical granulopoiesis is gradually replacing the fat tissue of the bone marrow space (Fig. 1a). The erythro- and megakaryopoiesis are not seriously reduced, but left in a sufficient amount, which is in contrast to the acute leukemias of ALL, AML or ANLL-type. The cellular differentiations of granulopoiesis is fairly preserved whereas precursors as promyelocytes and myelocytes are increased. Myeloblasts are not proliferating in remarkable amounts and their increase points towards a beginning transformation into a blastic crisis. Following this description the CGL may be defined as a neoplastic growth of only one cell lineage, while the other 2 or 3 lines remain at least superficially unchanged or may turn into a reactive hyperplasia of megakaryocytes in rare cases. This subgroup of typical CGL's amounts to 19% in our series of 718 patients (Table 1). From the CGL the group of myeloses should be distinguished which are characterized by an additional neoplastic proliferation of the megakaryopoiesis, thus resulting in a mixed cellularity from 2 cell lineages involved. The histology shows a remarkable numerical increase of megakaryocytes and their precursors (Fig. 1b). These cells are atypical with enlarged pleomorphic, non-pyknotic nuclei which differ from typical megakaryocytes even in CGL (Figs. 1c, d). The immature nuclei in a well developed mature cytoplasm cause a dissociation of the differentiation as formerly demonstrated in ultrastructural studies of bone marrow biopsies (Georgii and Thiele, 1976; Thiele et al., 1977a, b). The granulopoiesis is altered likewise, but mostly not to the same extend as in typical CGL. The erythropoiesis can be increased in some cases of early stages. The marrow mesenchyme with sinuses and reticulin fibers remains unchanged during the early stages of development, which was designated as stage I (Fig. 1b).

Based on the neoplasia of two cell lineages which do proliferate in a slow and fairly well differentiated way, we have called this entity a "chronic megakaryocytic granulocytic myelosis" – CMGM – in contrast to the one-line neoplasia "chronic granulocytic leukemia" – CGL – (Georgii and Thiele, 1976; Thiele et al., 1977b, c). The usual term of chronic myelogenous leukemia – CML – may probably include both entities the CGL and the CMGM too; since the latter can only be detected by a core biopsy of the bone marrow.

There are 2 other myeloproliferative diseases that should be strictly distinguished from this entity CMGM: Polycythemia vera and Idiopathic Thrombocythemia. The latter is a one cell line neoplasia of megakaryopoiesis while the other lines show an inconspicuous growth. The histopathology of bone marrow in Polycythemia vera is very similar to the CMGM, in spite of its very different clinical findings. Only by hyperplasia of erythropoiesis and of sinuses, which are increased in number and size, by the complete absence of stainable iron in histocytes and a lesser atypia in granulo- and megakaryopoiesis the experienced observer may reach the diagnosis of Polycythemia vera in non-treated cases.

The pathogenesis of CMGM may be observed by repeated biopsies dur-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ing the course of this disease. An increase of fine reticulin fibers in a discrete patchy pattern may be found by investigating silver stained slides in the polarizing light microscope. With an increasing number of reticulin fibers the disease is classified as stage II of CMGM; this shows a slow progress which may take many months, sometimes exceeding one year and more (Fig. 2a). This stage continues to proceed into a diffuse reticulin lattice which is extended all over the marrow space, polymerizing into collagen bundles which represent the onset of fibrosclerosis; this is defined as stage III of CMGM. The stage IV finally includes an additional endophytic growth of bone trabeculi while the reticulin changes into complete fibrosclerosis. Even in these final stages the neoplastic proliferation of megakaryocytes and granulocytes can be seen, which is, in regard to the proliferating cells, still similar to stages I and II (Fig. 2d).

Lymphonoduli aggregati consisting of loose assemblies of small lymphocytes, mostly localized in the centers of the marrow space and not at paratrabecular sites, can be found frequently in these CMGM's of the first 3 stages (Fig. 2a). These lymphonoduli are rare in the final stage IV of CMGM and very rare in pure CML's.

Chromosome Analysis

In these studies the Ph^1-chromosome was found in 75% (15 from 20) of CGLs and in about 70% (15 from 23) CMGM's of the stage I and II (Table 2). The myelofibroses and osteomyelofibroses, which are classified as CMGM III and IV, do show the same frequency as CGL's, i.e. 75% (15 from 20). The technique of Giemsa-banding displays a 9:22 translocation as the most frequent aberration. Besides there are some minor anomalies as aneuploidy and breaks without clonal evolution. The primary idiopathic myelofibrosis and Polycythemia vera were found to lack a Ph^1-chromosome with one exception in Polycythemia vera (Table 2).

Clinical Findings

The CGL differs from CMGM in clinical finding especially of the cell counts from the peripheral blood (Table 3). The values of leukocytes in CGL's are

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Fig. 1a–d
a Chronic granulocytic leukemia – CGL – well differentiated granulopoiesis and normal looking but increased megakaryocytes. **b** Chronic megakaryocytic granulocytic myelosis – CMGM – megakaryocytes are very increased, display severe atypias and are dislocated from their usual sites to paratrabecular areas, closely to the terminal sinuses. **c** CGL, higher magnification from Fig. a, neutrophil granulocytes metamyelocytes and eosinophils can be seen. Megakaryocytes do not show striking alterations. **d** CMGM with severe polymorphism of megakaryocytes and precursors; they are enlarged in size and abnormal (compare with same magnification as Fig. 1c)
Magnifications: a, b ×350; c, d ×875. Staining: Giemsa

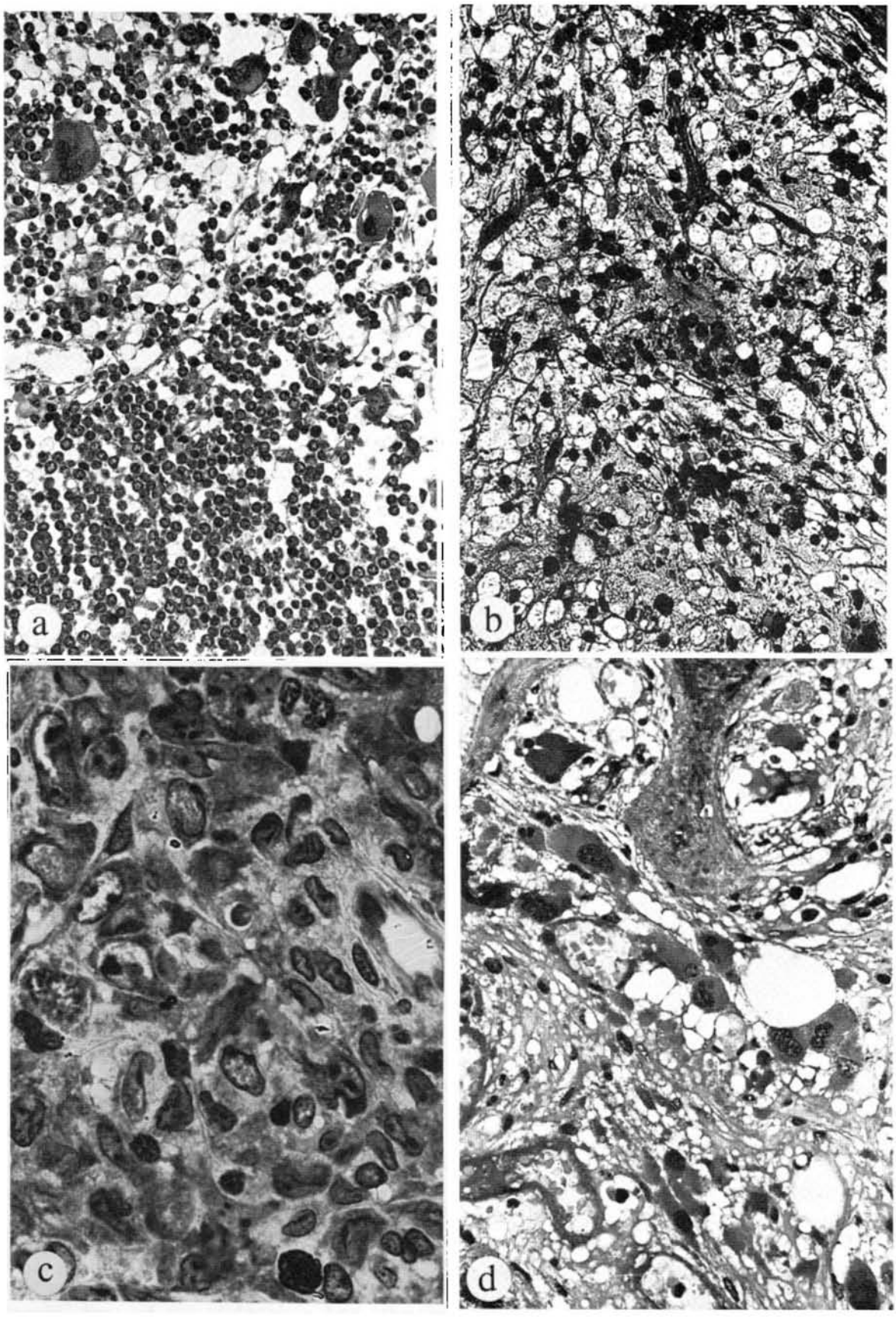
a
b
c
d

Table 2. The Philadelphia-chromosome in chronic granulocytic leukemia (CGL) compared with chronic megakaryocytic granulocytic myelosis (CMGM), primary myelofibroses, and polycythemia vera in a total of 74 patients

Histology	Ph^1-positive	Structural anomalies	Aneuploidy	No changes
Chronic granulocytic leukemia	15/20	1/20	1/20	3/20
Mixed myeloses (i.g. CMGM Ia. II)	15/23	1/23	2/23	5/23
Polycythemia vera	1/10	0/10	8/10	1/10
Myelofibroses (i.g. CMGM IIIa. IV)	15/20	0/20	0/20	5/20
Primary myelofibroses	0/1	0/1	1/1	0/1

significantly higher than in CMGM of stage I and II, and even compared with stages III and IV which resemble myelofibrosis and osteomyelofibrosis. The thrombocytes seem to be conspicuously increased, but this is not significant statistically. The leukocytic alkaline phosphatase is elevated in CMGM's compared with CGL's, which seems to be important. – There is a significant difference of the mean age from CGL to CMGM patients but not among the CMGM's themselves. The prolonged prediagnostic time points to an insiduous onset and retarded natural course of CMGM's which must be ascertained by further clinical studies.

The difference of some clinical complications may be understood from the varying cell counts in the peripheral blood and by the divergent cellular growth shown by histopathology of the bone marrow. Bleedings to a severe and sometimes letal extent were observed in CGL's only – 6/24 – whereas they are missed in all CMGM's – 0/47 –. A thrombotic diathesis was found in CMGM's stage I and II only – 5/24 – while stages III and IV – 0/23 – as the pure CGL – 0/24 – were free of this complication.

Blastic crisis of CMGM (see Fig. 2c) can occur during all of their four stages but they are much more frequent in pure CGL's: in a mean of 13,7%, i.e. = 41/298 patients – of all stages from CMGM compared to 48% – 55/115 – in CGL patients blastic crises were observed.

Fig. 2a–d
a CMGM, this is stage II with slight, scattered increase of reticulin fibers, which can be detected even in Giemsa staining by the widening of sinuses. Noticeable are the lymphocytes forming a nodular infiltrate (lower half of the illustration). **b** Idiopathic, primary myelofibrosis with hyperplastic erythropoiesis with maturing arrest, but no hyper – or neoplasia of granulo – or megakaryopoiesis. This should not be termed a myeloproliferative disorder. **c** Blastic crisis of CMGM with precursors of megakaryocytes and to a lesser amount of granulopoiesis. **d** CMGM stage IV, usually called osteomyelofibrosis with newly formed bone trabecula, fibrosclerosis, widened sinuses, atypical megakaryocytes, there are rests of erythropoiesis and almost no granulopoiesis is shown in this area.
Magnifications: a, b, d ×350; c ×875. Staining: Giemsa

Table 3. Values of cell counts from peripheral blood compared with histopathology of bone marrow from CGL's and myeloses of mixed cellularity, chronic granulocytic megakaryocytic myelosis (CMGM); also age of patients and time between beginning of clinical symptoms and diagnostic biopsy are compared

	CGL	CMGM Stage I	CMGM Stage I a. II	CMGM IIIa. IV i.g. Myelofibrosis
Leukocytes				
mean	160	26.6	25.3	15.5
range	8.6–780	1.1–270	1.1–270	1.3–77
n	24	32	75	76
Thrombocytes				
mean	364.5	762	551	241
range	45–986	29–3000	8–3000	1.7–1321
n	24	30	64	68
Erythrocytes				
mean	4.26	4.32	4.16	3.28
range	2.2–5.3	1.4–6.5	1.4–7.1	1.6–5.7
n	24	29	70	63
Alkal. phosphat. in leukocytes				
mean	24.9	121	144	116
range	0–188	3–343	0–360	0–393
n	21	23	49	37
Age				
mean	53.1		63.4	60
range	15–84		26–85	25–84
n	79		87	91
Time – month				
mean	17.4		29.1	38.4
range	1–74		1–146	1–264
n	24		63	64

Discussion

The myeloproliferative disease described here as a chronic megakaryocytic granulocytic myelosis – CMGM – has been known for a long time under the term of a leukemic megakaryocytic myelosis or leukemia and was described mainly in case reports (Rappaport, 1966, cit. in Georgii and Vykoupil, 1972, 1976). The majority of these cases were only detected in acute blastic phases and therefore most frequently counted among acute leukemias as could be shown by Bain et al. (1977). Modern techniques of obtaining the biopsies and processing the bone marrow with semithin section have changed this opinion. The chronic diseases are very frequent (Table 1). We conclude from our personal experience that most acute looking cases are actually blastic crisis of chronic diseases. Real acute megakaryocytic myeloses are extremely rare, if they do exist anyway. Furthermore the semithin sections as well as electron microscopy have shown that granulopoiesis is also involved in this myeloproliferation.

The demonstration of Philadelphia chromosomes in metaphases from bone marrow cells implies a specific aberration associated with chronic myelogenous leukemia – CML – (Rowley, 1976). In about 90% of patients the Ph^1-chromosome should be found, which is the result of a 9:22 translocation in over 90%, as Dr. Rowley has stated during this meeting. The chromosome analysis from these patients substantiate the pathogenetic relationship between CMGM and CGL. Indeed, this shows remarkably fewer Ph^1-positive patients in our material compared with standard findings. This result may probably be explained by our method of obtaining the specimens: this was done after extracting the core from the site of biopsy and not be squeezing the core itself. But the corresponding values of this marker chromosome between the CGL and CMGM-groups point toward the same pathogenetic pathway.

The terms myeloid metaplasia, myelofibrosis and osteomyelosclerosis (reviews by Gralnick et al., 1971; Rappaport, 1966; Ward and Block, 1971; Burkhardt, 1971; Lennert et al., 1975) and also the one of agnogenic myeloid metaplasia (Ward and Block, 1971) are enclosed in our conception of stages III and IV from CMGM (Fig. 2d). The terms hyperplastic panmyelopathy (K. Rohr, 1960, quoted from Lennert et al., 1975), panmyelopathy (Fischer and Schäfer, 1971) or panhyperplasia (Ward and Block, 1971) is covered by our definitions of stages I and II of CMGM (Figs. 1b, 2a). – However, here is no space to enter into a more detailed discussion concerning the relationships between myeloproliferative diseases with a secondary on the one and the so called primary myelofibrosis on the other side (Fig. 2d, versus 2b). If our suggestion is correct that there is a specific pathogenic pathway leading from chronic myelogenous diseases with mixed cellularity, i.e. CMGM, to myelofibrosis then it is only reasonable to summarize and head these terms under the four stages of one disease – CMGM.

In addition clinical findings extend and confirm our morphological results mentioned above. There are different courses and different complications of both these entities: there is a faster and inevidently leukemic course in CGL complicated by bleedings and blastic crises, not usually by myelofibroses. In CMGM there is a slow, inapparent, often a- or subleukemic course which can be complicated by thromboses and even by blastic crises, and is always terminated by myelofibroses. It is of great interest to investigate whether the lymphoid blastic crises are related to the CMGM's since histopathology frequently shows lymphonoduli in the bone marrow, while this can not be detected in CGLs.

Summary

Among the patients with chronic myeloproliferative diseases including clinical symptoms of chronic myelogenous leukemia – CML – two varying compartments with substantially differing histology of hemopoiesis were found: one with predominating granulopoiesis for which the usual term of

chronic granulocytic leukemia – CGL – seems adequate. The other with proliferation of granulopoiesis and megakaryopoiesis as a neoplasia with a mixed cellularity is observed to be different in its clinical course: there are often a leukemic or subleukemic cell counts, but mostly considerable increased platelets in the peripheral blood; there is a prolonged period of latency, a higher age group, an infrequent occurrence of blastic crisis and a regular outcome into myelofibrosis. This entity of chronic megakaryocytic granulocytic myelosis – CMGM – can be seen very frequently among myeloproliferative diseases. Among a total of 718 core biopsies from the bone marrow the CMGM-patients are up to 29% compared with 21% of the typical one-cell-line disease CGL. The Ph^1-chromosome may be presented in the CMGM-entity likewise.

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Bone Marrow Transplantation in Acute Leukemia: Current Status and Future Directions[1]

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Introduction

Acute leukemia is a neoplastic disease characterized by the abnormal proliferation and accumulation of immature hematopoietic cells. Progress in our understanding of this disease is reviewed in this volume. Significant recent advances in the therapy of acute leukemia include high remission rates in both acute lymphoblastic (ALL) and acute myelogenous leukemia (AML), the prevention of central nervous system leukemia in ALL, and development of moderately effective remission maintenance programs, particularly in ALL. Despite this progress, approximately 50 per cent of patients with ALL and over 95 per cent of those with AML will eventually die of resistant leukemia.

Recent studies at our institution and others have clearly demonstrated the feasibility of transplantation of normal hematopoietic stem cells in man. In view of this, and because of the disappointing results of chemotherapy in patients with acute leukemia who relapse, we studied the potential role of bone marrow transplantation in resistant acute leukemia. In this chapter I will briefly review some basic aspects of the biology and immunology of marrow transplantation and discuss its applicability to leukemia.

Cell Biology and Immunology of Bone Marrow Transplantation

The hematopoietic system is derived from pluripotent stem cells. These cells have several inherent characteristics relevant to marrow transplantation including self-renewal potential, differentiative capacity, and the presence of histocompatibility antigens (HLA) on their surface.

It is clearly possible to transplant hematopoietic stem cells in man. Requirements for engraftment include histocompatibility matching between donor and recipient, immunosuppression to prevent graft rejection, and a critical dose of marrow cells. The latter may relate to the clinical setting under which transplantation is performed rather than an inherent characteristic of stem cell(s) since a single cell may be capable of repopulating a congenitally anemic non-irradiated mouse under appropriate conditions.

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The human major histocompatibility complex (MHC), referred to as HLA, has been assigned to chromosome 6. The HLA locus has been further subdivided into the HLA-A, B, C, and D subloci. The first three are commonly defined by serologic technics while the HLA-D region is conventionally studied in the mixed lymphocyte culture (MLC) test (for review see [1]). Successful marrow transplants in man have been restricted to HLA-identical siblings with few exceptions. While HLA is of prime importance in determining graft outcome, other histocompatibility systems are undoubtedly involved. Little is known regarding these non-HLA systems and no attempt has been made to match for non-HLA antigens in clinical transplantation. This factor probably accounts for the high incidence of graft rejection and graft-versus-host disease (GVHD). There is a high degree of polymorphism in the HLA system. Since these antigens are inherited in a Mendelian fashion as codominant alleles, there is a reasonable possibility (25 per cent) of finding an HLA-identical donor within a family. In the general population the probability is in the range of one in 10.000. Because of this, most transplants have been performed between HLA-identical siblings.

Despite profound hematopoietic suppression, patients with aplasia and acute leukemia are capable of rejecting allogeneic grafts. Immunosuppression is therefore necessary to achieve sustained marrow engraftment. Pretransplant immunosuppression, referred to as *conditioning,* has utilized chemotherapy and radiation either singly or in combination. Since doses used in these regimens are supralethal, *rescue* with normal marrow is essential for survival.

The transplant procedure is relatively simple. Approximately one liter of bone marrow is removed from the donor by aspiration from the posterior iliac crests. A single cell suspension is prepared and infused *intravenously* to the recipient. The infused cells *home* to the marrow after a brief delay in the lungs and spleen. The usual dose is $1-5 \times 10^8$ nucleated marrow cells per kg. In most instances discrete clusters (colonies) of hematopoiesis are observed in the marrow with the first 2 weeks following transplantation [3]. These clusters are usually either erythroid or granulocytic, but mixed populations are occasionally observed. Peripheral white blood cells and platelets begin to rise within 2–3 weeks following transplantation and may return to normal levels by 1–2 months. Cytogenetic and gene marker studies clearly indicate that red cells, granulocytes, lymphocytes, platelets, monocytes, and hepatic and alveolar macrophages are of donor origin [4, 14, 16].

Following successful engraftment, the recipient is at risk to develop several immune-related problems including graft rejection, graft-versus-host disease (GVHD), post-transplant immunodeficiency, interstitial pneumonitis, and infectious complications (Table 1). Recurrent leukemia is an additional potential complication in leukemic recipients.

Graft rejection probably results from histoincompatibility between donor and recipient and may be facilitated by immunization of the recipient via blood transfusions. In some instances defects in the marrow microenvironment may be responsible for graft failure [5]. Graft failure occurs in

Table 1. Areas of investigation

Graft rejection
Resistant leukemia
Graft-vs-host disease
Immunodeficiency
Interstitial pneumonia
Infectious disease complications

20–40 per cent of patients with aplastic anemia but is rare in patients with leukemia. This may relate to either inherent differences between the two diseases or to the more intensive conditioning used in leukemics. Storb and coworkers have reported a correlation between graft rejection and both recipient anti-donor immunity and marrow dose [15], and we have reported a correlation with pre-transplant lymphocytotoxins [6]. Transfusions also contribute to graft rejection.

Graft-versus-host disease results from the introduction of immunocompetent donor cells into the immunosuppressed recipient. Principle target organs of GVHD include the lymphoid system, skin, liver, and gastrointestinal tract [17]. GVHD in man results from incomplete matching for non-HLA histocompatibility antigens. The loss of normal immune regulatory mechanisms and autoimmunity may also contribute. While GVHD initially results from immune stimulation, the end result is immunodeficiency. The incidence of GVHD following HLA-identical marrow transplantation is 70 per cent, and over one-half of these cases are fatal. The prevention and treatment of GVHD are problematic. Methotrexate is routinely given prophylactically to modify GVHD, but this is not completely effective. Attempts to prevent GVHD with antithymocyte globulin (ATG) or to treat it with ATG, corticosteroids, and other immunosuppressive drugs have been largely unsuccessful. While complete histocompatibility matching would theoretically prevent GVHD, this approach would further limit the number of potential candidates for bone marrow transplantation. The removal of immunocompetent cells from the marrow inoculum prior to transplantation by either physical or immunologic technics has appeal but has not been critically evaluated in man. The complete prevention of GVHD is not necessarily desirable since GVHD may have anti-leukemic effects.

Allogeneic marrow transplantation is followed by a period of immunodeficiency lasting several months to 1–2 years [7,20]. The cause of the immunodeficiency is multifactoral and includes abnormal or delayed lymphoid differentiation, GVHD, and the effects of immunosuppressive drugs. Post-transplant immunodeficiency is characterized by abnormalities of both T and B lymphocyte function including decreased antibody synthesis, decreased responsiveness to polyclonal mitogens, and inability to be sensitized to dinitrochlorobenzene (DNCB). Reactivity to alloantigens and skin graft rejection are normal. This immunodeficiency is correctable with time. This suggest that either a small number of lymphoid precursors are engrafted, or that their development is delayed. We have found no evidence of suppressor cells or factors in these patients [7].

Approximately 60–70 per cent of marrow graft recipients develop interstitial pneumonitis [11]. The incidence is higher in leukemic patients than in aplastics. One-half of cases are related to cytomegalovirus (CMV), 10 per cent to pneumocystis, and 10 per cent to other viruses. No etiology is identified in the remaining cases. It is likely that immunologic factors including immune stimulation, immunodeficiency, and GVHD play a critical role in the development of interstitial pneumonitis. Radiation and/or chemotherapy are probably not primary factors but may compromise resistance. In CMV pneumonitis, it is likely that both reactivation of latent endogenous infection and exogenous infection are important factors. Attempts to prevent or treat interstitial pneumonitis with antiviral chemotherapy (ara-A) have been unsuccessful. Studies of CMV immune globulin, or plasma and interferon, are currently underway at several centers.

Bacterial and fungal infections are an important complication of bone marrow transplantation [19]. These usually occur during the period of granulocytopenia immediately following the transplant and their magnitude is related to the intensity of the conditioning regimen. Most patients receive oral non-absorbable antibiotics for gastrointestinal tract sterilization, systemic antibiotics, and granulocyte transfusions. The value of prophylactic granulocyte transfusions and laminar air flow environments is controversial, but recent data suggest they may decrease the incidence of infection without a substantial effect on survival [2].

Current Results in Acute Leukemia

The survival of patients with resistant acute leukemia is poor with median survival of less than 6 months in several large series. Because of this, we and others have studied the potential role of allogeneic bone marrow transplantation in patients with resistant disease.

Transplantation in acute leukemia is difficult. In addition to the previously described immunobiologic problems, it is necessary to permanently eradicate the leukemic clone(s). A variety of chemotherapy-radiation therapy regimens have been developed to achieve this goal. Three representative regimens are indicated in Fig. 1 and remission and survival data in Figs. 2 and 3 [8,9,13,18]. Several important points emerge from these studies:

1. Leukemic relapse is common despite the use of supralethal levels of drugs or radiation; 2. The risk of relapse is high during the first 2 years but lower thereafter; 3. That with the possible exception of SCARI (see legend Fig. 1), more intensive conditioning has not been associated with a lower relapse rate; and 4. 15–20 per cent of patients with resistant disease may become long-term disease-free survivors. While this survival rate is not a satisfactory end result, it is probably superior to chemotherapy alone. It is noteworthy that immunologic problems rather than resistant leukemia are the major cause of death in some series. These problems may ultimately prove more soluble than resistant leukemia.

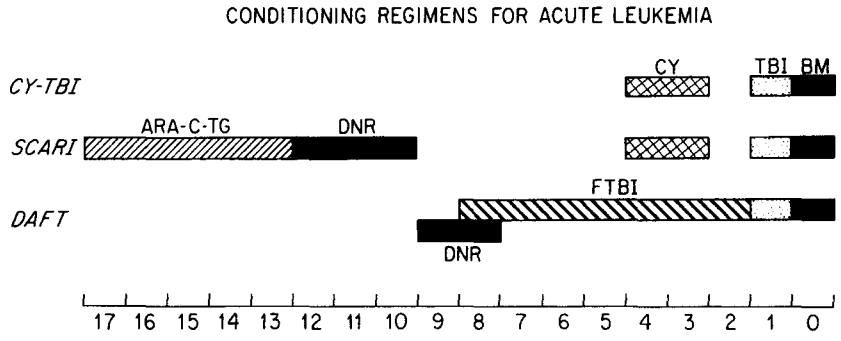

Fig. 1

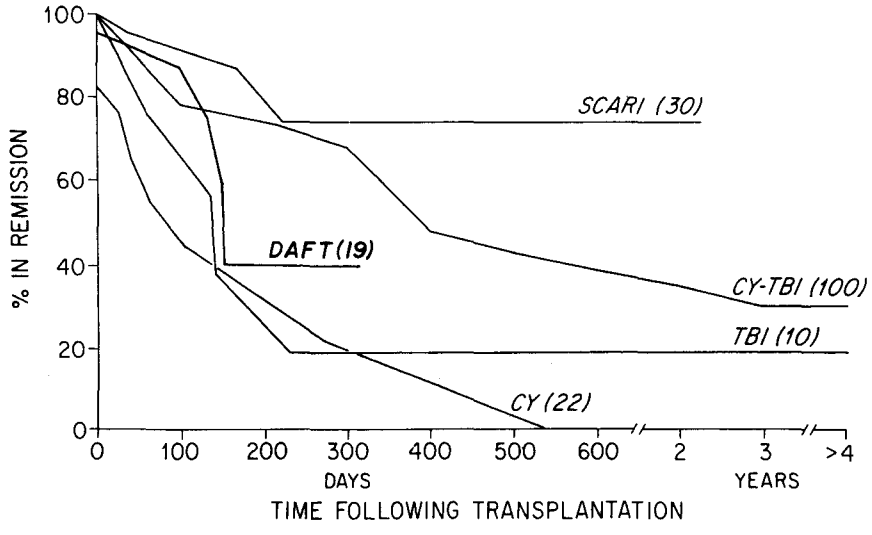

Fig. 2

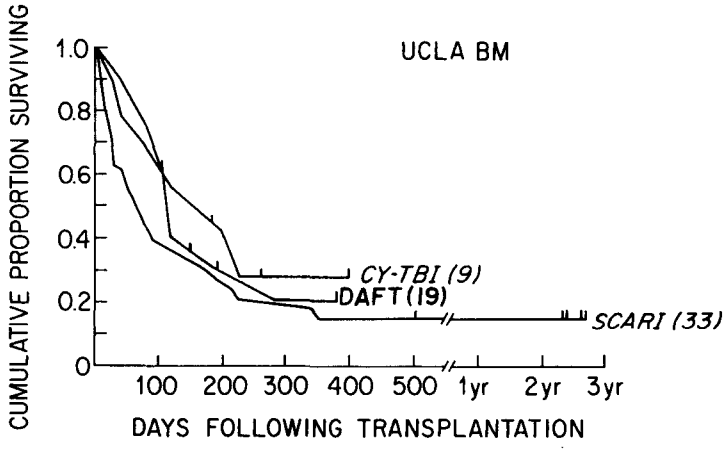

Fig. 3

Major features of bone marrow transplantation in leukemia are reviewed in Table 2. It should be emphasized that 98 per cent of relapses occur in recipient cells so that progress is dependent upon the development of more effective conditioning regimens. Potential approaches to this problem are indicated in Table 3. Perhaps the most promising are the development of more effective regimens and transplantation in remission. Preliminary data from Thomas and coworkers has indicated a low relapse rate in patients transplanted in remission. Finally, the introduction of new myelosuppressive drugs or innovative uses of radiation may improve the results of transplantation in acute leukemia.

Table 2. Leukemic recurrence following bone marrow transplantation

Indefinite time at risk
Predominantly in recipient cells
Maintains genetic markers of original disease
Residual normal hematopoiesis of donor origin

Table 3. Approaches to decreasing the rate of leukemic relapse

More effective chemoradiotherapy
Intensive chemoradiotherapy with optimal support facilities
Treat leukemic "sanctuaries"
Transplant before "resistance" develops
Combination of approaches

Future Directions

Future research in this field must concentrate on two critical problems:

1. More effective leukemia eradication and, 2. solutions to immunologic problems including GVHD, immunodeficiency, and interstitial pneumonitis. With regard to the first area, I have discussed the development of more effective conditioning regimens and transplantation in remission. GVHD is a difficult problem and it now seems clear that prevention is critical. Based on animal data, the *selective* elimination of immunocompetent cells from the graft by either physical or immunologic technics seems a logical step. Immunodeficiency is probably best approached by steps to facilitate the rate of lymphoid maturation. These might conceivably involve transplants of thymic epithelium of the use of thymic hormones. Progress in the area of interstitial pneumonitis will depend on an understanding of the etiologic and pathogenic mechanisms involved. Trials of CMV immune plasma and interferon are currently in progress. Development of more effective antiviral chemotherapy such as phosphonoacetic acid for CMV infection is clearly needed. Progress in GVHD and immunodeficiency may have a beneficial effect on interstitial pneumonitis. Finally, the possibility of lung-shielding should be considered in non-leukemic patients.

A recent area of considerable interest is autotransplantation using cryopreserved remission bone marrow (for review see [10]). Preliminary studies have clearly indicated that cryopreserved marrow can reconstitute a lethally radiated recipient but leukemic relapse has been a major obstacle. Whether this relates to residual leukemia in the patient or in the cryopreserved marrow is as yet uncertain. The concept of autotransplantation is of considerable theoretical interest since these patients would not be at risk to develop many of the immunologic problems associated with allogeneic transplantation such as GVHD. Autotransplantation could expand the applicability of marrow transplantation since most patients with leukemia lack an HLA-identical sibling donor. Leukemic relapse remains the major problem in

autotransplantation, and attemps to deplete clinically undetectable leukemic cells from remission marrow using either physical or immunologic technics need to be critically evaluated.

A final consideration is the use of HLA-matched *unrelated* donors for patients without HLA-identical siblings. Recent advances in histocompatibility testing, particularly HLA-D typing, make this a possibility. Opelz and coworkers have recently reviewed theoretical consideration involved in the development of donor pools for unrelated marrow transplantation [12].

Summary

Bone marrow transplantation is an experimental approach to the treatment of patients with acute leukemia, aplastic anemia, and other neoplastic and genetic diseases. To date, long-term disease-free survival has been achieved in a small proportion of carefully selected patients with resistant acute leukemia. While results are not optimal, they are acceptable in late stage patients where there are no effective alternates. Major problems in marrow transplantation for leukemia include tumor resistance and a spectrum of immunologic complications including GVHD, immunodeficiency, and interstitial pneumonitis. Potential approaches to these problems have been suggested. Progress in any one area would have a substantial impact on improving survival and extending the applicability of marrow transplantation to patients at an earlier stage of their disease.

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Impact of Specific Immunotherapy in Acute Myelocytic Leukemia

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Introduction

During the last ten years immunotherapy has become an important tool in the treatment of human leukemias. Mathé et al. [1,2] demonstrated the therapeutic value of irradiated allogeneic myeloblasts in combination with BCG in treating childhood lymphoblastic leukemia. Similar studies were conducted by Powles et al. [3] and Gutterman et al. [4] in patients with acute myelocytic leukemia involving chemotherapy with or without irradiated allogeneic myeloblasts plus BCG. These studies consistently show that immunized patients sustain a somewhat longer remission duration than those without immunization. Also, after the first relapse immunized patients are reported to have higher frequency and greater "ease" of reinduction. BCG has been used in conjunction with cultured leukemia cells in the immunization of patients with chronic myelocytic leukemia by Sokol et al. [5]. Under optimal conditions prolongation of median survival of CML patients was attained in patients who were treated with busulfan and immunotherapy as compared to controls who received busulfan alone. In an attempt to find a more standardized immunological adjuvant, Weiss et al. [6] conducted extensive studies with MER, the methanol extraction residue of BCG. They were able to demonstrate therapeutic advantage of MER, especially in murine leukemias.

Neuraminidase of Vibrio Cholerae origin has been used successfully in increasing the expression of tumor specific antigenicity of autochthonous and syngeneic tumors. This phenomenon is exclusively due to the enzymatic cleavage of surface membrane bound N-acetylneuraminic acid from the tumor cells [7]. The effectiveness of neuraminidase (N'ase) modified spontaneous and transplantable tumors as immunogen in both immunoprophylactic and chemoimmunotherapeutic experiments in syngeneic mice was positively established [8–11]. We have demonstrated that neuraminidase-treated E_2G leukemic cells which, like the AKR leukemia, are Gross virus induced, but are completely different at the H_2 genetic locus from the AKR mice, were as effective as the syngeneic leukemic cells in prolonging the survival of leukemic AKR mice [12]. This suggests the existence of a cross-reacting common viral membrane antigen, and would suggest that if similar etiology existed for human acute leukemia, it would not be essential to use autologous leukemia cells for immunization. These data provided the basis for using neuraminidase-treated allogeneic myeloblasts in human immunotherapeutic investigations. It will be demonstrated here that combined with

an effective remission inducing and sustaining chemotherapy in patients with acute myelocytic leukemia, neuraminidase modified allogeneic myeloblasts have an important therapeutic value when administered in a systematic program of chemoimmunotherapy.

Remission and Sustaining Chemotherapy

The chemotherapy protocol on this study is based in maximal chemotherapeutic reduction of leukemic burden. This is achieved by induction therapy with a regimen of cytosine arabinoside continuously administered intravenously for 7 days at 100 mg per square meter of body surface area per day, and daunorubicin at a dose of 45 mg per square meter of body surface area per day by direct injection on days 1, 2 and 3. This regimen has induced approximately 70 per cent of patients into remission. All patients were between the ages of 15 and 70. All received cyclical maintenance chemotherapy every 4 weeks. This consisted of 5 day courses of AraC in addition to 6-thioguanine, cyclophosphamide, CCNU, or daunorubicin sequentially with each course repeated at 4 months cycles.

Collection of Allogeneic Myeloblasts for Immunotherapy

Patients became eligible for collection of myeloblasts after satisfying the following criteria: Negative HA-A as determined by radioimmunoassay, no previous chemotherapy, total WBC higher than 25000/μl, and higher than 70% myeloblasts in the peripheral blood. The myeloblasts were obtained by leukophoresis. In the last five years we collected myeloblasts from 93 patients between the ages of 14 and 72 years and have not encountered any important side-effects during the two to four hour procedure. After leukophoresis, the myeloblasts were separated from contaminating red blood cells by sedimentation at 37°C. After sedimentation leukemic cells were mixed with special freezing media (free of calcium and magnesium) containing 15% autologous or AB plasma and 10% DMSO. The final cell concentration was $0{,}3-1{,}0\times 10^8$ cells/ml. Myeloblasts were frozen by programmed freezing at a temperature drop of 1,5°C per minute until – 38°C was reached, and then rapidly to 80°C. The frozen cells were immediately stored in the vapor phase of liquid nitrogen.

Treatment of Allogeneic Myeloblasts with Neuraminidase

Myeloblasts were thawed and were washed twice with mixed salt and glucose media at 4°C and further purified on a 22 per cent human albumin gradient, layered over 45% surcrose for the separation of viable from nonviable blast cells. After purification, blast cells were washed and incubated with N'ase at a concentration of 50 units of enzyme per 5×10^7 cells/ml in

sodium acetate buffer, for 45 minutes at 37°C. The cells were then washed and resuspended in physiological saline and used as immunogen within 30 minutes.

Immunization with Allogeneic Myeloblasts and MER

Immunization with neuraminidase treated allogeneic myeloblasts was performed by intradermal injections. In order to get maximum exposure to the immunogen, sites were widely spread in the supraclavicular, infraclavicular, arm, forearm, parasternal, thoracic, suprainguinal and femoral regions draining into several node bearing areas.

Dose dependent cellular titration was performed with each immunization with 0,5, 1,5, $2,5 \times 10^8$ and 0 cells. The total immunization load was about 10^{10} cells at 48 body sites. The injections of neuraminidase treated myeloblasts produced no local lesions other than the delayed type cutaneous hypersensitivity reaction (Table 1) and none of the patients developed chill, fever, or adenopathy. No hypersensitivity reaction was apparent at the site of injection of physiological saline, heat denatured neuraminidase, or supernatant of cell incubation media. In patients randomized to receive MER too, we used ten intradermal sites of 100 µg/.1 ml each totaling 1,0 mg of MER.

Table 1. Delayed hypersensitivity response to X-irradiated or neuraminidase treated myeloblasts

Immunization Cycles	Induration[a]						
	Number of n'ase treated myeloblasts injected per site $\times 10^8$				Number of X-irradiated myeloblasts injected per site $\times 10^8$		
	0.5	1.0	2.0	3.0	1.0	2.0	3.0
1	3.5 ± 1.5	6.2 ± 2	14 ± 4	18 ± 3	3.0 ± 1	5.4 ± 1.5	7.8 ± 2
6	7.1 ± 2	12.9 ± 3	19.8 ± 6.1	24 ± 6	4.2 ± 2.1	7.5 ± 3	8.4 ± 1.2
12	8.3 ± 2.4	14.1 ± 2.9	20.3 ± 5	25.1 ± 7	4.6 ± 1.6	7.2 ± 2.4	8.1 ± 2.5

[a] Mean induration in mm obtained from at least 40 injected sites. measured 48 hours after the intradermal injection of myeloblasts.
Standard error of mean

Impact of Specific Immunotherapy in Patients with Acute Myelocytic Leukemia

Based on experimental observations, a successful chemoimmunotherapy trial was conducted in patients with acute myelocytic leukemia. The interim analysis presented below is calculated by standard life table methods and is subdivided into several subsets. The data represents 91 patients with AML who were allocated in three groups following successful remission induction

using cytosine arabinoside and daunorubicin. Patients designated to receive immunotherapy were injected (i.d.) in approximately 48 sites every 28 days with 10^{10} N'ase treated allogeneic myeloblasts. For 27 of the 91 acute myelocytic leukemic patient, the remission duration on the chemotherapy alone was 243 days; for those receiving N'ase modified allogeneic myeloblasts as immunogen the mean remission was 686 days (Fig. 1). The difference in remission duration between the two treatment groups is highly significant: $p = .001$ using Breslow's, Logrank and Cox regression analysis. Combination of specific plus adjuvant immunotherapy did not act synergenistically in the treatment of AML patients. Fig. 2 shows the behavior of patients immunized with N'ase treated myeloblasts plus the full prescribed dose of MER with a mean remission duration of 336 days. This was compared to another group of patients, in whom, based on the demonstration of the presence of suppressor cells and supporting clinical evidence, the MER dose was attenuated or omitted. This modality provided considerable improvement in the remission duration (of 630 days) but still not has reached the level attained with N'ase treated myelobasts alone (see Fig. 1).

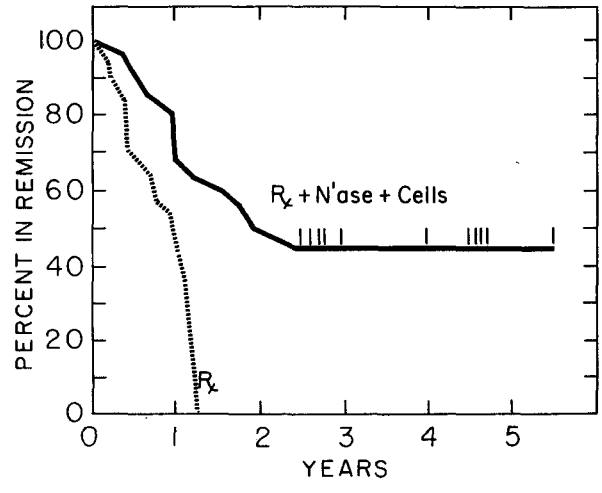

Fig. 1. Duration of complete remission in acute myelocytic leukemia in patients immunized with neuraminidase treated allogeneic myeloblasts.

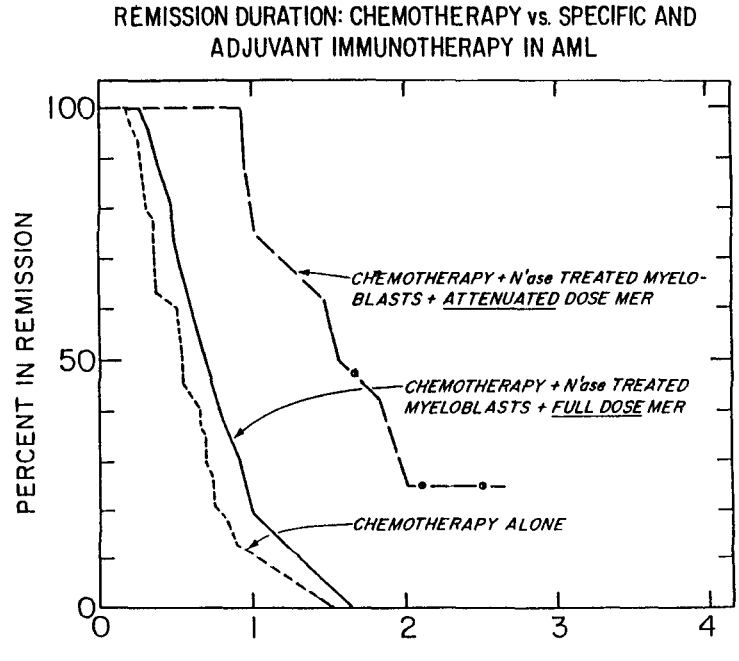

Fig. 2. Effect of chemotherapy and chemotherapy plus neuraminidase treated allogeneic myeloblasts plus MER on remission duration in patients with AML

However the difference between the control vs. cell + MER is significant at p = .03. It appears that inclusion of MER in this immunotherapy protocol adds no value to chemoimmunotherapy when added to N'ase treated allogeneic myeloblasts as immunogen in AML patients.

Response to Recall Antigens

The in vivo immunological status of the immunotherapy patients at various stages of their treatment was measured by DCH response to five recall antigens; PPD, mumps, candida, varidase and dermatophytin. Interpretation of the skin tests was based on the induration as measured in millimeters in two directions at 48 hours and considered positive if the diameter of induration exceeded 5 mm. Fig. 3 shows that there were significant improvements in the response to recall antigens in patients immunized with N'ase treated myeloblasts. However, patients who received N'ase treated myeloblasts plus MER, after an initial improvement, the DCH response to recall antigens gradually declined and was ultimately eradicated. The decline of in vivo response to antigens often preceeded subsequent relapse of those individuals who received full dose of MER in addition to N'ase treated myeloblasts.

Impact of Immunotherapy on the Immunological Status of AML Patients

On each immunization day, remission lymphocytes were isolated from freshly drawn heparanized blood by the Ficoll-Hypaque gradient method for the following in vitro assays: Surface markers by E and EAC rosettes,

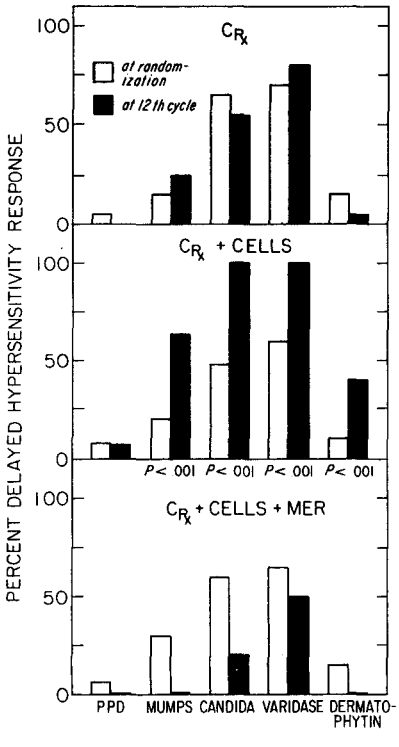

Fig. 3. Change of delayed hypersensitivity response to recall antigens during the course of chemoimmunotherapy

phytohemagglutinin (PHA) and pokeweed mitogen (PWM) induced lymphoblastogenesis and tumor leukocyte culture (MLTC) with immunizing allogeneic myeloblasts.

Although the quantification of E and EAC rosette forming lymphocytes from AML patients in the protocols were routinely performed, we are only showing in Table 2 two test periods: a) The initial E and EAC values at the time of randomization, and b) the impact of immunotherapy on the T and B lymphocyte surface markers. The median value for normal donors of E-rosetting PBL is 74,4%, with 1,986 as the number of absolute T-lymphocytes. For EAC rosetting the normal PBL values are 22,1% with 521 as the number of absolute lymphocytes. Patients at the time of randomization, still under recovery from induction and consolidating chemotherapy, have shown significantly lower percentage (49,2 and 51,7) and absolute number (412 and 487) of T-cells as well as lower percentage (16,2 and 16,5) and absolute number (169 and 195) of EAC rosetting lymphocytes.

Patients in both chemotherapy regimen showed a significant increase of T- and B-lymphocytes as compared to values at the time of randomization both in percentage and in absolute number.

Maximum lymphocyte blastogenesis was attained at 0,15 μg per well for PHA and 30 μg per well for PWM, for normal donors, as well as for the remission lymphocytes from patients in either of the therapeutic regimen. Lymphocytes obtained from patients receiving chemotherapy alone showed consistently lower degree of stimulation to both mitogens all through the observation periods. Lymphocytes obtained from AML patients who have been immunized with N'ase treated myeloblasts showed, despite the fact that they have been receiving chemotherapy, nearly normal lymphocyte function (Table 2). Countraiwise, patients immunized with N'ase treated myeloblasts plus MER have shown in the first six months of immunotherapy a continuous improvement in their response to mitogens but not to tumor cells. This was followed by a gradual decline in lymphocyte function. The fact that patients treated with N'ase treated myeloblasts plus MER have similar E-rosetting PBL as patients treated with cells alone, but have significantly altered in vivo and in vitro lymphocyte function (Fig. 4), raised the possibility of the presence of an inhibitory mononuclear cell population in the blood of such immunized patients. This hypothesis was tested and the data are summarized in Fig. 5. Isolated enriched T-cell fractions from normal donors or from patients immunized with N'ase modified myeloblasts gave similar uptake of H^3TdR as the unseparated PBL. However, isolated enriched T-cell fraction from AML patients who received N'ase treated myeloblasts plus MER, and have shown declining in vivo and in vitro immunological responses, gave 3–7 times greater H^3TdR incorporation in response to PHA than their unseparated PBL. The response of the enriched T-cells was strongly inhibited by addition of autologous but not normal donors' adherent mononuclear cells. These findings suggest that depression of cell mediated immunity is seen in most of the tested AML patients who received N'ase treated myeloblasts plus full dose of MER, but not among the patients immunized with N'ase modified myeloblasts alone,

Table 2. Lymphocyte function and surface membrane markers of remission lymphocytes in AML patients in the immunotherapy study

	PHA		PWM		MLTC[a]		MLTC[b]		E-Rosettes		EAC-Rosettes	
	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	max. stimul. c.p.m. × 10^3	SI	%	absolute number	%	absolute number
Normal subjects N 79	102.2 ±8.7	257	95.1 ±6.4	194					74.4	1,986 ±251	22.1	521 ±52
Randomization Chemotherapy N 25 After 8th course	41.2 ±5.1	93	37.8 ±5.3	67	15.1 ±3.4	29	19.2 ±4.9	36	49.1	412 ±17	17.5	146 ±6
	51.7 ±6.1	117	49.3 ±5.9	89	19.6 ±4.2	37	22.3 ±6.1	39	56.3	725 ±17	21.3	274 ±14
Randomization Immunotherapy with cells N 34 After 12th course	37.1 ±4.4	90	35.8 ±6.4	73	18.3 ±6.1	32	21.5 ±6.9	35	49.2	429 ±21	18.5	161 ±8.2
	79.5 8.2	212	75.3 ±7.6	142	28.7 ±8.5	59	20.5 ±3.2	29	70.1	1,122 ±120	20.2	323 ±27
Randomization Immunotherapy cells + mer N 19 After 12th course	38.3 ±5.4	85	37.1 ±4.9	79	17.6 ±4.1	28	18.7 ±3.5	31	51.7	487 ±45	22.3	210 ±21
	51.2 ±6.0	113	46.6 ±6.3	68	20.9 ±2.7	29	21.3 ±5	37	70.1	1,099 ±118	21.5	337 ±37

Allogeneic myeloblasts [a] used for immunotherapy or [b] not used for immunotherapy. the mixed tumor-leukocyte culture was performed: Myeloblasts incubated with mitomycin-C at 30 µg/ml of 2×10^6 cell suspension for 30 minutes at 37°C. After washing 2×10^5 myeloblasts were distributed in each of the replicate wells of the falcon microplates containing 10^5 responding lymphocytes per well in RPMI 1640 media supplemented with 20% heat inactivated autologous plasma. After 90 hours of incubation 1 µci ^{3}H-Tdr was added per well. Cultures were harvested 18 hours later with the addition of excess cold thymidine.

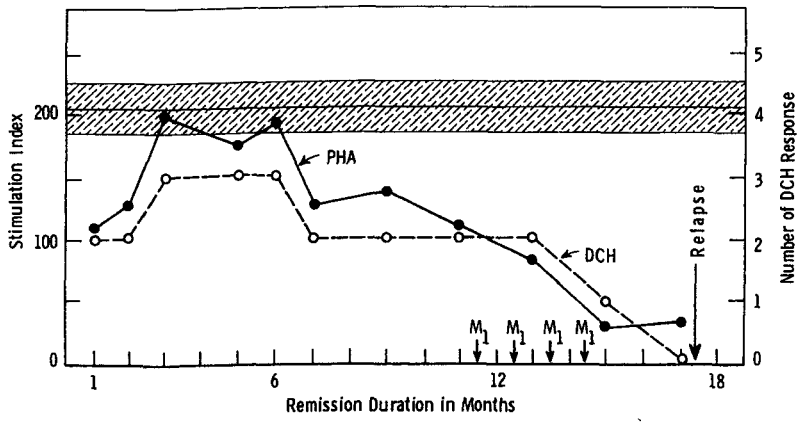

Fig. 5. Appearances of suppressor cell activity in AML patients treated with N'ase treated myeloblasts plus MER

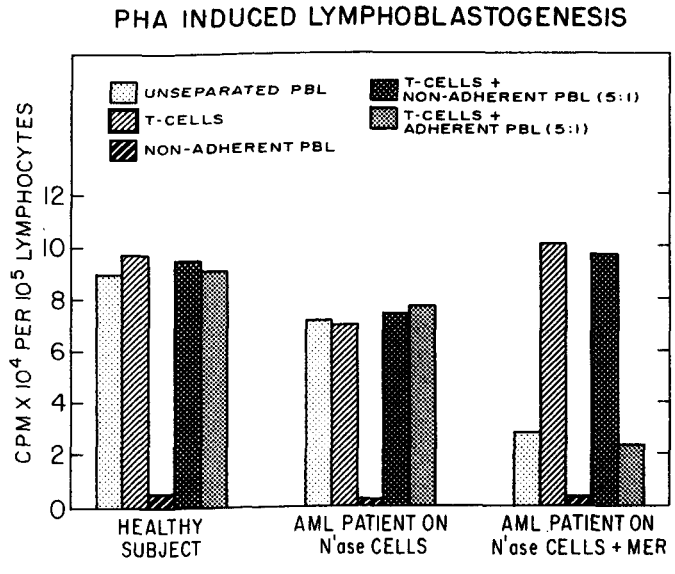

Fig. 4. Impact of N'ase modified myeloblasts plus MER on various immunological parameters in AML patients

maybe due to the suppression of certain T-cell functions by circulating monocytes affected by MER. The time of appearance of the apparent suppressor cell activity was different from patient to patient and omission of MER from the treatment in most cases prompted recovery of the patients' in vivo and in vitro immunological parameters and a gradual decrease of suppressor cell population.

Summary

Our studies clearly show that significantly longer remission duration was attained in groups of AML patients immunized with neuraminidase treated allogeneic myeloblasts as compared to patients who received chemotherapy alone or neuraminidase treated myeloblasts plus MER. It is clear that MER, albeit apparently active alone in certain other clinical studies impairs the immunotherapeutic value of neuraminidase treated allogeneic myelo-

blasts in AML patients. The in vivo and in vitro immunological test results reflect the host's immunological status in each arm of the protocol and correlate well with the duration of remission achieved with specific vs. combination of specific plus adjuvant immunotherapy.

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Treatment of Adult Acute Myeloblastic Leukemia

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The introduction of combination chemotherapy in the treatment of adult acute leukemia has had a profound impact on the response and duration of survival of patients with adult acute myeloblastic leukemia. In the past decade, the combination of cytosine arabinoside with an anthracycline antibiotic, either daunorubicin, adriamycin, or rubidazone, has been responsible to a large degree for the success.

In a study done at the M.D. Anderson Hospital in 1973, a combination of adriamycin, vincristine, Ara-C and prednisone (Table 1) was utilized for initial induction therapy for 58 adults between the age of 15 and 76 with a diagnosis of acute myeloblastic leukemia. 43 (74%) of the patients achieved a complete remission with 31 of 35 (89%) patients under the age of 50 achieving a complete remission [4]. Median duration of survival in this group of patients was 58 weeks. Nine patients remain alive between 4 and 5 years from diagnosis. These 9 are all in their original first complete remission and represents 9 of 31 Patients (29%) under the age of 50 who achieved remission.

Table 1. AD-oap. Acute myeloblastic leukemia

Entered	58		
Complete remission	43	(74%)	
Complete remission	<50	31/35	(88.5%)
	≥ 50	12/23	(51%)

Because of the initial success of this program, the same combination of agents was utilized in the Southwest Oncology Group for remission induction therapy. A total of 420 patients were entered, 224 achieved complete remission (54%). The survival of all patients and survival of those patients achieving a complete remission is shown in Fig. 1. The vertical lines on these survival curves represent patients still alive and can still effect the overall survival of these patients. The median duration of survival of all patients entered is 50 weeks and the projected median duration of survival of the patients who achieved complete remission is almost double this to 2 years [5].

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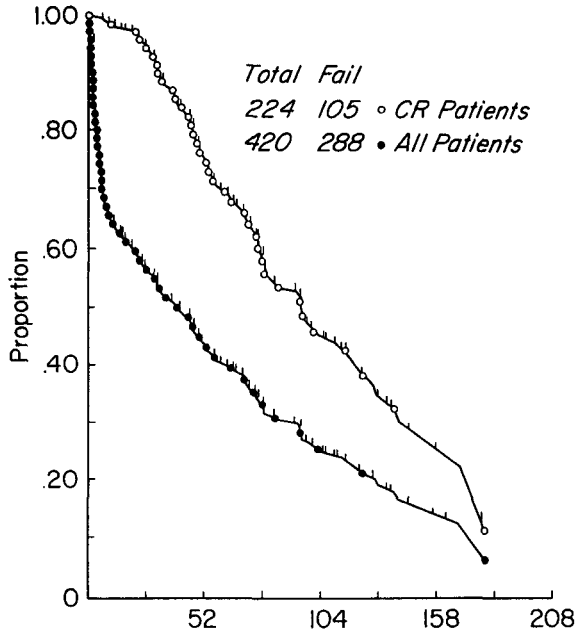

Fig. 1. Swog 7416/17. Acute myeloblastic leukemia survival from first treatment

In addition to adriamycin, rubidazone, a new anthracycline antibiotic related to daunorubicin, has been utilized in combination with cytosine arabinoside in the management of patients over the age of 50. The program is shown in Table 2. A total of 56 patients have been entered on this program with an overall response rate of 49% which is similar to the response rate seen in patients utilizing a combination of adriamycin, Ara-C, vincristine and prednisone [3].

Table 2. Roap. Acute myeloblastic leukemia. >50 years of age

Entered	57
Complete remission	28 (49%)

In an attempt to prolong remission, a late intensification program has been used in patients who have been in maintained complete remission for one year or longer. Late intensification therapy utilizes drugs that they have not been previously exposed to, the majority of the patients in this program received a combination of 6-mercaptopurine, methotrexate, vincristine and prednisone. A total of 62 patients have undergone late intensification therapy and 29 (47%) remain in unmaintained complete remission. The majority of the patients that relapsed, relapsed in the first six months and 31 of the 33 patients that have relapsed have done so within the first 24 months following discontinuous of chemotherapy (Table 3). The risk of relapse sub-

Table 3. Late intensification therapy in acute leukemia

Patients entered	62
Patients still in CR	29 (47%)
Relapsed by 6 months	21 (64%)
Relapsed by 24 months	31 (94%)

sequent to this time is less than 10% [1]. Of the 36 patients who are in continuous complete remission one year from complete remission, 9 (25%) had relapsed whereas 12 patients now have been discontinued from chemotherapy four years or longer from the late intensification and none of these have relapsed (Table 4). The overall risk of subsequent relapse after one year of continuous complete remission without chemotherapy is less than 10%.

Table 4. Duration of unmaintained remission after late intensification therapy

Duration of CR (Yrs from LI)	Number of Patients	Subsequent Relapses
1	36	9 (25%)
2	25	2 (8%)
3	18	1 (6%)
4	12	0

The survival in AML after relapse is short, the median duration of survival of patients with AML who have achieved a complete remission and subsequently relapsed, the survival from relapse is less than 20 weeks (Fig. 2). Only 5% of these patients are projected to be alive in one year. Because of these poor results following relapse, a program of autologous bone marrow transplantation has been introduced using marrow collected and stored from patients with acute myeloblastic leukemia during periods of remission. This therapy offers a viable alternative to reinduction chemotherapy. We have used a combination of piperazinedione and total body irradiation therapy.

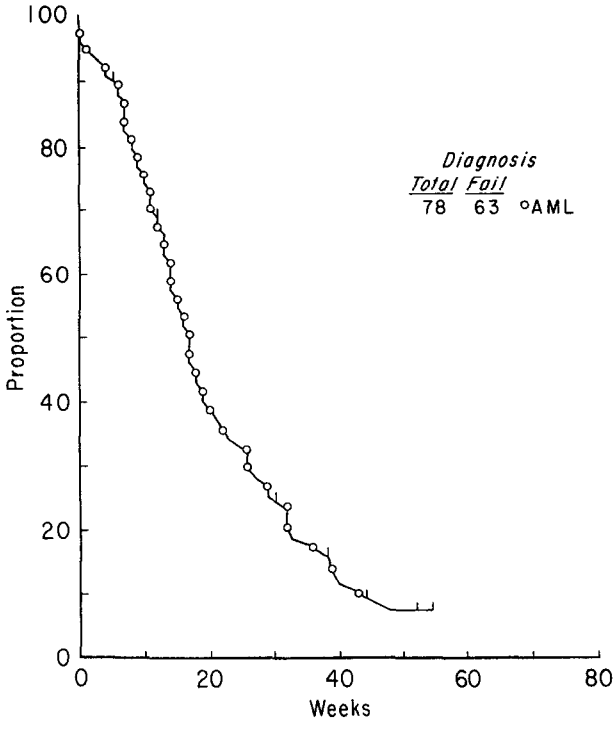

Fig. 2. Survival from relapse

This program has resulted in clearing of the leukemia and evidence of engraftment with normal myeloid recovery in 5 of 11 patients [2]. Survival of these patients, duration of remission and duration of bone marrow storage in months and interval between storage and relapse and the number of cells and the number of colony forming units reinfused is shown in Table 5.

Table 5. Autologous bone marrow transplantation

Number of Evaluable Patients	11
Number Showing Evidence of Engraftment	9
Number Achieving Complete Remission Survival (days) 17–320+	5
Interval Between Remission and Storage (months)	2–5 Median 12
Interval Between Storage and Relapse (months)	10–30 Median 19
Cells Transfused/kg b.w.	6.8×10^6–3.2×10^8 Median 1.2×10^8
CFU-C/10^5 Cells	0–15 Median 5

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Prediction of Therapeutic Response in Acute Myelocytic Leukemia

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Introduction

There have been many attempts to predict the outcome of antileukemic chemotherapy by characterizing the kinetic status or the drug uptake characteristics of a patient's leukemic cells. These attemps have for the most part been unsuccessful [1–4]. In this paper we will present recently acquired data from our laboratories which appears to be useful in predicting the outcome of therapy and will discuss some of the reasons for the failure of previous studies.

Methods

Patients were treated with a combination of adriamycin (ADR) (30 mg/m^2 IV d 1, 2, 3) and cytosine arabinoside (ara-C) (100 mg/m^2/d for 7 days by continuous infusion) [5,6]. Experimental methods are described in the referenced publications.

Results

Pharmacologic Assessment of Therapeutic Sensitivity
Cytosine Arabinoside Phosphorylation and Retention in Vitro

The 4-hour retention of cytosine arabinoside by the leukemic cells of 28 patients was measured (Rustum and Preisler, Cancer Res., submitted 1978). Patients could be divided into two groups: a high retention group-patients whose leukemic cells retained after 4 hrs >30% of their initial levels of ara-CTP, and those whose leukemic cell retention of ara-CTP was low (<20%). Table 1 gives the treatment results for these patients. Both groups were in-

Table 1

Patients	No.	CR	Cause of remission induction failure	Med. dur. CR-Wks.
Entire population	28	20	—	72
High ara-CTP retention (>30%)	13	11	2 pts. died during induction therapy	>78
Low ara-CTP retention (<20%)	15	9	3 pts. with resistant disease (7), 3 pts. died during induction	28

distinguishable with respect to characteristics such as age, initial white blood cell count, etc. It is clear that while the advantage of the high retention group over the low retention group with regards to remission rate was marginal ($p \geq 0{,}05$), the duration of remission for the former patients was much greater than for the latter ($p < 0{,}001$).

Estimation of Ara-CTP Levels Achieved in Vivo with Leukemic Cells

Methods have not as yet been developed which permit direct accurate assessment of the intracellular levels of ara-CTP which are reached during chemotherapy. The intracellular level of ara-CTP achieved during therapy has been determined indirectly by measuring the inhibition of ^{3}H-CdR incorporation into DNA (Rustum et al., unpublished data). An in vitro standard curve is generated for each patient's bone marrow cells prior to initiation of therapy using 0,0625 to 1,0 μg/ml ara-C. The intracellular ara-CTP level achieved in vitro at each concentration is correlated with the extent of inhibition of CdR incorporation into DNA and a standard curve drawn. Twenty-four hours after the initiation of therapy, bone marrow myeloblast cells are obtained from the patient and incubated with CdR-^{3}H to determine the extent of incorporation of CdR into DNA. The rate of incorporation of CdR into this specimen is then related back to the pretherapy in vitro standard curve and an estimation is made of the intracellular level of ara-CTP achieved after 24 hrs of therapy. The results summarized in Table 2 indicate that at an in vitro concentration of 0,0625 μg/ml ara-C the extent of inhibition varied from 8% to 93% in different patients. At 1,0 μg/ml this inhibition varied from 42% to 95%. These results also show that there is a close correlation between the amount of ara-CTP achieved intracellularly in vitro and the extent of inhibition of CdR-^{3}H incorporation into DNA prior to therapy and 24 hrs after the initiation of chemotherapy.

Biological Assessment of Determinants of Response

The development of in vitro clonogenicity assays has permitted the characterization of a population of progenitor cells which have hitherto been impossible to study directly. Since the leukemic CFU-c are a minority progenitor population hidden within the recognizable leukemic cells, studies carried out on the latter population of leukemic cells may not be reflective of the proper-

Table 2

Patient	In vitro percent Inhibition at 0,0625 μg/ml[c]	0,1 μg/ml	In vivo Inhibition[b]	Ara-CTP[a] (pm/10^7 cells)
1	8	45	22	2,8
2	60	83	68	18
3	94	96	93	30

[a] ara-CTP formed intracellularity when bone marrow myeloblasts were incubated with 0,0265 μg/ml ara-C.
[b] 24 hrs. after initiation of therapy.
[c] Ara-C concentration.

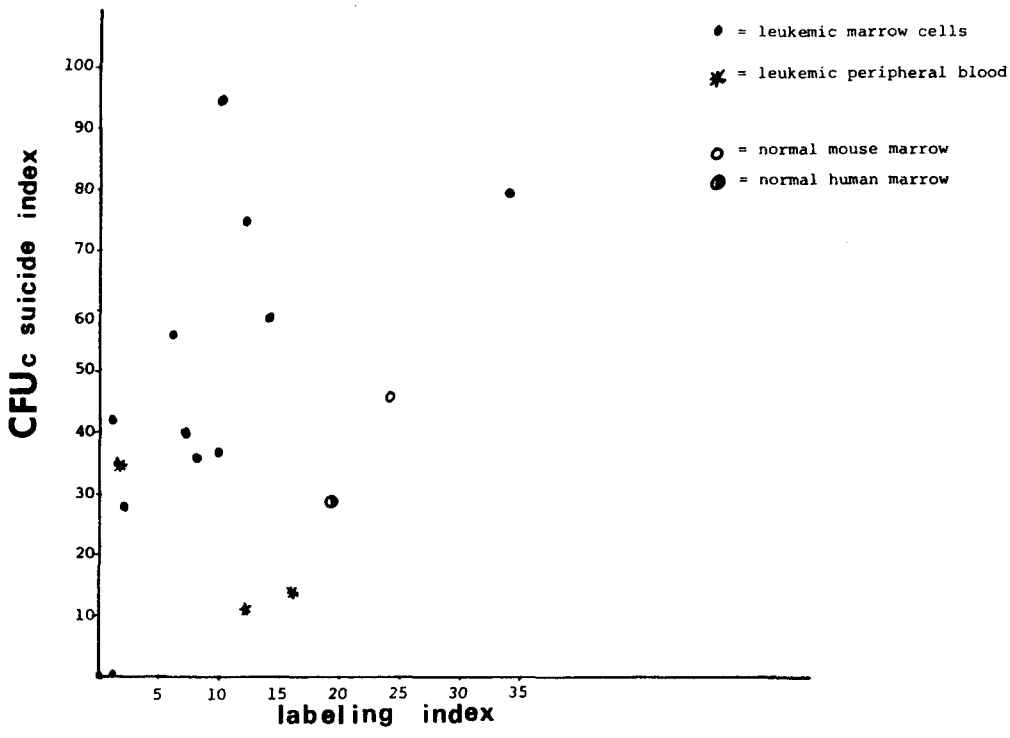

Fig. 1

ties of the leukemic progenitor cells. Support for this proposition has been found in our comparison of the labeling index of the recognizable leukemic marrow cells with the suicide index of the leukemic CFU-c (Preisler and Shoham, submitted to Cancer Res., 1978). Fig. 1 demonstrates that a simple relationship does not exist between the labeling index and the suicide index except in that the labeling index is consistently lower than the suicide index (usually $\frac{1}{2}$–$\frac{1}{7}$).

Estimation of Effects of Adriamycin in Vivo

Hoechst 33342 is a supravital dye which can be used to stain DNA for flow cytometric studies. While comparing leukemic cell DNA histograms before and 24 hrs after the initiation of antileukemia chemotherapy, we found that the degree of DNA fluorescence produced by H33342 was diminished in some patients (Preisler, H.D., Cancer Treat. Rep., in press). In vitro studies were carried out to further characterize this phenomenon. Fig. 2 illustrates one such study. With exposure to increasing concentrations of ADR, there was a progressive decrease in the DNA fluorescence produced by H33342. The effects of exposure to as little as 0,2 μg of adriamycin for 1hr could be detected. As with CdR uptake, we plan to construct a pre-therapy in vitro dose response curve to assess the potential sensitivity of a patient's leukemic cells to ADR. We will also incubate marrow cells with H33342 24 hrs after the initiation of therapy, measure the fluorescence produced and using the pretherapy dose response curve, estimate the in vivo effects of therapy.

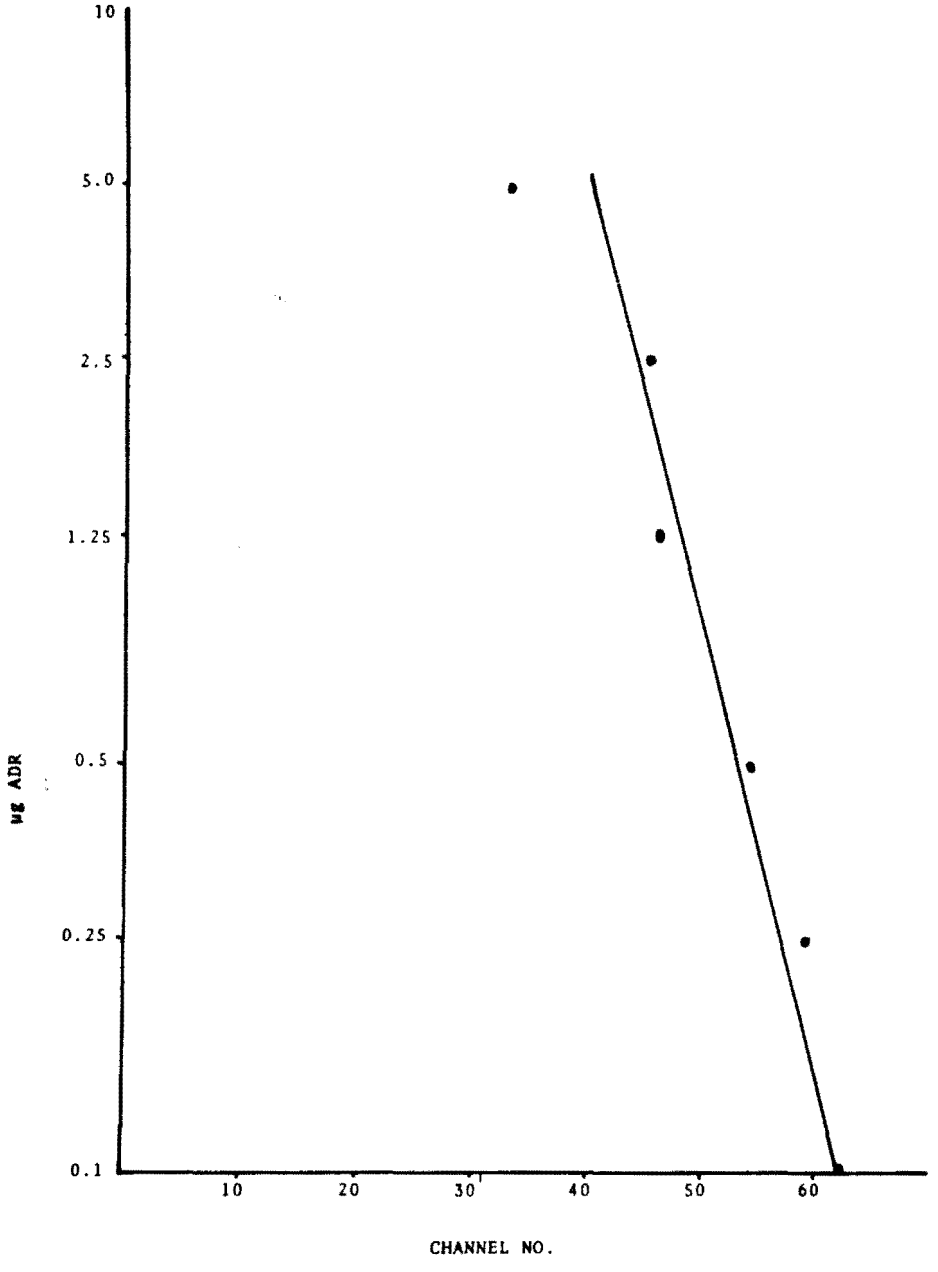

Fig. 2

Discussion

The effectiveness of antileukemia chemotherapy depends upon delivery of the drugs to the target cells, the uptake and activation (if necessary) of the drugs, and the presence of a sensitive metabolic pathway (the latter being dependent in some cases upon the cell cycle characteristics of the leukemic cells). We are attempting to obtain this information by measuring the uptake and activation of ara-C in vitro, deriving an estimate of the in vivo uptake of ara-C and ADR during therapy, and by measuring the labeling index and leukemic CFU-c suicide index prior to and 24 hrs after the initiation of therapy. The latter provides a measure not only of the kinetic status of leukemic CFU-c but also their sensitivity to the antileukemic therapy being employed. Hence, we are attempting to measure the relevant parameters which determine response to therapy.

Two theoretical but practical considerations warrant further discussion. Does measurement of drug uptake by leukemic marrow cells in toto accurately reflect the properties of the leukemic stem cell? This not a moot point since in all likelihood the majority of leukemic cells are end stage cells which are irrelevant with respect to therapeutic attack. The dissociation between the labeling index of the leukemic cells and the suicide index of the clonogenic cells clearly points out that the properties of the easily observable marrow leukemic cells are not necessarily reflective of the properties of the few but crucial stem cells. In our studies to date (and described in this paper), we have found an excellent correlation in previously untreated patients between response to therapy (remission duration – vide infra) and ara-CTP retention by marrow leukemic cells. By contrast, the correlation between ara-C metabolism and response for relapsed patients is not nearly so good and, in fact, in a few cases, high levels of ara-CTP retention have been associated with clinical refractoryness to therapeutic regimens which included ara-C. One possible explanation for this could be a discordance between the drug sensitivity of the leukemic stem cells and the easily observable leukemic cells – a discordance which developed during maintenance therapy. Alternatively, the leukemic cells of these patients may still activate and retain ara-C quite well but may no longer have a sensitive metabolic pathway. Measurement of DNA synthesis (using CdR incorporation) in the presence of ara-C will test this possibility.

The second point requiring discussion relates to evaluation of response to therapy. The end point of all previous studies has been an assessment of whether or not remission has been attained. Considering the aggressive therapeutic regimens currently in use, remission induction rate is an inappropriate end point since marrow aplasia is produced in the vast majority of patients and once this occurs, intercurrent problems relating to infection play a major role in determining whether or not a patient will survive for a sufficiently long period of time to permit regrowth of his bone marrow. Many patients whose leukemic cells are sensitive to therapy die during remission induction [6]. One of us has proposed a system for classifying failure to respond to remission induction therapy so that a distinction can be made with respect to patients who fail remission induction therapy because of leukemic cell resistance to the therapy employed as opposed to those patients who die of intercurrent problems and whose leukemic cells were sensitive to chemotherapy [7]. By contrast the duration of remission appears to be primarily determined by two factors: the number of leukemic cells persisting once remission occurs and the effect of maintenance therapy on residual disease – both of which are reflective of the drug sensitivity of the leukemic cells. Hence, the duration of remission and not the percent remission induction rate is a better measure of leukemic cell sensitivity to drugs. This theoretical construct agrees quite well with our data on ara-CTP retention.

Previous attempts to predict the outcome of antileukemia therapy have not been successful probably because they were based upon the measurement of a single parameter [3,4] (labeling index for example) or used the wrong clinical endpoint [1,2] (remission induction rate). We hope that by

simultaneously determining the relevant cell cycle parameters along with measurements of drug metabolism and the use of appropriate clinical parameters, we will avoid the pitfalls of previous studies and be able to predict the clinical responses of individual patients.

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Prognostic Factors in Adult Acute Lymphoblastic Leukemia

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Introduction

The introduction of three and four drug combination chemotherapy into the treatment of acute lymphoblastic leukemia (ALL) in adults has resulted in complete remission being achieved in approximately 70% of cases (Willemze, Hartgrink-Groeneveld, 1975; Jacquillat and Weil, 1973; Gee and Haghbin, 1976; Muriel and Pavlovsky, 1974; Atkinson and Wells, 1974; Einhorn and Meyer, 1975; Rodriguez and Hart, 1973; Whitecar and Bodey, 1972; Spiers and Roberts, 1975). In spite of the use of early central nervous system prophylaxis and continuous maintenance chemotherapy, however, the duration of complete remission remains considerably shorter than in childhood ALL. It is well documented that certain presentation features influence the prognosis in childhood (Henderson, 1969; Simone and Holland, 1972). We have, therefore, analysed the data from 42 adults in whom complete remission was achieved to determine which presentation features influence the prognosis in adults.

Materials and Methods

A. Patients

Between November 1972 and December 1976, 62 consecutive previously untreated adults with ALL were treated with combination chemotherapy at St. Bartholomew's Hospital. All patients received adriamycin, vincristine, prednisolone and L-asparaginase, as previously reported (Lister, Whitehouse, 1978), and complete remission was achieved in 43 (69%). One patient returned to India without maintenance therapy and subsequently relapsed. The remaining 42 cases form the basis of this analysis. All recieved early central nervous system therapy and continuous maintenance chemotherapy until relapse or for three years, whichever was shorter.

B. Diagnostic Criteria

The diagnosis of ALL was based upon conventional morphological criteria (Bennett and Catovsky, 1976) for May-Grunwald-Giemsa stained bone

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marrow smears, which showed at least 30% infiltration by agranular, Sudan Black negative blast cells. The periodic-acid-Schiff (PAS) stain was performed in all cases and considered positive if more than 5% of the blasts exhibited block or coarse granular activity. Cases with an occasional fine granular or negative reaction were also negative for napthol-As-Acetate esterase activity. Cytogenetic analysis showed that all patients were negative for the Philadelphia chromosome.

C. Treatment Programme

The treatment programme included three main elements: the induction and consolidation of remission, treatment of central nervous system (CNS) or CNS prophylaxis, and maintenance treatment.

1. Induction and Consolidation of Remission

At the start of the study we planned to give doxorubicin (adriamycin) and vincristine every week for a minimum of four courses regardless of the peripheral blood count. But the incidence of pancytopenia after the 2nd injection was so high that the schedule was modified and the 2nd course of doxorubicin and vincristine was given at least 14 days after the first. The interval between the later courses of doxorubicin and vincristine depended on the bone marrow findings.

Table 1. Treatment given for inducing and consolidation remission (OPAL[a])

Drug	Administration	Dose	Intervals of Treatment
Doxorubicin	Intravenous	30 mg/m^2	Days 0, 14, 28, 42[b]
Vincristine	Intravenous	1.4 mg/m^2 (max 2 mg)	Days 0, 14, 28, 42[b]
Prednisolone	By mouth	40 mg	Daily
Colaspase	Intravenous	10,000 IU/m^2	Days 0–14
Allopurinol	By mouth	200 mg three times a day	Daily until blasts cleared from blood

[a] Oncovin (vincristine), prednisolone, adriamycin (doxorubicin), and L-asparaginase (colaspase).

[b] Bone marrow was assessed on day 49; if leukaemic infiltration persisted two further injections of doxorubicin and vincristine were given about 14 days apart.

2. CNS Prophylaxis and Treatment

In the early part of the study lumbar puncture for CSF cytology was not performed until clinical and haematological remission had been achieved. Patients with no evidence of infiltration then proceeded to CNS prophylaxis. This consisted of cranial irradiation (2400 rads) given in 15 fractions over three weeks with concomitant intrathecal methotrexate 12,5 mg twice weekly for five doses during the same period. Analysis of the CSF findings in the first 28 patients who achieved complete remission indicated a high incidence of asymptomatic leukemia disease (Lister and Whitehouse, 1977). The first injection of intrathecal methotrexate was therefore introduced

during the induction of remission, when the platelet count reached $50 \times 10^9/l$ in the absence of circulating blast cells. The total number of doses of methotrexate was also increased to seven. Patients with proven CNS disease who were in clinical and haematological remission received more intensive radiotherapy and intrathecal chemotherapy. Cranio-spinal irradiation (2400 rads) was given in 20 fractions together with 5 doses of intrathecal methotrexate 12,5 mg followed by five doses of intrathecal cytarabine 50 mg given over four weeks.

3. Maintenance Treatment
This consisted of oral 6-mercaptopurine 75 mg daily, starting when complete remission had been achieved and always after allopurinol had been stopped. Once CNS therapy had finished oral cyclophosphamide 300 mg weekly and oral methotrexate 30 mg weekly were started together. The doses of all the drugs were adjusted to maintain the total white cell count at $3 \times 10^9/l$ and treatment was continued for three years and then stopped.

D. Cell Surface Marker Studies

The panel of membrane markers used included spontaneous sheep red blood cell rosette formation (for T cells), reactivity with anti-human immunoglobulin (for B cells) and with anti-ALL serum (for "common ALL" cells). Ficol-Triosil density gradient separation of peripheral blood or bone marrow samples was used to separate blasts and mononuclear cells.

The sheep red blood cell rosette (E-rosette) tests was performed by addition of a suspension of 1×10^6 test cells in 50 µl of medium with 50 µl of foetal calf serum (absorbed with sheep red blood cells) to 100 µl of a 2% suspension of sheep red blood cells which were neuraminidase treated (15 U/ml at 37°C for 30 minutes). The cells were centrifuged at 400 g for 5 minutes and left undisturbed at room temperature for 1 hour before gentle resuspension and counting in an haemocytometer.

The anti-immunoglobulin serum was a fluoresceinated $F(ab^1)_2$ preparation of sheep antibody to human IgG (courtesy of Dr. I. Chantler, Wellcome Research). It was used in a direct immunofluorescence technique by incubating 1×10^6 test cells in 50 µl of medium containing 0,02% sodium azide, with the anti-immunoglobulin at a 1 in 10 final dilution for 30 minutes at 4°C, washing cells 3 times and counting in suspension on a slide with a Zeiss Standard 16 phase contrast microscope with epifluorescence and narrow band FITC filters.

The anti-ALL serum has been previously described in detail (Brown and Capellaro, 1975). It was raised in rabbits against non-T, non-B ALL cells coated with antilymphocyte serum. After extensive absorption with normal haemopoietic cells, lymphocytes and acute myeloid leukemia cells, it was functionally specific for the majority of cases of non-T, non-B ALL and some cases of chronic myeloid leukemia in "lymphoid" blast crisis (Roberts and Greaves, 1978). This antiserum was used in an indirect immunofluorescence technique by incubation for 30 minutes at 4°C with 1×10^6 viable cells in suspension, washing cells twice and then incubation

for 30 minutes at 4°C with a goat anti-rabbit immunoglobulin antiserum which was fluorescein labelled. The cells were then washed 2 times before counting in suspension as above.

E. Statistical Analysis

Remission duration curves and graphic presentations were developed by standard life table formulae (Armitage 1971) and statistical significance was determined by the Log Rank analysis method (Peto and Pike, 1977). The significance of clinicopathological correlations was determined by the Mann Whitney U test.

Results

A. Overall Duration of Remission

The data from 42 patients are evaluable. Twenty two have relapsed. One elected to stop maintenance after 8 months and relapsed shortly thereafter. He has been analysed as not having relapsed, but as being in continuous complete remission for 8 months. One patient died at home during an influenza epidemic whilst in complete remission. The remainder continue in complete remission between 7 and 64 months. The median duration of complete remission was 21 months. Seven patients have already been in continuous remission more than 3 years.

B. Influence of Presentation Features on Remission Duration

1. Age

The age of the patients at presentation did not influence the duration of remission. The number of older patients is small, so statistical analysis would be unwise. However, only 2 patients out of 9 over the age of 40 have relapsed, both at 4 months: the remainder continue in remission between 4 and 46 months.

2. Bulk of Disease at presentation

I. Hepatosplenomegaly (Fig. 1)

Both the liver and spleen were clinically enlarged in 20 patients. Only 6 of these patients remained in complete remission, compared with 15 out of 22 patients in whom there was not hepatosplenomegaly. The duration of complete remission was significantly shorter for patients with hepatosplenomegaly ($p =< .001$).

II. Blast count at presentation

All 4 patients in whom the presentation blast cell count was greater than $100 \times 10^9/l$ had relapsed by six months. However, comparison of the duration of remission for patients with blast cell counts above and below $10 \times 10^9/l$ reveals no statistically significant difference.

3. Cytochemistry

The PAS reaction was positive in 20 cases and negative in 22. There was no

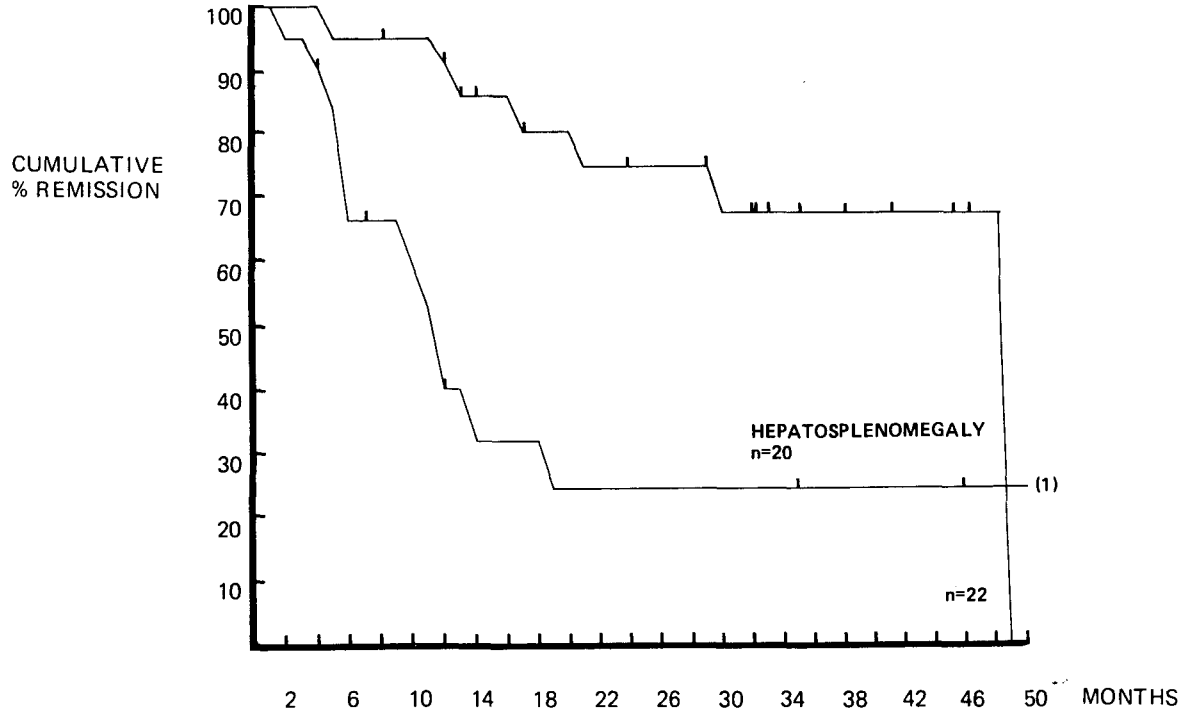

Fig. 1. Duration of complete remission in acute lymphoblastic leukemia. Influence of reactivity with anti-ALL serum

difference in the duration of complete remission between the 2 groups. Ten out of 20 patients in whom the reaction was positive have relapsed, the remainder being in complete remission between 12 and 64 months. Eleven out of 22 patients in whom it was negative have relapsed, the remainder being in complete remission 2 and 25 months.

4. Cell Surface Marker Studies

These were performed in only 29 cases in whom complete remission was achieved. The remaining 13 cases were not studied because they were treated before the techniques necessary were in routine use in our laboratory and not enough viable cells were stored to allow frozen samples to be tested. Thus the duration of follow-up of these cases is shorter than that of the whole study and the median duration of complete remission has not yet been reached.

Complete remission was achieved in only 3 out of 5 cases of Thy-ALL. Two have relapsed at 5 and 10 months, and the third continues in complete remission at 35 months.

The blasts from 16 of the remaining cases of unclassified or null ALL reacted positively with the anti-ALL serum. The duration of remission was significantly longer than that of the 10 cases of which did not react with the antiserum. Only 3 out of 16 anti-ALL positive common ALL cases have relapsed. The remainder continues between 7 and 46 months. Six out of the 10 anti-ALL, non-T, non-B cases have relapsed and only 4 remained in remission between 8 and 41 months (Fig. 2).

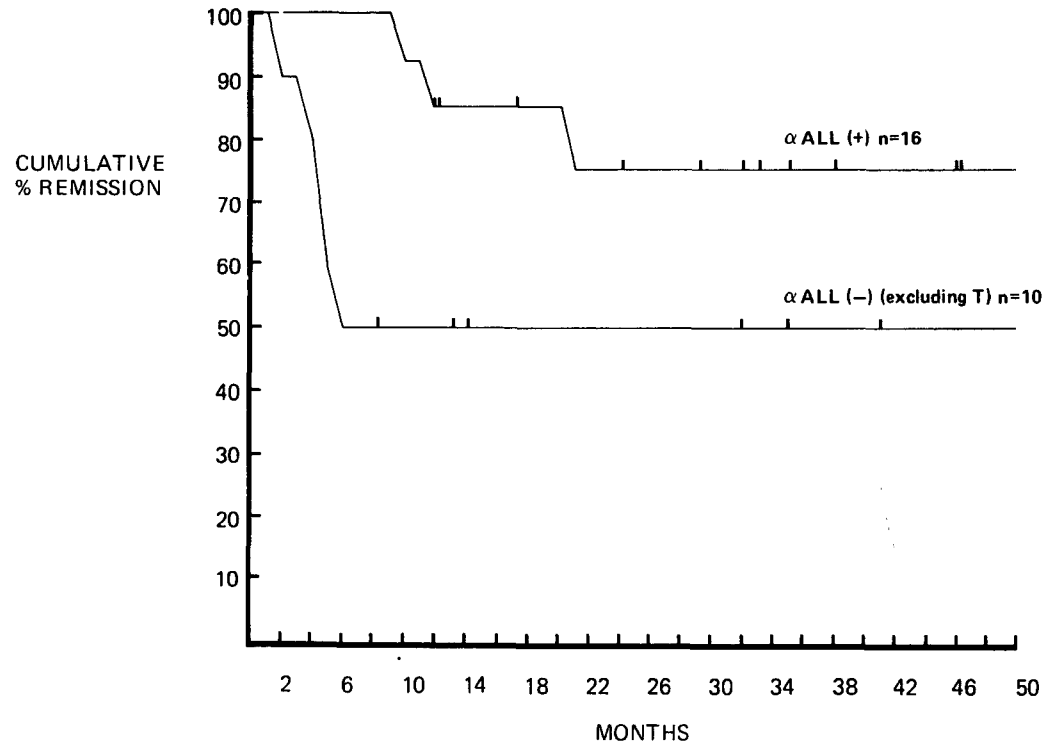

Fig. 2. Duration of complete remission in acute lymphoblastic leukemia. Influence of hepatosplenomegaly

Discussion

These results support the contention that the prognosis in ALL in adults is influenced by the extent of disease at presentation. The presence of hepatosplenomegaly was associated with a very short duration of remission. All patients with a very high blast count (greater than $100 \times 10^9/l$) had relapsed within six months, even though the previously significant adverse influence of a presentation blast count above $10 \times 10^9/l$ has not been confirmed.

The cell surface marker studies demonstrate a significant advantage for patients whose blast cells reacted with the anti-ALL serum. The number of patients was small and the findings should be interpreted with caution. However, the fact that the results are identical with those reported in childhood ALL reported by Chessels et al. Chessels and Hardisty (1977) suggests that our observations are valid.

The response to the initial therapy remains the most important prognostic factor, with survival being very significantly longer for patients in whom complete remission was achieved than for those in whom it is not. The recognition that the same prognostic factors apply to both childhood and adult lymphoblastic leukemia makes it possible to develop treatment programmes for adults on the basis of data obtained from childhood studies. This is most important since the number of adults with lymphoblastic leukemia is small and data are hard to obtain. The recognition that presentation features influence the prognosis must lead to the intensification of therapy for patients with adverse prognostic factors and also the avoidance of intensification of therapy for those patients in whom adverse prognostic factors are not found.

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Ultrastructure and Cell Marker Studies in Lymphoproliferative Disorders

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Surface marker studies have been shown to provide new objective criteria which help to define normal and leukemic cells. Methods for recognizing different subpopulations of lymphocytes are in sharp contrast to conventional morphological techniques, e.g. Romanowsky stained films and paraffin sections, which not only do not allow such distinction to be made, but fail sometimes even to demonstrate the lymphoid or myeloid nature of a neoplastic process. Transmission electron microscopy (TEM) increases the precision of morphological analysis of cell types by demonstrating in greater detail, and with less artefacts, such cell characteristics as nuclear chromatin condensation, nuclear shape, details of cytoplasmic organelles, inclusions, etc. The combination of TEM and surface marker studies is bound, therefore, to increase our power of identification and characterisation of the cells involved in lymphoproliferative disorders.

Materials and Methods

TEM and surface markers were performed in peripheral blood and/or bone-marrow samples from acute and chronic leukemias; lymph node biopsy specimens from non-Hodgkin's lymphomas were also tested. The disorders studied were: chronic lymphocytic leukemia (CLL), leukemic phase of diffuse poorly differentiated lymphocytic lymphomas (PDLL) and follicular lymphoma (FL), hairy-cell leukemia (HCL), prolymphocytic leukemia (PLL), acute lymphoblastic leukemia (ALL), and Sezary's syndrome. The B- and T-cell markers, namely, surface membrane immunoglobulins (SmIg) and rosetting tests with mouse and sheep RBC cells [2,4] were investigated in 194 cases. Cases of ALL were also investigated with an anti-ALL serum to detect the "common-ALL" antigen (Dr. M.F. Greaves, Imperial Cancer Research Fund) [5], and for the enzyme terminal transferase (TdT) (Professor A.V. Hoffbrand and K. Ganeshaguru, Royal Free Hospital) [7]. TEM was performed in 80 cases. In several instances cytochemical techniques for acid phosphatase, myeloperoxidase and immunoperoxidase were also studied at TEM level. For TEM the specimens were fixed in 3% glutaraldehyde and processed according to standard techniques. The material was embedded in Araldite and ultrathin sections were viewed with an AE1 6B electron microscope.

Results

The terms "differentiated", "poorly differentiated" and "undifferentiated" used to define certain conditions, will refer only to the morphological appearances.

Differentiated B-cell Disorders

These include B-cell leukemias: CLL, PLL, HCL and FL. In all the cases one or both of the B-cell markers used were positive.

Morphology

The predominant cell seen in *B-CLL* is a small lymphocyte with scanty cytoplasm, heavily condensed nuclear chromatin and inconspicuous Golgi apparatus (Fig. 1). Prolymphocytes predominate in *B-PLL;* they have a prominent nucleolus in the presence of peripheral chromatin condensation (Fig. 2). A spectrum of cells, depending on the degree of chromatin condensation and the shape of the nucleus can be seen in *B-PLL. FL* is characterized by cells with cleaved (notched) nuclei with variable degrees of chromatin condensation. Small cleaved cells are seen particularly in the peripheral blood in the cases associated with high WBC (Fig. 3); large cleaved cells with less chromatin condensation were predominant in the lymph nodes of the same cases.

Surface Markers

According to the density of SmIg, reflected in the intensity of the immunofluorescence reaction with polyvalent and monovalent antisera, cases were defined as having negative, weak, moderate or strong fluorescence. In B-CLL, 80% of cases had a weak to moderate reaction, and in 17% of cases SmIg were undetected; in contrast, 90% of B-PLL cases had a strong reaction. In FL, PDLL, and HCL, about half the cases had weak to moderate intensity and the rest had a strong reaction.

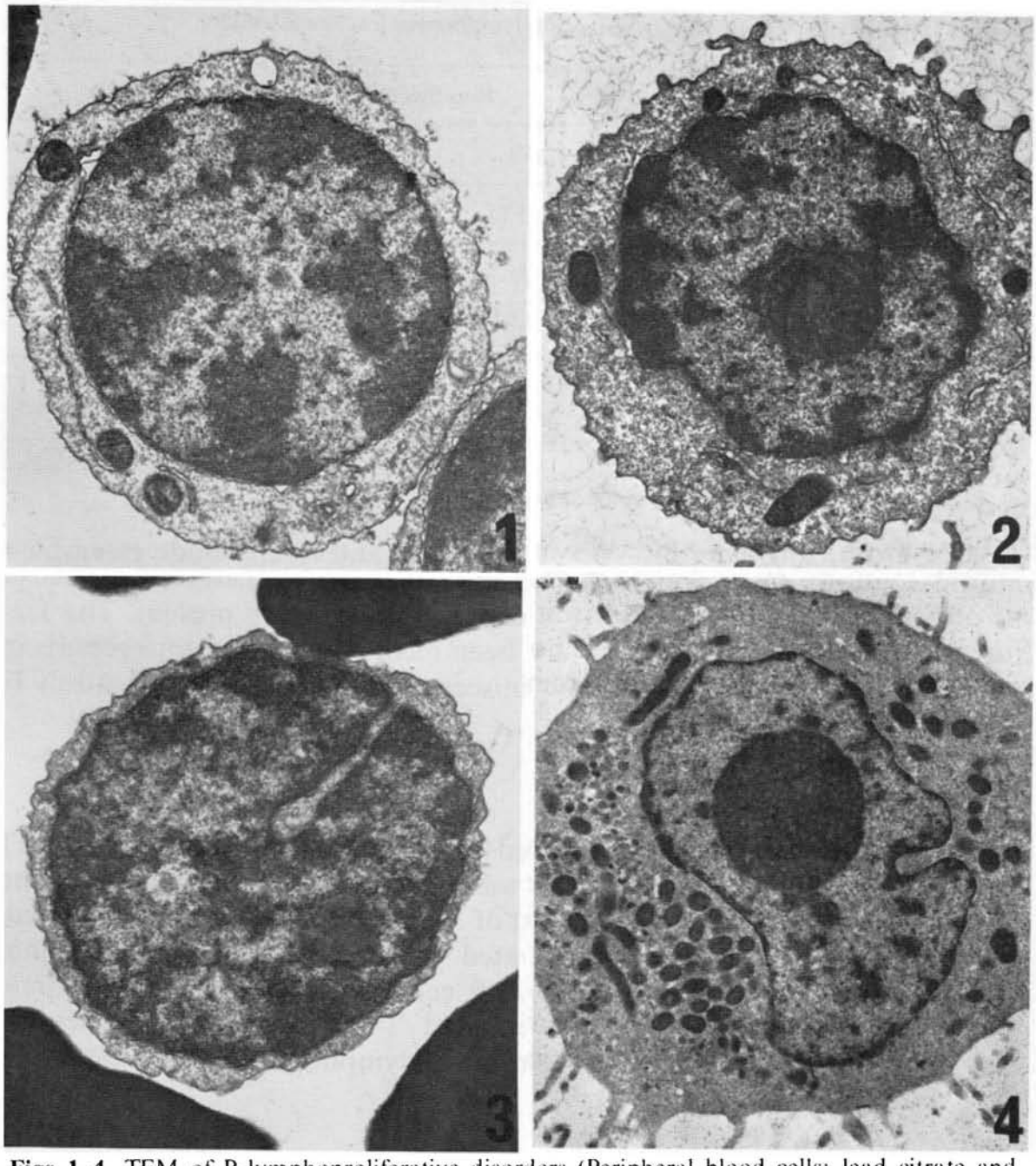
Results with the mouse RBC rosette test (neuraminidase-treated lymphocytes) are shown in Table 1. More than 50% rosettes were observed in the majority of B-CLL cases. In contrast, this was rarely the case in the other conditions where the common finding (73 to 87% of cases) was a low binding (<30% rosettes) of mouse RBC; only 1% of CLL cases showed a similar finding.

Differentiated T-cell Disorders

These include T-CLL (3 cases), Sezary syndrome (4 cases) and a case of T-cell lymphoma.

Morphology

A common feature of the cells of these cases was the irregular nuclear outline, a prominent Golgi zone, and the presence (in variable proportion) of



Figs. 1–4. TEM of B-lymphoproliferative disorders (Peripheral blood cells: lead citrate and uranyl acetate stain)
1. Typical lymphocyte in CLL (× 13000)
2. Prolymphocyte in PLL: prominent nucleolus and peripheral chromatin condensation (× 13000)
3. Small cleaved lymphocyte in FL (× 13000)
4. B-ALL. lymphosarcoma type. Larger size and no peripheral chromatin condensation main difference from PLL (× 11000)

electron dense granules in the cytoplasm, which showed acid phosphatase reactivity. The granules seen at E/M correspond to the azurophil granules seen at light microscopy [1]. In *T-CLL* the nucleus was frequently, but not always, irregular; rarely it resembled the Sezary cell (Fig. 5). In the Sezary cells the main feature was the very convoluted (cerebriform) nucleus (Fig. 6) with condensed chromatin with or without a prominent nucleolus. In the

Table 1. Mouse RBC rosettes in 166 cases of B-lymphoproliferative disorders

Disease	No. of cases	Rosettes in peripheral blood samples[a]		
		<30%	30–49%	≥50%
CLL	100	1 (1%)	21 (21%)	78 (78%)
PLL	20	16 (80%)	4 (20%)	—
FL	11	8 (73%)	1 (9%)	2 (18%)
PDLL & B-ALL	15	13 (87%)	—	2 (13%)
HCL	20	10 (59%)	7 (41%)	—

[a] More than 50% leukaemic cells in the samples tested

case of *T-cell lymphoma* the cells in the blood and lymph node resembled morphologically those seen in T-CLL and some had the cerebriform nucleus of Sezary cells (Fig. 7); electron dense granules were present. The features of this condition, which has not been recognized in previous reports of non-Hodgkin's lymphomas, are reminiscent of those described as Adult T-cell leukemia in Japan [10].

Surface Markers

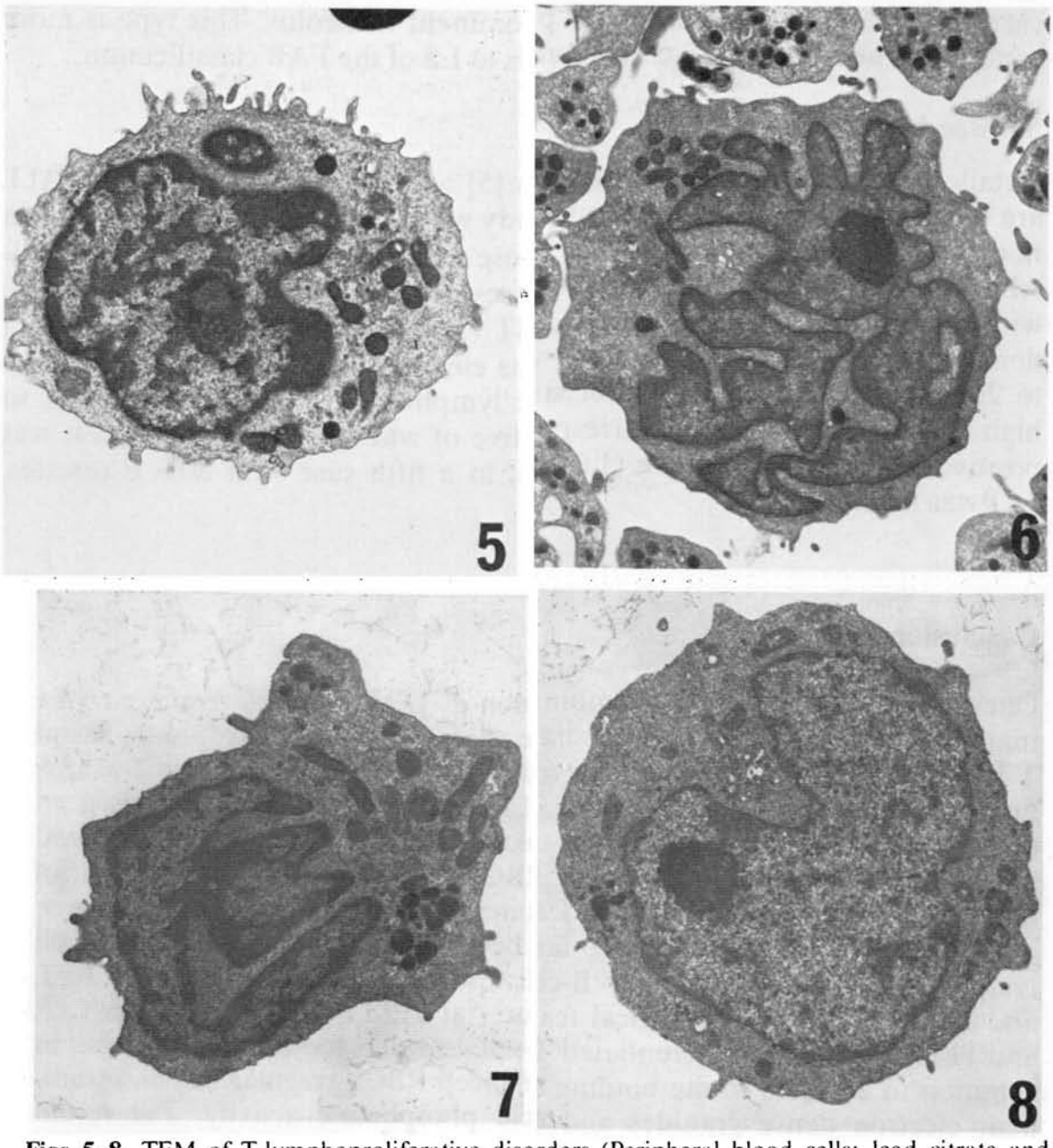
In all cases the leukemic cells formed rosettes with sheep RBC cells. TdT estimations showed that the enzyme was undetectable in the 3 T-CLL's, the case of T-cell lymphoma and in 2 out of 3 cases of Sézary syndrome tested. One of the last-mentioned had elevated values: 22,4 U/10^8 cells (normal values in bone marrow up to 1,5 U/10^8 cells) [7]. These findings contrast with those seen in the less differentiated T-cell disorders (see below). Similar results with TdT estimations in T-lymphoproliferative disorders were recently reported by Penit et al. [8].

Poorly Differentiated or Undifferentiated Disorders

These include conditions characterised by the proliferation of blast cells (with little or no heterochromatin): ALL, PDLL and lymphoblastic lymphoma.

Morphology

B-ALL cells have either features resembling those seen in Burkitt's lymphoma, namely, lack of nuclear maturation with fat globules and polyribosomes in the cytoplasm (L3 in the FAB classification), or those of PDLL with an irregular nuclear outline and a very large nucleolus (lymphosarcoma type) (Fig. 4). The latter cells are distinct from PLL (Fig. 2) because they are larger and have no nuclear chromatin condensation. In *T-ALL* and lymphoblastic lymphoma the nucleus is frequently irregular or convoluted with a



Figs. 5–8. TEM of T-lymphoproliferative disorders (Peripheral blood cells: lead citrate and uranyl acetate stain)

5. Lymphocyte with prominent nucleolus and electron dense granules in the cytoplasm in T-CLL (× 10000)
6. Typical Sézary cell with a prominent nucleolus (× 10000)
7. T-cell lymphoma cell with features resembling Sézary cells (× 11000)
8. Blast cell in a case of T-ALL with indented nucleus and a prominent Golgi apparatus (× 11000)

prominent Golgi zone (Fig. 8), where the acid phosphatase reaction is often localized. *Non-B, non-T ALL* includes two types of cells: 1. Small cells with a round or indented nucleus and inconspicuous nucleolus, some peripheral chromatin condensation and clumps of heterochromatin inside the nucleus. This type is seen more often in children and corresponds to L1 of the FAB classification. 2. Large blasts with more abundant cytoplasm, irregular nu-

clear outline, open chromatin and prominent nucleolus. This type is more common in adult ALL and corresponds to L2 of the FAB classification.

Surface Markers

Detailed accounts of surface markers [5] and TdT estimations [7] in ALL are given elsewhere. In the present study we observed high values of TdT in non-B, non-T ALL, particularly in those cases positive with Greaves anti-ALL serum [5,7]. The cells from 5 cases of adult ALL that were negative with the anti-ALL serum had high TdT values in 3 cases, and TdT was not demonstrable in 2. In T-ALL, TdT was elevated in the 3 cases tested (26,6 to 202 U/10^8 cells). In lymphoblastic lymphoma TdT values were not so high (4,5 to 15,4 U/10^8 cells) irrespective of whether the E-rosette test was positive (3 cases) or negative (1 case). In a fifth case with 80% E-rosettes, TdT was negative.

Conclusions

Our studies showed that the combination of TEM and surface and enzyme markers makes possible more accurate characterisation of neoplastic B and T lymphocytes than can be achieved with routine morphological assessment. B-CLL (the common form of CLL) can be distinguished clearly from other conditions with peripheral blood lymphocytosis by the typical morphology, the binding of mouse RBC rosettes and the weak pattern of SmIg. B-PLL also has well defined features by morphology and cell markers; its characteristic cells have not, so far, been found among the various B-cell lymphomas studied. The other B-cell disorders studied, PDLL, FL, HCL and B-ALL, have morphological features at TEM quite distinct from CLL and PLL. Cells in the differentiated T-cell disorders have several features in common in addition to the binding of sheep RBC: irregular nuclei, prominent electron dense granules and acid phosphatase activity. The recent report of Grossi et al. [6], describing the morphological features of the helper (T_M) and suppressor (T_G) T-lymphocyte subpopulations, is of great interest as the findings in the T_G cells resemble those seen in T-CLL. Two of our T-CLL cases were negative for the enzyme α-naphthyl acetate esterase, which appears to be characteristic of T_M cells [6], and one of them was positive for the Ia antigen, a finding also associated with T_G cells [9]. This, and the preliminary data of Uchiyama et al. [10] suggest that T-CLL may be a disorder of T-suppressor cells. TdT studies [7] help not only in distinguishing ALL from AML, but also between immature (poorly differentiated) and mature (differentiated) T-cell disorders. The combination of good morphology (TEM), surface markers, including Greaves's anti-ALL serum [5], TdT [7] and the acid phosphatase cytochemical reaction [3] permits a better identification and subsequent classification of most leukemias and non-Hodgkin's malignant lymphomas.

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Intermediate Dose Methotrexate (IDM) in Childhood Acute Lymphocytic Leukemia (ALL)

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Abstract

We employed three courses of intermediate dose Methotrexate (IDM) added onto a standard induction and maintenance program with the concept of both central nervous system (CNS) prophylaxis and simultaneous systemic intensification. Cranial radiation (RT) was not employed as CNS prophylaxis.

Fifty of 52 patients (to age 18) achieved complete remission. Time on study now ranges from 22–68 months with a median time of 33 months. We separated the children into standard risk and increased risk. We defined increased risk as a WBC over 30000/mm^3 at presentation and an age of less than two years or greater than 10 years at presentation. There have been 15 relapses on these 50 patients; 11 occurred in increased risk patients (of 22 increased risk patients) and four occurred in standard risk patients (of 28 standard risk patients). There were seven CNS relapses, six systemic relapses, one simultaneous systemic and CNS relapse and one testicular relapse. Toxicity to the IDM was small with the worst problem being mucositis. No leukoencephalopathy occurred. The control of hematological relapse is excellent and the avoidance of potential long-term complications noted is even of greater importance.

Introduction

In the last 15 years there has been a remarkable improvement in the actual "cure" of children with acute lymphocytic leukemia (ALL) [1–7]. Primarily, this improvement has been due to the use of central nervous system (CNS) "prophylaxis" as well as effective systemic chemotherapy [1–7].

In the early 1960's, with the availability of effective Systemic therapy, but prior to effective CNS prophylaxis, it became apparent that approximately 50% of these children would develop CNS leukemia [8]. Once they

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developed CNS leukemia, very few were cured. In the mid 1960's, effective methods of CNS prophylaxis were first employed to prevent overt CNS leukemia and eventual systemic relapse and death. The technique of cranial RT and intrathecal methotrexate (IT MTX) as CNS prophylaxis reduced the incidence of CNS disease to approximately 10% [1–3,5,6]. In 1968, Cancer and Leukemia Group B (CALGB) in Protocol 6801, utilized prophylactic IT MTX and found that instead of 50% developing overt CNS leukemia, only 23% of the children developed this complication [4].

However, cranial RT clearly has limitations, i.e., it cannot eradicate leukemic cells in sanctuaries other than the cranial cavity, e.g., the gonads, etc. The long-term toxicity from prophylactic cranial RT also was a growing concern. Therefore in 1972, we began a study with the following objectives: a) to prevent the development of CNS leukemia without employing cranial RT, b) to intensify systemic therapy and thus eradicate leukemic cells in other sanctuaries and thereby improve the "cure" rate. We based this study on pharmacologic data which demonstrated that intravenous IDM at a dose of 500 mg/m^2 was capable of adequately entering the cerebrospinal fluid (CSF) [9], and hopefully simultaneously penetrate other sanctuaries to a like degree. This report describes the clinical results of this study.

Materials and Methods

52 patients with newly diagnosed ALL were treated according to the protocol depicted in Fig. 1 which was instituted in the Department of Pediatrics at Roswell Park Memorial Institute (RPMI) in August 1972. Following induction with steroid, Vincristine, and L-Asparaginase, three courses of IDM were administered at three weekly intervals. IDM was given at 500 mg per m^2 one-third by intravenous (IV) push and two-thirds by IV infusion over 24 hours. IT MTX at 12 mg/m^2 was given from one-half to two hours after the initiation of IV MTX. 24 hours following completion of IV MTX, a single dose of citrovorum factor (leucovorin) was given at 12 mg/m^2. With moderately severe mucosal ulceration, the subsequent course of IDM was delayed until there was complete healing. The next IDM was then given at full dosage, but an additional dose of leucovorin at 12 mg/m^2 was given 72 hours from the start of IDM (48 hours after completion of IDM). Following high dose MTX, the patient received maintenance therapy consisting of daily oral 6-mercaptopurine and weekly oral MTX and pulse doses of steroid and Vincristine (Fig. 1).

All children with ALL or acute undifferentiated leukemia who could not be identified as acute myelocytic leukemia or acute monomyelocytic leukemia were entered on the study.

From August 1972 until August 1976, when the study was closed to patient accrual, 52 patients were entered, ranging in age from 6 months to 17 years (Table 1). There were 26 females and 26 males at presentation.

Patients were classified as standard risk or increased risk in terms of age or WBC at presentation, i.e., those patients less than two years or greater

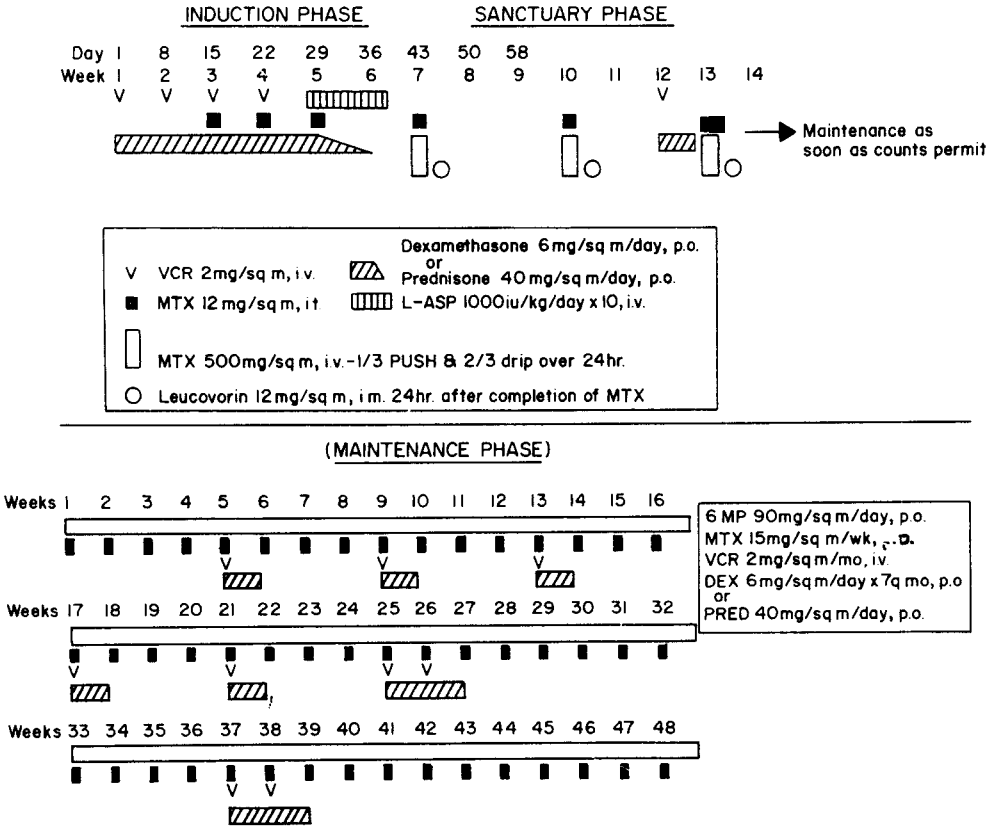

Fig. 1. Schema of treatment in ALL employing IDM

than 10 years of age and those patients who had a WBC greater than 30000 per mm^3 were defined as being increased risk (Table 1). In total, 24/52 children at diagnosis and 22/50 children who achieved complete remission were increased risk. Two children probably had CNS leukemia at diagnosis – one presented with papilledema and one had a right facial palsy of central type but when spinal taps were performed on these children two weeks later, no blasts were detected in the CSF.

Bone marrow aspirates were examined prior to the onset of therapy and

Table 1. Patient analysis at presentation

Number		52
Sex M:F		26:26
Achieved Complete Remission		50
CNS Leukemia at Diagnosis		2
Age	< 2 years	5
	> 10 years	11
WBC	> 100000/mm^3	5
	> 50000/mm^3	8
	> 30000/mm^3	12
Total Number with Increased Risk		22/50 (WBC > 30000/mm^3 or Age < 2 or > 10 years)

again at completion of the induction therapy and every 2–3 months thereafter, or at any time the peripheral blood was suspicious of a relapse. A remission bone marrow has normal granulopoiesis, thrombopoiesis, and erythropoiesis with fewer than 5% lymphoblasts and less than 40% lymphocytes plus lymphoblasts. Induction failure was defined as those patients not achieving a remission bone marrow (less than 5% blasts) by day 42.

For purposes of analysis, complete remission status was terminated by: 1. Bone marrow relapse (greater than 25% blast cells), 2. development of meningeal leukemia (>2 blast cells on cytologic preparations of the CNS or 10 cells/μl not attributable to chemical meningitis), 3. biopsy proven leukemic cell infiltration in extramedullary organs, and 4. death while in remission. Patients are taken off chemotherapy after four years of continuous sustained remission. There are now nine such patients.

All plots of remission duration were determined by actuarial life table analysis.

Results

The time on study now ranges from 22–68 months with a median time on study of 33 months.

Fifty of 52 patients (96%) achieved complete remission. The two induction failures were both in the increased risk group. To date, a total of 15 patients (30%) have relapsed (Table 2, Fig. 2). These included: 7 CNS relapses, 6 systemic relapses, 1 simultaneous systemic and CNS relapse, and 1 testicular relapse. Eleven of 22 increased risk patients (50%) and 4/28 standard risk patients (14%) have relapsed.

Of the four standard risk patients who relapsed, there were two CNS relapses, one systemic relapse and one testicular relapse. Two of the four have died, the remaining two (a CNS and the testes) are both disease-free at 15 months following retreatment.

Of the 11 increased risk patients who relapsed, five were in the CNS, five were systemic, and one was a combined simultaneous systemic and CNS relapse. Five are alive and six have died – all five deaths were in the group who relapsed systemically, and their survival from diagnosis ranged from

Table 2. Current analysis 6/1/1978

15 Relapses (of 50)	– 7 CNS – 6 Systemic – 1 CNS and Systemic – 1 Testes
Risk factor and relapse	– 11 (of 22) Increased Risk – 4 (of 28) Standard Risk
Time on study	– 22 to 68 Months
Median time on study	– 3 Months

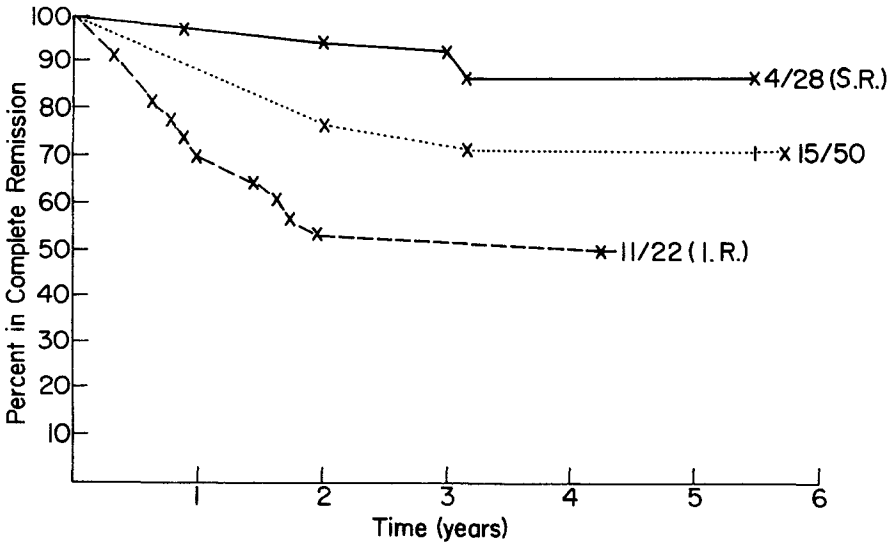

Fig. 2. Duration of complete remission employing IDM. The three curves are: Standard Risk Patients (top), Increased Risk Patients (bottom), and the Overall (middle)

9–17 months with a median of 12 months. Of the 5/11 patients at increased risk who suffered CNS relapse as the initial site of failure, 2/5 are currently disease-free at 33 and 9 months following retreatment.

One of the increased risk children who developed CNS leukemia was a 22-month-old male who presented with a central right facial palsy at diagnosis which subsequently disappeared with induction therapy and was thought to be due to CNS leukemia, but a spinal tap was not performed until two weeks later and there was no lymphoblasts in the CSF at this time. His CNS relapse occurred 23 months after diagnosis. A nine-year-old female who presented 48 months ago with frank papilledema was also thought to have CNS leukemia. The papilledema disappeared with induction therapy and again cytological confirmation from the CSF was lacking. She has remained in continuous complete sustained remission following the initial induction therapy.

Eight of the 50 children who entered complete remission have died and 84% currently remain alive with a median of 33 months after diagnosis (Fig. 3).

At present, of the 15 relapses, four have been successfully retreated and hopefully have a chance for cure. These four are comprised of three CNS relapses and one testicular relapse. Two were in the increased risk group and two in the standard risk group. Disease-free time intervals following retreatment in the CNS relapse group are 9, 17, and 33 months, and in the testicular relapse, 17 months. For CNS relapse, these patients were intensively retreated with steroids and Vincristine and IDM as before (i.e., 500 mg/m^2 on three occasions), but with simultaneous triple intraventricular chemotherapy through an Ommaya reservoir consisting of MTX at 12 mg/m^2 (maximal 15 mg/m^2), AraC at 25 mg/m^2, and Hydrocortisone at 6 mg/m^2 followed by maintenance intraventricular chemotherapy.

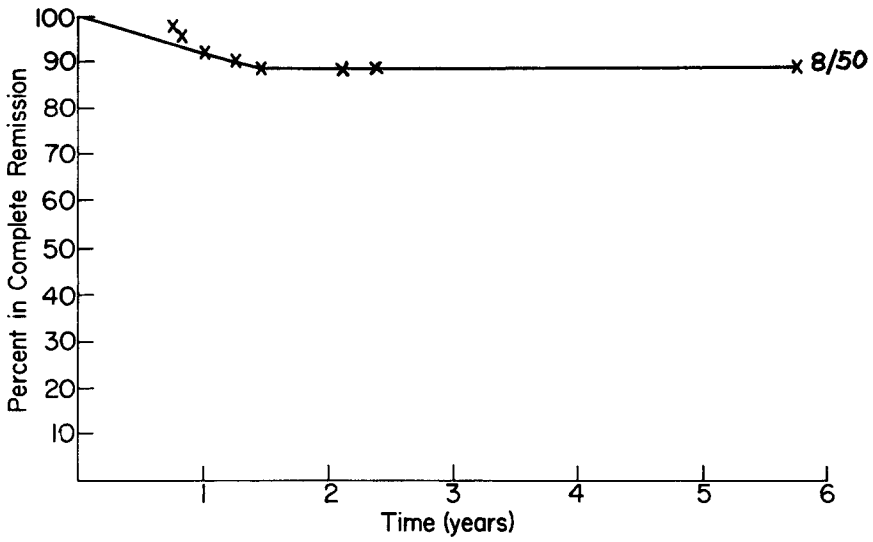

Fig. 3. Survival in 50 patients, treated with IDM, who achieved complete remission

Toxicity

The toxicity (Table 3) from the IDM included: 1. Vomiting occurring in 20/50 patients (40%) and was most pronounced during the first 2–4 hours after the institution of IDM, but occasionally persisting for 24–48 hours; 2. oral ulceration occurring in 20/50 patients (40%) with oral mucositis in 14 and pharyngitis in six patients. This was mild in 17/20, i.e., there were small ulcers which did not substantially interfere with oral intake and salivation; 3. hematologic toxicity occurring in 12 patients (24%) which, however, was minimal in its severity; there were no related clinical manifestations; 4. hepatic toxicity occurring in 11 patients (22%) as evidenced by increase of liver enzymes, particularly the SGOT. However, the peak SGOT was less than twice the normal level and returned to normal in all cases; and 5. transient maculopopular rashes occurring in three cases (6%) and lasting for several days. No case of renal toxicity was noted.

Table 3. Toxicity

Vomiting (with administration)	20/50	
Hematological		
WBC	2 ($<3000/mm^3$)	0 ($<1500/mm^3$)
Hgb	10 (<10 gm%)	0 (<8 gm%)
Platelets	0 ($<100000/mm^3$)	
Mucositis	14/50 (3 moderate and 11 mild)	
Pharyngitis	6/50	
Hepatic	11/50 (mild)	
Skin	3/50	
Renal	0/50	

The overall regimen has been very well tolerated. There has been no life-threatening toxicity and no deaths secondary to IDM. Furthermore, there have been no cases of leukoencephalopathy and no interstitial pneumonia associated with IDM. One adolescent experienced anaphylaxis with the first dose of L-Asparaginase. There have been neither infectious deaths nor toxic deaths for any patient while in remission on this study.

Discussion

This study was clinically based on the early work of Djerassi who demonstrated the effectiveness of high doses of MTX in ALL [10]. CALGB Protocol 6601 demonstrated that the greatest proportion of children remaining in complete remission were those who received the intensive cycles of IV MTX (18 mg/m^2) daily for five days every two weeks (i.e., they received 90 mg/m^2 as a total dose every two weeks) and reinduction pulses of Vincristine and Prednisone for a period of eight months [4]. In addition, CALGB Protocol 6801 demonstrated that "prophylactic" IT MTX during induction was important in preventing overt CNS leukemia [4]. Furthermore, Haghbin, et al. reported data suggesting that intensive systemic chemotherapy may decrease the incidence of CNS leukemia [11].

This study was pharmacologically based on the following: 1. Reports showing that IV IDM resulted in MTX levels of 10^{-7} M reaching the CNS axis and diffusing into the CSF [9]; 2. the studies of Oldendorf and Danson [12] using C^{14} sucrose in rabbits and Bourke, et al. [13] using C^{14}-5-fluro-uracil in monkeys demonstrated that the concomitant use of intrathecal with intravenous injection led to higher levels of drug in the CSF and more even distribution throughout the CNS than with either method alone, and the findings that when MTX is given only via lumbar puncture the distribution of MTX throughout the CSF is very variable [14]. Studies in man corroborate these animal observations, i.e., higher levels of CSF MTX are obtained with concomitant administration of IT and IV MTX than with either technique alone [14]. Thus, the technique employed in the present study of simultaneous IDM plus IT MTX enables one to more effectively bathe the CNS axis; and 3. the MTX levels following 500 mg/m^2 for 24 hours remain at 10^{-5} M in the serum for the 24-hour infusion period [9, 15]. It is thought that such levels will effectively "hit" leukemic cells in other potential sanctuaries such as the gonads, etc.

In large part, the clinical objectives of this study have been attained. Only 2/28 standard risk patients developed CNS leukemia (7%) and 7/50 of the entire population experienced this complication (14%). A recent study of children treated with prophylactic cranial RT and IT MTX or IT Cytosine Arabinoside showed that 53% developed abnormal findings as detected by computerized tomography (CT) [16]. These findings included: dilated ventricles, intracerebral calcifications, demyelination and dilation of subarachnoid space. Furthermore, a reduction in growth hormone secretion in children treated with prophylactic cranial RT has also been reported [17].

A comparable CT scan study has been undertaken in our population and only one child of 43 studied was clearly abnormal. This child presented with papilledema and probably had CNS leukemia at the time of diagnosis and demonstrated mild ventricular dilatation, but no calcification and no decreased attenuation coefficient was seen in any of the 43 cases.

The overall relapse rate in this study is 15/50 (30%) and the CNS relapse rate as the initial site of failure is 7/50 (14%). Thus, 7/15 relapses occurred in the CNS. This proportion is higher than that seen in studies where children received cranial RT plus IT MTX [18] or IT MTX alone because the systemic control is excellent. One possible explanation is that IDM is more effective in eradicating systemic leukemic rests in such areas as gonads, bone marrow, and liver.

Only one male child (1/25) developed testicular relapse. We attribute this to intensifying systemic therapy with IDM which presumably can eradicate disease in sanctuary sites such as liver, spleen and gonads.

A large study (CALGB Protocol 7111) recently reported by Jones [19] has demonstrated a protective value of cranial RT and IT MTX over IT MTX alone in preventing CNS leukemia, but no benefit in the overall complete remission rate. This was the result of an increased incidence of hematological relapse in the patients who received cranial RT. The British Medical Research Council also has observed a higher rate of hematological relapse in these patients receiving prophylactic craniospinal radiation than in those without CNS prophylaxis [20]. In the British study, the radiation-treated group, either cranial or craniospinal, had a greater lymphopenia which may reflect a perturbation of the immune system and thus lead to a greater systemic relapse [21,22].

The toxicity from IDM was minimal and easily tolerated. The control of bone marrow relapse (1/28) in standard risk patients, and in the entire population, 7/50 of the patients with ALL under observation through 68 months, is excellent (Fig. 3), The absence to date of CNS complications from this form of therapy, particularly when compared to the complications of cranial RT [16,17,23–25] are of great importance to children now being cured of this disease.

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Marrow Terminal Deoxynucleotidyl Transferase Activity in Adult Acute Leukemia

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Terminal deoxynucleotidyl transferase (TdT), a unique DNA polymerase that does not require a template, is found in thymocytes and marrow T-lymphocytes normally [1,2]. Shortly after these discoveries, McCaffrey et al. [3] described the presence of this enzyme in the leukemic cells of patients with acute lymphoblastic leukemia (ALL) and Sarin et al. [4] discovered that some patients with chronic myelocytic leukemia in blast crisis have TdT activity in leukemic cells. Currently, the data from a number of laboratories indicates that most patients with ALL have TdT-positive blasts and most patients with acute nonlymphocytic leukemia (ANLL) have TdT-negative blasts [5,6].

We have measured TdT in the bone marrow of 40 adult patients with acute leukemia prior to any therapy, 30 with ANLL and 10 with ALL by the method of Sarin and Gallo [4] as previously described. One unit of TdT activity equals one (n) ^{3}HdGMP incorporation/hr/10^{9} marrow cells. In this report, TdT results are correlated with the Wright's stained morphology of the bone marrow, Periodic acid-Schiff (PAS) reaction, Sudan black reaction and response to therapy.

Results

Marrow TdT results prior to therapy in adult patients with ALL are given in Table 1. All 10 patients had significant elevations of TdT ranging from 3,1 to 313,6 units, with a mean of 120 units. These results are consistent with those of others [5,6]. In 6 of 8 patients studied the PAS reaction was positive, and 5 PAS-positive patients achieved a complete remission with chemotherapy which always included vincristine and dexamethasone. Of 2 PAS negative patients, 1 achieved complete remission and the other did not.

Twenty-four of 30 ANLL patients had TdT activity $< 0,5$ units in bone marrow cells prior to therapy and 6 patients had levels ranging from 1,6 units to 26,3 units. The PAS reaction was studied in 15 TdT negative patients ($< 0,5$ units) and not done in 9. The reaction was positive in 5% to 75% of the marrow blast cells in 8 of the 15 patients studied and negative in 7. Of the 7 PAS negative patients who were also TdT negative, 4 achieved complete remission, one failed therapy, and 2 are currently undergoing treatment. Six of the 8 PAS positive TdT negative patients achieved com-

Table 1. Marrow terminal deoxynucleotidyl transferase in adult ALL

Patient	TdT[a]	PAS	SB	Response to Therapy
1.	292	+ +	–	CR
2.	3.1	+	–	CR
3.	124	–	–	CR
4.	83.5	+ +	–	CR
5.	30	ND	ND	CR
6.	13.7	+	ND	NR[b]
7.	220	–	–	NR[b]
8.	313.6	ND	ND	NR[b]
9.	105	+ + +	–	CR
10.	15.4	+	–	CR

[a] entries are (n) moles incorporation of ^{3}HdGMP/hr/10^9 cells

[b] died of infection while showing significant response to treatment TdT = terminal deoxynucleotidyl transferase, PAS = Periodic acid-Schiff stain, SB = Sudan Black stain, CR = Complete remission, NR = no useful response, ND = Not done

plete remission and 2 failed therapy. Initial therapy consisted of an anthracycline antibiotic and cytosine arabinoside for each patient.

The 6 TdT positive ANLL patients (> 0,5 units), patients 5, 11, 12, 20, 23, and 26 in Table 2 were similarly studied. Four of the 6 were PAS positive, and 2 were negative including the ANLL patient with the highest TdT activity (patient 26). These patients are described in more detail below.

Patient 5: This 45 year old lady was referred with a diagnosis of acute myelocytic leukemia on the basis of Wright's stain bone marrow morphology. Her white blood cell count was 130000/mm^3 with 92% blasts. The platelet count was 67000/mm^3. The spleen was palpable on physical examination and she had bilateral inguinal lymphadenopathy. Her blast cells were agranular, had abundant cytoplasm, and large amphophilic well defined nucleoli. The serum muramidase was 96 μg/ml, the PAS reaction was positive with fine granules staining in 68% of the marrow blast cells. The Sudan black reaction was positive in 80% of marrow cells. The correct diagnosis was felt to be acute myelomonocytic leukemia. The pretreatment bone marrow TdT determination was 7,9 units. The patient received 2 courses of daunorubicin and cytosine arabinoside without benefit. She then was treated with radiotherapy for a small bowel obstruction secondary to leukemic infiltration to which she responded. Subsequently she was treated with vincristine, methotrexate, L-asparaginase, and dexamethasone. Marrow TdT was still elevated, 1,8 units, just prior to initiation of this therapy. There was no response and the spleen increased in size. She was then treated with azacytidine and pyrazofuran to which she responded with a brief complete remission of less than 2 months. She has received additional drugs without benefit although she remains alive 9 months after her diagnosis.

Patient 11: This 48 year old man was referred with a diagnosis of acute myelomonocytic leukemia with a WBC of 34700/mm^3 with 62% blasts. The platelet count was 154000/mm^3. The marrow had 90% blasts with granules

Table 2. Marrow terminal deoxynucleotidyl transferase in adult ANLL

Patient	TdT[a]	PAS	SB	Organomegaly	Response to Therapy	Miscellaneous
1.	<0.5	–	+	No	CR	
2.	<0.5	–	–	No	CR	
3.	<0.5	+	+	No	CR	
4.	<0.5	–	+	No	CR	
5.	7.9	+ +	+	S+N+	NR	Normal Karyotype
6.	<0.5	+ +	+	No	CR	
7.	<0.5	ND	+	No	CR	
8.	<0.5	ND	+	No	NR	
9.	<0.5	ND	+	S+	PR	
10.	<0.5	ND	ND	No	CR	
11.	1.9	–	+	No	CR	
12.	1.7	+	+	No	CR	Normal Karyotype
13.	<0.5	–	+	No	CR	Auer Rods
14.	<0.5	ND	ND	No	NR	
15.	<0.5	+	+	No	NR	
16.	<0.5	+	+	No	CR	
17.	<0.5	ND	ND	No	NR	
18.	<0.5	ND	+	S+	CR	
19.	<0.5	+	+	No	CR	
20.	24.1	+ +	+	No	CR	
21.	<0.5	+	+	No	CR	
22.	<0.5	–	–	No	NR	
23.	1.6	+ +	+	S+	See Text	Auer Rods Normal Karyotype
24.	<0.5	+	+	N+S+	NR	Auer Rods
25.	<0.5	ND	ND	No	NR	
26.	26.3	–	+	S+	See Text	Normal Karyotype
27.	<0.5	+ +	+	No	CR	
28.	<0.5	–	+	No	TE	
29.	<0.5	ND	ND	No	NR	Auer Rods
30.	<0.5	–	+	No	TE	

[a] See table 1. PAS, SB, ND, CR, NR = Same as table 1. S+ = splenomegaly, N+ = lymphadenopathy, TE = Too early to evaluate

and abundant cytoplasm. The PAS reaction was negative and virtually all marrow blasts showed a fine granularity with the Sudan black reaction. There was no organomegaly. Marrow TdT prior to treatment was 1,9 units and serum muramidase was 50 μg/ml. The patient was treated with adriamycin and cytosine arabinoside and had a complete remission which continues for 2+ months.

Patient 12: This 66 year old man with acute myelomonocytic leukemia presented with a WBC of 13800/mm^3 and 50% blasts. The platelet count was 17000/mm^3. There was no organomegaly or lymphadenopathy. The marrow contained virtually 100% blasts and most were agranular but 15% had fine granules on Wright's stain. The blasts had abundant cytoplasm and many had convoluted nuclei with large, amphophilic, well defined single nucleoli. The PAS reaction was positive in 37% of marrow blasts (fine

granules) and the Sudan black reaction was positive in 61%. Serum muramidase assay was not done. The patient had a normal karyotype and a pretreatment marrow TdT of 1,7 units. He achieved a 3 month complete remission with adriamycin and cytosine arabinoside and is currently under treatment for relapse.

Patient 20: This 57 year old female presented with acute myelomonocytic leukemia with a WBC of 1300/mm^3 and 70% blasts. The platelet count was 134000/mm^3. There was no organomegaly. The Wright's stained bone marrow smear showed 58% blasts with agranular abundant cytoplasm. Serum muramidase was 29 μg/ml and TdT was 24,1 units. The PAS reaction was strongly positive with 81% of the blasts showing fine granules or clumps of PAS-positive material. Essentially all the marrow blasts were Sudan black-positive. The patient was treated with daunorubicin and cytosine arabinoside and obtained a complete remission of 5 months duration. At relapse the marrow TdT was 1,9 units and she was retreated with daunorubicin and cytosine arabinoside again, but showed no response. Subsequently, she failed vincristine and prednisone therapy and she is currently under treatment with azacytidine and pyrazofurin.

Patient 23: This 37 year old woman presented with acute myelomonocytic leukemia with a WBC of 9600/mm^3 and 3% circulating blasts. The platelet count was 32000/mm^3. The marrow contained 63% blasts. These cells examined with Wright's stain showed convoluted nuclei and abundant cytoplasm. Fine granules were seen in 10% of blast cells, and 25% of marrow blasts contained Auer rods. The PAS reaction was strongly positive with 69% of marrow blasts containing clumped PAS positive material. The Sudan black reaction was positive in 72% of marrow blasts. Serum muramidase was not done. The marrow TdT activity was 1,6 units. The patient was treated with vincristine and prednisone and had no response. However, after 2 weekly courses of that therapy, the marrow TdT activity was $< 0{,}5$ units and only 18% of marrow blasts gave a positive PAS reaction. Subsequently she was treated with daunorubicin and cytosine arabinoside and had a good partial response with an M2 marrow after the third course which lasted for less than 2 months. She was then treated with azacytidine and pyrazofurin and died without response, 9 months after her diagnosis.

Patient 26: This 31 year old female had a refractory anemia of unknown etiology for 3 months before the diagnosis of acute myelomonocytic leukemia was made. At the time of diagnosis she had a WBC of 2900/mm^3, with 2% circulating blasts, and a platelet count of 113000/mm^3. Her spleen was palpable. The bone marrow contained essentially 100% blasts with abundant cytoplasm containing no granules. The nuclei of the blasts were round and contained large, single amphophilic nucleoli. The PAS reaction was negative and 43% of the marrow blasts gave a positive Sudan black reaction. The patient had a normal karyotype and a serum muramidase of 50 μg/ml. The marrow TdT was 26,3 units. The patient achieved a complete remission after 2 courses of adriamycin and cytosine arabinoside, and is currently receiving maintenance therapy.

Discussion

These data confirm the results of others [5,6] that patients with ALL usually have marrow TdT activity and patients with ANLL usually do not. This study indicates that, in a small series of adult ALL patients TdT activity was uniformly present in PAS positive as well as PAS negative marrows. Furthermore, results of treatment were independent of PAS results. Marrow TdT activity in ANLL patients was also unrelated to PAS positivity. These results are consistent with those of Hutton and Coleman [5]. It is interesting to note, however, that all 6 ANLL patients with marrow TdT activity had a diagnosis of acute myelomonocytic leukemia based on Wright's stain morphology and supported by elevated serum muramidase activity in 4 of 4 patients in which it was determined. Many [7,8] but not all [9], TdT-positive ANLL patients previously reported have had acute myelomonocytic leukemia. One of our TdT positive patients had Auer rods, which are universally accepted as evidence for ANLL as opposed to ALL, and Hutton and Coleman found TdT marrow activity in some patients with Auer rods also [5].

The overall complete response rate for ANLL patients in this study was 61%. Four of the TdT positive patients with ANLL achieved a complete remission (66%) and another achieved a brief good partial response. Thus, in this study, TdT presence or absence did not influence the response rate for patients with ANLL. TdT positivity did not indicate responsiveness to vincristine and prednisone in patient 23 who failed that therapy and subsequently had a good partial response to standard ANLL therapy. Patient 20 also failed vincristine and prednisone therapy for relapse. Thus, TdT assays prior to therapy appear to be of no clinical value in ANLL patients. The value of serial determinations in monitoring disease activity is currently under study. It does appear, however, that ANLL patients with acute myelomonocytic morphology are more likely to have marrow TdT activity than other ANLL patients.

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Clinical Significance of TdT, Cell Surface Markers and CFU-C in 297 Patients with Hematopoietic Neoplasias*

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1. Introduction

Considerable progress has been achieved in recent years in the therapy of human leukemias and lymphomas. However, the less than 100 percent complete remission rates and the comparatively small number of long-term remissions in hematopoietic neoplasias of adults have stimulated efforts to detect additional clinical and cellular prognostic factors. Characterization of the cell phenotype in these disorders has proved to be a most promising approach in this context, based on the hypothesis that the neoplastic cell type is the most important determinant of clinical behavior [1–3,5,10,11,13,14,24]. Furthermore, cell marker analysis might contribute to our understanding of normal cellular differentiation assuming that most neoplastic cell types represent malignant proliferations "frozen" at distinct stages of the normal developmental sequence of the different cell lineages. Colony formation of myeloid committed stem cells in agar (CFU-c, 19), terminal deoxynucleotidyl transferase activity (TdT [4,6,7,12,15–18,22]) and cell surface marker analysis [1–3,5,8–11,13,14,24] have previously been shown to be of diagnostic and prognostic significance in human hematopoietic neoplasias. The study leading to the results described here, has been designed to evaluate a possibly increased discriminatory potential of multiple cell marker analysis in the diagnostic and prognostic evaluation of patients with hematopoietic neoplasias.

2. Materials and Methods

Studies on tissues from a total of 297 patients, predominantly from the adult Leukemia-Lymphoma Service of Memorial Hospital, have been evaluated. Diagnoses of leukemia were made on peripheral blood and bone marrow smears and in most cases confirmed by cytochemical stains. TdT determinations [18], CFU-c assays [19] and cell surface marker analysis [11] were carried out as described previously. Lymphomas were diagnosed on lymph node biopsies and classified according to Rappaport [20,21].

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3. Results and Discussion

3.1. *TdT Distribution in Normal Tissues*

Highest levels of TdT activity were detected in normal thymocytes and low activities in bone marrow mononuclear cells as has been described by others [6]. TdT activity containing cells from bone marrow could be enriched by SRBC-rosetting (×2), dessity separation (×5) and sedimentation at 1×g (×100). The latter procedure consistently produced two separate populations of TdT activity exhibiting cells at sedimentation velocities of 3.5 and 4.5 mm/h, with the 3.5 mm/h peak expressing high levels of the HTLA marker [23] after induction with thymopoietin (Fig. 1). Enriched human CFU-c and mouse pluripotent stem cell (CFU-s) fractions (N. Williams, R. Mertelsmann, unpublished) consistently exhibited no detectable levels of TdT activity.

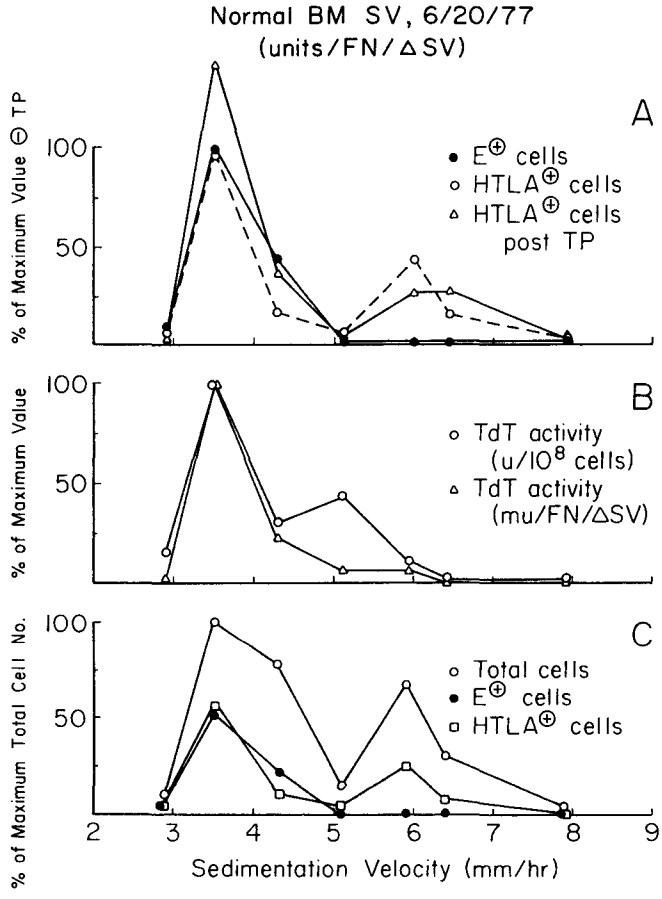

Fig. 1. Separation of normal human bone marrow mononuclear cells by sedimentation velocity at 1×g. Results are expressed in appropriate units per fraction per sedimentation velocity increment.
A. E^+ cells = cells forming rosettes with sheep erythrocytes; $HTLA^+$ cells = cells exhibiting the HTLA marker [23] before and after incubation in the presence of thymopoietin (post TP).
B. TdT activity expressed as specific activity per 10^8 cells and as absolute milliunits of TdT activity recovered per fraction and sedimentation velocity increment.
C. Total number of nucleated cells recovered and distribution of cells forming rosettes with sheep erythrocytes and of cells exhibiting the HTLA marker

3.2. *TdT, CFU-c and Cell Surface Markers in the Differential Diagnosis of Leukemias and Lymphomas*

Analysis of cell marker patterns, clinical diagnoses and TdT activities of 182 patients with acute leukemias (Table 1) and of lymphomas [16] demonstrated highest TdT activities in 70 cases of T and null cell acute lymphoblastic leukemia (ALL). These cases showed absent or low CFU-c formation with normal colony to cluster ratio, characteristic of lymphoid leukemias [19]. Twelve of 32 cases of acute phase chronic myeloid leukemia (CML) also exhibited high TdT activities and lymphoid cell marker characteristics (CML-LB) as did 2 cases of leukemic diffuse histiocytic lymphoma (DHL), 1 acute undifferentiated leukemia (AUL), 1 patient each with polycythemia vera and with a refractory anemia (myelodysplastic syndrome, MDS) who developed an acute leukemia (AL), and 5 cases with a morphological diagnosis of acute myeloid leukemia. Four cases with a morphological diagnosis of acute myelomonocytic leukemia (AMML) and cell marker data consistent with both, ALL and AML, probably represent a 2 "lineage" AL, as has been demonstrated by us in one of these patients [17]. Similar observations have been made by others in acute phase CML with simultaneous or subsequent demonstration of differ-

Table 1. TdT. cell surface markers and CFU-c in the differential diagnosis of acute leukemias[a])

Surface	TdT	CFU-c pattern	Clinical diagnosis	Cases studied n	TdT Specific activity PB mean	TdT Specific activity BM mean (μ/10^8 cells)
null	+	L	ALL	30	12.8	10.9
			LBL. leukemic	21	3.57	6.59
			CML. LB	12	23.6	32.7
			MDS. LB	1	—	15.7
			DHL. leukemic	2	—	10.6
			AUL	1	46.7	—
			AML	5	5.19	5.82
mono	+	AML	AMML. 2 clones?	4	—	3.48
T	+	L	ALL	14	14.2	17.8
			LBL. leukemic	5	11.4	—
T	–	L	ALL. LBL	2	<.05	<.06
null/mono	–	AML	AML. AMML. AMOL. EL	57	<.05	<.05
null		AML	AUL	3	<.01	<.01
null		AML	CML. MB	20	<.05	<.05
mono		AML	DHL	1	<.01	<.01
B	–	L	ALL	1	<.01	—
B	(+)	L	ALL	2	.14	—
null (pre-B?)	(+)	L	CML. LB	1	.26	.15

[a] for abbreviations see text

ent phenotypes [8,9]. No TdT activity was detected in 81 cases of leukemias exhibiting CFU-c and surface characteristics consistent with AML with clinical diagnoses of AML, acute monocytic leukemia (AMOL), AMML, erythroleukemia (EL), AUL, myeloblastic acute phase CML (CML-MB), and one case of "true" histiocytic lymphoma. No TdT activity was detected in 1 case each of T-cell ALL and lymphoblastic lymphoma (LBL) in leukemic phase, probably representing proliferations of more mature T cells. Two of 3 cases of B-cell ALL as well as 1 case of acute phase CML exhibited a lymphoid CFU-c pattern and low levels of TdT activity. Whether this represents low levels of TdT activity in early B-cells [25] or an admixture of cells containing high levels of TdT to a predominating TdT negative cell population is unknown at present.

No detectable TdT activity was observed in blood cells and, occasionally, low normal values in marrow cells from all patients studied with clinical and cell marker diagnoses of chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL), multiple myeloma (MM), Waldenström's macroglobulinemia (WMG), chronic phase CML, chronic myelomonocytic leukemia (CMMOL), and myelodysplastic syndrome. In several patients with CLL, HCL, CML, and myelodysplastic syndromes also lymph node, spleen and cells from involved body fluids were studied exhibiting very low to undetectable levels of TdT activity. One patient with CML who developed a TdT-positive acute phase, did not show any TdT activity in his peripheral blood cells 5 months prior to the acute phase. In the differential diagnosis of malignant lymphomas [16], determinations of TdT activity have been found most useful in confirming or ruling out a diagnosis of T or null cell LBL [18]. We have observed two cases of DHL exhibiting high levels of TdT activity [18] as has been reported by Donlon [4]. Most cases of so-called DHL represent B-cell proliferations, while "true" histiocytic lymphomas are rare (Table 2). Morphology does not appear to allow discrimination between these 3 different cellular types of DHL, which probably require different therapeutic approaches [10,11]. Most leukemias and lymphomas of T-cell lineage are of immature T-cell type, exhibiting high levels of TdT activity and carrying clinical diagnoses of ALL or LBL. While we have seen rare examples of TdT negative ALL and LBL of mature T-cell type, mononuclear cells from patients with Mycosis fungoides or Sezary's syndrome consistently do not exhibit TdT activity in blood, marrow or other involved tissues. In addition, 1 case of diffuse poorly differentiated lymphoma (DPDL) and DHL exhibiting a TdT-negative T-cell phenotype have been observed. Sixty-two cases of B-cell lymphoma with morphological diagnoses of diffuse or nodular histiocytic (DHL, NHL), mixed (DML, NML) or poorly differentiated lymphocytic (DPDL, NPDL), diffuse well differentiated lymphocytic lymphoma (DWDL), Burkitt's lymphoma (BL) and Hodgkin's disease (HD) were negative for TdT activity (Table 2). Two cases of ALL of Burkitt's type exhibited low levels of TdT activity in peripheral blood mononuclear cells, while 2 additional patients revealed undetectable levels in blood cells and on lymph node biopsies. Whether this indicates low TdT activity in some cases of Burkitt's lymphoma or an admixture of a TdT positive progenitor cell remains to be analyzed.

Table 2. Cell phenotypes observed in 297 patients with hematopoietic tumors[a]

CFU-c pattern	Cell surface	TdT	Clinical diagnoses (n)
"lymphoid"	"null"	+	ALL (30). LBL (21). CML-LB (12). MPS-LB (2). MDS-LB (17). DHL (2). AUL (1)
	"null"	+	AMML (5). *misleading morphology?*
	T	+	ALL (14). LBL (5)
	T	–	ALL (1). LBL (1). DPDL (1). DHL (1). Sezary's Syndrome (2)
	"null"	(+)	CML-LB (1). Burkitt's lymphoma (2)
	B	–	DHL/NHL/DML/NML (17). DPDL/NPDL (27). CLL/DWDL (14). PLL (1). MM (4). WMG (1). BL (2)
	"hairy"	–	HCL (8)
	?	–	HD (6)
myeloid	"null"	–	AML/AMOL/AMML (57). CML-MB (20). AUL (3). DHL (1). CML (21). CMMOL (1). MDS (8)
	"null"	+	AMML (4). *2 lineages involved?*

[a] for abbreviations see text

3.3. *Prognostic Significance of TdT in Leukemias and Lymphomas*

In AL patients with inconclusive morphology who received vincristine and prednisone because of some lymphoid features by morphology, and cytochemistry, 0/8 responses were seen in TdT negative cases as compared to 5/6 responses in TdT positive cases. One patient with probable 2-"lineage" AL achieved a complete remission (CR) on the ALL protocol (L-10M) while the second patient, after a partial remission (PR) on vincristine and prednisone achieved a CR on subsequent therapy with our protocol for acute non-lymphoblastic leukemia [17]. In acute phase CML, only 1 of 18 patients with myeloblastic CML by cell marker data and morphology, achieved a PR on chemotherapy in contrast to 4 CR and 1 PR out of 10 patients with lymphoblastic CML. Similar data of a controlled trial in acute phase CML have recently been reported by Marks et al. [15].

In 60 out of 62 patients with ALL or leukemic LBL, high levels of TdT were detected during active disease either before any chemotherapy was started or during remission induction. The 2 cases of TdT negative ALL, both of T-cell type, probably represent mature T-cell proliferations. It is of interest, that these two patients, as well as the patient with TdT negative DPDL of T-cell type, did not achieve a complete remission on vincristine and prednisone alone, but required cyclophosphamide and adriamycin. In 3 out of 6 patients with LBL without bone marrow involvement by morphological criteria, elevated TdT activity was observed in initial marrow samples. Two of these patients subsequently developed clinical marrow involvement suggesting an increased sensitivity of the TdT assay for detection of subclini-

cal marrow involvement as compared to cytological and histological techniques. During remission induction, lowest bone marrow TdT activities were observed in 7 patients who achieved long-term remissions (mean spec. act. 0.66 U/10^8 cells), while 6 patients who entered a CR of <1 year's duration showed a mean specific activity of 2.23 and 9 who did not enter CR of 5.25 U per 10^8 cells. However, there was a considerable overlap between specific activities from each group.

In continuous complete remission, 13 patients off chemotherapy exhibited a mean specific activity of 0.33 U/10^8 marrow cells, considerably higher than seen in normal controls (<0.1 U/10^8 cells). Patients with ALL in CR without relapse on chemotherapy showed a mean specific activity of 0.37 U/10^8 cells in contrast to patients who were studied during CR and relapsed subsequently, with a mean specific activity of 1.3 ($p < 0.05$). We have previously shown that marrow TdT activities in ALL in remission vary considerably over time, even in patients in CR off chemotherapy [18]. Further sequential studies are necessary in order to define criteria for impending relapse, because one single determination exhibiting a high or low level of TdT activity does not appear to allow prognostic conclusions.

Conclusions

1. TdT has been found to be a highly specific marker for immature cells of T-cell lineage in over 1500 samples from 297 patients with leukemias and lymphomas, and from normal controls. "True" exceptions must be rare.
2. Acute leukemias with cell marker and clinical features of ALL are not only seen in acute phase CML, but also in myeloproliferative and myelodysplastic syndromes terminating in an acute leukemia.
3. The majority of patients with leukemias and lymphomas exhibiting high levels of TdT activity will achieve complete remission on an ALL-type protocol irrespective of morphology.
4. The majority of patients with acute leukemias exhibiting low or undectable levels of TdT activity will not achieve complete remission on an ALL-type protocol.
5. Preliminary observations suggest that determination of TdT activity allows detection of subclinical bone marrow involvement in some cases of TdT positive lymphomas and that sequential analysis of TdT activities in marrow cells during remission induction and in complete remission might allow to predict early relapse in ALL.

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Biochemical Determinants for Antileukemia Drug Treatment

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The success or failure of chemotherapy for leukemia is determined biochemically by a combination of biochemical aberrations in the leukemic cells relative to their non-malignant counterparts, and biochemical perturbations created by antileukemic drugs. Examples of recent developments in our understanding of these factors include studies on the metabolism of cytosine arabinoside, the use of high-dose methotrexate, the relationship between "de novo" and "salvage" nucleic acid synthesis in leukemic cells, and the development of new drugs such as the adenosine deaminase inhibitor, 2'-deoxycoformycin.

1-β-D-arabinofuranosyl cytosine (Ara-C) requires phosphorylation by kinases to the triphosphate Ara-CTP, in order to exert its antileukemic effect by inhibiting DNA polymerase. The deamination of Ara-C, catalysed by cytidine deaminase, produces the inactive metabolite 1-β-D-arabinofuranosyl uracil. Early studies on the mechanism of action of Ara-C in man concentrated on enzymatic determinations of the kinases and deaminase, to reflect the potential for Ara-CTP formation in any given cell population. Steuart and Burke (1971) demonstrated an inverse relationship between cytidine deaminase activity and overall response to Ara-C, and that the development of resistance to this drug was associated with increased cytidine deaminase activity. Conversely Tattersall et al. (1974) claimed a determining role for deoxycytidine kinase activity, and Smyth et al. (1976) found no direct correlation between the ratio of kinase-to-deaminase activities with clinical response. It is likely that variation in experimental procedure accounts for some of the controversies in these various studies, but there are inevitable limitations inherent in indirect enzymatic studies of this nature. More recently attention has been focused on the direct measurement of intracellular levels of Ara-CTP. In a study of patients with acute and chronic leukemias, Chou et al. (1977) found that blast cells from patients with acute myeloid leukemia who were clinically responsive to Ara-C, produced two-fold more Ara-CTP than did cells from chronic myelocytic, acute or chronic lymphocytic leukemia, or normal subjects. Significantly increased levels of Ara-CTP were produced in the presence of tetrahydrouridine, a potent inhibitor of cytidine deaminase, but the development of acquired resistance to Ara-C was not associated with diminished formation of Ara-CTP. These findings indicate that the mere production of high levels of Ara-CTP is not of itself sufficient to determine therapeutic response to Ara-C. Recent studies by Rustum et al.

(1978) measured not only the formation of Ara-CTP in human leukemic cells, but also the time for which adequate levels were retained in the cells. Thus in 8 of 9 patients who achieved a complete clinical remission, high initial levels of Ara-CTP were produced, and greater than 9.7 pmoles/10^7 cells were retained at 4 hours incubation. In contrast, in 8 patients whose cells retained $<0{,}71$ pmoles Ara-CTP/10^7 cells at 4 hrs, only 4 attained complete remission, and the durations of the latter were shorter than with the former groups of patients. Further work on Ara-CTP retention is clearly indicated, but these results are encouraging in their potential for more accurate prediction of response to Ara-C treatment, than has previously been shown with the indirect enzymatic approach.

Methotrexate has long been known to be an active agent in the treatment of acute lymphocytic leukaemia. Recent developments in understanding the pharmacokinetics and mechanism of action of this drug, have led to its use in sufficiently high dosage to require "rescue" of vital host tissues following methotrexate infusion – either with folinic acid, or more recently with nucleosides. The rationale behind the administration of high concentration infusions of methotrexate is based on the concept of enhancing perfusion into "sanctuary" sites such as the testes and central nervous system (Stoffel et al., 1975) and enhancing the free intracellular methotrexate concentration – a factor recently shown to contribute towards maximal cytotoxic effect (Goldman, 1975; Bender and Makulu, 1976). Wang et al. (1976) have monitored the pharmacokinetics of methotrexate administered as a 24 hr intravenous infusion to patients with ALL, at doses of 500 mg/m^2, followed 24 hrs later by folinic acid. Methotrexate in the cerebrospinal fluid reached 1.2×10^{-7} M at 30 min, and remained constant for 24 hrs. Preliminary results indicate that methotrexate used systemically in this way may be effective in the prevention of central nervous system leukemia. Further results of these studies are presented elsewhere in this workshop (Freeman, 1978).

From the biochemical standpoint recent research has focused on alternative – more selective – rescue techniques following methotrexate infusions, than that provided by folinic acid. Evidence is accumulating to suggest that vital host tissues may be able to utilise pyrimidine and purine nucleosides preferentially over tumour tissues thus bypassing the metabolic lesion created by methotrexate, and restoring nucleic acid synthesis in the normal bone marrow and gastrointestinal tract, with less "rescue" of the tumour cells. Tattersall et al. (1975) demonstrated that the delayed administration of thymidine to BDF_1 mice bearing the L1210 leukemia was superior to folinic acid in preventing lethal methotrexate toxicity, whilst maintaining antitumour efficacy. Semon and Grindey (1976) using the L1210 system in DBA/2J mice, infused thymidine simultaneously with methotrexate and confirmed enhanced therapeutic selectivity. Extending these studies to man, Ensminger and Frei (1977) have shown that continuous thymidine infusion during and up to 48 hrs after methotrexate infusions, can prevent toxicity from doses of the antifolate of up to 6 g/m^2, although very large doses of the nucleoside were required. The above studies relate only to replenishment of thymidylate synthesis, but methotrexate is known also to inhibit de novo

purine synthesis. Thus the consequences of methotrexate depletion of reduced folate cofactors in any given target organ or disease will depend on the relative dependence of those tissues on pyrimidine and purine nutrition. Harrap et al. (1977) have recently published a study showing that the addition of a purine source (hypoxanthine) to thymidine rescue of methotrexate treated L1210-bearing mice, was superior to rescue with thymidine alone or to folinic acid. These studies indicate that nucleoside rescue is effective in preventing methotrexate toxicity in both animals and man, but selective rescue of host tissues versus tumour awaits confirmation in man. We are currently conducting a Phase I evaluation of pyrimidine-purine rescue in man, for future therapeutic comparion with conventional folinic acid.

Critical to the ability of host or tumour cells being able to utilise exogenous nucleosides is the activity of the "salvage" pathways for purine and pyrimidine re-utilisation. The "de novo" synthesis of purine and pyrimidine nucleotides – the immediate precursors of nucleic acid – involves multiple energy-consuming reactions starting from small molecular weight compounds such as glycine and aspartate, and resulting in the formation of inosinate from which the purine nucleotides dATP and dGTP can be produced, and uridylate the precursor of pyrimidine dCTP and TTP. The so-called "salvage" pathways refer to the reutilisation of pre-formed purine or pyrimidine nucleosides or bases, which by direct phosphorylation can yield the corresponding ribonucleotides and hence deoxyribonucleotides, with energy conservation to the cell. Controversy has existed for some time as to the reliance of human leukaemic cells on one or both of these pathways, but recent studies by Rustum and Higby (1978) and Rustum and Takita (1978) provide useful information on this issue.

Rustum has measured radiolabelled precursor incorporation and the ribonucleotide pools in cells from patients with chronic lymphocytic or myelocytic leukemia, during different phases of the diseases. A low ratio of ATP/IMP indicative of reliance on the salvage pathway was found in patients with stable, chronic leukemia, whereas the development of blast crisis was associated in the same patients with a shift to high ATP/IMP ratios indicating a change in metabolic dependence to the "de novo" synthetic route. Such information has a bearing on the choice of therapy for different stages of leukemia, and contributes to our knowledge of possible targets for the design of new antileukemic drugs. Interest in the design of inhibitors of salvage pathway enzymes has been stimulated in particular, by recent work on adenosine metabolism in malignant lymphocytes and the role of the enzyme adenosine deaminase.

A specific association between the activity of adenosine deaminase (ADA) and lymphocyte metabolism first became apparent with the description of severe combined immunodeficiency disease arising in children born with a congenital absence of this enzyme (Dissing and Knudsen, 1972; Giblett et al., 1972). Although ADA is normally present in all mammalian tissues, activity is highest in the lymphoid system and increases in antigenically stimulated lymphocytes (Hall, 1963; Hovi et al., 1976). The demonstration of greatly increased ADA activity in malignant lymphocytes (Smyth and Harrap, 1975;

Smyth, 1976; Smyth et al., 1978b) led to a search for effective inhibitors of this enzyme, which might by analogy with the severe lymphoid depletion associated with genetic deletion of the enzyme – exert a specific antilymphocytic effect of value in the treatment of lymphocytic leukemia. 2′-deoxycoformycin (DCf) is the most potent inhibitor of ADA, with a Ki of 1×10^{-12} M (Agarwal et al., 1977; Johns and Adamson, 1976). Toxicity studies in normal animals confirmed the hypothesis that effective inhibition of ADA results in severe lymphoid depletion (Smyth et al., 1978a) and led to a Phase I clinical trial which is currently in progress. The preliminary data from this toxicological evaluation indicates that inhibition of ADA with DCf exerts a selective antilymphocytic effect in man. In 8 patients with non-haematological malignancies and normal pre-treatment peripheral blood counts, administration of DCf at 0.25 mg/kg as a single dose resulted in greater than 50% decrease in circulating peripheral lymphocyte counts in 3 out of 4 patients, and greater than 90% decrease in all of 4 patients treated daily $\times 5$. Recovery to normal differential counts was seen by day 14. 4 patients with relapsed ALL having failed all conventional therapy have been treated with DCf at 0.25 mg/kg daily $\times 5$. In one of these heavily pre-treated patients there was no response, and in another the peripheral blast count had decreased by 70% on day 5 with respect to the pre-treatment value. However in the other two patients there was a complete clearing of peripheral blasts from pre-DCf values of 644×10^9/l and 82320×10^9/l respectively. In the latter case this dramatic change in peripheral activity was reflected by shrinkage of previous splenomegally and clearing of blasts from the marrow. Full details of this Phase I study will be published shortly.

In summary, recent research into the mechanism of action of existing antileukemic agents, and their biochemical consequences within the host, has yielded information that may improve the use of drugs such as cytosine arabinoside, and enhance the therapeutic selectivity of methotrexate. Studies of alternative metabolic pathways for the synthesis of nucleic acid by leukemic cells indicates that "de novo" synthesis or "salvage" reutilisation may operate at different stages in the leukemic process, and suggests possible targets for future development of antimetabolite drugs. Ongoing clinical trials with 2′-deoxycoformycin demonstrate that inhibition of the salvage enzyme adenosine deaminase results in selective lymphocytotoxicity, of potential value for immunosuppression and the treatment of lymphoid malignancies.

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Biological and Clinical Effects of a Partially Thiolated Polycytidylic Acid (MPC): A Potent Inhibitor of DNA Synthesis in RNA Tumor Viruses

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Single stranded polyribonucleotides are known to act as efficient templates for the viral DNA polymerases in the presence of complementary oligo-deoxy-ribo-nucleotide primer. Chemical modification of such templates would be expected to alter the interaction between the template and the viral enzyme [1–7]. This appears to be a very useful approach for designing specific inhibitors of viral DNA polymerases, which might find application in the chemotherapy of cancer [8,9].

Our efforts to develop compounds that inhibit viral DNA polymerase by interacting directly to the enzyme led to the discovery of polycytidylic acid analog, containing 5-mercapto substituted cytosine bases (Fig. 1), a partially thiolated polycytidylic acid [10–12]. This compound, abbreviated as MPC (mercapto-polycytidylic acid) was found to inhibit the oncornaviral DNA polymerase in a very specific manner [10–15]. The mode of action of this compound as an inhibitor of viral DNA polymerase, its biological effects on viral oncogenesis, and its clinical application in the treatment of childhood leukemia will be described here.

1. Inhibition of Oncornaviral DNA Polymerase by MPC

The inhibition of DNA polymerases from RNA tumor viruses by MPC was described earlier [10–15]. Partially thiolated polycytidylic acid preparations, MPC I–III (containing 1.7%, 3.5% and 8.6% 5-mercaptocytidylate units, respectively) inhibited the DNA polymerase activity of Friend leukemia virus (FLV) in the endogenous reaction as well as in the presence of poly-rA.$(dT)_{14}$, or poly rC.$(dG)_{12\text{-}18}$; the inhibitory activities were directly proportional to the percent of thiolation. A maximum inhibition was observed with preparations containing 15–17% of the thiolated cytosine bases.

1.1 Mode of Action

The mode of action of viral DNA synthesis by MPC was investigated by product analysis of the DNA polymerase reaction in the absence or in the presence of MPC, as described elsewhere [11]. The reaction mixtures were dissolved with Na-dodecyl sulfate (1%, wt/wt, final concentration), loaded

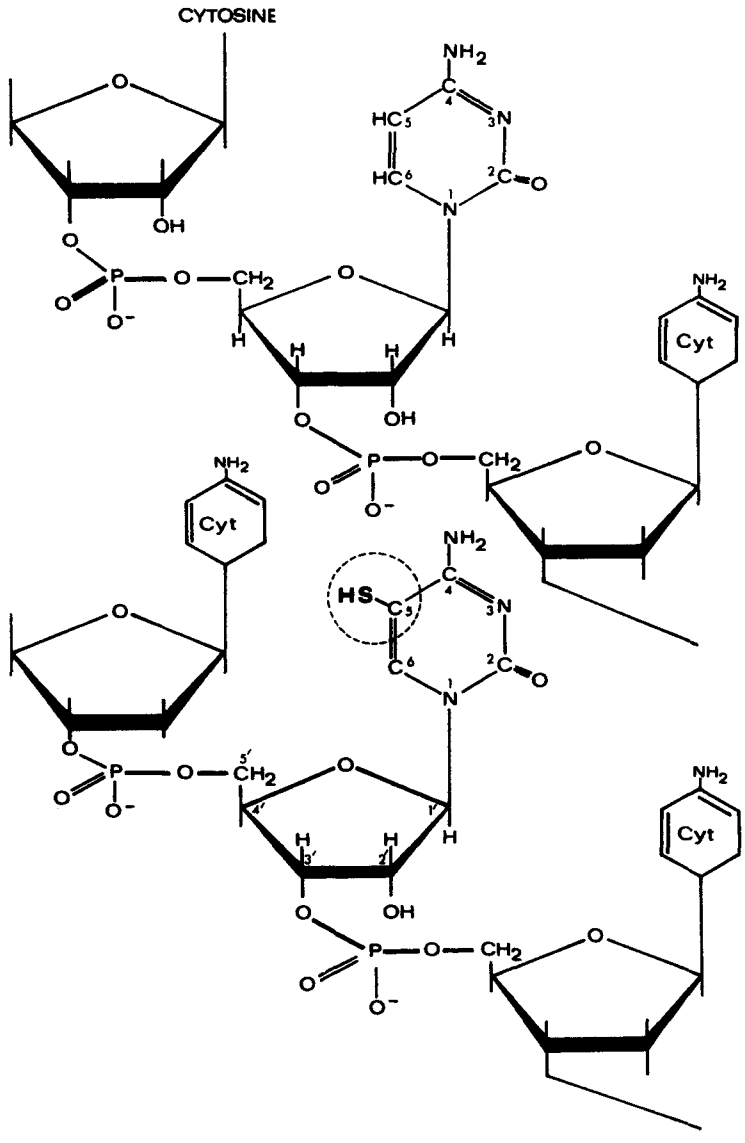

Fig. 1: Schematic presentation of the chemical structure of a partially thiolated polycytidylic acid (MPC)

on a hydroxylapatit column (1 g, Bio-Rad Lab., Munich), eluted with a Na-phosphate gradient (0. 05 – 0. 4 M), collected into about 40 tubes (total vol. approx. 100 ml), and the TCA-insoluble radioactivity collected on GF/C filters (Whatman) and counted in a liquid scintillation counter.

Analysis of the endogenous products of the detergent disrupted virions exhibits 3 DNA species: single stranded DNA (ss-DNA), RNA-DNA hybrids (hy-DNA) and the double stranded DNA (ds-DNA). As follows from Fig. 2, in the presence of MPC (open circles) there is an over-all inhibition of ^{3}H-dTMP incorporation, indicating that the formation of all the 3 species is blocked. This is to be expected since the inhibitor binds to the enzyme. This has been confirmed by ultracentrifugation studies in which the binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase was investigated.

In view of the fact that all of the oncornaviral DNA polymerases examined so far do require a primer-template-like double stranded secondary structure for the initiation of DNA synthesis, it is no surprise that single

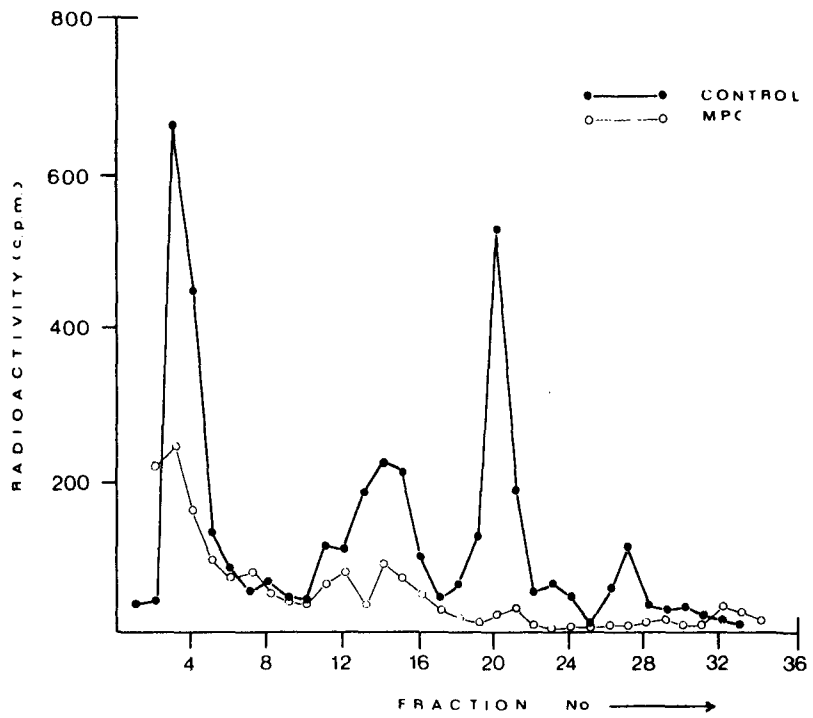

Fig. 2. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxylapatit column. Experimental details are described in the text. The first species to be eluted from the column contained ss-DNA, the second contained hy-DNA and finally, the ds-DNA, eluted in the last peak. The concentration of MPC in the reaction mixture was 20 μg/ reaction mixture

stranded synthetic polynucleotides (unprimed templates) can act as inhibitors of the polymerization reaction. This, presumably, is due to hydrogen bonding of the base sequences between the added polymer and the functional template. Thus, the specificity of inhibition by such polymers is not limited to the viral enzyme system only. On the other hand, minor modifications in the chemical structure of synthetic polynucleotides might be useful to develop inhibitors that interact directly with the enzyme but fail to be transcribed, i.e. they function as a "dead template" for the enzyme. The data from our laboratory have shown that the partially thiolated polycytidylic acid is functioning as a "dead template" in the DNA polymerase system of FLV [11]. The results of these studies can be summarized as follows: 1. The incorporation of ^{3}H-dGMP into DNA by the viral enzyme is stimulated to about 9-fold (compared to the endogenous value) in the presence of Poly rC.$(dG)_{12\text{-}18}$. However, under similar conditions a hybrid of MPC.$(dG)_{12\text{-}18}$ failed to stimulate the incorporation of ^{3}H-dGMP into DNA; 2. In the presence of MPC.$(dG)_{12\text{-}18}$, the increasing concentrations of poly rC.$(dG)_{12}$ in the reaction mixture have no effect on the activity of the enzyme; however, at higher enzyme concentrations the stimulatory effect of poly rC.$(dG)_{12\text{-}18}$ gradually reappears. These data indicate that the viral enzyme has higher binding affinity towards MPC than to its optimal template poly rC. The presence of zinc in reverse transcriptase makes it attractive to suggest that the mercapto group may undergo an interaction with zinc to form a stable complex.

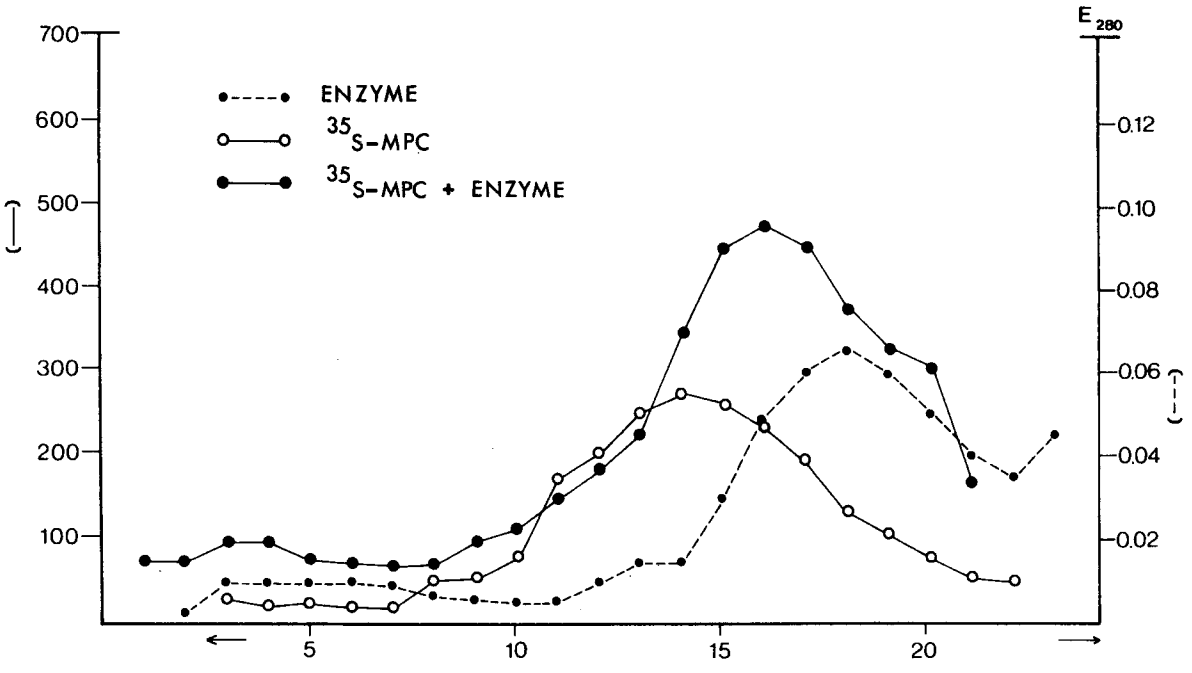

Fig. 3. Binding of ^{35}S-labeled MPC to a purified FLV-DNA polymerase. The specimen in 0.1 M Tris/HCl buffer (pH 7.4) were layered on linear gradients of 10–40% sucrose (RNase-free) in the same buffer and spun at 35,000 rpm (swingout rotor) at 4 °C for 20 hr. The gradients were dripped from below, fractions collected and after dilution, were analyzed for their radioactivity or absorbtion at 280 nm. The MPC preparation contained 10.1% of thiolated cytosine bases (sp. Act. 141 c. p. m./μg MPC)

1.2 Selectivity of MPC Action

In order to determine the selectivity of MPC action, further studies were conducted using DNA polymerases from different sources. As follows from Table 1, the viral DNA polymerases are most sensitive towards inhibition by MPC, whereas MPC is completely unable to inhibit the bacterial DNA poly-

Table 1. Evaluation of the inhibitory response of partially thiolated (SH = 13%). Polycytidylic acid on DNA polymerase from various sources

Source of DNA polymerase	Type of DNA polymerase	Template used	Compound required to inhibit 50% of the Reaction (μg/ml)
Human lymphocytes[a] (1788)	I	Poly(dA)·$(dT)_{12-18}$	30
	II	Poly(dA)·$(dT)_{12-18}$	38
Regenerating rat liver	I	CT-DNA	>100 (38% inhibition at 78 μg/ml)
E. coli K_{12}	I	Poly(dA-dT)	No inhibition >100
RMuLV[a]	Reverse transcriptase	Poly(rA)·$(dT)_{12-18}$	20
FLV	Reverse transcriptase	Poly(rA)·$(dT)_{12-18}$	19.2

[a] These studies were done by Dr. R. Graham Smith at the Laboratory of Tumor Cell Biology of the National Cancer Institute, Bethesda, Md.

merase. Enzymes from human lymphocytes are more sensitive towards MPC inhibition than the DNA polymerase I of regenerating rat liver. In spite of the fact that the experiments were carried out in different laboratories, a comparative evaluation shows that the viral enzymes are at least twice as sensitive as DNA polymerase from another source.

The studies on the selectivity of MPC action were substantiated using the cellular DNA polymerases (α, β and γ) and a reverse transcriptase from human spleen. We have recently discovered a reverse transcriptase in the spleen of patient with myelofibrosis [16,17], a preleukemic disease. This reverse transcriptase is antigenically related to DNA polymerases of primate RNA tumor viruses [16,17], the Simian sarcoma virus (SiSV) and Baboon leukemia virus (BaLV). This observation implicates the viral origin of the reverse transcriptase in myelofibrotic spleen (Fig. 4).

The effect of MCP (SH = 15%) on the activity of cellular DNA polymerases, and on the reverse transcriptase activity from human spleen is shown in Fig. 4. At a concentration of 1 μg/reaction mixture [cf ref. 16], none of the cellular enzymes was inhibited. In contrast, the reverse transcriptase activity was inhibited to approx 20%. At a concentration of 16 μg, the reverse transcriptase activity was inhibited to 80%, whereas the cellular enzymes lost only 25–40% of their activities. Kinetic studies on the DNA-polymerase reaction, catalyzed endogenously by SiSV, revealed that MPC inhibition is of a non-competitive nature (Fig. 5).

2. Effects of MPC on Oncogenesis by RNA Tumor Viruses

To measure the effect of MPC (SH = 8.6%) on the production of splenomegaly by Friend leukemia virus (FLV), we first incubated the cell-free extracts of spleen from mice (Groppel strain) with 100 μg/ml of MPC at 37 °C for 1 hr. In the control group, where no compound was used, the cell-free spleen suspension was preincubated with Tris/HCl buffer, pH 7.4, the solvent for MPC. The aliquots of this suspension were injected (0.2 ml, LD_{90}) into each group, consisting of 10 animals. The spleen weights were analyzed on the 8th or the 12th day after infection [8]. There was a 60% reduction of spleen weights (arithmatic mean of five individual values) in the MPC-treated group, measured on the 8th day after FLV-infection. However, no differences were observed on the 12th day. This is probably due to fact that at this MPC concentration the whole of virus is not inactivated, so that the residual active virus particles lead to potentiation of leukemogenesis.

The studies reported above were extended using MPC in-vitro and in-vivo. The animals were divided into four groups of five each (donors): 1. Group 1 was injected with a viral suspension (citrate plasma from FLV-infected animals, dose LD_{90}) preincubated with Tris/HCl buffer, pH 7.6 for 30 min. at 37 °C; 2. Group 2 was injected with the viral suspension, as in 1, but preincubated with MPC (200 μg per 0.2 ml of suspension) at 37 °C for 30 min. These animals received in addition, on day 5 and day 9 (post infection) 50 μg of MPC, injected intraperitoneally; 3. Group 3 was treated similar to group

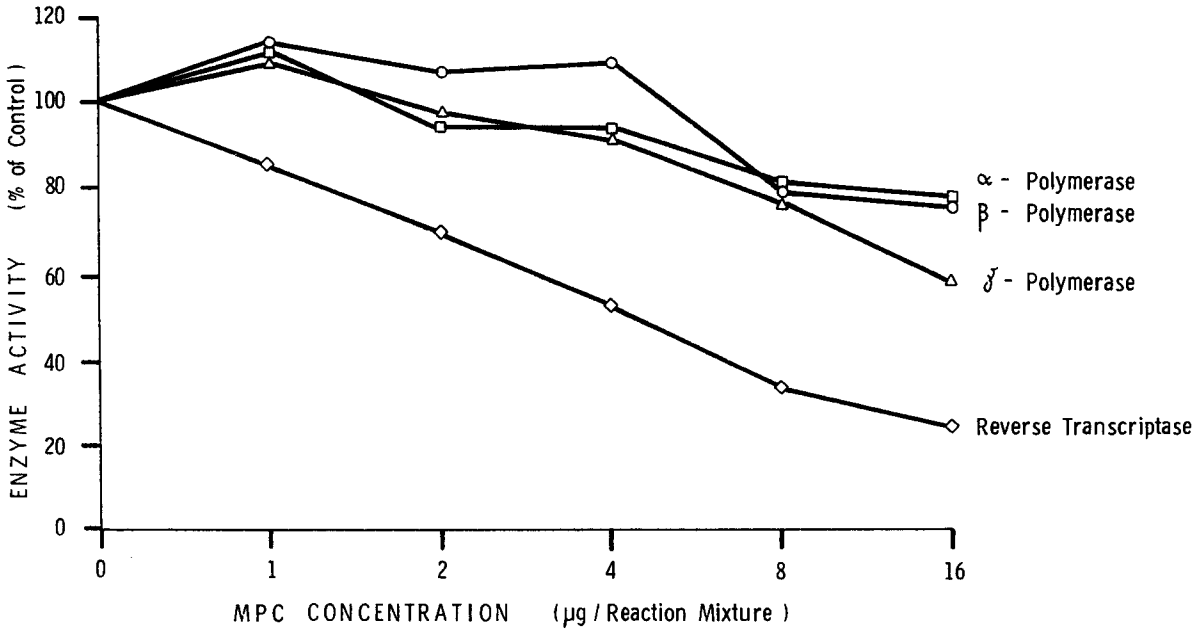

Fig. 4. Effect of MPC (SH = 15%) on the activity of cellular DNA polymerases (α, β and γ), and on the reverse transcriptase activity from human spleen of a patient with myelofibrosis. Bivalent cation and template specificities for cellular DNA polymerases and reverse transcriptase from human myelofibrotic spleen are described elsewhere [16]

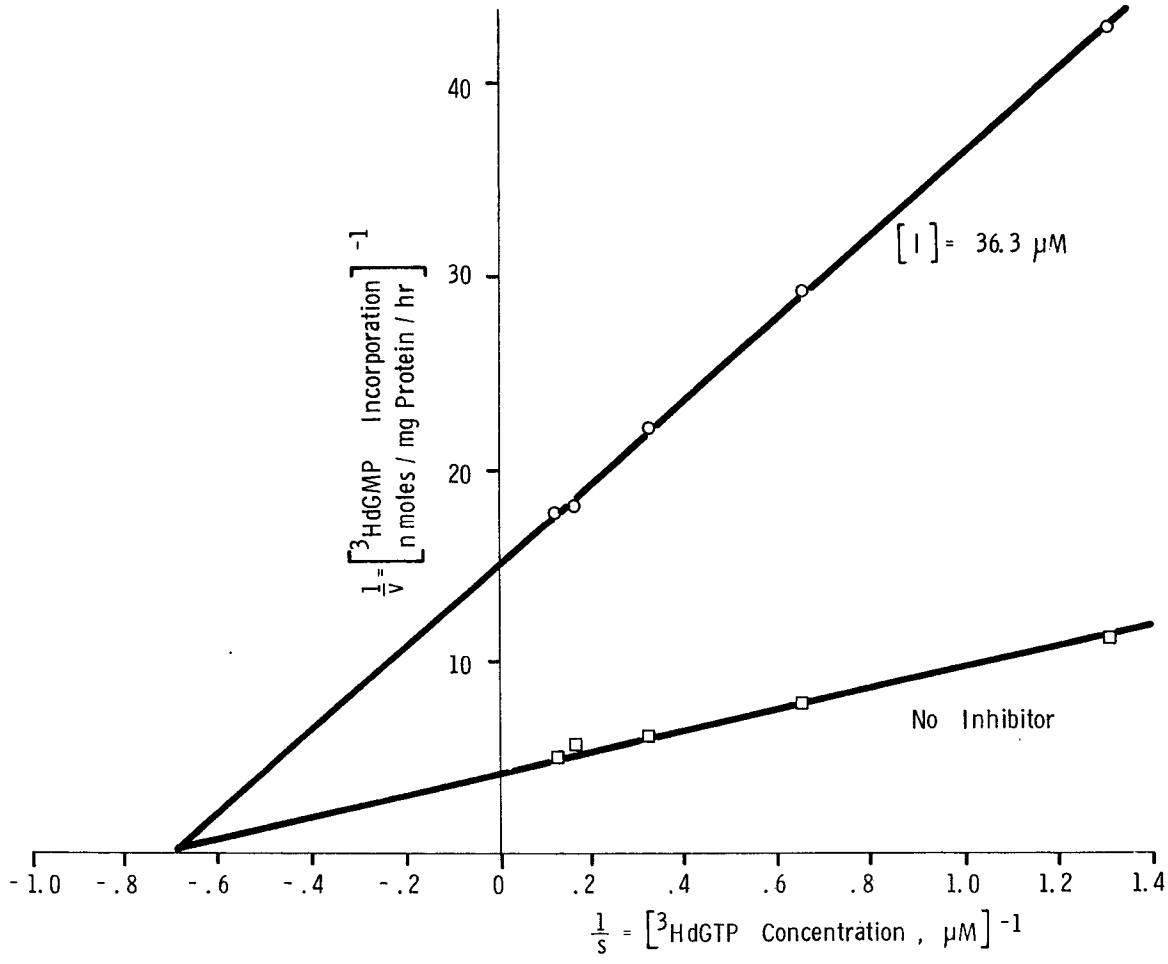

Fig. 5. Lineweaver-Burk plot the kinetics of DNA polymerase reaction, catalyzed endogenously by Simian Sarcoma Virus (SiSV). The reaction conditions have been described by Chandra and Steel [16]

1, except that the viral suspensions were preincubated for 2 hrs.; 4. Group 4 was treated in a similar manner as group 2, except that the viral suspensions were preincubated for 2 hrs at 37°C. On the 10th day, animals were sacrificed and spleen extracts were prepared, as described elsewhere [8]. The spleen extract from each mouse was then analyzed individually, with respect to their leukemogenic potentiality. Each "donor" spleen specimen was reinjected to a different "receipient" mouse (20 in total), and the leukemogenesis was followed, as shown in Table 2.

Table 2. Assay for leukemogenic potential of spleen extracts from FLV-infected mice after their in-vitro/vivo treatment with MPC

Treatment of donor mice	Leukemogenesis in recepient mice after infection with spleen extract[a]		
	No. of positive Total no. of mice	Mean survival Time (days)	Mean spleen Weight (g)
Virus Suspension[b] (0.2 ml) + Tris buffer (37°C, 30 min)	5/5	47	2.41
Virus Suspension + 200 μg of MPC (37°C, 30 min) + 50 μg MPC, i.p. (day 5 & 9)	2/5	123[c]	1.05 (1.78, 2.10, 0.52, 0.41, 0.44)
Virus Suspension + Tris buffer (37°C, 2 hr)	5/5	52.2	1.80
Virus Suspension + 200 μg of MPC (37°C, 2 hr) + 50 μg MPC, i.p. (day 5 & 9)	1/5	110 4 (123)[c] 1 (97)	0.38 (0.74, 0.29, 0.34, 0.22, 0.31)

[a] Cell-free spleen extracts were prepared (see reference 8) from spleens of individual donors on the 10th day after being challenged with the virus, or other treatments as shown.
[b] Citrate plasma from FLV-infected mice was used as the source of visus (LD_{90}).
[c] The experiment was terminated on day 123 and all animals were sacrified on this day. Therefore, the term "mean survival period" does not apply to these animals.

All animals in groups 1 and 3 developed splenomegaly and died between 40–60 days; whereas, in the MPC treated groups, of the 10 animals only 3 showed signs of splenomegaly. In group 2, 2 animals had splenomegaly but, in spite of that, all animals survived till the 123rd day, at which time our experiment was terminated. Similarly, in the last group 4 animals survived till the 123rd day; one died on the 97th day. The spleen weights, shown in the last column, also exhibit large differences between the MPC-treated group, and the control group. In another study we have analyzed the effect of MPC on normal mice of the same strain. We failed to observe any effect of MPC on the spleen weights of non-infected mice.

In another biological study we have analyzed the in-vivo effect of MPC on leucocytes of mice infected with the active Friend virus (Fig. 6). Within 12–24 hrs. after MPC injection (50 μg/mouse) a dramatic fall in the leucocyte count of animals infected with FLV was observed; MPC failed to reduce the leucocyte number in mice not-infected with the virus. It is interesting to note

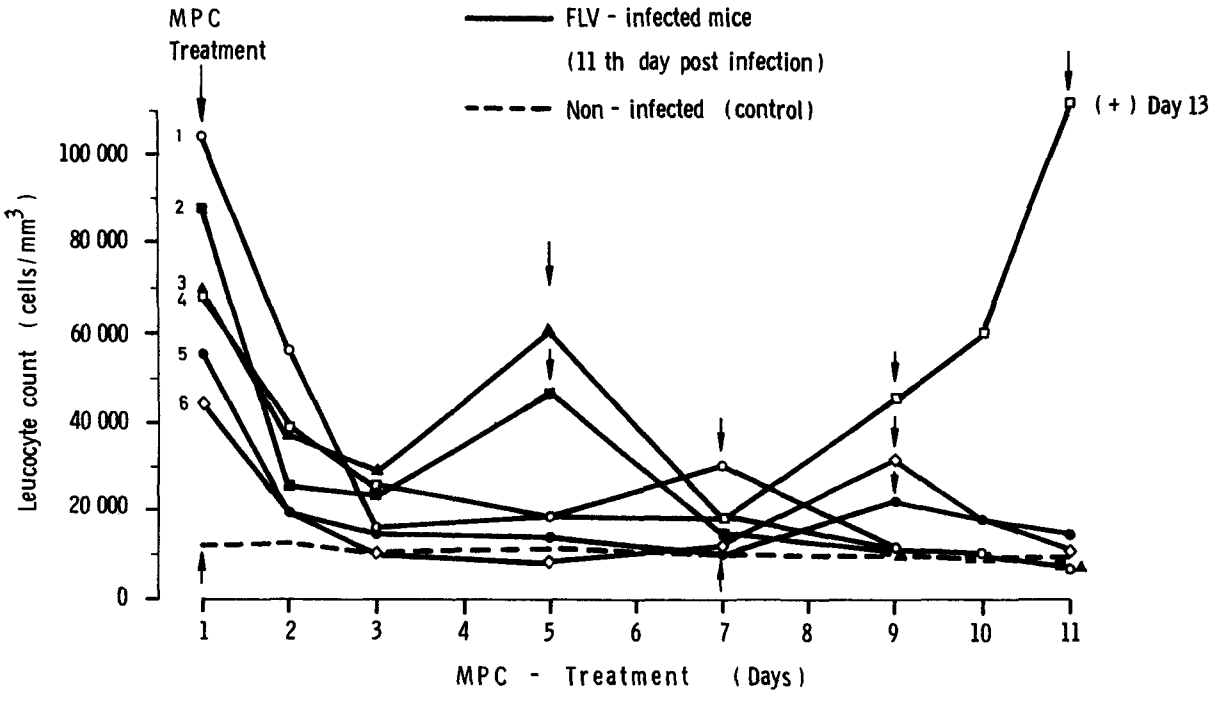

Fig. 6. In-vivo effect of MPC (SH = 15%) on the leucocyte number of mice infected with the active Friend Leukemia Virus (FLV). Arrows indicate the day of MPC treatment. MPC was injected intraperitoneally (50 μg per mouse). The MPC treatment was started on the 11th day postinfection with FLV. The hatched line indicates the leucocyte number of a control mouse treated with MPC. The control group (non-infected) had 5 animals, but the effect of MPC was similar to the one represented by the hatched line

that in one of the infected animals MPC failed to suppress the leucocyte number;on the contrary, there was a gradual increase in leucocyte number. This animals died on the 13th day of MPC treatment. Unfortunatly, we were not able to analyze the spleen of this animal. It is therefore difficult to interpret the reasons for failure of MPC effect in this animal.

3. Clinical Trails with MPC in the Treatment of Childhood Leukemia

Clinical data of patients submitted to MPC trials are shown in Table 3. Of the 18 cases treated with MPC, were in the terminal phase of the disease. These patients were resistent to all previous chemotherapeutic regimes which involved drugs, such as prednisone, vincristin, daunorubicin, L-asparaginase, Ara-C, 6-mercaptopurine, methotrexate, cyclophosphamide and actinomycin D.

MPC used in our clinical trials contained 15% of thiolated cytosine bases. The lyophilized product (MPC) was dissolved in 0.1 M Tris/HCl buffer, pH 7.6, and diluted with 0.9% NaCl before use. This solution was sterilized by passing through a membrane filter (Millipore GmbH, Neu Isenburg, Germany). It was kept at 4 °C and used immediately, or within the next five days; solutions older than 5 days were reprecipitated, purified on the column and resterilized. In our clinical trials, MPC (sterile) was given intravenously at a dose 0.5 mg/kg body weight. The injections were given once a week.

Table 3. Clinical data of patients with ALL and AML submitted to MPC trials

No.	Init.	Age	Sex	Diag.	Stage	Results
1	D.M.	8	♀	ALL	3rd rel.	PR, WBC ↓
2	T.I.	6 6/12	♀	ALL	2nd rel.	?
3	B.M.	6 7/12	♂	ALL	2nd rel.	?
4	M.M.	8 4/12	♂	ALL	2nd rel.	CR
5	J.O.	3 6/12	♂	ALL	2nd rel.	?
6	N.A.	10	♀	ALL	2nd rel.	CR
7	B.C.	5 8/12	♀	ALL	3rd rel.	CR
8	N.N.	7 11/12	♂	ALL	3rd rel.	?
9	M.A.	12	♂	ALL	1st rel.	?
10	M.I.	8 6/12	♂	ALL	1st rel.	?
11	K.C.	11 3/12	♀	ALL	4th rel.	?
12	K.K.	10 9/12	♀	ALL	5th rel.	PR
13	L.J.	7	♂	ALL	3rd rel.	WBC ↓
14	F.D.	2 3/12	♂	ALL	init. ph.	WBC ↓
15	S.B.	7 11/12	♀	ALL	init. ph.	WBC ↓
16	H.B.	12 5/12	♀	ALL	init. ph.	WBC ↓
17	S.N.	4	♀	AML	init. ph.	WBC ↓
18	W.H.	5 6/12	♂	AML	init. ph.	WBC ↓

Of the 13 terminal cases, complete remission was achieved in 3, and a partial remission achieved in 2 other cases. Fever, occasionally accompanied by shivering, was frequently observed under MPC treatment in the first hour after injection. However, these symptoms never lasted more than the first hour, and no other sideeffects could be observed.

On the basis of our experience with MPC on terminal cases, we were motivated to give MPC a clinical trial in the beginning of leukemia. A monotherapy with MPC, as devised for terminal cases is, however, not possible. We therefore decided to introduce MPC (0.5 mg/kg body weight) therapy in the beginning of treatment of cases which at the time of diagnosis had leucocytosis. This initial treatment, a single injection of MPC, was then followed up by polychemotherapeutic protocol, adopted by the university hospitals in Berlin, Frankfurt and Münster [see ref. 18]. As shown in Fig. 7, 24 hrs. after MPC injection, there was a significant reduction leukemic cells in all the cases. Five of these seven children had ALL, and two AML.

The status of this drug in the chemotherapy of fresh leukemic cases is not known, since monotherapy with MPC in such cases has not been done. The fact that under the pressent polychemotherapeutic protocols one can frequently achieve longterm remissions, hinders one ethically to use MPC as a monotherapeuticum in fresh cases. However, its use in the initial phase of the acute disease, and its use as a monotherapeutic agent in terminal cases are quite encouraging. On the basis of our to-date experience with MPC we could summarize by saying: a) MPC is useful to initiate the therapy in freshly diagnosed acute leukemic cases, b) it has shown promise as an effective drug in the treatment of leukemic cases in the terminal phase, and c) it could

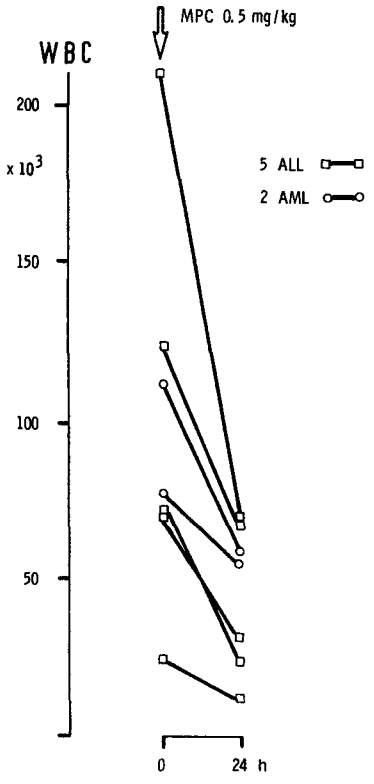

Fig. 7. MPC trials in the initial treatment of freshly diagnosed leukemic cases. Details are given in the text

be used in the remission maintenance therapy. This aspect is yet to be investigated. This, as a matter of fact, is the rationale for its therapeutic application, since it is a potent inhibitor of reverse transcriptase.

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Summary of Clinical Poster Sessions

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The first of six presentations in this poster session to be discussed is that of Preisler and Rustum [1]. This paper demonstrates the efficacy of anthracycline derivatives given as a pulse injection daily for 3 days in combination with a continuous seven day infusion of cytosine arabinoside for the treatment of acute nonlymphocytic leukemia (ANLL). This regimen and regimens similar to it [2] are among the best treatments for this disease at the present time. A number of investigators have achieved complete responses on the order of 65–70% with such treatment [3–5]. Preisler and Rustum attempt to determine whether the intracellular retention of activated Ara-C in vitro by a patient's bone marrow leukemic cells correlates with remission rate and duration. Unfortunately, the authors did not find an improved remission rate in those patients who retained phosphoralated cytosine arabinoside better than others. However, there was a highly statistically significant correlation between phosphorolated cytosine arabinoside retention and remission duration. This is an important finding not only because it may allow one to predict in broad strokes what remission duration is likely to be in a given patient, but the data strongly suggest that the induction therapy given to a patient with ANLL is the most important determinant of remission duration. Others have also suggested this may be so [6]. A number of investigators have recently questioned the value of intensive maintenance therapy in this disease and the results have been variable [7,8]. Perhaps further studies of this kind will allow for more selective and therefore more successful therapy in terms of remission induction and duration of response. Similar work by others [9] suggests that this may already be partly true for solid tumors.

The paper by McCredie et al also concerns ANLL treatment [10]. This paper further confirms the efficacy of the anthracycline-cytosine arabinoside combination with different doses and schedules of the drug. The combination of adriamycin, vincristine, cytosine arabinoside, and prednisone produced a 74% complete response rate at M.D. Anderson Hospital and a 54% complete response rate in the Southwestern Oncology Group. Group studies always give poorer results than studies performed at single institutions that specialize in the treatment of the disease entity in question. Substituting rubidazone [11], a new anthracycline currently undergoing trial in France and in the United States, for adriamycin in older patients produced essentially the same complete response rate as that of the adriamycin containing combination. Therefore, there is no advantage gained by substituting

rubidazone for adriamycin. It is somewhat disappointing that this new anthracycline derivative is not more active than the two (adriamycin and daunorubicin) already in widespread use. A good deal of analog research has occurred in cancer chemotherapy with the hope that refinement of a molecule with proven activity might lead to a greater therapeutic index. Unfortunately, this has not been the case thus far with the anthracyclines, the nitrosoureas, the antipurines and the antipyrimidines, or the actinomycin-mythramicin antibiotics. Perhaps this is not too surprising when one considers that analog research in the aminoglycoside antibiotics and, indeed, in the cardiac glycosides has not yielded a major improvement over the respective parent compound.

The study of McCredie et al. includes a new approach to remission maintenance. The late intensification program at M.D. Anderson Hospital has been reported previously [12]. The present data suggest that roughly half of the patients who undergo this program remain in unmaintained complete remission for significantly longer periods of time than reported with other relatively intensive maintenance programs. The data also suggest that if the patient remains in continuous complete remission for 24 months following the discontinuation of late intensification therapy, he has a 90% chance of remaining in complete remission. We will watch these data with intense interest over the next months and years with the hope that they continue to be as impressive as they are at the present time.

The McCredie paper also describes another novel approach to remission maintenance, use of autologous bone marrow transplantation using marrow collected and stored from patients with ANLL during periods of remission. Too few patients have been entered on this aspect of the study to date to allow full evaluation or even significant optimism at this point. One might not be surprised, however, if this method ends in failure. Spiegleman et al. [13] have shown that even during complete remission reverse transcriptase may be isolated from what appear to be normal granulocytes in the peripheral blood of ANLL patients. Therefore, one might expect that the viral etiologic agent, if there is one, might also be present in the morphologically normal appearing cells used for these autologous transplants. One cannot be too optimistic about transplanting cells that may harbor pathologic time bombs.

The third paper of this series is that of Freeman et al. [14] concerning intermediate dose methotrexate in childhood acute lymphocytic leukemia (ALL). The purpose of Freeman's study is to prevent the development of CNS leukemia without employing cranial radiotherapy by administering relatively high doses of parental methotrexate that will result in therapeutic CSF levels of the drug. Additionally, the goal of the study is to eradicate leukemic cells in other sanctuaries such as the testes. In the study, children are induced with a combination of steroid, vincristine, L-asparaginase and intermediate doses of methotrexate (500 mg/M^2). The methotrexate is followed by citrovorum factor. The idea of this study has merit. More and more undesirable effects of commonly employed methods of CNS prophylaxis (intrathecal methotrexate and cranial irradiation) are coming to light with time [15, 16]. It might have been more reasonable, however, to design the study so

that L-asparaginase was given after the high-dose methotrexate. In this way L-asparaginase might not only be used as a therapeutic agent with activity against ALL but as a methotrexate reversal agent which would obviate the need for citrovorum factor [17]. This might be an important consideration since high dose parenteral methotrexate would still enter CSF and brain, but L-asparaginase would not. Variable amounts of citrovorum factor enter CSF.

The results of this study are of interest. Complete remission was achieved in 96% of patients but there were 7 CNS relapses according to the paper. For reasons that are totally unclear to me, two patients who presented with CNS leukemia are included in this study (one relapsed with CNS leukemia). It would have been perfectly reasonable to exclude patients from admission to the study if they presented with CNS leukemia since one of the goals of the study is to evaluate this regimen for its potential to prevent CNS leukemia.

One can conclude from the study that this method of CNS prophylaxis probably is better than no prophylaxis at all. However, it is not clear that it is equal to or superior to more standard cranial irradiation and intrathecal methotrexate administration. In addition, the fact that intrathecal methotrexate was also given to these patients makes my interpretation of these results even more difficult since others [18] have suggested that intrathecal methotrexate alone may be sufficient.

The paper by Lister et al. [19] concerns 62 adult patients with ALL who received adriamycin, vincristine, prednisone and L-asparaginase in a program based on lessons learned from childhood ALL. The complete response rate in the study was 69%. This is one more paper that indicates that combinations of anthracyclines, vincristine, glucocorticoids, and L-asparaginase and other drugs can produce complete responses in adults with ALL on the order of 70%, as previously reported by Henderson [20] and Capizzi [21]. This study and ones similar to it represent significant advances in the treatment of this disease. Cranial irradiation and intrathecal methotrexate were given as CNS prophylaxis. A standard dose of methotrexate not related to body surface area was used intrathecally in all patients. This makes sense since almost all adults have a CSF volume of approximately 150cc. irrespective of body surface area. Such practice will decrease the number of inadvertant overdoses of intrathecal methotrexate [22]. As maintenance therapy, patients received oral 6-mercaptopurine oral methotrexate and oral cyclophosphamide. I question the usefulness of cyclophosphamide in this regard since data in children from St. Jude's Children's Hospital in Memphis has shown that as one multiplies the number of drugs used during maintenance, one multiplies the complications to be expected and does very little to improve the remission duration [23]. In my opinion, cyclophosphamide is a drug with marginal activity in ALL and is more likely to be hazzardous than helpful. I cannot help but wonder whether the one patient who died at home during an influenza epidemic while in complete remission may not have been a cyclophosphamide casualty. The maintenance therapy was continued for three years and then stopped. I agree with the concept, but would have been more pleased to see half the patients randomized to dinscontinue maintenance and the other half randomized to have treatment continued. That kind of comparative informa-

tion about a crucial question, that is whether or not one can safely take an acute leukemia patient in remission off maintenance therapy at some period in time needs to be developed. The remission duration median of 21 months in this study represents some of the best data of this kind. The fact that 7 patients have already been in continuous complete remission for more than three years is encouraging, but not different from the early observations in other studies of similar design.

In an attempt to identify features on presentation that might influence remission duration, the authors found that age was not a factor as have other investigators. They did find, as have others, that patients with hepatosplenomegaly on admission have, on the average, shorter remission durations than patients with less disease bulk, and they found that patients who have extremely high white counts on admission relapse relatively quickly. Cytochemical and cell surface marker studies gave the same results that have been reported by others for childhood ALL.

This study confirms the fact that adult ALL behaves much the same as childhood ALL and responds to the same kind of therapeutic manipulations. The question then comes up as to whether or not separate studies need to be designed for children and adults with ALL. It would appear from these data and other studies that children and adults might be treated on the same protocols with the same stratifications applied to both.

The paper by Catovsky et al. [24] concerns ultrastructure and cell marker studies in lymphoproliferative disorders. The authors contend, and rightly so, that surface marker studies and electron microscopic studies increase the accuracy of subclassification of the acute leukemias. They carefully define the morphology and cell surface marker study results in various B and T cell disorders. They conclude that these studies are useful and no one doubts this. I do take issue, however, with their idea that prolymphocytic leukemia is a disorder separate and distinct from chronic lymphocytic leukemia (CLL). It seems to me most likely that what has been called prolymphocytic leukemia by these authors is simply middle or endstage CLL. Many patients with CLL end up with lymphocytes in the marrow and peripheral blood that are younger than those which were demonstrated at the time of diagnosis. An occasional patient with CLL terminates with what appears to be a blastic crisis based on the immature nucleolated morphology of the leukemic cells at or near the time of death [25]. I see no scientific or clinical reason for separating this disorder out from CLL since the treatment for Catovsky's prolymphocytic leukemia is not different from that of resistant CLL.

The last paper in this series is by Mertelsmann et al. [26], and it concerns marker studies in hematologic malignancies. The authors characterized malignant cells by terminal deoxynucleotidyl transferase (TdT) activity, CFS-c assays, and cell surface marker studies. They have found, as have others [27] that TdT activity is highly specific for lymphoid neoplasms and that TdT positivity approaches 100% in acute lymphocytic leukemia. In addition, approximately one-third of patients with the blast phase of chronic myelogenous leukemia (CML-BC) have TdT positive blasts. An extremely important observation from this study is that acute leukemia patients whose

blasts are TdT positive almost always respond to vincristine and prednosine, whereas TdT negative patients do not. This correlation of TdT with response remains valid even when the standard morphological examinations are inconclusive. In addition, the majority of the CML-BC patients who responded to chemotherapy were TdT positive. It must therefore be concluded from this study and similar ones [27] that bone marrow TdT assay is a powerful aid in determining type of acute leukemia and in predicting response to therapy. This study also defined 4 cases of morphological acute myelomonocytic leukemia associated with cell marker data consistent with a double stem cell lineage. Other recent evidence suggests a stem cell capable of both myeloid and lymphoid differentiation as the site of the major lesion in some acute leukemias [28]. The fact that myeloid-appearing cells in CML blast crisis can exhibit TdT activity [27] suggests that such a common stem cell may be deranged in many cases of CML-BC. The absence of the Philadelphia chromosome in lymphocytes found in the chronic phase of CML has been considered evidence against a common lymphoid-myeloid stem cell in nature. However, cytogenetic examination of lymphocytes obtained during the chronic phase of the disease from patients who have sustained the chronic phase for 5 to 10 years may shed some light on this important question. In those patients, all lymphocytes would have been formed since the development of the CML, whereas many lymphocytes present at the time of diagnosis would have been formed prior to the advent of the disease with its marker chromosome. Such studies as these and those of Mertelsmann may eventually lead to a better understanding of the origin of some hematologic malignancies.

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Progress in Acute Leukemia 1975–1978*

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During the three years which have elapsed between the second and third Wilsede conferences, the clinical management of acute leukemia has become almost routine. Every clinic has become the advocate of combination chemotherapy for remission induction and maintenance, early treatment of the central nervous system at least for childhood disease, and an appreciation of the need for supportive care measures and attention to metabolic abnormalities in the preparation for and sustenance of patients during chemotherapy. This has clearly led to the happy prospect of more patients receiving better care and thus a better chance for long-term survival and possible cure from this once uniformly uncured disease.

This confirmity of approach, however, has serious implications concerning therapeutic research in acute leukemia. First, it tends to obscure the fact that most patients continue to die of their disease; and second, it reflects the leveling off of significant advances in treatment for a sufficient time (3 to 5 years) for the average competent physician to "catch up" with the avant garde. Thus it highlights, among other things, the failure of non-specific immunomodulation (with BCG, MER, and the like) to significantly alter the final outcome of leukemia, and the continued urgent need for more, and more specific, new compounds to break through the toxicity barrier, especially during the remission consolidation and maintenance phases. Finally, given the success of primary treatment of "standard" acute lymphoblastic and acute myeloblastic leukemia of children *and* adults, necessarily greater attention is now commanded by the late toxic effects of drugs, and especially that which remains the most devastating malignancy of all: the acute leukemia which follows (is caused by?) previous bone marrow disease and injury.

Current Therapy and Results

The results of recent successful treatment regimens in acute leukemia are reviewed elsewhere in these proceedings by Freeman, Lister, McCredie, Preisler, and Wiernik. For acute lymphocytic leukemia (ALL), the three-drug combination of vincristine, prednisone, and asparaginase will induce

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almost all good risk patients into complete remission (good risk factors being 2–10 years of age, no previous treatment, and the lack of lymphoblast differentiation into those cells which bear T and B cell surface markers). These same drugs will induce remissions in older patients and those with T cell markers in approximately 75 percent of initial trials. The addition of a fourth drug (daunorubicin) increases the effectiveness of the regimen, at least in adults (Lister et al., 1978; Henderson et al., 1978). In both adults and children maintenance treatment and central nervous system prophylaxis appear necessary for optimal results; although the long-term administration of intensive maintenance treatment has led to increased toxicity, late complications, and deaths in remission; and has compared unfavorably in terms of survival with maintenance regimens employing fewer drugs and/or less frequent drug administration (Pinkel, 1978; Simone et al., 1976; Henderson, 1974). On the other hand, short-term intensification during established remissions may be advantageous (Bodey et al., 1976; McCredie et al., 1978; Freeman et al., 1978).

For acute myelocytic leukemia (AML) combinations of cytosine arabinoside and anthracycline antibiotics given in such a manner that myelosuppression is sustained for 10–14 days or more appears central to an optimal response, given present drugs (Yates et al., 1974; Preisler et al., 1977; Silver et al., 1977; McCredie et al., 1976 and 1978) and support techniques. Although most concede its value, the exact role and necessary duration of remission treatment remains uncertain, as does the administration of treatment to the central nervous system in AML.

Using these treatments survivals of greater than 50 percent have been achieved in ALL in children (Pinkel, 1978; Jones et al., 1977) and up to 25 percent in active (i.e., neither secondary nor smouldering) AML and ALL in adults at several centers (Henderson et al., 1977; McCredie et al., 1975; McCredie et al., 1978).

On the other hand, poor risk patients with acute leukemia continue to do poorly. These are the patients with T cell or B cell disease, extensive extramedullary leukemic infiltrates at diagnosis, at the extremes of age, or most strikingly, those with a history either of preexistant bone marrow disease, or chemotherapy and/or radiation therapy antecedent to the development of leukemia. Unfortunately, given our limited ability to treat them successfully, the incidence of poor prognosis patients appears to be increasing. Until adequate therapies are devised, it is still critical that such patients are identified early to prevent 1. their imbalanced inclusion into comparative treatment studies or 2. their inopportune and premature treatment with intensive drug regimens having little chance of inducing benefit. In this regard, it is unfortunate but true that attempts to increase response and survival with superintensive treatment have rarely proved of benefit, while frequently adding morbidity. For such patients the failure to normally reconstitute hematological and immunological function underlies most failures. Accordingly, the development of drugs or other agents with precise specificities for malignant cells and the sparing and/or reconstitution of normal blood cell progenitors appears to be the areas of greatest promise and highest priority in the immediate future.

At the heart of the problem of specificity is the question of the reliable identification of the progenitors of leukemic and normal cells. Numerous previous attempts to identify and monitor these cells, their function, their replication, and their drug sensitivity have been at best only partially successful. The striking acquisition of new knowledge and techniques for measurement of myeloid and lymphoid cells is the most heartening advance of current years in the study and management of leukemia and allied diseases. Antisera specific for stages of differentiation and the identification of specific surface receptors for antibodies, hormones and antigens have permitted in situ identification and in some instances sorting of specific subpopulations of cells, which can then be studied for replicative activity, drug uptake and effects, and clonogeneicity, thus for the first time permitting sensitivity to drugs to be reliably assessed. Given proper reagents and improvements in sorting technology, it may be possible either in vivo or more likely in vitro, to separate malignant and normal cells from cell mixture, e.g., peripheral blood leukocytes or bone marrow cell suspensions. All this is within the capability of current laboratories and their associated clinics within the next 3–5 years.

Once isolated one from another, it will be possible to assess not only the presence of differentiation related (and/or tumor related) markers, but also function, and clonogenicity and drug susceptibility. Proliferation and differentiation to a more mature state is not sufficient to identify a stem cell, the test of which is self-replication simultaneous with the spawning of differentiate functional progeny. (By analogy it has been noted that there are in fact two cells maintaining immunologic memory, both will respond rapidly to recall antigens but while most will form effect cells at the expense of their own survival, one class will in addition self-replicate, thus renewing memory.) It is the latter class of cell that is obviously most significant in long term propagation of any tissue or function, and it is this type of cell toward which the major search must be directed, and with which assays such as those described by Preisler and Rustum (1978), Greaves (1978) and others can be attempted with assurance of benefit. At the same time, alternate assessment of their involvement in a malignancy can be accomplished by detailed cytogenetic analyses, such as those described by Rowley (1978), idiotypic directed antisera (Broder et al., 1975; Fu et al., 1978), or enzyme allotype studies such as those reviewed by Fialkow (1978). Such studies will, doubtless, continue to amaze us with their demonstration of the ontogenetic scope of normal and malignant tissues which appear otherwise homogenous.

Despite its early promise and despite demonstrations of histological and therapeutic activity, immunotherapy has to date not been noted to increase the probability of long-term survival and cure (Hersh, 1978). However, the preliminary studies reported at this conference by Bekesi (Bekesi, 1978; Silver, 1977) suggest that inoculation of enzyme-treated human leukemic cells may be efficacious, perhaps through the mediation of a leukemia associated or specific immune reaction. Both this premise, and the clinical utility of neuraminidase treated cell immunization, remain to be proved, but results to date are impressive, not only in the observed differences between the survivorship of control versus chemotherapy plus neuraminidase-treated-cell

inoculation groups, but also in the shape of the curves which indicate for the latter group that the risk of relapse is diminishing with time. Such survival curves are in contrast to those observed following BCG or MER treatments (Mathe's series remaining a notable exception) and encourage the hope that this form of treatment will effect cures.

The role of bone marrow transplantation in relapsed patients is now obvious. There is no approach in advanced refractory leukemia which can duplicate that which has been demonstrated by the major transplantation groups in terms of response and survival (Gale, 1978; Storb, 1977). It would appear that the worth of engraftment lies in its permission of supralethal drug and/or irradiation dosages, rather than in any immunological effect (e.g., the graft versus leukemia effects postulated by Boranek, 1968) since identical-twin or autologous stem cell grafting coupled to intense cytolytic regimens appear to afford results comparable to allogeneic transplantation (Grace and Gale, 1977; Bruckner et al., 1977; Fefer et al., 1977). This is of major imporance in providing a rationale for transplantation of stored autologous marrow, freed by sorting either of leukemic cells on the one hand, or allogeneic immunocytes responsible for both graft-versus-host and graft-versus-leukemic reactions on the other. Stem cell replacement could thus become available to all patients, rather than remaining restricted to a small minority of individuals with leukemia.

Finally, as always, the therapist looks forward to the development and discovery of new agents especially those with tumor specificity. Science is providing knowledge of receptors, mediators, and the like which control devision and differentiation, and are thus choice targets for chemical attack. One such example of a new semisynthetic agent is metholated polycytidylic acid, one of a series of "antitemplates" synthesized by Bardos and Ho (Ho and Bardos, 1977) in Buffalo, and shown by Chandra et al. (1978) to potently inhibit mammalian DNA polymerases, including reverse transcriptase extracted from human tissue. Preliminary trials in children with recurrent ALL have demonstrated the activity of this compound in a clinical setting (Kornhuber, 1978). The possible utility of this class of agent will be under investigation during the coming months, along with trials of new anthracyclines, vinca alkaloids, and antimetabolites all selected on the basis of reduced major organ toxicity and, thus, greater specificity than their clinically utilized congeners. Also to be tested are naturally synthesized compounds (or activities) such as immune RNAs and interferons. The possibility of using natural metabolic intermediates, induced in the patients own cells by certain inciting principles, e.g., the 2′5′A oligonucleotides described in these meetings by Kern (1978), is a particularly attractive approach combining as it does the potential advantages of tumor *and* host specificity.

The investigations of the past 3 years, and the spirit of current investigations as manifested in these recent Wilsede meetings, augers well for rapid advances in the direction of truly specific and safe treatments for acute leukemia and allied diseases within the near future. They also emphasize the importance of studying human patients directly, rather than by analogy, attesting that "The proper study of man is man".

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Regulatory Interactions in Normal and Leukemic Myelopoiesis*

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1. Introduction

In vitro culture studies have revealed complex hemopoietic cell interactions involving local and systemic signals controlling cell proliferation and differentiation. Phenotypic characterization of myeloid leukemia utilizing in vitro culture methodologies suggests that subtile regulatory imbalance may play a major role in clonal dominance of transformed pluripotential stem cells or committed granulopoietic progenitor cells. The emerging complexity of potential bioregulatory networks in both normal and neoplastic hemopoiesis has almost exclusively been demonstrated in vitro, however the concept of "dependence" as applied to retention of regulatory responsiveness by neoplastic myelopoietic cells is of sufficient practical and theoretical importance to justify detailed consideration of hemopoietic regulators in the context of human leukemia.

2. Regulatory Networks in Normal and Leukemic Myelopoiesis

I. Control of the granulocyte-macrophage progenitor cell (CFU-c)

Myeloid leukemic cells can be cloned in semi-solid culture medium, in suspension cultures or in diffusion chambers implanted into mice. Confirmation of the leukemic origin of the cells derives from morphology, karyotypic analysis, biophysical characteristics and in vitro clonal growth patterns. As has been extensively documented, normal granulocyte macrophage progenitor cells (CFU-c) exhibit an absolute dependence upon provision of an appropriate source of colony stimulating factor (CSF). Since a major source of CSF resides within the hemopoietic system itself, either from mitogen stimulated lymphocytes or monocytes and macrophages, any consideration of leukemic cell responsiveness to CSF must also include recognition that CSF may itself be a product of leukemic cells. Cells from patients with leukemia can be cloned without addition of exogenous CSF. However, growth is not autonomous and cell separation procedures have demonstrated the presence of endogenous CSF producing cells which could be separated from leukemic colony forming cells [1]. Early studies suggested that CFU-c from patients with myeloid leukemia are heterogeneous in their responsiveness to exogenous

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CSF with acute myeloid leukemic cells generally more responsive than remission or normal CFU-c and chronic myeloid leukemic cells exhibiting decreased sensitivity to stimulation [2]. The question of altered responsiveness of leukemic cells to stimulating factors is, however, complicated by interactions between inhibitory and stimulatory cell populations within leukemic bone marrow and the known functional heterogeneity of CFU-c subpopulations and differing species of CSF.

Recognition that normal and neoplastic monocytes and macrophages can produce CSF and that this factor is an absolute requirement for proliferation and differentiation of normal or neoplastic granulocyte-macrophage progenitor cells introduces the problem of mechanisms designed to counterbalance this positive feedback drive. Limitation of CSF-dependent myelopoiesis may theoretically be mediated by activities inhibiting CSF production by mononuclear phagocytes, by direct inactivation of the CSF molecule or by alteration in the responsiveness of the CFU-c. Evidence for the latter was provided by the observation that the E series prostaglandins (PGE_1, PGE_2) profoundly inhibited normal and leukemic CFU-c proliferation in vitro [3]. This inhibition was prevented by preincubation of bone marrow with the dibenzoxapine hydrazide prostaglandin antagonist SC-19220 providing evidence for a PG receptor on the CFU-c. PGE mediated inhibition was also partially counteracted by increasing the concentration of CSF in the culture system suggesting a dualistic modulation of CFU-c proliferation [3]. The central regulatory role of macrophages in myelopoiesis was further indicated by studies which showed that blood monocytes and tissue macrophages were a major biosynthetic source of prostaglandin E as detected by radioimmunoassay [4] and that this synthesis correlated with the production of a diffusible, dialyzible, non species specific inhibitor of CFU-c proliferation [5]. The apparently paradoxical observation that macrophages produced two activities with mutually antagonistic roles at the CFU-c level can be resolved by three observations.

1. While macrophage activation by agents such as endotoxin or zymosan leads to increased synthesis of CSF and PGE, the former occurs as an acute response peaking within 1–3 hours whereas the latter increase is delayed for 18–24 hours [6].

2. The temporal dissociation between peak induction of CSF and PGE synthesis suggested that PGE biosynthesis may be dependent upon the earlier increase in CSF. This possibility was confirmed by the observation that exposure of macrophages to increasing concentrations of a pure source of CSF led to a subsequent dose dependent increase in PGE synthesis and that this response was also seen with macrophages from endotoxin resistant C3H/HeJ mice [5, 6].

3. By employing sedimentation velocity separation of normal peritoneal cells, it was shown that the cells which produced CSF and synthesized PGE resided within the cell population which was adherent, α-naphthyl esterase positive and phagocytosed latex beads. A subpopulation of these macrophages constitutively produced CSF but little or no PGE ("Helper" macrophages) and were clearly separable from a population of larger macrophages which

constitutively produced PGE and little CSF ("Suppressor" macrophages) [6]. Of particular significance was the observation that the CSF producing – "helper" macrophage population could be induced to extensive PGE production following exposure to endotoxin or an exogenous source of CSF [6].

The relevance of these observations to leukemogenesis resides in the fact that macrophages can function in a helper or suppressor mode to influence myeloid leukemic cell proliferation. Suppression of leukemic cell proliferation by activated macrophages may, for example, be relevant to non-specific host defense against neoplasia. Relatively low numbers ($1-2\times 10^5$/ml) of activated mouse peritoneal macrophages are capable of producing a reversible cytostatic block on a variety of human and murine lymphoid and myeloid leukemic cell lines [7]. This cytostasis was detected by inhibition of leukemic cell growth, ^{3}HTdR incorporation, cloning efficiency and by cytofluorometric analysis. In all cases, cell contact was not required and the diffusible macrophage derived inhibitory activity was prostaglandin E.

While it is often thought that leukemic transformation involves an irreversible maturation block with loss of differentiation function, it is becoming increasingly clear that leukemic cells can, under appropriate conditions, be induced to express various differentiated functions. One example of this is retention by neoplastic myelomonocytic, monocytic and macrophage cell lines of the capacity to constitutively or inducibly synthesize and secrete CSF and prostaglandin E. As can be seen in Table 1, three of the five murine cell lines, the myelomonocytic leukemia WEHI-3, the spontaneous macrophage tumors SK2-2 and PU5.18 and the human adherent cell line SPGcT, constitutively elaborated both CSF and PGE, and like normal macrophages, the augmentation of CSF production following short term incubation with lipopolysaccharide (LPS) was linked to stimulation of PGE synthesis. In contrast to most normal macrophages, the RAW-264 Abelson virus induced macrophage tumor and the Balb/c macrophage cell line J774 did not constitutively produce either CSF or PGE; however, induction of synthesis of both activities followed treatment with LPS. Furthermore, the addition of a source of CSF induced both RAW-264 and J774 to synthesize PGE at levels very similar to those induced by LPS alone. CSF and PGE synthesis by RAW-264 could also be induced by agents other than LPS, such as zymosan, purified protein derivative (PPD) and Poly I-Poly C which also stimulate CSF and PGE production by normal peritoneal macrophages [6]. In this context, treatment of the neoplastic macrophage cell lines with hydrocortisone blocked the induction of CSF and PGE synthesis as well as induction of such functional properties as latex phagocytosis, antibody-dependent phagocytosis of sheep RBC and antibody-dependent lysis of tumor targets; however, hydrocortisone had no effect if these were constitutive properties of the neoplastic cells [8].

II. Granulocyte mediated inhibition of myelopoiesis

The concept of granulocyte mediated negative feedback regulation of myelopoiesis has received some experimental support, however this remains a contentious area due to difficulties in demonstrating in vivo correlates of in vitro phenoma. The end stage mature granulocyte, the polymorphonuclear

Table 1. Production of colony stimulating factor (CSF) and prostaglandin E (PGE) by murine and human monocyte-macrophage cell lines

Cell line	CSF[a]		PGE[b]		
Mouse	Control	+ LPS	Control	+ LPS	+ CSF
WEHI-3	84 ± 4	161 ± 18	173 ± 91	1040 ± 85	863 ± 35
SK2.2	16 ± 3	91 ± 9	296 ± 13	3462 ± 149	nd
PU5.18	12 ± 2	94 ± 12	283 ± 37	2385 ± 312	nd
RAW 264	0	111 ± 1	0	305 ± 25	gceoe± 25
J774	0	128 ± 10	0	1033 ± 61	1670 ± 88
Human					
SPGcT	100 ± 3	nd	3129 ± 150	nd	nd
U937	0	0	0	0	nd

[a] Colonies/7.5×10^4 marrow cells/0.1 ml of 24 hr. conditioned medium
[b] Picograms of PGE determined by radioimmunoassay

neutrophil (PMN), has been reported to produce chalone-like activities which can suppress ^{3}HTdR uptake in proliferating myeloid cells [9] or can alter the structuredness of the cytoplasmic matrix of such cells [10]. More recently, we have shown that PMN and their products indirectly inhibit CFU-c in vitro by decreasing the production and release of CSF by monocytes and macrophages [11]. The basis of the assay for this type of inhibitory activity involves density separation of bone marrow or blood leukocytes in order to obtain populations of cells enriched for CFU-c and CSF producing cells but depleted of mature granulocytes. When cultured in agar, endogenous production of CSF results in "spontaneous" colony formation independent of an exogenous source of CSF. Addition of mature granulocytes, granulocyte extracts or granulocyte conditioned medium to such cultures reproducibly suppresses spontaneous colony formation by an inhibitory action on CSF production by monocytes and macrophages [11]. Investigations of this PMN inhibitory activity in patients with myeloid leukemia and myeloproliferative disorders have demonstrated a marked quantitative defect [12, 13]. Interestingly, this defect persisted in many patients satisfying standard criteria for complete remission and could not be correlated with chemotherapy [12]. In a study of PMN mediated inhibition of in vitro myelopoiesis in 58 patients with chronic myeloid leukemia at all stages of the disease, a double defect in negative feedback regulation was observed [13]. PMN from the patients were quantitatively deficient in inhibiting normal or leukemic colony formation but, in addition, monocytes or macrophages from leukemic patients were less sensitive than normal cells to inhibition by activity derived from normal PMN. Again this decreased responsiveness was a quantitative rather than a qualitative difference and CSF production by leukemic monocytes and macrophages of human and murine origin can be effectively suppressed by high concentrations of granulocyte inhibitor [13, 14].

The inhibitory activity in serum free human granulocyte lysates is labile and rapidly inactivated at 37°C due to protease activity [15]. Partial purification of the inhibitor was obtained by ultracentrifugation, DEAE-sephadex

chromatography, SDS polyacrylamide gel electrophoresis and isoelectric focusing. Using this purification procedure, a 1×10^6 fold purification was obtained and the inhibitor was identified as a glycoprotein with a molecular weight of 80000–100000 and isoelectric focus points of pH 6.0–6.5. This inhibitory factor was therefore similar in many respects to lactoferrin, the iron binding protein first isolated from milk and also present in epithelial secretions and mature granulocytes [16]. Purified lactoferrin from human milk was tested in the spontaneous colony assay system against normal human marrow and in the native form (8% iron saturated) was inhibitory at 10^{-15} M, indeed the fully iron saturated form inhibited at 10^{-17} M whereas the apoform (depleted of iron) was only active at concentrations $> 10^{-7}$ M [17]. Serum transferrin, a biochemically similar iron-binding protein which is antigenically distinct from lactoferrin, was only minimally inhibitory at concentrations $> 10^{-6}$ M. Separation of the granulocyte inhibitory factor and lactoferrin by isoelectric focusing confirmed that the regions of inhibitory activity corresponded in both to a pH of 6.5. In addition, the purified immunoglobulin fraction of rabbit anti-human lactoferrin antiserum, but not anti-transferrin, inactivated the capacity of both lactoferrin and granulocyte inhibitor to block CSF production [17]. It has been postulated that mature granulocytes limit CSF production by eliminating bacteria [18], however, mature granulocytes in the presence of bacterial products and in response to phagocytic challenge release large quantities of their intracellular contents of lactoferrin and suppression of CSF production under these circumstances is probably mediated via lactoferrin. The observed quantitative deficiency of lactoferrin in the mature granulocytes of patients with acute and chronic myeloid leukemia and myeloproliferative disorders [19] further substantiates our contention that this iron binding protein is involved in a primary regulatory dysfunction associated with neoplastic myelopoiesis.

III. The role of stimulatory or inhibitory activities unique to the leukemic state

The theoretical possibility exists that the growth advantage of the leukemic clone is due to selective proliferation of leukemic cells in response to a macromolecule which is not growth stimulating to normal myelopoietic cells; alternatively, leukemic cells may produce an inhibitory activity specifically suppressive to normal but not leukemic hemopoiesis. Precedents for both possibilities have been reported. Conditioned medium from human embryo cell cultures (WHE-1CM) appeared to be specific for leukemic myeloid cells since it stimulated continuous growth and differentiation in suspension culture of human acute and chronic myeloid leukemic cells but had no influence on bone marrow or blood cells from normal donors either in suspension culture or in semi-solid media [20].

Evidence for a leukemia specific inhibitory activity (LIA) has been provided by Broxmeyer et al. [21,22]. Extracts and/or conditioned medium of bone marrow or blood cells from 76 of 85 patients with acute leukemia of a variety of morphological types inhibited colony formation of normal CFU-c by 28 to 90%. The activity was not related to treatment since untreated as well as treated patients possessed cells with inhibitory activity. Extracts from cells

of 41 of 47 patients with CML and 6 of 6 patients with chronic lymphoid leukemia were also inhibitory to normal CFU-c and titration of inhibitory activity suggested that extracts obtained from patients with chronic leukemia had lower levels of inhibitor than observed in acute leukemia [21,22]. The finding that the varied leukemic states contain the same type of inhibitory activity suggests that there may be a common link between the diseases which manifest as abnormalities in different hemopoietic cell lines and which are associated with profound suppression of normal myelopoiesis. Maximum inhibitory activity was present in medium conditioned by leukemic cells after three days, exceeding the inhibitory activity obtained by direct extraction and indicating active synthesis of the inhibitor [21,22]. The specificity of the inhibitory activity resided in its capacity to inhibit normal CFU-c but not leukemic CFU-c from patients with acute and chronic myeloid leukemia. The inhibition of normal CFU-c was cell cycle specific since pulse exposure of normal marrow to high specific activity ^{3}HTdR, washing and then pulsing with leukemic cell extract resulted in no greater inhibition than after ^{3}HTdR alone.

The cell type in the bone marrow and blood of patients with leukemia which produces the inhibitory activity has been characterized as belonging to a minority population of non-adherent, non-phagocytic cells of low density (< 1.070 gm/cm^3), slowly sedimenting (2–6 mm/hr) and present in the sheep RBC rosetting population which is E^-, EAC^-, Ig^-, EA^+ and Ia^- as determined by complement cytotoxicity with rabbit anti-human Ia-like antibody [12,23]. This cell population can be distinguished from the vast majority of the leukemic CFU-c and the blast cell population of the marrow. It therefore remains unclear as to whether leukemic inhibitory activity is a direct product of a subpopulation of leukemic cells or an abnormal reactivity of non-leukemic cells imposed by the leukemic state.

3. Neoplastic Transformation of the Pluripotential Stem Cell

Short term in vitro clonal assay systems have proved invaluable in providing information on the characteristics of both normal and leukemic progenitor cells restricted to the various hemopoietic cell lineages. Such in vitro systems have the disadvantage of not supporting pluripotential stem cell maintenance and consequently can provide little information on the processes involved in the differentiation of the pluripotential stem cell into the committed compartments. Furthermore it is now very clear that in a variety of human neoplastic hemopoietic diseases such as chronic myeloid leukemia, many varieties of acute myeloid leukemia, certain preleukemic states, polycythemia vera and primary myelofibrosis, the transformed target cell is the pluripotential stem cell. Recent developments in tissue culture methodology now permit extensive proliferation of mouse pluripotential stem cells (CFU-s) for many months in a bone marrow suspension culture system [24,25]. The key to successful stem cell replication in vitro appears to be the requirement for establishing an adherent layer of bone marrow cells containing a variety of cell types representative of bone marrow stromal elements [26]. This continu-

ous bone marrow culture system provides a unique model for defining the process of leukemic transformation from the outset, particularly as it may involve phenotypic changes in pluripotential stem cells. Dexter et al. [27] have reported that infection of continuous marrow cultures with an NB-tropic pool of Friend leukemia complex virus resulted in chronic production of spleen focus forming virus (SFFV) with in vivo erythroleukemia inducing capacity. However, no evidence of in vitro erythroleukemia was obtained but sustained proliferation of CFU-s was observed in virus infected cultures and after 9–10 weeks these stem cells were atypical forming granulocytic colonies of all stages of maturation in the spleen. Using a cloned stock of NB-tropic Friend strain of helper C type virus free of SFFV (F-MuLV), we have observed that many of the phenotypic changes first reported by Dexter et al. [27] using the Friend complex were produced by the F-MuLV helper virus alone [28,29]. These changes were first evident after 10 weeks of marrow culture and were not simply attributable to virus replication since high titers of F-MuLV were produced continuously throughout the culture period. The changes involved prolonged replication of CFU-s and CFU-c with greatly increased cloning efficiency of the latter with in vitro maturation defects observed both in the colonies and cultured marrow cell population.

The evidence for transformation of pluripotential stem cells is based on the detection of replicating CFU-s in 25% of transformed agar colonies and in cell lines derived from such colonies maintained in simple suspension culture for many weeks. Unlike the observation of Dexter et al. [27], the spleen colony morphology appeared normal and lethally irradiated mice reconstituted by F-MuLV transformed bone marrow remained alive and well for many months [28,29]. Mortality from leukemia in such reconstituted mice was only evident after 5–6 months. It is highly probable that CFU-c were also target cells for F-MuLV transformation since many transformed agar colonies did not contain CFU-s but continuously replicated new CFU-c. Since CFU-c self renewal was not seen in control colonies, one phenotypic sign of CFU-c transformation may be acquisition of extensive self renewal capacity on the part of a cell population normally dependent on differentiation from a pluripotent stem cell compartment.

Phenotypic changes were also observed in cultures infected with, and replicating, SFFV in the presence of F-MuLV. These were less obvious than in the F-MuLV infected cultures suggesting the possibility that SFFV may be interfering with stem cell transformation due exclusively to the F-MuLV "helper" virus.

In contrast to the influence of F-MuLV, marrow cultures infected with pseudotypes of Kirsten sarcoma virus with Rauscher virus showed a rapid loss of stem cell and progenitor cell production associated with transformation of the macrophage component of the adherent marrow microenvironment [29]. While it is possible that a wider spectrum of potential hemopoietic target cells may exist for K-MSV and that the Rauscher helper virus may transform stem cells in a manner analogous to F-MuLV, the rapid transformation of the adherent layer precludes the persistence of stem cells and progenitor cells which might otherwise have undergone transformation.

The role of C type viruses in the development of human leukemia remains an intriguing but as yet unresolved issue. The isolation of a type-C virus (HL-23V) from human acute myelogenous leukemia cells after sustained exponential leukocyte growth in vitro [20] has further drawn attention to the need for an in vitro hemopoietic system to investigate the transformation potential of such virus isolates. We have developed two systems which may prove useful in this regard [29]. The sustained replication of human CFU-c and production of myeloid cells for some weeks in continuous human bone marrow culture strongly suggests that pluripotential stem cell replication is being supported. The system, however, remains less than optimal when compared with the duration of stem cell production in the murine system. Two obvious differences between the species have become apparent from a comparison of the cultured bone marrow. Firstly, the adherent layer established with the primary inoculum of human marrow is deficient in the foci of giant fat-containing cells so characteristic of the murine system [26]. Fat-containing cells do, however, develop within focal areas of adherent marrow cells but unlike the mouse, these latter progressively become spherical and detach [29]. A second difference is that while CSF production in the mouse marrow culture is undetectable, high levels of CSF are produced in long term human marrow culture. The accumulation of CSF may compromise the continuous replication of stem cells by favoring conversion of the culture to a predominantly macrophage morphology. Attempts to sustain human stem cell replication on murine bone marrow adherent layers proved unsuccessful presumably due to species restrictions on cell interactions and the known species specificity of various regulatory macromolecules such as CSF. As part of a phylogenetic analysis, we have been able to establish that the Tupaia, one of the most primitive living prosimians, was hematologically very similar to man but established an adherent marrow environment similar to that seen in the mouse [29]. The extensive accumulation of fat-containing foci and absence of endogenous CSF production indicated that Tupaia adherent marrow could provide a suitable microenvironment for human stem cell replication. This possibility remains to be established since all attempts to coculture human marrow on Tupaia adherent layers have been thwarted by the continuous replication at high levels of Tupaia CFU-c as confirmed by colony cytogenetic analysis. A single inoculum of Tupaia marrow appears to be sufficient to sustain continuous replication of CFU-c and differentiating granulocytic cell production far in excess of six months [29]. This production is independent of a second feeding with either fresh Tupaia or human bone marrow and indicates that this in vitro prosimian marrow culture system exceeds even that of the mouse in sustaining prolonged stem cell replication. The development of marrow culture systems in species other than the mouse should provide further insight into the control of stem cell replication and the role of these cells as potential targets for viral transformation.

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Leukemic Inhibition of Normal Hematopoiesis*

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Introduction

The effects of human leukemic blasts on normal in vitro granulocyte-macrophage colony-forming cells (CFU-C) [2,19] have been studied with conflicting results [4,5,8,9,11,13,18,25]. In some studies inhibition of normal human CFU-C by leukemic blasts was seen [4,5,13,18] while other studies failed to show inhibition [8,9,25]. These discordant results may, in part, result from histocompatibility differences as suggested by Bull et al. [4].

We have utilized a syngeneic murine model, the C1498 acute myeloid leukemia of C57B1/6J mice [6,7] to further study the effects of leukemic blasts on normal hemopoietic stem cells. In these studies we have employed coculture of leukemic blasts both in-vitro [2,19] and in-vivo diffusion chamber (DC) cultures [1,22]. In preliminary studies we have also evaluated interactions of human leukemic blasts with normal human CFU-C.

Methods

Female CF1 mice were used as host mice for diffusion chamber (DC) cultures and female C57BL/6J mice were used as sources for normal marrow cells and as tumor bearers for the C1498 acute myeloid leukemia.

Stem Cell Assays

Murine granulocyte-macrophage progenitor cells (CFU-C) were assayed by a modification of a double layer soft agar technique [3,21] utilizing sera from endotoxin injected mice [20] or murine lung conditioned media [23] as sources of colony stimulating activity (CSA). Pluripotent stem cells (CFU-S) were assayed according to the method of Till and McCulloch [12,27] except that assay mice received 950 R from a cesium 137 source (118 R per minute). C57BL/6J cells were assayed in irradiated C57BL/6J mice. Burst-forming unit erythroid (BFU-E) and colony forming unit erythroid (CFU-E) stem cells were assayed by a plasma clot technique as previously described [28]. Human CFU-C were cultured by the method of Robinson and Pike [26].

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* Preliminary reports of some of the observations in this paper appeared in abstract form in Blood 44, 952 (1974) and Exp. Hemat. 4, 84 (1976) (suppl.)

In Vivo Diffusion Chamber

Normal C57BL/6J marrow, C1498 cells or mixtures of the two were grown in diffusion chambers [1,22] implanted into unirradiated or irradiated (950 to 1000 R) CF_1 host mice and differentiated and stem cell recovery assessed after 2–14 days. Normal marrow cells were implanted at $0{,}25-0{,}50\times 10^6$ cells per diffusion chamber and C1498 cells at the same levels; normal marrow-C1498 mixtures consisted of $0{,}05\times 10^6$ C1498 cells plus $0{,}45-0{,}50\times 10^6$ marrow or $0{,}125-0{,}25\times 10^6$ C1498 cells plus $0{,}25\times 10^6$ marrow cells. The effect of diffusible factors from C1498 cells was assessed by growing the leukemic cells adjacent to normal marrow cells, but separated from them by a cell impermeable 0,22 micron millipore filter in double diffusion chambers. In each experiment normal marrow was implanted into one of the chambers of the double diffusion chamber and the opposite chamber was implanted with either normal marrow or C1498 leukemic cells at the same concentration or with a higher concentration of normal cells; the recovery of differentiated and stem cells from the chambers with normal marrow grown adjacent to these groups was then assessed. The test chambers were implanted with $0{,}25-0{,}5\times 10^6$ normal marrow cells while the adjacent chambers were implanted with either $0{,}25-0{,}5\times 10^6$ normal marrow, $0{,}25-0{,}5\times 10^6$ C1498 cells (C1498) or $2{,}5\times 10^6$ normal marrow cells. Cocultures of human leukemic blasts (previously cryopreserved at −196°C in 10% DMSO) with normal marrow from HLA-MLC compatible siblings were carried out in a similar manner.

Cell Separation Experiments

C1498 cells were separated by unit gravity velocity sedimentation by the method of Miller and Phillips [16]. Cells were pooled and different pools assessed for their effect on normal marrow growth when mixed directly with normal marrow cells in diffusion chambers.

Probability figures were calculated using Student's T Test.

Results

The C1498 cells were unresponsive to CSA in in-vitro soft agar culture. In repeated experiments neither cluster (3–50 cells) nor colony formation was noted after 8–10 days growth. Mixing of from 0,25 to $0{,}5\times 10^5$ normal marrow cells with varying concentrations of leukemic cells in in-vitro agar cultures inhibited CFU-C growth (Fig. 1). Similarly direct mixtures of normal marrow and C1498 blasts in DC cultures inhibited CFU-C recovery (Fig. 2) but normal differentiated cell recovery was only inhibited with higher input concentrations (33–50%) of C1498 cells ($61 \pm 9\%$ of control over 5–12 days of culture, $p < 0{,}01$). This inhibition correlated in general with total C1498 cell recovery from DC (Fig. 3).

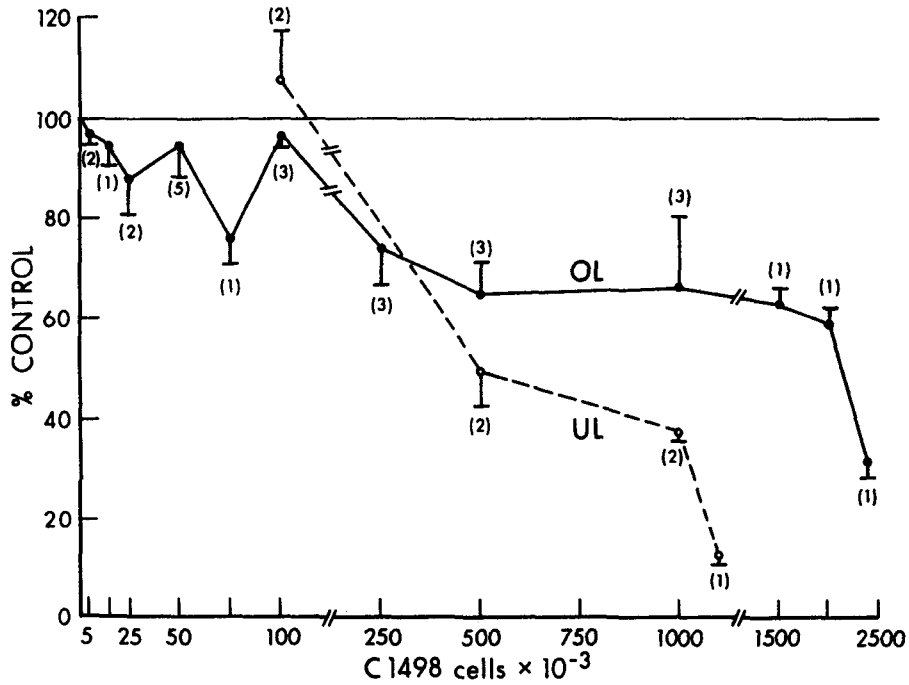

Fig. 1. The effect of C1498 cells on normal C57BL/6J marrow CFU-C in in-vitro soft agar cultures. Varying numbers of C1498 cells were added to cultures of from 0.25–0.50 × 10^5 normal marrow cells and CFU-C growth compared to cultures without added leukemic cells. C1498 cells were either mixed directly with normal marrow cells in the overlayer (OL) or with the source of CSF in the underlayer (UL) of the double layer agar cultures. The results are expressed as a per cent of control ± one standard error of the mean (SEM). The numbers in parenthesis represent the number of separate experiments for each concentration of C1498 cells. These data are derived from a total of 11 separate experiments. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**.)

Similar diffusion chamber mixing experiments were carried out with subpopulations of C1498 cells separated by unit gravity velocity sedimentation and the degree of inhibition compared to that seen with unseparated leukemic cells (Table 1). Inhibition was most marked with the larger more rapidly growing C1498 cells (pools 1–2) as compared either to unseparated or smaller C1498 cells (pools 3–4) mixed at the same per cent with normal marrow. There was a general but not exact correlation of CFU-C inhibition with total numbers of C1498 cells derived from the various pools (Fig. 4).

Transmembrane Experiments

The effect of either C1498 cells or normal marrow cells cultured transmembrane from normal C57BL/6J marrow cells on CFU-C and CFU-S recovery after varying periods of double diffusion chamber growth is presented in Fig. 5.

The CFU-S, and to a lesser extent the CFU-C, were inhibited when normal marrow cells were cultured adjacent to C1498 cells. The mean recovery of CFU-S grown across from C1498 cells in diffusion chambers for 5–14 days was 45 ± 7% of control ($p < 0{,}002$) and that of CFU-C was 72 ± 7% of control

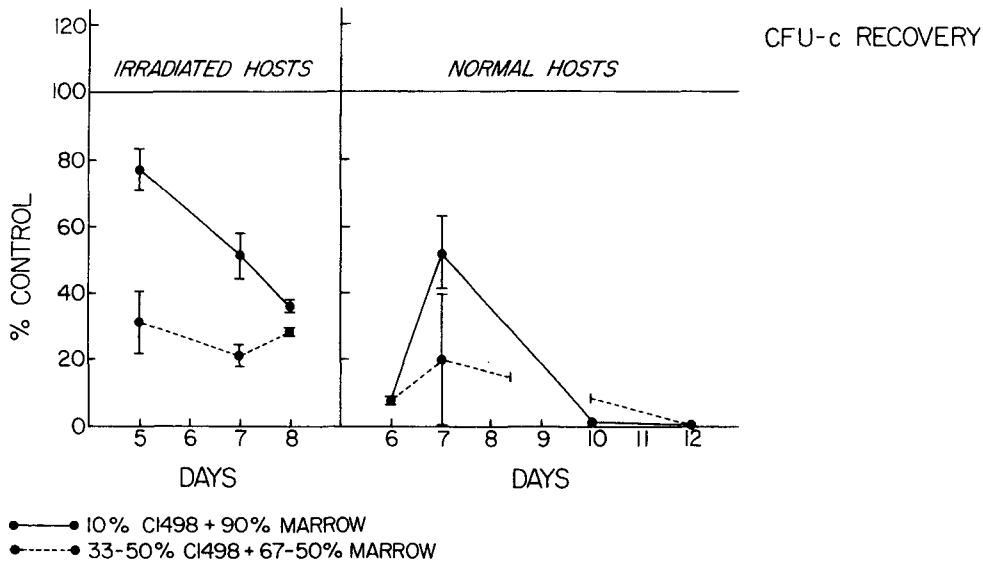

Fig. 2. The effect of C1498 cells mixed with C57BL/6J cells on CFU-C recovery from DC cultures. The number of CFU-C recovered from DC with 10–50% C1498 cells mixed with normal marrow cells at varying times after surgical implantation expressed as a per cent of control ± one SEM. The number of cells plated for the in vitro assay of CFU-C varied from experiment to experiment; in 13 of 15 experiments the numbers of C1498 cells plated were not sufficient to account for the observed degree of inhibition. In 7 of 15 experiments the number of C1498 cells plated in-vitro was below 118947, a number unlikely to give significant in-vitro inhibition (see Fig. 1). These data are from 15 separate experiments (4 separate surgical implantations); the data at day 5 are the mean of 2 separate experiments for each concentration of C1498 cells. A total of 195 DC were utilized in these experiments

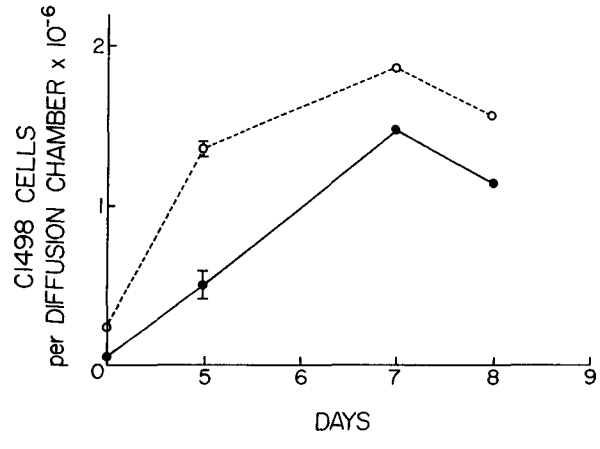

Fig. 3. Blast recovery per chamber from chambers with 10% (solid line) or 50% (dashed line) C1498 cells mixed with normal marrow. These studies utilized irradiated CF_1 hosts and are from the same experiments presented in panel 1 (Fig. 2). The data are presented as number of C1498 cells per chamber ± 1 SEM. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

($p < 0{,}02$). Stem cell recovery was not effected by a 10 fold higher input of normal marrow in the opposite chamber.

In general, chambers implanted with C1498 cells or high concentration normal marrow had equivalent cell yields. Chambers implanted with $0{,}25 \times 10^6$ C1498 cells yielded 1,0, 1,8, 3,0 and 5,0 million cells on days 5, 7, 8 and 14, respectively, while chambers implanted with $2{,}5 \times 10^6$ normal marrow cells yielded 2,3, 3,4 and 1,3 million cells on days, 7, 8 and 14 of growth, respectively. There was no demonstrable inhibitory effect of C1498 cells on total cell yield from adjacent chambers implanted with $0{,}25 \times 10^6$ normal marrow (mean 104 ± 19% of control, 5–14 days growth) but there was a suggestion of augmented recovery of cells from normal marrow grown opposite high concentration marrow (mean 154 ± 28% of control, 7–14 days of growth). There

Table 1. The effect of C1498 cells separated by velocity sedimentation on normal marrow CFU-C recovery from diffusion chamber mixtures (per cent of control)

Exp.	Days diffusion chamber growth	% Unseparated C1498 cells	% Separated C1498 cells			
		3%			3%	
			Pools 1	2	3	4
1	5	133 ± 6	89 ± 3	122 ± 7	186 ± 3	178 ± 8
	7	79 ± 7	69 ± 2	70 ± 5	94 ± 6	—
					5%	
2	5	71 ± 2	39 ± 2	50 ± 7	60 ± 4	65 ± 1
	7	51 ± 7	10 ± 1	22 ± 1	68 ± 1	129 ± 3

The results are expressed as a per cent of control ± 1 SEM. A total of 160 and 144 diffusion chambers were evaluated in exp. 1 and 2, respectively. The mean number of control CFU-C implanted into $0{,}45 \times 10^6$ diffusion chambers in these 2 experiments was 1324, while the mean number recovered on days 5 and 7 of diffusion chamber growth was 1044 and 1665, respectively, the range of sedimentation velocities in exp. 1 and 2 for pools 1, 2, 3 and 4 were 10,1–6,9, 6,9–4,7, 4,5–2,8 and 2,7–0,73 mm/hr, respectively

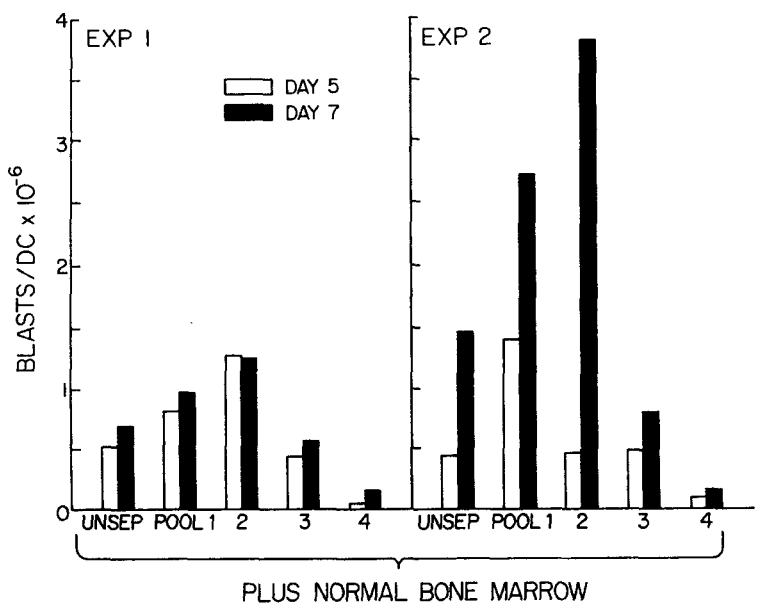

Fig. 4. The number of C1498 blasts per diffusion chamber from unseparated and separated C1498 cell-normal marrow mixtures after 5 to 7 days of diffusion chamber cultures. These results are from the same experiments presented in Table 1. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

was also no significant shift in differentiation when normal marrow was grown adjacent to the various groups. The over-all (5–14 days) mean percentage of granulocytes in the normal marrow groups ranged from 63,6 to 66,1 and that of macrophages from 32,6 to 35,2.

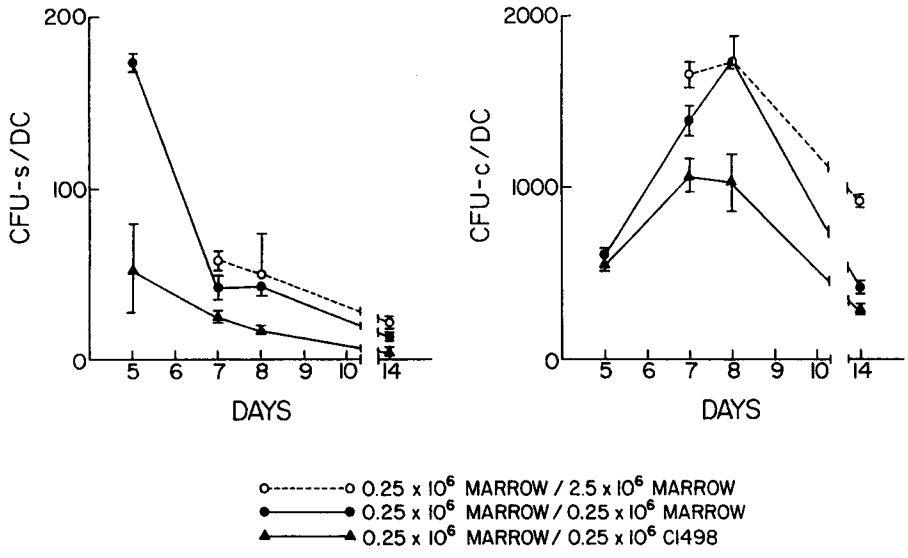

Fig. 5. Transmembrane effects of C1498 cells or high concentrations of C57BL/6J cells on CFU-S and CFU-C recovery in double diffusion chamber cultures. C57BL/6J marrow cells implanted at a concentration of $0{,}25 \times 10^6$ cells per chamber (test chamber) were grown adjacent to C1498 cells implanted at the same cell level or C57BL/6J marrow implanted at $0{,}25 \times 10^6$ or $2{,}5 \times 10^6$ cells per diffusion chamber and the recovery of CFU-C and CFU-S assessed from the test chambers at varying times after implantation. These data are from 5 separate experiments for C1498 and 4 for $2{,}5 \times 10^6$ NL marrow cell studies (5 and 4 separate surgical implantations, respectively) in which cells from 84 control and 75 experimental double diffusion chambers were evaluated. The data at day 8 represents the mean from 2 separate experiments. Host CF_1 mice were pre-irradiated with 1000 R on the day of surgery. Data are expressed as stem cell per diffusion chamber ± one SEM. (Reprinted from Quesenberry et al. by permission from N. Engl. J. Med., **24**)

In preliminary experiments in-vitro coculture of C1498 cells in plasma clot cultures with normal C57BL/6J marrow resulted in inhibition of BFU-E but not CFU-E growth (Table 2).

Table 2. C1498 leukemic cell inhibition of normal C57BL marrow erythroid stem cells

Group	% Control	
	CFU-E/10^6 cells	BFU-E/10^6 cells
Normal: C1498 (1:2)	99 ± 5	102 ± 5
Normal: C1498 (1:10)	106 ± 8	61 ± 12

Coculture of blasts from a patient with acute myelomonocytic leukemia with HLA-mixed leukocyte culture compatible sibling marrow cells in DC cultures in irradiated mice resulted in inhibition of normal CFU-C growth (Fig. 6). The effects of subpopulations of blasts separated by velocity sedimentation was also assessed and it appeared that the smallest slowly sedimenting cells were most inhibitory.

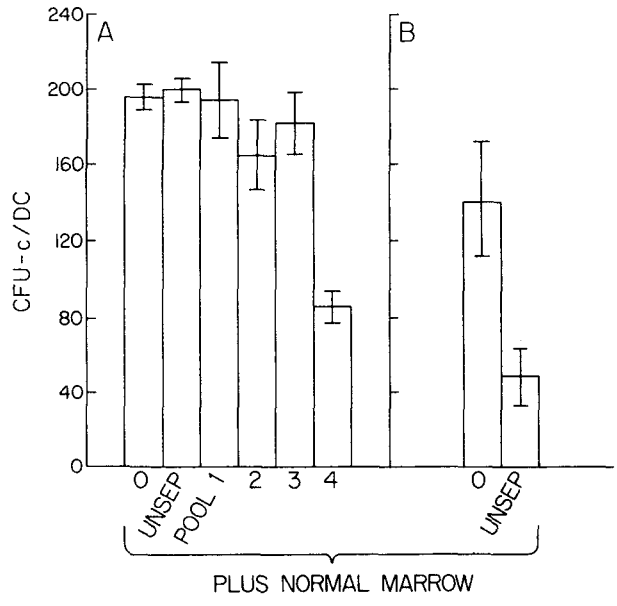

Fig. 6. Inhibition of histocompatible (HLA-MLC) normal Human CFU-C by marrow cells from a patient with acute myelomonocytic leukemia (AMML). Panel A shows the number of normal human CFU-C recovered after 4 days of diffusion chamber culture when unseparated AMML cells or AMML cells separated into four pools by velocity sedimentation were mixed directly with a marrow from an HLA-MLC compatible sibling in diffusion chambers. These AMML cells did not form colonies. 0.05×10^6 unseparated AMML or pool 2 and 3 cells or 0.02×10^6 pool 1 and 4 cells were mixed with 0.45×10^6 normal marrow cells in DC and CFU-C recovery compared to that seen with 0.45×10^6 normal marrow cells in DC alone (0 group). Inhibition of CFU-C recovery by pool 4 cells is apparant. Pools 1–4 had sedimentation velocities of 10.68–7.74, 7.17–5.02, 4.49–1.99 and 1.52–0.1 mm/HR. respectively. Results are expressed as the number of CFU-C per DC ± one SEM. Panel B shows the number of normal CFU-C recovered after 4 days of DC culture when 0.25×10^6 unseparated AMML cells were mixed in DC with 0.25×10^6 normal sibling marrow cells (unseparated group) or when 0.25×10^6 normal marrow cells were cultured alone in DC

Discussion

The present experiments indicate that C1498 leukemic blasts inhibit normal murine marrow hematopoietic stem cells. Inhibition of normal CFU-C and BFU-E (but not CFU-E) was demonstrable when C1498 cells were mixed with C57BL/6J marrow cells in in-vitro cultures (Fig. 1 and Table 2).

A decreased recovery of CFU-C was also noted when leukemic cells were mixed with normal marrow cells in in-vitro diffusion chamber cultures (Fig. 2), and experiments utilizing the double diffusion chamber technique, in which the C1498 and normal marrow cells are separated by a cell impermeable membrane, showed that both CFU-C and CFU-S were inhibited (Fig. 5) and that cell contact was not necessary for this inhibition to occur.

Inhibitory effects on differentiated cell production were only demonstrable with relatively high leukemic-normal cell input ratios (33–50% C1498 cells) and were not seen with a lower ratio (10% C1498 cells) or in the transmembrane experiments. These effects on differentiated cell production in diffusion chamber cocultures of C1498 and normal marrow are similar to those reported by Miller et al. [14]. Our results differ from those of Miller et al.

[14, 15] in that we found inhibition at the CFU-C, as well as the CFU-S level, and demonstrated that cell contact was not necessary for this inhibition.

Cocultures of subpopulations of C1498 cells separated by unit gravity velocity sedimentation and normal marrow indicated that the large rapidly growing leukemic cells (pool 1) were most inhibitory with little or no inhibition observable with the smallest slower growing leukemic cells (pools 3 and 4) (Table 1). Although there was not an exact correlation with total numbers of chamber leukemic cells, the striking differences in the inhibitory effects of the larger and smaller C1498 cells seem most likely to be based predominantly on differences in growth rate and final diffusion chamber leukemic cell concentration, rather than any other unique characteristic of the separated leukemic cell subpopulation.

The double diffusion chamber studies on the transmembrane effects of C1498 cells on normal marrow stem cells indicate that a substance released by the leukemic cells mediates stem cell inhibition. The cell recovery after 7–14 days of in-vivo culture from diffusion chambers implanted with $2{,}5 \times 10^6$ normal marrow cells approximated that from chambers with C1498 cells implanted at $\frac{1}{10}$th that level, suggesting that there was more cell death in the chambers with high concentrations of normal marrow, yet there was no demonstrable inhibition by these cells. These results suggest that the inhibition seen with C1498 cells was not due simply to cellular degradation products. The inhibitory substance which is released by C1498 cells could be unique to leukemic blasts, a by-product of malignant cells in general or simply relate to rapidly proliferating cells.

In toto, the present data indicate that murine leukemic blasts inhibit normal marrow stem cells by elaborating a diffusible inhibitory substance. Consistent with these observations are the studies of Handler et al. [10] showing that rat myeloid leukemic cells can condition media with substances inhibitory to normal CFU-C. Furthermore, in the present studies this inhibition appears to be most marked at the pluripotent stem cell level with lesser degrees of inhibition seen with increasing degrees of differentiation within the myeloid pathway. These data are consistent with the concept that leukemic inhibition acts primarily on CFU-S with effects on CFU-C, BFU-E and differentiated granulocytes possibly being of a secondary nature.

Preliminary studies from our laboratory indicate that human HLA-mixed lymphocyte compatible leukemic blasts can inhibit normal CFU-C recovery from diffusion chamber cultures and that it may be possible to isolate a subpopulation of inhibitory cells from marrow cells from patients with acute myelomonocytic leukemia (Fig. 6). These studies, along with the studies on human CFU-C inhibition noted above [4, 5, 13, 18] and Jacobson's studies on human leukemic inhibition of normal human marrow stem cells in plasma clot diffusion chamber cultures [11] suggest that it may be possible to extrapolate the present results to the mechanisms underlying the myelosuppression in humans with acute leukemia.

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Clonal Diseases of the Myeloid Stem Cell Systems

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The structure and kinetics of the hematopoietic stem cell compartment have long been the subject of considerable speculation. Based on morphologic observations of normal and abnormal human marrow and of a perturbed system in experimental animals, primarily the rabbit, in 1938, Downey [1] concluded there was a stem cell capable of giving rise to all hematopoietic tissue. He believed this cell in turn gave rise to a lymphoid stem cell and to a myeloid stem cell. The myeloid stem cell could give rise directly to erythroid, megakaryocytic and monocytic cell lines and in turn produced a tertiary stem cell which could generate neutrophils, eosinophils and basophils. With the development of functional assays for clonal cell growth in vivo and in vitro and through the use of chromosome marked clones this suggested structure has proved to be correct in substance although certain minor variations are indicated. Most definitive studies of the structure of the stem cell compartment are in mice [2] but, in general, the data generated in human diseases suggest that the human stem cell structure is the same as that of the mouse. In Fig. 1, one current "best guess" is shown. There seems little doubt that at least 3 concatenated precursor compartments exist for all myeloid cells. Whether there are still more intermediate stages and whether or not most cells forming colonies in vitro are stem cells (i.e. capable of self-replication) remain open question.

Studies from Phillips Laboratory [2] using irradiation induced chromosomally marked clones injected into W/W^v mice have shown the presence of an hematopoietic stem cell which is totipotent for all hematopoietic cells, lymphoid as well as myeloid (THSC) and distinct from a cell pluripotent for myeloid tissue (PMSC). This latter cell produces spleen colonies in irradiated recipients and is, therefore, also known as a colony forming unit cell (CFU-S), but whether other classes of cells will also produce spleen colonies is unknown. The structure of the lymphoid compartment as derived from the THSC will not be discussed.

A cell which is restricted to the production of neutrophil-monocyte colonies in vitro, colony forming unit – culture, (CFU-C) has characteristics distinguishing it from the CFU-S. Among a variety of differences including a much higher percent in DNA synthesis [3], perhaps the most convincing is the presence of normal CFU-C in the W/W^v/mouse in the face of very abnormal behaving CFU-S [4]. Cells apparently restricted to production of erythroid (burst forming unit, BFU-E) [5] megakaryocytic (CFU-meg) [6] and eosinophilic (CFU-Eos) [7] are also demonstrable by in vitro analysis.

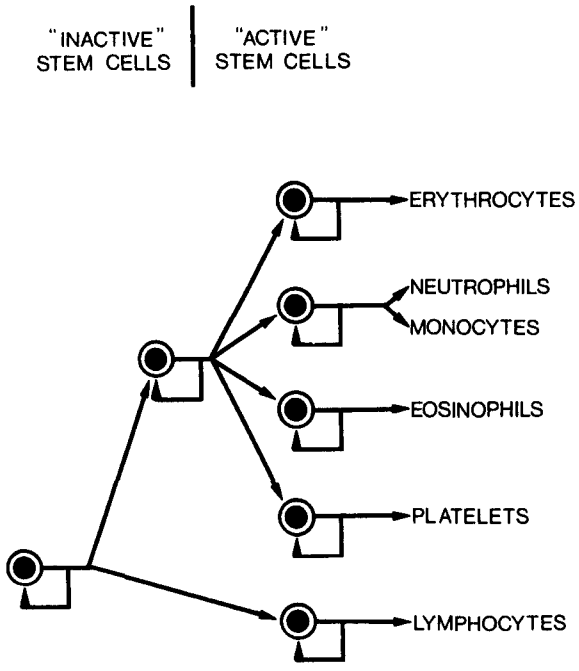

Fig. 1. A model of the Hematopoietic stem cell system. Three concatenated cell systems are presented. "Active" refers to the fact that a high percentage of cells forming colonies in vitro are in DNA synthesis while the more pluripotent spleen colony forming cell compartment has few cells in DNA synthesis

At the present time, human diseases of the stem cell system appear to involve either the THSC or the PMSC (CFU-S) compartment, although specific defects in more committed compartments may possibly explain diseases of a single cell line such as Diamond-Blackfan anemia or certain forms of congenital neutropenia. However, no clonal markers have been identified in these conditions.

There is evidence that the myeloid leukemias (ML) acute (A) and chronic (C) involve a wide spectrum of hematopoietic tissue. In the case of AML the most common morphologic expression is by an increase in myeloblasts. However, the cells often have some monocytic features as well and any of the myeloid cell lines may appear as the predominant morphologic expression in AML (Table 1). The very frequent myeloblast-monocyte morphologic mix-

Table 1. Morphologic expressions of myeloid leukemia

AML	CML	in blast crisis
√	√	Myeloblastic
		Maturation
√	√	Promyelocytic
√	√	Promyelocytic (Eosinophil)
√	√	Promyelocytic (Basophil)
√	√	Monoblastic – monocytic
√	√	Myelomonocytic
√	√	Erythroblastic
√	√	Megakaryoblastic
√	√	Any mixture of above
(X)	√	Lymphoblastic
X	√	Any mixture of above

ture may reflect the immediate common origin of these cells. To date there has been no means of separating individual precursors for these cell lines in the in vitro clonal assays. When colonies of human or murine cells are grown in semi-solid media in the presence of colony stimulating factor, mixed colonies of neutrophils and monocytes occur [7]. Is AML one basic disease involving a pluripotent myeloid stem cell or a series of diseases involving the specific progenitors; such as, CFU-C or BFU-E, etc? Put another way, if these are induced by an oncornavirus, what is the primary target cell?

There are a number of fairly simple clinical observations which bear on this question. First of all, there is almost never a shifting myeloid-lymphoid picture in AML and a mixed myeloid-lymphoid presentation of AML is not recognized. AML is defined arbitrarily in this paper as excluding any patient in whom the Ph^1 chromosome is present for I have observed an apparently mixed myeloid-lymphoid blastic pattern in patients presenting with Ph^1 positive acute leukemia. Such patients are defined, again somewhat arbitrarily, as presenting in the blastic those of CML (see below). Thus, the THSC does not appear to be involved in AML. However, a shifting morphologic expression within myeloid cell lines does occur. Perhaps the most commonly recorgnized shift is in the patient who presents with a predominantly erythroblastic picture but proceeds to develop an increasing predominance of myeloblasts or myelomonoblasts. Even when the predominant cell is a myeloblast at the time of diagnosis, megaloblastic erythroid precursors and abnormally small megakaryocytes are often present if the marrow smear is searched with diligence. This suggests that more than one myeloid cell line is involved in the AML process and suggests that the target cell is the PMSC rather than the more differentiated CFU-C systems. This is supported by chromosomal studies in which marker chromosomes in the myeloblastic cells have also been found in erythroid cells [8].

Clinical observations in CML suggest the target cell may be the THSC. During the chronic phase, abnormalities of all of the myeloid cell series may be observed suggesting that the leukemic clone is at least feeding through the PMSC. During acute transformation, all of the morphologic spectrum seen in AML may appear and, furthermore, a lymphoblastic or even a mixed lymphoblastic-AML picture may develop [9]. The Philadelphia chromosome is found in erythroid precursors, megakaryocytes, monocytes and eosinophils as well as in neutrophil precursors [10]. Uniformity of G-6 PD isozymes in the myeloid series of patients whose non-hematopoietic cells are heterozygous confirms the clonality of the disease and again indicates involvement of more than one cell in the myeloid series [11]. Furthermore, such heterozygotes may also have certain lymphocyte populations homozygous for the isoenzymes, strongly suggesting that the THSC is the target cell [12].

Polycythemia Rubra Vera (PRV) and idiopathic myelofibrosis (IMF) are also diseases in which there is clinical evidence for disturbance in cell production of all of the myeloid cell systems. Analysis of G-6 PD isozyme data is compatible with the concept that these diseases are also clonal diseases of myeloid stem cells [11]. In paroxysmal nocturnal hemoglobinuria there is evidence for abnormality of neutrophils and platelets as well as for red blood

cells suggesting that this also might be a disease of the pluripotent myeloid stem cell [13]. As yet, there is little data which will allow one to make a guess as to whether these diseases are at the level of the THSC or the PMSC. However, the report of the development of acute lymphoblastic leukemia in a patient with PRV [14] favors the THSC rather than the PMSC being the affected cell.

Still other diseases, such as aplastic anemia and cyclic neutropenia are diseases which appear to involve myeloid stem cells, although it seems unlikely that they are clonal.

How does a single stem cell take over the entire production of the myeloid system? In most patients, chromosomal and isozyme data indicate that all cell production is from the clone and in vivo evidence for persistent growth of normal stem cells is lacking. Evidence relative to the question of whether normal cells are still present is discussed below. As a generality, when we observe a clone of cells which is growing with seeming inappropriateness and eventually leading to death we make a diagnosis of a malignant neoplasm. For this reason most now consider PRV and IMF as well as AML and CML to be malignant neoplasms. The "neoplastic" cell, in this case a neoplastic THSC or PMSC, must have some form of relative growth advantage as compared to the normal cells and secondly, its growth must in turn somehow be suppressive for growth of the comparable compartment of normal cells. Theoretically, these two characteristics could be independent phenomenon or might be mediated by the same mechanism.

In any system which I've been able to envision which would allow the neoplastic cell to take over the myeloid system, there must be an abnormality in that cell with respect to its response to normal, physiologic factors regulating the system. This abnormality could range from complete autonomy of growth (a cell which would continue to grow without regard to the presence or absence of physiologic regulators) to subtle defects; such as, one in which the neoplastic cell was simply more sensitive to growth stimulators or less sensitive to growth inhibitors than is the normal cell. In either event, the normal cells could become repressed by a variety of mechanisms. As the neoplastic clone expanded the normal control system might recognize the expanded neoplastic stem cell system and repress the normal one or the neoplastic clone could even produce inhibitors of the normal.

Undoubtedly spurred on by the observation that most megaloblastic anemias, once widely thought to be closely allied to leukemia, were due to vitamin deficiency, a long standing hypothesis has been held by many that a least certain "leukemias" may represent faulty regulatory systems rather than intrinsic neoplastic abnormality of the cell identified as "leukemic". In my opinion, the demonstration that these are clonal diseases, coupled with the demonstration that the normal counterpart cells are either absent or repressed rules out this hypothesis as a primary cause of the disease. There may be abnormalities of the regulatory system as well, but I think these must be considered secondary to the primary neoplastic process rather than as playing a causative role.

Just as the primary direct evidence for clonality of disease comes from

chromosome and isozyme data, so does the evidence for the presence of some residual normal stem cells.

Is a chromosome abnormality an accurate marker as to whether or not a cell is part of the clone of human leukemia? This question cannot be answered with certainty, but there is growing evidence, if of an inferential nature only, that it does not. I think all would agree that all cells bearing the Ph^1 abnormality are part of the clone in CML and that cells bearing a consistent chromosome abnormality in AML, PRV or IMF are part of that clone. It is the converse situation where serious questions must be raised; it is not clear that a cell not bearing the chromosome abnormality is not part of the clone. A number of pieces of evidence suggest that only a portion of the clone carries the chromosome abnormality.

Perhaps the strongest evidence suggesting that this is true are the somewhat discrepant findings with respect to chromosome abnormalities and isozyme studies in PRV and IMF [11, 15]. The discrepancies may be due to the fact that both studies have been done in a very limited number of patients and parallel studies have not been done in the same patient, but discrepancies are there none-the-less. All isozyme studies to date in patients with active PRV and IMF have indicated that all myeloid cells analyzed from the patients are part of the clone. However, in those patients in whom a chromosome abnormality has been found it often is present in only a portion of the analyzed myeloid tissue [16]. Similarly, when a chromosome defect is present in AML it often is not present in all analyzed myeloid tissue, even when virtually 100% of the myeloid cells appears to be leukemic on stained smears. This is also true for changes other than the Ph^1 in blastic crisis of CML [17] and quite discordant changes in chromosome defects and morphology may be observed in blastic crisis [18].

Although most patients with CML have the Ph^1 chromosome in all analyzed myeloid metaphases, some do not. The general assumption is that the latter patients are chimeric, i.e. have persistence of both normal and leukemic cells, an assumption which may or may not be true. When chromosome analysis was carried out on granulocyte-macrophage colonies grown in vitro, Ph^1 negative colonies were found in some patients in whom all direct metaphases had been positive [15]. This suggested that normal stem cells were still present, but that they were dormant in vivo. However, when Fialkow and coworkers (see Fialkow's paper in this symposium) analyzed G-6 PD isozymes in individual G-M colonies from patients with CML, no colonies were found which were not part of the clone. Resulution of this seeming discrepancy will require further studies in which both chromosomes and isozymes are analyzed in colonies from the same patient.

For the above reasons, the use of the lack of chromosome markers to prove the persistence of normal stem cells in these clonal diseases may be questioned. Keeping that in mind, there is none-the less fairly strong evidence that normal stem cells persist in these diseases but probably in a quiescent state. First, and perhaps foremost, with respect to the strength of the evidence is the development of remission in AML. Suffice it to say that virtually all current evidence points to remission in AML representing the re-

growth of the normal myeloid system while the clone has been reduced and held in check by therapy. Evidence is also quite strong for the persistence of normal PMSC in PRV. All myeloid tissue taken directly from the patient for isozyme analysis has apparently been part of the clone. However, when colonies of erythroid tissue have been grown from the same patient, some have been isozymically heterozygous (see Adamson's paper in this symposium). This is compatible with the previously expressed concept that the expanding neoplastic clone induces repression of normal cells in vivo, but that they are still present. As noted above the situation is not so clear in CML.

In summary, there is strong evidence that CML, AML, PRV and IMF are clonal diseases of a pluriopotent hematopoietic stem cell and suggestive evidence that PNH is such a disease. There is strong evidence that CML is a disease of the THSC and suggestive evidence that PRV may involve this cell; AML more likely is a disease of the PMSC and IMF is a clonal disease of one of these two cells. The nature of the growth advantage enjoyed by the abnormal cell as compared to the normal cell is unknown. However, the data in hand strongly suggest that normal stem cells, while still clearly present in some of the diseases, are quiescent and not producing mature cells in most patients. Existing data appears to rule out the possibility that any of these diseases is due to faulty regulation by factors external to the stem cell itself.

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Intramedullary Influences on in Vitro Granulopoiesis in Human Acute Myeloid Leukemia*

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Summary

Provision of granulocyte-monocyte colony stimulating activity by human bone marrow (CSA_{BM}) was determined in 21 patients with acute myeloid leukemia (AML) utilizing in vitro culture techniques to assess intramedullary cellular interactions on human granulopoiesis. CSA_{BM} production of these patients was compared to that from normal marrow by testing the capacity of conditioned mediums from adherent marrow cells to promote granulocyte-monocyte colony formation in agar of relatively light density nonadherent human marrow target cells.

Morphologic, cytochemical, density and phagocytic characteristics of normal marrow cells suggested that CSA_{BM} production was provided by mid-density adherent cells including those of the monocyte-macrophage series. Significantly decreased CSA_{BM} provision was found in 62% of patients with AML at diagnosis or relapse. Only 33% of these patients entered chemotherapy-induced complete remission, in contrast to an 88% remission rate in the patients with normal CSA_{BM}. Sequential studies in 9 patients during complete remission showed normal or increased CSA_{BM}, which generally decreased concomitant with relapse. These findings suggest that adequate CSA_{BM} provision may be essential for sustaining normal granulopoiesis in AML and may reflect persistence of a normal marrow monocyte-macrophage population. Monitoring this parameter appears useful for evaluating microenvironmental influences on granulopoiesis and assessing prognosis in AML.

Microenvironmental influences within bone marrow and spleen have been shown to be critical for hemopoietic stem cell proliferation and differentiation in experimental animals (McCulloch, Siminovich et al., 1965; Trentin, 1971; Gallagher, McGarry et al., 1971; Knospe and Crosby, 1971; Chamberlin, Barone et al., 1974; Matioli and Rife, 1976; Cline, LeFevre et al., 1977). Histologic and functional studies by these investigators have demonstrated that locally active cell-derived factors provide stromal influences contributing to the support of hemopoiesis.

In vitro marrow culture techniques have permitted analysis of factors involved in the regulation of granulopoiesis by evaluating the ability of

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granulocytic progenitor cells (CFU-C) to form granulocyte-macrophage colonies in agar under the necessary influence of the humoral stimulatory substance termed colony stimulating activity (CSA) (Rickard, Shadduck et al., 1970; Metcalf, 1973). Human marrow cells require cellular sources of CSA for their in vitro proliferation, whereas murine marrow is stimulated as well by CSA present in serum and urine (Foster, Metcalf et al., 1968; Metcalf and Stanley, 1969; Pike and Robinson, 1970; Metcalf and Moore, 1975). Recent studies in mice have shown that marrow CFU-C proliferation is related predominantly to intramedullary CSA elaboration by cells firmly adherent to the inner surfact of hemopoietic bone (Chan and Metcalf, 1972; Chan and Metcalf, 1973). Thus, local production of CSA within the marrow plays a major role in influencing granulopoiesis.

Cellular sources of CSA are also present within human marrow and can be selectively harvested by their adherence and density characteristics (Haskill, McKnight et al., 1972; Messner, Till et al., 1973; Moore, Williams et al., 1973; Senn, Messner et al., 1974). We have employed these physical separation techniques to evaluate marrow cell-derived CSA levels in normal subjects and patients with acute myeloid leukemia (AML) in order to determine the possible role of human marrow CSA provision as a microenvironmental stimulus for granulopoiesis.

Methods

The methodology for these studies has recently been described in detail (Greenberg, Mara et al., 1978). The buoyant component of aspirated human marrow cells were obtained by Hypaque-ficoll density centrifugation. These cells were then permitted to adhere to plastic tissue culture dishes (Messner, Till et al., 1973). The nonadherent cells were rinsed off and the remaining adherent cells were incubated in modified McCoy's medium containing 15% fetal calf serum and 0.5 mM 2-mercaptoethanol for 7 days at 37°C. With regard to the kinetics of the CSA production, an initial rise within 1–2 days occurred, followed by a fall and then a more marked sustained rise by 5–7 days of cellular incubation. After this incubation, the conditioned medium was harvested and stored at –20°C until use. Target normal human marrow cells were provided by obtaining buoyant cells less dense than 1,068 g/cm^3, utilizing the bovine serum albumin neutral density (density cut) procedure (Greenberg, Mara et al., 1976; Heller and Greenberg, 1977). These cells were then permitted to adhere to tissue culture dishes and the nonadherent buoyant cell population was harvested and used as target cells. In selected experiments continuous albumin density gradients were performed. Cells and test conditioned mediums were incubated for 7–10 days in agar culture at 37°C and colonies were counted, as previously described (Greenberg, Nichols et al., 1971; Greenberg, Mara et al., 1976). Colonies consisted of more than 50 cells, with granulocytic-monocytic differentiation. Quantitative estimates of effective CSA concentrations were obtained by performing titration curves of conditioned mediums. For standardization, the number of colonies produced

by these concentrations were compared to those stimulated by a stable leukocyte conditioned medium CSA source. The data were analyzed by curve-fitting computer programs (Greenberg, Bax et al., 1974).

Results

A sigmoid-shaped dose response curve of CSA values was obtained with increasing numbers of adherent and total marrow cells. Plateau levels of CSA occurred at approximately $3–15 \times 10^5$ adherent cells. Most normal specimens provided this number of adherent cells, with approximately 9% of the marrow cells being adherent. All of the CSA was provided by the adherent cell population, and specifically no CSA was provided by the nonadherent target marrow cells. Plasma from normal marrow or peripheral blood had no demonstrable CSA when nonadherent target cells were used, whereas the presence of the adherent cells permitted colony formation to occur. This indicated that substances present in normal serum enhance CSA production by endogenous CSA-producing cells rather than providing CSA itself. Control plates lacking a CSA source had no colony formation. Density distribution profiles showed that the CSA-producing cells represented a subpopulation of the adherent cells, with a peak density of 1.066 g/cm^3.

The morphologic, cytochemical and phagocytic characteristics of the adherent marrow CSA-producing cells were assessed, and showed 84–87% of these cells to be α-naphthyl acetate esterase positive, to morphologically resemble monocytes, and to be capable of phagocytosing latex particles. These data suggest that mid-density monocytes and macrophages contribute a major portion of the marrow CSA.

With these studies providing a background for quantitating and characterizing the normal marrow CSA-producing cells, we turned our attention to patients with AML. All AML patients received the same chemotherapeutic induction regimen (daunomycin, cytosine arabinoside, and 6-thioguanine) and maintenance program (monthly cytosine arabinoside and 6-thioguanine), as previously reported (Embury, Elias et al., 1977). In comparison with 16 control subjects, significantly low marrow CSA levels were found in 13 of 21 AML patients at diagnosis or relapse. Only 4 of the 13 patients (33%) with low marrow CSA entered complete remission, whereas 7 of 8 patients (88%) with normal CSA did achieve complete remission ($p < 0.01$). These data have recently been more completely described (Greenberg, Mara et al., 1978). All 4 patients in partial remission and 42 of 46 in complete remission had normal marrow CSA values. In comparison with control subjects, low CSA values were particularly found in patients with acute myeloblastic as opposed to myelomonocytic leukemia. These two entities were distinguished by previously defined morphologic criteria (Hayhoe and Cawley, 1972). It should be emphasized, however, that this method of categorization is less sensitive and specific than current cytochemical techniques (Bennett, Catovsky et al., 1976). Other clinical parameters and patterns of marrow colony formation were evaluated, and showed no significant correlation with complete remission

rates or marrow CSA levels. Further experiments showed the absence of inhibitors of CSA production or dilution of CSA-producing cells in the AML marrow. Sequential studies of 3 patients with AML in stable remission demonstrated persisting normal marrow CSA and CFU-C values (obtained monthly, just prior to the chemotherapy pulses). In contrast, in 6 patients in remission when these studies were begun who subsequently relapsed, marrow CSA values decreased within 2 to 3 months of relapse. Marrow CSA values paralleled marrow CFU-C during remission until relapse, with normal CSA values persisting longer than CFU-C.

Discussion

In these studies we have quantitated marrow cell CSA provision, characterized the marrow CSA-producing cells, and assessed alterations of marrow cell CSA levels in normal subjects and patients with AML. Alteration of marrow CSA levels in AML correlated well with the patients' clinical status and prognoses. Patients at diagnosis or relapse had significantly decreased marrow CSA values, particularly patients with acute myeloblastic leukemia and those failing remission induction. Low marrow CSA was a significant negative prognostic indicator, since only 33% of patients with this finding entered drug-induced complete remission, whereas complete remission occurred in 88% of patients with normal CSA.

Sequential investigations showed that marrow CSA provision was generally normal in patients during stable remission. In contrast, low or progressively decreasing marrow CSA values occurred in patients with impending relapse. Marrow CFU-C and CSA correlated well in these patients during remission, with a decrease in marrow CFU-C occurring earlier than low marrow CSA in impending relapse. Prior studies (Greenberg, Bax et al., 1974) have shown that the relatively nontoxic maintenance chemotherapy regimen utilized was not associated with decrements of CFU-C one month post therapy. The prolonged persistence of marrow CSA may relate to relatively longer life span of monocyte-macrophages in comparison with granulocytic progenitor cells (Van Furth, 1970). The sequential marrow CFU-C patterns found during remission of AML are similar to those previously reported from this and other laboratories (Greenberg, Nichols et al., 1971; Bull, Duttera et al., 1973; Heller and Greenberg, 1977).

These findings that normal marrow CSA values were associated with both achievement and persistance of complete remission suggest that adequate marrow CSA provision may be essential for sustaining normal granulopoiesis in AML following induction chemotherapy. The absence of CSA in plasma from marrow or peripheral blood further implicated local cellular sources as being important providers of CSA. Thus, marrow CSA production may provide a measure of intramedullary influences involved in regulating granulopoiesis. Histologic examination of human marrow has indicated that granulopoiesis occurs within the marrow parenchyma (Weiss, 1970), where granulocytic precursors and cells of the monocyte-macrophage series are in

proximity. Thus, it is possible that short range interactions could occur in this microenvironment between adherent-cell elaborated stimulatory substances and granulocytic precursors. In addition to CSA production by these aspirable marrow cells, recent studies from our laboratory have shown that stromal cells firmly adherent to endosteal human bone also produce CSA. The relative contribution of those two intramedullary sources for CSA production is currently being assessed. In this model, long range influences could also participate in the response to major perturbations such as infection or antigenic challenge.

Monitoring marrow CSA provision appears useful for evaluating microenvironmental influences and intramedullary cellular interactions on granulopoiesis, and assessing prognosis and clinical status in AML.

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Analysis of Myeloproliferative Disorders Using Cell Markers in Culture

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A. Introduction

We have used cell markers to study aspects of marrow regulation in selected patients with hematological neoplasms. While this report focuses on studies of normal individuals and of patients with polycythemia vera, the principles are widely applicable to any one of a number of disorders.

The analysis takes advantage of naturally-occurring cellular mosaicism in selected females. The marker used is that of the X chromosome-linked isoenzymes of glucose-6-phosphate dehydrogenase (G-6-PD) and has been detailed by Fialkow ([3] and see chapter in this text).

These studies have demonstrated that the peripheral blood elements in G-6-PD heterozygotes with chronic myelogenous leukemia [5], polycythemia vera [1], and agnogenic myeloid metaplasia with myelofibrosis [7] contain only a single isoenzyme type while extracts of skin fibroblasts demonstrate two isoenzyme types. This has led to the conclusion that these disorders arise at the level of a pluripotent stem cell, and, at least at the time of analysis, the peripheral blood elements probably represent the products of a single clone.

In an extension of these studies, we have cultured bone marrow cells from such patients to determine if progenitors could be detected which did not bear the isoenzyme type found in peripheral blood cells. Because such precursors must exist in the marrow in small numbers, we took advantage of the fact that these cells have marked proliferative potential when cultured under appropriate conditions, and we have employed methods for the growth of both granulocytic and erythroid colonies. Such an analysis, therefore, might detect the products of normal stem cells and, if so, provide an estimate of the ratio of abnormal to normal progenitors in the marrow and give insight concerning mechanisms of normal hematopoietic regulation.

B. Methods and Materials

I. Subjects

Hematologically normal G-6-PD heterozygotes were identified and served as controls. Heterozygosity was established by isoenzyme analysis of peripheral blood cells.

Two G-6-PD heterozygotes with polycythemia vera were studied. These patients, at the time of diagnosis, had pancytosis with splenomegaly. At the time of analysis, peripheral blood red cells, granulocytes, and platelets ex-

hibited only isoenzyme type A on electrophoresis [1]. Direct analysis of full thickness skin biopsies, in contrast, demonstrated approximately equal amounts of isoenzyme types A and B. Heterozygosity was repeatedly confirmed by analysis of cultured skin fibroblasts from both patients.

II. Marrow culture

To determine whether products of presumably normal stem cells were present in the marrow of the patients with polycythemia vera, we cultured cells from both individuals and exposed them to increasing concentrations of erythropoietin (ESF). Similar studies were carried out on marrow cells of the normal controls. Bone marrow cells were aspirated in a routine fashion into heparinized tissue culture medium and cultures established as previously published [10, 11]. Between days 6 and 12, colonies containing hemoglobinized cells appeared in the methylcellulose. These colonies were plucked from the medium using fine capillaries and placed on cellulose acetate strips soaked in buffer and subjected to electrophoresis [11]. In later studies, colonies derived from different classes of erythroid progenitors (CFU-E and BFU-E) were analyzed independently.

C. Results

I. Normal subjects

Erythroid colonies harvested from the normal marrow cultures exhibited either A or B type isoenzyme but rarely both [11]. The incidence of colonies containing a double isoenzyme phenotype was no greater than would be predicted on the basis of two spatially related progenitors. As shown in Table 1, the ratio of colonies containing one isoenzyme type versus the other reflected the ratio of isoenzyme types determined by direct analysis of peripheral blood elements. This was true irrespective of the concentration of ESF used and whether CFU-E- or BFU-E-derived colonies were analyzed. Recently, it has also been shown that CFU-C-derived colonies, as well, reflect the isoenzyme ratio observed in circulating granulocytes [12].

Table 1. The ratio of A and B isoenzymes of G-6-PD in peripheral blood cells and marrow-derived erythroid colonies from hematologically normal G-6-PD heterozygotes

	Peripheral blood	Colonies	
	A:B	A	B
Normal 1	50:50[a]	38[b]	37
Normal 2	60:40	51	25

[a] Ratio of isoenzyme types determinated by electrophoresis

[b] Actual number of colonies typed as A or B

II. Polycythemia vera subjects

When marrow from patients with polycythemia vera is cultured in semisolid medium in the absence of ESF, so-called endogenous erythroid colonies appear [6,9,13]. However, as higher concentrations of ESF are added to culture, an increased number of erythroid colonies is seen [9,13]. When these colonies were analyzed for G-6-PD isoenzyme type, those appearing in cultures having low concentrations of ESF contained only the isoenzyme type found in the peripheral blood, type A (Fig. 1). However, at higher concentrations of ESF, increasing numbers of erythroid colonies were detected which contained isoenzyme type B. At the highest concentrations of ESF, up to 25% of the colonies typed for isoenzyme B and thus, presumably, arose from normal stem cells. Although the initial study did not distinguish between colonies derived from CFU-E and BFU-E, a repeat study of one of the patients has demonstrated that normal (isoenzyme type B) colonies arise primarily from BFU-E. Essentially no normal colonies arose from CFU-E [2].

Also appearing in the cultures were limited numbers of granulocyte-macrophage colonies arising from granulocyte colony forming units (CFU-C). Their appearance was apparently stimulated by the release by marrow cells of

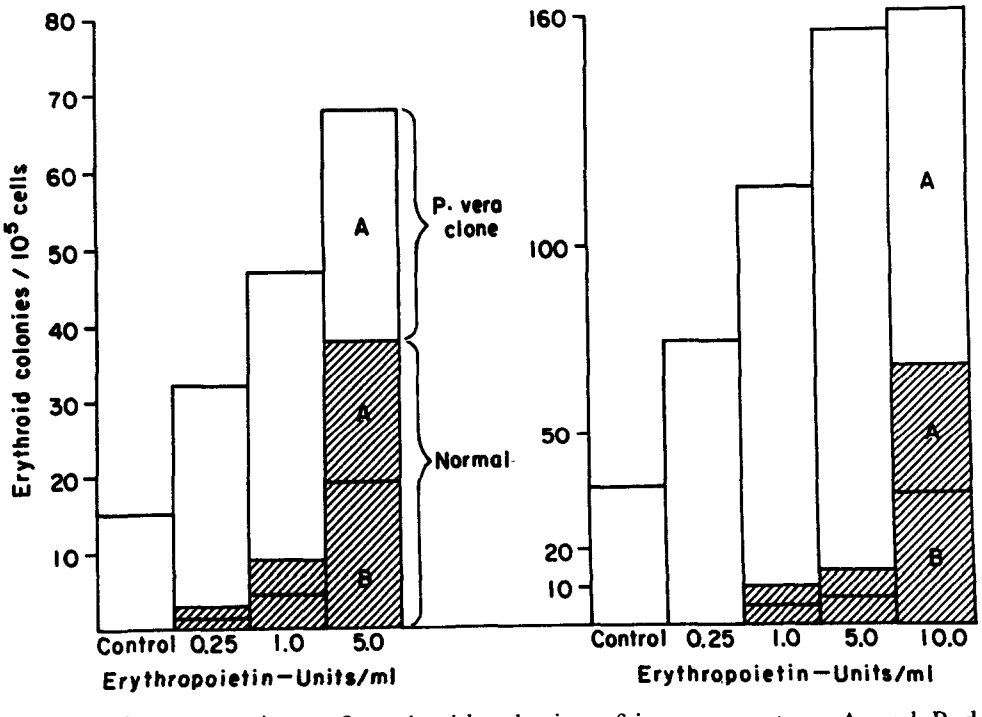

Fig. 1. The proportions of erythroid colonies of isoenzyme type A and B determined from marrow cultures of two G-6-PD heterozygotes with polycythemia vera. In cultures containing no added erythropoietin, only colonies bearing the isoenzyme type found in the peripheral blood were observed. As erythropoietin concentrations increased, an increasing percentage of colonies with isoenzyme type B was found. The shaded area denotes the calculated percentage of expected normal colony forming units based on the number of type B colonies detected and the assumption that an equal number of normal type A colonies exist in heterozygotes with balanced expression of the two G-6-PD isoenzyme types.

endogenous colony stimulating factor (CSF). A restricted number of these colonies were also harvested and analyzed. As shown in Table 2, CFU-C-derived colonies containing isoenzyme type B were also detected in the cultures of these patients, although a detailed CSF dose/response curve was not done and culture conditions had not been adjusted to optimize granulocyte/macrophage colony growth.

Table 2. The isoenzyme types of granulocyte/macrophage colonies arising in marrow cultures from two G-6-PD heterozygotes with polycythemia vera

	Isoenzyme type	
	A	B
Subject 1	20[a]	9
Subject 2	22	1

[a] Number of colonies typed as A or B

D. Discussion

The demonstration of the origin of certain hematologic disorders at the level of the pluripotent stem cell and the observation that peripheral blood elements are predominantly the products of a single clone suggest that in the course of the disease there is clonal expansion. What is uncertain, however, is whether such expansion is associated with the elimination of normal stem cells and their progeny or the suppression of growth of such progenitors. In order to approach this question, we cultured bone marrow cells from patients with polycythemia vera who are also heterozygotes for a naturally-occurring marker of cellular mosaicism, the X-linked isoenzymes of G-6-PD. The method used employed the growth of marrow cells in culture. These assays detect the presence of progenitors capable of marked proliferation and differentiation. The advantages of such a colony forming assay are several, but perhaps the most relevant to this analysis are the facts that recognizable differentiated cells can be detected in culture and that the progeny reflect an inherent property of the parent cell – that of the G-6-PD being expressed by the remaining active X chromosome. Thus, through in vitro amplification, it is possible to detect progenitors which may have arisen from stem cells not of the abnormal clone, some of which, by definition, should not contain the dominant G-6-PD isoenzyme type.

For this analysis to be valid, however, there are two requirements. First, the various colony types must be able to be analyzed by available techniques and, second, culture conditions should not favor the growth of progenitors having one isoenzyme type over the other. Studies in hematologically normal G-6-PD heterozygotes confirmed the validity of the approach. The results of such studies reveal that erythroid and granulocytic colonies can be individually recovered from methylcellulose and their G-6-PD isoenzyme type determined with a high degree of fidelity. Since virtually all colonies expressed only one isoenzyme type, either A or B, it could be concluded that such colo-

nies arose from single cells. In addition, the ratio of isoenzyme types obtained from the analysis of individual colonies reflects the ratio determined directly from the peripheral blood cells of the normal heterozygotes. This was true whether such erythroid colonies were derived from CFU-E or BFU-E and has recently been shown to be true for granulocyte/macrophage colonies. Finally, increasing concentrations of ESF failed to alter the isoenzyme ratios, suggesting that, at least in cultures from hematologically normal subjects, the conditions did not favor the growth of colonies of one isoenzyme type over the other.

The analysis of the cultures from the polycythemia vera patients provided several insights. First, only one isoenzyme type, type A, was detected in the peripheral blood of such individuals. Endogenous erythroid colonies also contained this same isoenzyme. With increasing concentrations of ESF, however, up to 25% of colonies were detected which contained type B isoenzyme. This implies that up to half of all colonies formed at high concentrations of ESF arose from presumably normal stem cells. This estimate of the incidence of normal colonies is derived from the fact that in a G-6-PD heterozygote with a balanced (approx. 50:50) expression of isoenzymes, there should be a normal A type colony for every type B colony which is found. Such an estimate is allowed by the observation in normals of concordance of ratios of isoenzyme activities in tissues of various types, including skin and blood [4]. If one makes such an estimate, as indicated in Fig. 1, it can be seen that half of the erythroid colonies detected were presumably normal.

This estimate of the percent of normal progenitors is far greater than the level of sensitivity of the electrophoresis technique used to determine isoenzyme activity. This implies that if such progenitors underwent terminal differentiation and maturation in vivo, it should have been possible to detect their products in circulation. The fact that normal cells were not detected suggests that the progenitors are somehow suppressed, perhaps by the abnormal clone, in their final maturation steps. Whatever the nature of this suppression, it appears to influence not only erythropoiesis but granulopoiesis as well (Table 2).

The mechanism by which suppression of normal progenitors is carried out is unknown. Although speculative, it is possible that such suppression is achieved by regulatory substances acting over short distances [8] rather than an abnormal product of the neoplastic clone. Such regulatory factors may be normal products; however, only the normal progenitors are capable of recognizing and responding appropriately to such growth controllers. Thus, the proliferative advantage of the abnormal clone would involve a lack of responsiveness to growth controllers rather than increased sensitivity to stimulating factors.

Further studies in culture of such patients may permit definition of the factors regulating marrow growth and also provide the opportunity to examine the influence of different forms of therapy and time on the relative numbers of normal and abnormal progenitors.

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Morphologic Characteristics of Human Blood Cells in Semi-Solid Marrow Cultures

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Blood cells proliferated and differentiated in vitro should be classified according to the classical hematologic nomenclature only after unequivocal identification by classical hematologic techniques. This has been done by Maxinow [6] and others at the beginning of this century, more than 50 years ago, when they established their in vitro methods of bone marrow in semi-solid media. In contrast, modern experimental hematologists sometimes use the nearly 100 years old classification of human blood cells which is rather uncritical to characterize blood cells grown in vitro.

Materials and Methods

8870 colonies of bone marrow cultures from 301 patients have been investigated cytologically (Pappenheim staining), cytochemically (peroxidase, acid phosphatase, α-N-esterase) or immunocytochemically (FITC conjugated anti human immunoglobulins).

Agar cultures were prepared using the double layer agar technique of Pike and Robinson [12]. Plasma clot cultures were prepared according to Axelrad [13] and Tepperman [14], modified by Hellwege [5]. Instead of erythropoietin placenta conditioned medium [4] was used to stimulate the granulopoietic cell differentiation and proliferation.

Results

1. Agar gel culture (Table 1): We mainly found colonies with macrophages, always strongly acid phosphatase positive, and monocytes, weakly peroxidase positive. In addition, strongly peroxidase positive pure eosinophilic colonies and mixed colonies of monocytes and eosinophils were observed. Besides this, we found plasma cells in colonies from leukemic patients and two normal persons, and blast cells only in leukemic patients. Using

* Supported by the Deutsche Forschungsgemeinschaft

Table 1. Cytological classification of colonies (%) grown in soft agar with leukocyte feeder layer

	number of patients	number of colonies analyzed	monocytes[a]	macrophages	eosinophils[b]	plasma cells[c]	blasts
normal and leukemic patients in remission	184	1978	77.5	8	6.5	7	1[d]
ALL untreated	21	164	81	10	4	4	1
AML untreated	12	74	19	—	32	41	8
	217	2216					

[a] mostly monocytes and macrophages
[b] 50% pure eosinophils, 50% mixed colonies with eosinophils
[c] mixed colonies with plasma cells (demonstrated by immunofluorescence)
[d] blast cell colonies from leukemic patients in remission

immunofluorescence techniques, we were able to show that plasma cells in vitro produce immunoglobulins [1,2,11].

2. *Plasma clot culture* (Table 2): In contrast to agar gel culture, we only found very few macrophage colonies, but mainly monocytoid (Fig. 1b) and eosinophile (Fig. 1a) cells in plasma clot cultures. The monocytoid cells were peroxidase positive (Fig. 1b, c, d) and therefore belong to the myeloid cell lines. However, they could not clearly be identified as neutrophils since the stainings were rather diffuses and not granular (Fig. 1b). In contrast to bone marrow cultures from normal persons we found a higher amount of peroxidase negative colonies (Table 3, Fig. 1e) in leukemic patients. In addition, we found further evidence for abnormal differentiation as a rather weak peroxidase reaction and an apparent vacuolization of the cytoplasm (Fig. 1f). Quantity and size of colonies were also diminished in leukemic patients. The percentage of cluster in relation to colonies became normal about two years after having finished therapy (Table 3).

Table 2. Cytological classification of colonies (%) grown in plasma clots with placenta conditioned medium

	number of patients	number of colonies analyzed	pure eosinophile colonies	colonies of peroxidase positive monocytoid cells[a]	peroxidase negative colonies	mixed colonies of eosinophils and monocytes
normal bone marrow	22	2612	31.4	65.0	0.8	2.8
ALL in therapy	33	1914	38.0	45.8	15.0	1.2
ALL in remission and without therapy	29	2182	28.0	63.0	8.3	0.7
	84	6708				

[a] very rarely macrophages

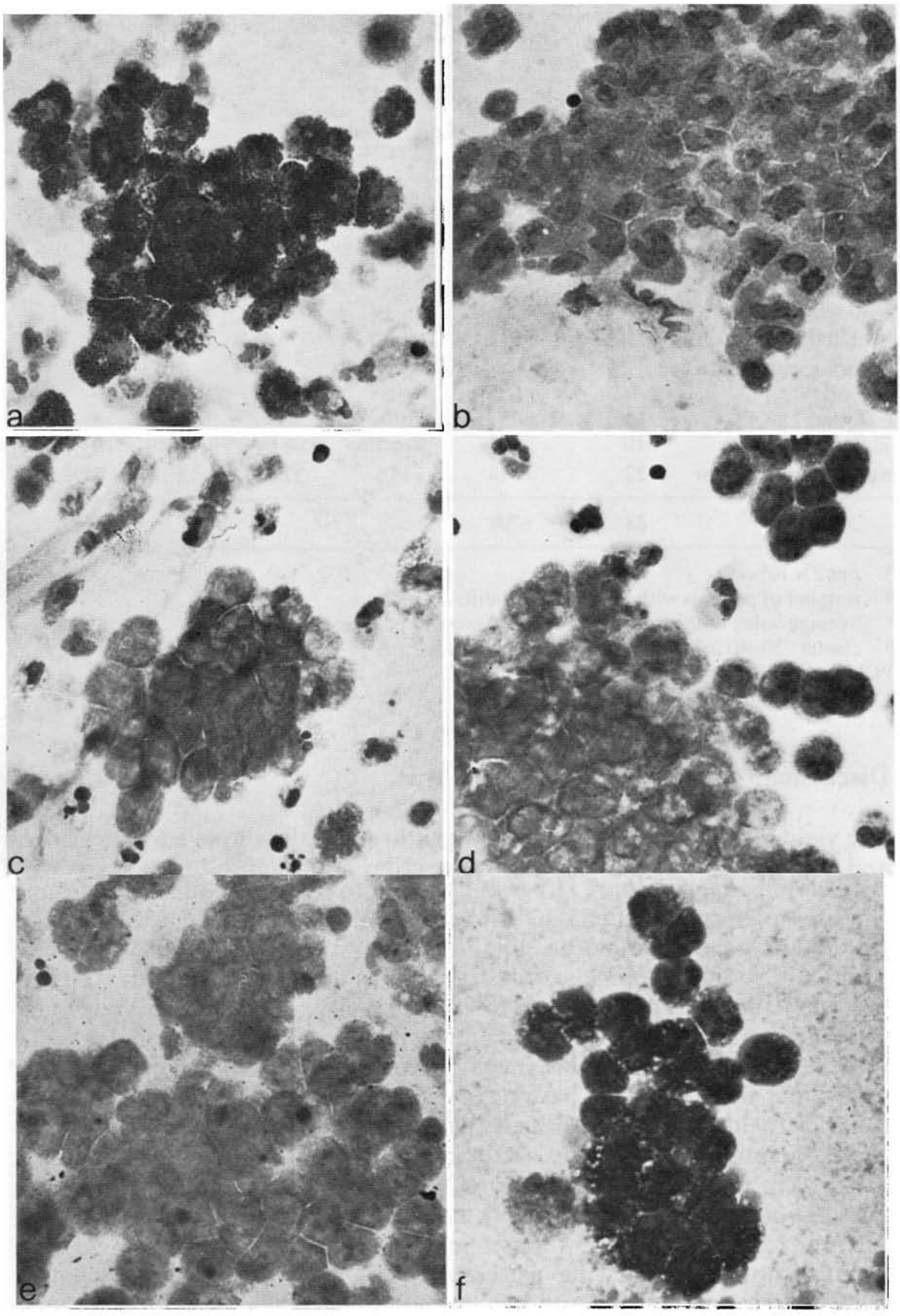

Fig. 1. Colonies of plasma clot cultures from human bone marrow **a)** peroxidase positive eosinophile colony **b)** peroxidase positive monocytoide colony **c)** small peroxidase positive colony (cluster) **d)** part of a large peroxidase positive colony **e)** peroxidase negative colony **f)** eosinophils with vacuoles **e + f** = colonies of bone marrow cultures from leukemic patients

Table 3. Quantitative and qualitative criteria in plasma clot cultures from all patients in different stages of the disease

	number of patients	number of colonies analyzed	colonies[a,c] without PCM	colonies[a,c] with PCM	[c,d] cluster colony	unnormal cell diff.[b]
relapse without therapy	4	10	0	<1		
relapse with therapy ≲ 9 weeks	8	301	1.1	13.2	4.8	5
remission with therapy 9–40 weeks	10	701	4.6	24.6	4.0	8
40–130 weeks	15	912	4.5	18.4	4.3	6
remission without therapy ≲ 1 year	9	630	8.1	18.3	4.7	4
1–2 years	10	751	7.2	24.6	2.7	4
≲ 2 years	10	801	6.1	26.6	1.3	4
normal bone marrow	22	2612	14.5	37.0	1.8	0
	88	6708				

[a] per 5×10^4 cells
[b] number of patients with unnormal cell diff.
[c] average value of the patients' individual growth patterns
[d] cluster: 20–40 cells to avoid background faults
PCM = placenta conditioned medium

Discussion

The yield of colonies in bone marrow cultures usually shows a wide variation and therefore is no reliable diagnostic criterion in the follow up control of leukemic patients. Moore [7,8,9,10] has improved the significance of bone marrow culture techniques by introducing the cluster-colony relationship in AML, which we found possible higher in risk patients. The plasma clot technique for granulopoietic cells described in this paper allows an even better qualitative interpretation of growth criteria. We describe in this paper additional qualitative growth criteria for bone marrow cultures from leukemic patients. These are peroxidase negative colonies (Fig. 1e) and cells showing unnormal differentiation patterns as vacuolization (Fig. 1f) and a rather weak peroxidase reaction. These criteria are of additional value for the diagnostic identification of bone marrow from normal and leukemic patients. They were found in bone marrow cultures from patients in remission even after as many as six years without therapy. It is reasonable to assume that there is a pathological alteration of bone marrow stem cells in these patients which is not reflected by the usual myelogram and colony number counting in bone marrow culture. Further follow-up studies will have to show whether these additional criteria might become significant with regard to diagnostics of preleukemic states and therapy of leukemic patients and whether the plasma clot method will prove to be more efficient than other in vitro techniques.

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Differentiation Ability of Peripheral Blood Cells from Patients with Acute Leukemia or Blast Crisis in Chronic Myelocytic Leukemia*

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Introduction

The diffusion chamber (DC) system of in vivo culture is useful for testing human leukemic peripheral blood cells for several reasons. With this technique, stem cells apparently preceding the granulopoietic progenitor cells (CFU-c) can be detected. Arguments for this come from diffusion chamber studies on normal human peripheral blood cells where an absolute increase in CFU-c was found (Hoelzer et al., 1976a) and also from DC culture of human bone marrow where CFU-d (cells that form granulocytic colonies in DC) as precursors of CFU-c could be distinguished on the basis of different velocity sedimentation profiles and different ^{3}H-thymidine suicidal fractions (Jacobsen et al., 1978). Differentiation of cells in the DC system occurs not only into the granulopoietic/macrophage series but also into megakaryocytes and, even if rarely, into erythropoiesis, as well as into lymphatic cell lines. Furthermore, the cultures can be maintained over long periods; in our present experiments leukemic cells have been cultured for up to 2 months and Hodgkin cells for even longer (Boecker et al., 1975). Thus the DC system seems to be suitable for sustaining the growth of leukemic cells of an earlier "stemness" than normal CFU-c and seems also to promote differentiation into haemopoietic cell lines other than granulopoiesis.

On this basis, the DC culture is here used to study the differentiation ability of peripheral blood cells from patients with various forms of acute leukemia or with blast crisis in chronic myelocytic leukemia (CML).

Materials and Methods

Peripheral blood samples obtained from patients with acute myelocytic leukemia (AML), acute lymphoblastic leukemia (ALL), acute undifferentiated leukemia (AUL) or blast crisis in CML were separated by the Isopaque-Ficoll technique and the mononuclear cell fraction was cultured in DC as already described in detail (Hoelzer et al., 1974). In most cases, blast cells constituted more than 80% of this fraction, the rest being mainly lymphocytes.

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The cells (5×10^5 in 0.1 ml) were filled into DC with filter pore size 0.22 μm implanted into the peritoneal cavity of mice pre-irradiated with 650 R or 750 R and the chambers were then re-implanted weekly into new pre-irradiated recipients. In harvesting, the clot formed in DC was dissolved by pronase treatment and from the resulting single cell suspension the total nucleated cell number per chamber was determined, smears were made and stained with Giemsa or peroxidase reaction, an aliquot prepared for cytogenetic analysis and a further aliquot used for assay of CFU-c content by agar colony culture.

Results

The total growth of nucleated cells in DC of AML blood cells was in general well above normal values, whereas growth from ALL cells is much less than normal (Hoelzer et al., 1977). In contrast to the normal pattern, which has an initial decrease in total cell number and an increase between days 6 and 13 of culture, for most cases of AML the cell number starts to increase immediately after implantation and continues to increase over the whole culture period, in some cases up to 6 or 7 weeks. There is not only a proliferation of blast cells, as demonstrated by ^{3}H-thymidine studies (Hoelzer et al., 1976b), but also a development of large numbers of granulopoietic cells.

The absolute number of granulopoietic cells developing from AML cells was far in excess of that from normal blood cells (Fig. 1), except in one pa-

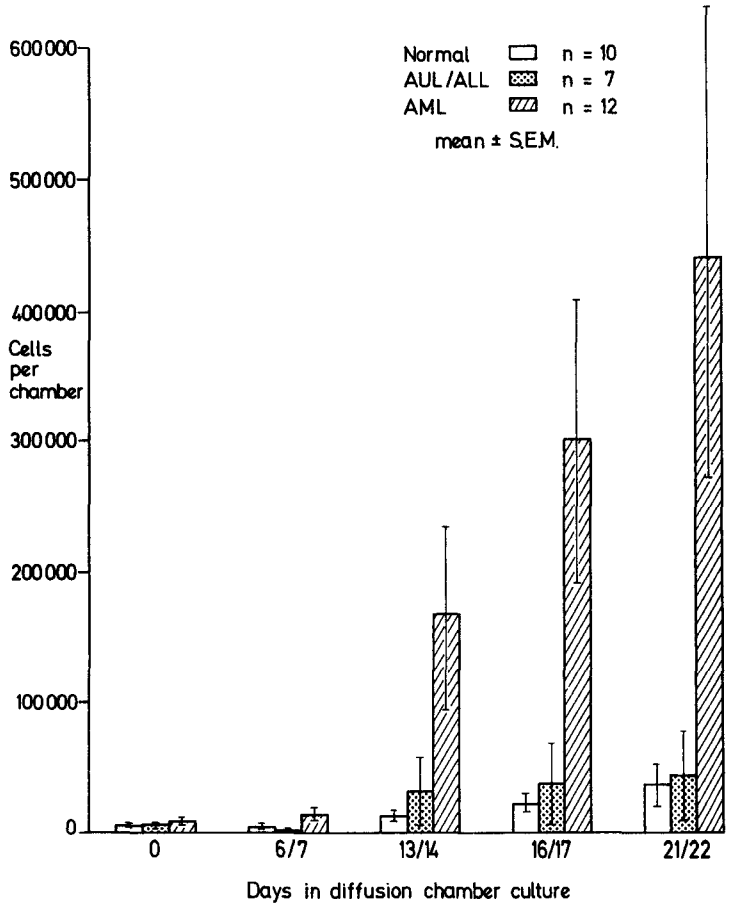

Fig. 1. Absolute numbers of granulopoietic cells per diffusion chamber at the time of implantation (day 0) and during culture for peripheral blood cells from normal persons and for patients with AML or AUL/ALL

tient. Sometimes the newly appearing granulopoietic cells were of abnormal morphology, but their granulopoietic nature could be confirmed by the peroxidase reaction or Sudan black staining. Although in some cases maturation was restricted to the myelocyte stage, in 6 out of 12 cases of AML terminal differentiation up to segmented granulocytes was seen, exceeding the numbers from normal persons. In addition, cells from one AML patient developed predominantly into mature eosinophils.

In cultures of blood cells from patients with ALL or AUL, the number of granulopoietic cells which developed was of the same order as from normal blood cells, and it might well be that they were descendants of normal stem cells which had been suppressed in vivo but could grow in DC culture conditions.

Peripheral blood cells from patients with *blast crisis in CML* had a similar growth pattern to AML cells. As shown in Fig. 2, there was a continuous pro-

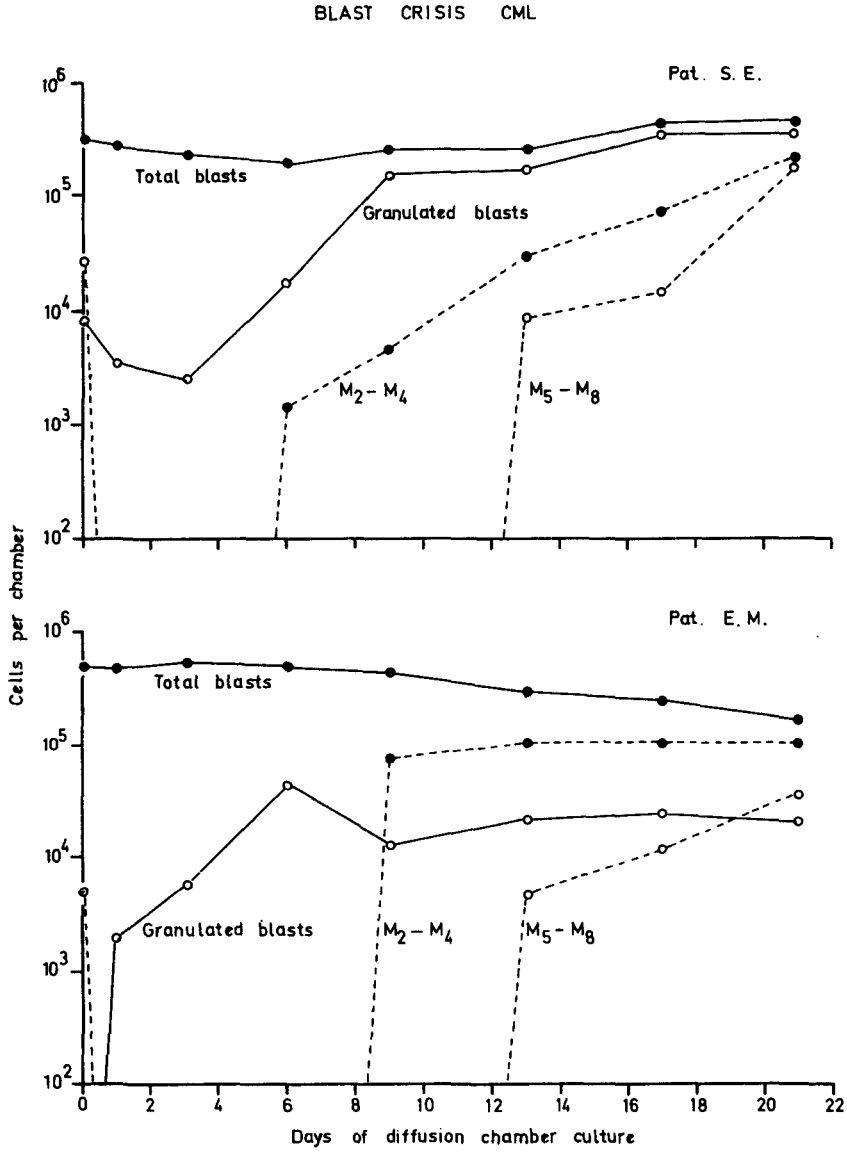

Fig. 2. Growth in diffusion chambers of peripheral blood cells from two patients with blast crisis in CML. The numbers of blast cells, appearance of granulated blast cells (immature blast cells with azurophilic granules in the cytoplasm), immature proliferating (M_2–M_4) and mature (M_5–M_8) granulopoietic cells are given

liferation of blast cells. Granulated blast cells appeared in one patient (E. M.) and increased soon after implantation in the second patient (S. E.). The immature granulopoietic precursors (M_2–M_4) in the cell inoculum disappeared immediately after implantation and reappeared within a week; after about 12 days of culture, mature granulopoietic cells (M_5–M_8) were observed. From the sequence of events it seems likely that the blast cells renewed themselves and also differentiated into granulated blasts with subsequent differentiation into immature and finally mature granulopoietic cells.

Discussion

The principal point of these investigations is whether the granulopoietic cells which developed from AML cells were derived from leukemic cells or from remaining normal stem cells. Although there is no conclusive proof that they were leukemic descendants, several arguments strongly indicate that they were at least partially of leukemic origin.

The main argument is the *absolute number of granulopoietic cells* from patients with AML, exceeding normal values by up to 40-fold. In most of these cases, the cell inoculum comprised over 80% blast cells, so that an exclusively normal origin for the granulopoietic cells would require a vastly increased proportion of normal circulating stem cells in the lymphocyte population, a somewhat unlikely event for leukemic patients. A further consideration is the *early appearance* of granulopoietic cells in cultures of AML cells. Whereas from normal blood cells, granulopoiesis was not observed before day 5, in some cultures of AML cells granulopoietic cells appeared almost immediately (days 1–3) after implantation. It must therefore be assumed that leukemic cells, although of immature morphology, require only one or two steps of division to become recognisable granulopoietic cells.

The *cytogenetic analyses* contribute only partial evidence for the leukemic origin of the granulopoietic cells. The cells harvested from DC showed hyperdiploid marker chromosomes in 5 out of 13 patients. However, it should be mentioned that in all cases numerous diploid metaphases were present and also hypodiploid metaphases, the latter being probably technical artefacts.

With other culture systems it has been demonstrated in recent years that differentiation is possible in some cases of AML. Reports of this in the agar colony culture system with an exogenous source of stimulation come from Paran et al. (1970), Robinson and Pike (1971), and Barak et al. (1974) although often with abnormal morphology (Moore et al., 1973). A certain extent of differentiation was also observed in the liquid culture system (Gold and Cline, 1973). In continuous long-term culture, one AML cell line (H-60) was established which showed continuous differentiation into granulopoiesis without additional stimulation (Collins et al., 1977) and the authors suggest that a sub-population of leukemic blast cells might secrete factors regulating proliferation and differentiation. In the diffusion chamber culture system, other workers have failed to demonstrate differentiation from leukemic bone

marrow cells (Fauerholdt and Jacobsen, 1975; Steele et al., 1977) and it is open to question whether this might have been due to inhibition by a particular fraction of bone marrow cells, to inadequate stimulation of the host animals by a lower radiation dose or to the use of cyclophosphamide. However, in DC culture of peripheral blood cells from a patient with AML maturation of leukemic cells could be confirmed by the presence of Auer rods in the non-proliferating granulopoietic cells (Boecker et al., 1978).

The investigation described here provides evidence that in nearly all cases of AML, differentiation into recognisable granulopoiesis is possible, with terminal differentiation up to mature neutrophils in half the patients studied. Similarly, it seems that in the myeloid type of blast crisis in CML a differentiation into the granulocytic series can occur.

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Modification of the Proliferative and Differentiation Capacity of Stem Cells Following Treatment with Chemical and Viral Leukemogens

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1. Introduction

Following treatment with a leukemogen, a variable length of time, from weeks to years, elapses before the onset of clinically apparent leukemia. The events occurring during this time are still a mystery, but as one approach to this problem we have been investigating the mechanism(s) whereby leukemogens modify the behaviour of the haemopoietic stem cells and the consequences of such alterations in terms of the proliferation and differentiation potential of such treated populations. Since the stem cells are probable "targets" for many leukemogens, our understanding of leukemogenesis, in many respects, lies in the answer we can give such questions.

These studies have been greatly facilitated by the development, in the last few years, of suitable systems whereby the pluripotent haemopoietic stem cell (CFU-S) can be maintained in vitro for several months (Dexter and Testa, 1976; Dexter et al., 1977) and where the progeny of such stem cells can be induced to undergo proliferation in soft-gel media to form the variety of haemopoietic elements – the lymphocytes, granulocytes, megakaryocytes and erythroid cells (Metcalf et al., 1975b; Sredni et al., 1976; Bradley and Metcalf, 1966; Pluznik and Sachs, 1966; Metcalf et al., 1975a; Stephenson et al., 1971). The development of these systems, and the demonstration that under appropriate conditions haemopoietic cells can be readily transformed in vitro (Rosenberg et al., 1975; Rosenberg and Baltimore, 1976; Dexter and Lajtha, 1976; Dexter, Scott and Teich, 1977) provide valuable tools in elucidating both normal and abnormal haemopoiesis. In this respect we show the effects of several chemical and viral leukemogenic agents upon haemopoietic stem cell proliferation and differentiation in bone marrow cultures and demonstrate that such treatments can induce a variety of abnormal haemopoietic conditions in vitro.

2. Materials and Methods

2.1. Bone Marrow Cultures: These have been described in detail elsewhere (Dexter and Testa, 1976; Dexter et al., 1977). The content of a single mouse femur are flushed into culture flasks containing 10 ml of Fisc[illegible]'s medium

+25% horse serum (Flow Labs.). No attempt is made to obtain a single cell suspension and the cultures are maintained at 33°C or 37°C (as indicated) and fed weekly by demi-depopulation. After three weeks, the adherent layer established is inoculated with growth medium containing a further 10^7 syngeneic marrow cells. These are considered the cultures proper, and are subsequently maintained on a feeding regime of demi-depopulation at weekly intervals. The non-adherent cells removed in the growth medium are assayed for stem cells and the "committed" progenitor cells using established procedures (Dexter and Testa, 1976).

2.2. Mice: BALB/C, C57BL/6, DBA/2 and (C57BL/6×DBA/2) F_1 (BDF_1) mice were used. Cultures were established using femoral bone marrow of mice 6–8 weeks of age.

2.3. Chemical Leukemogen Treatment: 6–8 week old BDF_1 mice were treated with a single i.v. injection of methylnitrosourea (MNU) dissolved in ice cold 0,9% NaCl. Three weeks later, some mice were killed, and adherent layers established from the femoral bone marrow cells. Three weeks later a further batch of the same mice were killed, the femoral marrow cells removed and 10^7 cells inoculated onto the established adherent layers.

2.4. Viral Leukemogen Treatment: These included the NB-tropic pool (F-B strain) of Friend leukemia virus complex, provided by F. Lilly. Abelson virus (provided by E. Lennox) and the FBJ osteosarcoma virus (provided by Dr. C. Reilly). Infectivity was measured by established techniques (Rowe et al., 1970; Scher and Siegler, 1975).

2.5. Leukemogenic Capacity of Cultured Cells: Non adherent cells removed at the time of refeeding were injected into syngeneic adult mice. Occasionally neonates were used. The animals were palpated twice weekly for signs of splenomegaly or lymphodenopathy. When leukemia was suspected, the animal was killed – tissue was removed for microscopic examination – and the suspected leukemic cells were retransplanted.

3. Results

3.1. Characteristics of Normal Bone Marrow Long Term Cultures: These have been described in detail elsewhere (Dexter et al., 1977). Cultures are characterised by maintenance of proliferation of stem cells for 10–12 weeks, and concomitant production of CFU-C. Initially there is an extensive granulopoiesis, followed by production of mononuclear phagocytic cells. The cells do not produce leukemia when injected into adult or neonatal syngeneic recipients.

3.2. Culture of MNU-treated Bone Marrow Cells: The characteristics of these cultures are shown in Table 1. An initial granulopoiesis is followed by an ac-

Table 1. Characteristics of long term cultures of MNU treated bone marrow cells

Weeks cultured	Cell Count $\times 10^5$	CFU-S	Morphology B	G	Mo
1	32.0	1000	16	80	1
5	20.0	370	11	85	2
7	12.0	190	30	66	4
10	8.0	234	37	61	3
17	7.0	150[a]	25	0	75
22	5.0	0	20	0	80

[a] Atypical, small undifferentiated
B = Blast cells G = Granulocytes (all stages) Mo = Phagocytic mononuclear cells

cumulation of blast cells (for at least 22 weeks) and mononuclear phagocytes. Apparently normal CFU-S are maintained for the first 10–12 weeks (able to form erythroid, granulocytic and megakaryocytic spleen colonies and protect mice from potentially lethal radiation) – but at later times (12–17 weeks) the spleen colonies formed contain undifferentiated blast cells. Injection of cells cultured for 0–12 weeks does not produce leukemia, whereas injection of cells cultured for 13–22 weeks will regularly produce a transplantable leukemia (Table 2). These leukemias, which are of donor origin (chromosome analysis), show no detectable θ antigen or surface Ig, and are best classified as null-cell type.

Table 2. Culture of MNU treated bone marrow cells. Leukemogeneic potential

Weeks cultured	Virus production[a]	Leukemic mice/ mice injected	Average latent period (weeks)
0	ND	0/30	—
1–5	$8\times 10^1 - 2\times 10^3$	0/59	—
6–12	$1\times 10^2 - 2\times 10^3$	1/20	26
13–17	$4\times 10^2 - 8\times 10^2$	10/21	22[b]
18–24	$2\times 10^2 - 2\times 10^3$	9/17	22[b]

[a] PFU/ml culture medium
[b] θ and sIg negative, TdT positive Donor origin (chromosome analysis)

The cultures were regularly assayed for the production of infective virus and found to be low level producers from the first weeks. However, there was no correlation between the production of infectious particles and the ability of the cultured cells to produce leukemia.

3.3. Infection of Marrow Cultures with Leukemia Viruses: The results of these studies are summarised in Table 3. Infection of genetically susceptible BDF_1 cultures with Friend leukemia virus complex generates three biologically distinct "variants", depending upon whether the whole viral complex (SFFV and LLV) or only the LLV component, is replicated. When both viral

Table 3. Biological effects of infection of long term bone marrow cultures with various murine leukemia viruses

			Characteristics				
Cultured cells	Virus inoculated	Replication	Left[a] shift	Extended CFU-C production	Transformed agar CFC	Extended CFU-S production[b]	Leukemic potential
BDF_1	FLV	1. SFFV LLV	+	+	–	+	Viral (Erythroleukemia)
		2. LLV	+	+	–	+	Non-leukemic
		3. LLV	+	–	+	–	Myelomonocytic leukemic
C57	FLV	LLV	–	–	–	–	Non-leukemic
BALB/c	Abelson	Abelson	–	–	+	–	Lymphoid leukemic
DBA/2	FBJ	FBJ	+	–	+	–	Myelomonocytic leukemic

[a] increase in "promyelocytes"
[b] Atypical-limited differentiation

components are replicated the cultures are characterised initially by an extensive granulopoiesis, followed by a shift to the left (especially an increase in promyelocytes), an extended maintenance of CFU-C (normal in their responsiveness to colony stimulating activity – CSA) and prolonged production of CFU-S. For the first 8–10 weeks these CFU-S are apparently normal-forming erythroid, megakaryocytic and granulocytic colonies, but after 10 weeks they become limited in their differentiation capacity and form spleen colonies containing *only* granulocytes and megakaryocytes. Injection of the cultured cells into syngeneic mice gives rise to an erythroleukemia of host origin (presumably representing infection and transformation of "erythroid" host cells by the viral complex liberated from the cultured cells).

Sometimes, following FLV infection, only the LLV component is replicated causing: –

a) an initial granulopoiesis, followed by a shift to the left, loss in CFU-S, production of transformed CFU-C (which grow independent of CSA and form compact myelomonocytic colonies) and injection of the cells into adults or neonates produces a rapidly progressing leukemia of myelomonocytic type. The cells grow autonomously of the adherent layer and can be established as permanently growing cell lines.
b) Initial extensive replication of normal CFU-S and CFU-C followed by a shift to the left, extended production of normal CFU-C, and proliferation of CFU-S, again limited in their differentiation capacity. These cells will *not* produce leukemia (even when injected into neonates) – and can also be established as permanent cell lines.

Infection of C57BL/6 cultures with FLV, although leading to extensive LLV replication, does not produce the biological effects described above.

As previously reported (Teich and Dexter, 1978) infection of susceptible cells with Abelson virus leads to a rapid transformation and accumulation of leukemic blast cells. Initially, the growth of these cells is dependent upon the marrow derived adherent population – but subsequently the cells develop autonomously, and will grow in the absence of known stimulating factors.

Finally, we have recently found that infection of DBA/2 cultures with FBJ osteosarcoma virus may give a situation similar to that seen after FLV infection *i.e.* a shift to the left and the development of a leukemic myelomonocytic cell population.

4. Discussion

The results demonstrate that following treatment with leukemogenic chemicals or viruses a series of changes occur in the proliferative and differentiation capacity of stem cells – often terminating in leukemic transformation. *All* cultures initially (first few weeks) show normal granulocyte maturation and production of normal CFU-S and CFU-C. Injection of the cells, at this time, *will not produce leukemia.* Subsequent changes depend upon the treatment given and – in the case of viruses – the response of the cells to the leukemogen. MNU treated marrow cultures show a production of atypical

"CFU-S" and an accumulation of undifferentiated blast cells. These changes in the culture seem to parallel the ability of the cells to produce leukemia, since maximal leukemic activity is demonstrated by cells cultured for 13 to 24 weeks. This indicates that a period in culture is "essential" for some change to occur in a potentially leukemic cell population. However, the long latent period in vivo indicates that after culture "conditioning", an essential part of the leukemic process is "host-conditioning". Of some interest is that the leukemia inducing ability of the cultured cells is not simply related to the expression of endogenous virus.

In the Friend virus infected cultures, in particular those showing only replication of the LLV component, proliferation of apparently normal CFU-S and CFU-C is followed by either a myelomonocytic leukemic transformation or production of "atypical" CFU-S with extensive replication capacity in vitro. While these cells do not produce leukemia in neonates, little is so far known of their capacity to reconstitute (in the long term) the haemopoietic systems of mice. Will they, for example, produce a chronic granulocytic leukemia after several weeks? A major obstacle in such studies is their limited differentiation capacity (being defective for erythropoiesis – and probably for lymphopoiesis – and the consequent difficulty in keeping reconstituted irradiated mice alive) but studies currently underway using hypertransfused mice, housed under sterile conditions, should give us some information on this point. Nonetheless, FLV cultures (as with MNU cultures) again show this pattern of initial normal non-leukemic haemopoiesis fallowed by the development of leukemic cell populations or production of atypical stem cells. Similarly when infection of cultures with Abelson leukemia virus or FBJ osteosarcoma virus is performed.

It will be of great interest to determine whether these changes are brought about by virally induced malfunction in the adherent haemopoietic microenvironment or in the stem cells themselves. Using appropriate combinations of genetically resistant environments and susceptible stem cells, such studies are now possible. Also, we need to define more clearly the role of viruses in such transformation. Is the original virus active in transformation or do such events represent a recombinational process between the virus and part of the host cell genome (xenotropic virus?) as suggested by Troxler et al., 1977. Certainly, increasing evidence for the role of such recombinations has emerged (Elder et al., 1977) and analysis of any recombinants produced in the long term marrow cultures is currently being performed.

Of particular interest is our finding – and others previously reported (Moore and Dexter, 1978) of the progressive changes occurring in the cultures prior to transformation. Hopefully, these are a reflection of similar processes operating in vivo, but which can now be analysed in our in vitro system.

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Characterization of Myelomonocytic Leukemia Cells Induced by in vitro Infection of Bone Marrow with FLV

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A. Introduction

The development of systems in which pluripotent haemopoietic stem cells and committed progenitors proliferate and differentiate in vitro has allowed the analysis of factors involved in the regulation of normal haemopoiesis (Bradley and Metcalf, 1966; Pluznik and Sachs, 1966; Axelrad et al., 1974; Stephenson et al., 1971; Dexter and Testa, 1976) and of events associated with leukemia, both in experimental systems (reviewed by Metcalf, 1977) and in patients (e.g. Moore, 1974).

A recent report (Dexter et al., 1977) shows that in vitro infection of mouse bone marrow cells with murine leukemia viruses affects the proliferation and differentiation of the stem cells, and in particular, that infection with Friend leukemia virus complex (FLV) induces extensive proliferation and differentiation of pluripotent stem cells and committed granulocytic progenitor cells, which maintain normal characteristics for several weeks. The present work reports the occurrence of leukemic cells of myelomonocytic type 2 weeks after in vitro infection of mouse bone marrow cells with FLV.

B. Materials and Methods

I. Initial Bone Marrow Culture

This technique has been described fully elsewhere (Dexter and Testa, 1976; Dexter et al., 1977). Donor mice were 8 week old ♀ BDF_1. Briefly, the content of a mouse femur was flushed into a culture bottle containing 10 ml of Fischers medium plus 25% horse serum and antibiotics. Replicate cultures were kept at 33 °C in an atmosphere of 5% CO_2 in air for a period of 3 weeks to allow the development of an adherent layer which is essential for the maintenace of haemopoiesis (Dexter et al., 1977), and were fed weekly by replacing one half of the growth medium with fresh medium. After that time, a fresh inoculum of 10^7 syngeneic bone marrow cells was added to each culture, followed by infection with FLV within 2 hours (NB-tropic pool, F-B strain) as described by Dexter et al. (1977). The cultures were maintained by weekly feeding as described above.

II. Isolation of the Leukemia Cell Line

Cells collected 14 days after the infection with FLV were plated in 0,8% methylcellulose in alpha medium plus 30% foetal calf serum enriched with bovine serum albumin, transferrin and sodium selenite (Gilbert and Iscove, 1976) as described previously (Testa and Dexter, 1977). After 10 days of incubation at 37°C, these cultures contained atypical colonies which were aspirated, pooled and subcultured as a single cell suspension in similar growth medium, minus methylcellulose, in culture bottles gassed with 5% CO_2 in air and also maintained at 37°C. The cells were subcultured when saturation growth was reached. A summary of the isolation procedure for this cell line, identified as 427E-MC, is shown in Fig. 1.

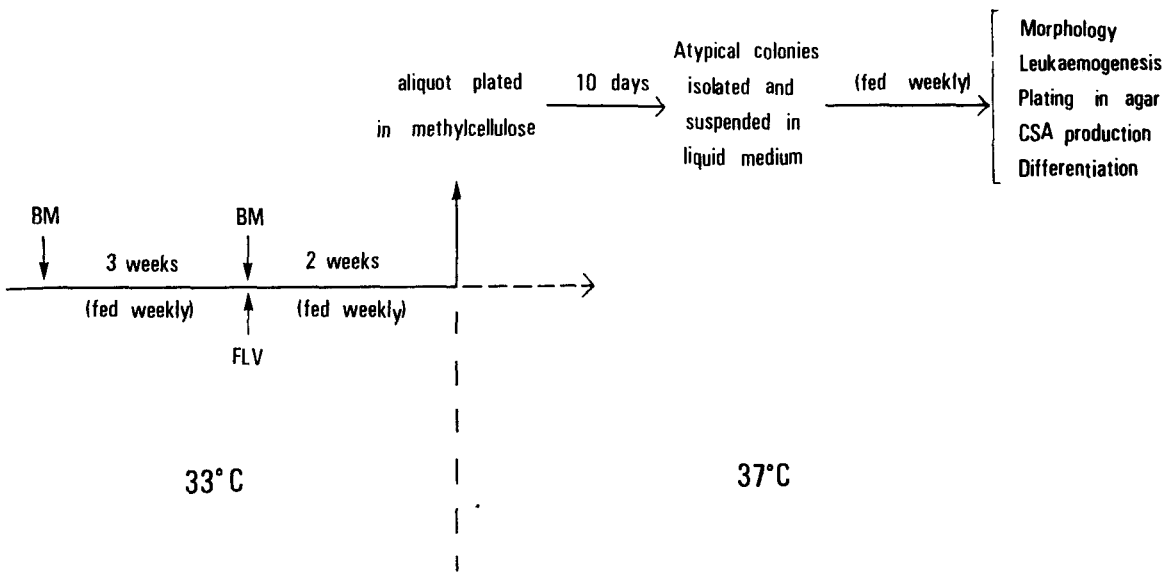

Fig. 1. Isolation of 427E-MC cells

III. Cloning Studies

The cells to be assayed were plated in 0,3% agar in Fischers' medium plus 25% horse serum, with or without mouse heart conditioned medium as an exogenous source of colony stimulating activity (CSA, which is essential for the growth of granulocytic colonies derived from normal progenitor cells). Cultures were incubated in a humidified atmosphere of 5% CO_2 in air and colonies of more than 50 cells were scored after 7 days.

IV. Induction of Leukemia by Cultured Cells

Cells harvested from the suspension cultures (B.II.) were injected into syngeneic neonatal or adult mice, at doses between 10^6–5×10^6 cells per mouse. The mice were killed within a week of the observation of the growth of a local tumour at the site of subcutaneous inoculation, or, in the case of i.v. injection, when they appeared ill. Leukemia was confirmed by histological examination of the haemopoietic tissue.

C. Results

I. Characteristics in Suspension Culture

These are summarized in Table 1. The 427E-MC cells grow in suspension in the absence of an adherent layer (see B.I.) with a doubling time of 12–16 hours to a density of $1–2 \times 10^6$ cells per ml, and have a high plating efficiency in agar, even in the absence of exogenous CSA. They produce CSA which stimulates the growth of granulocytic colonies from normal bone marrow cells. Morphologically, 20–40% are classified as blasts, a similar proportion as promyelocytes and the rest as myelocytes and metamyelocytes, with some monocytic characteristics.

Only the lymphatic leukemia helper virus (LLV) of the FLV complex replicates in this culture.

Table 1. Characteristic of 427E-MC cell line

Maximum number of cells/ml	$1–2 \times 10^6$
Morphology	Blasts, promyelocytes, myelocytes
Differentiation to segmented granulocytes	Not observed
Virus production:	
LLV (lymphatic leukemia helper virus)	Yes
SFFV (spleen focus-forming virus)	Not observed
CSA production	Yes
Plating efficiency in agar	10–30%
Induction of leukemia	Yes

II. Characteristics in Agar

These are summarized in Table 2. Colonies derived from 427E-MC cells are spherical, compact, and contain up to 5×10^3 cells after one week of growth. Both the morphology of the cells (similar to that found in suspension cultures) and the plating efficiency are similar in the presence or absence of exogenous CSA. The colony cells have high replating efficiency in agar and induce leukemia when injected into mice.

Table 2. Characteristics of in vitro colonies derived from 427E-MC cells

Appearance of colonies	Compact
Morphology of colony cells	Blasts, promyelocytes, myelocytes
Differentiation to segmented granulocytes	Not observed
Dependance on exogenous CSA	No
Replating efficiency	10–15%
Induction of leukemia	Yes

III. Leukemia Inducing Ability

Injection of 427E-MC cells into neonatal and adult mice results in the induction of leukemia which reaches an advanced state at the time shown in Table 3.

Table 3. Leukemia-inducing ability of 427 E-MC cells

Mice	*No. of leukemic mice* No. inoculated	Inoculation	No. of cells	Time of observation (days)
Neonatal	8/8	s.c. or i.p.	10^{-6}–2×10^{6}	19–41
Adult	3/4	s.c. or i.v.	5×10^{6}	35–96

D. Discussion

Stimulation of granulopoiesis showing normal characteristics with regard to growth and differentiation has been reported after injection of FLV into mice (Golde et al., 1976) and for several weeks after in vitro infection of bone marrow cultures (Dexter et al., 1977). In the latter report, myeloid leukemic transformation was observed in one instance 12 weeks after the infection, although in contrast with the present work, normal differentiation and CSA dependance were observed when the cells were cloned in agar.

Our results confirm that cells other than erythroid precursors may be the target of FLV, and indicate that a long latency period is not an absolute requirement for malignant transformation to lake place. It is of interest that in spite of the capacity of the bone marrow culture system (B.I.) to support the growth of early erythroid precursor cells (Testa and Dexter, 1977), no erythropoietin-independent erythropoiesis was observed at the time at which the 427E-MC line was isolated.

Acknowledgement

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Epstein Barr Virus (EBV) and Complement (C_3) Interactions With Human Lymphoid Cells

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EBV is a herpesvirus associated with several lymphoproliferative diseases in man [1]. It is the causative agent for infectious mononucleosis (IM), [2], and is associated with African Burkitt's lymphoma (BL) and carcinoma of the nasopharynx [3]. In addition, EBV has the ability to transform normal human (and some simian) lymphocytes in vitro, into continuously growing cell-lines [4]. Permanent lines can also be established directly from BL biopsies or IM lymphocytes [5]. Cell-lines established in either way were shown to be of the B lineage [6], and trials to infect non-B cells by EBV were unsuccessful [7]. Thus, EBV has a very restricted tropism in that it is able to infect and/or transform human (and certain simian) B lymphocytes, possibly due to specific viral receptors expressed on such cells only.

While scoring different human lymphoma cell-lines for presence of EBV-receptors, an interesting correlation was noticed between the expression of C_3-receptors and EBV-receptors [8]. These were simultanuesly present or absent, and in no case, was only one of them expressed. When receptor positive cells were incubated with EBV preparations, binding of EAC (indicator for C_3-receptors) was partially blocked. Incomplete inhibition of EBV binding to cells could be acheived by preincubating them with C_3 and two layers of antibodies. These results indicated that the EBV-receptor and the C_3-receptor were not identical but closely related structures on the membrane. EBV-receptors and C_3-receptors co-capped on the cell-membrane [9]. Neither of these receptors co-capped with other independent membrane markers such as IgM, Fc-receptors and $beta_2$ microglobulin indicating that the association observed was highly exclusive and specific.

In another experiment we have employed an EBV absorption bioassay [10] in which the binding of an infectious virus to EBV-receptors is measured quantitatively by testing the residual supernatant capacity to superinfect Raji cells, as manifested by the induction of Early Antigen (EA) synthesis. Incubation of cells with appropriate ligands for 4–6 hours at 37° led to a selective stripping of different membrane components including EBV-receptors and C_3-receptors [11].

It was shown that stripping of C_3-receptors drastically reduced the EBV absorbing capacity of the cells with similar efficiency to EBV-receptor stripping, while stripping of $beta_2$ microglobulin, IgM or Fc-receptors had no effect on the EBV absorptive capacity whatsoever.

Using the quantitative virus absorption bioassay it was also possible to compare the extent of EBV-receptor and C_3-receptor expression on different cell-lines. A very good correlation was found between the ability of the cells to absorb EBV and to bind C_3 [12].

A positive correlation between EBV receptors and C_3-receptors expression was also found among different subpopulations of normal human lymphocytes from peripheral blood [7]. Using a combined cell separation procedure [13] fractions enriched for B, T and non T non B (0) cells were obtained. The B cell enriched fraction had the highest virus absorptive capacity with the strongest expression of C_3-receptors. About 30% of the 0 cells expressed C_3-receptors and also exhibited a significant EBV binding capacity. A minor fraction of T cells had also C_3-receptors (about 10%) but did not show EBV binding activity. This is in line with the fact that many other non-B cells expressing C_3-receptors (e.g. erythrocytes, granulocytes, and monocytes) do not show any EBV binding activity, and association of C_3-receptors with EBV-receptors is found exclusively on B (and some 0) lymphocytes.

Taken together, the above results suggest that EBV has adapted the C_3-receptor on human B lymphocytes, to serve as its own receptor. It seems that the binding site of C_3 is not identical with that of EBV and the receptor association reflects adaptation to a structure associated with the C_3-receptor of the human B lymphocyte, which is missing from other C_3-receptor bearing cells.

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Anatomy of the RNA and Gene Products of MC29 and MH2, two Defective Avian Tumor Viruses Causing Acute Leukemia and Carcinoma: Evidence for a New Class of Transforming Genes

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Abstract

The RNA species of the defective avian acute leukemia virus MC29 and of the defective avian carcinoma virus MH2 and of their helper viruses were analyzed using gel electrophoresis, fingerprinting of RNase T_1-resistant oligonucleotides, RNA-cDNA hybridization and in vitro translation. A 28S RNA species, of 5700 nucleotides, was identified as MC29- or MH2-specific. MC29 RNA shared 4 out of about 17 and MH2 RNA at least 1 out of 16 T_1-oligonucleotides with several other avain tumor virus RNAs. In addition MC29 and MH2 RNAs shared 2 oligonucleotides which were not found in any other viral RNA tested. 60% of each 28S RNA could be hybridized by DNA complementary to other avian tumor virus RNAs (group-specific) but 40% could only be hybridized by homologous cDNA (specific). *Src* gene-related sequences of Rous sarcoma virus were not found in MC29 or MH2 RNA. The specific and group-specific sequences of MC29, defined in terms of their T_1-oligonucleotides, were located on a map of all T_1-oligonucleotides of viral RNA. Specific sequences mapped between 0,4 and 0,7 map units from the 3' poly(A) end and group-specific sequences mapped between 0 and 0,4 and 0,7 and 1 map units. The MC29-specific RNA segment was represented by 6 oligonucleotides, two of which were those shared only by MC29 and MH2 RNAs. In vitro translation of MC29 RNA generated a major 120000 dalton protein and minor 56000 and 37000 dalton proteins. The 120000 dalton protein shared sequences with the proteins of the avian tumor viral *gag* gene, which maps at the 5′ end of independently replicating viruses. Since a *gag* gene-related oligonucleotide was also found near the 5′ end of MC29 RNA, we propose that the 120000 MC29 protein was translated from the 5′ 60% of MC29 RNA. It would then include sequences of the defective *gag* gene as well as MC29-specific sequences.

Since both MC29 and MH2 lack the *src* (sarcoma) gene of Rous sarcoma virus, it is concluded that they contain a distinct class of transforming (*onc*) genes. We propose that the specific sequences of MC29 and MH2 represent

all, or part of, their *onc* genes because the *onc* genes of MC29 and MH2 are specific and represent the only known genetic function of these viruses. If this proposal is correct, the *onc* genes of MC29 and MH2 would be related, because the specific RNA sequence of MC29 shares 2 of 6 oligonucleotides with MH2. It would also follow that the 120000 dalton MC29 protein is a probable *onc* gene product, because it is translated from MC29-specific (and group-specific) sequences and because both MC29- and MH2-transformed cells contain specific 120000 and 100000 dalton proteins, respectively.

Introduction

MC29 and MH2 are avian RNA tumor viruses that cause acute leukemia, carcinoma and also transform fibroblasts in culture [1,2,3,4,5,6]. This oncogenic cell transformation is due to a transforming gene, termed *onc* [7], which has not been defined genetically or biochemically. Both viruses require a helper virus for replication because they are defective in all three replicative genes of the avian tumor virus group *gag* (for internal group-specific antigen), *pol* (for DNA polymerase) and *env* (envelope glycoprotein) [7]. The viral genome was identified as a 28S RNA species of 5700 nucleotides because it is absent from pure helper virus and because the sequence of 28S RNA remains conserved when propagated with different helper viruses [8.9.10.11]. Hybridization with DNAs complementary to avian tumor virus RNAs (cDNAs) have distinguished two sets of sequences in each RNA. One set comprises 60% of the RNA which is related to other, independently replicating members of the avian tumor virus group including nondefective Rous sarcoma virus (ndRSV) [8–11]. This set is termed group-specific and probably represents defective replicative genes as well as conserved regulatory and structural elements [8,9,10]. The second set comprises the specific sequences of MC29 and MH2 RNA, which represent 40% of each RNA and is a likely candidate for the *onc* gene of these viruses. Since neither MC29 nor MH2 RNA contains sequences related to the *onc* gene of ndRSV, which is termed *src*, it has been suggested that these viruses contain distinct *onc* genes [8–11]. These and similar studies on avian [9–15] and murine [16–18,23] viruses have suggested that within a given RNA tumor virus family, transforming genes may differ whereas replicative genes are relatively conserved.

It is the purpose of this study to biochemically define the *onc* genes of MC29 and MH2 in order to structurally and functionally compare them to each other and to the *src* gene of RSV. The *src* gene of ndRSV has been unambiguously defined by analyses of *src*-deletion mutants and *src*-recombinants as a sequence of about 1500 nucleotides that segregates with sarcomagenicity [13,15,19]. Moreover the *src* gene was mapped near the 3′ end of viral RNA [14,15,19] and appears to be translated into a protein of 60000 daltons [20,21]. Due to their defectiveness in all three replicative genes [4,5,6] *onc*-deletion mutants and recombinants of MC29 and MH2 would lack biologically detectable genetic markers in replicative genes. Further, the specific sequences of MC29 are not expected to recombine readily with other

avian tumor viruses lacking them, because analysis of tumor virus recombination has demonstrated that efficient recombination only occurs between closely related, allelic sequences [22]. Therefore, the approach that was used to define *src* of ndRSV cannot yet be used to define the *onc* genes of MC29 and MH2 or to define the *onc* genes of other defective transforming viruses.

In this paper we describe an alternate, more biochemical, approach to the definition of the *onc* genes of MC29 and MH2. We identify (for MC29 and MH2) and locate (only for MC29) on the viral 28S RNAs strain-specific and group-specific sequences, we then investigate, in the case of MC29, the proteins encoded by these sequences with an interest in identifying a protein involved in cell transformation. Preliminary work has been described [9–11] and recently a more complete comparison of MH2 and MC29 has been published [40].

Results

MC29 and MH2 Contain Specific 28S RNA Species which Share Specific Oligonucleotides

A 28S RNA species that is physically and chemically distinct from the 34S RNA of the helper virus has been found in several defective virus-helper virus complexes of MC29 and of MH2 [8–11]. To demonstrate that the 28S RNA species in each virus complex was specific to the defective transforming MC29 or MH2 virus, different MC29 and MH2 pseudotypes have been investigated for the presence of 28S RNAs. It can be seen in Fig. 1 that three different MC29 pseudotypes, MC29 (ring-neck pheasant virus [RPV]), MC29 (MC-associated virus of subgroup A [MCAV-A]) and MC29 (MCAV-B) each contained a 28S RNA species in addition to the 34S RNA of the respective helper virus. The same was true for the MH2 pseudotypes, MH2 (MH2AV of subgroups A and C [A+C]) and MH2 (RAV-7) (Fig. 1). By contrast, the RNA of helper viruses RPV [8], MCAV-A, MH2AV-A+C and RAV-7 contained only a 34S RNA species (not shown). It was concluded that different pseudotypes of MC29 as well as of MH2 each share physically indistinguishable 28S RNA species.

We have investigated the large RNase T_1-resistant oligonucleotides of the 28S RNAs of MC29 and MH2 as specific diagnostic markers of each viral RNA. This is experimentally complicated because owing to their defectiveness these viruses only replicate in the presence of a helper virus. Hence viral RNA is a mixture of defective and helper virus RNAs, the ratio of which may vary with a given infected culture [8]. If 28S RNA is present at equal or higher concentration than 34S RNA in this mixture, it can be detected and isolated by preparative electrophoresis as is shown in Fig. 1. However, 28S RNA prepared in this fashion is still contaminated by degraded 34S RNA, the degree of contamination depending on the relative amount of 34S RNA present in the mixture and on the integrity of each RNA species. Degraded

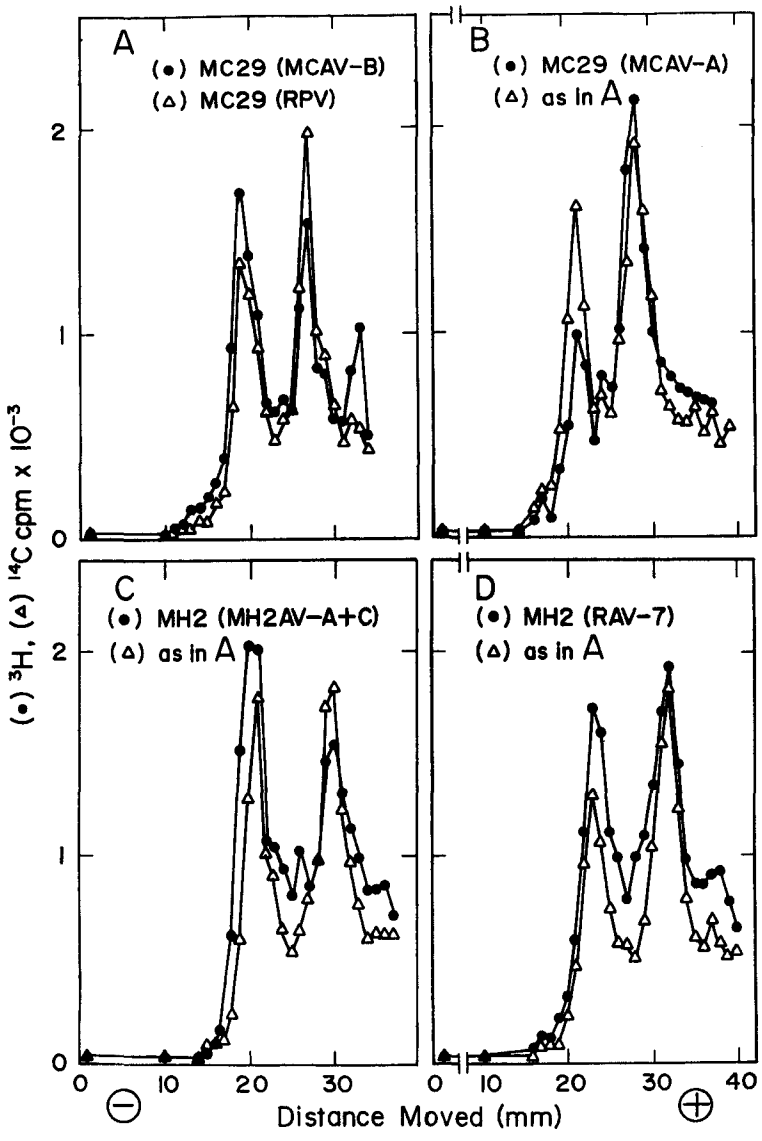

Fig. 1. The RNA monomers of different avian acute leukemia virus MC29-pseudotypes and of different avian carcinoma virus MH2-pseudotypes after electrophoresis in 2% polyacrylamide gels. Preparation of viral RNA and conditions for electrophoresis have been described [8]. Each RNA was electrophoresed with MC29 (ring-neck pheasant virus of subgroup F [RPV]) RNA standards containing a 28S MC29 and a 34S RPV RNA species [8]. (A) RNA of MC29 (MCAV-B). This pseudotype of MC29 was propagated on chick embryo fibroblasts and was obtained from C. Moscovici, who had received it via R. Smith from J. Beard. (B) RNA of MC29 (MCAV-A). This pseudotype of MC29 was also propagated on chicken fibroblasts and was obtained from C. Moscovici, who had received it from K. Bister and P. K. Vogt. (C) RNAs of MH2 (MH2AV-A and C) and (D) of MH2 (RAV-7). These pseudotypes of MH2 were prepared by one of us (P. K. Vogt) and had been propagated on chicken macrophage cultures

34S RNA that has the size of 28S RNA would coelectrophorese with intact 28S RNA of MC29 or MH2. Thus in order to distinguish 28S RNA-specific oligonucleotides from oligonucleotides derived from fragmented 34S RNA present in a given pool of 28S RNA, we have fingerprinted both RNA species. By subtracting from the 28S RNA oligonucleotides that the 34S and 28S RNAs have in common, we arrived at a minimal estimate of 28S RNA-specific oligonucleotides. A complete catalogue of 28S RNA-specific oligonucleotides must also identify those oligonucleotides that the 28S RNA

of a defective virus may have in common with the 34S helper virus RNA. Determination of such oligonucleotides was approached by analyses of 28S RNA pools that are little contaminated by 34S RNA (as was the case for RNA from MC29 (RPV) propagated by the quail Q8-cell line [8]) or by identifying specific oligonucleotides from different 28S RNA pools of a given defective virus that was propagated with different helper viruses.

Fingerprint analysis of 28S MC29 RNA prepared from MC29 (RPV) is shown in Fig. 2A and that of 34S RPV RNA has been published previously [8]. A comparison indicates that of all the T_1-oligonucleotides numbered in Fig. 2A, 28S MC29 RNA shares three, i.e., nos. 4, 9 and C with RPV RNA (8, 9, Table 1). Since the relative concentration of no. 4 was lower than that of all others in Fig. 2A and varied with different preparations of 28S MC29 RNA, we conclude that it was derived from contaminating RPV RNA (therefore it is in parentheses in Fig. 2A). Henceforth, the oligonucleotides of 28S RNA pools that are thought to derive from contaminating helper virus RNA are parenthesized in the Figures and Tables. Oligonucleotides shared with helper viruses that are present at equimolar ratios are believed to be integral parts of the 28S MC29 RNA shown in Fig. 2A. This was directly proven in the case of oligonucleotide no. 9. This oligonucleotide is a conserved element of the *gag* gene of other avian tumor viruses, which maps near the 5′ end of viral RNAs [27, 28]. If this oligonucleotide were present in a 28S RNA pool of MC29 (RPV) RNA as part of a RPV fragment, this fragment would have to include the 5′end of the 34S RNA but would lack the 3′ poly(A) end. Therefore only the poly(A)-tagged species of a 28S RNA pool of MC29 (RPV) RNA had been fingerprinted in Fig. 2A. The result that oligonucleotide no. 9 was present indicates that it was an integral part of 28S MC29 RNA. Its presence at a slightly lower than equimolar concentration in poly(A)-selected 28S RNA (Fig. 2A) was expected, because the 5′ end of the RNA would be preferentially lost in a poly(A) selection (compare Figs. 2A and 3A below). Analyses of the RNase A-resistant fragments of the large T_1-oligonucleotides of the 28S and the 34S RNA species of MC29 (MCAV-B) shown in Figs. 2B and C have not yet been completed. Preliminary results indicate that the 28S RNA species share several, large oligonucleotides with 28S MC29 RNA isolated from MC29 (RPV). These homologous oligonucleotides were given the same numbers in Fig. 2A and B. In addition, the 28S RNA species of MC29 (MCAV-B) contained oligonucleotides which appeared to have chromatographic counterparts in the 34S helper virus RNA of MC29 (MCAV-B) shown in Fig. 2C. Some of these probably derived from contaminating 34S helper RNA. In addition 28S MC29 RNA may also have acquired some helper viral oligonucleotides by recombination.

To identify MH2 RNA-specific oligonucleotides, 28S RNA pools from MH2 (MH2AV-A and C) and from MH2 (RAV-7) were compared to each other and to those of their 34S helper virus RNAs (Fig. 2E-H) as described above for MC29 RNA. MH2-specific oligonucleotides are numbered without parentheses in Fig. 2E, F and Table 2. Further work is necessary to determine whether some of the oligonucleotides shared by 28S MH2 and 34S MH2AV-

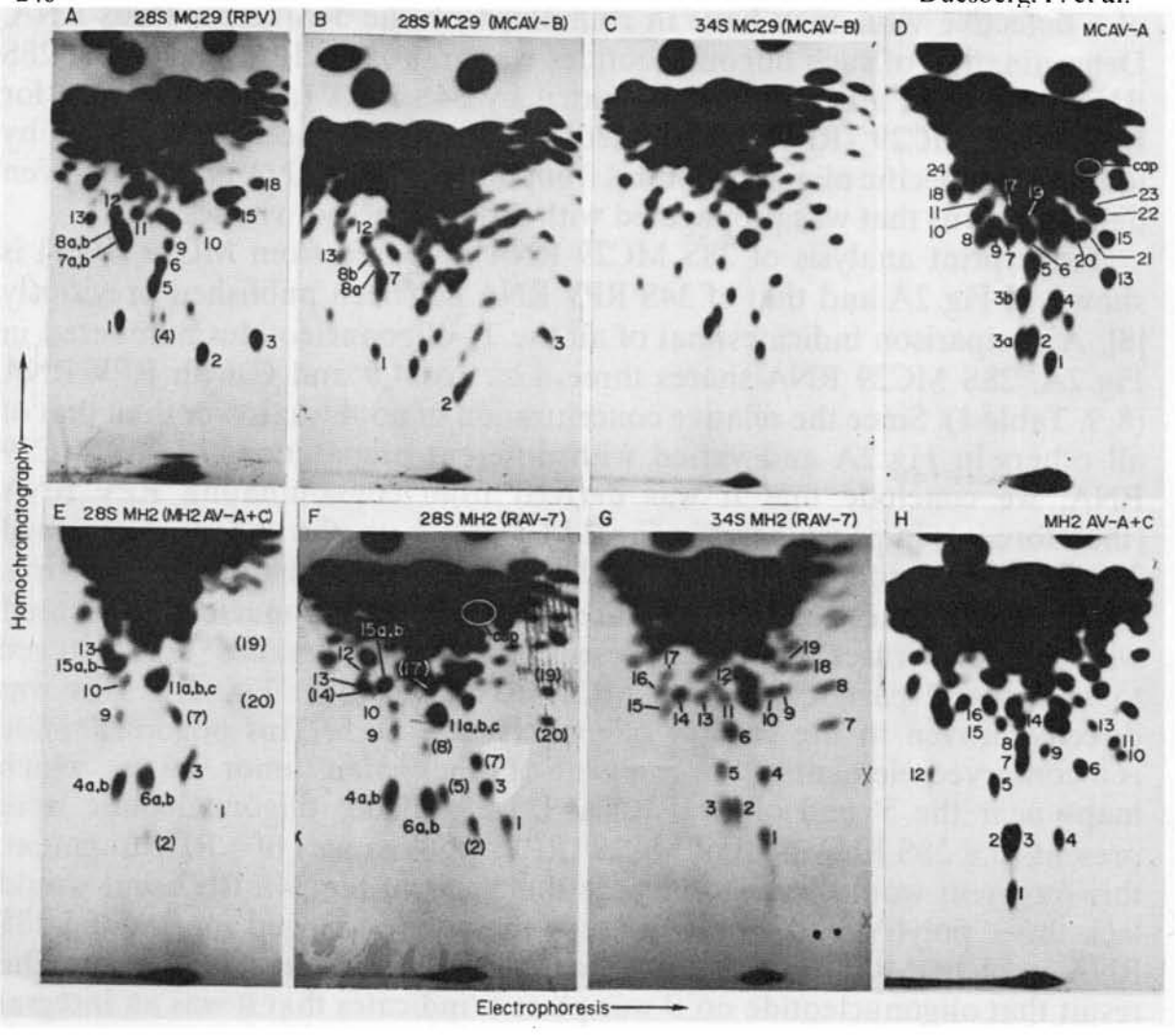

Fig. 2. Autoradiographs of RNase T_1-digested viral [^{32}P] RNA components after 2-dimensional electrophoresis-homo-chromatography (fingerprinting). Preparation of viral RNA components and conditions of fingerprinting have been described [8, 14]. Numbers identify large RNase T_1-resistant oligonucleotides, or spots consisting of more than one oligonucleotide. Numbers of oligonucleotides of 28S RNA pools which are probably derived from degraded 34S helper virus RNA are parenthesized. Cap designates the 5′ terminal capped oligonucleotide. The following RNAs were fingerprinted: (A) The poly(A)-containing 28S MC29 RNA prepared electrophoretically from MC29 (RPV) RNA as in Fig. 1 and chromatographed on oligo(dT)-cellulose [14]. (B) the 28S RNA of MC29 (MCAV-B), electrophoretically prepared from MC29 (MCAV-B) RNA as for Fig. 1. (C) the 34S MCAV-B RNA electrophoretically prepared from MC29 (MCAV-B) as in (B). (D) the 60–70S RNA of MCAV-A prepared from virus propagated in chicken fibroblasts. (E) the 28S RNA of MH2 (MH2AV-A and C), prepared as for (A, B). (F) the 28S RNA of MH2 (RAV-7) also prepared as for (A, B). (G) the 34S RAV-7 RNA prepared from MH2 (RAV-7) RNA as described for (C) and (H) the 50–70S RNA of MH2AV-A and C. A mixture of these two helper viruses was propagated in chicken fibroblasts

A and C or 34S RAV-7 RNAs are integral parts of 28S MH2 RNA or are derived from degraded helper virus RNA. Analysis of 28S RNA was hampered by difficulties with propagating sufficient radioactive virus in chicken macrophages [5] for biochemical analyses of RNA and because virus from transformed chicken or quail fibroblast cultures contained, in over thirty cases tested, > 3 times more 34S than 28S RNA, and was thus unsuitable for studying 28S RNA.

Table 1. Composition of T_1-oligonucleotides of 28S MC29 RNA[a]

28S MC29	RNase A digestion products	28S MH2[b]	MH2AV-A&C[b]	MCAV-A[b]	PR-B[b]	RPV[b]
Spot No.[c]				Spot No.		
1	2U, 3C, G, 2 (AC), (AU), 2 (AAC)					
2	7U, 7C, G, (AC), 3 (AU), (AAC), (AAU)					
3	8U, 2C, G, (AAC), <1 (AAAN)					
(4)	5U, 8C, 3 (AC), (AU), (AAC), (AAG)					1
5	3U, 5C, 2 (AC), (AU), (AAU), (AAAC), (A_4G)					
6	2U, 8C, 3 (AU), (AG), (AAAU)					
7a	2U, 6C, G, 2 (AC), (AU), (AAC)					
7b	3U, 8C, (AC), (AAG)	9				
8a	2U, 2C, G, 2 (AC), (AAAC)					
8b	2U, 4C, (AC), (AG), (AAU), (AAAC)	10				
9	5U, 6C, (AAG), (A_4C)	11a,b,c?		5a,b	8	5
10	3U, 2C, 2 (AU), (AG), (AAU)					
11	2U, 4C, G, 2 (AC), (AU), (AAC)	15a,b	16	7	14	
12	U, 4C, 2 (AC), (AU), (AG), (AAC)					
13	U, 3C, G, 2 (AC), (A_4N)					
15	4U, 4C, G, (AC), (AU), (AAU)					
18	5U, 3C, G, (AU), (AAU)		22			
C	G, (AC), (AU), (AAU), (AAAN)	C	C	C	C	C

[a] Oligonucleotides numbered as in Figs. 2 and 3 were eluted from fingerprints shown in these Figs. and from fingerprints of poly(A)-tagged RNA fragments (not shown) prepared to derive the oligonucleotide map shown in Fig. 3 as described [14]. RNase A-resistant fragments of T_1-oligonucleotides were determined following published procedures [8, 14] except that digestion with RNase A was in 10 µl at 0,4 mg RNase A per ml for 3 hr.

[b] The columns marked on the right indicate 28S MH2, MH2AV-A&C, MCAV-A, PR-B and RPV-oligonucleotides numbered as below and in refs. 8–11, 14, 15, with the same composition as the respecitve counterparts of MC29 RNA.

[c] Numbers as in Fig. 2A

A comparison of the oligonucleotides of 28S MC29 and MH2 RNAs indicates that the two RNAs share about 5 out of 12–15 large oligonucleotides (see Tables 1 and 2). Three of these common oligonucleotides are also shared with other avian tumor viruses. However two are only shared by MC29 and MH2, i.e., nos. 7b and 8b of MC29 and nos. 9 and 10 of MH2. Hence it is conceivable that these oligonucleotides are part of the functionally related, specific *onc* genes of these two viruses.

It has been argued that (defective) transforming viruses are generated by recombination of a helper virus with an unknown (defective) virus preexisting in the cell or with cellular genetic elements [16–18, 23, 24]. The helper viruses isolated from the original stocks of MC29 and MH2 would appear to be likely candidates for one parent of such recombinational events. It would then be expected that the defective recombinant virus shares more sequences with its progenitor than with other possible helper viruses. To test this, the RNA of a cloned helper, isolated from the original MC29 stock, i.e.,

Table 2. T_1-oligonucleotides of 28S MH2 RNA[a]

28S MH2	RNase A digestion products	MC29[c]	RAV-7[c]	MH2A-A&C
Spot No.[b]				
1	U, 2C, G, 5 (AU), (AAU), (AAAC)			
(2)[b]	9U, 7C, 2 (AC), 2 (AU), (AG), (AAC), (AAU)		1	4
3	4U, 5C, G, 3 (AU), 2 (AAU), (AAAU)			
4a,b	3U, 9C, 2G, 4 (AC), 3 (AU), 3 (AAC), (AAAC)	1[d]		
(5)	6U, 9C, 2 (AC), (AU), (AAC), (AAG)		2	2
6a,b	9U, 16C, 2G, 2 (AC), 2 (AU), 2 (AAC), (AAAC), (A_5N)			
(7)	5U, 5C, G, 2 (AC), 3 (AU)		4	6
(8)	4U, 5C, (AC), (AAU), (AAAC), (AAAG)		5	
9	3U, 10C, (AC), (AAG)	7b		
10	2U, 3C, (AC), (AG), (AAU), (AAAC)	8b		
11a,b,c	10U, 15C, G, 3 (AC), 5 (AU), (AG), (AAG), (AAAG), (AnN)	9?		
12	2U, 4C, 2 (AC), (AU), (AG)			
13	U, 3C, 2 (AC), (AAG), (AAAU)			
(14)	4C, 2 (AC), (AU), (A_4G)		15	
15a,b	2U, 4C, G, 2 (AC), (AU), (AG), (AAC), (AnN)	11?		
(17)	2U, 4C, G, 2 (AC), (AU)		11	
(19)	10U, 7C, G, (AU)		8	
(20)	6U, 2C, G, 3 (AU), (AAU)		7	
cap[e]	class IV	cap		cap

[a] Oligonucleotides were eluted from fingerprints shown in Fig. 2 and their RNase A-resistant fragments were determined as described previously [14] and for Table 1. Some, but not all oligonucleotide numbers are the same as those used previously for more preliminary analyses of MH2 RNA [9, 10]. Due to scarcity of MH2 [^{32}P] RNA, some numbers are semi-quantitative, combining scintillation counting of RNase A-resistant fragments and reflecting visual estimates from autoradiograms.

[b] Numbers as in Fig. 2E and F. Oligonucleotides with homologous counterparts in RAV-7 or MH2AV-A&C helper virus RNAs are parenthesized (see text).

[c] Number denotations of homologous oligonucleotides found in MC29, RAV-7 and MH2AV-A&C RNAs (see Tables 3 and 4)

[d] 4a is MC29 no 1, see ref 40.

[e] see ref 22 for composition

MCAV-A, was fingerprinted (Fig. 2D) and the RNase-A resistant fragments of large oligonucleotides were compared to those of 28S MC29 RNA isolated from MC29 (RPV). It can be seen in Tables 1 and 3 and Fig. 2 that the two RNAs share only about three of their large T_1-oligonucleotides. In addition both RNAs as well as RPV RNA have the same 5′ terminal cap-oligonucleotide (marked in Fig. 1) [22]. The same was true for the relationship of 28S MH2 RNA to MH2AV-A and C RNAs, two helper viruses isolated from the original stock of the virus. These helper viruses did not share more oligonucleotides with MH2 RNA than with RAV-7 (compare Fig. 2 and Tables 2, 4 and 5). We conclude on the basis of oligonucleotide homologies, that 28S MC29 and MH2 RNAs are not more closely related to their original helper viruses than to the other helper viruses tested.

Table 3. Composition of T_1-oligonucleotides of MCAV-A RNA[a]

MCAV-A	RNase A digestion products	PR-B[c]
Spot No.[b]		
1	7U, 5C, (AC), (AU), (AG), (AAC), (AAU)	
2	6U, 10C, 2 (AC), (AU), (AAC), (AAG)	4
3a	4U, 7C, G, (AC), (AU), (AAAC)	6
3b	3U, 5C, (AC), (AAU), (AAAC), (A_4G)	
4	6U, 6C, G, 3 (AC), 3 (AU)	
5a,b	8U, 9C, G, 2 (AC), (AU), (AAG), (A_4C)	8
6	3U, 6C, G, 3 (AC), 2 (AU)	
7	2U, 5C, G, 2 (AC), (AU), (AAC)	14
8	4C, 2 (AC), (AU), (A_4G)	
10	U, 5C, 3 (AC), (AAG)	18
11	3C, (AU), (AG), (A_5N)	20b
13	6U, 2C, G, 3 (AU), (AAU)	
15	8U, 5C, G, (AU)	
18	5C, G, 4 (AC)	
21	5U, G, (AC), 2 (AU)	
22	5U, 3C, G, (AU), (AAU)	18
23	3U, 2C, G, 2 (AU), (AAU)	
24	5C, <2 (AC), (AG)	

[a] As in Table 1.
[b] As in Fig. 2D.
[c] Oligonucleotides of the same composition were found previously in PR-B (14) and MC29 (see Table 1)

Table 4. Composition of T_1-oligonucleotides of MH2AV-A and -C RNA[a]

MH2AV-A and C	RNase A digestion products	PR-B[c]
Spot no.[b]		Spot no.
1	7U, 10C, G, 3 (AC), (AAC)	
2	5U, 10C, 2 (AC), (AU), (AAC), (AAG)	4
3	6U, 10C, G, (AC), (AU), (AAAC)	6
4	8U, 6C, 2 (AC), 2 (AU), (AG), (AAC), (AAU)	
5	3U, 6C, G, 2 (AC), (AU)	
6	6U, 6C, G, 2 (AC), 4 (AU)	
7a,b	4U, 3C, 2 (AC), 2 (AU), (AG), (AAU), (AAAN), (A_4N)	
8	6U, 6C, (AAG), (A_5N)	
9	6U, 9C, G, (AAC)	
10	8U, 3C, 4 (AU), (AG), (AAU)	
11	6U, 2C, G, 4 (AU), (AAC)	
12	U, 2C, G, (AC), 2 (AAAN)	
13	4U, 3C, G, 2 (AU), (AAAN)	
14	5U, 4C, C, (AC), (AAAC), (AAAG)	
15	4U, 4C, (AC), (AAAC), (A_4G)	
16	2U, 5C, G, 2 (AC), (AU), (AAC)	14

[a] Determined as in Table 1.
[b] Numbers refer to oligonucleotides spots shown in Figure 2H.
[c] Oligonucleotides of the same composition were found in PR-B (14)

Table 5. T_1-oligonucleotides of RAV-7 RNA

Spot no.	RNase A digestion products[a]
1	7U. 4C. 2 (AC). 2 (AU). (AG). (AAC). (AAU)
2	4U. 7C. 2 (AC). (AU). (AAC). (AAG)
3	6U. 6C. G. (AC). (AU). (AAAC)
4	5U. 5C. G. 2 (AC). 3 (AU)
5	4U. 5C. (AC). (AAU). (AAAC). (AnN)
6	6U. 6C. 3 (AC). (AU). (AAG). (AAAN)
7	6U. 2C. G. 3 (AU). (AAU)
8	10U. 7C. G. (AU)
9	3U. G. (AC). 2 (AU)
10	4U. 1C. (AC). (AU). (AnG)
11	2U. 4C. G. 2 (AC). (AU)
12	2U. 4C. G. 2 (AAU). (AAAN)
13	2U. 4C. G. 2 (AC). (AU). (AAC)
14a,b	3U. 5C. 3 (AC). (AU). AG). (AAC). (AAAN)
15	4C. 2 (AC). (AU). (AnG)
16	2C. 2 (AC). (AAG)
17	2C. (AU). (AG). (AnN)
18	4U. 2C. G. (AU). (AAU)
19	2U. C. G. 3 (AU). (AAU)

[a] Oligonucleotides were prepared as for Tables 1–4. RAV-7 RNA was isolated from MH2 (RAV-7) virus as described in Fig. 1 and in the text. Quantitation of RNase A-resistant fragments was based on visual estimates of autoradiographs

The Relationship of 28S MC29 and MH2 RNAs to Each Other and to the RNAs of Other Avian Tumor Viruses Measured by Hybridization

To determine whether 28S MC29 and 28S MH2 RNA contain *src*-specific nucleotide sequences of avian sarcoma viruses and to investigate their relationship to each other and to the RNAs of helper-independent avian RNA tumor viruses, the RNAs were hybridized to various cDNAs. All hybridizations were carried out with an excess of cDNA and at increasing cDNA to RNA ratios to reach plateau values of maximal hybridization. Under our conditions, maximal hybridization of 28S MC29 RNA with homologous MC29 (RPV) cDNA and of Prague RSV-B (PR-B) RNA with homologous cDNA was about 93% (Table 6). To determine whether 28S MC29 and MH2 RNAs contain *src*-specific sequences each RNA was first hybridized to cDNA from PR-B which contains *src* and then to cDNA from transformation-defective (td) PR-B which lacks *src* [13–15]. It is seen in Table 6 that approximately the same percentage (62 to 66%) of each RNA was hybridized by each cDNA. The MC29 and MH2 RNA sequences hybridized by PR-B and td PR-B cDNA were not additive, because a mixture of these two cDNAs did not hybridize more than each by itself (Table 6). The PR-B cDNA used was shown to include *src*-specific sequences, because it was able to hybridize 13% more PR-B RNA than td PR-B cDNA (Table 6). This was the expected difference, because the *src* gene corresponds to about 13% of the

viral RNA [13]. It follows that MC29 RNA and MH2 RNA lack *src*-specific sequences. About 62 to 66% of MC29 and MH2 RNAs are related to the RNAs of PR-B, td PR-B and other independently replicating avian tumor viruses. Therefore these sequences are termed group-specific. At least 31% (i.e., 93 minus 62) or 1700 nucleotides of each RNA would appear to be specific for MC29 or MH2, respectively. This is considered a minimal estimate because each electrophoretically prepared 28S RNA was contaminated with degraded helper virus RNA (c.f., Figs. 1 and 2). Helper virus RNAs including RPV RNA, 34S MH2AV-A and C RNA were 60 to 80% homologous to the RNAs of PR-B, td PR-B and of other avian tumor viruses (Table 6).

To test whether the specific sequences of MC29 and MH2 RNA (defined as those which did not hybridize with PR-B or td PR-B cDNA) are related to each other, 28S MH2 RNA was hybridized to MC29 (RPV) cDNA. About 64% of the RNA was hybridized (Table 6). If annealed with MC29 (RPV) and PR-B cDNAs, about 70% of 28S MH2 was hybridized. It follows that approximately 30% of the 28S MH2 RNA is unrelated to MC29 and PR-B RNA. The result that about 5–10% more 28S MH2 RNA was hybridized by MC29 (RPV) and PR-B cDNAs together than by each cDNA alone, may indicate that each of these cDNAs contains sequences related to different sequences of MH2. It is conceivable that PR-B cDNA would hybridize with segments of the above defined group-specific sequences of MH2 not represented in MC29 (RPV) cDNA and MC29 cDNA with segments of MH2-specific RNA not represented by PR-B cDNA. A specific relationship between MC29 and MH2 is consistent with the finding that MH2 and MC29 RNAs share two oligonucleotides not found in any other avian tumor virus tested here (see above).

To test further the notion mentioned above that defective transforming viruses are recombinants of a helper virus and an unknown parent, sequence homologies between the RNAs of MC29 and of its original helper virus, MCAV-A as well of MH2 and of MH2AV-A and C were determined. It is seen in Table 6 that 28S MC29 RNA is hybridized by MCAV-A cDNA to approximately the same extent, i.e., 61%, as by cDNA prepared from other avian tumor viruses. The same was true for 28S MH2 RNA and MH2AV-A and C cDNA (Table 6). The MCAV-A cDNA as well as the MH2AV-A and -C cDNA used, hybridized 94–96% of their homologous viral RNAs (Table 6). It is concluded that MC29 and MH2 are not more closely related to their original helper viruses than to other avian tumor viruses tested. This conclusion is consistent with the results described above that the T_1-oligonucleotides of MC29 and MCAV-A as well as those of MH2 and MH2AV-A and C RNAs are not more closely related to each other than to those of other avian tumor virus RNAs.

Mapping MC29-Specific and Group-Specific Sequences of MC29 RNA

To locate MC29-specific and group-specific sequences on MC29 RNA the following strategy was used: First the RNase T_1-resistant oligonucleotides of

Table 6. Hybridizations[a] of viral RNAs with viral cDNAs[b]

RNA	cDNA	% Hybridization at various cDNA/RNA ratios mean ± S.D. 5:1	20:1	50:1	100:1	150:1
28S MC29[c]	MC29 (RPV)	65 ± 6	84 ± 4	88 ± 1	93 ± 7	96
	RPV	46 ± 3	61 ± 0	66 ± 3	69 ± 4	
	PR-B	40 ± 2	54 ± 7	61 ± 8	61 ± 8	
	tdPR-B	50 ± 2	55 ± 6	61 ± 6	62 ± 7	
	PR-B + tdPR-B[h]	50	62 ± 6	60	61	
	MCAV-A	55	60 ± 3	61		
	PR-B + MCAV-A[h]	61	60 ± 5			
34S RPV	MCAV-A	64	65			
	MC29 (RPV)	48	81	80	80	
	tdPR-B	35	58			
34S MCAV-A	MCAV-A	91 ± 6	94 ± 5			
	MC29 (RPV)		49	80	80	
	RPV			75		
	PR-B		66	70		
28S MH2[d]	PR-B			60,5 ± 1	64	66,5 ± 5
	tdPR-B			58 ± 5	59,5	65,5 ± 2
	PR-B + tdPR-B[h]			62 ± 3		
	MC29 (RPV)			61,5 ± 5	61,5	64 ± 1
	MC29 (RPV) + PR-B[h]			67 ± 3		70,5 ± 6
MH2[e]	MH2AV-A and C			58 ± 5	60 ± 2	
34S MH2AV[d]	PR-B			79,5 ± 8		
	tdPR-B			79 ± 8	78	
	PR-B + tdPR-B[h]			86,5 ± 2		
	MC29 (RPV)			70 ± 5	75	
	MC29 (RPV) + PR-B[h]			84,5 ± 4		
MH2AV-A and C[f]	MH2AV-A and C			75 ± 4	96	
PR-B[g]	PR-B		93 ± 0	93 ± 1		
	tdPR-B		81	81		
	PR-B		94 ± 0	92 ± 1		
	tdPR-B		79	81		
RAV-7[e]	MH2AV-A and C				83	

[a] Each reaction mixture contained about 1 ng of ^{32}P RNA (2000 cpm/ng) and 5–100 ng of ^{3}H cDNA (50 cpm/ng). Hybridizations were in 4–5 µl of 70% deionized formamide/0,3 M NaCl/0,03 M Na citrate/15 mM Na phosphate, pH 7,0 and 0,05% $NaDodSO_4$ at 40° for 12 hr. Percentage nuclease-resistance is expressed as the radioactivity recovered in aliquots digested with nuclease relative to that found in undigested aliquots. Each value is the mean of 2 or 3 experiments using, in some cases, independent preparations of RNA and cDNA. Digestion was with RNases A (5 µg/ml), T_1 (10 units/ml) and T_2 (10 units/ml) for 30 min at 40° in 0,3 M NaCl/0,03 M Na citrate, pH 7,0. The background of nuclease-resistance of an aliquot heated at 100° in 0,01 M Na^+ was $<0,5\%$. Many of these data are from refs. 8, 9, 10. Ratios of cDNA to RNA represent maximal values for a given RNA sequence because cDNAs are nonequimolar mixtures of viral sequences and include viral double-stranded DNA as well as some cellular DNA species.

[b] cDNA was prepared as described previously [8, 9].

[c] Prepared by gel electrophoresis from MC29 (RPV) [8] as shown for Fig. 1.

the MC29-specific and of the group-specific segments of MC29 RNA were determined. MC29-specific RNA segments were recovered from RNA-DNA hybrids formed between viral RNA and MC29-specific cDNA. Group-specific segments of MC29 RNA were obtained from hybrids formed with cDNAs from other avian tumor viruses [10, 11, 26]. The location of the RNA segment to which a given oligonucleotide belonged was then deduced from a map of all large oligonucleotides of MC29 RNA. An oligonucleotide map describes the location of each large oligonucleotide relative to the 3′ poly(A)-coordinate of viral RNA. The location of a given oligonucleotide is deduced from the size of the smallest poly(A)-tagged RNA fragment from which the oligonucleotide can be obtained [14].

MC29-specific cDNA was prepared by hybridizing MC29 (RPV) cDNA to an excess of unlabeled RNA of RPV and of PR-B under conditions of moderate stringency (Fig. 3). In this way all but the MC29-specific sequences of this cDNA were converted to heteroduplexes leaving only MC29-specific cDNA single-stranded. This cDNA was then hybridized in a second step with 50–70S MC29 (RPV) [^{32}P] RNA essentially under the conditions described above. However incubation was for a shorter time, to minimize displacement of unlabeled RNA from heteroduplexes present in our preparation of MC29-specific cDNA by related or identical sequences of MC29 [^{32}P] RNA. After digestion of unhybridized MC29 (RPV) [^{32}P] RNA with RNases A and T_1, the resulting hybrids were prepared by chromatography on Biogel P100. Subsequently the hybrid was heat-dissociated and the [^{32}P] RNA was digested with RNase T_1. The resulting MC29-specific T_1-oligonucleotides were detected by fingerprint analysis (Fig. 3B) and their RNase A-resistant fragments were determined (Table 1).

The remaining oligonucleotides of MC29 RNA (Fig. 3A) are expected to derive from group-specific RNA segments, sequence-related to the RNAs of other members of the avian tumor virus group. Group-specific RNA sequences were identified as follows: 28S MC29 [^{32}P] RNA was prepared electrophoretically from 50–70S MC29 (RPV) RNA [8]. The RNA was then hybridized to an excess of PR-B and RPV cDNA as above (Fig. 3C). After hybridization the reaction mixture was treated with RNase T_1 to degrade unhybridized RNA. RNase A was not used for this purpose in order to preserve small MC29-specific oligonucleotide segments which are part of larger group-specific polynucleotide segments of MC29 RNA hybridized with PR-B and RPV cDNAs. Thus mismatches involving oligonucleotide segments with fewer than two Gs would register as complete hybrids in our conditions. Such mismatches are expected because neither RPV nor PR-B are immediate predecessors of MC29 and because group-specific sequences of avian tumor virus RNAs defined by hybridization are known to differ if compared by

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[d] Prepared from MH2 (MH2AV-A and C) propagated in macrophages as for c.
[e] Prepared from MH2 (RAV-7) as for c.
[f] 60–70S RNA from cloned MH2AV-A and C was used.
[g] Shown electrophoretically to be free of tdPR-B RNA [8].
[h] Each cDNA was present at the DNA to RNA ratio indicated.

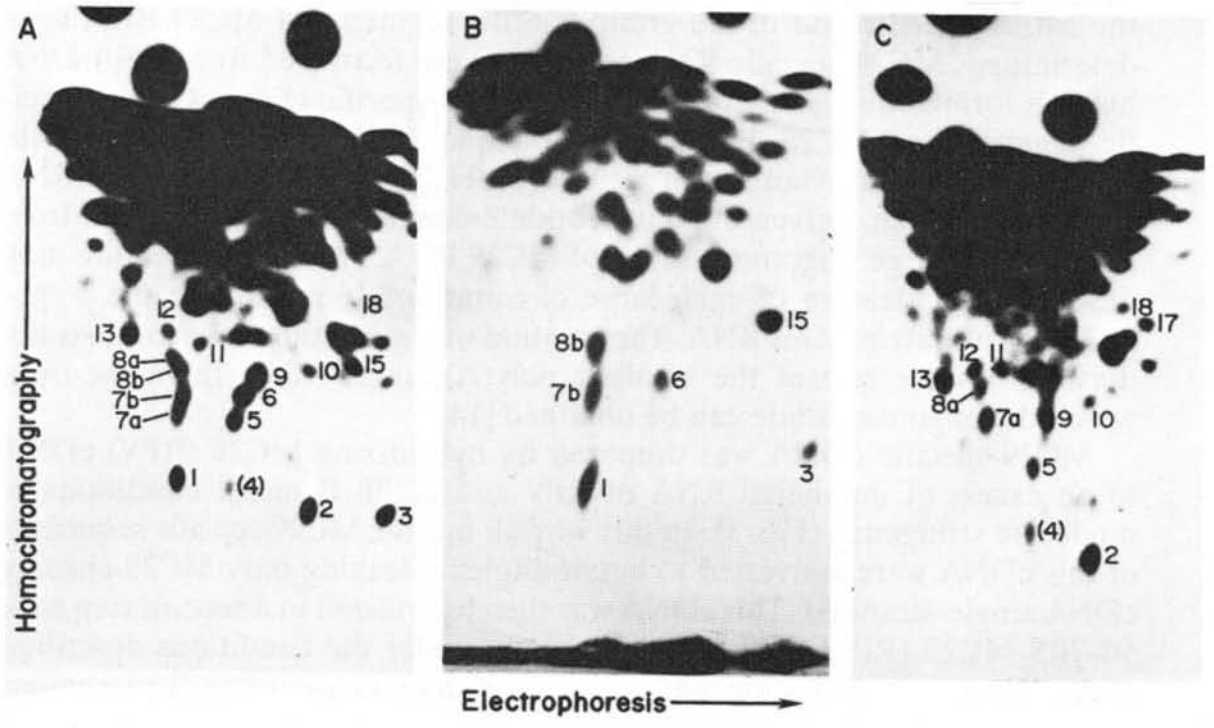

Fig. 3. RNase T_1-resistant oligonucleotides of whole 28S MC29 RNA (A). of MC29 RNA sequences that are not sequence-related to other avian tumor virus RNAs (MC29-specific) (B), and of MC29 RNA sequences that are sequence-related to other avian tumor virus RNAs (group-specific) (C). 28S MC29 [^{32}P] RNA was prepared electrophoretically from 50–70S MC29 (RPV) RNA, digested with RNase T_1 and subjected to 2-dimensional electrophoresis-homochromatography as described [8.14]. Oligonucleotides were numbered as in Fig. 2 and previously [8–11]. In cases where double-spots were resolved, distinct oligonucleotides were denoted alphabetically (Fig. 2. Table 1). Oligonucleotide no. 4 is in parenthesis because it is thought to derive from contaminating RPV RNA [8] rather than from MC29 RNA (see Fig. 2). (B) To prepare MC29-specific sequences from MC29 RNA. 50–70S MC29 (RPV) [^{32}P] RNA was hybridized to MC29-specific cDNA. MC29-specific cDNA was made by incubating 2 μg MC29 (RPV) cDNA [8.19] with 15 μg RPV RNA and 12 μg PR-B RNA for 12 hr at 40°C in 10 μl 50% formamide containing 0,45 M NaCl, 0.045 M Na citrate and 0.01 M Na-PO_4 pH 7.0. Subsequently, 1.5 μg of MC29 (RPV) [^{32}P] RNA (5×10^6 cpm/μg) was added in 20 μl of the above formamide buffer and incubation was continued for 1 hr. After digestion for 30 min at 40°C in 200 μl of 0,3 M NaCl, 0,03 M Na citrate containing 5 μg/ml RNase A and 50 units/ml RNase T_1, the resistant hybrid was isolated from the void volume of a Biogel P100 column ($12 \times 0{,}6$ cm) equilibrated in 0,1 M NaCl, 0,01 M Tris pH 7,4, 1 mM EDTA and 0,2% Na dodecylsulfate. Hybrid was extracted 3 times with phenol in the presence of 30 μg carrier yeast tRNA, then ethanol-precipitated, heat-dissociated in buffer of low ionic strength, digested with RNase T_1 and subjected to fingerprint analysis as above. The oligonucleotides from MC29-specific RNA segments so identified are underlined in the oligonucleotide map shown in Fig. 4. (C) To prepare avian tumor virus group-specific sequences of MC29 RNA, 0,25 μg of electrophoretically prepared 28S MC29 [^{32}P] RNA (Fig. 1, refs. 8,9,10) (2×10^6 cpm/μg) was hybridized with 1 μg of PR-B and 1 μg of RPV cDNAs for 12 hr in 25 μl of 70% formamide, 0,3 M NaCl, 0,03 M Na citrate, 0,02% Na dodecylsulfate and 0,015 M Na-PO_4 pH 7,0. The reaction product was heated to 50°C for 1 min in 0,15 M NaCl, 0,015 Na citrate pH 7,0 and treated with RNase T_1 (but not with RNase A) and otherwise as described for (B)

fingerprinting T_1-oligonucleotides [14,15,27,28]. This is because fingerprinting detects specific oligonucleotides even in RNA sequences which differ by only a few percent of their nucleotides and which are closely related if compared by RNA-cDNA hybridization. The T_1-oligonucleotides of the result-

ing hybrid are shown in Fig. 3C and Table 1. They represent RNA sequences of MC29 RNA that are closely related to but not identical with sequences of PR-B and RPV RNA. It can be seen in Figs. 3 and 4 that the T_1-oligonucleotides of MC29 RNA fall into two non-overlapping sets, those representing MC29-specific and those representing group-specific sequences of MC29 RNA.

An oligonucleotide map of 28S MC29 [^{32}P] RNA was derived by fingerprinting poly(A)-tagged fragments of electrophoretically purified 28S MC29 RNA (Fig. 4). The approximate distance of each large oligonucleotide, numbered as in Figs. 2 and 3, is given in nucleotides as well as in relative map units, each originating at the 3′ poly (A) coordinate. The specific oligonucleotides (underlined in Fig. 4) mapped together between 0,4 and 0,7 units with some uncertainty about the relative order of nos. 2 and 8b. Oligonucleotides of group-specific sequences (not underlined) are found at the 5′ end and in the 3′ half of viral RNA. The group-specific sequences of the 5′ end included oligonucleotide no. 9, previously identified as a conserved element of the *gag* gene of other avian tumor viruses (see above) [27,28]. The 3′ half contained oligonucleotide no. 11, found previously at the *src* border of *env* in other virus RNAs [14,15,27,28] and the highly conserved C oligonucleotide which maps near the 3′ end of avian tumor virus RNAs [14].

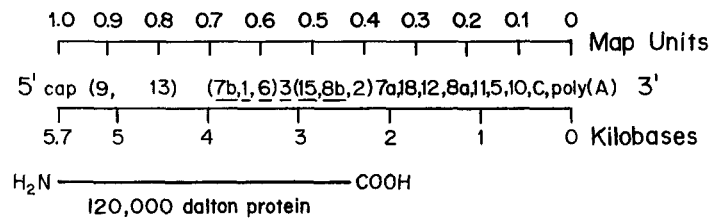

Fig. 4. Oligonucleotide map of 28S MC29 RNA. 28S MC29 [^{32}P] RNA (approx. 6×10^6 cpm) was prepared electrophoretically from 50–70S MC29 (RPV) RNA. The RNA was degraded by incubating 3 equal aliquots for 3, 6 and 9 min, respectively, in 0,05 M Na_2CO_3 at pH 11,0 and 50°C. Fragments were combined and poly(A)-tagged species selected on oligo(dT)-cellulose and fractionated into different size classes as described [14]. The T_1-oligonucleotides of 6 size classes of RNA fragments differing by approximately 1000 nucleotides from each other, were fingerprinted (not shown). Oligonucleotides of fragments were identified by their chromatographic properties and by their RNase A-resistant fragments (Table 1), and are numbered as in Fig. 2, 3 and Table 1. The resulting order of oligonucleotides is plotted on 2 scales, one denoting the approximate distance of an oligonucleotide from the 3′ poly(A)-coordinate in kilobases, the other denoting it in relative map units. When the relative order of oligonucleotides was uncertain, they are shown in brackets. Oligonucleotides from strain-specific sequences of MC29 RNA (Fig. 1B) are underlined and those from group-sequences (Fig. 1C) are not underlined. The bottom line represents the MC29-specific, 120000 dalton protein P120^{mc}. It is drawn in a position that is colinear with the RNA segment from which it was probably translated based on data described in the text and previously [10,11]

In Vitro Translation of MC29 RNA

To identify the products of the MC29 RNA genome, the RNA was translated in a cell-free system. Using this technique it has been possible to identify the *gag*, *pol*, *src*, and possibly also the *env* gene products of RSV [21,29,30,31].

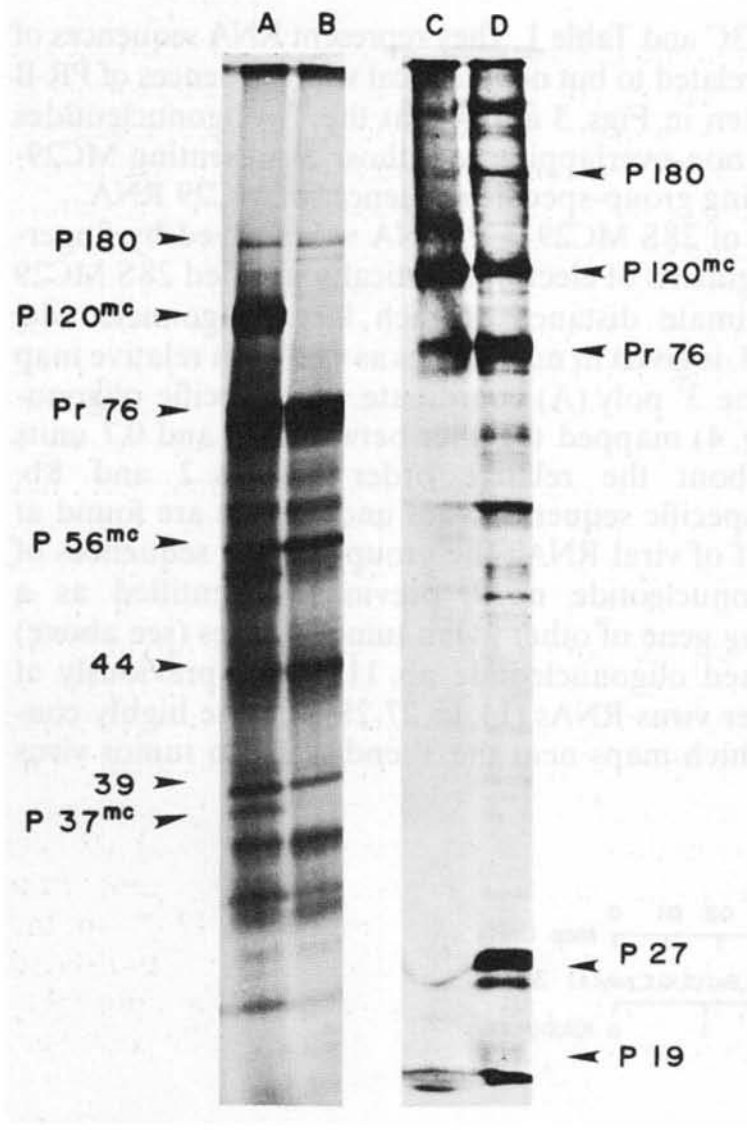

Fig. 5. Electrophoretic analysis of in vitro translation products of viral RNAs. RNAs were translated in the messenger-dependent rabbit reticulocyte lysate. [^{35}S]-methionine was present at 400 μCi/ml (600–1200 Ci/mmol). Products were analyzed by electrophoresis on a 12.5% polyacrylamide slab gel. The gel was autoradiographed after fixing and drying [4, 10, 11]. Tracks A and B show the products of total heat-denatured, poly(A)-selected (14) 50–70S virion RNAs from MC29 (RPV) and RPV (Track A), and from RPV alone (Track B). Track C shows the cell-free translation-products of MC29 (RPV) and RPV RNA (as in Track A), and Track D shows the proteins precipitated with antibody to disrupted avian myeloblastosis virus (AMV) (which contains mainly antibody to *gag* protein) from MC29 (RPV) infected cells. Immunoprecipitations were carried out as described [4, 20]. The viral proteins precipitated by anti-AMV serum were not precipitated by control serum, and P120^{mc} was not synthesized in RPV-infected cells (not shown)

MC29 (RPV) 50–70S RNAs were heat-denatured, poly (A)-selected, and translated in the messenger-dependent rabbit reticulocyte lysate [10, 11]. Products were analyzed by polyacrylamide gel electrophoresis. To identify the products specified by the MC29 RNA, the products of total poly (A)-selected MC29 (RPV) RNAs (Fig. 5, track A) were compared with those of RPV RNA alone (Fig. 5, track B). A number of products were in common between the two tracks, and these were assumed to be products of the RPV RNA.

These include a 180000 dalton-molecular weight polypeptide (P180), which is believed to be the *gag-pol* gene product [31]; Pr76, the primary product of the *gag* gene [30,31,32] and a number of smaller products, most of which are immunoprecipitated by antiserum to the *gag* protein, P27, and were only synthesized from full-length 34S RNA, suggesting that they are premature termination products of the *gag* gene [11].

In addition to these products there were three polypeptides which are specific to MC29 RNA. They have molecular weights of 120000, 56000, and 37000 daltons on this gel system, and will be denoted as $P120^{mc}$, $P56^{mc}$, and $P37^{mc}$ (Fig. 5). $P120^{mc}$ was a major product of the MC29 (RPV) RNA mixture, and was synthesized with the same order of efficiency as Pr76 (since they have a similar number of methionine residues [11]). A protein of similar size was recently found in MC29-infected cells and was shown to contain serological determinants of the viral *gag* gene proteins [4]. To test the relationship of the two proteins, $P120^{mc}$ was compared electrophoretically to its presumed counterpart precipitated from MC29-infected cells with antibody to disrupted avian myeloblastosis virus (AMV), which includes antibody to *gag* proteins. It can be seen that both proteins were electrophoretically identical (Fig. 5C, D). In addition $P120^{mc}$ synthesized in vitro was specifically immunoprecipitated by antiserum to P27 of AMV, the major *gag* gene protein, indicating that it contains determinants of *gag* proteins (not shown). $P120^{mc}$ was not recognized by antisera against products of the *pol* and *env* genes (not shown). Further evidence that the in vivo and in vitro-made $P120^{mc}$ are the same has been obtained recently [11]. We conclude that the $P120^{mc}$ translated in vitro from viral RNA and that found in MC29-infected cells are probably the same and that $P120^{mc}$ contains *gag*-related and MC29-specific peptides.

Discussion

The RNA and Gene Products of MC29 and MH2

The finding that different pseudotypes of MC29 or MH2 contained physically and chemically very similar or identical 28S RNAs, but 34S RNAs that varied with the respective helper virus, proved that the 28S RNAs are MC29- or MH2-specific. Each 28S RNA contained 30–40% of specific nucleotide sequences, which only hybridized with homologous cDNAs and 60–70% of sequences which hybridized with cDNAs of other avian tumor viruses which were termed group-specific. *Src* gene-related sequences were not detected in 28S MC29 or MH2 RNA.

In the case of 28S MC29 RNA, the specific sequences, identified by the large T_1-oligonucleotides they contain, mapped about 0,4 to 0,7 map units from the poly (A) end of the RNA (Fig. 4). The observation that MC29 and MH2 share two specific oligonucleotides, which in MC29 RNA mapped in a contiguous, MC29-specific RNA segment (Fig. 4), suggests that the specific sequences of the two viruses are related. This relationship has since been extended to three oligonucleotides [see Table 2 and ref 40].

In vitro translation of MC29 RNA generated one major 120000 dalton protein product, and two minor proteins of 56000 and 37000 daltons. The 120000 MC29 protein included protein sequences serologically related to the *gag* gene of other avian tumor viruses. Since one *gag* gene-related oligonucleotide, i.e., no. 9, was found near the 5′ end of MC29 RNA and since the *gag* gene of other avian tumor viruses maps near the 5′ end of their RNAs [27,28] it appears plausible that the *gag* gene-related portion of the 120000 MC29 protein was translated from the 5′ end of the RNA and that the remainder was translated from the MC29-specific sequences of the viral RNA (Fig. 4). Our recent observation that only full-length 28S MC29 RNA can be translated into $P120^{mc}$ [11] also argues for a 5′ map location of this protein, because eukaryotic mRNAs only effectively use one initiation site near the 5′ terminus [33,34,35].

The group-specific sequences that MC29, MH2 and other avian tumor viruses have in common are nearly indistinguishable, if compared by hybridization, but are distinct in each viral RNA if analyzed by the more sensitive method of fingerprinting, which detects single base changes. Since MH2 and MC29 do not express replicative genes, we can only speculate on the function of the group-specific sequences of their RNAs. Some of these sequences must play direct roles in virus replication by providing specific sites for packaging of viral RNA into helper virus proteins, for reverse transcription of viral RNA and for dimer linkage of 28S RNA monomers [6,8,9,10]. The *gag*-related, group-specific sequences of MC29 are translated into $P120^{mc}$ and may as such be involved in transformation (see below). Further analyses of $P56^{mc}$ and $P37^{mc}$ are necessary to determine whether their sequences overlap with $P120^{mc}$ or with each other or whether they correspond to distinct segments of viral RNA, since MC29 RNA may code for approximately 200000 daltons of protein.

What is the Onc Gene of MC29 and MH2?

Since neither MC29 nor MH2 contain *src* gene-related sequences, there are two different hypotheses as to which RNA sequences of these viruses represent their *onc* genes. One suggests that the specific sequences constitute the *onc* gene, while an alternative hypothesis suggests that their defective replicative genes i.e., group-specific sequences function as transforming genes. We prefer the first hypothesis for several reasons.

The idea that specific RNA sequences apparently unrelated to the replicative genes might be specific *onc* genes is proven for RSV [13,14,15,19] and has also been postulated for defective murine sarcoma [16,17,18,23,24] and acute leukemia viruses [9,10,36]. The existence of specific sequences in MC29 and MH2, which are related to each other, but unrelated to the other avian tumor virus RNAs tested, suggests that these sequences may belong to a family of related genes, possibly the functionally related [1–5,9,10] *onc* genes of MC29 and MH2. A specific *onc* gene for this class of viruses also corresponds with the distinct transformed phenotypes of MC29- or MH2-infected fibroblasts, which differ from those of RSV-transformed cells

[1–5,37]. The result that MC29-specific and group-specific sequences are translated into a specific protein, $P120^{mc}$, and that the same protein is also found in transformed cells [4] (and not in large quantity in the virion [unpublished]) suggests that this protein may be involved in cell transformation. A possibly analogous non-structural protein of 120000 daltons, that contains *gag*-related and specific peptides has been found in cells transformed by MH2 [5] and by the defective Abelson murine leukemia virus [38].

Further work correlating the specific RNA sequences of MC29 and MH2 with the specific determinants of their $P120^{mc}$ proteins is expected to support the hypothesis that this class of protein may be involved in transformation. Moreover, it is important to determine whether the 56000 and 37000 molecular weight MC29 proteins synthesized in vitro are also synthesized and functional in MC29-infected cells. While a definite answer to the question of whether the specific sequences of MC29 or MH2 and the $P120^{mc}$ proteins are involved in transformation can only be given if genetic variants become available, our data allow us to conclude that the *onc* genes and gene products in different prototypes of the avian tumor virus family, are different. Thus MC29 and MH2 must transform cells with gene products and possibly by mechanisms that differ from those of RSV.

Our data, that viruses with specific oncogenicity carry specific *onc* genes does not exclude roles for other viral genes, including those of the helper virus, in determining the oncogenic spectrum of a defective transforming virus. For example, the oncogenic spectrum of a defective virus should be greatly influenced by the *env* gene of its helper virus which provides the envelope glycoproteins for the defective virus. Since the cellular receptors for viral envelope glycoproteins differ greatly among different animals [39] and even among different target cells of the same animal [25] different helper viruses may deliver the same *onc* genes into specific target cells and thus cause a different form of cancer.

Acknowledgments

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Studies on the Structure and Function of the Avian Sarcoma Virus Transforming Gene Product

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A. Introduction

RNA tumor viruses quickly and efficiently transform cells and are therefore extremely useful agents for the study of the molecular events in oncogenesis. It is well established that the product(s) of a single avian sarcoma virus (ASV) gene *(src)* is responsible for the induction and maintenance of cell transformation in vitro and tumor production in infected animals [6]. We have carried out experiments designed to identify this product by techniques that require no assumptions concerning its mechanism of action. This approach was necessary because we anticipated that the *src* protein would be present at relatively low levels in the transformed cells, therefore making direct identification impossible. For example, the precursor to the major virion structural proteins is present at such low levels it can be identified only by immunoprecipitation [5]. Furthermore, since there are such a variety of biochemical changes reported to occur in transformed cells, it is difficult to predict at what level the *src* gene product might disrupt normal cellular processes. This, in turn, makes an accurate forecast concerning its function unlikely.

Recent work in our laboratory has resulted in the identification of a phosphoprotein with a molecular weight of 60000 that appears to be the product of the ASV *src* gene. A summary of that evidence is presented in Table 1. Because most of the experimental results that support these statements have been published [2,3,12,13], they will not be reviewed here. We feel that it is consistent with our results to designate the 60000 molecular weight phosphoprotein, $p60^{src}$, the product of the *src* gene.

Table 1. Evidence that p60 is the product of the avian sarcoma virus transforming gene

I.	A transformation-specific phosphoprotein with a molecular weight of 60000 is found in ASV-transformed cells by immunoprecipitation with antiserum from tumor-bearing rabbits.
II.	The polypeptide p60 is not found in cells infected with ASV mutants which have a deletion in the transforming gene.
III.	Immunoprecipitation of p60 does not depend on antibody directed against virion proteins.
IV.	The p60 polypeptides found in all avian and mammalian cells transformed by the Schmidt-Ruppin strain of ASV are biochemically similar.
V.	In vitro translation of viral RNA which contains the transforming gene produces a p60 immunologically and biochemically similar to that found in transformed cells.

The major tools employed in these studies were a) cell-free translation of that region of the viral RNA which contains the *src* gene and b) immunoprecipitation carried out with antiserum from rabbits bearing ASV-induced fibrosarcomas (TBR serum). The latter approach is one that has proved useful in the identification of non-structural proteins encoded by DNA-containing tumor viruses. The availability of antiserum that recognizes the product of the ASV *src* gene provided an essential reagent for the design of experiments for its purification and the characterization of its function. We will describe here recent results concerning the function of the ASV *src* gene product.

B. Results

I. Preliminary Observations

We were able to prepare antiserum that was monospecific for $p60^{src}$ and carry out immunofluorescence studies and found that transformed cells generally exhibited a diffuse cytoplasmic fluorescence without a strong association with any specific structures (Paula Steinbaugh and Joan S. Brugge, unpublished data). The nucleus of transformed cells was negative as was the cytoplasm of uninfected cells or cells infected with ASV mutants which have *src* gene deletions. Consistent with these observations were studies which showed that $p60^{src}$ appeared in cytoplasmic but not nuclear fractions when transformed cells were fractionated with detergents prior to immunoprecipitation. Additional information showed that $p60^{src}$ was readily solubilized in the absence of detergent by sonication of transformed cells. Taken together, these observations suggested to us that $p60^{src}$ might be an enzyme localized in the cytoplasm. In considering enzymes known to play a role in regulation of cellular functions, protein kinases were ready candidates. We could speculate, for example, that *src* encoded a protein kinase, which when expressed at relatively high levels produced a cellular alteration(s) that results in the transformed phenotype.

II. Correlation of the Immunoprecipitation of $p60^{src}$ and Protein Kinase Activity

To determine if immunoprecipitates containing $p60^{src}$ exhibited protein phosphorylating activity, immune complexes (formed with the protein A-bearing bacterium *Staphylococcus aureus* [7]) were resuspended directly in a protein kinase reaction mixture [4]. These complexes were incubated with [γ-^{32}P] ATP in the presence or absence of exogenous substrates. The reaction was terminated by adding SDS-containing buffer, the bacteria were separated from the IgG and antigens, and the latter subjected to SDS/polyacrylamide gel electrophoresis. Under these assay conditions exogenous substrates, such as histone and casein were not phosphorylated, but IgG was phosphorylated. The phosphorylated IgG was found to contain a single tryptic phosphopeptide and exclusively phosphothreonine.

Table 2. Correlation of the protein kinase activity and the immunoprecipitation of $p60^{src}$

Virus	Cell	Trans-formation	$p60^{src}$	Protein kinase activity
—	Chick	–	–	–
RAV-2	Chick	–	–	–
td-SR-ASV	Chick	–	–	–
SR-ASV	Chick	+	+	+
SR-ASV	Hamster	+	+	+
SR-ASV	Hamster revertant	–	–	–
—	Vole	–	–	–
SR-ASV	Vole	+	+	+
Pr-ASV	Chick	+	–	–
B77-ASV	Chick	+	–	–
BH-ASV (RAV-50)	Chick	+	–	–

Uninfected cell cultures or cultures infected with various strains of ASV were either radiolabeled with [^{35}S] methionine (for detection of $p60^{src}$) or left unlabeled (for detection of protein kinase activity). Cells were lysed and each extract (1800–3600 μg of protein) was immunoprecipitated with TBR serum and a portion of the bacteria-bound immune complexes was incubated in the protein kinase reaction mixture.
The reaction mixtures (25 μl) contained 20 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, and 0.4–1.2 μM [γ-^{32}P] ATP (1000 Ci/mMol). After termination of the reaction by heating to 95° for 1 min. in SDS-containing buffer and pelleting of the bacteria, the supernatant was subjected to electrophoresis in a discontinuous SDS/polyacrylamide slab gel [9]. Autoradiography was used to detect the presence (+) or absence (–) of ^{35}S-methionine-labeled $p60^{src}$ and phosphorylated IgG.

The immunoprecipitation of $p60^{src}$ by the rabbit antiserum used in these experiments is strain-specific. Antibody produced in rabbits bearing tumors produced by Schmidt-Ruppin (SR) ASV does not immunoprecipitate a transformation-specific polypeptide from chick cells transformed by other strains of ASV. This fact permitted us to test the specificity of, and association of, the protein phosphorylating activity with the presence of $p60^{src}$ in a variety of circumstances. The results of these experiments are summarized in Table 2. These results show that protein kinase activity is immunoprecipitated from cell extracts only when $p60^{src}$ is also immunoprecipitated. The activity is not found in untransformed cells or in cells infected with strains of ASV which lack a *src* gene and which therefore do not cause cellular transformation. Furthermore, no activity is found in immunoprecipitates of extracts of transformed cells when we are unable to immunoprecipitate $p60^{src}$ because of the specificity of the TBR serum. However, when antiserum from tumor-bearing marmosets [10], which crossreacts with the *src* gene product encoded by other strains of ASV (unpublished data), is used in this assay, protein phosphorylation is found in the precipitates (Table 3). No enzymatic activity is ever observed in complexes formed with normal serum [4].

A further indication that the protein kinase activity is the result of expression of the ASV *src* gene is shown by the observation that the expression of the phosphorylation activity observed is thermosensitive in chick cells infected with a temperature-sensitive *(ts)* mutant in the *src* gene. Parallel cultures of chick cells infected with nondefective (nd) SR-ASV and the SR-ASV

Virus	$p60^{src}$	Protein kinase activity
SR	+	+
PrC	+	+
B77	+	+
Bryan	+	+

Table 3. Immunoprecipitation of $p60^{src}$ and protein kinase activity from transformed chick cells by serum from tumor-bearing marmosets

ts mutant NY68 [6] were transformed and then grown for 16 hours at either 35° or 41°. Cell extracts were prepared from each of the four cell cultures, immunoprecipitated with TBR serum, and then analyzed for protein kinase activity. The results show (Table 4) that cells infected with nondefective ASV yielded slightly more (2-fold) phosphorylating activity when grown at 41° compared to those grown at 35°. This is in sharp contrast to NY68-infected cells which show a dramatic decrease in phosphorylation activity when grown at the nonpermissive temperature, 41°.

Table 4. Growth temperature-dependent expression of src protein-immunoprecipitated phosphorylating activity in chick cells infected with a ts transformation mutant of ASV

Virus	Growth temperature, °C	*Phosphorylating activity* ^{32}P incorporated, fmol/mg protein	Normalized values
SR-ASV (nd)	35	16.6	1.00
SR-ASV (nd)	41	35.8	2.16
SR-NY68	35	19.8	1.00
SR-NY68	41	1.7	0.09

Parallel cultures of chick cells infected with nd SR-ASV and cells infected with the ts transformation mutant of SR-ASV, NY68, were maintained at either the permissive (35°) or nonpermissive (41°) temperature. Cell extracts were prepared from the four cultures, samples were taken for determination of protein content, and the remainder was immunoprecipitated with TBR serum as described in Table 2. Phosphorylating activity by the bacteria-bound immunoprecipitated complexes was also determined. The resulting activity values, determined by quantitation of the phosphorylated IgG bands from a polyacrylamide gel, are normalized with respect to the amount of cell extract protein used for immunoprecipitation and the activity present in the respective cells grown at 35°.

III. In Vitro Synthesis of $p60^{src}$ that Functions in the Phosphotransferase Reaction

Radiolabeled $p60^{src}$has been synthesized in cell-free extracts programmed by the 3′ third of viral RNA, the region of the genome which contains the *src* gene, but not by similar RNA from a mutant which has a deletion in the *src* gene [12, 13]. Because the in vitro and in vivo products of the *src* gene were structurally similar [13] we also tested the in vitro translation product for phosphotransferase activity. The 70S RNA from nondefective and from

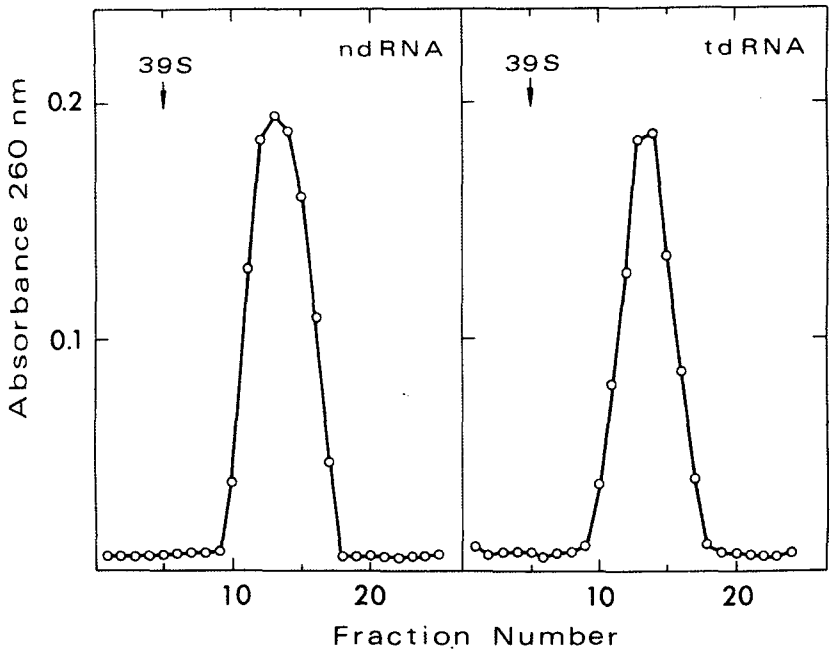

Fig. 1. Sedimentation profile of nd and td poly A-containing virion RNA selected fro translation. RNA selected from a previous sucrose gradient was precipitated with ethanol. dissolved in EDTA 1 mM. Tris-HCl 0.01 M. pH 7.2. heated at 80°C for 2 min. quick chilled and sedimented for 105 min through a 20 to 5% (W/V) sucrose gradient containing 0.1 M NaCl at 10°C and 45 000 rpm in a Beckman SW 50.1 rotor. The RNA in fraction numbers 12–15 was ethanol-precipitated and used for cell-free translation as described in the legend to Fig. 2

transformation-defective (td) virions was purified, heat denaturated and the poly A-containing RNA was purified by oligo-dT cellulose chromatography. The 3′ third of the genome was selected from this RNA by sucrose gradient sedimentation. This RNA was resedimented (Fig. 1) and the RNA in fraction numbers 12–15 was used to program messenger RNA-dependent reticulocyte lysates as described in the legend to Fig. 2. The ^{35}S-methionine-labeled polypeptide translation products analyzed directly by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis show that p60^{src} is translated only from nd RNA (Fig. 2, tracks 1–3). No p60^{src} is translated from td RNA although this RNA stimulated protein synthesis as well as did the nd RNA, both about 10-fold above background. We have not studied the nature of the polypeptides synthesized in response to td RNA, except that one migrates with Pr76, the product of the ASV *gag* gene.

TBR serum was used to immunoprecipitate the in vitro translation products by the same procedures used to precipitate p60^{src}-associated protein kinase from transformed cell extracts (Table 1 and reference [4]). The radiolabeled polypeptides found in immunoprecipitates are displayed in Fig. 2, tracks 4–7. TBR serum precipitates p60^{src} and a small amount of Pr76 from the products synthesized in response to nd RNA (track 5). A small amount of Pr76 is found in the immunoprecipitate of the products synthesized in response to nd RNA (track 5) and to td RNA (track 7). This indicates that radiolabeled virus-specific polypeptides synthesized in cell-free extracts are antigenically similar to those found in transformed cells [2,3].

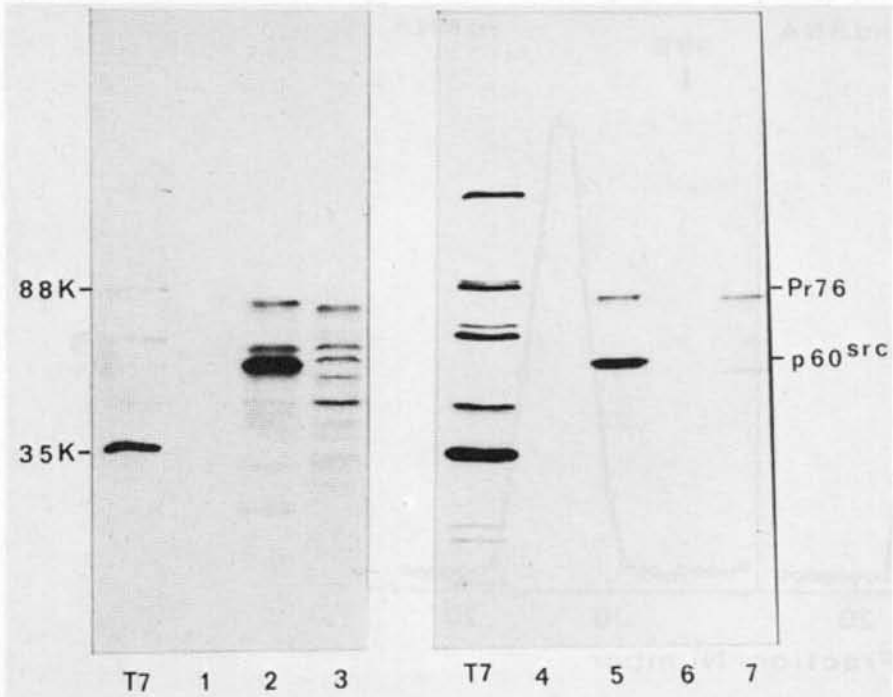

Fig. 2. Fluorogram of SDS-polyacrylamide gel electrophoresis analysis of ^{35}S-methionine-labeled polypeptides synthesized in cell-free extracts programmed by the RNA shown in Fig. 1. Messenger-dependent reticulocyte lysates were prepared as described by Pelham and Jackson [11] and the conditions of translation were as described [13] except that the final concentrations were 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM Mg acetate, 100 μM unlabeled methionine, and 0.5 μCi μl^{-1} ^{35}S-methionine (700 Ci mM^{-1}, New England Nuclear). Three picomoles of nd RNA was added to each of two 300-μl reaction-mixtures, and 3 picomoles of td RNA was added to one 300 μl reaction-mixture, and the reaction-mixtures were incubated at 30°C for 40 min. At this time samples were taken for the determination of incorporation, for polyacrylamide gel analysis, and one-half of each reaction was used for determination of enzymatic activity. Stimulation of 0.3 N KOH (15 min at 37°C)-resistant and 10% TCA-precipitable radioactivity was 10-fold above background for the lysate programmed with nd RNA and 9-fold for tha programmed with td RNA. The direct analysis of these translation products is shown in tracks 1, no RNA, 2, nd RNA and 3, td RNA. Immunoprecipitation of the translation products was carried out with normal rabbit serum or TBR serum and the immunoprecipiated polypeptides are shown in tracks 4, nd RNA, normal serum, 5, nd RNA, TBR serum, 6, td RNA, normal serum, and 7, td RNA, TBR serum. Immunoprecipitations were carried out as previously described [2] using the procedure of Kessler [7]. Prior to electrophoresis, samples were boiled in sample buffer (0.07 M Tris-HCl, pH 6.8, 11.2% glycerol, 3% SDS, 0.002% bromophenol blue, 5% β-mercaptoethanol). T_7 virion proteins are included as molecular weight markers

A portion of the immune complexes formed with the translation products shown in Fig. 2 was also resuspended directly in kinase reaction buffer, and the transfer of phosphate from γ-labeled ATP to the heavy chain of IgG was analyzed by SDS-polyacrylamide gel electrophoresis of the reaction products. As seen in Fig. 3, only the products of translation of nd RNA immunoprecipitated with TBR serum yielded phosphorylated IgG (Fig. 3, track 4). Translation of the same amount of td RNA resulted in no detectable enzymatic activity with immune serum (Fig. 3, track 3). These results, as well as those given in Tables 2 and 3 and those previously published, indicate directly that only immune complexes which contain p60^{src} display phosphotransferaste activity.

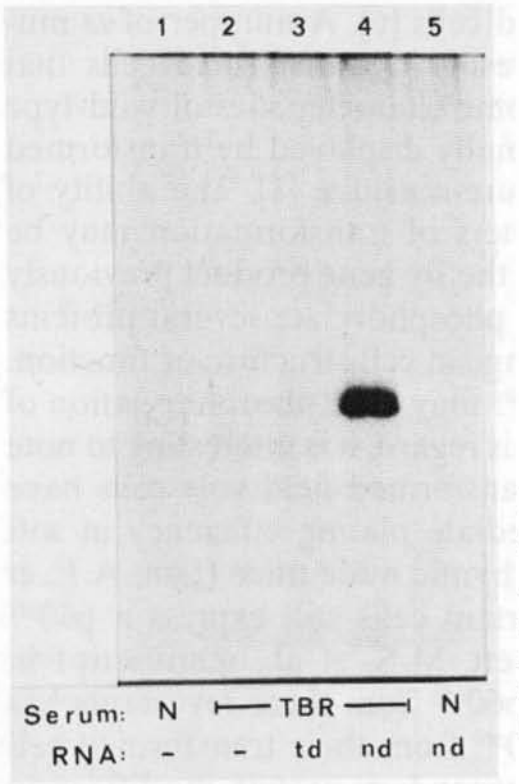

Fig. 3. Autoradiogram of SDS-polyacrylamide gel analysis of phosphorylated proteins. A portion of the bacteria-bound immune complexes obtained after protein synthesis and immunoprecipitation was resuspended directly in 25 µl of protein kinase reaction-mixture (Table 2). The phosphorylation of IgG was determined by SDS-polyacrylamide gel electrophoresis as described (Table 2, reference [4]).

C. Discussion

The results that we have presented in this communication and elsewhere demonstrate that a product of the ASV *src* gene is a phosphoprotein of molecular weight 60000 and that this protein appears to have protein kinase activity. Biosynthetic radiolabeling enables detection of only one transformation-specific polypeptide, $p60^{src}$, in the avian and mammalian cells transformed by ASV in which we have observed immunoprecipitable protein kinase activity. However, the possibility does exist that a cellular kinase specifically associates with $p60^{src}$ and is responsible for the observed enzymactic activity. This seems unlikely since such a cellular kinase would have to be present in both avian and mammalian cells as well as in reticulocyte cell-free extracts. Furthermore, additional experiments (M.S. Collett, unpublished data) support the association of the protein kinase activity with $p60^{src}$. The enzymatic activity and $p60^{src}$ cosediment during glycerol gradient centrifugation and coelute from ion exchange columns and immunoaffinity columns. The purification of the enzyme from cells infected with nondefective or *ts* virus will more clearly resolve these questions.

The fact that expression of protein phosphorylating activity is thermosensitive in *ts* mutant-infected cells suggests that the $p60^{src}$-associated protein kinase is directly involved in the transformation process and that ASV may transform cells by aberrant phosphorylation of cellular proteins.

In view of the well-documented role of protein phosphorylation in the regulation of cellular processes [14], this activity alone may be sufficient to initiate and maintain neoplastic transformation. Such a function of the transforming gene product would serve to explain many of the observations made

concerning the behavior of ASV-transformed cells [6]. A number of *ts* mutants of ASV have been described which result in transformed cells that display at the nonpermissive temperature some characteristics of wild-type transformed cells while other functions normally displayed by transformed cells are absent and thus are still temperature-sensitive [1]. The ability of these mutants to dissociate certain parameters of transformation may be related to the pleiotropic cellular response to the *src* gene product previously described [6]. The $p60^{src}$ protein kinase may phosphorylate several proteins and each in turn may produce a unique change in cell structure or function. Mutations occurring at different sites in $p60^{src}$ may alter phosphorylation of one cellular target but not that of others. In this regard, it is interesting to note that morphological revertants of SR-ASV transformed field vole cells have been isolated [8] which still exhibit intermediate plating efficiency in soft agar and the ability to produce tumors in athymic nude mice (Lau, A. F. et al.. manuscript in preparation). These revertant cells still express a $p60^{src}$ protein which is enzymatically active (Collett, M. S. et al., manuscript in preparation). However, peptide analysis of $p60^{src}$ from these revertants has revealed a slight change, relative to the $p60^{src}$ from their transformed cell counterparts, in either primary structure or secondary protein modification of a single peptide (Collett, M. S. et al., manuscript in preparation). Such an alteration in $p60^{src}$ may reduce its ability to phosphorylate a particular cellular target protein, consequently producing a partial reversion of the transformed phenotype. Alternatively, a mutation in one of the cellular target proteins may render it resistant to $p60^{src}$-mediated phosphorylation. Direct identification of the proteins in normal cells phosphorylated by $p60^{src}$ will serve to clarify these issues. Moreover, possible functions for the product of the ASV transforming gene other than protein kinase should also be investigated.

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The in vitro Translation of Rous Sarcoma Virus RNA and Function of the Viral Protein During the Viral Replication

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Abstract

The *gag* gene and *pol* gene of the Rous sarcoma virus are translated *in vitro* from the 35S viral RNA. The *env* gene cannot be translated *in vitro* from the 35S RNA. For the *in vitro* translation of the *src* gene. 3' end fragments of the viral RNA are used.

The *gag* protein p15 has a proteolytic activity and specifically processes its own protein precursor pr76. The *gag* protein p19 suppresses the *in vitro* translation of the *pol* gene.

The replication of RNA tumor viruses was thought to be strictly dependent on their host cell activities. There is recent evidence, however, that some parts of the replication of avian RNA tumor viruses are independent of their host functions; they are controlled by a feedback mechanism of their viral gene products.

Which viral proteins are involved and what are their functions?

In order to determine which viral proteins have functions during the replication cycle, a cell-free system was used to synthesize the viral proteins; a) to prove that those proteins are in fact coded by the viral genome, b) to work in a system which is free of host cell contamination.

In a cell free system from mouse ascites Krebs II cells the 35S RNA of Rous sarcoma virus (RSV) has been translated into a precursor of the viral group specific antigen *(gag)* protein, pr76 [1], and a precursor of the reverse transcriptase *(pol)*, pr180 [2]. Both protein precursors are not processed when synthesized in vitro, even after long incubation periods. Upon addition of *gag* protein p15 however (which is purified in 6,0 M GuHCl on an agarose column) the precursor pr76 is cleaved within 30 min into the *gag* proteins p27, p15, p12 and pr32 which is a protein precursor to p19 (Fig. 1) [3].

This cleavage is very similar to that observed in the RSV infected cells. Fig. 2 shows that the *pol* precursor pr180 is cleaved as well by the *gag* protein p15 [2].

These results indicate a new way of how precursors can be processed: By a specific cleaving activity which is contained in one of their cleaved end products. Since our results were obtained in vitro, a question arises:

Is this in vitro cleavage an artefact or does p15 play a physiological role in the intracellular processing?

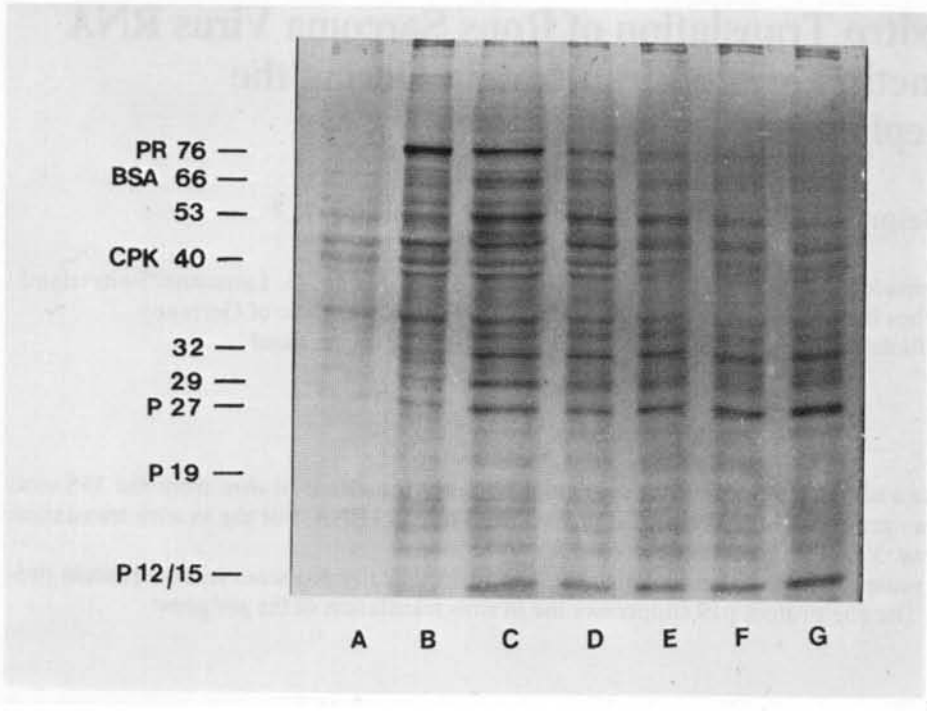

Fig. 1. In vitro processing of pr76 by p15. Autoradiograph of SDS-gel electrophoresis of ^{35}S-methionine labeled polypeptides synthesized in a cell free system directed by 35S RSV RNA (1.5 μg/20 μl). (A) no RNA added. (B) plus RNA. synthesis for 60′ (C–G) further incubation with unlabeled methionine in presence of p15 for (C) 5 min. (D) 10 min. (E) 15 min. (F) 30 min. (G) 4-fold amount of p15 was added and incubated for 10 min

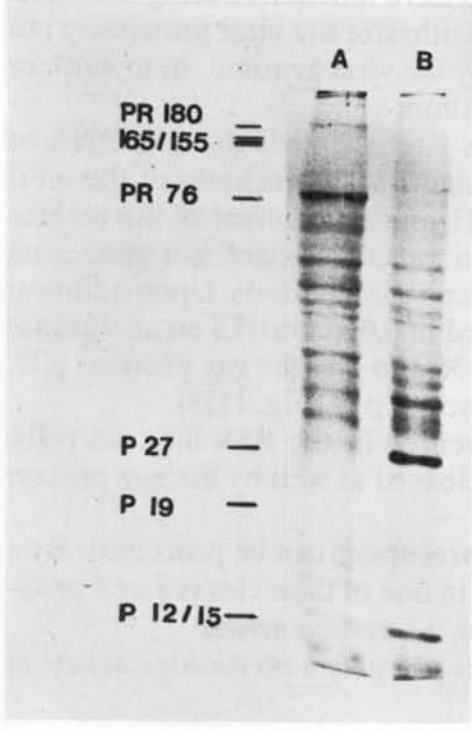

Fig. 2. Autoradiograph of in vitro products as in Fig. 1 (A) incubation for 90 min (B) further incubation for 30 min with unlabeled methionine in presence of p15

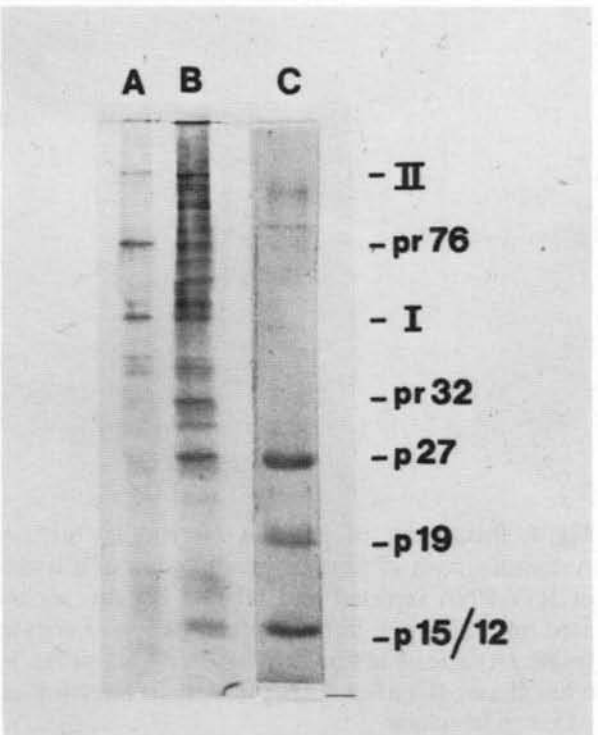

Fig. 3. Intracellular processing of pr76 by fusion-injection of p15. – Autoradiograph of immunoprecipitation of ^{35}S-methionine labeled hamster cell lysate separated on SDS-gel. (A) Mock injected cells (B) p15 injected cells (C) viral markers

The answer is given by the following two experiments:

a) Purified protein p15 was injected into a RSV transformed hamster [4] cell line which is not permissive for RSV replication but contains small amounts of uncleaved *gag* precursor pr76. The injection was carried out by the fusion-injection technique [5] in which p15 is loaded into erythrocytes by a minor osmotic shock and those loaded erythrocytes are then fused to ^{35}S-methionine labeled hamster cells [6]. After two hours of incubation the cells had been lysed and anti-*gag* serum added. Processed *gag* proteins had been precipitated from the p15 injected cell lysate (not from the mock-injected control cells (Fig. 3) which shows that pr76 has in fact been intracellulary processed by the protein p15 [6].

b) A further experiment in oocytes confirms the intracellular cleavage by p15. The *gag* precursor pr76 can be synthesized in frog oocytes upon injection of 35S RSV RNA. The precursor is consecutively very slowly processed within the oocytes and the cleavage is completed after 72 hrs [7]. – If p15 is involved in the cleavage, the reaction time should be significantly decreased by injecting p15 in the pr76 containing oocytes: In our experiment oocytes had been injected with RSV RNA; precursor pr76 was synthesized during a 4 hrs pulse with ^{35}S-methionine after injection (Fig. 4A), the 4 hrs pulse was succeeded by a 6 hrs chase: almost no cleavage was detectable; the pulse was succeeded by injection of p15, and a 6 hrs chase: most of the precursor pr76 is cleaved into the *gag* proteins (Fig. 4C) as it

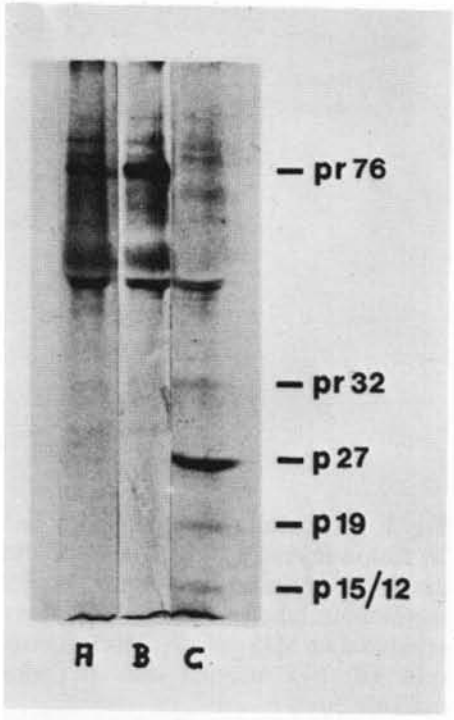

Fig. 4. Processing of pr76 in oocytes by p15. – Autoradiograph of immunoprecipitation of a lysate of RSV RNA injected and labeled oocytes separated on a SDS-gel. Immunoprecipitation of oocyte lysate (A) after 4 hrs pulse, (B) after 4 hrs pulse + 6 hrs chase. (C) after 4 hrs pulse, then injection of p15 + 6 hrs chase

had been found by immunoprecipitation with anti *gag* serum (von der Helm, K., and Rungger, D., manuscript in prep.).

Conclusion: RSV *gag* protein p15 is involved in both in vitro and in vivo processing of the *gag* precursor protein pr76. The processing of pr76 is essential for the assembly and thus for the replication of the virus. –

Furtheron we studied viral proteins other than p15 for possible involvement in the viral replication and made an interesting observation: the *gag* proteins p12 and p19 are known to bind specifically to the viral RNA [8,9]. When p12 is added to a cell free system, the translation of the RSV RNA is almost completely suppressed. When p19 is added to such an in vitro system, the translation of only the *pol* gene is inhibited, i.e. the synthesis of pr180 is specifically suppressed while the pr76 precursor is synthesized (Fig. 5).

In infected cells about 10 times less pr180 precursor than pr76 precursor is found. We suggest that the *gag*-protein p19 controls the synthesis of the pr180 precursor during viral replication. –

The *gag* and *pol* gene, and not the *env* nor *src* gene can be translated in vitro from the 35S RSV RNA. However, RNA fragments of the 3′ end of the viral RNA, separated into two size classes of about 15–20S and 20–22S, both containing the *src* gene, could be translated in vitro [10,11,13].

The translation products of the 15–20S RNA are polypeptides of 25, 35 and 43K dalton and the translation product of the 20–22S RNA is predominantly a polypeptide of 60K dalton. Rabbit antiserum against the 60K polypeptide [12] (a kind gift of R. Erikson) precipitates the 60K polypeptide in vitro made from the 20–22S RNA (Fig. 6B) [13] and from the 15–20S RNA predominantly the 25K and to a lesser amount the 35K and 43K polypeptides (Fig. 6C). The 3′ end RNA fragments from a transformation defective (td)

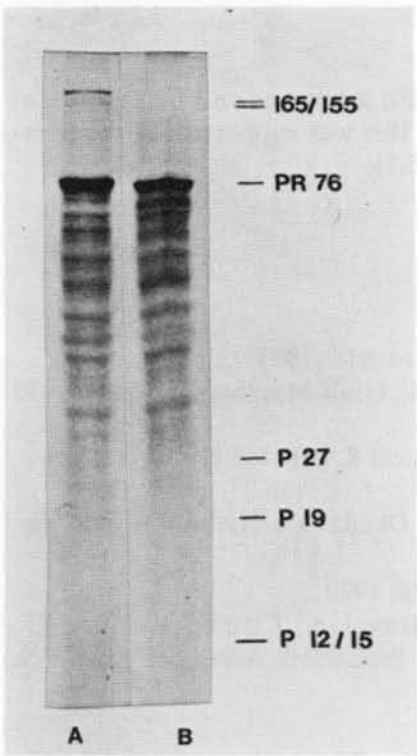

Fig. 5. Autoradiograph of in vitro products as in Fig. 1. In vitro translation of 35S RSV RNA in absence (A) and presence (B) of protein p19

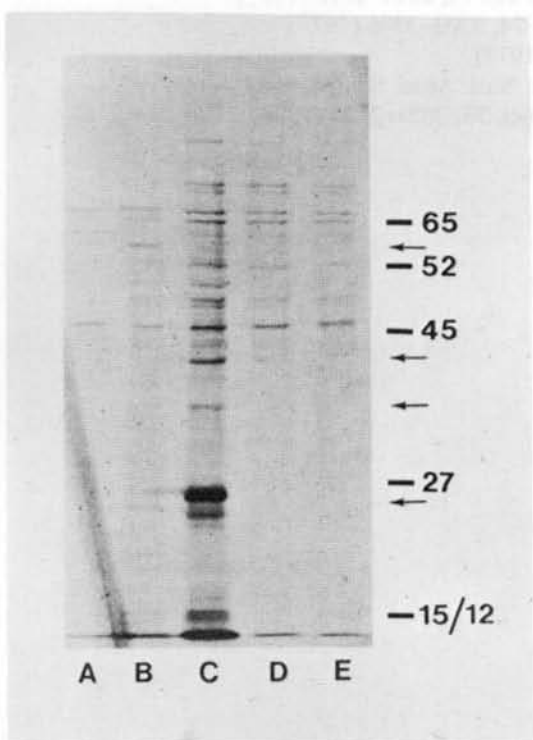

Fig. 6. Autoradiograph of immunoprecipitation of in vitro translation of 3′-end src-containing fragments of RSV-RNA: (B) 24–20S (C) 20–15S, and (td) RSV RNA: (D) 24–20S (E) 20–12S. (A) endogenous background of in vitro system. All samples are immunoprecipitated with antiserum against src product

mutant of RSV which has a deletion in src, were not translated in vitro (Fig. 6D and E) implying that the in vitro made polypeptides are in fact *src* gene products. The functions of the smaller polypeptides are not known. It is implied that the *src* gene product plays a role during the transformation of cells. Collett and Erikson found that the 60K polypeptide synthesized in vitro as well as in vivo have a protein kinase activity [14].

Acknowledgements

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Regulation of Translation of Eukaryotic Virus mRNAs

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A. Introduction

One of the best studied examples of the regulation of translation is the inhibition of host cell protein synthesis following viral infection (Bablanian, 1975; Smith and Carrasco, 1978). In several virus/host systems the bulk of viral proteins are synthesized under conditions in which the initiation of translation of cellular mRNAs is strongly inhibited.

We have raised the possibility that changes in the concentration of ions in the cellular cytoplasm following viral infection could specifically block cellular protein synthesis. We showed for picornavirus infected cells that the cellular membrane of the host becomes leaky at the time viral protein synthesis begins (Farham and Epstein, 1963; Carrasco, 1978). Moreover, optimal translation of picornavirus RNA in vitro occurs under ionic conditions in which the synthesis of cellular proteins is strongly restricted (Carrasco and Smith, 1976). In the present work, we show that the higher optimum of monovalent ions required for in vitro translation is also observed with mRNAs from papovaviruses, togaviruses, rhabdoviruses and myxoviruses. These observations are discussed in the general context of viral development, the shut-off of host protein synthesis and as a possible explanation for the differential inhibition of viral and cellular mRNA translation following exposure of cells to hypertonic medium (see Koch et al. in this volume).

B. Results

1. Papovaviruses

The in vitro translation of the mRNAs present in the cytoplasm of polyoma infected 3T6 cells is illustrated in Fig. 1. The monovalent ion optimum for the synthesis of actin (a cellular protein) and VP1 (a viral protein) differ by 30 mM K^+, indicating that the polyoma 16S mRNA is more efficiently translated in vitro at higher potassium concentrations. This result was obtained using either the chloride or acetate salt of potassium. These results indicate: a) that in vitro changes in the concentration of monovalent ions do not produce an indiscriminate inhibition on the translation of all mRNAs; ions have to be regarded as specific effectors of protein synthesis, because they inhibit the translation of some mRNAs and at the same time stimulate translation of

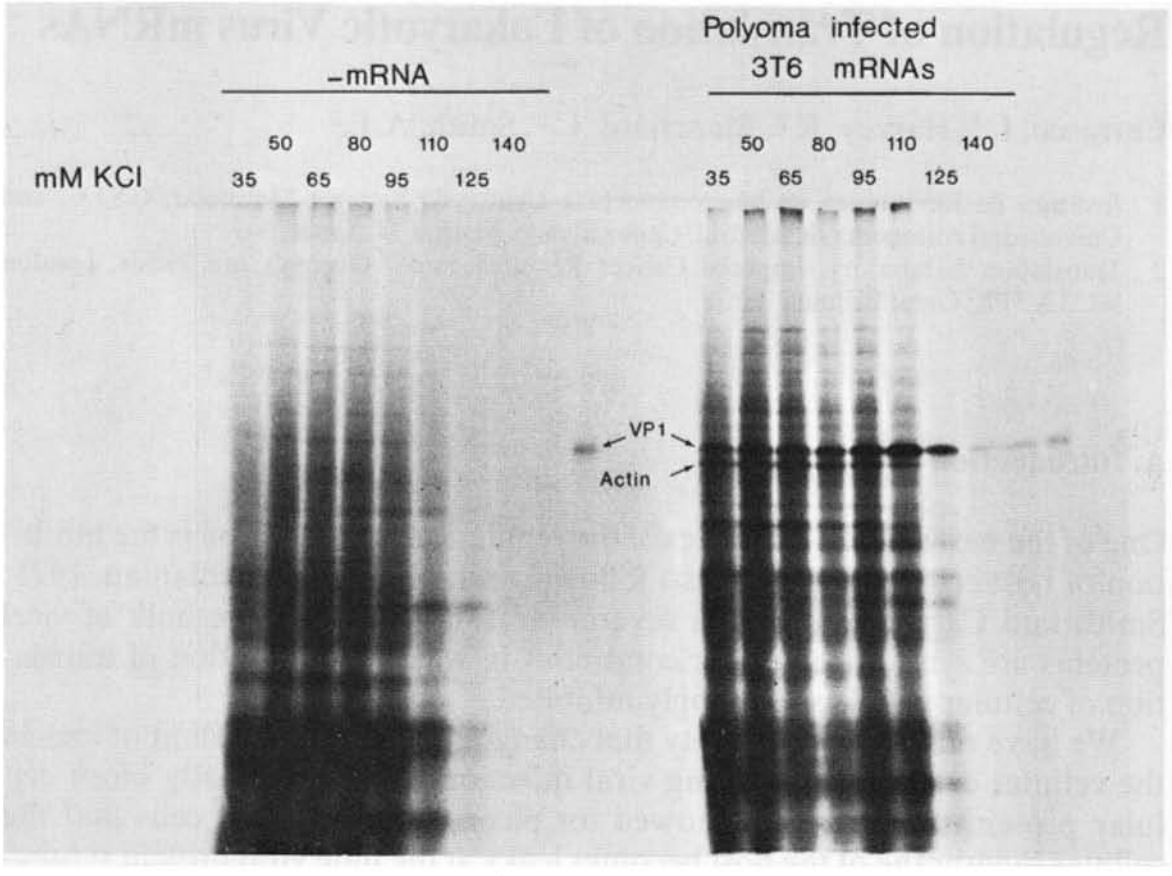

Fig. 1. Analysis by polyacrylamide gel electrophoresis of the products synthesized in the wheat germ system in response to mRNAs from 3T6 cells infected with polyoma virus. Effect of the KCl concentration

others and b) that from the relative synthesis of two proteins in vitro we cannot deduce directly the actual proportion of their mRNAs in the cell-free system.

It has been suggested that the difference in monovalent cation requirement between picornavirus and host cell mRNAs reflects the difference in the size of the mRNAs. If this hypothesis were correct, it would follow that those mRNAs requiring a high concentration of monovalent cations for optimal translation are large and code for high molecular weight proteins. The present results do not support this conclusion, because the synthesis of VP1 has a higher monovalent ion requirement as compared to the synthesis of actin, yet both proteins have a similar molecular weight and similar sized mRNAs (see also the results obtained in section B 2).

In vitro changes in the concentration of divalent cations (magnesium or calcium) did not produce a differential effect on the synthesis of VP1 and actin. This finding indicates to us that the changes of monovalent ions are more likely to be involved in the shut-off of host protein synthesis than divalent cations.

2. Togaviruses

Semliki Forest virus has a 42S genomic RNA which is translated early during infection to produce non structural proteins. Later, coat proteins are synthes-

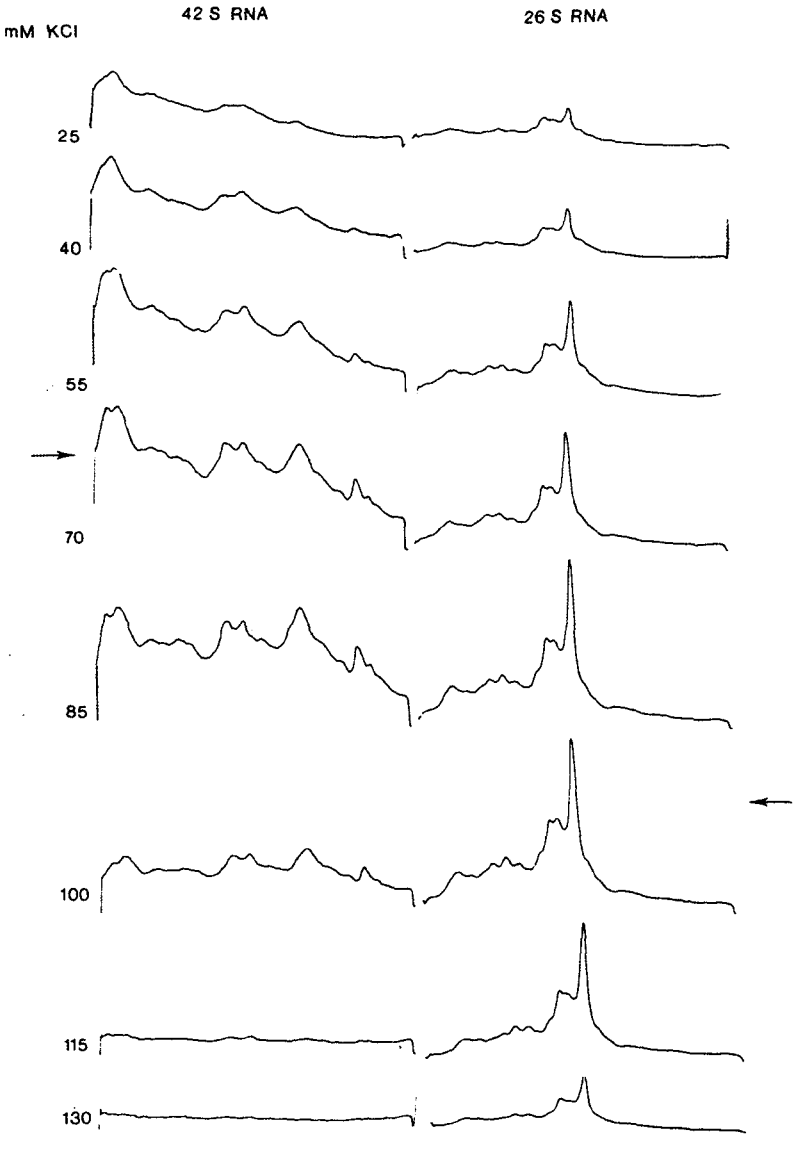

Fig. 2. Effect of the KCl concentration on the in vitro translation of Semliki Forest Virus 42S and 26S mRNAs. The arrows in the scans indicate the optimum for translation

ized from the subgenomic 26S mRNA, and at the same time, the synthesis of host and viral non structural proteins are inhibited in parallel (Lachmi and Kaariainen, 1977). This system provided us with a good model to test our hypothesis. Since the viral 26S mRNA is translated when shut-off occurs, the model predicts it should have a high optimum of monovalent ions for translation. On the other hand the viral 42S mRNA which is translated before host protein synthesis is inhibited should have a lower optimum of monovalent ion concentration for in vitro translation similar to that of host cell mRNA. Fig. 2 shows that indeed this is the case. Under the ionic conditions in which maximal synthesis of coat proteins occurs the translation of the genomic 42S mRNA is severely inhibited.

3. Rhabdoviruses and Myxoviruses

Vesicular stomatitis virus (VSV), a rhabdovirus, is probably the best studied species in class V viruses (Smith and Carrasco, 1978). It produces a drastic inhibition of cellular protein synthesis after infection (Wertz and Youngner, 1972). A similar inhibitory effect on translation is observed when susceptible cells are infected by influenza virus, a myxovirus. The in vitro translation of the mRNAs from these two viruses is shown in Fig. 3. Again, the KCl optimum for the in vitro translation of influenza and VSV mRNAs is higher than the optimum required to translate cellular mRNAs.

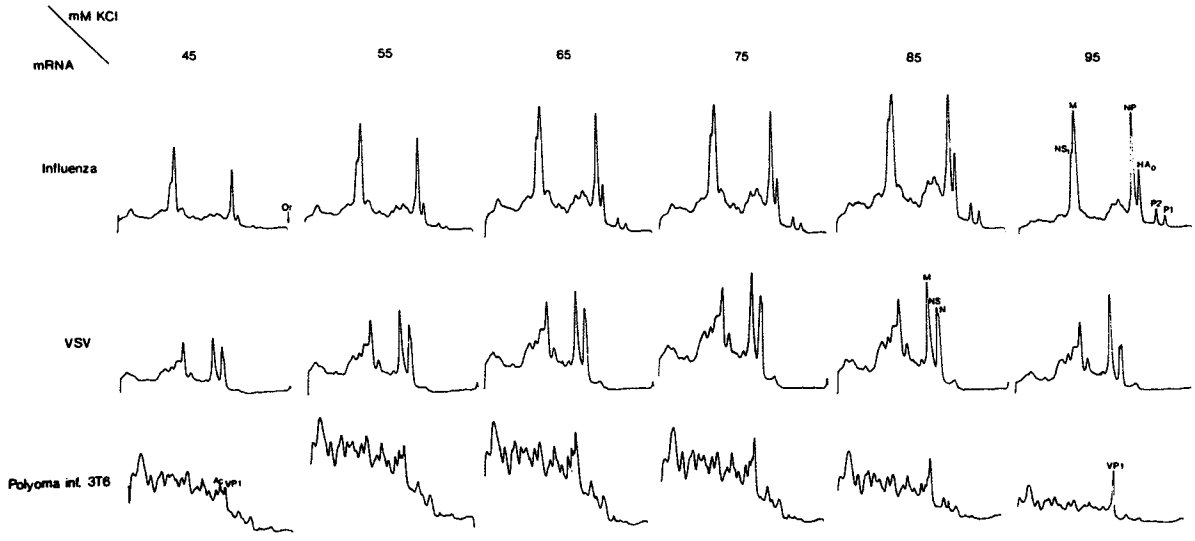

Fig. 3. Effect of the KCl concentration on the in vitro translation of VSV and influenza mRNAs. The products synthesized in the wheat germ system were analysed by PGE and the autoradiogram was analysed in a densitometer

C. Discussion

Several theories have been advanced to explain the regulation of translation in eukaryotic organisms (Lodish, 1976). Nowadays the existence of initiation factors specific for different mRNAs is considered unlikely. Recently evidence has accumulated which indicates that the phosphorylation of initiation factors and ribosomal proteins occurs in vitro. However, as yet there is little direct evidence to show that these modifications are responsible for the in vivo regulation of translation, observed under different physiological conditions.

It is well known that ions are involved in the regulation of a great many biological processes and metabolic reactions. The results shown in this work indicate that changes in monovalent ions in vitro are able to produce effects that mimic those observed in vivo after viral infection. In addition, we also know that the membrane of many viral infected cells becomes leaky to ions and small metabolites at the time as the bulk of viral proteins are synthetized (Carrasco, 1978; Contreras and Carrasco, manuscript in preparation). These

results also suggest that the effects of hypertonic medium in some virus infected cells can be explained in a very simple way: hypertonic medium produces a higher concentration of monovalent ions in the cellular cytoplasm and thus favours the translation of some viral mRNAs, and inhibits the translation of other cellular mRNAs. Whether changes in salt concentration are directly involved in shut-off, or whether other effects such as competition between mRNAs or distruction of specific initiation factors play a role, remains to be established.

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Control of Eukaryotic Protein Synthesis by Phosphorylation

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Phosphorylated proteins as physiological effectors may have a much broader role than previously assumed. Many specific regulatory signals may activate one or more protein kinase that phosphorylate and thereby modulate the activity of key enzymes in various pathways. The sarcoma gene of avian sarcoma virus appears to code for a protein kinase that may disrupt normal differentiation to cause transformation in vitro and sarcoma induction in vivo [1]. Examples of other kinase systems are considered in a recent review article [2]. Some hormones and neurotransmitter substances are known to act through cAMP-dependent protein kinases to regulate carbohydrate and lipid metabolism as well as the characteristics of the cell's surface. However, recent work has focused on cAMP-independent protein kinases. Regulation of virus assembly provides a clear example of a cAMP-independent kinase system. Phosphorylation of viral core protein, p12, changes its ability to bind to viral RNA in either Rauscher leukemia virus [3] or simian sarcoma-associated virus [4]. Translational regulation of protein synthesis provides another example of a pathway that is regulated by cAMP-independent protein kinases. Here we will consider regulation of protein synthesis by cAMP-independent protein kinases with emphasis on examples in which a correlation between phosphorylation and inhibition of peptide initiation can be demonstrated.

Eukaryotic Peptide Initiation

Regulation of eukaryotic protein synthesis is known to occur at steps of peptide initiation. This multi-step process is schematically outlined in Fig. 1. Though the general scheme is widely accepted, the actual function of most of the initiation factors involved is not yet understood nor is the exact point of GTP hydrolysis firmly established [cf. 6]. Most of the initiation factors consist of one peptide chain ranging in molecular weight from 15000 to 150000 [7]. Two initiation factors have a more complex structure; eIF-3 appears to be a high molecular weight complex consisting of 9–11 proteins [7,8] and eIF-2 contains 3 subunits with molecular weights of 34000, 48000 and 52000 daltons [7,9].

Phosphorylation has been reported for several eukaryotic initiation factors [10,11] and for proteins of the small as well as the large ribosomal subunit [12,13], but a correlation to or apparent change in function or activity has to

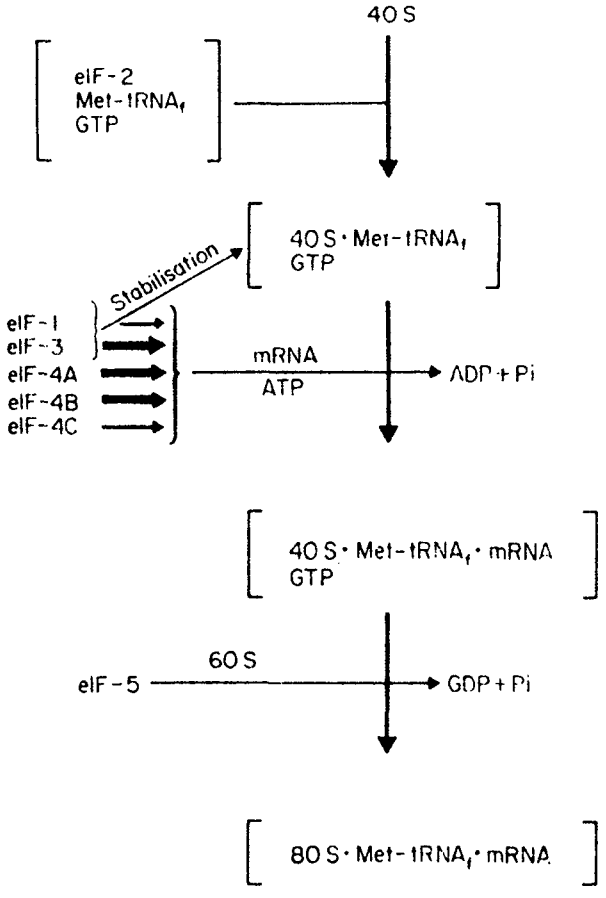

Fig. 1. Assembly scheme of the initiation complex. The relative importance of factors for mRNA binding is indicated by the thickness of the arrows. This figure is taken from ref. [5]

be demonstrated yet in these cases. However, phosphorylation of the smallest subunit of the initiation factor eIF-2 has been shown to inhibit protein synthesis as will be described in detail in the next paragraphs.

Control of Protein Synthesis in Reticulocytes by Phosphorylation

As described in a previous contribution to this series [14], protein synthesis in reticulocytes is regulated by the availability of hemin. Incorporation of ^{14}C leucine into protein in a cell-free reticulocyte lysate system will stop after a few minutes of incubation unless hemin is added to the reaction mixture. Inhibition of protein synthesis in the absence of hemin appears to be achieved by preventing the formation of a stable 40S ribosomal subunit · Met-tRNA$_f$ initiation complex [15, 16]. An inhibitory protein(s) may be isolated from the reticulocyte postribosomal supernatant incubated for several hours in the absence of hemin [17] or briefly in the presence of N-ethylmaleimide [18, 19]. This protein (complex) has been called the hemin-controlled repressor (HCR; ref. [15, 17]).

Preparations of partially to highly purified HCR have been shown to contain protein kinase activities for the smallest subunit of the initiation factor eIF-2 [20–22] and for proteins of 40S ribosomal subunits [21]. The actual mechanism by which phosphorylation and inhibition is interlocked is an area of intensive studies at present. Also, the mechanism by which the protein kinase(s) are activated in the absence of hemin is not understood yet. Papers

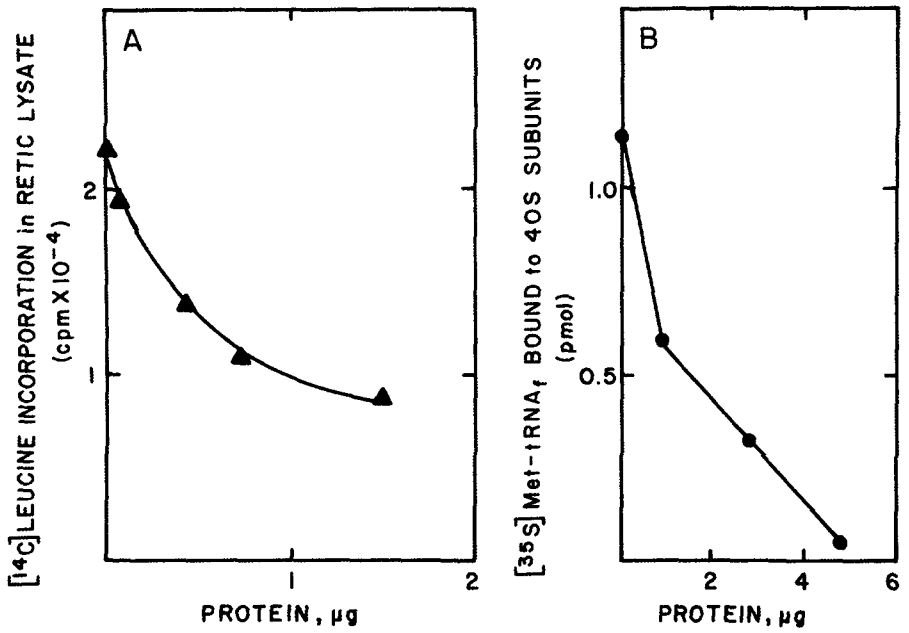

Fig. 2. Inhibition of protein synthesis in reticulocyte lysate and inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits by an HCR preparation.
A Protein Synthesis in rabbit reticulocyte lysates was carried out as described [25]. A 100-µl reaction mixture contained 20 µl lysate; ^{14}C leucine was used at a specific radioactivity of 40 Ci/mole. HCR was added in the amounts shown.
B Binding of ^{35}S Met-tRNA$_f$ (2 Ci/mmol) to reticulocyte 40S ribosomal subunits was performed as described previously [26]. The initiation factor eIF-2 (2.1 µg of protein) was preincubated with an HCR preparation in the amounts indicated and 0,5 mM ATP, then hexokinase and glucose was added for a second incubation. Finally, Met-tRNA$_f$ binding to 40S subunits was determined as detailed [26]

from S. Ochoa's laboratory claim the involvement of a cAMP-dependent protein kinase in the activation process (ref. [23] and earlier papers cited therein); Evidence is accumulating that cAMP is not involved in the activation of HCR [19, 19a].

Binding of Met-tRNA$_f$ to 40S ribosomal subunits that is dependent on eIF-2 and GTP can be measured directly in a partial reaction of peptide initiation [24]. We demonstrated that an HCR preparation will inhibit this reaction using reticulocyte 40S ribosomal subunits [25]. Furthermore, preincubation of either eIF-2 or reticulocyte 40S ribosomal subunits with an HCR preparation and ATP, followed by degradation of residual ATP, will inactivate these components for the subsequent Met-tRNA$_f$ binding reaction [26]. This reaction has to be carried out with a GTP-analog, GMP-P (CH_2)P, to prevent further phosphorylation. An experiment of this type is shown in Fig. 2B. HCR (protein kinase)-dependent phosphorylation of eIF-2 resulting in inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits (Fig. 2B) is compared with inhibition of ^{14}C leucine incorporation in a cell-free reticulocyte lysate system by the same preparation of HCR (Fig. 2A). Whether phosphorylated eIF-2 is impaired for direct interaction with 40S subunits or for interaction with another (initiation) factor [27] has yet to be demonstrated.

Translational Inhibitors from Uninduced and DMSO-Stimulated Friend Leukemia Cells: Comparison to the Reticulocyte HCR

Friend leukemia cells (FLC) are capable of undergoing partial erythroid differentiation by the addition of various organic compounds such as dimethylsulfoxide [28], butyric acid [29] or hexamethylene bisacetamide [30] to the growth medium.

Protein synthesis in uninduced FLC appears not to be regulated by hemin. The data in Fig. 3 indicate that hemin has no effect on the rate of protein synthesis in FLC that do not synthesize hemoglobin. Incorporation of ^{3}H leucine into peptide chains was compared in the absence and presence of hemin. Desferal [31] was used in the control condition to remove trace amounts of free iron ions which may be present in the incubation medium. This compound itself showed no effect on protein synthesis (data not shown). FLC that had been treated with dimethylsulfoxide (DMSO) for 5 days synthesized hemoglobin as judged by their red color and benzidine staining (about 41% positive; viability over 85%). Fig. 3 indicates that protein synthesis in these hemoglobin producing cells is dependent on hemin. In its absence, ^{3}H leucine incorporation over a period of 2 hr is reduced by about 40%.

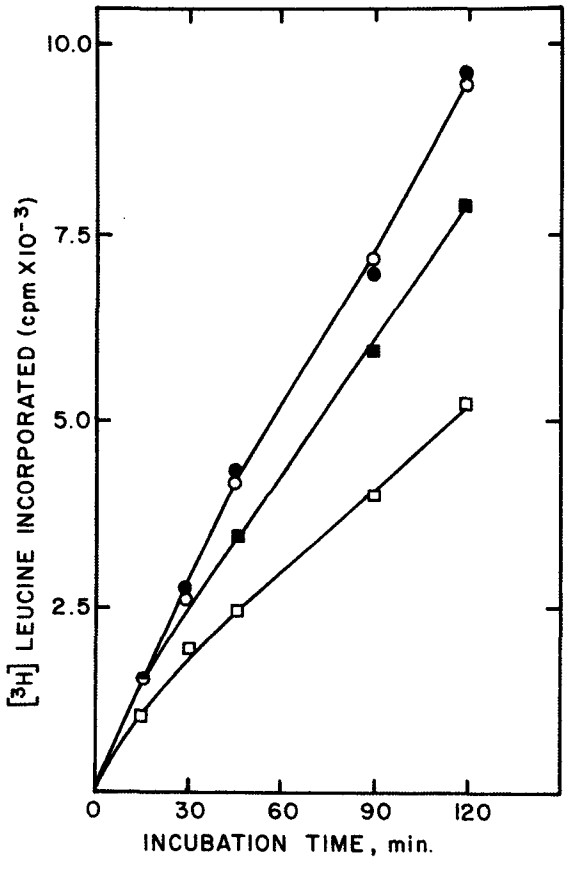

Fig. 3. Effect of hemin on ^{3}H leucine incorporation in vivo in unstimulated or DMSO-stimulated FLC. Protein synthesis was determined by ^{3}H leucine incorporation (30 μM, 8000 Ci/mol) in uninduced FLC or FLC that had been treated for 5 days with 1,5% DMSO (cf. [33]). About 3×10^7 cells in a final volume of 0,55 ml were incubated either with 10 μg hemin or 10 μg desferal (deferoxamine mesylate, Ciba Pharmaceutical Co., Summit, NJ). Aliquots of 20 μl were withdrawn at the indicated times, placed into 2.5 ml ice-cold NKM-solution and filtered through Millipore filters. These were washed with NKM, then with 5% trichloroacetic acid, and its radioactivity determined by liquid scintillation counting.

○——○ = uninduced FLC + desferal
●——● = uninduced FLC + hemin
□——□ = DMSO-stimulated FLC + desferal
■——■ = DMSO-stimulated FLC + hemin

A translational inhibitor has been isolated from uninduced FLC [32]. This inhibitor has functional similarities to HCR but differs in physical properties. Pretreatment of the postribosomal supernatant fraction from FLC was not necessary to detect inhibitory activity. In search for HCR-like inhibitors that

may be present in the inactivate form in the postribosomal supernatant of either uninduced or DMSO-treated FLC, these fractions were incubated with N-ethylmaleimide (NEM), a compound known to activate HCR [18,19]. Untreated or NEM-treated postribosomal fractions from uninduced or DMSO-stimulated FLC were processed in parallel and chromatographed on DEAE cellulose and phosphocellulose to partially purify inhibitory activity. Inhibitory activity was assayed in the reticulocyte cell-free lysate system. One unit of inhibitory activity is defined as the amount of protein that reduces ^{14}C leucine incorporation by 50% in the reticulocyte lysate system used. The results presented in Table 1 demonstrate that the inhibitor fraction from DMSO-induced FLC exhibits a higher specific activity after the postribosomal supernatant of these cells was incubated with NEM suggesting activation of an HCR-like translational inhibitor. An authenic HCR fraction from reticulocytes that was carried through the same chromatography steps showed 7–8 fold higher inhibitory activity when assayed under the same conditions (Table 1). The four different inhibitor fractions isolated from FLC as listed in Table 1 possess strong protein kinase activity for the smallest subunit of eIF-2 as shown in Fig. 4. Consequently, they inhibit the binding of Met-$tRNA_f$ to reticulocyte 40S ribosomal subunits as does HCR (cf. Fig. 2B). In Fig. 5 this inhibition of the Met-$tRNA_f$ binding reaction is compared with the inhibitor fractions isolated from uninduced and DMSO-stimulated FLC. In both cases the postribosomal supernatant was treated with NEM. The experiment was done in the same way as described in Fig. 2B: eIF-2 was incubated under protein kinase conditions with the inhibitor fraction and ATP, residual ATP was destroyed by the use of glucose and hexokinase. Then the activity of eIF-2 was measured in the Met-$tRNA_f$ binding reaction. The results (Fig. 5) show that eIF-2 is inactivated depending on the concentration of the inhibitor used. Furthermore, the inhibitor fraction derived from DMSO-stimulated FLC exhibit stronger inhibitory activity than the one from uninduced FLC. These findings are in agreement with the results shown above in Table 1.

Table 1. Inhibition of protein synthesis by different inhibitor preparations

Source of Inhibitor Fraction	NEM Treatment	Specific Activity (units/mg)
Uninduced FLC	–	88
Uninduced FLC	+	88
DMSO-induced FLC	–	90
DMSO-induced FLC	+	222
Reticulocytes	–	0
Reticulocytes	+	1904

Inhibitor fractions were prepared from the postribosomal supernatant by chromatography on DEAE cellulose and phosphocellulose as described [31]. Activity was assayed in the reticulocyte lysate system as outlined in the legend to Fig. 2

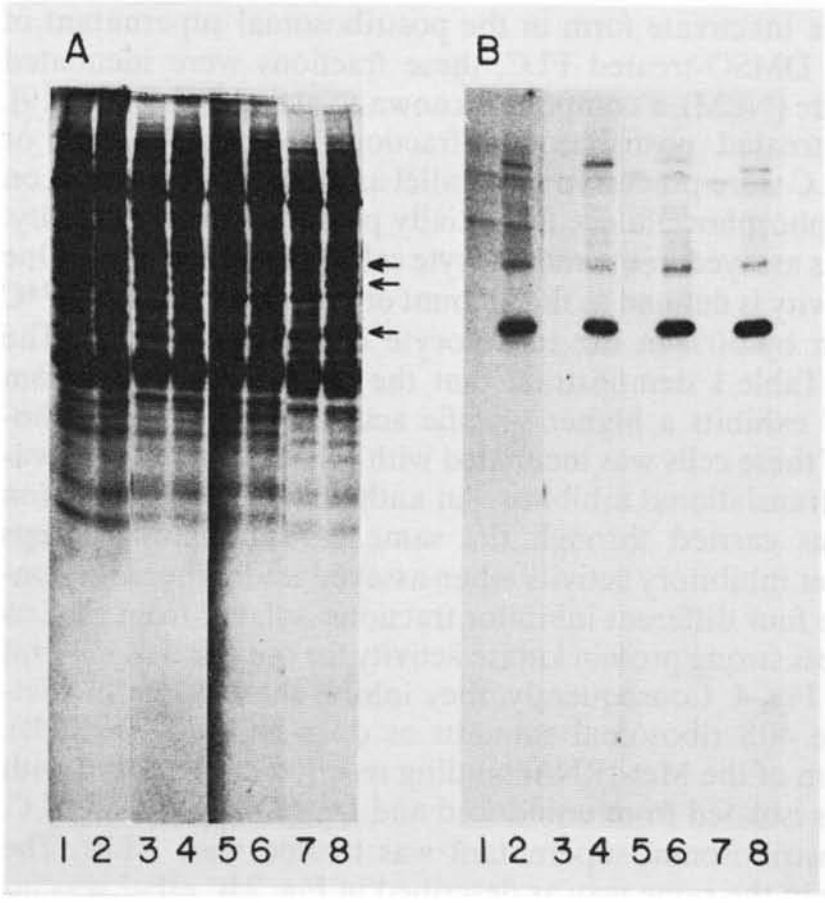

Fig. 4. Protein kinase activity of the FLC inhibitor fractions. Samples containing the different inhibitor fractions described in the text (2,5 units each) were incubated without or with 2.5 μg eIF-2 or 0.2 mM ^{32}P ATP (650 Ci/mol) and analyzed by SDS-polyacrylamide gel electrophoresis [26].

A Shows the gel stained with Coomassie brilliant blue. The arrows indicate the positions of the 3 subunits of eIF-2.

B Shows the autoradiogram. The following samples were loaded on slots 1–8: 1 = 31 μg of untreated, uninduced FLC inhibitor; 2 = as 1, plus eIF-2; 3 = 31 μg of NEM-treated, uninduced FLC inhibitor; 4 = as 3, plus eIF-2; 5 = 28 μg of untreated, DMSO-induced FLC-inhibitor; 6 = as 5, plus eIF-2; 7 = 11 μg of NEM-treated, DMSO-induced FLC-inhibitor; 8 = as 7, plus eIF-2

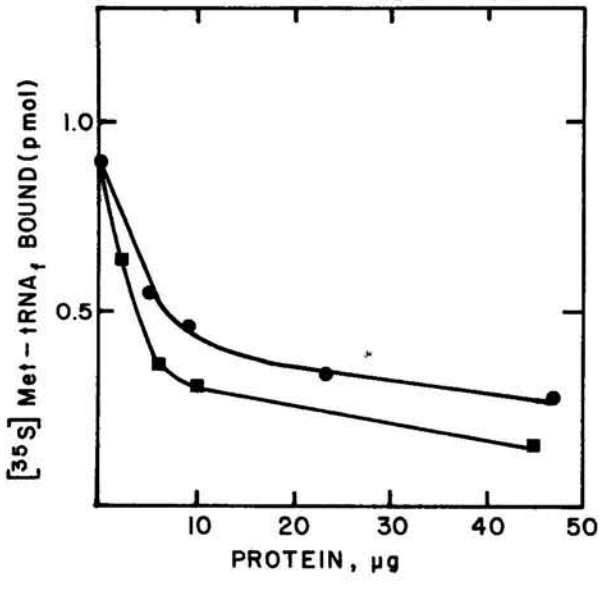

Fig. 5. Inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits by FLC inhibitors. The experiment was performed as described in the legend to Fig. 2B except that FLC inhibitors in the amount indicated substituted for HCR.

●——● = NEM-treated, uninduced FLC-inhibitor;

■——■ = NEM-treated, DMSO-induced FLC-inhibitor

Discussion

Inhibition of peptide initiation is known to provide translational regulation in reticulocytes. This inhibition occurs at the step in which Met-tRNA$_f$ is bound to 40S ribosomal subunits and is mediated by a protein kinase(s) that phosphorylate the smallest subunit of the initiation factor eIF-2 and proteins of the 40S ribosomal subunit. Regulation of protein synthesis may be unique in reticulocytes (and other hemoglobin-synthesizing cells) in that the level of hemin controls the activity of these protein kinases.

Regulation of protein synthesis by phosphorylation (and counteracting phosphatases that have yet to be described) may be a more general principle. Inhibitory protein kinases that phosphorylate the smallest subunit of eIF-2 have been partially purified from FLC as detailed above and have been described to occur in rat liver [34] or Ehrlich ascites cells [35]. Also, a protein kinase that phosphorylates the same subunit of eIF-2 appears to be involved in the inhibition of protein synthesis observed after addition of double-stranded RNA to reticulocyte lysates [20] or to interferon-treated cell extracts [36–38].

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Cell-free Protein Synthesis and Interferon Action: Protein Kinase(s) and an Oligonucleotide Effector, pppA2′p5′A2′p5′A

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Interferon, or more correctly the interferons, for it is now clear that there are several antigenic types of interferon molecule even within a species, are glycoproteins which are produced by cells in response to virus infection. On exposure to interferon, cells develop an antiviral state in which the replication of a wide spectrum of viruses is inhibited: which is, of course, the basis for our interest in interferon. (A comprehensive account of the interferons is given in [4].)

Human interferon was shown some years ago to alleviate the effects of influenza and the common cold and more recent human clinical trials have shown it to have a marked beneficial effect in the treatment of osteogenic sarcoma, chronic hepatitis and herpetic infections in immunosuppressed patients. The first full double-blind, placebo-controlled human clinical trial in immunosuppressed patients has just been completed and the results are positive: interferon does work [11]. Interferon, therefore, is being and will increasingly be used clinically. It would be good to know a little more about what it does and how it does it.

Interferon is not directly antiviral. Interferon treatment is always of the cell. It is active in very small amounts, probably only a few molecules per cell are required to trigger the response. After interferon treatment it takes several hours for the antiviral state to develop during which time there is a requirement for RNA and protein synthesis. The simplest interpretation of the data is that interferon induces the cell to produce a protein or proteins which are the actual antiviral agents. Over the years the work of a number of groups has indicated that in different cell-virus systems the antiviral affect of interferon may be expressed at the level of viral RNA synthesis, viral protein synthesis, uncoating of the virus, or, in the case of the RNA tumour viruses, at the level of virus maturation or release. Moreover interferon is not an exclusively antiviral agent. It induces changes in the cell membrane and inhibits the growth of many cell lines. It was this antigrowth property, incidentally, rather than any putative viral aetiology that provided the rationale for the osteogenic sarcoma trial ([5, 12] for recent reviews of interferon action).

Against this rather complex background we chose to analyse the clear cut inhibition of protein synthesis seen in certain cell-free protein synthesising systems from interferon–treated mouse L-cells. More particularly, we first became involved with double-stranded RNA (dsRNA) in the interferon system when it seemed possible to us that the inhibition of protein synthesis

that we and others had observed in interferon-treated, vaccinia-virus infected L-cells and cell-free systems was triggered by viral dsRNA. There was, therefore, some sort of logical progression into the work on dsRNA and most important a possible link between the results with dsRNA and events in the interferon-treated, virus-infected cell and cell-free system.

In accord with this, some years ago, we observed that protein synthesis in cell-free systems from interferon-treated cells shows an enhanced sensitivity to inhibition by dsRNA [8]. Most importantly, this enhanced sensitivity has exactly the same dose response curve as the antiviral effect in the intact cell. In other words as soon as you begin to pick up an antiviral effect in the intact cell you can detect the enhanced sensitivity to dsRNA in the cell-free system [8]. It was of interest, therefore, to determine the basis for this latter phenomenon.

To cut a long story short when one incubates an extract from interferon-treated cells with dsRNA and ATP one activates a protein kinase (s) which, by analogy with the work of a number of groups with rabbit reticulocyte lysates ([3] for example), is thought to phosphorylate one of the initiation factors (elF-2) involved in the initiation of protein synthesis. This interferon mediated kinase has been observed by a number of groups working in mouse, human and chick cell systems ([10, 14, 15] L. A. Ball personal communication) and it is impossible to overemphasize its potential significance in mediating the multiplicity of effects attributed to interferon. The difficulty, as always with a kinase, is to know which phosphorylations to look for and the significance of those observed. For example, the interferon mediated kinase also phosphorylates histones [14] and it is obviously tempting to speculate that this may have some physiological significance in the effects of interferon on transcription and cell growth. Meanwhile it is reasonable to assume that the kinase does indeed phosphorylate elF2 and that this plays a part in the inhibition of protein synthesis observed. It may not, however, be the only or even the major factor involved, for when one incubates interferon-treated cell extracts with dsRNA and ATP one also activates an enzyme (2–5 A synthetase) responsible for the synthesis of the unusual oligonucleotide pppA2′p5′A2′p5′A (which for convenience will be referred to as 2–5 A) which is effective at subnanomolar concentrations in the inhibition of protein synthesis in cell-free systems.

Interestingly both the 2–5 A synthetase and the kinase will bind to a column of dsRNA itself bound to sepharose. Accordingly we've used this property to purify and fractionate these enzymes and the way is now open for an investigation of the effect of each individually on protein synthesis in the cell-free system. Meanwhile we have used the 2–5 A synthetase in its stable column-bound state to synthesize and radioactively label the 2–5 A with α- or γ^{32}P- or ^{3}H-ATP [6, 9]. An analysis of the radioactive inhibitor yielded the structure pppA2′p5′A2′p5′A in which the 2′–5′ linkage is unusual not having been reported previously for biologically synthesized material [9].

Here, therefore, we have an inhibitor of a type not previously described. Furthermore, it is not unique to the interferon-treated mouse L-cell-free system. What is almost certainly the same inhibitor is also synthesized by an

enzyme fraction from interferon-treated chick cells and, interestingly in this system the 2–5A synthetase has also been detected in smaller amounts in control cell extracts [1]. In addition, from our own work we know that the 2–5A system also operates in rabbit reticulocyte lysates [7]. The 2–5A preparations from rabbit reticulocyte lysates and interferon-treated mouse L-cell systems have the same specific biological activity in the inhibition of protein synthesis in cell-free systems [6,7]. Moreover, preparations from both sources have now been subjected in parallel to the same detailed analysis as we have already published for the 2–5A from interferon-treated cell extracts and it is beyond reasonable doubt that they are the same.

The availability of reticulocyte derived material on a larger scale has enabled us to carry out proton and phosphorus NMR analyses of the purified 2–5A. Although these studies are not yet complete sufficient has been done to confirm the major aspects of the structure proposed. Ultimate proof of this structure will come from chemical synthesis. This is in process and preliminary results suggest that chemically synthesized 2–5A shows the same specific biological activity as the biologically synthesized material.

How then does 2–5A work? Its high potency (it is effective at subnanomolar concentrations in the cell-free system) combined with the time lag in the development of the response to it, suggests that it is not directly inhibitory but that it, in turn, sets in train a sequence of events leading to the observed inhibition [6,7,9]. Indeed it is now clear from our own work and that of a number of other groups that at least one of the things that it does is to activate a nuclease ([2,13] C. Baglioni, L.A. Ball, M. Revel, C.E. Samuel and their respective collaborators, personal communication).

Interestingly the 2–5A, the nuclease and the inhibition of protein synthesis are all unstable. On addition of exogenous 2–5A to cell-free systems a nuclease is activated and protein synthesis is inhibited. Meanwhile the 2–5A is degraded, nuclease activity diminishes in parallel and, in the absence of a 2–5A regenerating system, protein synthesis will resume on freshly added mRNA as efficiently as in corresponding controls. The enzyme which degrades 2–5A appears to have activity similar to that of snake venom phosphodiesterase yielding AMP or ATP as the products of digestion (L.A. Ball, B.R.G.W. and I.M.K., unpublished data, M. Revel personal communication). It is possible that it is the induced nuclease which carries out this degradation providing a self-regulatory feed-back mechanism whereby the 2–5A activates the nuclease responsible for its own destruction, but this remains to be established. What is clear, however, is that cells have a mechanism (s) for coping with 2–5A and the components interacting with 2–5A all appear subject to cyclic activation and deactivation, which is exactly what one might expect for a delicately balanced control system. Interferon pretreatment of the cell, incidentally, appears mainly, if not exclusively, to enhance the synthesis of 2–5A, its activity and fate are essentially identical in interferon-treated and control cell extracts.

We routinely synthesize and assay 2–5A in the cell-free system. It is also active, however, in intact cells. Although one requires more of it than in the cell-free system, the 2–5A is still potent, a concentration of just over 10 nM

being sufficient to bring about a 50% inhibition of protein synthesis in suitably permeabilised cells.

Finally, I would like to emphasize that the interferon mediated sensitivity to dsRNA is an enhanced sensitivity. This, the potency of the 2–5A and the fact that the 2–5A synthetase is found in a variety of both interferon-treated and control systems makes it very tempting to speculate that, in addition to any role that it may play in the antiviral action of interferon, the 2–5A system (with or without enhancement by interferon) may be involved in the regulation of normal cell growth or development.

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Alterations in Translational Control Mechanisms in Friend Erythroleukemic Cells During DMSO Induced Differentiation

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Presently many laboratories are involved in studies on the regulation of the biosynthesis of proteins at the level of translation [1,2]. We have reported previously that membrane mediated events are involved in the regulation of biosynthesis of proteins [3]. Several physiological conditions (hyperosmolarity of the growth medium, hypertonic salt, DMSO, ethanol) induce a rapid increase in the number of 80S ribosomes and a concomitant decrease in the size and number of polysomes indicating that protein synthesis is affected at the level of initiation [4,5,6]. The quantitative decrease in the amount of protein synthesis is accompanied by an extensive alteration in the pattern of protein synthesis as observed upon pulse labeling of treated and untreated cells with radioactive amino acids.

When overall protein synthesis in cells in culture is inhibited by more than 80% by the hypertonic initiation block (HIB), the synthesis of one major cellular protein, actin, is reduced to a level of 10%. In contrast, the synthesis of some proteins is unchanged or less reduced indicating that the translation of some species of mRNA i.e. actin are more sensitive to and other species of mRNA are more resistant to HIB.

Thus the relative translational efficiencies (RTE) of viral and cellular mRNAs can differ over a wide range, and as such mRNAs can be classified in a hierarchical order according to translational efficiencies. Based on the example of actin stated above, we would assign an RTE of 0,5 to actin and an RTE of 1 to mRNAs from which the relative synthesis of the corresponding protein remains unaltered by HIB conditions.

Friend virus transformed mouse erythroleukemia cell lines have attained widespread use as a model system for the in vitro study of differentiation [7,8]. In this paper we describe studies concerned with the RTEs of several viral mRNAs and selected cellular mRNAs in Friend virus induced erythroleukemia cells. We also report on the effect of induced differentiation on translation control mechanisms in these cells.

Materials and Methods

Cell Cultures

Friend virus producing erythroleukemia cell line FSD-1-F4 was generously provided by W. Ostertag, Max-Planck-Institut für Experimentelle Medizin, Göttingen.

Labeling of Cells and Extraction of Proteins

The synthesis of viral proteins was analyzed by pulse labeling of cells with [^{35}S] methionine, followed by lysis of the cells and selection of virus specific proteins by incubation with antisera directed against viral proteins and subsequent separation by polyacrylamide gel electrophoresis (PAGE), autoradiography and densitometry [9,10,11]. Protein synthesis was quantitated by the method of Mans and Novelli [12].

Results

Identification of Viral and Cellular Proteins and Determination of RTEs of the Corresponding mRNAs

In previous publications [9,10] we reported on the identification by immunoprecipitation of a number of Friend virus specific proteins in several Friend erythroleukemia cell lines isolated by Ostertag, in the Eveline cell line and in the original cell line 745 characterized by Dr. Friend. Our recent investigations were performed with the Ostertag cell line F4–N. Additional virus specific proteins are present in this cell line based upon data obtained by immunoprecipitation with specific antisera against viral proteins.

Cell extracts prepared as described previously [10] from 60 min [^{35}S] methionine pulse labeling experiments were first exposed to normal goat antiserum, then to *Staphylococcus aureus* strain Cowan I (S. aureus), followed by exposure to anti p30 goat serum, again to *S. aureus* and finally to anti gp70 antiserum and to *S. aureus.* Labeled proteins specifically adsorbed to the three *S. aureus* precipitated samples were eluted and separated by PAGE as previously described [10]. Proteins which react preferentially or exclusively with one of the specific sera (see Fig. 1, legend) are considered virus specific. Cellular proteins (especially actin) contaminated all 3 antigen-antibody-*S. aureus* complexes to a comparable extent.

We have determined the RTE of cellular and viral mRNAs by quantitation of the amount of synthesis of corresponding proteins under isotonic and various HIB conditions. Fig. 1 shows densitometer tracings of autoradiographs from Page separations of [^{35}S] pulse labeled proteins from Friend cells after immunprecipitation and adsorption and desorption to *S. aureus.* The apparent molecular weights of the proteins in kilodaltons are indicated above each peak. We have previously shown that a quantitative analysis of the synthesis of individual proteins can be achieved by densitometry of autoradiographs [11]. One can readily see that the synthesis of various proteins in the non-induced cells was differentially affected by HIB. Actin migrates with an apparent molecular weight of 44 K in the present gel system. The synthesis of actin is highly sensitive to HIB indicating that in Friend cells – as in other tissue culture cells – the RTE of the mRNA coding for actin is very low. The mRNAs coding for the cellular proteins with a molecular weight of 19 and 20 K have an RTE of approximately 1, that is, the synthesis of the 19 and 20 K proteins was inhibited by HIB to the same extent as overall cellular protein

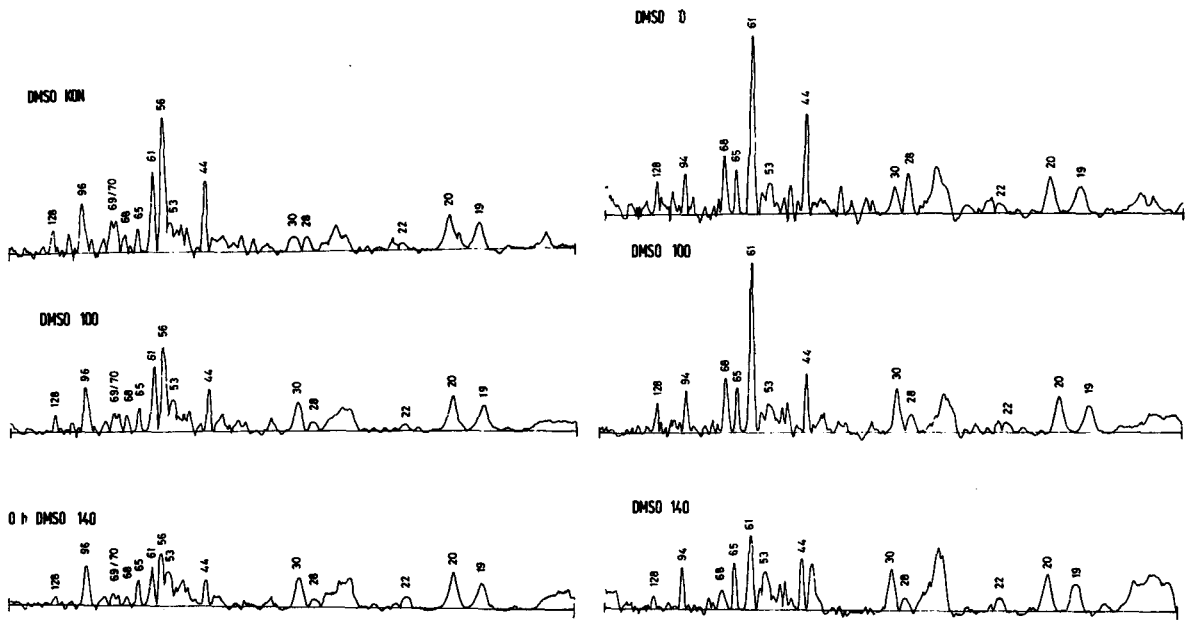

Fig. 1. Densitometry tracings of autoradiographs of SDS gel electrophoresis of S. aureus adsorbed immuncomplexes from friend cell extracts pulselabeled with [^{35}S] methionine. The approximate molecular weights in kilodaltons of the labeled protein peaks is based on the migration of unlabeled molecular weight markers and on the migration of labeled vesicular stomatitis virus proteins. The cells were preincubated for 25 min with medium containing one-twentieth the normal concentration of methionine. The osmolarity of the growth medium was increased by addition of extra NaCl a) 0 mM (0), b) 100 mM (100), c) 140 mM (140) which resulted in an overall inhibition of protein synthesis by a) 0%, b) 50%, c) 80%. Therefore, culture b) and c) received 2 and 10 times more [^{35}S] methionine than culture a) in order to label the proteins in the cell extracts to a comparable extent [11].
Left panel: Cell extract treated with anti gp70 serum
Right panel: Cell extract treated with anti p30 serum

synthesis. We utilized the relative intensity of the labeling of these proteins bands as standards for the estimation of the RTEs of viral and cellular mRNAs from autoradiograms of labeled cell extracts or immune precipitates. The results presented in Table 1 indicate that the RTE of some RNA tumorvirus mRNAs in this system are lower than the RTEs of cellular mRNAs. In fact, the RTE of mRNAs coding for gp70 and gp56 are as low as

Table 1. Relative translational efficiencies of cellular and viral mRNAs in Friend erythroleukemia cells

cellular proteins							
MW (Kd)	128	44	22	20	19		
RTE	0,4	0,5	2,0	1,0	1,0		
gp70 specific proteins							
MW (Kd)	96	70	56				
RTE	0,9	0,3	0,3				
p30 specific proteins							
MW (Kd)	94	68	65	61	53	30	28
RTE	1,0	0,4	1,1	0,4	1,4	1,4	0,4

the RTE of the actin mRNA. This observation posed some very interesting questions concerning the mechanism of translational control in this system, since there is no apparent correlation in the amount of viral mRNAs and the corresponding viral proteins synthesized (Racevskis, Koch and Ostertag, unpublished).

Increase in Sensitivity to HIB During DMSO Induced Differentiation in Friend Erythroleukemic Cells

Induction of differentiation in Friend erythroleukemic cells causes a severe reduction in the growth rate of the cells [7]. The division time of the cells increases from 13 hours to 24 hours by 3 days after induction with DMSO (Weber and Koch, unpublished). In order to determine and compare the RTEs of mRNAs at various times after induction of differentiation we analyzed the incorporation of ^{35}S methionine into proteins under isotonic and various HIB conditions. The results presented in Fig. 2 confirm previous

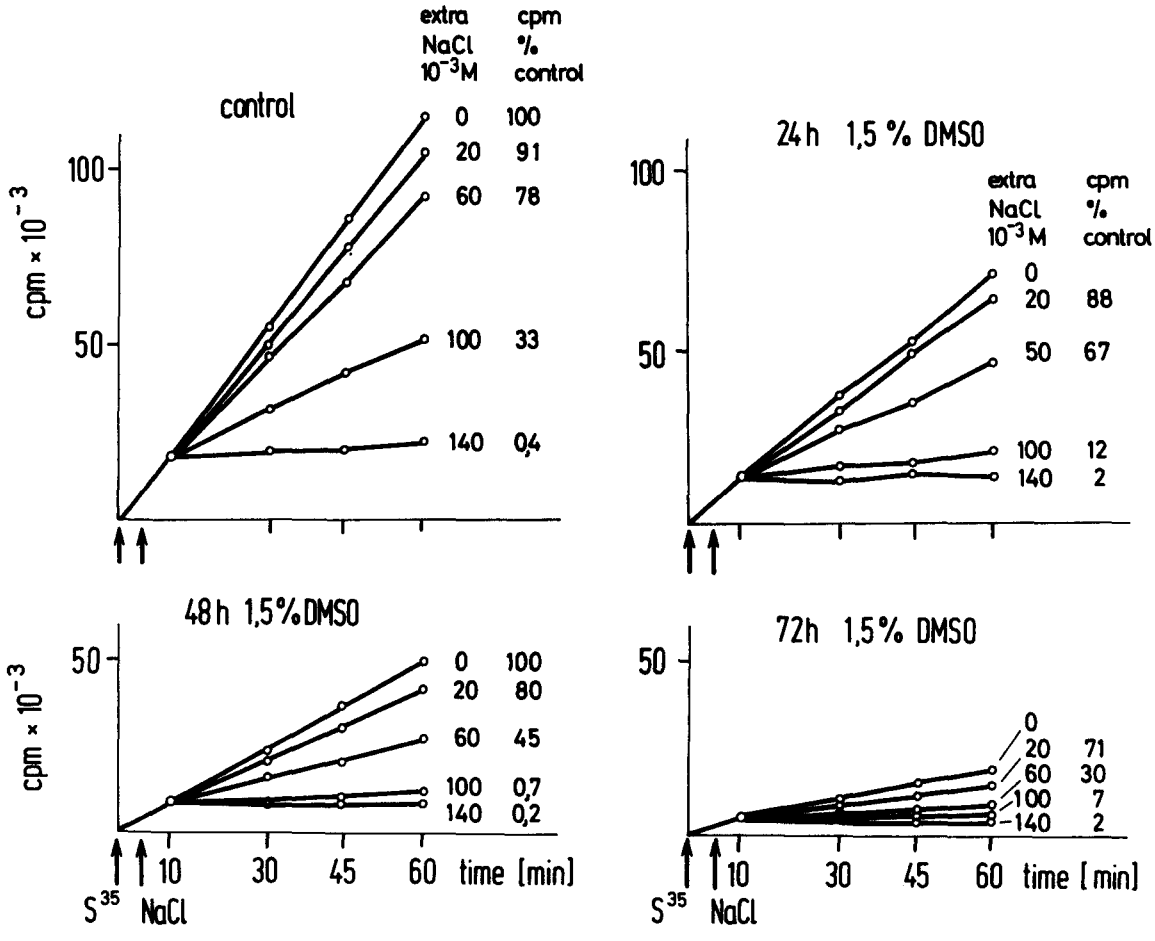

Fig. 2. Effect of HIB on the incorporation of [^{35}S] methionine into proteins at various times during DMSO induced differentiation of Friend-erythroleukemic cells. In order to exclude nutritional effects the cells were fed with fresh medium every 24 hours during the course of the experiments. [^{35}S] methionine was added to several aliquots of the culture (1.0×10^6 cells/ml) and the osmolarity of the growth medium was increased by addition of different amounts of extra NaCl. Incorporation of [^{35}S] methionine into proteins was determined as described [12]

findings on reduced incorporation of amino acids during differentiation [15]. In addition, they indicate that the cells become progressively more sensitive to HIB with increasing time after induction of differentiation, that is, a smaller increase in osmolarity causes a more dramatic inhibition of protein synthesis.

Alterations in Translational Control Mechanisms in Friend Erythroleukemic Cells During DMSO Induced Differentiation

The question arose as to whether the inhibition of cellular protein synthesis and the intracellular concentration of a given polypeptide changes coordinately after induction of differentiation and also whether coordinate changes in RTE and the yield of the corresponding proteins occur.

The experiments described in Fig. 1 were repeated at daily intervals after induction of differentiation with DMSO, and the results are summarized in Table 2. RTEs of mRNAs can remain constant (i.e. for cellular proteins 20 and 19 K), decrease (i.e. cellular 128 K) or increase (mRNA for some viral proteins, and most dramatically for a cellular protein with a molecular weight of 22 K). An RTE over 1 indicates a relative increase in the amount of synthesis of a given protein under restricted condition of polypeptide chain initiation. The cellular protein 22 K with an RTE of 4 at 72 hours after induction accumulates under conditions of 80% inhibition of overall protein synthesis at a rate of 80% of the normal rate. An actual increase in the synthesis of a given protein under HIB conditions was observed for the synthesis of globin in Friend cells by utilizing two different experimental approaches both the quantitative determination of the hemoglobin content by the ben-

MW (Kd)	RTE		% Change in yield of intracellular protein from 0–72 h
	0 h	72 h	
cellular proteins			
128	0,4	0,3	180
44	0,5	0,4	130
22	2,0	4,0	100
20	1,0	1,0	100
19	1,0	1,0	100
gp70 proteins			
96	0,9	1,0	160
70	0,3	0,6	110
56	0,3	0,6	120
p30 proteins			
94	1,0	0,8	70
68	0,4	0,9	230
65	1,1	1,2	130
61	0,4	0,8	60
53	1,4	2,4	110
30	1,4	2,5	160
28	0,4	0,4	130
25	1,8	2,8	110

Table 2. Changes in RTE of mRNAs and in the relative amounts of corresponding proteins during DMSO induced differentiation

zidine technique (Bilello, unpublished) and from autoradiographs of HIB treated induced cells. It is important to mention that in spite of the dramatic increase in the RTE of the mRNA coding for the cellular protein 22 k during differentiation, this protein does not accumulate at a higher rate under isotonic conditions in differentiated cells.

Summary

Induction of erythroid differentiation in Friend erythroleukemia cells causes a reduction in the rate of protein synthesis and a prolonged growth cycle of the cells. A study of the relative translational efficiencies (RTE) of viral mRNAs and selected host cell mRNAs revealed unexpected low RTEs of the mRNA coding for the gp70 protein and RTE of the mRNA coding for the p30 protein. The RTEs of viral and of some host mRNAs show significant changes during differentiation. The rate of synthesis of corresponding proteins are altered non-coordinately, indicating transcriptional and/or posttranscriptional regulation of mRNA, as well as amplified regulation at the level of translation of mRNA.

Discussion

A great number of laboratories are engaged in studies to delineate the pleiotropic effects which are triggered by inducers of erythroid differentiation in Friend erythroleukemia cells. Nevertheless, little is known so far on the role of the regulation of protein synthesis at the level of translation and on the fate of newly synthesized proteins (post translational modification, cleavage and degradation). We have previously reported on alterations in the processing pathway and in the rate of processing of viral precursor proteins in the Friend cell line 745 [10]. The experiments presented here show that the decreased rate of overall protein synthesis observed during differentiation is accompanied by drastic alterations in the rate of synthesis of some proteins and in the RTE of mRNAs. Changes in the RTE of an mRNA and in the rate of synthesis of the corresponding proteins are not coordinate, indicating that a lowered RTE may be counteracted by an increased availability of active mRNA. Likewise the effect of an increase in the RTE of mRNAs might be reduced by a decreased amount of availability of an mRNA. Regulation of protein synthesis at the level of translation might also involve other factors than amount of mRNA and RTE of mRNA. This view is supported by the observation that early vaccinia virus mRNAs with high RTEs are present but not translated at later times in the replicative cycle [13]. Comparable results have been obtained in frog virus infected cells [14]. In this study we were able to demonstrate both alterations in the RTEs of several mRNAs and a non-coordinate change in the accumulation of corresponding proteins. While other investigators have reported on the existence of specific regulatory mechanisms for the translation of a given mRNA it is difficult for us to imagine how such a model could

be consistent with our findings in regard to the multitude of changes. Thus we would like to suggest that more generalized alterations in the translational machinery must occur during differentiation and the ultimate alteration in the pattern of proteins synthesized is dependent upon multiple factors at both the transcirptional and post transcriptional levels.

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Inhibition of Polypeptide Chain Initiation by Inducers of Erythroid Differentiation in Friend Erythroleukemic Cells*

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Friend virus transformed erythroid cells can be isolated and maintained serially in cell culture [1,2]. Such erythroleukemic cell cultures can be induced to differentiate along the erythroid pathway by exposure to a variety of compounds including DMSO [1–3], aprotic solvents [4], fatty acids [5], purines and purine analogs [6] and the cardiac glycoside ouabain [7]. Phenotypic changes observed in Friend cells after inducer treatment appear to parallel the expression of genes typical of the normal differentiating cell. A multitude of erythroid functions are induced including the induction of heme pathway enzymes [8], globin mRNA synthesis [2,3,9,10], and the synthesis of erythrocyte specific proteins [11–13]. The extent of hemoglobin synthesis can be as high as 20–25% of the total cellular protein synthesis in induced cells, compared to less than 1% in uninduced cells.

The observation that a variety of inducers could affect a marked alteration in gene expression prompted us to study protein synthesis in Friend cells after treatment with a variety of inducers. We were particularly interested in determining whether there was a common mode of action whereby these inducers could affect the specific synthesis of the unique spectrum of erythrocyte proteins observed during differentiation of erythroleukemic cells.

Materials and Methods

Cell Culture

The origin and maintenance and labeling of Friend erythroleukemia cell cultures is essentially as described in Racevskis and Koch [14]. Protein synthesis was quantitated by the method of Mans and Novelli [15].

Hemoglobin Assay

The hemoglobin content of cell extracts was measured utilizing the benzidine technique described by Luftig et al. [16].

Results and Discussion

In Friend erythroleukemic cell cultures addition of 10 to 15% DMSO to the tissue culture medium results in a rapid, reversible inhibition of cellular protein

* Supported by Stiftung Volkswagenwerk.

synthesis accompanied by the complete breakdown of polyribosomes. DMSO concentrations effective in inducing cell differentiation (1–2%) cause a sustained inhibition of protein synthesis and a shift in the polyribosome profile wherein there is a substantial decrease in the percentage of mRNA associated ribosomes (Bilello et al., manuscript in preparation). We have observed a similar sustained inhibition of the pattern of protein synthesis with a series of other known inducers (Table 1). Since we have measured protein synthesis by incorporation of radiolabeled methionine into protein, we were concerned that changes in amino acid uptake or pool size may have colored the interpretation of our data. As can be seen in Table 2, amino acid transport was reduced upon the addition of DMSO. The reduction in amino acid transport occured early after addition of inducer, but was not substantial enough to explain the decreased protein synthesis in treated cells. Other inducers appear to affect changes in amino acid and hexose transport and may reflect a general effect of inducers at the cell membrane (17, Ostertag personal communication).

Since we had observed inhibition of initiation in Friend cells treated with 10–15% DMSO we wanted to determine whether initiation was inhibited during the course of Friend cell induction. Polyribosome profiles were analysed in F4–6 cells two hours after the addition of DMSO, HMBA or butyric acid since at this time point inhibition of protein synthesis was established and amino acid uptake was only moderately lowered. After treatment of F4–6 cells more than 10% of the mRNA associated ribosomes were shifted to the

Table 1. Effect of inducers on Friend erythroleukemic cell protein synthesis

Addition	Concentration mM	Hemoglobin[a] μg/10^8 cells	Protein synthesis[b] % Control	
			24 hrs	72 hrs
F4–6				
None	—	14	—	—
DMSO	192	155	34	33
HMBA	5	239	51	34
Butyric Acid	1	178	42	37
Hemin	0.1	189	69	—
Hypoxanthine	2.5	230	65	—
Ouabain	0.1	121	56	—
B8				
None	—	44	—	—
DMSO	192	240	56	41
HMBA	5	341	33	31
Butyric Acid	1	502	56	46
Hemin	0.1	276	107	47
Hypoxanthine	2.5	108	115	48
Ouabain	0.1	406	104	56

[a] Hemoglobin production measured in cell extracts 3 days after addition of the compound as described in the Materials and Methods

[b] Protein synthesis as measured by incorporation of ^{35}S methionine into hot TCA insoluble material 24 or 72 hrs after addition

Table 2. Alteration of amino acid transport subsequent to the addition of DMSO to Friend erythroleukemic cell cultures

Time in culture hrs	Treated cells cpm/mg protein	Untreated cells cpm/mg protein	% $T/_U$
0	13 006	14 213	91.5
2	11 315	14 641	77,2
4	10 426	14 931	69,8
6	10 629	14 490	73.7
12	9 009	15 482	58,3

Cells were labeled with ^3H amino acid mixture for 10 minutes, washed 3 times with Earles Balanced salt solution. Cell extracts were precipitated with 10% TCA and TCA soluble counts were determined. Total cell protein was determined in an aliquot by the Lowry method.

monosome region of the gradient indicative that ribosomes previously associated with mRNA were no longer actively engaged in protein synthesis. Polyribosome profiles indicative of reduced initiation have been observed throughout the course of induction. These results suggested that inhibition of the initiation of protein synthesis is an early event which is sustained during the course of induction.

Table 3 summarizes a series of experiments designed to access whether initiation and/or elongation inhibitors could induce erythroleukemic cell differentiation. Treatment of Friend cells with the elongation inhibitors puromycin, cycloheximide or emetine had no effect upon the induction of hemoglobin synthesis. In contrast hemoglobin synthesis was induced upon exposure to ethanol or hypertonic medium which inhibit initiation [18]. Initiation inhibitors induce higher levels of hemoglobin synthesis in B8 cells,

	Addition	Hemoglobin μg per 10^8 cells at day 3
Exp 1	none	13
	DMSO 192 mM	290
	Sucrose 50–200 mM	13–22
	Emetine 10–50 μg/ml	13–34
Exp 2	none	8
	DMSO 192 mM	310
	Puromycin 0,1–10 μg/ml	2–11
	Cycloheximide 0,1–5 μg/ml	1–19
	Ethanol 1–2%	77–84
Exp 3	none	16
	DMSO 192 mM	127
	Excess KCl 25–75 mM	30–100
	Excess NaCl 25–75 mM	32–81
Exp 4[a]	none	44
	DMSO 192 mM	240
	Excess KCl 50 mM	161
	Excess NaCl 75 mM	100

Table 3. Protein synthesis Inhibitors: Effect upon hemoglobin synthesis in uninduced Friend erythroleukemic cells

[a] Experiment 4 utilized B8 cells which have a high background synthesis of globin and globin mRNA

cells which have a higher background level of globin mRNA in the uninduced state [10]. Inhibition of initiation in such cells leads to a rapid increase in hemoglobin synthesis. Only initiation inhibitors which induced sustained inhibition of cell protein synthesis were capable of induction; sucrose or DEAE dextran were incapable of either induction or sustained inhibition of protein synthesis in Friend cells.

Our findings indicate that inducers of erythroleukemic cell differentiation inhibit protein synthesis at the level of initiation. Different mRNAs often differ in the rate of formation of the initiation complex. Under conditions of lowered initiation, translation of high efficiency mRNAs i.e. globin mRNAs are favored relative to other mRNAs with lower initiation frequencies [18,19]. Other experiments have demonstrated that exposure of erythroleukemic cells to high concentrations of NaCl or KCl increased the synthesis of globin relative to other cell proteins. Thus the ability of inducer moieties to inhibit initiation may serve to provide the optimum conditions for globin synthesis which is the major protein synthesized in the differentiated erythroid cell.

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Possible Role of the Friend Virus Life Cycle in Differentiating Friend Leukemia Cells Treated with Interferon

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A. Introduction

Friend erythroleukemia cells (FLC) are proerythroblasts chronically infected with the Friend virus complex (FLV, composed by LLV and SFFV). They undergo erythroid differentiation accompanied by synthesis of heme and hemoglobin (Hb), accumulation of globin mRNA and erythrocyte-specific membrane changes upon treatment with DMSO and other polar solvents [6].

Data collected in the past 2–3 years have convincingly shown widespread pleiotropic effects of interferon (IF). As reviewed elsewhere [1], inhibitory as well as stimulatory effects of IF on various types of cells have been reported. As for the antiviral activity of IF, it has been shown that in cell chronically infected wi RNA tumor viruses, IF treatment suppresses extracellular virus production, but does not abolish intracellular virus antigens expression [4]. We have been studying the action of DMSO and IF upon growth and differentiation of FLC, on one hand, and the effect of IF on the FLV genome expression, on the other hand.

B. Control of FLC Differentiation by Interferon

In previous papers we have described in detail the effects exerted by high doses of IF on FLC growth, cell cycle and erythroid differentiation [9,11,12].

FLC growth is inhibited, in a dose-dependent fashion, by the administration of IF doses higher than 500 U/ml. Cell cycle parameters also change with respect to G_1 and G_2 phases, that are almost doubled in length. Removal of IF results in a prompt resumption of growth potential and normal cycle parameters. Similar data were obtained when DMSO-stimulated FLC were treated with IF. Under conditions allowing at least 2–3 cell doublings in the presence of DMSO, Hb synthesis was reduced by 90%, but globin mRNA only by 30%, in FLC treated with DMSO + IF. It is apparent that the observed transcriptional effect of IF, although a novel one for a "cellular" mRNA, cannot fully explain the magnitude of the translational inhibition. In addition, these globin mRNAs (from DMSO- and from DMSO + IF-treated FLC) are indistinguishable from one another for a) base sequence, as deter-

mined by Tm, b) size, analyzed by 99% formamide, 4,5% polyacrylamide gel electrophoresis, and c) ability to direct the synthesis of globin-size materials in homologous (S_{30} lysates) and heterologous (wheat-germ) cell-free protein-synthesizing systems. Nonetheless, DMSO + IF-treated FLC do not synthesize appreciable amounts of globin α and β chains, although they do apparently produce significant amounts of a protein(s), more cationic than β chain, immunoprecipitable with a monospecific globin antiserum.

IF doses lower than 100 U/ml do induce, instead, a substantial increase of Hb-producing cells in DMSO-stimulated cultures [1]; data in Table 1 demonstrate that this effect is mediated by increase of globin mRNA levels. Preliminary results, moreover, indicate that the administration of low doses of IF stimulates erythroid differentiation of FLC *per se*, i.e. in the absence of any induction by DMSO; increased amounts of globin mRNA have been observed in IF-treated FLC as compared to untreated FLC (Table 1).

Table 1. Erythroid differentiation and viral gene expression, on day 3 of culture, of FLC given low dosages (25 U/ml) of IF on day 0

Treatment	Benzidine-positive cells (%)	Globin mRNA copies/cell	LLV-specific RNAs (% of cytoplasmic RNAs)
none	1	157	0,022
+ IF	12	556	0,015
+ DMSO	41,7	1641	0,044
+ DMSO + IF	50,2	2434	0,009

C. Life Cycle of FLV in FLC Treated with Interferon

The extracellular virus release is impaired by IF [4], whereas the synthesis of FLV-specific proteins is not blocked, so that intracytoplasmic accumulation of such proteins occurs [3].

We have analyzed cytoplasmic RNAs extracted from IF-treated FLC, with/without simultaneous treatment with DMSO, by molecular hybridization with cDNA probe complementary to the LLV component of FLV. Results are shown in Table 1: while in DMSO-treated FLC there is a significant increase of viral RNAs, IF treatment alone barely reduces the virus-specific cytoplasmic RNA content. The combined treatment, DMSO + IF, instead, significantly decreases the viral RNA concentration as compared both to untreated and to DMSO-treated FLC.

Possible explanation of these results could be: either IF operates at both an early (transcription) and a late (assembly) step of virus cycle, or, alternatively, the intracellular levels of virus RNAs are controlled by the accumulated viral proteins in a "feedback-type" inhibition.

D. Discussion

In conclusion, we have reported here a set of data, apparently unrelated to one another, namely the peculiar inhibitory action of IF on the life cycle of FLV, and the "pendulum" type of effect on FLC differentiation. It seems to us, however, that there is a possibility of giving a unifying interpretation.

I. Recent evidence [5, 14], contrary to what has been assumed so far, suggests that the LLV component of the FLV complex is able to induce erythroid leukemias on its own in susceptible newborn mice. The question raises as to what role SFFV is playing in the biology of the FLV system, particularly in the as yet unknown mechanism(s) triggering on erythroid differentiation in vitro.

One possible answer could be the following one: SFFV induces high levels of polycythemia in susceptible mice. Whether or not the SFFV interaction(s) by itself with its target cells results also in leukemic transformation is still not clear. It seems reasonable, however, to assume that its ability to induce polycythemia in vivo may play a critical role in providing the lymphatic leukemia virus component (LLV) with a much larger subpopulation of "erythroid-committed" precursor cells. These cells would then become available for the transforming event. Such transformation, therefore, would be the result of the interaction of these cells with LLV. An erythroleukemia would then ensue as a consequence of a combined and sequential action of the two viruses.

This is in agreement with the finding that CFU-E's from mice injected with the polycythemic strain of FLV (FLVp, composed by LLV and SFFV) are a mixture of erythropoietin-dependent and erythropoietin-independent cells, whereas CFU's from mice given the anemic strain (FLAa, only LLV composed) are all erythropoietin-dependent (Peschle and Rossi, unpublished data). According to the above mentioned hypothesis, the conversion of the CFU-E's from erythropoietin-dependency to erythropoietin-independency would be accomplished by the SFFV component. This hypothesis would also explain why the SFFV-deprived preparations of FLVp induce "late" lymphatic leukemias, whereas it does not account for the fact that the anemic strain FLVa, which is free of SFFV from the origin, is also able to cause erythroleukemias, and only these, in vivo. Minor, and as yet undetected, differences between the two strains of FLV-LLV may be responsible for this apparent discrepancy.

II. In the in vitro system (the Friend cells), treatment with DMSO or with other inducers causes a pronounced erythroid differentiation accompanied by increased production of FLV complex (LLV and SFFV). It is very interesting to compare the kinetics of the increased production of SFFV with that of LLV in the framework of the "induced" erythroid differentiation. The stimulation of LLV by DMSO is quantitatively small (2–5 fold) and occurs later (3–4 days after induction) [2, 10]. Such LLV stimulation, therefore, follows the erythroid differentiation, whose early parameters (spectrin, glycophorin, nonhistone chromatin changes) have been detected much earlier [6, 8, 13]. The SFFV increase, instead, is a very early and much more pro-

nounced phenomenon [10], and it seems to occur prior to the appearance of the early markers of erythroid differentiation, suggesting that it might be the real "trigger" of differentiation.

III. This hypothesis would also help in understanding the IF "pendulum" type of action on erythroid differentiation. It could be due, of course, to some inherent properties of the IF molecules, but one may also offer an alternative explanation, based on interactions between IF and the two components of the FLV complex. The relative sensitivity of SFFV and LLV to IF may be different. Differences in the susceptibility of viruses to IF have been reported [7]. We postulate that LLV is sensitive even at low doses of IF, whereas SFFV is sensitive only to high doses (above 500 U/ml). At low doses only LLV would be inhibited with a relative enrichment of SFFV, and consequently with some enhancement of erythroid differentiation. At high doses, instead, SFFV would also be blocked, with a consequent inhibition of erythroid differentiation.

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Genetic Analysis of Human Cell Surface Structures

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Many studies indicate that cell surface components are important in the control of cellular growth and function and that alterations of these structures occur in the malignant state. The extreme complexity of the human cell surface makes difficult the exact determination of the role of specific cell surface structures in the malignant process. The strategy we have employed to simplify the genetic analysis of human cell surface structures takes advantage of two experimental facts.

1. When human cells are fused with the Chinese hamster ovary cell the resulting hybrids selectively lose human chromosomes (Kao and Puck, 1970) and it is possible to prepare human-Chinese hamster somatic cell hybrids which contain only a single human chromosome (Jones et al., 1972; Kao et al., 1976; Moore et al., 1977). Therefore human cell surface structures due to individual human chromosomes can be studied. Fig. 1 illustrates the presence of the single human chromosome number eleven in one of these hybrids.
2. Antisera elicited in experimental animals after the injection of human cells are toxic to human cells in the presence of complement but do not kill the Chinese hamster ovary cell (Oda and Puck, 1961). As illustrated in Fig. 2

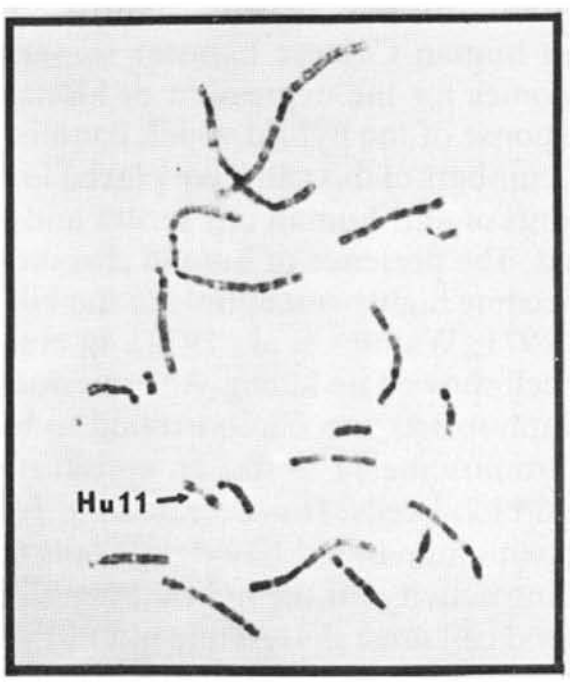

Fig. 1. Chromosomes of a human-Chinese hamster somatic cell hybrid containing all of the Chinese hamster ovary cell chromosomes and human chromosome 11

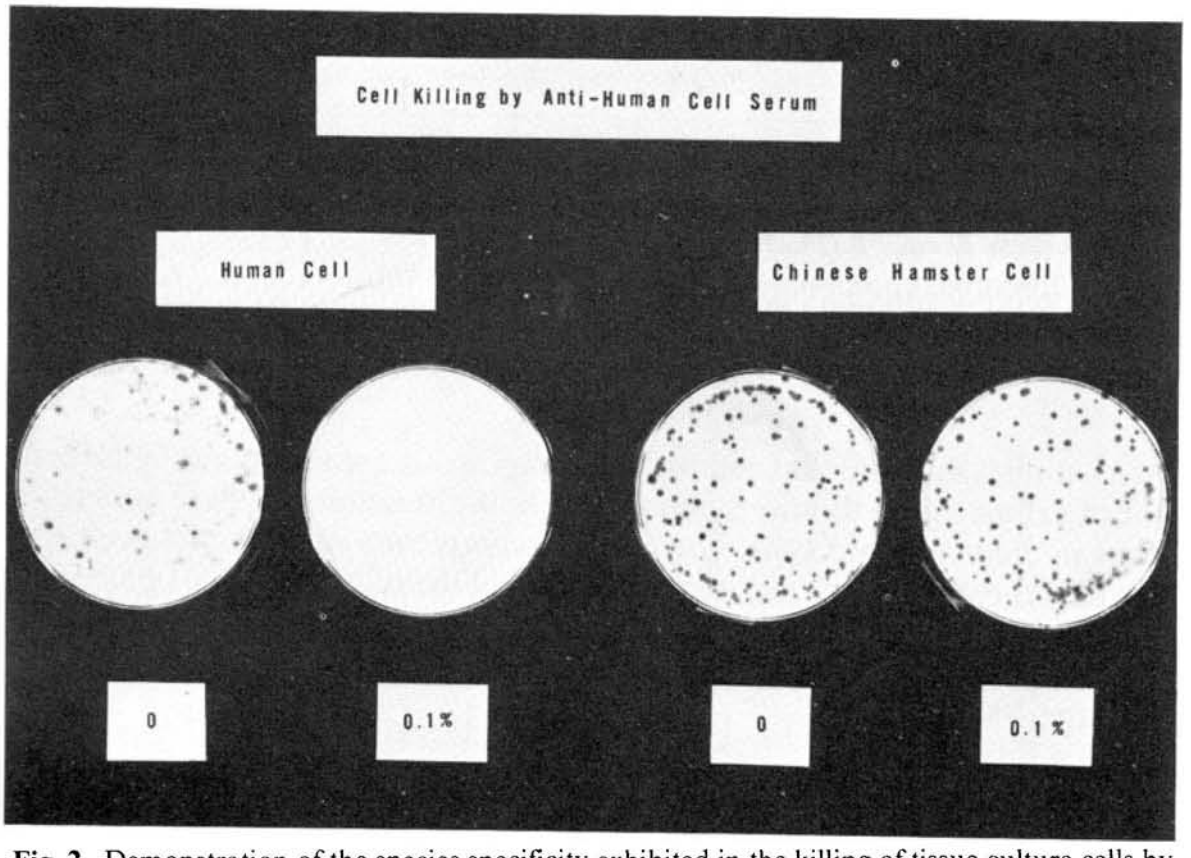

Fig. 2. Demonstration of the species specificity exhibited in the killing of tissue culture cells by antiserum. An antiserum to cultured human lymphoblasts was prepared by injecting these cells into a sheep. The antiserum kills human cells but the same concentration of antiserum has no effect on the plating efficiency of Chinese hamster cells. Normal rabbit serum was the source of complement which was present in all plates

when human cells are placed in a petri dish containing growth medium alone and are incubated the cells form large distinct colonies. In the presence of an antiserum produced against human cells all of the plated cells are destroyed. The Chinese hamster cell formed equal numbers of colonies in the presence and absence of the anti-human cell serum.

Consequently it becomes possible to test human-Chinese hamster somatic cell hybrids with single human chromosomes for the expression of human cell surface antigens. Fig. 3 shows the response of the hybrid which contains only human chromosome 11 when equal numbers of this cell were placed in a series of dishes to which increasing amounts of anti-human cell serum and a constant amount complement were added. The presence of human chromosome 11 in such hybrids causes them to become highly susceptible to the killing action of the antiserum (Puck et al., 1971; Wuthier et al., 1973). In contrast the parental Chinese hamster ovary cell showed no killing. An antiserum prepared in the horse against human lymphoblasts was demonstrated to be highly lethal to the hybrid containing chromosome 11 as did an antiserum prepared in the rabbit against human red blood cells. However, exhaustive adsorption of the antilymphoblast serum with human red blood cells fails to remove more than a small part of the killing activity for the hybrid (Wuthier et al., 1973). Therefore, the human red blood cell must share some but not all of the lethal antigens common to this hybrid and the human lymphoblast. It

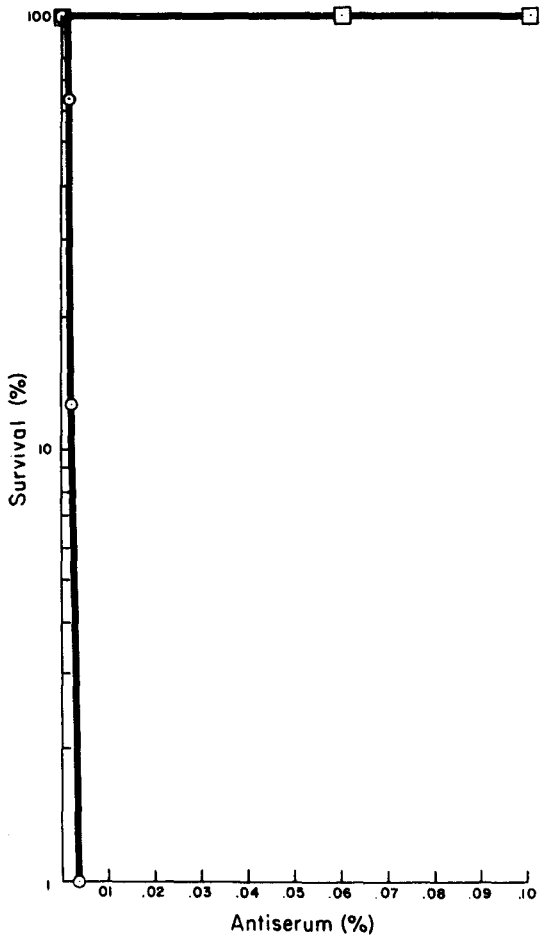

Fig. 3. Demonstration that sheep anti-human red blood cell serum kills the human-Chinese hamster somatic cell hybrid which contains the single human chromosome 11 (circles ⊙) but fails to kill the parental Chinese hamster ovary cell (squares ⊡)

has been possible using these different antisera to demonstrate that at least three human antigens (a_1, a_2 and a_3) are specified by human chromosome 11, and by mutagenesis and immunoselection to prepare clonal stocks of variants containing various combinations of the three antigen markers (Jones et al., 1975; Jones and Puck, 1977). For example a population of hybrid cells is treated with the mutagenic agent, ethyl methanesulfonate, and after removal of the mutagen the survivors which represent 10–20% of the original population are allowed to grow for several days. They are then placed in complement plus sheep anti-human red blood cell serum which has anti a_1 and a_3 activity. Clones which develop under these conditions are isolated and grown into large cultures and their resistance to anti a_1 and a_3 serum confirmed. They are also tested with anti a_2 serum and if they are killed their phenotype is by definition $a_1^- a_2^+ a_3^-$. Clones with other possible phenotypes have been prepared in a similar manner by selection in the appropriate antiserum.

Some of these variant clones were shown to contain deletions of specific segments of human 11 (Kao et al., 1977; Jones and Kao, 1978). These proved useful in the regional mapping of the antigen markers and of two human enzymes, lactate dehydrogenase A and acid phosphatase 2 which have also been assigned to this chromosome (Boone et al., 1972; Bruns and Gerald, 1974). Table 1 summarizes the phenotypic and cytogenetic characterization of some of these clones. Analysis of these data has led to the assignment of the a_1 and a_3 antigens to the short arm of chromosome 11, in the region 11pter

Table 1. Characteristics of clones isolated from the hybrid with human chromosome 11 and used for regional gene mapping

Clone number	Mutagenic agent used	Identified deletions in human chromosome 11	Presence of human markers				
			a_1	a_2	a_3	LDH-A	ACP_2
Parent (J1)	none	none	+	+	+	+	+
7	X-ray	pter → p11	−	+	−	−	−
9	UV	pter → p12	−	+	−	−	+
11	ICR-191E	qter → q13	+	−	+	+	+
23	X-ray	pter → p13	−	+	−	+	+

LDH-A – lactate dehydrogenase A
ACP_2 – acid phosphatase 2

→ 11p13 and the a_2 antigen to the long arm in the region 11qter → 11q13. The regional assignment for the human gene for lactate dehydrogenase A is 11p12 → 11p13 and for acid phosphatase 2, 11p11 → 11p12. Other variants which lack specific antigens without detectable chromosomal changes are being subjected to further genetic analysis. It has been demonstrated that the a_1 phenotype is made up of different complementation groups, mutation in any one of which results in a_1^- behavior. The distribution of these antigens on various normal and malignant cell types is being investigated. The human red blood cell has a_1 and a_3 antigenic activity on its cell surface but not a_2. Normal peripheral blood lymphocytes, cultured lymphoblasts and human amniotic fluid fibroblasts have a_1, a_2 and a_3. It has been found that a_1 is immunologically related to the glycophorin fraction of human red blood cells (Moore et al., 1976).

The ability to identify human cell surface components and to establish their corresponding genetic elements should provide a link in understanding the role of the cell surface in malignancy.

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Effect of Vitamin A on Plasminogen Activator Synthesis by Chick Embryo Fibroblasts

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Abstract

Low concentrations of Vitamin A stimulated plasminogen activator synthesis (PA) in chick embryo fibroblasts (CEF). It caused a dose dependent and reversible increase in PA synthesis in both normal CEF and CEF infected with a temperature sensitive mutant of Rous Sarcoma virus (RSV-Ts68). Both induction and deinduction of PA could be inhibited by Actinomycin D.

Vitamin A also accentuated the morphological changes associated with transformation in the Rous Sarcoma virus infected cells. The effects of Vitamin A on PA synthesis were essentially similar to those of the known tumour promoter, phorbol myristate acetate (PMA).

Both Vitamin A and PMA were found to act synergistically with sarcoma gene expression as far as PA synthesis was concerned.

Introduction

Vitamin A has been shown to inhibit squamous metaplasia in organ cultures (Sporn et al., 1976) and to promote the development and growth of experimental tumours (Polliack and Sasson, 1972; Polliack and Levij, 1969). It has also been shown to induce proteolytic activity in chick embryo limb buds cultured in vitro (Lucy et al., 1961; Fell et al., 1962). Wigler and Weinstein (1976) and Wilson and Reich (1979) have shown that the potent tumour promoting agent phorbol myristate acetate (PMA) acts as a strong inducer of the serine protease, plasminogen activator (PA).

In this paper we present the results of experiments that document the stimulatory effect of Vitamin A on PA synthesis in vitro and define certain aspects of the inductive response in normal chick embryo fibroblasts (CEF) and CEF infected with a temperature sensitive mutant of Rous Sarcoma virus (Ts68).

Materials and Methods

Primary cultures of chick embryo fibroblasts (CEF) were prepared as described by Rifkin and Reich (1971) and maintained and propagated in Eagle's minimal medium supplemented with 5% or 10% fetal bovine serum (FBS).

For experiments CEF were seeded at 10^6 cells/60 mm petri dish as tertiary cultures in Eagles medium containing 5% FBS. The cells were allowed to adhere overnight before the experiment was started and drugs were added to the cultures in 5 ml of fresh medium.

Cells were lysed by the addition of 0,1 ml of 0,1% Triton X-100 per 10^6 cells. Lysate aliquots containing 0,3–6 μg of protein were assayed for PA activity using the ^{125}I-fibrin method (Strickland and Beers, 1976). One unit of PA activity is defined as the amount of enzyme that will catalyse the release of 5% of the total trypsinizable radioactivity in 1 hr at 37°C.

Results

a) Morphology and growth

The addition of 10^{-6}M retionoic acid (RA) to Ts-68 infected cells accentuated the morphological changes of transformation normally observed when such cells are shifted from the restrictive temperature (41°C) to the permissive temperature (37°C). The clustering and loss of cell adherence observed were similar to the changes described by Ossowski et al. (1973) and Ossowski et al. (1974) in SV40-transformed hamster fibroblasts.

The addition of RA to normal or Ts-68-infected CEF at 37°C or 41°C had little effect upon the rate of cell growth.

b) Plasminogen activator

The addition of RA to cultures of normal CEF stimulated a rapid, dose dependent rise in intracellular PA activity (Table 1). In the presence of 10^{-6}M RA this rise was evident 1,5 hr after addition and increased to a maximum at 6 hr that was approximately ten-fold higher than in corresponding RA-free cultures, after which intracellular levels fell gradually. Lesser concentrations

Table 1. Effect of retinoic acid on plasminogen activator induction in chick embryo fibroblasts

	Addition	Plasminogen activator (U/mg protein)
	None	60
Retinoic acid	10^{-6}M	795
Retinoic acid	10^{-7}M	698
Retinoic acid	10^{-8}M	594
Retinoic acid	10^{-9}M	430
Retinoic acid	10^{-10}M	60

Normal chick embryo fibroblasts were seeded at 10^6/60 mm petri dish at 37°C. 24 hr later medium alone or medium containing retinoic acid was added to the cultures. 6 hr later duplicate petri dishes were removed and assayed as described in Methods.

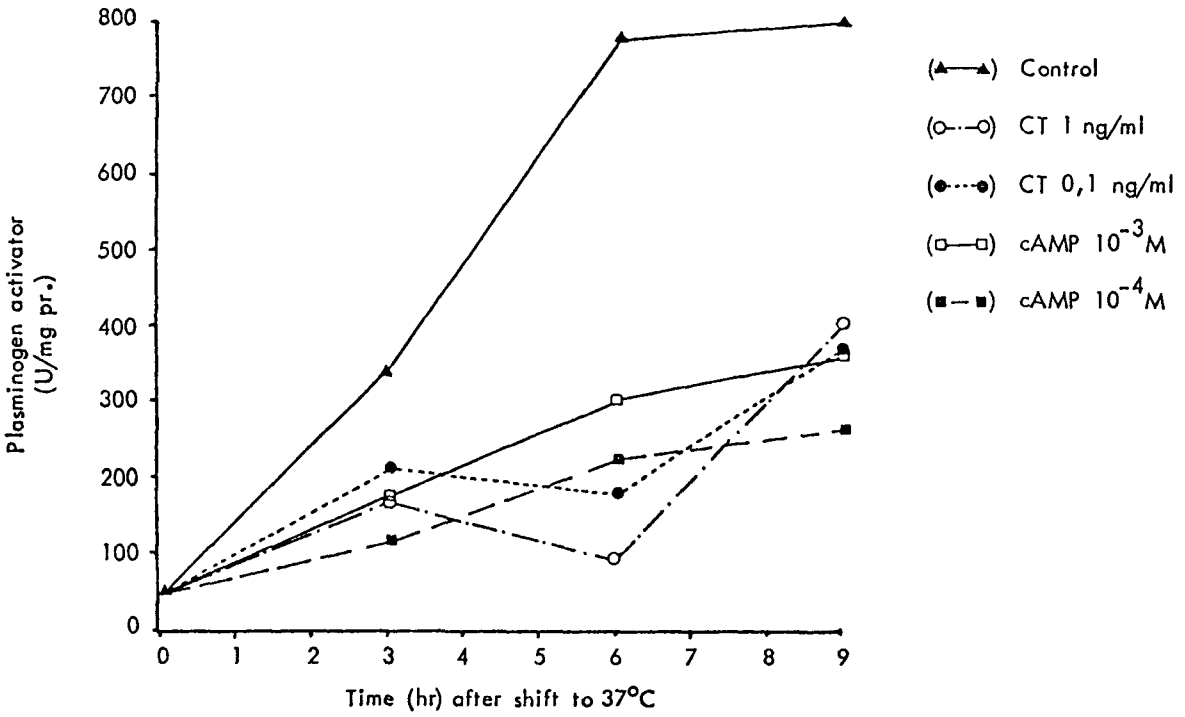

Fig. 1. The effect of cholera toxin and cyclic nucleotides on plasminogen activator induction in Ts-68-infected cells after shift to the permissive temperature for transformation. Ts-68-infected cells were seeded at 10^6/60 mm petri dish at 41°C. 6 hr later cholera toxin at 0,1 ng/ml or 1,0 ng/ml was added to some of the cultures. 18 hr later the medium was removed and replaced with fresh medium at 41°C containing no addition (▲——▲); cholera toxin 0,1 ng/ml (●----●); cholera toxin 1,0 ng/ml (O–·–O); cyclic AMP 10^{-3}M (□——□) or cyclic AMP 10^{-4}M (■——■). The dishes were replaced at 41°C for 1hr, after which some of the cultures were shifted to 37°C. 3, 6 and 9 hr later duplicate plates were removed from 41°C and 37°C and assayed for intracellular plasminogen activator as described in "Methods"

of this compound were also effective, with a significant rise in PA apparent after 3 hr exposure to 10^{-9}M RA (Wilson and Reich, 1978).

Addition of RA to Ts-68-infected cells at both permissive (37°C) and non-permissive (41°C) temperatures also caused a substantial increase in enzyme activity. The magnitude of the increase in enzyme activity observed when RA and viral transformation were acting together at 37°C was far greater than that observed when either was acting alone. The effects of RA and sarcoma gene expression on PA induction were therefore synergistic rather than additive, in analogy with the pattern previously found for phorbol esters (Weinstein et al., 1977; Wilson and Reich, 1979).

Removal of RA from the medium was followed by a progressive decline in PA activity. This fall was significantly inhibited by treatment with Actinomycin D indicating that, as in the case of de-induction after PMA withdrawal (Wigler and Weinstein, 1976) or following temperature shift to non-permissive conditions (Rifkin et al., 1975) the suppression of PA synthesis following RA withdrawal is an active process requiring synthesis of new RNA. Essen-

tially similar reversibility was observed when RA was removed from Ts-68-infected cultures incubated at 41°C or 37°C.

When normal CEF were treated with cycloheximide (10 μg/ml) or Actinomycin D (1 μg/ml) for 30 min prior to addition of RA, the rise in cellular enzyme activity was completely inhibited. Similar results were obtained with Ts-68-infected cultures at 37°C or 41°C. Thus both RNA and protein synthesis are required for enzyme induction by Vitamin A.

Retinoic acid, retinol and Vitamin A acetate, in that decreasing order, induced PA synthesis in cultures of normal CEF. The action of all three compounds was inhibited by Actinomycin D.

Since 3′-5′ cyclic nucleotides mediate numerous cellular processes, and these compounds, or substances which modify their metabolism are known to have effects on PA levels (Strickland and Beers, 1976; Vassalli et al., 1976) a number of experiments were performed to examine the effects of cAMP and cholera toxin on the induction of PA synthesis in CEF.

As seen in Fig. 1 cAMP at concentrations of 10^{-3} M and 10^{-4} M and cholera toxin at 0,1 ng/ml and 1,0 ng/ml, inhibited the increase in PA levels associated with a shift from restrictive to permissive temperatures.

In contrast, neither cAMP nor cholera toxin inhibited the induction of PA by retinoic acid (Wilson and Reich, 1978) indicating that the control of RA-induced PA synthesis, in contrast to the virally induced increase in PA levels, is not related to the cellular concentration of cyclic nucleotides.

Discussion

Studies of a series of phorbol derivatives (Weinstein et al., 1977) have shown that the potency of these compounds for inducing PA in CEF cultures closely parallels their potency as tumour promoting agents in mouse skin carcinogenesis. The results presented here suggest that Vitamin A derivatives may have an in vitro tumour promoting action that corresponds with their in vivo action in the RSV-chicken system (Polliack and Sasson, 1972) or the DMBA-hamster cheek pouch system (Polliack and Levij, 1969) but it is difficult to reconcile with studies that have ascribed antineoplastic activity to retinoids (Sporn et al., 1976). These diametrically opposed views and conflicting reports on the action of retinoids in pre-neoplasia may well reflect differences in the way in which different species or tissues respond to Vitamin A.

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The Molecular Biology of Differentiation and Transformation: An Emerging Field Theory

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It appears to me that finally, a glimmer of insight is to be seen in the basic mechanism of oncogenic transformation. The emerging picture is based on fundamental observations from a large number of sources. We have been privileged to see the results of some of the currently brightest research in the mosaic during this workshop. The framework for the emerging picture is based on the recognition within recent years that cellular differentiation is a social event among cells in which one cell type triggers the differentiation of another. This principle hardly would have been a revelation to the early embryologists. Perhaps the foremost, technically accessible problem of biology at this time is to understand the specific biochemical mechanisms by which these processes are implemented within the cell.

The Emerging Field Theory

Certain cells exert their function in inducing differentiation in target cells either through cell-to-cell physical contact or from a distance. In both cases, specific substances, frequently glycoproteins, from one cell interact with highly specific receptors on the target cells. If the triggering substance is released from the cell it may be commonly known as a growth substance or hormone. These factors may be active at chemically incredibly low concentrations. At this meeting Malcolm Moore has reported that lactoferrin acts as a factor for the growth of colonies of myeloid cells and that the active component is effective at concentrations of 10^{-14} M! For the differentiation factors to be effective at these concentrations, the binding constant for interaction with the receptor must be extremely large. In most cases it appears that binding of no more than a few molecules per target cell is adequate to elicit the response. In general, a special system will be required to amplify the signal received at the cell surface to a level that it can be effective on intracellular reactions. It is not clear whether or not part or all of the differentiation factor molecules themselves must be somehow transported to the nucleus to effect transcriptional control. The relation of the cellular components involved are schematically depicted in Fig. 1.

A proper relation in time during development as well as space is implicit in the scheme. Differentiation may occur only during a transient period when cells exhibiting the appropriate recognition sites are exposed to the

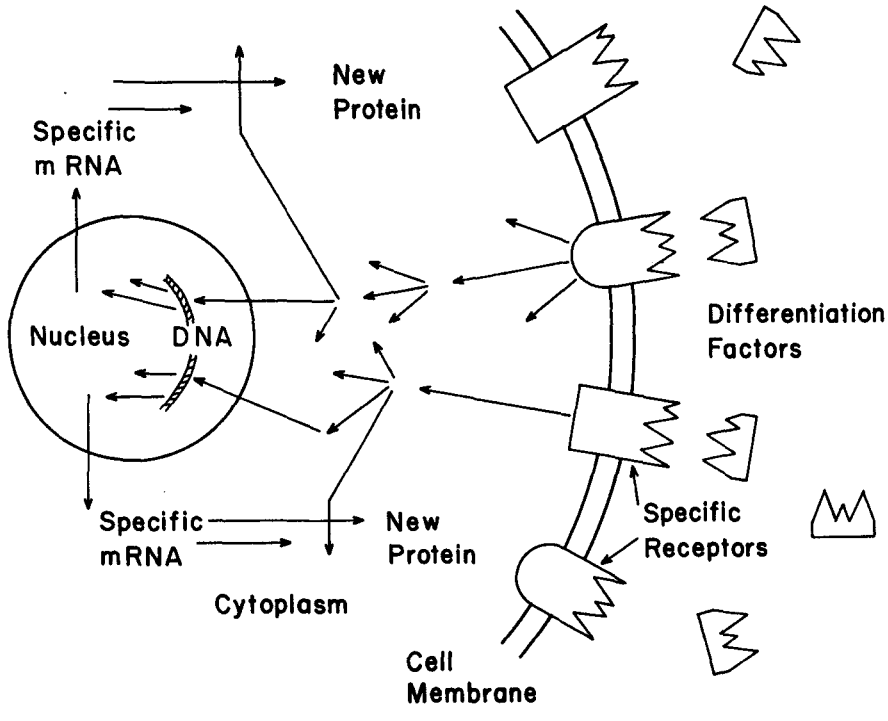

Fig. 1. Diagramatic representation of the cellular components and reactions involved in differentiation

proper signal molecules. Undifferentiated cells may be maintained in the adult organism, then triggered to differentiate into a mature or terminal cell type in which cell division is limited or completely stopped. Differentiation of blood cells provides a classical example of such a system. Thus the function of the cellular genome during differentiation might be equated more correctly with a library than a computer. Information stored in the DNA is expressed when the proper request is received rather than at a fixed point in a free-running program.

Recent evidence appears to indicate that at least in some and probably most situations, the immediate, causal alteration that results in oncogenic transformation involves a lesion in the information retrieval system rather than DNA itself.

Theoretically the lesion might occur at any point from the differentiation factors themselves to the synthesis of a new protein. A number of specific mechanisms can be envisioned. I divide them into four classes based on the location of the lesion:

I. Defective or Deficient Differentiation Factors

A differentiating cell might not be exposed to the proper factors, perhaps because of time-space relationships with other cells or because of a lesion in the cells producing the appropriate differentiation factor(s). In this case the oncogenic cell might be normal in its capacity to recognize and respond to differentiation factors which are not present in its environment. Certain teratocarcinoma appear to be excellent candidates for this class. Exciting

experiments involving implantation of normal embryonic and teratocarcinoma cells into pseudo-pregnant mice to form normal, chimeric or allophenic progeny strongly support such a model (K. Illmensee, and B. Mintz, 1976). Hopefully, additional successful experiments in this area will be reported shortly.

II. Defective Recognition Sites

Another theoretically possible situation might involve numerically inadequate or defective recognition sites on the target cell. Chemically inert, surface reactive materials such as asbestos may function as carcinogens by this mechanism. Such cells might be induced to differentiate if the concentration of the critical factors could be elevated to a level at which they could induce a non-proliferative state. There are a number of reports involving leukemias that may reflect this situation (L. Sachs, 1974; R.C. Gallo et al., 1977) or possible Type I defects. Certain neuroblastomas that can be stimulated to differentiate with cAMP (K.N. Prasad and A.W. Hsie, 1971) may fall into this category. Neuroblastomas that can be stimulated to differentiate in the presence of glia cells (C.P. Reynolds and J.R. Perez-Polo, 1975) and pheochromocytomas in which neurite proliferation can be stimulated with nerve growth factor (A.S. Tischler and L.A. Greene, 1975) might involve lesions at the level of either the differentiation factor or the cellular receptor.

III. Defective Intracellular Signal Transmission

The next level of lesion that can be envisioned involves transmission of the signal received at the cell surface to the transcriptional and translational machinery of the cell. Some virus-induced transformation appears to provide an example of a lesion at this level. Rat kidney cells that have been transformed by Rous sarcoma virus can be caused to revert to a normal phenotype by inhibiting protein synthesis, presumably by blocking the synthesis of an unstable product of the transforming gene (J.F. Ash et al., 1976). The src gene appears to code for a protein kinase that may interfere with intracellular control mechanisms as mentioned below and considered by Ray Erikson at this meeting. It appears that avian and murine leukemia virus carry transforming genes that are quite different from the src gene and code for other proteins that may disrupt the intracellular transmission of the signal in different ways. Murine erythroid cells transformed by Friend leukemia virus provide a system that may belong to this class. Differentiation can be induced in these cells by the simple expedient of growing them in tissue culture in the presence of dimethylsulfoxide (C. Friend et al., 1971) or a number of other aprotic solvents.

In all of the examples given above the transformed cells can be induced to undergo differentiation if they are exposed to the proper external stimulus. The block in differentiation can be overcome! In effect, the cells can be cured

of their "transformed" condition! The point of fundamental importance is that transformation has not involved an irreversible loss of genetic information or a structural change in DNA that precludes further differentiation.

IV. Defective Malignant Cell DNA

Yet another class of oncogenic transformation may involve structural alterations of DNA so that the affected cells lack genetic information required for terminal differentiation. Certain genetically determined cancers may fall into this class. However, it should be noted that some imaginal disc tumors of *Drosophila* that clearly are inherited according to the principles of Mendelian genetics may revert to a differentiating state (E. Gateff, 1978a). Genetically inherited imaginal disc tumors fall into two classes. Those that appear to be irreversibly transformed and others that are capable of differentiation when they develop in close contact with wild type cells (E. Gateff, 1978b). It appears likely that the latter class may involve a mutation that affects the production of a differentiation substance produced in non-malignant cells and thus probably should be classified as a Type I transformation.

X-ray induced leukemias may provide another example of a Type IV transformation in which radiation has resulted in damage of genes required for a late stage of differentiation which has occurred in an undifferentiated cell type. The damage may not be expressed for a relatively long time until the defective cells are induced to start along a differentiation pathway. Thus transformation resulting from changes in the DNA of the malignant cells fall into a fundamentally different category than the three classes considered above, in that they involve seemingly irreversible loss of essential genetic information.

Session Highlights

Peter Duesberg

Dr. Duesberg presented evidence from technically elegant experiments indicating that the transforming capability of avian acute leukemia virus MC29 and avian carcinoma virus MH2 is related to a specific 1,5–2,0 kilobase nucleotide sequence and that the sequence is not closely related to that of the src gene of Rous sarcoma virus. The nucleotide sequence appears to be near the *gag* gene which is located at the 5′-end of independently replicating virus. The results demonstrate clearly the technical capacity of existing techniques to study the structure of transforming genes. Dr. Duesberg suggested the intriguing possibility that transforming genes of these viruses may be host cell genes that have been integrated into the viral genome. They may code for regulating proteins, such as protein kinases, that no longer respond to the normal cellular control systems. Dr. Duesberg's findings were complimented by results presented by Thomas Graf who reported that a specific transforming gene in avian erythroblastosis virus appears to be distinct from the trans-

forming genes of both the avian acute leukemia virus and the Rous sarcoma virus. Dr. Graf also indicated that the transforming genes of avian erythroblastosis virus and avian myelocytomatosis virus may be acquired host cell genes that function in hemopoetic differentiation. He suggested that the product of these genes may induce leukemic transformation by a non-functional interaction with a cellular receptor, thereby competitively inhibiting the unmodified differentiation product of the host cell.

Ray Erikson

Perhaps the most exciting development in recent years in the area of the mechanism of transformation are the results from Dr. Erikson's laboratory, involving characterization of the src gene product. His group has used immunological procedures to detect the protein formed from the src gene of avian sarcoma virus. Of paramount importance is the observation that the protein appears to be a protein kinase that will phosphorylate IgG. It is likely that phosphorylation of IgG is an in vitro artifact. Thus far, there is no direct demonstration of what the substrate(s) in vivo for the kinase might be, however, there is strong indirect evidence suggesting that polymerization of cytoskeletal elements might be involved. Phosphorylation of a cytoskeletal protein appears to cause depolymerization of the cytoskeletal elements (W. Birchmeier and J. Singer, 1977) resulting in changes in the cell membrane that may trigger the physiological changes characteristic of the transformed state. An initiation factor of protein synthesis, eIF-2, also appears to be a possible candidate for the natural substrate of such a kinase. Clearly more work is required to firmly establish the src gene product as a protein kinase, however the data appear to be sufficiently reliable at this point to make this an extremely promising and no doubt intensely competitive area for future work.

Considered togehter, these and data from other sources appear to indicate that peptide(s) formed from specific nucleotide sequences, transforming genes, carried by certain types of virus is the immediate causal agent for oncogenic transformation. There appears to be nothing special about the virus itself or the transforming genes beyond their capacity to code for these special proteins. Furthermore, the nucleotide sequence of the transforming genes are different and apparently code for different peptides. It appears likely that these products will cause transformation by different specific mechanisms.

Gisela Kramer

Data indicating that the src gene product is a protein kinase, has evoked special interest in this area. Dr. Kramer has described cAMP-independent protein kinases that inhibit translation in Friend leukemia cells and rabbit reticulocytes. Activity of cAMP-dependent protein kinases is promoted by binding of cAMP to the regulatory subunit thereby causing it to dissociate from the catalytic subunit of the holoenzyme. However, virtually nothing is known about the molecular mechanism by which cAMP-independent kinases are regulated. The so-called heme-controlled repressor, HCR, from rabbit reticulo-

cytes is held in an inactive from in the presence of heme. Protein kinase activity with high specificity for an initiation factor of protein synthesis, eIF-2, and inhibitory activity for protein synthesis, is generated in the absence of heme both in vitro and in intact cells. An eIF-2 specific protein kinase that appears not to be regulated by heme has been isolated from Friend leukemia cells that have not been stimulated to differentiate by dimethylsulfoxide. It has been speculated that this kinase might be involved in the block in differentiation. It is not known whether or not the kinase is coded by the viral or host cell genome. After stimulation by dimethylsulfoxide, Friend cells appear to gain the capacity to be regulated by heme.

Ian Kerr

Dr. Kerr described what appears to be a different type of system to amplify the signal received at the cell surface. He has shown that interferon treatment of intact cells potentiates the synthesis of an adenine trinucleotide with a very unusual 2′- to 5′-phosphodiester linkage. Double-stranded RNA also is involved in the synthesis of this compound. Interferon is a species-specific glycoprotein that appears to have highly specific cell surface receptors. The target for the unusual adenine trinucleotide is not known but may be a ribonuclease that has been implicated frequently in interferon action. Also, interferon appears to activate a cAMP-independent, eIF-2 specific protein kinase that is physically distinguishable from the heme-controlled repressor. The physiological relation between the trinucleotide and the protein kinase is unclear. Experimentally interferon provides one of the most useful, and biochemically well-characterized examples of an intracellular regulatory system that is triggered by a specific interaction at the cell surface. The interferon system may come to serve as a model for this type of control.

Gebhard Koch

One of the fundamental conceptual problems of translational control involves specificity for the synthesis of specific proteins. How can phosphorylation of a peptide initiation factor that apparently is used during the translation of all mRNA species differentially affect the synthesis of specific proteins? A partial answer to this question has come from Dr. Koch's laboratory. His group has shown that different species of mRNA are translated with very different efficiencies. The relative proportion of products formed from different mRNA's can be altered by changing parameters such as salt concentration that affect the overall rate of the synthetic reaction. For instance viral mRNA typically is translated with high efficiency relative to cellular proteins. However, a reduction in the overall rate of protein synthesis frequently causes a dramatic reduction in the relative proportion of viral and host cell proteins that are formed.

The Future

I believe any detailed prediction of future developments in molecular biology and biochemistry are likely to be wrong or at least incomplete, probably to a major extent. However, there are key problems that must receive continual consideration, and several areas that appear to be ripe for investigation. The concepts inherent in the mechanism of normal differentiation reflected in Fig. 1 will be tested and retested in many systems in the forthcoming years. The most crucial problem for an understanding of normal differentiation is the molecular mechanism(s) by which signals received at the cell surface activate transcription from specific genes. Is part or all of the protein differentiation factor taken into the cell and used in the activation process itself, perhaps at the DNA level as appears to be the case with steroid hormones, or are intermediate reactions involved? For most systems, the physical and chemical characterization of the differentiation factors and their specific receptors presents a severe technical problem in working with the very small quantities that are available. In many cases the assay systems used to monitor isolation are not quantitative and are no more than marginally satisfactory. This presents a formidable problem, especially when the biological response depends on two or more specific components, as frequently appears to be the case. The development of better assay systems, especially in vitro systems involving specific biochemical reactions rather than the response of intact cells, is critical to satisfactory progress in this area.

With respect to the sequence of intracellular reactions triggered by growth substances, two problems or areas stand out as being both technically feasible and crucially important. The first problem involves the mechanism by which signals received at the cell surface are amplified and transmitted to target reactions in the cytoplasm and nucleus. It appears that there are likely to be a number of alternative mechanisms to cascade systems involving protein kinases for amplification of the signal received at the cell surface. The small nucleotide described by Ian Kerr that is produced as part of the interferon and double-stranded RNA system seems to be part of such a system. It is likely that other types of amplification mechanisms will be found. The second problem involves regulation of cAMP-independent protein kinases. A number and perhaps a great many cAMP-independent protein kinases may be involved in amplification and transmission of cell surface signals. The enzyme system that is activated by double-stranded RNA and interferon is an excellent example. What is the specific molecular mechanism by which such enzymes are activated and do they function in cascade sequences?

With respect to transformed cells, the search for differentiation factors and conditions with which transformed cells can be induced to either stop dividing or differentiate to a non-dividing form appears to be the key problem. However it is frought with technical limitations that may limit progress until they are resolved. The transforming genes and their products are ripe for investigation and an investigative effort will be made in a number of laboratories. Are transforming genes really cellular genes that have been integrated into a viral genome in such a way that they no longer respond to

the normal control systems within the cell? What is the biochemical mechanism by which the product of transforming genes disrupt differentiation and induce the physiological changes associated with transformation? It appears possible that the next Wilsede workshop may include hard answers to some of these problems and a consideration of substantiated models of the molecular mechanism by which oncogenic transformation occurs. Eventually, I believe such insight will provide the basis for a rational therapy to cure leukemia at the cellular level.

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Leukemic Cell Phenotypes in Man: Relationship to 'Target' Cells for Leukemogenesis and Differentiation Linked Gene Expression

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Established cell lines derived from malignant cells are proving to be of considerable value in the investigation of differentiation and controls of gene expression. This is especially evident in the leukemias; the Friend virus-induced erythroleukemia (Harrison, 1977), avian erythroleukemia (see Graf, this book) and myeloid leukemias in rats (Lotem and Sachs, 1974) and a recently described human myeloid line (Collins et al., 1978), all having a maturation arrest which can be reversed by various agents and to some extent controlled by viruses (see papers by Gallo, Ruscetti and Graf in this book).

During the past few years we have developed reagents for an immunological analysis of acute and chronic leukemias in man and permanent lines established from these cells (reviewed in Greaves, 1975; Greaves and Janossy, 1978; Thierfelder et al., 1977). Part of the incentive has been to provide a routine immunodiagnostic service; this is now operational and effectively identifies subgroups of Acute Lymphoblastic Leukemias (ALL) which are distinct in terms of prognosis (Chessells et al., 1977; Greaves, 1978; see also Pinkel, D., this book). A longer-term interest has been to use ALL cells and their cell line derivatives for analysis of early lymphoid cell development and diversification. Table 1 lists the antisera and enzyme assays that we use to characterize leukemic cells. All the antisera are directed against cell surface determinants and their binding is detected by immunofluorescence and with the Fluorescence Activated Cell Sorter (FACS) as previously discussed (Greaves, 1975; Greaves et al., 1976).

1. Subgroups of Acute Lymphoblastic Leukemia and Lymphoid Lineage Differentiation

Fig. 1 illustrates the heterogeneity of ALL as revealed by the various immunological markers and enzyme assays. Four major groups are identified and within these fairly clear subsets. The four subgroups have different prognosis in both children (Chessells et al., 1977) and adults (Lister et al., 1979). These profiles are assembled from the analysis of over 1000 acute leukemias and we believe that they reflect relative stable maintenance of normal gene expression by the major subclones in these leukemic patients, i.e. the phenotype of each type is appropriate for the developmental level at which maturation arrest has occurred and the markers are in essence differentiation-, or matura-

Table 1. Markers used to Phenotype Human Leukemic Cells[a]

[a] Details of these markers are reviewed in Greaves (1975) and Greaves and Janossy (1978). References for individual markers are also given in the text of this paper

Membrane markers	
Antisera:	Anti-ALL
	Anti-p28,33 ('Ia')
	Anti-HuTLA (T cell antigen)
	Anti-M (Myelomonocytic antigen)
	Anti-L (Lymphoid antigen)
	Anti-SmIg (membrane immunoglobulin)
Sheep erythrocyte rosettes	
Mouse erythrocyte rosettes	
Cholera toxin	
Enzyme markers	
Terminal deoxynucleotidyl transferase (TdT)	
Hexosaminidase isoenzyme (HEX-I)	
Acid phosphatase (AP)	

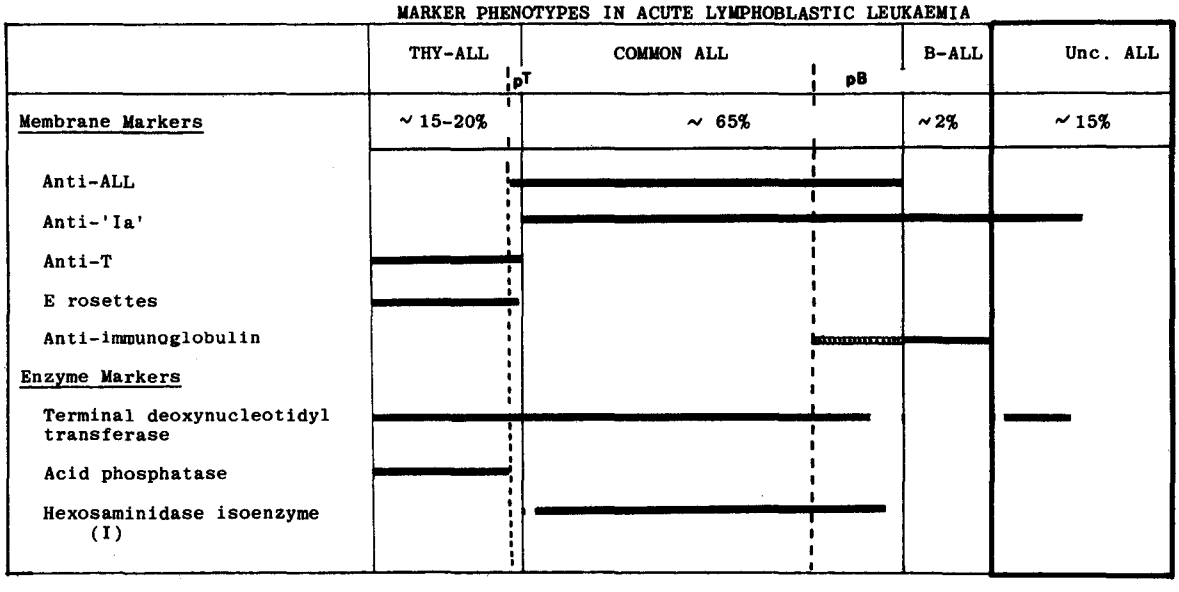

Fig. 1. Major phenotypes observed in Acute Lymphoblastic Leukemia (ALL). The solid horizontal bars are proportional to the number of patients positive for the given marker. Over 1000 patients (~90% untreated, 10% in relapse) have been tested with anti-ALL, anti-SmIg and E rosettes and between 100 and 500 tested with other markers. Results from children (80% of cases) and adults have been pooled to construct the above profile; the size of subgroups is, however, different in children and adults. In children 75% of ALL's are anti-ALL serum positive and 15% of T-ALL's whereas in adults (> 16 years) only 50% are anti-ALL positive and 10% are T-ALL's. Thus, the unclassified category is larger in adults.
Unc: Unclassified ALL ($cALL^-$, T^-, E^-, $SmIg^-$)

tion-linked. As discussed elsewhere (Greaves and Janossy, 1978) there is no evidence that any of these antisera detect leukemia-specific antigens; furthermore we have questioned whether any such antigens exist with any consistency (Greaves and Janossy, 1978).

It seems plausible that the subgroups of ALL identified represent virtually all the early lymphoid lineage maturation compartments, the subgroups being overlapping rather than completely distinct. A 'polarity' of the groups can be revealed by the overlap of individual characteristics. Thus a subset of T-ALL ($\sim 10\%$) have only a partial T or thymic phenotype (see Fig. 1) and express the cALL antigen, albeit weakly. We refer to these as 'early' or pre-T's. Similarly within the major or common ALL subgroup are leukemic cells with 'early' or pre-B features, i.e. cytoplasmic IgM but no detectable cell surface Ig (Vogler et al., 1978; Greaves, Verbi, Vogler and Cooper, unpublished observations). The 'unclassified' or 'null' subgroup in turn has phenotypic overlap with the cALL group by expressing (in some cases but not all) TdT and Ia antigens. We suspect therefore that these profiles may reflect fairly accurately normal patterns and sequences of gene expression in lymphocyte ontogeny. To establish this hypothesis experimentally requires identification of normal precursors with equivalent phenotypes and an in vitro system in which sequential maturation linked phenotypes can be induced (see below).

ALL is principally a proliferation of immature lymphoid cells. Several types of T and B neoplasms exist, however, where the majority of cells are 'frozen' at levels equivalent to relatively mature lymphocyte subsets. This point has been emphasized by Salmon and Seligmann (1974) in the context of B cell leukemias (CLL), lymphomas and myeloma and seems also likely to be correct for T lineage malignancy. In Fig. 2 we have constructed a tentative cell lineage map in which the leukemic phenotypes we have observed are placed in position and sequence according to a possible lineage order of gene expression.

2. Molecular Nature and Cellular Selectivity of Expression of the cALL Antigen

In hemopoietic malignancy the cALL antigen is detected only in those which are considered lymphoid or undifferentiated, i.e. AUL, cALL and Ph^1 positive 'lymphoid' blast crisis of CML (Greaves et al., 1977; Roberts et al., 1978). Cross-absorption studies indicate that the same antigenic determinant is found in these different lymphoid leukemias (Roberts et al., 1978); in addition the antigen has the same general molecular characteristics when isolated from Ph^1 positive 'lymphoid' blast crisis, cALL and T-ALL cell lines (Sutherland et al., 1978). The cALL antigenic determinant is found on a single glycosylated polypeptide with an apparent molecular weight of 100,000 daltons in SDS polyacrylamide gel electrophoresis. This molecule is released or secreted by leukemic cells and leukemic cell lines and runs in the same position with and without reducing agent. All of the molecules bind to ricin lectin (which 'recognises' terminal galactose residues) but only

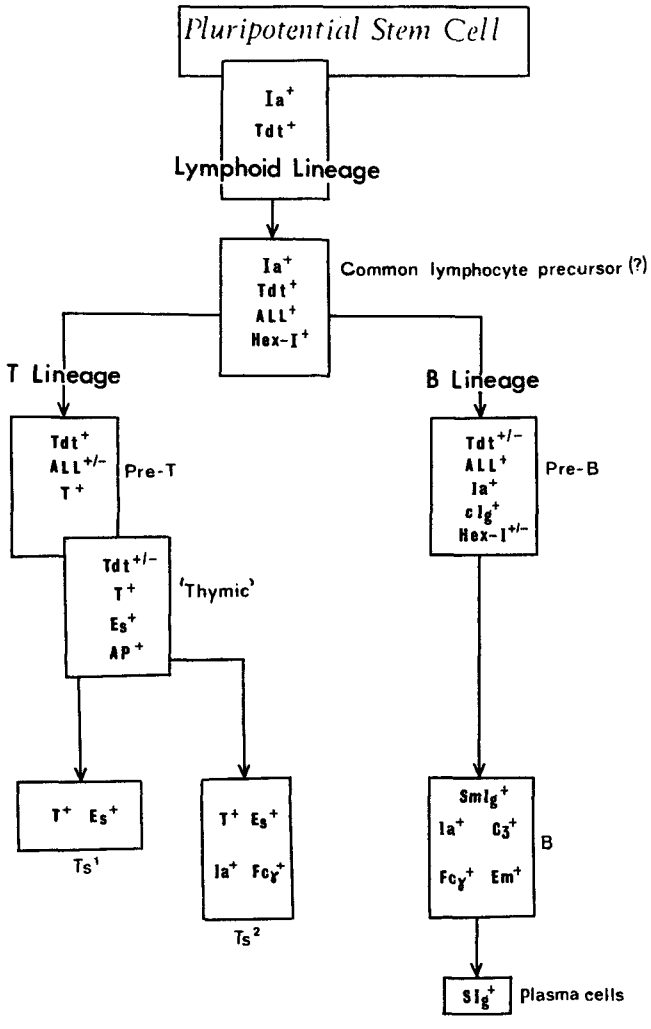

Fig. 2. A hypothetical map showing the sequence of gene expression in the lymphoid lineages as suggested by analyses of phenotypes of leukemic cells
+ positive8 – negative
See Table 1 for abbreviations.

Fcγ	Receptor for IgG
C3	Receptor for complement component C3
Em	Receptor for mouse erythrocytes
CIg	Cytoplasmic Immunoglobulin
SmIg	Surface membrane Immunoglobulin
SIg	Secreted Immunoglobulin

a fraction to lentil lectin (-terminal glucoside or mannoside residues); its isoelectric point is 5.6 (Smart et al. 1979).

Recently we have been able to identify a normal 'lymphoid' cell with ALL^+, Ia^+, T^-, $SmIg^-$ phenotype of cALL cells (Greaves and Janossy, 1978). We had previously failed to find such cells in normal adult marrow but now find them in fetal hemopoietic tissue, regenerating marrow (e.g. post-transplantation for aplastic anemia or post-chemotherapy for malignancy) and in neonatal, lymphoproliferative non-malignant 'leukemoid' reactions. Although the ALL antigen has not been isolated from these sources and shown to be identical with the antigen on leukemic blasts we conclude that the cALL antigen is most probably a normal gene product of lymphocyte precursors.

3. Acute Myeloid Leukemia (AML) Associated Membrane Antigens and Myeloid Lineage Differentiation

90% of the AML cases we have tested (~100) have been positive for the M (myeloid) antigen and negative for the ALL antigen (M^+, ALL^-) as are cases of CML (10/10) and a proportion of CML in blast crisis (17/25) (Roberts et al., 1978; Roberts and Greaves, 1978). In the majority (~90%) of cases of AML (Schlossmann et al., 1976; Janossy et al., 1977a) and Ph^1 positive CML

in blast crisis (Janossy et al., 1977a and b) the myeloblasts are also Ia or p28,33 positive; the granulocytic cells in CML itself are Ia negative (Janossy et al., 1977a and b). In our series 10/10 AML were Hex-I negative (Ellis et al., 1978) and 17/17 TdT negative ($\sim$0·2 μ/10^8 cells, Hoffbrand et al., 1977). The major phenotype of AML is therefore: M^+, Ia^+, ALL^-, Hex-I^-, TdT^- and, in addition, $HuTLA^-$, E^-, AP^+ (see in Tables 1 and 2). Myeloblasts in AML may sometimes be SmIg positive (k plus λ) due to adsorption of IgG to Fcγ receptors. There is often considerable variation in the levels of M and Ia antigens on cells from individual patients with AML or CML in blast crisis. This heterogeneity can be evaluated by the Fluorescence Activated Cell Sorter and has been shown to correlate with the degree of morphological maturation within the granulocyte lineage (Roberts and Greaves, 1978; Janossy et al., 1977a and b). Parallel studies on normal marrow and blood samples indicate an essentially similar relationship (Roberts et al., 1978; Janossy et al., 1977b; Winchester et al., 1977) suggesting that membrane marker expression on leukemia clones is maturation-linked and essentially normal. In our laboratory absorption of three rabbit and one rhesus monkey anti-Acute Myeloid or Myelomonocytic sera with normal bone marrow (M. Greaves, unpublished observations) removed all activity against leukemic cells indicating that these particular sera contained no leukemia specific antibodies. Mohanakumar and colleagues (Mohanakumar et al., 1975; 1976) have reported different results with various primate antisera.

The finding of anti-Ia(p28,33) binding to AML as well as ALL cells was unexpected and intriguing. Previously similar 'anti-B' cell sera were thought to be leukemia-specific; these studies probably gave inadequate consideration to cellular controls since several normal cell types are positive with anti-B

Table 2. Acute lymphoblastic leukemia associated antigen Expression in hemopoietic malignancy

Positive:	non-T,non-B ALL (85% of childhood ALL, 55% of adult ALL, total studied: 500) Thy-ALL (10%, pre-T) (12) pre-B-ALL (25) Ph^1+ AL/CML in 'L' blast crisis (30) AUL (50%, 15)
Negative:	Acute Myeloid, Monocytic, Myelo-monocytic leukemia (130) Chronic Myeloid Leukemia (25) Ph^1+ CML in 'M' blast crisis (55) Thy-ALL (90%, 110), B-ALL (8) Chronic Lymphocytic Leukemia (B,T (25)) B lymphomas, myeloma (30) Sezary syndrome (5) Erythroleukemia (7)

Taken from Roberts and Greaves, 1978, Greaves et al., 1977, and the unpublished observations of Greaves, Roberts and Janossy.
Number of cases studied in brackets

or 'Ia'-like sera (Greaves and Janossy, 1978). The presence of Ia^+(p28,33) molecules on AML cells is rationalized by correlation with the maturation status of these cells. This can be clearly demonstrated by an investigation of Ia antigen expression on normal myeloid precursors in vitro (M-CFUc) (see Moore, M., in this book). When Ia(p28,33) positive cells from normal bone marrow are selected on the Fluorescence Activated Cell Sorter using sterile techniques it can be shown that the majority of CFUc in man express the Ia antigen (Greaves et al., 1978; Janossy et al., 1978). It has also been shown that 'anti-B' cell sera and complement eliminate M-CFUc (Cline and Billing, 1977; Kaplan et al., 1978); although this latter approach is technically less satisfactory the result accords with expectations.

4. Levels of Maturation Arrest and 'Target' Cells for Leukemogenesis in Man

The phenotype described for leukemic cells and the corresponding level of maturation arrest does *not* necessarily identify the 'target' cell. A block to further maturation may occur at the level of 'target' cells (e.g. in some Avian leukemias, see Graf, this book). However, there is evidence that this need not be the case. This point is best exemplified by chronic myeloid leukemia (CML) which is a hemopoietic stem cell disorder associated with a specific chromosomal change [t(22Q$^-$): the Ph^1 or Philadelphia chromosome] (see papers by Rowley and Fialkow in this book). This Ph^1 positive clone is usually dominant in the granulocytic, monocytic, erythroid and platelet lineages even though overt leukemia is only usually evident in the granulocytic series. Attempts to identify the Ph^1 chromosome in PHA stimulated (T) lymphocytes have generally failed. This would appear to place the 'target' cell at the level of a myeloid stem cell with all hemopoietic options excluding lymphocytes. Recent evidence indicates, however, that in some cases at least the target cell might in fact be the pluripotential stem cell (Janossy et al., 1976). CML frequently enters an acute phase or 'blast crisis'; in a minority of cases the cells seen in this stage of disease resemble ALL cells which prompted Boggs to propose that this might indicate that CML was a pluripotential stem cell disorder (Boggs, 1974). Morphology, of course, can be misleading. Detailed phenotypic analysis of CML in blast crisis, however, supports the pluripotential stem cell thesis (Janossy et al., 1977a; Greaves and Janossy, 1978). Some blast crises are TdT positive (Sarin et al., 1976; McCaffrey et al., 1976; Hoffbrand et al., 1977) and more than 30 cases have been identified in our own series which share the cALL phenotype (i.e. $cALL^+$, Ia^+, TdT^+, $SmIg^-$, T^-, E^-, M^-; cf. Table 1 and Fig. 1) (Janossy et al., 1977a; Hoffbrand et al., 1977). The ALL and Ia antigens on Ph^1 positive cells are completely cross-reactive with those of Ph^1 negative ALL cells (Janossy et al., 1977a) and have the same general molecular characteristics (Sutherland et al., 1978; see above). Cell separation studies indicated furthermore that the Ph^1 chromosome was indeed present in the 'ALL' type cells in blast crisis (Janossy et al., 1978). We have suggested (Janossy et al., 1976; Greaves and Janossy, 1978) that the sharing of a common phenotype is indirect but compelling evidence for the

following two points: 1. That common ALL and some blast crises of CML have a maturation arrest at the same or closely-related level of early lymphoid lineage development and, 2. that, saving for second independent malignancies arising, these cases of CML *must* have been initiated in a pluripotential stem cell. It might be argued that since we have no instance (in over 100 cases examined) of a mature T or B cell phenotype arising in a CML that the credibility of this interpretation rests on the assumption that the cALL phenotype is exclusive to the lymphocyte lineages. At present this is difficult to establish unequivocally although we know that M-CFUc are ALL antigen negative (Janossy et al., 1978).

Recent observations have, however, provided direct evidence for involvement of the B cell lineage in Ph^1 positive CML. Out of 20 cases of blast crisis of CML investigated 3 had a 'pre-B' phenotype with cytoplasmic IgM (Greaves et al., 1979); similarly, the Ph^1 positive cell line (NALM-1) established from a case of CML in blast crisis also has a 'pre-B' phenotype.

This observation accords with recent data from Fialkow in which enriched B cell populations from a patient with Ph^1 positive CML appeared to be monoclonal with respect to Glu-6-P-D enzymes (see Fialkow, this book).

The developmental level of maturation arrest observed in leukemia is therefore very much a reflection of the competence of the dominant subclone to respond to maturation-linked signals – a critical factor which might alter radically as the disease progressively evolves (Nowell, 1976); in this respect CML in man finds an approximate parallel in the Friend virus-induced erythroleukemias of mice (Jasmin et al., 1976). Myeloma in man provides another similar instance. The myeloma Ig idiotypic antigens have been identified in pre-B cells in several patients (M. Cooper, personal communication) and, in one instance, on T as well as B cells (Preud'homme et al., 1977). These observations suggest that myeloma need not be a transformation of plasma cells but can be initiated in early B cells, a common T plus B progenitor or even earlier in the lineage sequence.

5. Leukemic Cell Lines

We have recently characterized a large series of leukemia and normal cell lines with respect to the antigenic markers discussed above (Table 1). These studies were carried out in collaboration with many different colleagues who established the original lines (see footnote to Table 3 and references therein). Table 3 is a summary of cALL antigen expression in cell lines; other details of the phenotypes can be found in the references given. The cALL antigen expression in cell lines parallels that observed in uncultured leukemic cells except that it is present in 2 B-cell lymphoma lines and unexpectedly in five out of six T-ALL lines (Minowada et al., 1978). One of the latter, MOLT-4, has no detectable cell surface ALL antigen but synthesizes and releases the 100K dalton polypeptide bearing the ALL antigenic determinant into the culture medium (Sutherland et al., 1978). The Ph^1 positive line NALM-1 established from a case of blast crisis of CML in a young girl has the cALL

Table 3. Acute lymphoblastic leukemia associated antigen Expression in leukemic cell lines

	Positive	Negative
1. *ALL*		
common ALL	3	0
Thy-ALL	5	1
1. *Ph^1+ CML-bc*	1	1
3. *B cell lines*		
B-ALL	1	3
Burkitt's Lymphoma	1	6
Non-Burkitt's Lymphoma	1	8
Myeloma	0	2
Normal B cells	0	20
4. *Others*		
Histiocytic lymphoma, Myelomonocytic leukemia, neuroblastoma	0	5

From Minowada et al., 1978, Rosenfeld et al., 1977, plus unpublished observations of Greaves, Nilsson and Schneider

phenotype characteristic of 'lymphoid' blast crisis (Minowada et al., 1977). In contrast the line K562 which was also established from Ph^1 positive blast crisis of CML lacks all of our markers including the 'myeloid' antigen. The latter antigen is, however, expressed on HL-60, a pro-myelocytic line (Boss and Greaves, unpublished; see also Gallo, this book) and U-937, a histiocytic lymphoma line (Roberts and Greaves, 1978). RAJI and NALM-6 are interesting lines in having a dominant 'pre-B' like phenotype, i.e. no or very little cell surface Ig but positive for cytoplasmic IgM (Greaves and Minowada, unpublished observations). It is important to note that all the ALL lines are difficult to establish and all those currently available are derived from patients in relapse. Also, it is probably very significant that all lines derived from malignant hemopoietic cells have aneuploid karyotypes. We hope to be able to use some of the ALL lines to investigate early lymphocyte differentiation in man. So far we have not been able to induce phenotypic alterations in these lines using ligands effective in other systems (e.g. DMSO, butyric acid) (Greaves and Verbi, unpublished observations).

6. Summation: Leukemic Cell Phenotypes and Hemopoietic Differentiation in Man

The analysis of an extensive series of acute and chronic leukemias of man with a panel of antigenic and enzymatic markers has provided us with some

insight into the heterogeneity of these leukemias and their biological properties. Irrespective of the outcome of our more academic objectives it is already clear that the subgroups of leukemias defined by these tests (e.g. in ALL and CML blast crisis) are distinct in terms of prognosis and therefore are important in providing useful aids in diagnosis and the allocation of patients to appropriate therapy groups.

The main conclusion to be drawn from this study to date is that the phenotypes appear to reflect normal gene expression which is appropriate for the developmental level at which maturation arrest has occurred. Leukemia-specific antigens, if they exist at all, have not been revealed by these studies. By definition the leukemia-associated membrane antigens are *differentiation* antigens whose expression is restricted in terms of cell lineage and maturation levels. It is important to recognise that some antigens, e.g. the cALL and p28,33 antigens may be restricted to infrequent hemopoietic precursors in particular lineages; their expression in leukemia might readily be misinterpreted as indicative of either fetal gene derepression (i.e. 'onco-fetal' antigens) or the presence of unique leukemic determinants.

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Characterization of Antigens on Acute Lymphoblastic Leukemias

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Introduction

Detectable differences in surface membrane characteristics of normal lymphocyte populations have facilitated the classification of leukemic cells according to cell origin and stage of differentiation. Recent reports show that distinct leukemic cell populations do not only express markers found on normal cell populations. Using specific antisera new antigenic determinants on cells of common acute lymphoblastic leukemia (cALL) and cells of some chronic myeloid leukemias (CML) in blast crisis have been identified [1,11]. The discovery of a cALL-antigen allows the identification of further subgroups of ALL, which has already been described as a heterogenous disease by clinical and morphological criteria [4]. The nature of the cALL-antigen and the question of its occurence during normal hemopoietic development is still speculative. Our studies investigate the distribution of cALL-antigen in correlation with common markers on normal cells, cell lines and leukemic cells of a major group of ALL patients and analyze the effect of anti-cALL antibodies on hemopoietic precursor cell populations.

Material and Methods

Normal and leukemic blood cells: The preparation of cells from peripheral blood and tissues has been described in detail elsewhere [12].

Leukemic Cell Lines: NALM was established from Ph^+CML in blastic crisis [6], REH from E^+ALL [13], U-698M from a lymphosarkoma using the spongestan grid technique [8] and MOLT-4 [5] and JM [14] from E^+ALL. All lines were kindly provided to us by the authors.

Indicator Systems: Cytotoxicity, complement fixation, indirect immunofluorescence, rosette formation and colony forming unit (CFU-c) test were used for the analysis of the different cell populations and have been described previously [7,12,15]. *Anti-cALL-globulin (AcALLG):* anti-cALL serum was produced by immunizing rabbits with cALL cells (negative for surface Ig, T-antigen and E-rosettes). The antiserum was absorbed with liver/kidney homogenate, normal spleen cells, peripheral blood lymphocytes, CLL cells,

and selected lymphoblastoid cell lines of the B-cell typem Further details including the purification of the globulin fraction are described elsewhere [10, 12, 17].

Anti-T-cell globulin (ATCG): ATCG was prepared from rabbit anti-human thymocyte globulin by absorption with liver/kidney homogenate different CLL-cells of the B-cell type and B-lymphoblastoid cell lines [12]. The globulin fraction (ATCG) was isolated as described for AcALLG. ATCG showed a specific reaction for cells of the T-lymphocyte series and no further crossreactions with other blood cells [9, 12].

Results and Discussion

Crude antisera against cALL cells (ALL-cells without markers such as E-rosette formation, T-cell antigen, and SMIg) were not able to discriminate between normal blood and leukemic blasts. Subsequent absorption with liver/kidney homogenate, normal lymphocytes, and CLL-cells eliminated the crossreaction with normal lymphocytes and granulocytes. Nevertheless, this antiserum still gave a clear reaction with the immature precursor stages of the myelopoietic and erythropoetic differentiation of normal bone marrow and several lymphoblastoid B-cell lines. These crossreactions of the antiserum were undesirable, since they complicated the determination of specific antigen-positive cells in the bone marrow. Additional absorption of the antiserum with B-lymphoblastoid cell lines, eliminated the crossreactions with bone marrow cells but still left a highly specific reactivity to cALL cells. This was obviously caused by antigens which occured both in a series of normal bone marrow cells and in B-lymphoblastoid cell lines, and which were also found on cALL cells in addition to the cALL antigen itself [11]. All subsequently described investigations were conducted with an antibody preparation isolated from the crude antiserum by absorption with all the cell types specified and followed by a subsequent purification process resulting in the specific AcALLG.

Analysis of normal cell populations with AcALLG revealed that a detectable amount of cALL-antigen was not found in any of the cell sources examined, such as thymocytes, peripheral blood lymphocytes, granulocytes, and bone marrow cells. The reaction of AcALLG with thymus, spleen and bone marrow cells prepared from 3 and 6 month fetuses was also negative. These indicator systems cannot exclude, with absolute certainty, the presence of a very small cALL-positive cell population among a largely negative one. There are indications, that ALL-antigen may be present to a greater extent in early precursor blood cells where it acts as a differentiation antigen [1]. To investigate this question the effect of AcALLG on precursor cells was examined in a colony-forming test (CFU-c) (Fig. 1). Bone marrow was incubated with AcALLG in the presence of complement and then cultivated using in an agar layer technique [7]. The CFU-c-reduction was measured in comparison with a bone marrow preparation incubated with normal rabbit globulin without any activity. While non-absorbed globulin against cALL cells eliminated all the CFU-c up to log 2 titer 7, highly-specific AcALLG did not cause any

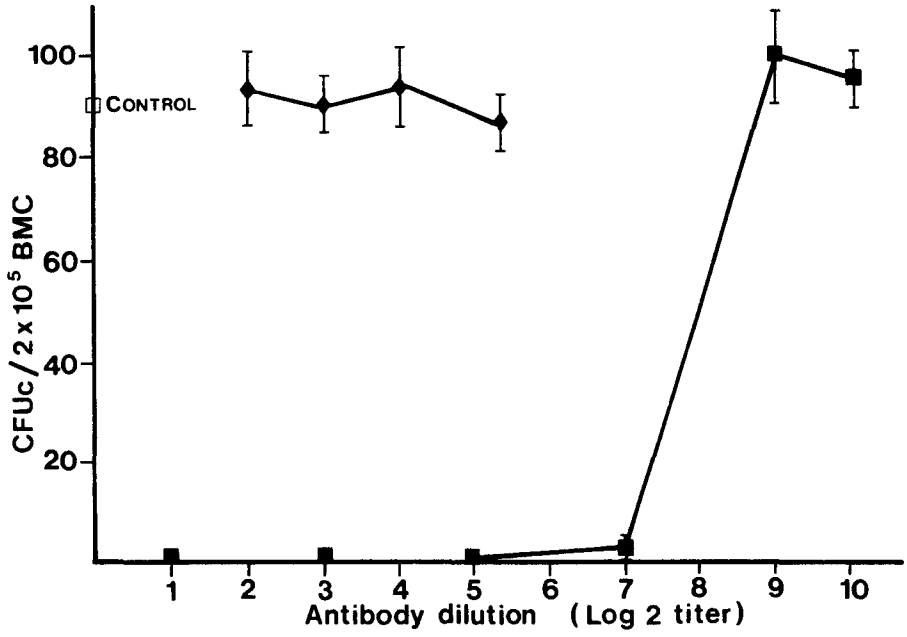

Fig. 1. Effect of unabsorbed (■——■) and absorbed (♦——♦) anti-cALL-Globulin on CFU-c of human bone marrow in the presence of complement

detectable CFU-c reduction, even in higher concentrations. Therefore cALL-antigen seems not to be expressed on progenitor cells growing in this test system.

We then analysed the leukemic cells of 174 patients with ALL for the expression cALL-antigen and common markers such as E-rosette formation, T-cell antigen and surface Ig (SIg) (Table 1). 13 leukemias without detectable surface markers remained unclassified (AUL). The leukemias of the remaining 161 patients could be classified in 6 groups: 56 childhood and 8 adult ALL carried only cALL-antigen and were therefore of common ALL-type, 35 childhood and 4 adult ALL expressed both cALL-antigen and T-cell antigen. The latter phenotypic pattern was expressed by the NALM and REH cell line. We found simultaneous expression of cALL-antigen and SIg in 1 childhood ALL. A similar expression of markers was found in the lymphosarcoma cell line U698M. 15 childhood and 9 adult ALL expressed only the T-cell antigen, 23 childhood and 6 adult ALL showed additional E rosette formation. The latter pattern was also found in cell lines like MOLT of JM. 2 leukemias expressed SIg and were identified as B-ALL. Some of the leukemias classified as cALL may possibly belong to a pre-B form described by Vogler et al. [18] showing cytoplasmatic Ig expression, which was only detected in fixed cell preparations. These data show that at least 6 clearly definable subgroups of acute lymphoblastic malignancies can now be identified. The pattern of differentiation markers of each subtype may allow the stage of development of the leukemias to be deduced. It is still not completely clear, whether cALL antigen is a genuine differentiation antigen reflecting a regular cell development, or whether it is the reflection of pathologic changes taking place in this phase due to an agent able to transform the cells. There is no experimental evidence to support the hypothesis that cALL-antigen is an

Table 1. Expression of cell surface markers (cALL-antigen, T-cell antigen, spontaneous rosette formation and membrane immunoglobulin) in patients (132 children and 29 adults) with ALL and lymphoid cell lines. Analysis of the populations was performed in several test systems like complement fixation, immunofluorescence, cytotoxicity, rosette formation and immunoautoradiography

ALL Classification[a]	Children No. (%)	Adults No. (%)	Phenotypic pattern cALL antigen	T-cell antigen	SRBC rosettes	Surface Ig
c-ALL	56 (42.4)	8 (27.6)	⊕	—	—	—
c/T-ALL	35 (26.5)	4 (13.8)	⊕	⊕	—	—
Pre-T-ALL	15 (11.4)	9 (31.0)	—	⊕	—	—
T-ALL	23 (17.4)	6 (20.7)	—	⊕	⊕	—
c/B-ALL	1 (0.8)	–	⊕	—	—	⊕
B-ALL	2 (1.5)	2 (6.9)	—	—	—	⊕
Lymphoid cell lines	Origin					
NALM	CML blast crisis		⊕	⊕	—	—
REH	c-ALL		⊕	⊕	—	—
U698-M	Lymphosarcoma		⊕	—	—	⊕
MOLT-4	T-ALL		—	⊕	⊕	—
JM	T-ALL		—	⊕	⊕	—
IFH-16	PBL[b]		—	—	—	⊕
IFH-20	PBL		—	—	—	⊕

[a] 13 cases of acute leukemias (6 children, 7 adults) remained unclassified (AUL) and were not listed

[b] Established by infection of normal peripheral lymphocytes with EBV

exogenous virus-coded antigen of any of the known oncogenic viruses ([2], unpublished observations). The lack of cALL-positive cells in normal human bone marrow and fetal liver in our experiments may only reflect the scarcity of cells carrying this antigen during a transitory stage of hemopoietic development. Our findings show that in a defined number of ALL-cases as well as in several lymphoid cell lines the cALL-antigen can be detected together with T-cell antigen or SIg, which means that the cALL-antigen is expressed by cells committed to differentiate along the T or B cell axis. Leukemic cells, carrying cALL-antigen only, may represent a differentiation stage of a common lymphoid precursor cell (LyP) not yet committed to one of the two pathways of differentiation. On the other hand our data do not support the postulate of Janossy and coworkers that cALL antigen is expressed on pluripotent stem cells (PSC) [3]. The proliferative activity of colony-forming and diffusion chamber stem cells was not affected by an incubation with AcALLG. Both test systems analyze the growth of early precursor cells of human bone marrow. The diffusion-chamber provides a suitable milieu for the growth of certain pluripotent stem cells [15] and in the CFU-c assay mainly myeloid precursors give rise to colonies. Additional investigations in the CFU-c test have been performed with bone marrow cells cultured in diffusion chambers. The growth pattern of these cells indicate an influx from pluripotent stem cell

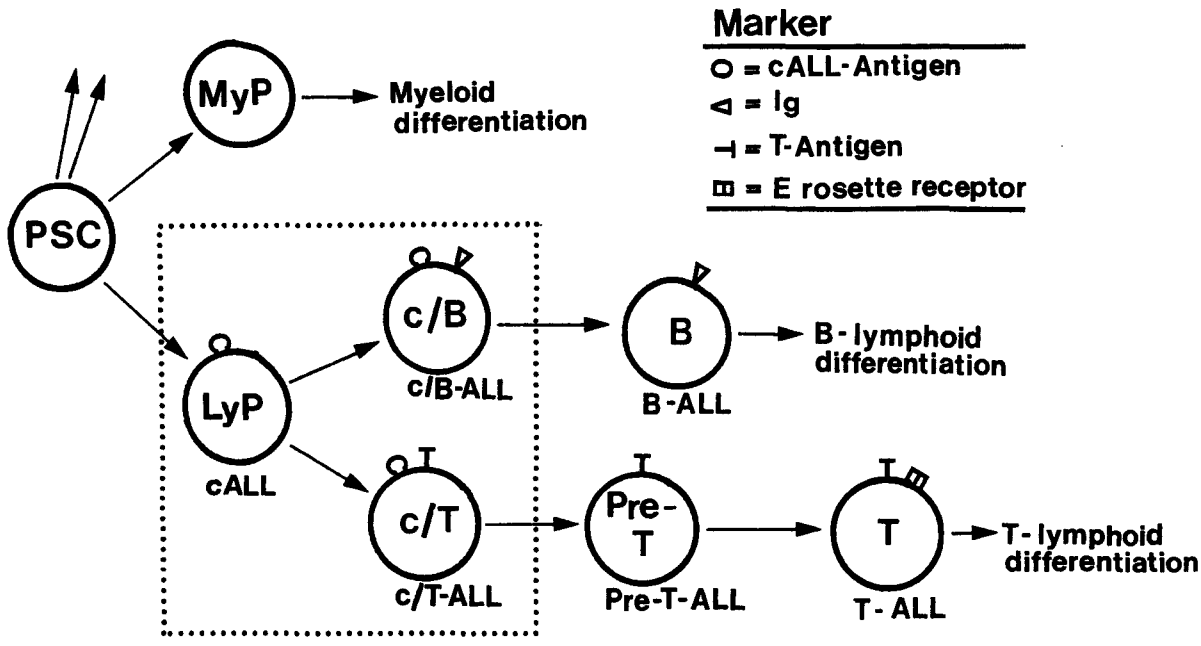

Fig. 2. Different ALL phenotypes as counterparts of differentiation steps in a possible scheme of normal hemopoietic development (abbrevations explained in the text)

pool in addition to the limited self replication of the committed myeloid precursor cells. Colony development under these conditions is not influenced by incubation treatment with AcALLG prior to the chamber implantation as reported by Netzel et al. [7] which may exclude the elimination of stem cells by cALL-specific antibodies. On the basis of the reported findings and comparative investigations of normal fetal cells (Thiel et al., submitted) the following differentiation scheme can be proposed (Fig. 2). If ALL cells and cell lines represent arrested stages of normal hemopoiesis, cALL antigen is expressed transitorily on cells of early lymphopoietic development (LyP, c/B, c/T).

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Common All Associated Antigen from Cell Surface and Serum: Molecular Properties and Clinical Relevance*

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Introduction

Detection of distinct cell surface markers has brought up remarkable improvements not only in diagnosis and classification of leukemic diseases, but also has gained immediate clinical relevance concerning therapeutic aspects. Especially, such markers may give information about the antigenic phenotype reflecting the origin of distinct leukemic cells from different stem cell compartments and their degree of maturation.

Recently a cell surface antigen has been detected, which so far seems to be highly specific for common ALL (cALL) cells. Interestingly, in some cases this antigen has also been found on T-ALL cells and on cells appearing in blastic crises of CML [7]. Nevertheless, the detection of this common ALL associated antigen (Ag cALL) has been proved to be useful in clinical practice especially in combination with other routinely applied tests for cell surface markers, e.g. E-rosetting, immunoglobulines and C-receptors. We here describe the solubilization and partial physico-chemical characterization of the cALL associated surface antigen. Furthermore, a glycoprotein exhibiting the cALL antigenic specificity could be isolated from the sera of two patients in active relapses of common ALL.

Material and Methods

Chemicals

Phosphate buffered saline (PBS, 10 mM phosphate buffer, pH 7,2, 0,14M NaCl, 0,02% NaN_3). Sodium deoxycholate was purchased from Merck, Darmstadt. Ficoll and Sepharose CL-6B were products of Pharmacia, Uppsala. Ultrogel AcA 34 was from LKB, Bromma. The reference proteins, myoglobin (purest, from horse), ovalbumin (crystallized two times, from chicken egg) were from Serva, Heidelberg, and bovine serum albumin was from Behring-

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werke, Marburg. Blue dextran 2000 was from Pharmacia, Uppsala, and DNP-α-alanine from Serva, Heidelberg. Aldolase (from rabbit muscle) was from Boehringer Mannheim. β-Lactoglobin (purest, from milk) was from Behringwerke, Marburg.

Cells and Antisera

Peripheral blood lymphocytes (PBL) and bone marrow cells (BMC) were prepared from the following groups of donors: healthy persons, patients suffering from different infectious diseases, non-leukemic hematological disorders, and various types of leukemia, the latter classified according to standard clinical, hematological and immunological criteria [9,11].

In analytical experiments Ficoll isopaque purified cALL-cells of patient T.I. were used as standard targets. Batches of these cells, labelled to 98% by anti-cALL-serum (A-cALLS), were preserved by freezing as described earlier [8]. A-cALLS were raised in rabbits according to Greaves et al. [4] and heat inactivated at 56°C for 30 min. Standard absorptions of A-cALLS with AB-erythrocytes, human AB-serum crosslinked by glutardialdehyde, tonsillar lymphocytes, normal bone marrow cells, cells of acute myelocytic leukemia and normal peripheral blood cells were performed as described in detail recently [8]. Indirect immunofluorescence was used to check for completeness of absorption. The IgG-fraction of the so absorbed A-cALLS was prepared by ammonium sulfate precipitation and subsequent anionic exchange chromatography on DEAE-52 [6].

The specificity of the A-cALLS after the final absorption step is ascertained by using different target cells.

Tetra-ethyl-rhodamine (TRITC) conjugated goat anti-rabbit globulin (TRITC/GARG) was from Nordic Immunologic Laboratories/Tilburg.

Preparation and Solubilization of cALL Cell Membranes

Human ALL-cells (10^9) were homogenized in 20 ml 0,02M Tris, pH 8,0, 0.25M sucrose at 4°C using a Potter Elvejhem homogenizer (3 × 1 min at 1400 rpm). The homogenate was sonicated for 3 × 1 min (MSE sonicator) at 18 microns/min while kept on ice. The sample was then centrifuged for 20 min at 10000 g and the supernatant chromatographed on a Sepharose Cl-6B column (100 × 2,5 cm; flow rate: 40 ml/hr, fraction volume: 10 ml). The material eluted with the void volume containing the cALL plasma membranes was solubilized as follows: 10 ml samples were dialysed step-wise first against 2% (w/v) sodium deoxycholate (6 hr at room temperature) and then against 0,2% sodium deoxycholate (12 hr at 4°C). The dialysed samples were concentrated by ultrafiltration (Amicon PM 10) to a volume of 2,5 ml. The solubilized material was chromatographed on an Ultrogel LKB AcA 34 column (1,0 × 90 cm; flow rate: 15 ml/hr, fraction volume: 3,0 ml), equilibrated with 0,14M NaCl, 10 mM Tris, pH 8,0, 0,02% NaN_3 and 0,2% sodium deoxycholate.

Preparative and Analytical Chromatography of the Ag cALL from Serum of cALL Patients

For analytical chromatography of Ag cALL the 20–50% ammonium sulfate precipitate of 20 ml serum of a cALL patient was dissolved in 20 ml PBS and subjected to gel filtration on AcA 34 (90×5 cm; buffer PBS; flow rate: 60 ml per hr. fraction volume: 5 ml). 30 ml of the combined fractions containing the Ag cALL activity (peak II, Fig. 5) were further purified on agarose bound lentil lectin columns (1,6 mg protein/ml agarose, column size 8,0×0,9 cm, flow rate: 16 ml/hr, fraction volume: 10 ml). Before eluting the antigen with 30 ml 0,2 M α-Methyl-D-Mannoside the column was washed with 50 ml PBS. Analytical gel filtration was performed on an AcA 34 column (90×2,5 cm; buffer: PBS; flow rate: 16 ml/hr). The apparent molecular weight was calculated from Kd versus logarithm of molecular weight of the reference proteins (Fig. 6).

Testsystem

Testing of the absorbing capacity of Ag cALL solubilized from plasma membranes of serum of either cALL patients, healthy control persons or patients suffering from different other leukemic diseases was performed as described in detail recently [8].

The chromatographed solubilized fractions of the plasma cell membranes and of the serum preparations were dialysed against distilled water for 12 hours and lyophilized subsequently. The single fractions were then dissolved in 100 µl PBS. For absorption 20 µl samples each were mixed with 2 µl A-cALLS (final dilution 1 : 11). The mixtures were first incubated for 20 minutes at room temperature and then for 5 hours in the cold. Insoluble material was removed by centrifugation. The supernatants were tested for residual Ag-cALL antibodies by indirect immunofluorescence using (TRITC/GARG) with standard cALL-cells as targets as described previously in detail [8]. The absorbing capacity was calculated according to the formula:

$$\% \text{ absorbed activity} = 100 - \frac{\% \text{ stained cells after absorption}}{\% \text{ stained cells before absorption}} \times 100$$

Unspecific binding was prevented by performing the indirect immunofluorescence assay in the presence of 0,2% sodium azid and Heparin (500 U/ml) 20 µl/ml [2]. Applying this technique the background of unspecific staining was less than 0,2%.

Polyacrylamid-Gel-Electrophoresis (PAGE)

The PAGE in slab gel was performed according to Maizel [10]. The samples (4 µg protein) were applied to 10% polyacrylamid gels and subjected to electrophoresis (35 m A, 3 hours at room temperature). The protein was stained by coomassie blue. The following proteins were used for calibration: Aldolase, Ovalbumin, β-Lactoglobulin and Myoglobin.

Results

Reactivity of Different Human Target Cells to Rabbit A-cALLS

The suitably absorbed A-cALLS showed a high specificity for cALL-cells (Table 1). No binding has been observed to cells of other leukemic diseases and non-leukemic hematological disorders with two exceptions: 1. Ag cALL positive cells were found in bone marrow and, in further course of the disease also in PBL of a two year old boy suffering from CML. 2. In a new born male patient with Trisomie 21 and leukemoid reaction 60% of peripheral leuko-

Table 1. Binding of standard A-cALLS[a] to various human blood cells

Cell type	Number of cases	Positive reaction
1. Normal cells		
peripheral blood lymphocytes	4	0[b]
bone marrow cells	4	0
2. Tumor cells		
common ALL	32	30[c]
T-ALL	7	0
B-ALL	1	0
acute myelocytic leukemia (AML)	9	0
chronic myelocytic leukemia (CML)	7	1
chronic lymphatic leukemia (CLL)	4	0
juvenile chronic myelocytic leukemia (JCML)	2	0
acute monocytic leukemia (AMOL)	2	0
acute myelocytic-monocytic leukemia (AMML)	1	0
osteomyelofibrosis/sclerosis (OMF/S)	2	0
3. Leukemoid reaction		
trisomy 21	1	1
4. Infectious diseases		
mononucleosis	3	0
measles	1	0
tonsillitis	2	0
pneumonia	1	0
pertussis	2	0
5. Non-leukemic hematological diseases		
panmyelopathy	3	0
idiopathic thrombocytopenias	2	0
thalassemia	1	0

[a] Standard absorption was performed with: AB-erythrocytes, glutardialdehyde fixed human AB-serum, tonsillar lymphocytes, normal bone marrow cells, AML cells and peripheral blood lymphocytes.

[b] 0 means no more than 0,2% of positive cells were found. This is in the range of the background found with normal rabbit serum used as control in all immunofluorescence assays.

[c] In our cases of positive reactions at least 30% of the mononuclear cells were stained with the standard absorbed A-cALLS. In leukemias the percentage of the so labelled cells was in most cases close to that of cells identified as leukemic cells by standard morphological, cytological and cytochemical methods

cytes had expressed Ag cALL. Four weeks later we detected only 4% of Ag cALL positive cells in the peripheral blood of this boy and after another two weeks all Ag cALL bearing cells had disappeared.

Solubilization and Chromatography of Membrane Ag cALL

In pilot experiments it was found that sodium deoxycholate, up to a final concentration of 2% (w/v) did not influence the antigenicity of the structure under study to any significant extent. Therefore we used this procedure as a standard method to solubilize cALL plasma membranes. The solubilized membrane antigens were chromatographed on LKB AcA 34 in the presence of 0,2% sodium deoxycholate and tested for their ability to absorb the antibodies against Ag cALL out of A-cALLS. As shown in Fig. 1, the antigenic activity of Ag cALL was detected in three peaks: The bulk of the antigenic activity was found with fractions 37–42 (peak III). A small amount of this antigenicity eluted with fractions 31–33 (peak II). In addition there was antigenic activity associated with unsolubilized material, which eluted with the void volume. By comparison with reference proteins (see legend Fig. 2), the antigenic molecules of peak II and III were estimated to have an apparent molecular weight of 110000 (peak II) and 55000 (peak III) respectively (Fig. 2).

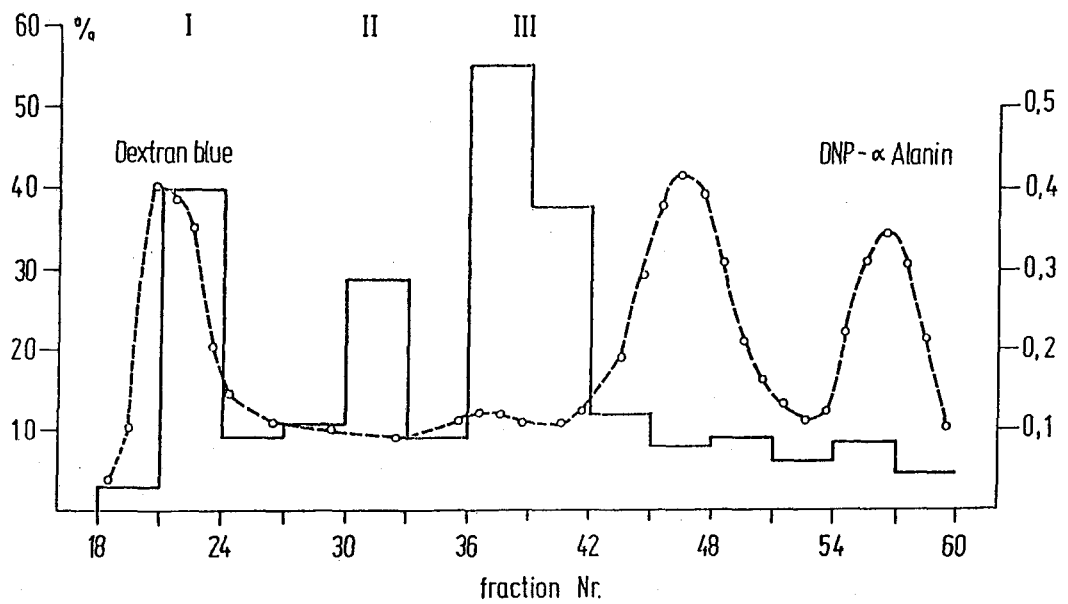

Fig. 1. Analytic absorptions of standard absorbed A-cALLS with solubilized and chromatographed common ALL membrane fractions. The elution volume was collected in 42 fractions (18–60). Every three of these fractions were pooled, lyophilized and resuspended in 100 µl HBSS. For absorption 20 µl of these fractions were incubated with 3 µl standard absorbed A-cALLS (final dilution 1:8). Absorbed antibodies ○——○ E 280 nm

Absorption of A-cALLS with Normal and cALL-Serum

Sera of two cALL patients having Ag cALL positive cells in their peripheral blood were taken for partial characterization of the serum Ag cALL. As may

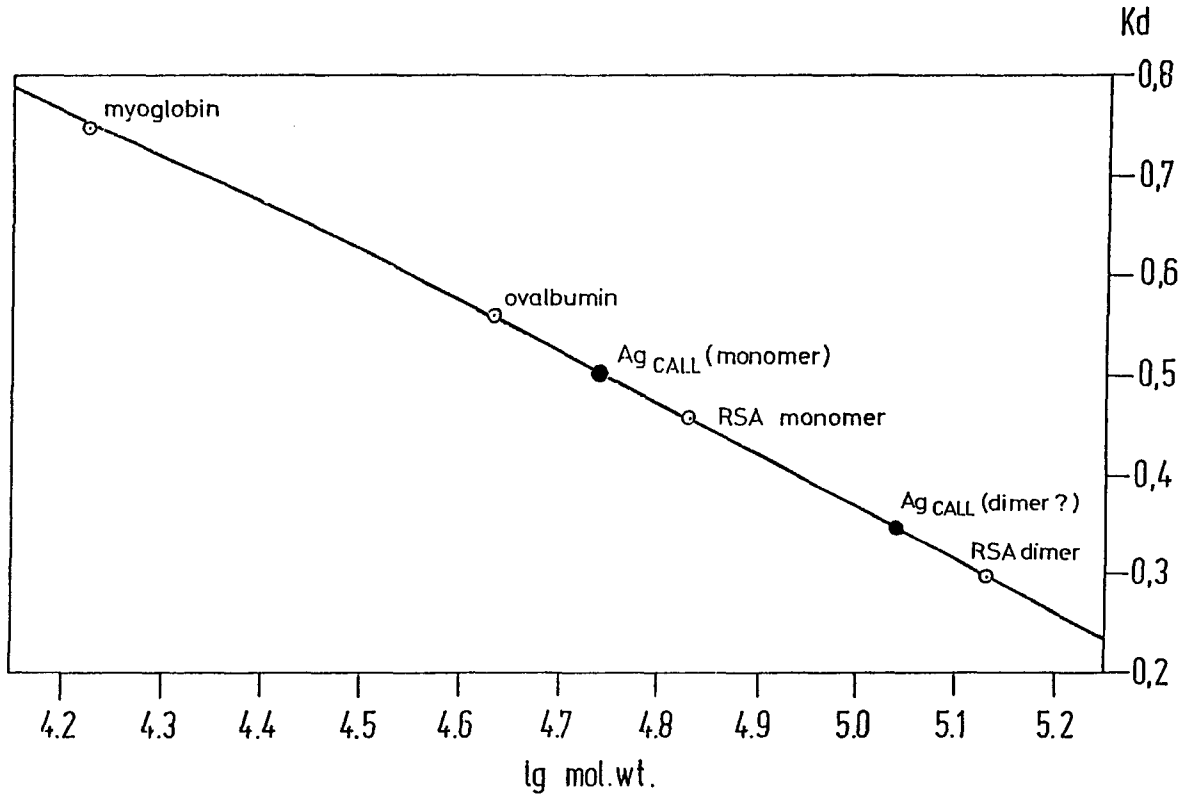

Fig. 2. Calculation of the apparent molecular weight of membran Ag cALL on an AcA 34 column equilibrated with 0.2%; sodium deoxycholate BSA = bovine serum albumin. The molecular weight was calculated from the plot of Kd versus lg molecular weight

be seen from Fig. 3 both sera removed cALL specific antibodies out of A-cALLS. No absorbing effect was seen when sera from normal persons as well as from patients with T-ALL, CLL, AML, AMML and CML were used instead.

Ammonium Sulfate Fractionation and Analytical Chromatography of Serum Ag cALL

Most of the serum cALL associated antigenicity could be precipitated at 30–50% ammonium sulfate saturation. A smaller amount was also found to be pre-

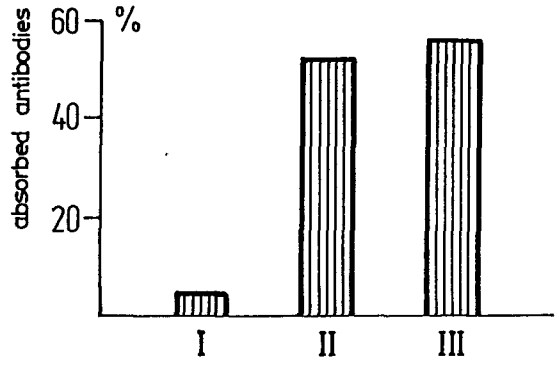

Fig. 3. Analytical absorption of A-cALLS with normal serum and serum of cALL patients. I normal serum; II serum from patient T.F., 2×10^4 cALL cells/µl; III serum from patient T.I., 6×10^4 cALL cells/µl

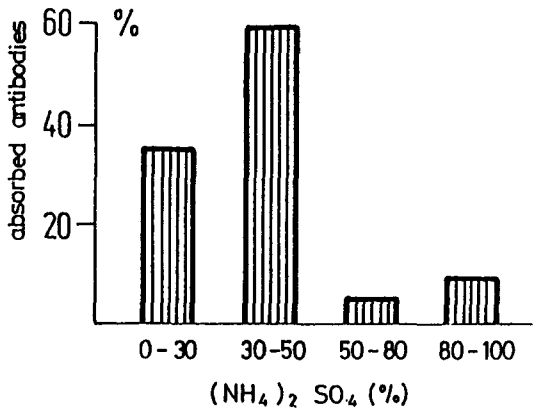

Fig. 4. Fractional precipitation of serum Ag cALL by ammonium sulfate at pH 7,5. 1 ml serum from cALL patients was diluted 1:10. 60 min after adding the desired amount of $(NH_4)_2SO_4$ the sample was centrifuged for 10 min at 12000×g. The sediment was then dissolved in 1 ml PBS and dialyzed for 12 hr against this buffer. 20 µl of the single fractions were used for absorption

cipitated between 0–30% (Fig. 4). By chromatography of the ammonium sulfate precipitated and redissolved cALL-serum fractions on LKB AcA 34 it was found that the elution diagram was characterized by two antigen active peaks (Fig. 5). Peak I eluting with the void volume and peak II between fraction 148–187 corresponding to a molecular weight of 125000 (Fig. 6). After rechromatography of peak II under identical experimental conditions again two antigen active peaks were observed both having the same positions as shown in Fig. 5. The combined fractions of peak II were applied on to an agarose lens culinaris hemagglutinin A column. As may be seen from Fig. 7 the antigen activity bound completely to lens culinaris lectin and could be

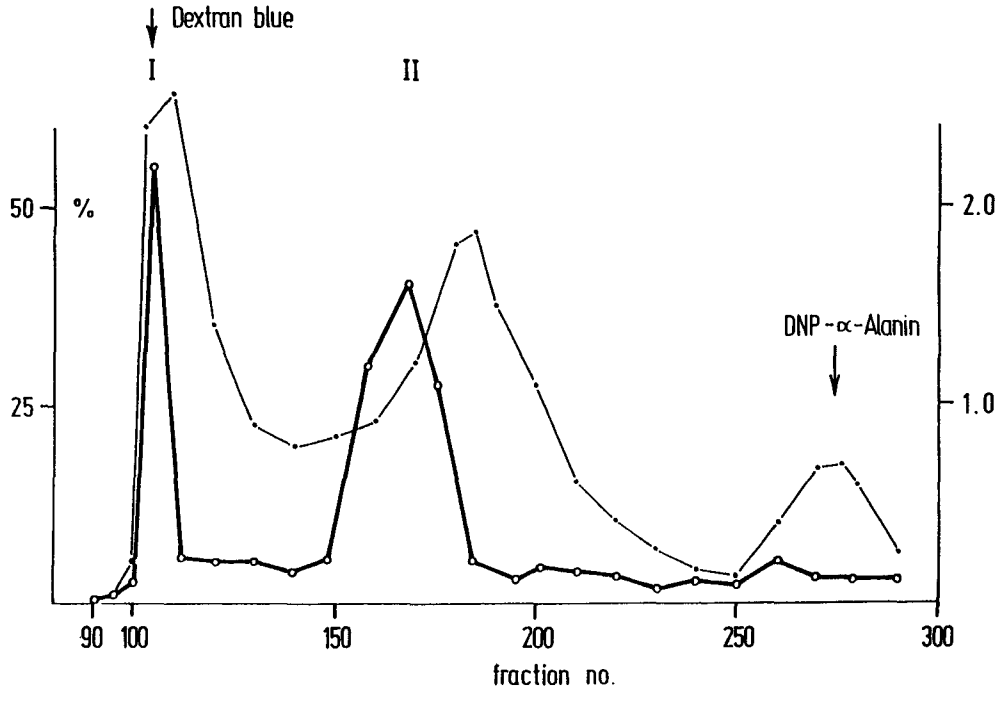

Fig. 5. Chromatography of the 20–50% ammoniumsulfate precipitated Ag cALL from cALL serum on LKB AcA 34; ●——● E 280, ○——○ absorbed antibodies

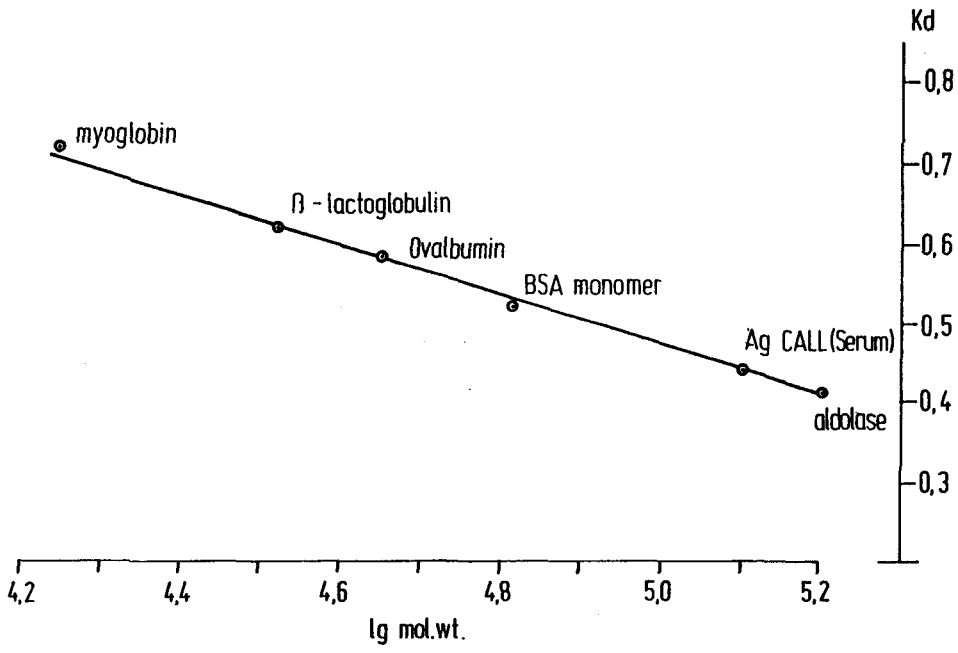

Fig. 6. Calculation of the apparent molecular weight of serum Ag cALL by means of AcA 34 column chromatography. BSA = bovine serum albumin. The molecular weight was calculated from the plot of Kd versus lg molecular weight

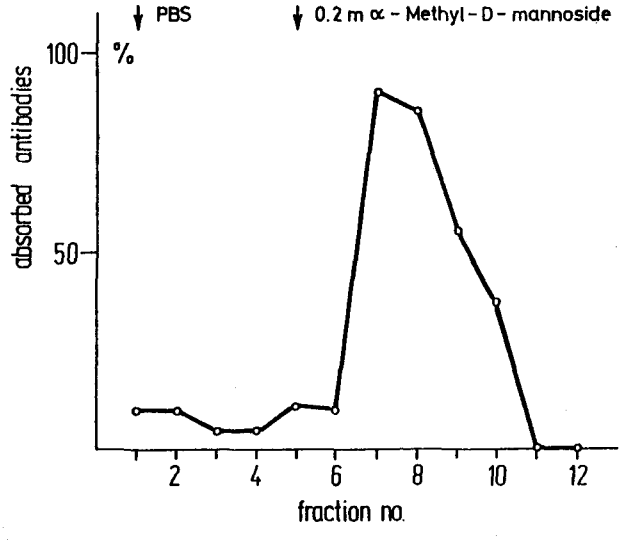

Fig. 7. Affinity chromatography of serum Ag cALL on agarose lens culinaris hemagglutinin A. 30 ml of the combined fractions of Peak II (Fig. 5) after gel chromatography were applied

specifically eluted by 0,2M a α-Methyl-D-Mannoside. The SDS (0,1%) PAGE of this material showed a single major band running at 140000 daltons under unreducing conditions (Fig. 8).

Discussion

Our experiences concerning the appearance of Ag cALL on the surface of leukemic cells and especially on those of cALL are in good agreement with those of Greaves et al. [5] and Rodt et al. [13]. The detection of Ag cALL on blastoid cells from a patient with Trisomie 21 apparently having a leukemoid

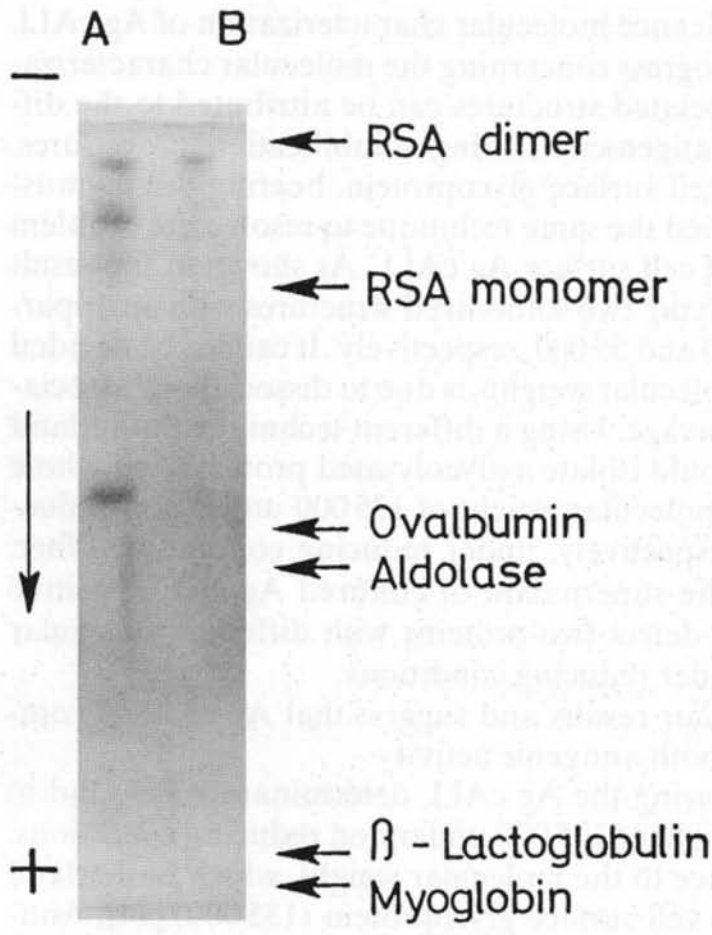

Fig. 8. SDS-Polyacrylamid gel electrophoresis on 10% Slab gels of serum Ag cALL preparations before (left column A) and after (right column B) affinity chromatography on agarose lens hemagglutinin A

reaction suggests that the Ag cALL might be not confined to cALL cells and certain other leukemic diseases. Evidence for this suggestion is also gathered by Roberts et al. [12], demonstrating binding of A-cALLS to rare cells in fetal, neonatal and regenerating bone marrow. Although consequently A-cALLS seems to be not specific for cALL cells, it may be used for clinical purposes with great advantage. This may be demonstrated by the following clinical examples.

On examining the bone marrow of a CML case when first admitted to the clinic, we found no Ag cALL positive cell in peripheral blood (700000 leukocytes/mm^3), but 7% of the bone marrow cells bound A-cALLS. After 6 months the patient developed a blastic crisis. 60 per cent of peripheral blood cells now were found to be Ag cALL positive blast cells. Up to this blast crisis the patient had been treated according to a CML regimen. This was now replaced by an ALL therapy concept, by which the blastic crisis could be controlled within a few days. By this observation it might be concluded that imminant blastic crisis can be detected by frequent monitoring of CML cases with anti-cALLS. Moreover, it should be considered if a combined CML-ALL therapeutic protocol applied at the first appearance of even a few Ag cALL positive cells may prevent blastic crises. In six cases where the clinical, morphological and cytochemical findings were inconclusive, an relapse affecting the CNS could be ascertained by demonstrating Ag cALL positive cells in the CSF even in samples with cell counts as low as 4 cells/mm^3.

Because of its clinical significance molecular characterization of Ag cALL is of great interest. The little progress concerning the molecular characterization of plasma membrane associated structures can be attributed to the difficulties of preserving their antigenicity during solubilization procedures. Recently we isolated another cell surface glycoprotein, bearing the thymus-brain antigen [1]. Now we applied the same technique to resolve the problem of isolating antigenically intact cell surface Ag cALL. As shown in the result section we succeeded in identifying two solubilized structures with an apparent molecular weight of 110000 and 55000, respectively. It cannot be decided whether the 2:1 ratio of the molecular weights is due to dissociation/association events or to enzymatic cleavage. Using a different technique Sutherland (communicated by Greaves) could isolate a glycolysated protein from whole cell extract with an apparent molecular weight of 135000 under non-reducing conditions and 100000, respectively, under reducing conditions. When investigating Ag cALL from the supernatant of cultured Ag cALL positive leukemic cell lines they could detect two proteins with different molecular weight (100000 and 38000) under reducing conditions.

These data are supporting our results and suggest that Ag cALL is composed of two subunits having both antigenic activity.

The serum glycoprotein bearing the Ag cALL determinant was found to have an apparent molecular weight of 125000 under non reducing conditions. This result is in good accordance to the molecular weight, which Sutherland et al. found for the unreduced cell surface glycoprotein (135000) [14]. Antigenic and lectin specificity correspond to the data presented by Brown et al. [3] and Sutherland et al. [14]. Interestingly, in our experiments additional cALL antigen activity was found in the void volume after gel chromatography of serum Ag cALL on AcA 34. The observation that after rechromatography of the combined fractions of peak II (mol. weight 125000) parts of the antigen activity again eluted with the void volume suggests its tendency to associate under the chosen experimental conditions. The apparent molecular weight of the associated molecules of the Ag cALL is presently under study.

The appearance of soluble Ag cALL in the serum indicates shedding or secretion of this material under in vivo condition. These suggestions are supported by the fact that the cALL antigen is found under in vitro conditions in the culture supernatant of native leukemic cells, cell lines derived from leukemic patients and released by the MOLT-4 cell line, which has no detectable cell surface Ag cALL [14].

The binding of nearly equal amounts of anti-Ag cALL antibodies by the sera of the patients (T.I. 54 years old ♀ 60000 leukemic cells/mm^3; T.F. 8 years old ♂, 20000 leukemic cells/mm^3) although differing in their peripheral leukemic cell count by three fold indicates different releasing activity for Ag cALL probably depending on the type and state of the cALL disease. If the serum Ag cALL is common to a vast proportion of acute lymphocytic leukemias the detection of this antigen in the serum might possibly improve diagnosis and therapeutic monitoring of common ALL, especially if it could indicate subclinical disease.

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Monoclonal Antibodies to Human Cell Surface Antigens

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Köhler and Milstein's method for producing monospecific antibodies by fusing antibody-producing and myeloma cells to derive hybrid cell lines secreting monoclonal antibody considerably facilitates the analysis of the cell surface [1]. In particular, it is possible to produce monoclonal antibodies to a large number of human cell surface antigens. These antibodies will be suitable for immunocytochemical labelling studies, and will also allow the purification of substantial quantities of the major molecules of the cell surface with a view to their characterisation. Furthermore, using interspecific somatic cell hybrids, the genes coding for surface molecules can be assigned to particular chromosomes and conversely a panel of assigned surface antigens would be extremely useful as markers in somatic cell genetics.

We have applied the above approach to an analysis of the cell surface of normal and leukaemic leukocytes using cell membrane for immunization. A

Table 1. Specificity of monoclonal antibodies to human cell surface antigens

Cell fusion experiment reference no.	Immunization		Number of hybrid specificities defined			
	Tissue	Preparation	Species[a] common	Leukocyte associated	Erythrocyte specific	Differentiation antigens
W6[b]	Tonsil lymphocytes	Sucrose gradient purified membrane	4[c]	—	2	—
Gen Ox 4	AML cells	Sucrose gradient purified membrane	8	3	—	—
Gen Ox 5	ALL cells	Tween 40 membrane	2	1	—	—
Gen Ox 7	CLL cells	Tween 40 membrane	5	3	—	B cell associated
Totals:	*Antibody of given specificity*		19	7	2	1
	No of hybrids tested		29	29	29	29

[a] Some antigens defined were not present on erythrocytes
[b] Reference [2]
[c] W6-32 is against an antigenic determinant shared between products of the HLA A, B and C loci

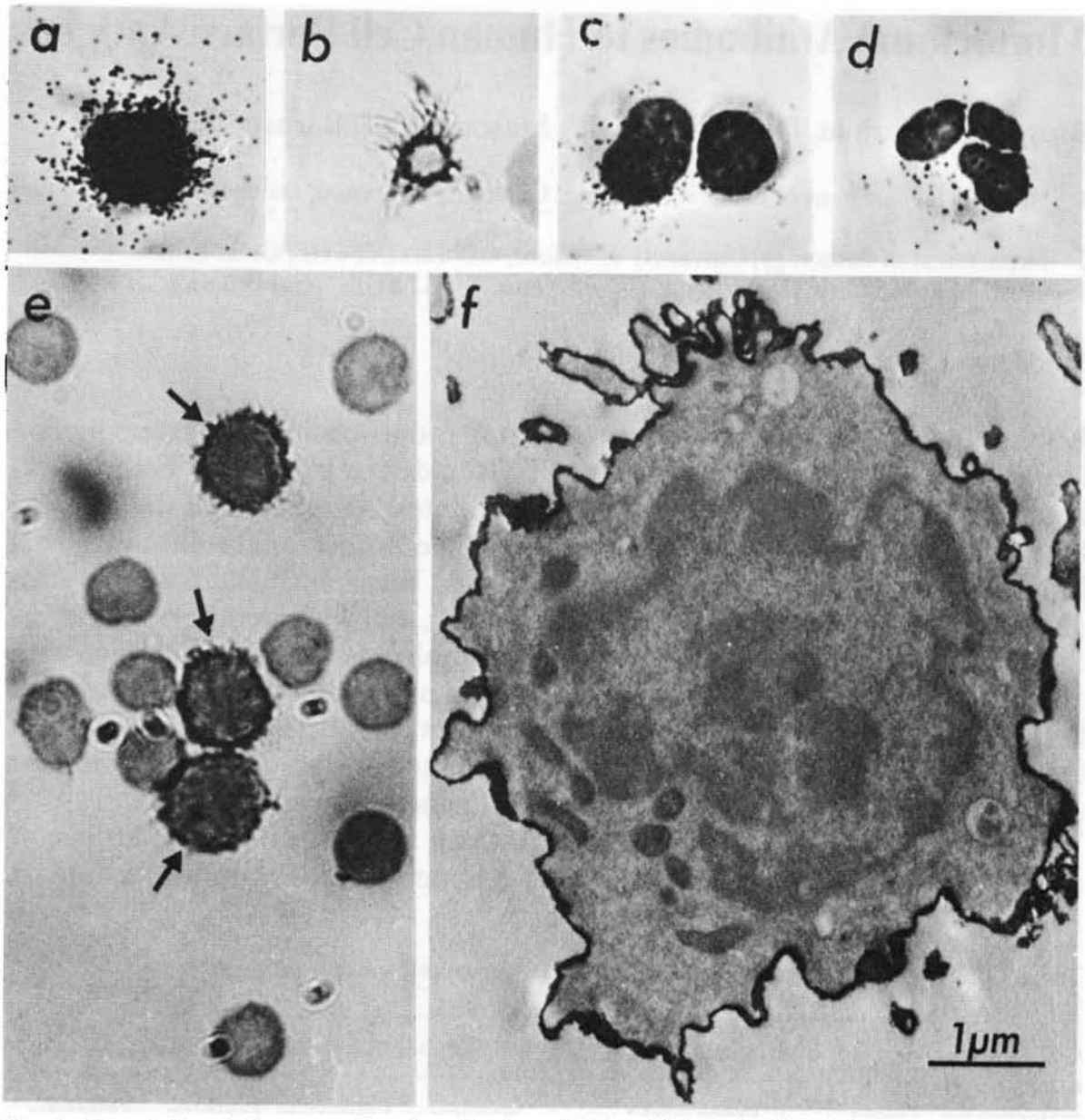

Fig. 1. Reactivity of the monoclonal antibody to HLA with lymphoid and bone marrow cells.
a) Bone marrow lymphocyte
b) Platelet
c) myelocyte and normoblast
d) polymorphonuclear leukocyte
e) thymocytes, positive cells are indicated by the arrows
f) tonsil lymphocytes
a), c) and d) are autoradiographs and the binding is demonstrated in b), e) and f) using an immunoperoxidase procedure. f) shows the surface membrane distribution of HLA, the cells were fixed in paraformaldehyde and glutaraldehyde before incubating with the monoclonal antibody

number of hybrids secreting antibody have been identified and their individual specificities characterized. The results are shown in Table 1. Many of the antibodies recognize antigens of wide tissue distribution, but a number of leukocyte associated, erythrocyte specific and putative lymphocyte specific antigens have been identified. Further clones are at present being investigated.

The monoclonal antibody W6-32 is against an antigenic determinant shared between products of the HLA, A, B and C loci [2]. By immunofluorescent,

immunoenzymatic and autoradiographic techniques, the cellular distribution of HLA A, B and C antigens, both on cell suspension and tissue sections, has been investigated. The differential expression of these antigens on developing haemopoietic and lymphoid cells has been semi-quantitatively analysed by grain counting of autoradiographs [3]. The results are shown in Fig. 1 and Table 2. Lymphocytes and platelets were very densely labelled and myeloid

Table 2. The distribution of HLA on human lymphoid. bone marrow and peripheral blood cells

Tissue	Reactivity
Peripheral blood	
Polymorphs	+ +
Lymphocytes	+ + + +
Erythrocytes	–
Platelets	+ + + +
Bone marrow	
Lymphocytes	+ + + +
Myeloid precursors	+ + +
Erythroid precursors	±
Thymus	
Lymphoid cells	85%–/15%+ + +
Tonsil lymphoid cells	+ + + +

precursors showed more labelling than mature neutrophils. Erythroid precursors, although very weakly labelled, were clearly positive, in comparison with bone marrow erythrocytes which were negative except for a small percentage (11%) which were presumed to be reticulocytes. In the thymus HLA negative, thymocyte-antigen positive cells [4] can be distinguished from HLA positive, thymocyte-antigen negative cells. By using immunofluorescent techniques on tissue sections, Mason, Christonsson and Biberfeld have shown the former to be cortical thymocytes and the latter medullary cells. The immunoperoxidase technique has the advantage that antigens can be demonstrated at the electron microscope level. Fig. 1f shows a continuous surface membrane distribution of HLA on tonsil cells.

These antibodies will also allow the purification of substantial quantities of the major molecules of the cell surface. W6-32 antibody precipitates radioactive molecules of apparent m.wt 43000 daltons and 12000 daltons from lysates of BR18 cells which have been lactoperoxidase labelled with I^{125} [2]. Furthermore, an antibody affinity column of W6-32 bound equally the HLA A, B and C antigens (Parham, Barnstable and Bodmer: In preparation) and can be used to isolate substantial quantities of the antigen.

By using monoclonal antibodies against cell surface antigens, genes or gene clusters coding for surface molecules can be identified. Rodent-human somatic cell hybrids tend to undergo a relatively rapid loss of human chromosomes and then become karyotypically stable. Human genes can, therefore, be assigned to specific chromosomes by correlating the presence or absence

Table 3. Genetic analysis of cell surface markers

Antibody	Chromosome
W6-32	6
W6-34, 45, 46	11
Gen Ox 4-17, 21	
NA/1-33	13
Gen Ox 4-1	14
Gen Ox 4-7, 24	22

Monoclonal antibodies have also been produced against β_2-microglobulin, a marker for chromosome 15 (Brodsky, Parham, Barnstable and Bodmer. In preparation)

of a particular gene product with the presence or absence of a particular cytologically identified chromosome. The results presented in Table 3 represent a summary of the characterisation of a number of monoclonal antibodies on a panel of somatic cell hybrids.

Ten antigens have been assigned, to date, to particular chromosomes. Four different specificities are coded for by chromosome 11 and markers are also available for chromosomes 6, 13, 14 and 22. The gene coding for the antigen SA-1, described by Buck and Bodmer, is on chromosome 11 and is probably a glycosyl transferase. Of obvious interest is whether a cluster of genes involved in cell surface carbohydrate structure is located on chromosome 11.

Monoclonal antibodies against assigned surface markers could also be used to select hybrid derivatives with or without particular chromosomes, for example by the use of the fluorescence activated cell sorter. The efficiency of these selections and the reliance that can be placed on their results depend upon the quality of the reagents detecting the cell surface antigens. Monoclonal antibodies are ideal in this respect.

These results clearly demonstrate that it is possible to produce monoclonal antibodies to a large number of human cell surface antigens suitable for immunocytochemical and genetic studies. However, many of the antibodies recognize antigens of wide tissue distribution if cell membrane is used for immunization. Therefore, alternative sources of antigen, for example purified glycoproteins, may provide monoclonal antibodies against differentiation antigens of greater interest.

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Elicitation of Anti-Leukemia Cytotoxic Responses*

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A major goal in our understanding of leukemia involves the study of cell surface antigens on the leukemic cells that are not present on patients' normal cells of the same type. These antigens, if they serve as tumor rejection type antigens, are commonly referred to as tumor associated transplantation antigens. One approach to the study of these antigens, which is also of potential interest with regard to cellular immunotherapy of leukemia, involves attempts to generate cytotoxic cells against autologous leukemia cells. The approaches that we have used have largely been based on earlier and concurrent studies involving recognition and response to alloantigens in the mixed leukocyte culture (MLC) and cell mediated lympholysis (CML) assays. Two general approaches have been used, both of which are presented in this paper. In each case the background studies of the allogeneic system are given as a reference.

Methods Employed

The mixed leukocyte culture (MLC) test [1,2] is most commonly assayed by studying the incorporation of radioactive thymidine into the dividing cells in the responding cell population. Stimulating cells are treated with x-irradiation or mitomycin-C [3] so that they will present their foreign antigens to the responding cells but will not themselves respond, i.e. incorporate radioactive thymidine. It is now recognized that both helper T lymphocytes (T_h) and cytotoxic T lymphocytes (T_c) respond proliferatively in a mixed leukocyte culture; in addition, in all probability suppressor T lymphocytes (T_s) also respond. In fact, it is not critically established whether the T_h cells do themselves proliferate in a mixed leukocyte culture. However, proliferation of the responding cells has most commonly been associated with a helper response in that the requirement for T_h activity, in terms of the development of cytotoxic cells, is present. Whereas in a primary mixed leukocyte culture, more than 90% of the dividing cells belong to the T lymphocyte subclass cells that

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carry the Ly 1 differentiation antigen [4,5] (which we associate with helper T lymphocyte activity), it would probably be dangerous to equate proliferation to T mediated "help" under all MLC-like conditions.

Cytotoxic T lymphocytes (T_c) are generated in mixed leukocyte cultures and can be measured in the cell mediated lympholysis assay [6–8]. The T_c cells are tested for their ability to lyse radioactive sodium chromate (^{51}Cr) labeled target cells that carry the sensitizing antigens.

In addition to the generation of T_h and T_c cells in an MLC, suppressor T lymphocytes are also generated. These cells are usually detected by their ability to suppress autologous responding cells from differentiating into T_c in response to unrelated alloantigens in a fresh MLC.

The Three-Cell Approach to Generating Killer T_c Cells

In vitro T lymphocyte reactivity to allogeneic cells in man appears to be restricted almost entirely, in a primary sensitization system, to a response against antigens of the major histocompatibility complex, HLA. Within the HLA complex, as with the mouse homolog of that complex (H-2), there are two types of antigens which are commonly referred to as LD (lymphocyte or MLC defined) and CD (cytotoxicity or CML defined) antigens insofar as these antigens are recognized by the T lymphocytes. The LD antigens in man are associated with the HLA-D locus and in mouse with the H-2 I region; the CD determinants are associated with the HLA-A, -B and -C loci in man and primarily with the H-2 I region in mouse [9]. The finding of greatest note with regard to the principle emerging from studies involving allogeneic cells as it relates to the generation of cytotoxic cells against autologous leukemia cells is the following. If responding T lymphocytes of one individual are stimulated with cells that differ by CD determinants but do not differ by LD antigens, then the cytotoxic response which develops against the CD determinants is markedly weaker than when the stimulating cells include both an LD and a CD stimulus [9].

These findings are illustrated in a schematic fashion in Table 1. The ap-

Table 1. A three-cell experiment for the generation of cytotoxic T lymphocytes

Sensitizing MLC	HLA difference between responding and stimulating cell	^{3}H-TdR incorporated	% CML target cell		
			A	B	C
AB_m	HLA-D	+ + + +	–	–	–
AC_m	HLA-A, -B and -C	–	–	–	–
AB_mC_m	HLA-A, -B, -C and -D	+ + + +	–	–	+ + +

Typical results of CML studies in a family in which a recombinational event has taken place. Siblings A and B are identical for the HLA-A, -B, and -C loci but differ for HLA-D. Siblings A and C differ for the three SD loci, HLA-A, -B, and -C but are identical for HLA-D. In the three-cell experiment. A is simultaneously stimulated with the cells of sibling B and the cells of sibling C. [Reprinted from Bach. F. H.: Ann. Rev. Genet. **10**, 319–339 (1976)].

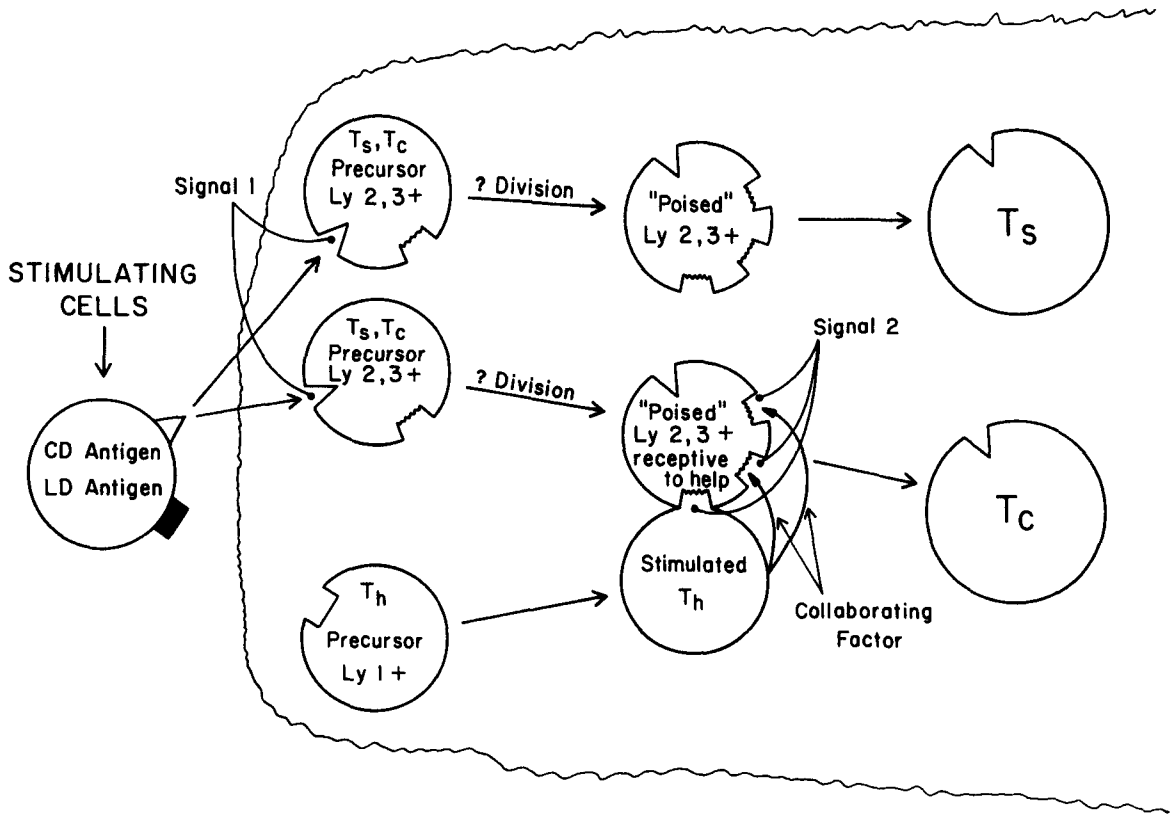

Fig. 1. A model illustrating the signals involved in the development of T_c in a primary response. This represents a modification of the model presented previously [9]

parent basis for this phenomenon, which we have referred to as LD-CD collaboration, is that functionally different populations of T lymphocytes respond preferentially to LD and CD determinants. It is the T_h cells that primarily recognize LD antigens and the T_c cells that recognize the CD determinants. In a manner that is not clearly understood at the present time, the reaction of T_h cells to the foreign LD determinants on the allogeneic cell provides "help" to the precursor cytotoxic T lymphocytes that have recognized the allogeneic CD determinants. This results in the development of a stronger cytotoxic response. The cellular model, for which extensive experimental support exists, depicting response to LD and CD determinants is shown in Fig. 1.

The greater cytotoxic response engendered when both foreign LD and CD determinants are present as stimulating determinants can be obtained by using stimulating cells from a single individual who differs from the responding individual by both LD and CD determinants. Alternatively we can use the "three-cell protocol" wherein the responding lymphocytes are simultaneously cultured with stimulating cells from one individual who differs by CD determinants and with stimulating cells from a second individual who differs by LD determinants [9–11]. The precursor T_c cells of the responding cell population presumably recognize the CD determinants on the stimulating cells of one individual (depicted as C in Table 1) whereas the T_h cells respond to the LD determinants on the cells of a second individual (depicted as in-

dividual B in Table 1). It is the interaction between the T_h and T_c cells in this "three-cell protocol" which leads to the amplified cytotoxic response. In some situations, if the stimulating cells are presented in such a way that they present their CD determinants without "any" LD stimulus, no cytotoxic response is generated. However, if third party LD different, helper-stimulating cells are added, a cytotoxic response is developed against the stimulating cells that differ from the responder with regard to CD, but not LD, determinants. This last observation is particularly important for our interpretation of the data with leukemia.

Application of Three-Cell Protocol to Leukemia

Leukemic blast cells from an individual who has active disease at the time of taking the cells do not, as a general rule, stimulate a cytotoxic response in the remission, normal lymphocytes of that same individual. This is illustrated in Table 2 [12]. On the assumption that the leukemic blast cells may in fact express a CD-like target determinant which the responding autologous cells can "see" but to which they cannot respond because there is no helper stimulus available, we have used the "three-cell" approach. We have added stimulating cells of an unrelated individual who differs from the patient with leukemia by HLA-D determinants (i.e. LD antigens) to a mixture of responding lymphocytes and autologous leukemia stimulating cells. Under these conditions, we

Table 2. In vitro generation of lymphocytes cytotoxic for autologous acute myelomonocytic leukemia cells by a "three-cell" protocol

Responding lymphocytes	Stimulating cells	Effector/target cell ratio	% Specific ^{51}Cr release ± S.D.			
			Leukemic cells	Remission marrow cells	Normal lymphocytes from A	Normal lymphocytes from B
C (patient's lymphocytes)	L_x (patient's leukemia cells) + A_x (normal allogeneic lymphocytes)	40:1	17,8 ± 6,5	3,5 ± 1,4	44,0 ± 4,1	1,8 ± 2,3
C	L_x	40:1	0,0	0,0		
C	A_x	40:1	2,9 ± 1,1	0,45 ± 1,6	60,7 ± 10,1	2,2 ± 2,2
B (normal individual)	L_x	20:1	17,5 ± 12,0	14,7 ± 2,0	–0,7 ± 3,4	–0,4 ± 2,8
B	A_x	20:1			27,7 ± 2,3	–0,2 ± 3,4

Effector cells were collected 7 days after stimulation, and incubated with 1×10^4 ^{51}Cr-labelled target cells at the designated effector: target cell ratios. The % specific ^{51}Cr release was measured after a 6 hour incubation of effector cells and target cells at 37°C. [Reprinted from Zarling et al.: Nature **262**, 691–693 (1976)]

Table 3. Generation of cytotoxic lymphocytes against an HLA identical sibling's acute lymphocytic leukemia (ALL) cells by a "three-cell" protocol[a]

Responding cells	Stimulating cells	% Specific ^{51}Cr Release ± S.D.[b] Target cells	
		Sib 2 lymphocytes	Patient's ALL blasts
Sib 1	Media	- 16,0 ± 5,7	9,8 ± 2,0
Sib 1	Sib 2_m	- 9,4 ± 5,7	6,8 ± 1,5
Sib 1	Z_m	- 6,5 ± 5,7	21,4 ± 2,0
Sib 1	Pt. ALL $blasts_m$	- 10,6 ± 5,7	8,6 ± 1,8
Sib 1	Pt. ALL $blasts_m + Z_m$	- 7,2 ± 5,9	38,7 ± 1,9
Z	Pt. ALL $blasts_m$	46,6 ± 8,9	46,2 ± 3,0

[a] The ALL patient whose blasts are studied here (Pt. ALL blasts), and his siblings, Sib 1 and Sib 2, were found identical for HLA-A and HLA-B haplotypes following serological typing of all siblings at both parents. Individual Z is unrelated..

[b] Six days following mixed leukocyte culture of Sib 1's or 2's lymphocytes with mitomycin-$treated_{(m)}$ stimulating cells, the cell mediated lysis experiment was performed using a ratio of 20 effector cells: ^{51}Cr labeled target cell. Sensitization of Sib 1's cells with mitomycin-treated HLA identical ALL blasts together with mitomycin-treated allogeneic cells (Pt. ALL $blasts_m + Z_m$) resulted in the generation of a strong cytotoxic response against the patient's blasts but not against the other sibling's (Sib 2) lymphocytes. [Reprinted from Sondel et al.: J. Immunol. **117**, 2197–2203 (1976)].

have found that in a certain number of cases, we have been able to generate cytotoxic T lymphocytes that are capable of lysing the autologous leukemia cells but do not lyse normal target cells of the same individual.

An alternative approach to this problem has been to use an HLA identical sibling as the responding cell donor and ask whether these cells can generate a cytotoxic response against the leukemic cells of the HLA identical sib. (Lymphocytes of normal HLA identical siblings do not stimulate each other to produce a cytotoxic response.) As with the autologous situation, in most instances in which this protocol is attempted, when just the leukemic blast cells are used as the stimulating cells, no cytotoxic response is generated. On the other hand as depicted in Table 3 [13], a cytotoxic response is generated in some cases when the three cell approach is utilized. These findings using the three-cell approach have received extensive confirmation from the recent studies of Lee and Oliver [14].

Pool Sensitization for Generating T_c Cells

Several years ago, we noted that if cells from 20 unrelated individuals, chosen at random, are mixed in a "pool" in equal numbers, then the resulting "pooled stimulating cell" would cause a very strong proliferative response in lymphocytes from any one individual, the response being at least as strong and in most cases stronger than that induced by any one allogeneic stimulating cell [15]. Subsequent studies by Sondel et al. [16] and Martinis and Bach [17] demonstrated that stimulation with the pooled stimulating cells apparently resulted

Table 4. Lysis of autologous hairy cell leukemia cells by T cells sensitized to pooled allogeneic normal cells[a]

Responding cells	Stimulating cells	% ^{51}Cr Release ± S.D. Target cells			
		Patient 1's splenic leukemic cells	Patient 1's peripheral leukemic cells	Patient 1's peripheral T cells	Individual A's peripheral T cells
Exp. a					
P1 (patient 1)	P1-L_x (patient 1's leukemia cells)	1,7 ± 2,3		0,5 ± 3,0	
P1	$pool_x$	24,5 ± 1,8		0,8 ± 2,5	
Exp. b					
P1 (patient 1)	P1-L_x (patient 1's leukemic cells)	−1,0 ± 0,5			
P1	A_x	5,3 ± 2,6			37,7 ± 3,9
P1	$pool_x$	19,0 ± 3,7	21,1 ± 0,8	−1,5 ± 2,7	36,4 ± 5,6
A (norm. individ.)	P1-L_x	32,8 ± 2,6			
A	$pool_x$	29,5 ± 2,8	45,5 ± 3,1	15,8 ± 2,7	−9,2 ± 3,4
		Patient 2's splenic leukemic cells	Patient 2's peripheral leukemic cells	Patient 2's peripheral T cells	Individual A's peripheral T cells
Exp. c					
P2 (patient 2)	P2-L_x (patient 2's leukemic cells)	−2,6 ± 1,7			
P2	$pool_x$	18,1 ± 5,6	27,2 ± 3,6		
A (norm. individ.)	P2-L_x	25,2 ± 4,1	21,0 ± 2,6	16,8 ± 4,9	−3,6 ± 2,0
Exp. d					
P2 (patient 2)	$pool_x$	18,9 ± 1,5		−0,3 ± 2,5	
B (norm. individ.)	$pool_x$	32,7 ± 2,9		41,0 ± 5,4	

[a] T cells from normal individuals A and B and from the patients with hairy cell leukemia (P1 and P2) were isolated as described in the methods and the T cells were stimulated with the leukemia cells (P1-L_x and P2-L_x), with cells from a single normal individual (A_x) or with normal cells pooled from 20 individuals ($pool_x$). The ratio of stimulating cells to responding T cells was 1 : 1 in this experiment, however, in no case when the patients' T cells were stimulated with autologous leukemia cells at ratios of 1 : 1 to 2 : 1 was cytotoxicity against the leukemia cells detected (data not shown). T cells from the normal individuals and the patients, that were cultured in the absence of stimulating cells, were not cytotoxic for the leukemic cells (−2,3 ± 1,4 to 0,6 ± 2,0% ^{51}Cr release). [Reprinted from Zarling et al.: Nature 274, 269–271 (1978)].

in activation of essentially all cells that recognize LD determinants as well as all cells that recognize CD determinants that differ from those expressed on the responding cells. The latter finding was based on the demonstration that the cytotoxic T lymphocytes that are generated by stimulation with the pool killed virtually any allogeneic cell. Subsequent studies in our laboratory demonstrated that sensitization with the pool also led to the development of cytotoxic cells capable of killing autologous lymphoblastoid cell lines derived by Epstein-Barr virus transformation [18].

We have studied the ability of pool sensitized cells to lyse autologous leukemia cells in two patients with hairy cell leukemia. Results from those studies, shown in Table 4 [19], demonstrate that in these two cases of leukemia the pool sensitized cells are able to cause significant lysis of the leukemia cells but not autologous normal lymphocytes.

The reasons why pool sensitization is an effective method of generating T_c cells against autologous lymphoblastoid cell line or leukemia targets in at least some cases are not understood. Several possibilities exist. First, it may be that the CD determinants present on the allogeneic cells cross-react with the "CD-like" determinants recognized as foreign on the autologous abnormal cells. Alternatively it may be that pool sensitization leads to some form of "polyclonal" activation in that all clones of lymphocytes of different functional subcategories are activated by pool stimulation; there is no evidence to support such a suggestion for pool stimulation. Whether the target antigens recognized after pool-sensitization are tumor-associated antigens associated with the leukemogenic process, normal differentiation antigens expressed on the leukemic cells as they are on the precursors from which leukemia was derived, or are derepressed normal histocompatibility antigens is not clear.

Summary

We have presented the rationale for the in vitro approaches that we have taken for generating cytotoxic lymphocytes capable of lysing autologous leukemia cells or leukemia cells from HLA identical siblings. Two different approaches have been used, both of which are based on earlier findings concerning the antigenic and cellular interactions involved in the generation of strong cytotoxic responses to alloantigens in mixed leukocyte culture.

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Histocompatibility Antigens and T Cell Responses to Leukemia Antigens

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Despite the enormous number of publications on the subject, there is little solid evidence in man for tumour specific antigens which are both antigenic and immunogenic compared with the ease to which such antigens have been demonstrated for transplantable tumours in experimental animals. The failure to demonstrate such antigens in a recent study of spontaneously occurring tumours in laboratory mice has been interpreted as evidence against the existence of tumour antigens in spontaneous tumours in man. Such data cannot exclude the possibility that there may be weakly immunogenic antigens undetected by the systems under test.

The use of immunological stimulation with one molecule to augment response to an otherwise non-immunogenic molecule is not new. In experimental animals the use of carrier molecules to stimulate antibody response to hapten are standard procedures and the interdependence of T and B cells in these responses is well documented. It is usual in these systems for the hapten to have to be attached to the carrier molecule. In contrast for cellular responses to transplantation antigen which also have been shown by Eijsvoogel [1] to require two signals (i.e. a serologically defined HLA antigen and a lymphocyte activating HLA D antigen) it has been clearly demonstrated that it is not necessary for the two signals to be presented on the same stimulating cell. Using three cell experiments i.e. two stimulators, one differing for HLA A, B and C antigens and one differing for HLA D antigens from the responder, Eijsvoogel [1] demonstrated that the two types of antigen can be present on separate cells and still produce cytotoxic lymphocytes which retain specificity for the target HLA antigen but did not react to the cell which only carried the lymphocyte activating HLA D antigen.

Applying this principal to patients with acute leukemia, Sondell et al. [4] demonstrated that HLA identical siblings of patients with acute leukemia developed significantly greater cytotoxicity against the patient's leukemia blast cell if primed in vitro with the patient's blast and a third party allogeneic lymphocyte, than when they were primed with the patient's blast cell alone. Zarling et al. [5] reported one patient where it was possible to generate significant cytotoxicity of remission lymphocytes against autologous blasts by priming with the autologous blast cell mixed with allogeneic lymphocytes. Lee and Oliver [2] recently applied this technique to study patients receiving either BCG or BCG plus allogeneic blast cells as remission maintenance immunotherapy. They found that 10 out of 14 patients studied were able to

generate significant T cell mediated cytotoxicity against the specific autologous blast cell when primed in vitro with the specific blasts in presence of a third party allogeneic cell. This cytotoxicity was specific for the leukemia as no cytotoxicity was demonstrable against PHA transformed autologous remission lymphocytes (or autologous remission bone marrow in the one case tested). Investigation of the nature of the antigen on the third party helper cell using lymphoblastoid cell lines showed that the cytotoxic cells could only be generated if the helper cell was HLA D antigen different from those of responder but not if they were only different for HLA A and B locus antigens. Using this technique it was not possible to use cross killing experiments against allogeneic blast cells to investigate whether the target antigen was present on allogeneic blast cells. This was because the use of a third party allogeneic cell in the priming mixture always led to production of anti-HLA cytotoxic cells in addition to anti-leukemia cytotoxic cells. However results from four indirect approaches (in vitro priming with allogeneic blast cells and testing on the autologous blasts [2], cross over experiments with pairs of patients [2], absence of any difference in the level of cytotoxicity in patients who have received BCG or BCG plus allogeneic blast cell immunotherapy [3], cold target inhibition experiments [2]) suggest that the target antigen is not present on allogeneic blasts.

Recent experiments have been directed to investigate the nature of the target antigen on the autologous blast cell. To exclude the possibility that the target antigen was a normal myeloid or T or B lymphocyte differentiation antigen, anti-leukemia cytotoxic cells were tested against autologous remission bone marrow and autologous remission T or B enriched lymphoid sub populations (see Table 1). There was no evidence from these experiments for the target antigen being a normal differentiation antigen, though to be absolutely certain of this it would be necessary to use purified stem cells from remission bone marrow which would be technically extremely difficult to do.

The experiments in Table 2 attempt to exclude the possibility that the cytotoxicity demonstrable in this assay was due to cross reactivity with normal HLA antigens generated by priming in the presence of an allogeneic

Table 1. Evaluation of normal T and B lymphocytes as targets for anti leukemia CML effector cells

Priming stimulus	Targets					
	Autologous T lymphocytes		Autologous B lymphocytes		Autologous leukemia blasts	
	1	2	1	2	1	2
Autologous B lymphocytes	1[a]	ND	3	ND	0	ND
Autologous B lymphocytes + Allogeneic lymphocytes	0	ND	5	ND	0	ND
Autologous leukemia blasts + Allogeneic lymphocytes	0	0	8	0	21	13

[a] % specific ^{51}CR release

Table 2. Specificity studies on anti-leukemia effector cells

	Targets							
	Autologous remission lymphocyte		Autologous leukemia blast		Allogeneic lymphocyte		Daudi	
	a	b	a	b	a	b	a	b
GP	0	0	0	25	0	0	ND	20
EB	0	0	0	0	0	0	ND	40
JK	0	0	0	46	0	0	ND	70
TW	0	0	0	25	0	0	ND	90
MB	2	0	0	44	13	8	ND	45
LF	ND	0	ND	18	ND	0	ND	23

[a] Remission lymphocytes cultured 6 days without priming.
[b] Remission lymphocytes primed with autologous blasts plus Daudi.

lymphocyte. The experiments shown make use of the fact that Daudi does not express serologically defined HLA A, B or C locus antigens, but can help in recognition of autologous leukemia antigen. These experiments which demonstrate that effector populations with anti leukemia activity do not kill allogeneic normal cells are in contrast to those of Zarling et al. [6]. They reported two patients with hairy cell leukemia where cytotoxic cells produced by priming the patient's lymphocytes with a pool of allogeneic normal cells generated significant cytotoxicity against their own leukemia cell. It is possible than an adequate number of normal cells were not tested using our assay or that the target antigen in AML is different from that in hairy cell leukemia. Further investigation of this is clearly required to clarify the difference.

Conclusions

The use of allogeneic cells to facilitate recognition of a non-immunogeneic leukemia antigen opens up new avenues for development of immunotherapy in human tumours. The fact that the target antigen has not been demonstrated on allogeneic tumours invalidates most of the recent studies of immunotherapy using allogeneic tumour cells.

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T Cell Function in Myelogenous Leukemia

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Introduction

There are several previous reports of proliferative reactions between remission lymphocytes and autologous or allogeneic cyopreserved blasts, in a proportion of patients with granulocytic and lymphocytic leukemias [1,2]. More recently, T lymphocytes cytotoxic for autologous leukemic blasts have been generated in vitro by a method involving allogeneic help [3–5]. These results suggest that leukemia specific antigens may exist on leukemic blast cells, and are immunogenic for autologous T lymphocytes.

If more detailed specificity studies confirm this interpretation, which would be compatible conceptually with the central dogma of tumour immunology derived from animal experiments, the immunotherapy of human leukemia with specifically immune autologous T cells becomes justifiable experimentally. For this approach to be practicable, large numbers of leukemia-specific T cells will be required.

T lymphocytes from normal donors have been maintained in culture for up to 13 months using a factor obtained from conditioned medium (LyCM) derived from phytohaemagglutinin (PHA) – stimulated normal human lymphocytes [6]. We have used this culture system to grow, selectively, T lymphocytes from the peripheral blood of patients with granulocytic leukemias in the untreated acute phase of the disease, and report here preliminary studies on the function of the cultured cells.

Methods

1. T Cell Culture

The procedure for T lymphocyte culture has been described previously [6,7]. Briefly, peripheral blood leukemic cells, obtained by leukophoresis, were seeded into 16 × 125 mm plastic tubes (Falcon Plastics, Oxnard, Calif.) at a concentration of $1-2 \times 10^5$ per ml of RPMI – 1640 medium supplemented with 20% heat inactivated fetal calf serum and 20% of four-fold concentrated LyCM (Associated Biomedic Systems, Buffalo, N.Y.). Cell concentration was maintained between 3×10^5 and 1×10^6 per ml by subculturing the cells in the same medium.

2. Mixed Leukocyte Culture

One way mixed leukocyte reactions (MLRs) were set up as described previously [7], using mitomycin-C treated autologous or allogeneic stimulating cells.

Results and Discussion

Fresh unfractionated peripheral blood white cells from only 11 of 26 patients with untreated acute phase myelogenous leukemias responded to allogeneic stimulation in the MLR.

Cells from a total of 15 patients were fractionated on nylon wool columns (Table 1). Alloresponsive cells were detected in either non-adherent or adherent populations in 3 out of 9 patients whose unfractionated cells were unresponsive. In another 3 patients, 8-03, 7-126 and 7-127, the magnitude of the allogeneic response of both non-adherent and adherent fractions was greater than that of the unfractionated population.

Table 1. Allogeneic and autologous responses of nylon fractionated leukaemic and normal peripheral blood white cells

Patient	Diagnosis	% Recovery		Allogeneic response			Autologous response
		Nylon non-adherent	Nylon adherent	Unfractionated	Nylon non-adherent	Nylon adherent	Non-adherent vs. Adherent
Normal		47,9	14,3	+	+ +	±	+
8- 03	CGL	7,6	24,7	+	+ +	+ +	+
7-126	AML	51,5	31,7	+	+ +	+ +	–
7-127	AML	46,1	19,0	+	+ +	+ +	–
7-105	AML	24,0	72,5	+	+ +	ND	–
7-119	AML	75,9	24,0	+	+ +	±	+
7-129	CGL	36,0	35,3	+	–	+	–
7-112	AML	50,0	26,7	–	+	–	+
7-133	AML	43,3	25,5	–	+	–	+
7-115	AML	14,7	19,0	–	–	+	–
7-107	AML	79,5	21,5	–	–	ND	+
7-113	CGL BC	34,0	37,3	–	–	–	–
7-117	CGL BC	64,0	23,3	–	–	–	–
7-110	AML	57,6	42,4	–	–	–	–
7-130	AML	54,7	17,3	–	–	–	–
7-131	AML	61,3	24,0	–	–	–	–

In 5 of the total of 15 patients whose cells were fractionated on nylon wool, non-adherent cells were stimulated by autologous nylon adherent cells. However, a similar autologous MLR was detected between the nylon wool fractions from all 10 normal donors tested.

In the residual 5 patients no allogeneic or autologous reactions were seen in the unfractionated or in either nylon wool fraction.

Samples from 13 patients were cultured in the presence of LyCM. Before culture, samples from the majority of patients with granulocytic leukemias had fewer than 5% E-rosette positive cells. In the presence of LyCM, the proportion of E-rosette positive cells after 12–17 days of culture is between 36 and 91%. After 26–36 days in culture, the percentage has increased to between 61 and 93%.

Cells cultured from all 13 donors showed significant alloresponses after culture (Table 2). Samples from 4 patients, whose fresh uncultured cells were unresponsive, all showed an allogeneic response after between 7 and 29 days in culture. The appearance of alloresponsive cells after culture in LyCM provides functional evidence that the E-rosette positive cells present after culture of leukemic peripheral blood are indeed T lymphocytes.

Cultured T cells from 8 patients were tested in a one way MLR for their response to autologous blast cells. In only 4 cases, however, was a significant response detected. Ia-like antigens have been detected serologically on a variety of leukemic blast cells [8], and Ia-bearing human B cells can stimulate histocompatible human T cells [9]. Evidence for in vivo immunization by leukemia-specific antigens therefore requires analysis of the specificity of the autologous proliferative reactions observed in vitro.

The present results indicate that T lymphocytes can be cultured from the peripheral blood of leukemic patients in the acute phase of the disease, which mount an allogeneic blastogenic response comparable to that of cultured T cells derived from normal donors. The appearance of such functional T cells

Table 2. Response of leukemia-derived cultured T lymphocytes to allogeneic stimulation in a one-way mixed lymphocyte reaction

Patient	Diagnosis	Days in culture	Stimulation index
7-11	AML	62	0,65
7-18	AML	35	5,92
7-22	Sezary syndrome	27	21,72
7-23	CGL	21	9,90
7-35	AMML	19	1,32
7-56	CGL	26	1,80
7-66	Acute undifferentiated leukemia	19	28,08
7-69	AML	12	156,41
7-82	ALL	11	10,80
7-92	AML	29	27,56
7-93	CGL blast crisis	29	13,89
7-97	AML	7	1,18
7-101	AML	21	41,34

after culture away from the leukemic microenvironment suggests that there is no functional defect in the residual circulating T cells in vivo. The autologous reactivity observed in some patients suggests that leukemic T cells may have the potential of becoming immunized by antigens on the leukemic blast cells. The availability of cultured T cells derived from leukemic donors offers the possibility of in vitro boosting of any such primary immunity, and autologous immunotherapy with in vitro boosted cultured T cells.

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Recognition of Simian Sarcoma Virus Antigen by Human Sera

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Abbreviations

RIA: Radioimmunoassay; IEM: Immuno-electron microscopy; SSV: Simian Sarcoma Virus; SSAV: Simian Sarcoma Associated Virus; gp70 SSV (SSAV): The 70000 d virus envelope glycoprotein of SSV (SSAV); NHS: Normal human serum

A. Introductory Remarks

Previous work has indicated the presence of antibodies in human sera, capable of reacting with the envelope antigens of murine and primate C-type viruses [1–5]. These antibodies are present in the majority of all adult sera tested to date, but are absent in cord blood. These, plus related observations (discussed in [3,6]) are compatible with the idea, that horizontally transmissable viruses, which are immunologically related to, but not identical with the currently known C-type tumor viruses, may have elicited the anti-viral antibodies.

Because no human type C tumor virus strains have so far been isolated, it is imperative that the precise nature of the anti-viral immune response is defined and documented beyond any doubt. This is of considerable importance, as two laboratories have been unable to detect anti-tumor virus antibodies in man [7,8]. We have therefore tried to think of ways which, in addition to the sensitive RIAs previously used by us, would indicate the presence of specific anti-viral antibodies in man.

In this communication, we present evidence that the anti-viral human antibodies belong to the immunoglobulin G class, and that they bind with differing affinities to the SSV (SSAV) gp70, the prime target antigen used for the human antibodies. Furthermore, evidence is presented showing that a recently developed immuno-electron microscopical method (IEM) seems suitable to confirm previous positive data, and that sero-epidemiological investigations may enable us to easily detect sera with anti-viral antibody titers.

B. Experimental Approaches

Virus Antigens

Simian sarcoma virus (Simian sarcoma associated virus) SSV (SSAV) is a mixture of two C-type primate tumor viruses first isolated from a woolly monkey fibrosarcoma in 1971. The major envelope glycoprotein gp70 of SSV (SSAV) was purified [9] and kindly made available by Drs. H.-J. Thiel and W. Schäfer, Max-Planck-Institut für Virusforschung, Tübingen.

Sera

The human sera employed in this study originated either from blood donors from the Tübingen area or were obtained through the international services of Behringwerke, Marburg. IgG and IgM were purified according to published procedures. Anti-human IgG and IgM antisera were prepared in goats.

Hyperimmune goat anti-SSV (SSAV) or anti-gp70 SSV (SSAV) control sera were obtained from Dr. F. Deinhardt, Pettenkofer-Institut, Universität München, and from Dr. J. Gruber, Office of Logistics and Resources, National Cancer Institute, Bethesda, respectively.

Radioimmunoassays

A detailed description of our indirect radioimmunoassay procedure has been published previously [3,6]. Anti-immunoglobulin antisera were used to cross-link and sediment antigen-antibody complexes.

Antibody Affinity Determinations

The relative affinity (K_R, litres/mole) were measured by a double antibody precipitation method, as previously described [10], using 10 µl of serum. Briefly, the assay consisted of the determination of free and antibody-bound antigens at equilibrium over a range of antigen concentrations from 2.5 to 20 ng. Total antibody-binding site concentration was obtained by extrapolation to infinite free antigen concentration of a Langmuir plot of the reciprocal of the bound antigen versus the reciprocal of the free antigen.

$$\frac{1}{b} = \frac{1}{k} \cdot \frac{1}{c} \cdot \frac{1}{Abt} + \frac{1}{Abt}$$

where b = bound antigen, c = free antigen, k = affinity and Abt = total antibody binding sites.

Antibody affinity was calculated from this curvilinear plot as the reciprocal of the free antigen concentration when half the antibody-binding sites were bound to antigen.

Immuno-peroxidase (I-POD) Labeling

Besides conventional conjugates of POD and anti-IgG antibody [11], a newly developed indirect labeling technique using protein A-POD was applied in

evaluating the immunoreactivity of human and control animal sera. This conjugate was prepared using glutaraldehyde (GA) in a two-step procedure involving GA-activation of the peroxidase (Boehringer, Mannheim) in the first step, removal of unreacted GA by gel filtration and reaction of the activated POD with protein A (Pharmacia, Uppsala).

A detailed characterization of this conjugate and its potential use in comparison with other types of I-POD and immunoferritin conjugates will be presented elsewhere (H.G. and W.V., manuscript in preparation).

Normal rat kidney cells infected by SSV (SSAV) (NRK-SSV (SSAV) cells) were grown for 24 to 48 hrs in Microtest No. 1 plates (Falcon Plastics, Oxnard, Calif.) and processed for immuno-labeling after GA fixation as published previously [12]. Briefly, human as well as hyperimmune and negative animal control sera where used undiluted, except for the goat anti-SSV (SSAV) which was applied at a 1:20 dilution. These primary sera as well as the anti-immunoglobulin G POD-conjugates were each incubated for 30′ at 37°C with the NRK-SSV (SSAV) cells. The catalytic activity of the bound enzyme was localized according to Graham and Karnovsky [13] using diamino-benzidine and H_2O_2 (Merck, Darmstadt). Following post-fixation in 1% OsO_4 the cultures were dehydrated in ethanol, embedded in situ in Epon and processed as published previously [12].

C. Demonstration of Anti-Viral Human Antibodies

Previous experiments [3] had indicated that the predominant anti-viral antibody activity in exposed animals is directed against so-called "interspecies-specific" determinants on the envelope glycoprotein antigens (gp70s) of the mammalian C-type viruses. These interspecies-specific determinants are shared by viruses originating from different natural host species. A typical titration curve of normal human sera with the gp70 of SSV (SSAV) is shown in Fig. 1. It can be seen that even the titers of sera which react well (e.g. NHS No. 29 and No. 30), are relatively low compared to titers observed after infection with classical horizontally infectious viruses, e.g. rhino- or influenza-viruses. NHS No. 41 (▲ in Fig. 1) is unusual in that it possesses the highest titer by far, and recognizes more than the interspecies-specific determinants on gp70 SSV (SSAV). Since this serum is from a laboratory worker who used to work with SSV (SSAV) we have reason to suspect that the relatively high titer is the result of laboratory exposure to this virus.

In Table 1, the precipitation of gp70 SSV (SSAV) by whole sera is compared to that of immunoglobulins isolated from the sera. The precipitation by isolated immunoglobulin G can account for the entire anti-viral serum reactivity. Only in four out of over one hundred sera, could a slight anti-viral IgM reactivity be observed (R.K. and C. Schmitt, unpublished observation). The association of the anti-viral immunity with the immunoglobulin G class is not only confirmed by the use of isolated IgG, but also by the use in the RIAs of goat anti-human IgG antisera which by themselves have no anti-viral or anti-IgM activities.

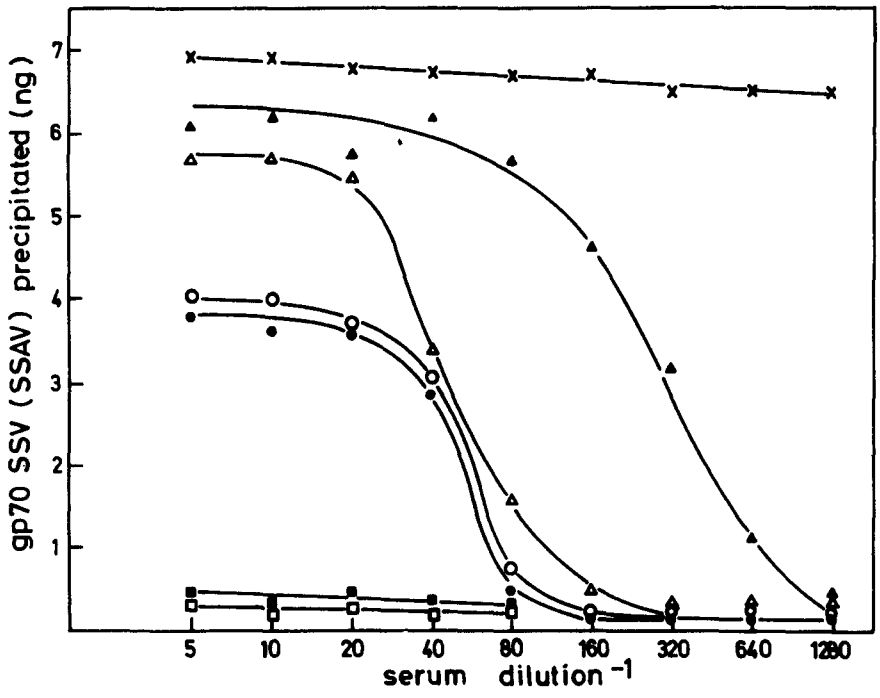

Fig. 1. Titration of human sera with gp70 SSV (SSAV). Goat anti-SSV (SSAV) antiserum: X. Human sera: NHS 41: ▲; NHS 24: △; NHS 29: ○; NHS 30: ●; NHS 63: ■; normal goat serum: □. Input antigen: 7,72 ng gp70 SSV (SSAV)

Preliminary sero-epidemiological investigations yield an interesting pattern of the distribution of positive sera (Table 2). Whereas about 50% of the sera from the Tübingen area react clearly with gp70 SSV (SSAV), the percentage and titers fall drastically when sera from, for example Japan and Egypt are tested. In contrast, sera from South America so far possess unusually high titers.

Table 1. Comparative immunoprecipitation of gp70 SSV (SSAV) by human sera and immunoglobulins

Serum	Serum source	Precipitation by whole serum[a] (ng)	IgG (ng)	IgM (ng)
normal goat serum	goat	<0,5	<0,3	<0,3
goat anti-SSV (SSAV)	goat	4,86	4,42	<0,3
NHS 41	laboratory worker	4,07	3,68	<0,3
NHS 64	laboratory worker	1,10	0,7	<0,3
NHS 24	blood donor	3,22	3,17	<0,3
NHS 29	blood donor	2,66	2,72	<0,3
NHS 30	blood donor	2,22	2,46	<0,3
NHS 63	blood donor	<0,5	<0,3	<0,3

[a] Radioimmunoassay using 10 µl (1:40 final dilution) whole human serum or the equivalent amount of IgG or IgM. Input antigen: 5,15 ng gp70 SSV (SSAV)

Table 2. Immunoprecipitation of gp70 SSV (SSAV) with human sera from different continents

Sera	Serum source	Origin of serum	gp70 SSV (SSAV) precipitated (ng)[a]	number positive[d] sera/ number tested
normal goat serum	goat	Germany	<1,0	
goat anti-SSV (SSAV)	goat	Germany	20,9	
NHS 41	laboratory worker	Germany	17,9[b]	
NHS 42	blood donor	Germany	<1,0[c]	
S1		Japan	1,28	
S2			3,01	
S3			1,35	
S4			1,60	
S5			1,45	
S6			1,01	
S7			1,33	
S8			2,03	
				3/9
S19		Egypt	1,05	
S20			1,30	
S21			0,95	
S22			0,98	
S23			1,76	
S24			1,05	
S25			1,35	
S26			2,48	
S27			1,28	
S28			1,47	
				2/10
S33		South America	5,91	
S34			4,08	
S35			5,07	
S36			11,30	
S37			6,34	
S38			9,57	
S39			3,37	
S40			4,04	
S41			4,20	
S42			6,24	
				10/10

[a] excess gp70 SSV (SSAV) precipitated by 10 µl (1:40 dil) human serum
[b] highest titer normal human serum
[c] negative titer normal human serum
[d] positive sera (10 µl, 1:40 final dilution) precipitate >1,5 ng gp70 SSV (SSAV)

When the affinites of antibodies in sera from blood donors and patients with various diseases were measured, it was found that a proportion reacted as high affinity sera with K_R values between 5×10^8 and 2×10^9 (litres × mole) (Table 3). Scatchard plots of some of the sera listed in Table 3 are shown in Fig. 2.

Table 3. Average antibody affinities of human sera to purified envelope glycoprotein (gp70) of SSV (SSAV)

Serum	Serum source	Average affinity K_R (litres/mole)
normal goat serum	goat	Not calculable
goat anti-SSV (SSAV)	goat	$2{,}4 \times 10^9$
NHS 22	laboratory worker	$1{,}9 \times 10^9$
NHS 24	blood donor	$1{,}0 \times 10^9$
E 5	teratocarcinoma patient	$1{,}3 \times 10^9$
E 33	teratocarcinoma patient	$1{,}0 \times 10^9$
M 102	melanoma patient	$5{,}1 \times 10^8$
L 38	systemic lupus erythematosus patient	$5{,}0 \times 10^8$

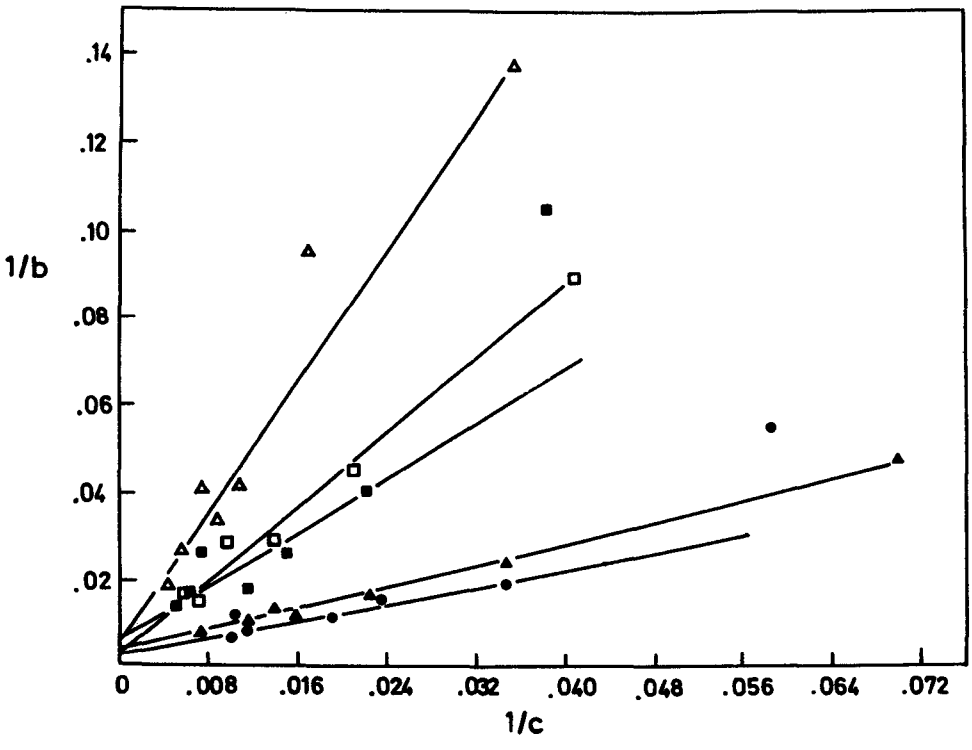

Fig. 2. Relative affinity, (K_R), of anti-gp70 SSV (SSAV) antibody in various human and animal sera. Horizontal axis: 1/c (reciprocal of free antigen), vertical axis: 1/b (reciprocal of bound-antigen). ●: goat anti-SSV (SSAV), 1 : 100 initial dilution. ▲: NHS 41; ■: NHS E5; △: NHS L38; □: NHS M102

In an effort to demonstrate anti-viral immunity by additional techniques, IEM was employed for the detection of anti-viral antibodies. Human sera which reacted well in RIAs also react with the protein A-POD technique (Figs. 3 and 4a). Conversely, RIA-negative human and animal control sera remained negative (Fig. 4b).

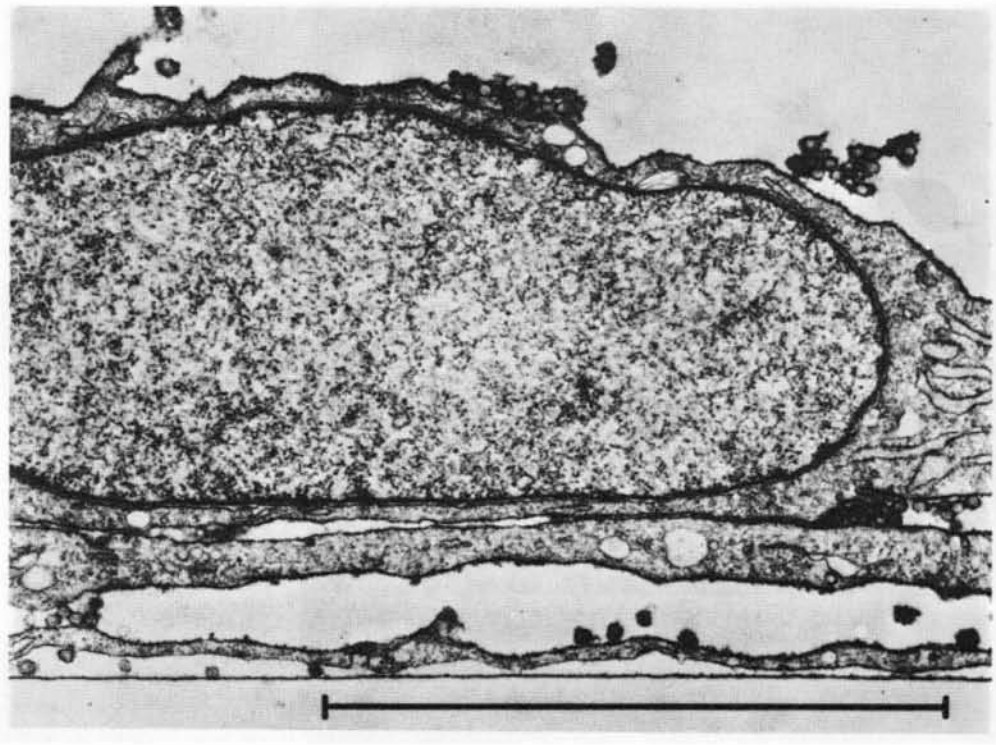

Fig. 3. Low power micrograph showing section of three cells and aggregates of virus particles. Virus and cell surface show electron dense label after incubation with NHS No. 41 and protein A-POD reaction. Bar represents 5 μm; Magnification: × 15000

D. Discussion and Future Prospects

In this communication, additional data are presented which support previous evidence for a widespread human immune reactivity against C-type tumor virus related antigens. The antibodies which react via interspecies-specific determinants with the prototype viral antigen, the gp70 of SSV (SSAV), are here shown to belong to the IgG-class. RIAs, in which purified gp70 SSV (SSAV) was recognized by purified human IgG, and where the cross-linking was achieved with the purified IgG-fraction from goat anti-human IgG (data not shown here) have been performed. IgM reactivity is found only rarely, if at all, and in the few cases where anti-viral IgM activity is observed, the titers are low.

Preliminary sero-epidemiological studies indicate that the percentage and titers of positive human sera may be quite different in different areas of the world. It is obvious, that the limited number of serum samples from overseas (approx. 100), which have been tested so far precludes any premature conclusions. Nevertheless, the differences in activity between sera from e.g. Egypt and South America, are striking. These studies will be extended.

Anti-viral activity has previously been demonstrated for both human antibodies and lymphocytes [5]. Usually, RIAs as well as cytotoxic assays and lymphocyte stimulation techniques have been used. The successful IEM approach with human antibodies made visible by the use of protein A-peroxid-

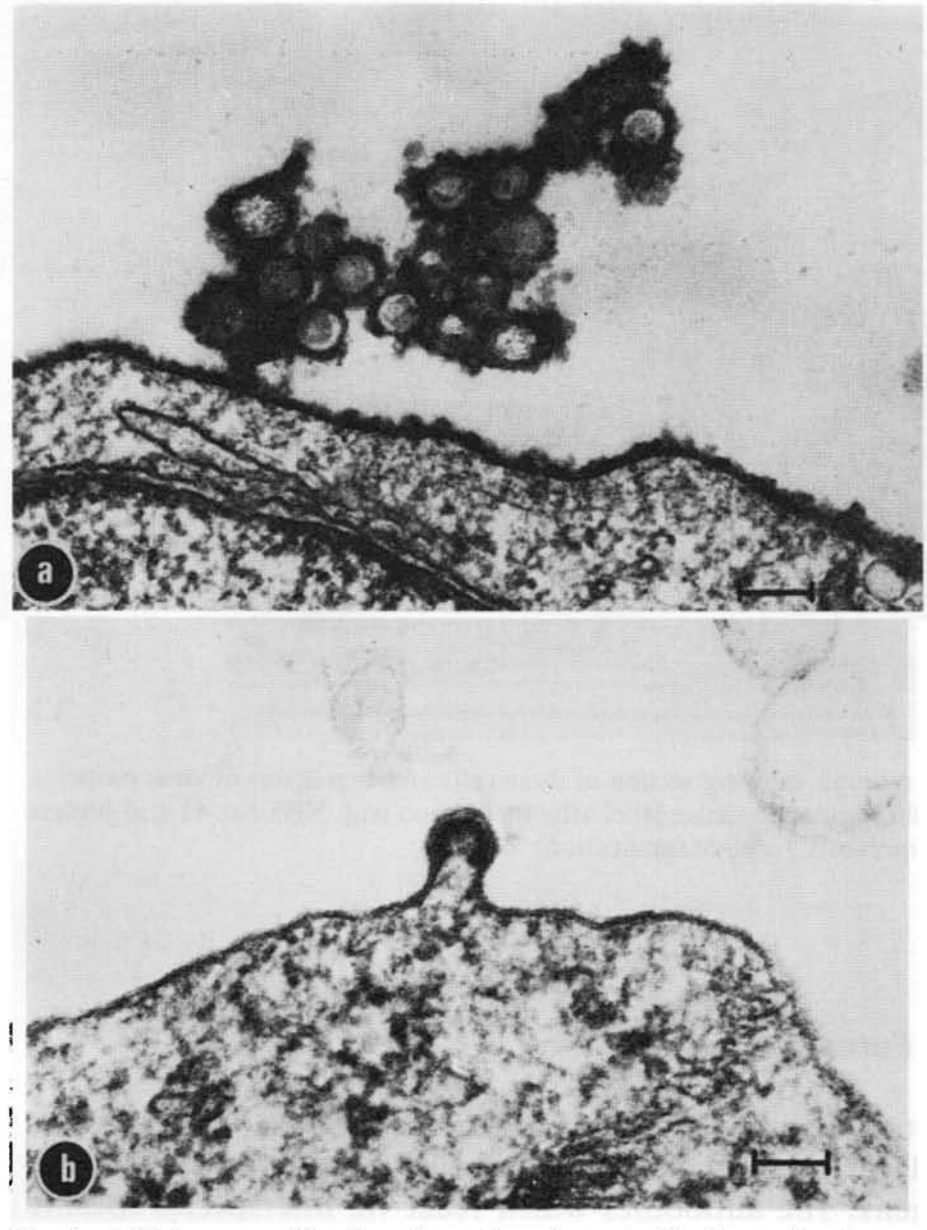

Fig. 4. a) Higher magnification of a section shown in Fig. 3 revealing heavy electron-dense reaction products indicating positive immune reactions. b) Appearance of NRK-SSV (SSAV) cells and budding virion after incubation with normal rabbit serum and protein A-POD reaction. Bar represents 100 nm. Magnification: × 100000

ase conjugates adds another method to the arsenal of refined immunological techniques which detect anti-viral human antibodies. These data confirm earlier results by Aoki and co-workers [1] who used ferritin-tagged antibodies to monitor the absorption of human antibodies to tumor viruses and virus-infected target cells.

One of the strongest pieces of evidence supporting the specificity of the antibody-binding to tumor virus antigens can be derived from the (preliminary in number) affinity data. The high average affinity constants observed with a proportion of human sera indicate that whatever the antigens were that induced the human antibodies, they must have been very similar in antigenicity to the determinants detected on tumor virus envelope antigens.

In closing, however, we would not like to leave the interested but experimentally uninvolved reader with the impression that we are here arguing for the presence of horizontally transmissible C-type tumor viruses which have yet to be identified. Even though this certainly still represents a viable alternative – and there are a number of possibilities why horizontal virus transmission may have been missed so far – other mechanisms are easily conceivable which may have induced an anti-viral immunity in man. Such alternatives have been discussed in some detail previously [6, 14]. Suffice it to say that it cannot be excluded at present that antigens may exist which fortuitously cross-react with tumor virus antigens. To our knowledge, however, no such cross-reacting antigens have yet been demonstrated for any animal C-type tumor virus.

Our present investigations are directed towards elucidating the anti-viral immune status in groups of patients with various diseases, notably tumors. A constant change in immunity in a given group of patients, if observed, would then justify a detailed search for viral foot-prints in the corresponding diseased tissues, e.g. in neoplasms.

Acknowledgements

We would like to thank Drs. W. Schäfer and H.-J. Thiel, Max-Planck-Institut für Virusforschung, Tübingen, for their generous gift of purified gp70 SSV (SSAV). We also gratefully acknowledge the expert technical assistance of Mrs. U. Mikschy, Mrs. C. Baradoy and Mr. H. Reupke. Part of this work was supported by the Deutsche Forschungsgemeinschaft with grants No. Ku 330/3 and Wa 139/10 to R.K. and Ge 281/2 to H.G.

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Studies of Human Serological Reactivity with Type C Virus and Viral Proteins

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Introduction

The persistent controversy surrounding possible association of type C viruses with human malignancies is the result of a number of not always compatible observations in many laboratories. Thus, while a number of reports of virus-like particles (occasionally with associated infectivity), reverse transcriptase, virus-like RNA, viral proteins, and proviral DNA sequences in human tissues exist [1], the results must be weighed in relation to negative findings by other investigators seeking similar evidence [2].

Our approach to the question of human exposure to type C RNA viruses has been to investigate human sera for naturally occurring antibodies to such agents [3]. Others have followed a similar tactic [4,5]. It is generally accepted that, even if humans are naturally infected with a type C virus, overt virus replication, such as occurs in cats or gibbon apes, is a rare event, if it occurs at all. With this in mind, we have employed a very sensitive radioimmunoprecipitation (RIP) assay in a search for type-C virus-reactive human antibodies, which we assumed might only be detectable in relatively low titers.

We observed that many human sera are in fact reactive with surface antigens of a particular group of type C viruses and capable of precipitating intact radiolabeled virions [3]. By this assay approximately 75% of normal human sera precipitate significant amounts of purified leukemia viruses of woolly monkey (SSV-SSAV), murine (Rauscher-MuLV), and cat (FeLV) origin; in contrast, tests on endogenous type C viruses of the cat (RD114) and baboon (BEV), on an avian leukosis virus (RAV-2) and on two enveloped RNA-containing non-type C viruses (VSV and Sindbis virus) revealed no appreciable titers (for representative data see Fig. 1). Demonstration of activity in the RIP assay with purified IgG and $F(ab')_2$ fragments of IgG indicated that binding of virus is mediated by antibody. The observed reactivity cannot be attributed to the presence of any known heterophil antibody specificity or to the presence of antigens specific to tissue culture cells [3].

Evaluation of Evidence for Viral Specificity in the RIP Assay

The restricted reactivity of the human antibodies for a limited group of type C viruses (Fig. 1) was strongly suggestive of some viral specificity. Comparative RIP titers for a number of human sera with the above viruses have shown that in general, sera react with SSV-SSAV, R-MuLV, and FeLV to roughly

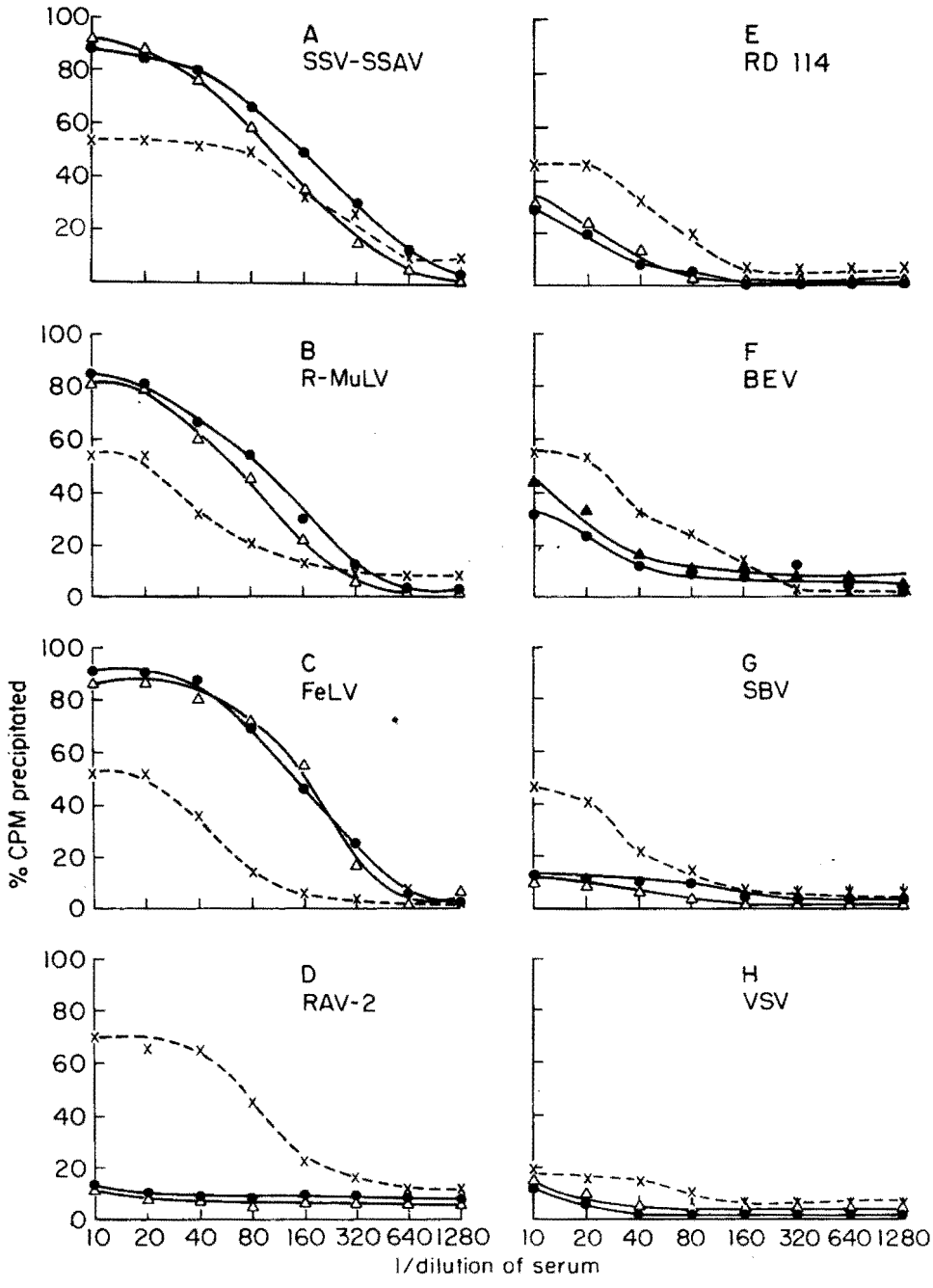

Fig. 1. RIP titrations of two normal human sera (●, △) and rabbit anti-FCS serum (x) with 1 μg intact radiolabelled virus: (A) ^{3}H-amino acid labelled SSV-SSAV, 4000 cpm, (B) ^{3}H-amino acid labelled R-MuLV, 3200 cpm, (C) ^{3}H-leucine labelled FeLV-AB, 4100 cpm, (D) ^{3}H-amino acid labelled RAV-2, 8000 cpm, (E) ^{3}H-amino acid labelled RD114, 2000 cpm, (F) ^{3}H-leucine labelled BEV, 3200 cpm, (G) ^{3}H-leucine labelled SBV, 3500 cpm, (H) ^{3}H-leucine labelled VSV, 3700 cpm. Titrations were performed as described in Snyder et al. [3]

similar titers. The similarity holds whether titers are high or low. The relationship between the reactive antigens in these viruses was studied in quantitative absorption experiments in which increasing amounts of unlabelled virus were tested for the ability to compete with 1 μg of labelled SSV-SSAV (left panel, Fig. 2) and unlabelled R-MuLV (right panel) for binding to antibody in a limiting dilution of a human serum. Unlabelled SSV-SSAV, R-MuLV, and FeLV competed completely in both assays, whereas RD114 competed

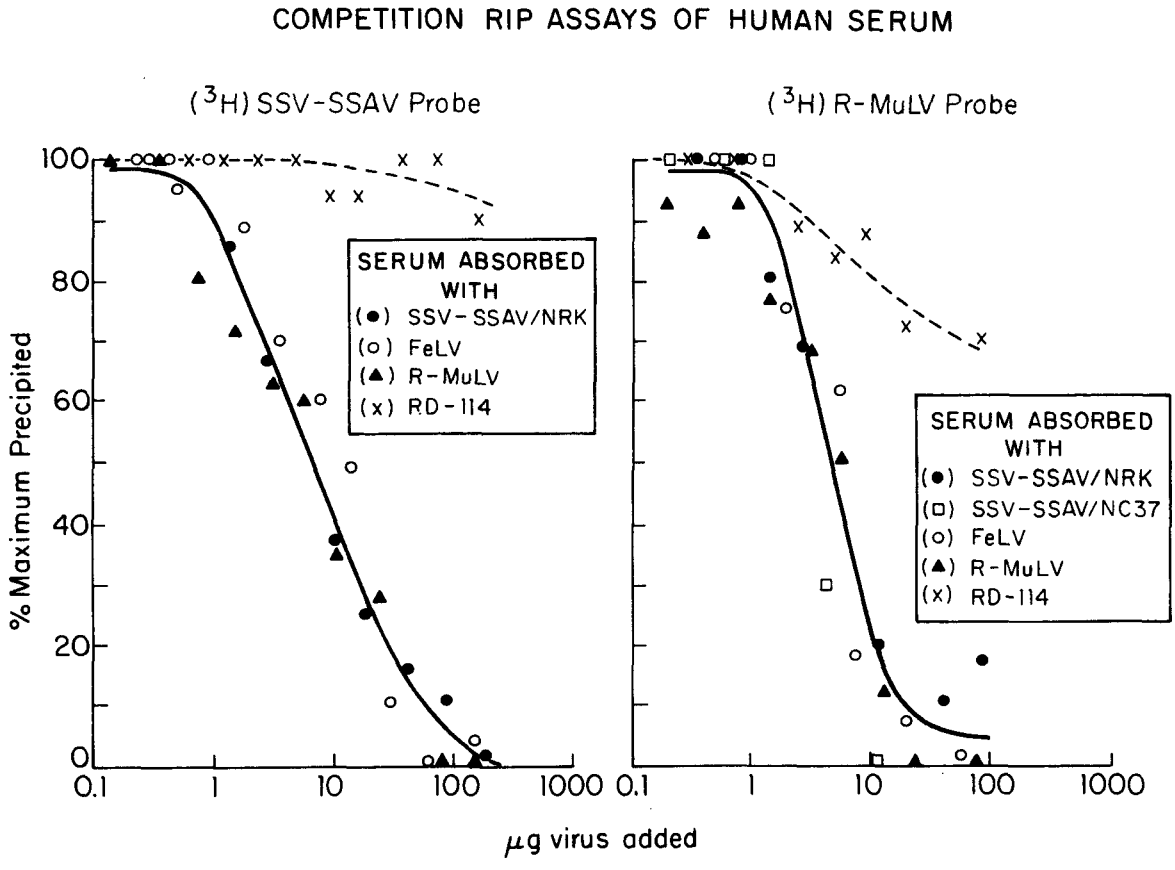

Fig. 2. Competition RIP assay of antibodies in a limiting dilution of a normal human serum for binding 1 μg ^{3}H-amino acid labelled SSV-SSAV (left panel) or R-MuLV (right panel) while in the presence of the amounts of unlabelled virus preparations indicated on the abscissas

only slightly and then only at the highest concentrations tested. The data suggest that the human antibodies being measured are directed against determinants which resemble, however distantly, the broadly cross-reactive viral envelope antigens of the exogenously infectious type C viruses SSV-SSAV, R-MuLV, and FeLV rather than antigens of endogenous viruses belonging to the RD114/BEV class.

Additional evidence of viral specificity was provided by the demonstration that a fraction of SSV-SSAV containing predominantly viral gp70 completely absorbed human serum activity in a RIP assay while fractions containing other viral structural proteins did not [3]. It should be noted also that the competition experiments of Fig. 2 are consistent with the known antigenicities of the respective gp70s [6]. Recently we purified SSV-SSAV which had been radiolabeled in tissue culture to high specific activity with ^{3}H-glucosamine (10^5 cpm/μg), lysed the virus with NP40 and centrifuged it at 100000 g for 1 hr. Labeled viral gp70 in the supernatant served as a probe for analysis of human sera for antibodies. A panel of human sera were analyzed for 1. RIP reactivity against intact SSV-SSAV and 2. precipitating activity against

the glucosamine-labeled gp70 probe. The sera tested were obtained from Dr. R. Kurth (Tübingen) and contained some samples capable of precipitating a purified SSV-SSAV gp70 probe (Kurth, personal communication). While none of these sera reacted with our gp70 probe obtained by gel filtration in 6M guanidine-HCl [3], our new results show a strong correlation between the ability to precipitate intact virus and the ability to precipitate detergent solubilized gp70 (Fig. 3). Partial purification of the gp70 from this supernatant by phosphocellulose chromatography (0,1–0,2M salt elution) did not result in any loss of the antigen detected by the human sera. Further purification is still required to establish unequivocally that viral gp70 is involved in these reactions. Interestingly (and not unexpectedly) observed titers with the intact virus RIP assay were higher than could be observed by using a solubilized gp70 probe, suggesting that the former system can be more sensitive (Fig. 3).

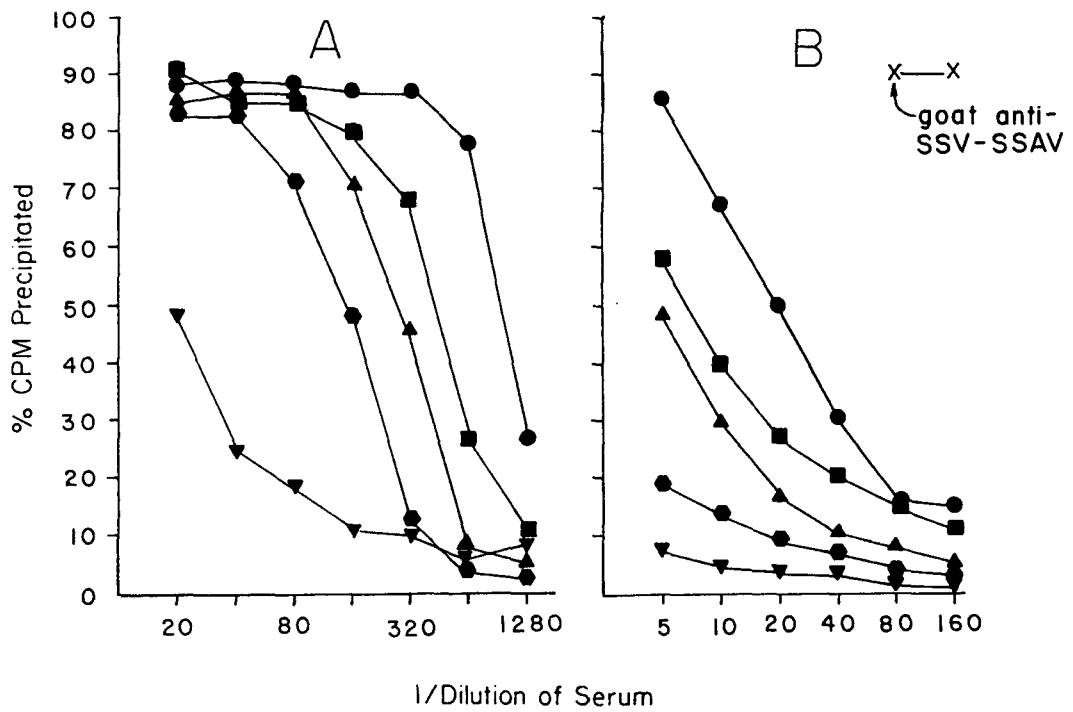

Fig. 3. Left: RIP titrations of 5 normal human sera with 1 μg intact radiolabeled SSV-SSAV. Right: Radioimmunoassay titration of the same sera with a solubilized ^{3}H-glucosamine-labeled gp70 probe from 50 ng SSV-SSAV (approximately 5 ng gp70)

Evidence for a Limited Role of Fetal Calf Serum (FCS) Components in the RIP Reaction

RIP titration experiments with a rabbit anti-FCS serum indicated the presence of FCS components in a subfraction of our virus preparations (Fig. 1). Human serological reactivity with SSV-SSAV was shown to be diminished to a limited extent in quantitative absorption tests with FCS proteins. However

human sera quantitatively absorbed with FCS proteins retained RIP activity with a reduction in titer only averaging approximately 50%. By either 1. alcohol precipitation, followed by concanavalin A chromatography and Sephadex G-150 filtration of FCS protein or 2. chromatography of serum proteins over a human IgG-containing immunoaffinity column, a glycoprotein of approximately 55000 MW has been identified which is a minor constituent of FCS (<0,1% of total protein) [7]. This serum component is thus reactive with a particular class of human antibody, distinguishable by absorption tests from the class of antibody which reacts with type-C virions [3].

Seroepidemiology

In preliminary seroepidemiological studies we have observed that the RIP reactivity in normal sera varies significantly as a function of age: we find that cord sera are barely reactive, maximum titers are reached between 5–10 years of age, a new minimum is reached at about 20 years of age, and there is a slow rise in titer thereafter. In experiments involving small numbers of individuals the sera of patients with neoplastic or autoimmune disease, have, in general, shown no marked differences in titer compared with sera from normal individuals. Studies of natural antibodies to type C viruses, especially in inbred mice, have shown that a complex group of genetic factors can affect immune responses to any particular virus class. Thus further investigations including family studies are planned to identify the basis of human serum reactivity with type-C viruses.

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A Search for Type-C Virus Expression in Man

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Introduction

Two immunological approaches can be used to search for type-C virus expression in man. Firstly, viral proteins present on intact cell membranes can be detected by using specific anti viral sera in cytotoxic or immunofluorescence assays. Secondly, an immune response to the viral antigens can be detected by looking for antibody specific for type C viral proteins. Both types of studies have yielded positive and negative reports.

Leukemic lymphocytes (Metzgar et al., 1976), a human fibrosarcoma cell line in early passage (Smith et al., 1977) and kidney sections from systemic lupus erythematosus (SLE) patients (Panem et al., 1976; Mellors and Mellors, 1976) are reported to possess antigens cross reactive with either SiSV or BaEV/RD114.

Assays for human antibodies against whole or detergent disrupted primate viral particles such as SiSV, GALV or BaEV, have yielded positive results (Snyder et al., 1976; Aoki et al., 1976; Kurth et al., 1977); whereas, other studies using purified proteins such as SiSV p30 and gp70 (Stephenson and Aaronson, 1976), NZBp30 (Charman et al., 1975), Mason Pfizer monkey virus p30 (Charman et al., 1977) or FeLV gp70 and p30 (Krakower and Aaronson, 1978) have produced completely negative results.

We have surveyed fresh peripheral blood lymphocytes from normal persons and a selection of leukemic patients using a panel of anti type C sera including high titer rabbit antisera raised against the SiSV component of HL23 V-1 (Teich et al., 1975) and BaEV.

We have also used a sensitive cell monolayer radioimmunoassay to look for differential binding of sera from normal individuals to pairs of virus infected and uninfected cell lines (Hogg, 1976). The major advantages of this latter approach are that viral antigens may be presented on cell membranes in a manner analogous to the way they are presented "in vivo" and that sera can easily be assayed for binding affinities on many types of virus infected/uninfected cells.

Materials and Methods

Anti Type C Viral Sera

Rabbit antisera specific for the SiSV component of HL23 V-1 and BaEV were raised by inoculating rabbits intramuscularly with sucrose gradient

banded virus (1 mg × 6 months). Other sera were obtained from sources listed in Table 1. Each antiserum was titrated by immunofluorescence on the appropriate virus infected and uninfected cell lines and was adsorbed with the uninfected cell line until the uninfected cell was fluorescence negative. This usually required 2 to 4 adsorptions at a ratio of one part cells to four parts serum.

Table 1. Leukemia screening sera (fluorescence)

	Adsorbed with
Rab anti-BEV (Pepsin)	NC37, 8155
Rab anti-HL23-SSV (Pepsin)	KNRK
NRS (Pepsin)	NC37, 8155
	KNRK
Rab anti-disrupted Mol-MuSV/MuLV	TO EF
Goat anti-Rauscher p30 (NCI 2S-658)	MEF
Goat anti-FeLV (Jarrett)	FEA
Goat anti-SSAV (NCI 5S-295)	NC37, Tonsil
NGS	Appropriate cells

Immunofluorescence Assay

Antisera were used at dilutions of 1:20 to 1:80. $(Fab^1)_2$ fragments of fluorescent goat anti-rabbit immunoglobulin (f-GaRIg) were obtained from Robert Sutherland (I.C.R.F.) and used at a dilution of 1:20. Fluorescent rabbit anti-sheep immunoglobulin (Miles-Yeda Ltd.) which was cross reactive with goat immunoglobulin was also used at a dilution of 1:20.

Cell Monolayer Radio Immunoassay

These assays were done as described by Hogg (1976). The cell monolayers were fixed for 10 minutes with 0.25% glutaraldehyde in phosphate buffered saline (PBS), then washed with 2% foetal calf serum (FCS) in PBS before use. This fixing procedure preserves the antigenicity of Moloney-MuLV gp70 and p30 (N. Hogg, unpublished).

Human Peripheral Blood Lymphocytes (PBL) and Sera

Blood samples were obtained from laboratory workers and from leukemic patients at St. Bartholemew's Hospital, London. Upon arrival the PBL were prepared by Ficoll-Hypaque density gradient centrifugation and used either immediately or after overnight storage at 4°C in an immunofluorescence assay. Sera were obtained from four selected individuals and stored at −20°C.

Results

Fresh human peripheral blood lymphocytes (PBL) were tested by immunofluorescence for the presence of type C viral proteins using rabbit antisera to HL23 V-1 and BaEV viruses. In most cases, the PBL were also examined with

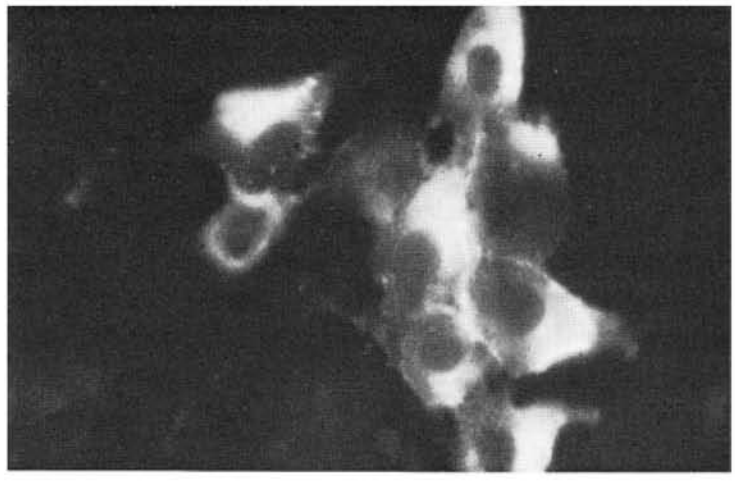

Fig. 1. Reaction of rabbit anti HL23 V-1 (1:40 serum dilution) with KNRK-HL23 V-1 cells (immunofluorescence). The same antiserum was negative when reacted with uninfected KNRK cells

antisera specific for feline leukemia virus (FeLV), subgroup A and B several murine leukemia viruses (MuLV) and other sera shown in Table 1. To date, 30 samples from a variety of leukemic patients (ALL, AMC, CML and others) plus 19 normal controls have been examined. No virus positive cells have been detected. The activity of each antiserum was tested with laboratory cell lines infected with the appropriate virus. For example, the reaction of rabbit anti HL23 V-1 with KNRK cells infected with HL23 V-1 is shown in Fig. 1. Uninfected KNRK cells show no fluorescence with this antiserum.

The second approach has been to look for antibody to type C viral proteins using a sensitive cell monolayer radioimmunoassay to detect differential binding to pairs of virus infected and uninfected cell lines in the sera of normal individuals. One of the selected individuals has been shown to possess anti-primate virus antibodies in a conventional radioimmunoassay (Kurth et al., 1977). A titration of rabbit anti HL23 V-1 and rabbit anti BaEV on appropriate infected and uninfected cell monolayers is shown in Fig. 2, demonstrating that this type of assay is as sensitive as a conventional radioimmunoassay. Antibody binding to BaEV, HL23 V-1, FeLV (A and B) and AKR MuLV infected cell lines was assessed. There were dramatic differences in antibody titers between individuals (1:10 to $>$ 1:270). However, the titers were similar on both infected and uninfected cell lines (Figs. 3.1 and 3.2). Adsorption of sera with uninfected cells (KNRK and 8155) reduced antibody titers to virus infected cells to background levels (Figs. 3.1 and 3.2). If the sera were titrated in the presence of increasing quantities of FCS (5, 10, 25, 50 percentage) the titers also dropped to background levels (Fig. 3.3).

Discussion

PBL samples from 30 leukemic patients and 19 healthy persons were examined by immunofluorescence for antigens related to HL23 V-1, BaEV and other type C viruses and *no* positive cells were detected. In another study, PBL from 20 SLE patients were also negative when examined with the same panel of sera (N. Hogg and N. Zvaifler, unpublished). Therefore, either viral antigens were not present in sufficient quantity to be detected or our antisera were not specific for the relevant antigens.

Human sera were tested for differential binding to pairs of virus infected and uninfected cell monolayers. Although antibody titers varied dramatically between individuals, each serum had an equivalent titer on both types of cells and this binding could be eliminated by adsorption of the serum with uninfected cells or by doing the assay in 50% FCS. Therefore, the only type of antibody that we are at present able to detect has specificity for a FCS component which is adsorbed to tissue culture cells similar to that described by Irie et al. (1974) and Snyder and Fox (1978).

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Tumor Cell Mediated Degradation In Vitro of Antitumor Antibodies

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Introduction

An in vivo interaction between tumor cells and humoral immune components was strongly supported by the findings that malignant tumor cells of human and animal origin are coated in vivo with Ig molecules. In some tumor host systems it was possible to show that at least some of the tumor bound Ig molecules expressed antibody activity against the tumor cells [13].

The type and expression of such an immune interaction between tumor cells and immune components may be the outcome of the mutual effects which both parties might exert on each other. Cellular factors which may play an important role in such an interaction on behalf of the tumor cells, are lysosomal enzymes which exhibit pronounced activity within [1, 10], and in the close vicinity of the tumor cells [12]. Such enzymes originating from mouse and human tumor tissue, have the capacity to degrade in vitro the corresponding Ig [6, 14].

In vitro studies on the interaction between antitumor immune factors and tumor cells [5] mainly emphasized its outcome on the viability and cellular functions of those cells. It is, thus, the interest of the present study to demonstrate how tumor cells and tumor cell components may affect the structure and function of antibodies upon such an interaction.

A. Degradation of Cytotoxic Antibodies by Tumor-Derived Lysosomal Extracts (LE)

I. The Effect on Antibody Structure and Function

The IgG fraction from rabbit antisera with cytotoxic activity against a mouse plasmacytoma (RaPCT-IgG) was incubated at pH 3,8 for 4–6 hr at 37°C, with lysosomal extracts (LE) from solid plasmacytomas of BALB/c mice (PCT) [7].

The complement-dependent cytotoxic activity of LE treated RaPCT-IgG was markedly reduced after the treatment when compared to RaPCT-IgG incubated under the same conditions but in the absence of LE (pH treated control) and native RaPCT-IgG (Table 1).

A kinetic study showed that with incubation time, increasing amounts of LE treated RaPCT-IgG were required to obtain 50% cytotoxicity, indicating

Table 1. The effect of plasmacytoma (PCT) derived lysosomal extracts on the complement-dependent cytotoxicity of rabbit IgG anti-PCT antibodies (RaPCT-IgG)

Exp. No.[a]	Reciprocal of RaPCT-IgG dilution giving 50% cytotoxicity		
	Lysosome treated[b]	pH treated control[c]	Native
1	2–4	128–256	256
2	16	64	64–128
3	2	128–256	256–512
4	2–4	256	128–256
5	<1	64–128	64–128
6	4–8	128–256	128–256
7	2–4	128–256	128–256
8	4–8	16	64
9	2–4	128–256	128–256

[a] Individual experiments were done with a different antiserum pool. IgG protein concentration was constant within each experimental group.
[b] Globulin incubated with lysosomal extracts (LE) at pH 3,8 for 6 hrs at 37°C. The reaction mixture contained an 8-fold excess of IgG protein over LE protein.
[c] Globulin incubated at pH 3,8 for 6 hrs at 37°C without LE

a time dependent process which generated non-cytotoxic products during the incubation.

Physicochemical changes of LE treated RaPCT-IgG were observed by following the precipitability at different ammonium sulfate (AS) concentrations, and the gel filtration pattern of ^{125}I labelled IgG.

A differential precipitation at 40% and 70% saturation of AS revealed that essentially all of the protein bound radioactivity in the pH treated control preparations precipitated at 40% saturation of AS, whereas only 60–80% of the LE treated IgG could be precipitated. Ten to 30% of the radioactive material in the LE treated preparations precipitated when the AS concentration was increased to 70% saturation.

^{125}I labelled LE treated and ^{131}I labelled pH treated control preparations of RaPCT-IgG were filtered through Sephadex G-100 columns. The filtration pattern of LE treated RaPCT-IgG revealed a shift towards the lower molecular weight range.

The incubation of RaPCT-IgG with LE was, thus, observed to render the antibodies less cytotoxic to the tumor cells and generated molecules, which compared to the untreated RaPCT-IgG showed a decreased precipitability at AS and slower gel filtration characteristics.

Attempts were made to spearate and study the nature and activity of those degradation products.

II. The Properties of the Degradation Products

Separation of the degradation products from LE treated RaPCT-IgG was carried out by an initial step of differential AS precipitation at 40% and 70% saturated AS solutions followed by gel filtration on Sephadex G-100 of the resulted precipitates.

The fraction precipitated by 40% saturated AS solution (LE treated RaPCT-IgG-40) emerged as a single distinct peak following filtration. The IgG in this fraction retained its antigenic characteristics and cytotoxic potential.

The fraction which could be precipitated by a 70% (but not a 40%) saturated AS solution (LE treated RaPCT-IgG-70) was separated upon filtration into two major dispersed peaks, which were divided into six subfractions (I–VI). Subfractions I–IV, which emerged first, contained antigenic IgG but lacked any expression of antigenic Fc. All of the above subfractions were incapable of mediating complement-dependent lysis of PCT cells.

The LE treated RaPCT-IgG subfractions precipitated at 70% saturated AS solutions seem, therefore, to contain IgG fragments devoid of Fc expression in terms of both antigenicity and biological activity.

Some of these subfractions could, however, compete with native, cytotoxic RaPCT-IgG for cellular antigens. Preincubation of viable cells with 50 μg of the LE treated RaPCT-IgG-70 subfractions which emerged first from the column and designated I and II, protected the treated cells from lysis when subsequently subjected to native, cytotoxic RaPCT-IgG and complement (Table 2). These subfractions seem, therefore to maintain their antigen binding capacity.

Table 2. The specific blocking activity of rabbit IgG anti-plasmacytoma (RaPCT-IgG) treated with lysosomal extracts (LE)

IgG source[b]	% Blocking of cytotoxic RaPCT-IgG[a]			
	LE treated IgG subfraction added[c]			
	I	II	III	IV
RaPCT	62	87	0	6
RaDNP-BSA	0	0	0	0

[a] Blocking was achieved by preincubation of target cells with 50 μg of the tested subfractions prior to the addition of native RaPCT-IgG which lysed 80% of the cells in the presence of complement.

[b] LE treated IgG was obtained from rabbit antisera directed against plasmacytoma cells (RaPCT) and a conjugate of DNP-bovine serum albumin (RaDNP-BSA).

[c] LE treated IgG subfractions were obtained by precipitation by 70% saturation of ammonium sulfate followed by filtration through a Sephadex G-100 column

B. Degradation of Antibodies by Viable Tumor Cells in Culture

The results presented here indicate that tumor cells contain proteolytic enzymes capable of degrading antitumor antibodies as well as other IgG molecules in cell free systems. We tested next whether or not such a proteolytic potential is expressed by the viable cell under physiological conditions.

Viable or formalin fixed EL-4 lymphosarcoma cells from C57B1/6 mice were sensitized with ^{125}I-labelled IgG isolated from rabbit antisera against EL-4 cells (RaEL-4-IgG) and washed of excess unbound IgG. The sensitized cells were incubated at 37°C or 4°C in a Marbrook type culture apparatus [8], composed of a large external chamber which housed an internal one. The chambers which contained culture media were separated by a dialysis membrane and the sensitized cells were placed in the small inner chamber. At the end of the incubation we monitored the radioactivity level bound to the cells, in the cell culture medium (supernatant), and in the external chamber (dia-

lysate). Degradation of IgG into dialysable low molecular weight fragments should be detected by the appearance of radioactive materials in the external chamber.

A time-dependent study of the degradation of IgG by viable and formalin fixed cells showed a progressive generation of radioactive materials of low molecular weight. Up to 40% of the labelled proteins initially bound to viable cells were degraded at 37°C into dialysable fragments, concomitant with a gradual loss of radiolabelled antibody from the cells (Table 3).

Table 3. Degradation of IgG by viable cells in culture

	IgG present in culture compartments (% of initially cell bound radioactivity)[a]			
	Viable EL-4 cells		Formalin fixed EL-4 cells	
	2 hrs[b]	24 hrs	2 hrs	24 hrs
Cell bound[c]	79	30	78	75
Cell supernatant[c]	17	27	21	24
Dialysate[c]	4	43	1,0	1,0

[a] Viable and formalin fixed EL-4 cells were sensitized with ^{125}I-labelled rabbit IgG anti-EL-4 cells. The cells were washed of excess IgG and incubated in a Marbrook type culture at 37°C.

[b] 2 and 24 hrs after the onset of the experiment samples were taken and monitored.

[c] At the end of each incubation period the radioactivity bound to the cells (cell bound), present in the cell culture medium (cell supernatant) and in the external culture chamber (dialysate) was monitored and expressed as percent of initial cell bound radioactivity

The disappearance of functional antibodies from the surface of sensitized cells was confirmed by measuring the residual cytotoxicity of RaEL-4-IgG upon incubation with cells in culture.

EL-4 cells were sensitized with a cytotoxic preparation of RaEL-4-IgG and incubated at 37°C and 4°C for 24 hr. At the end of the incubation cell cytotoxicity was determined following the addition of guinea pig complement. The cytotoxicity level of the IgG preparation which initially yielded 90% killing of the cells dropped to 25% killing after incubation at 37°C and 70% at 4°C.

Attempts to locate the site for antibody degradation indicated that the process occurred at the cell level. A pair labelled mixture composed of ^{125}I-RaEL-4-IgG and ^{131}I-RaDNP-BSA-IgG was added to viable cells in a short term culture and incubated for 24 hrs at 37°C. The data presented in Table 4 indicated that only a fraction of the specific RaEL-4 antibody was degraded by the viable EL-4 cells as revealed by the appearance of low molecular weight fragments in the dialysate. The non-related anti-DNP-BSA incapable of binding to the cells remained intact in the cell supernatant.

Whether degradation occurred on the cell surface or within the cells was examined next. It was found that a certain proportion of the ^{125}I-labelled RaEL-4-IgG remained bound to the sensitized cells after 24 hrs of incubation (see Table 3). These bound antibodies could be eluted from the cells by a low

Table 4. Preferential degradation of rabbit anti-EL-4 antibodies by cells in culture

	IgG present in culture compartments[b] (% of radioactivity added)	
	^{125}I-RaEL-4-IgG[a]	^{131}I-RaDNP-BSA-IgG[a]
Cell bound	6,5 ± 0,3	1,5 ± 0,8
Cell supernatant	82,5 ± 1,2	97,0 ± 0,2
Dialysate	11,0 ± 1,3	1,5 ± 0,5

[a] Viable EL-4 cells were incubated in culture in the presence of an IgG mixture which contained IgG from rabbit antisera against EL-4 cells labelled with ^{125}I (^{125}I-RaEL-4-IgG) and against DNP-bovine serum albumin labelled with ^{131}I (^{131}I-RaDNP-BSA-IgG). Cultures were incubated for 24 hrs at 37°C.

[b] At the end of the incubation, radioactivity levels on the cells (cell bound) in the cell culture medium (cell supernatant) and in the external culture chamber (dialysate) were monitored and expressed as percent of radioactivity added

pH buffer treatment. A comparison of the amounts of elutable antibody with the amount of non-elutable antibody revealed a relative increase in the amount of non-elutable antibody as a function of incubation time. It was possible to reveal the existence of the intracellular radiolabelled material by its release upon cell lysis, which indicated that endocytosis of the antibody was responsible for the non-elutable antibody fraction. Most of the released intracellular material could not be precipitated by AS solutions of 50% saturation, which suggested an intracellular location of degraded IgG fragments. Such fragments are released thereafter and detected as dialysable materials in the external culture chamber.

Discussion

Proteolytic enzymes of tumor cell origin play a very important role in the invasiveness and metastatic spread of malignant cells [9]. It was important, therefore, to study the effect they may have on humoral immune components.

In the model system used in this study, it was shown that in a cell free system tumor derived lysosomal enzymes deprived antitumor antibodies of their capacity to mediate complement dependent cytotoxicity, probably by degrading their Fc fragment. These antibodies, however, could specifically block humoral cytotoxicity at the level of the target cell. In other studies it had been shown that alloantibodies rendered non-cytotoxic by lysosomal extracts could specifically protect target cells from lysis by alloimmune lymphocytes [3].

The study was extended to investigate the capacity of viable tumor cells in short term culture to express their proteolytic activity and degrade antitumor antibodies under physiological conditions.

The results show that the elimination of antibody from antibody-coated viable cells under metabolism permissive physiological conditions in culture,

is associated with antibody degradation. As a consequence of this process low molecular weight degradation products were generated, but no blocking factors capable of abrogating humoral cytotoxicity could be detected.

The tumor site serves as an interaction ground for immune effector mechanisms, which infiltrate the tumor site [4, 13], and tumor-derived proteases. If indeed the described process occurs in vivo, as suggested by the findings of partially degraded IgG molecules on tumor cells and in their close vicinity [2, 11], then the consequence of such an interaction between proteases and antitumor components will be a continuous consumption of the latter, resulting in their selective depletion.

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Evidence Supporting a Physiological Role for Endogenous C-Type Virus in the Immune System

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The discovery that sequences coding for C-type viruses were endogenous to the genome of a variety of vertebrates raised several important questions. Principal among these were the relationship of endogenous viruses to oncogenic C-type viruses and their putative function during normal differentiation and development of their host cells. Recently a possible link between endogenous virus expression and the development of spontaneous leukemia has been proposed by the work of Hartley and Rowe with AKR mice [1]. Reasons for the retention and expression of these viruses in normal cells, however, have been lacking.

A few years ago we reported that several B-cell mitogens induce the expression of endogenous virus in murine spleen cell cultures [2,3]. These mitogens (lipopolysaccharide *E. coli*, tuberculin, lipoprotein *E. coli*) also trigger DNA synthesis and the differentiation of precursor B-cells into immunoglobulin-secreting end cells [4]. Recently we have shown that with hot thymidine suicide and inhibitor experiments, the stimulation of DNA synthesis is a prerequisite for virus production [5]. With these facts in mind, we began an examination of the molecular mechanism of virus induction as well as the relationship between virus expression and the immune response. Here we discuss these data in light of our hypothesis that the expression of endogenous viral antigens plays a physiological role in the humoral immune response of mice.

The molecular mechanism by which mitogens induced virus was investigated by assaying for viral transcripts in lymphocytes. DNA complementary to viral RNA was synthesized by the viral endogenous reverse transcriptase and used as a virus-specific probe. Hybridizations between control lymphocyte RNA and cDNA showed a low level of viral transcripts, homologous to approximately 25% of the probe (Table 1). This expression of viral sequences in unstimulated control lymphocytes was found both with BALB/c cells, which release virus following mitogen stimulation, as well as with cells from 129 mice, which do not release virus. BALB/c lymphocytes treated with mitogens increased their expression of virus sequences significantly (Table 1). We conclude from these results that expression of viral sequences and, presumably, viral protein(s) is a trait common to murine lymphocytes and that additional sequences are synthesized in response to mitogen induction.

The fact that mitogens mimick antigenic stimulation led us to hypothesize that virus expression may reflect a physiological process necessary in the gen-

Table 1. Endogenous viral transcripts in murine lymphocytes [9]

Source of RNA	Treatment	Saturation hybridization values[a]
Indicator fibroblast line	control	8%
	virus producing	70%
BALB/c spleen cells	control	23%
	+ LPS/BU[b]	55%
129/J spleen cells (uninducible strain)	± LPS/BU[b]	25%

[a] DNA complementary to induced virus was hybridized to saturation with the indicated RNAs in excess RNA reactions.
[b] Lipopolysaccharide E. coli in combination with bromodeoxyuridine

eration of immunoglobulin-secreting cells. We tested this hypothesis by injecting mice with a rabbit antibody directed against endogenous virus. Such animals showed reduced numbers of immunoglobulin-secreting cells following antigenic stimulation [6,7]. Experiments with this immunosuppressive serum are summarized in Table 2. Immunosuppression was observed with different antigens, in all mouse strains tested, and was also observed in in vitro systems. Absorption experiments confirmed the viral specificity of the immunosuppressive component. Since $F(ab')_2$ fragments were equally effective in immunosuppression, cytotoxicity can be ruled out as the mechanism of immunosuppression. Systems measuring cellular immune responses were, however, not affected. Surprisingly, the antibody was only immunosuppressive when administered early during the 4–5 day immune response against sheep red blood cells. This suggested that it interfered with the events which lead to triggering the B-cells to divide, possibly in T–B-cell cooperation. This conclusion was supported by recent experiments using the KLH-DNP carrier-helper system, performed in collaboration with Dr. Peter Erb, which revealed that activated T-helper cells were one target of the serum. A second target was the B-cell, since the serum also suppressed the T-independent anti-

Table 2. Immunosuppression by rabbit antiserum directed against BALB/c endogenous xenotropic virus

		References
Response suppressed:	sheep red blood cells, horse red blood cells, KLH-DNP, non-soecific polyclonal anti-DNP	6, 7 and unpublished results with Dr. P. Erb
Strains suppressed:	BALB/c, C57BL, AKR, DBA, 129	7
Specificity:	effect absorbed by purified virus, no effect on cellular immunity	6, 7 and unpublished results with A. Brownbill
Mechanism:	independent of complement, acts early during the immune response, possible blocking of T–B-interaction	7 and unpublished results

DNP response against bead-coupled DNP (unpublished results). These results are consistent with data from Wecker and coworkers who demonstrated the presence of viral glycoprotein gp70 on T- and B-cells participating in an anti-KLH-DNP response [8].

It was concluded that blocking of viral structures present on T-helper cells and antigen reactive B-cells suppresses the immune response. Two alternative mechanisms are consistent with our data. Blocking of viral antigens could lead to steric hindrance of a functional structure. Alternatively, viral antigen itself may represent a structure necessary for the generation of a humoral immune response. The present data do not allow us to distinguish between these explanations. However, the hypothesis that endogenous virus plays a physiological role remains a plausible interpretation.

Our studies on the induction of endogenous virus in mouse lymphocytes have established several features of this phenomenon. When mitogens stimulate B-cells of inducible mouse strains to synthesize DNA and differentiate, endogenous viruses are activated. Virus induction requires cellular DNA synthesis in the stimulated cells. These lymphocytes then transcribe new viral sequences not previously found in the population. However, unstimulated cells contain viral sequences that may represent memory cells as yet unactivated.

The expression of endogenous viral antigens on lymphocytes does not appear to be fortuitous since antisera against these viruses block humoral immune responses. These data are consistent with viral antigens mediating B-cell activation either through a T-cell interaction or directly. On T- and B-cells for example, viral antigens could be involved in cell-cell recognition. Alternatively, they could be secreted from the T-helper cell and deliver a biochemical signal necessary for B-cell activation. Should this hypothesis prove correct, it is conceivable that understanding how viral genes function in lymphocyte activation will also shed light on how leukemia viruses are involved in cell transformation.

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Immunological Aspects

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Three immunological areas were represented in this session. The one dealt with the detection and characterization of leukemia-specific or leukemia-associated antigens; the second with the detection of antibodies directed against antigens associated with leukomogenic viruses in animals; the third topic dealt with two aspects of tumor-host relations.

1. Membrane Antigens of Leukemic Cells

Rodt and his associates described an ALL-associated antigen (or group of antigens) detectable by a xenogeneic antiserum reagent. This reagent, after absorption, reacted only with ALL cells but not with a variety of normal adult or fetal cells. Cells from 46 patients out of 66 assayed were shown to be positive. However, it would be desirable to obtain data on the biochemical or immunochemical nature of the antigens.

Greaves described this morning the occurrence of an ALL-associated antigen which, in view of its presence on certain fetal cells and in regenerating haemopoietic tissue, was described as a differentiation rather than an ALL-specific antigen. In spite of this prima-facie difference between the antigens described by the two groups, it would be of interest to find out whether or not the Rodt antigen corresponds to the differentiation antigen found by Greaves. It would be important to determine whether or not an absorption of Greaves' antiserum with cells originating from regenerating haemopoietic tissue would removenactivity against these cells but leave a residual activity towards the leukemic cells. This situation would fit that described by Rodt. On the other hand, the possibility cannot be excluded that by using more sensitive assays with their antiserum, the Rodt group will be able to detect activity with some fetal cells.

Kabisch and his colleagues discovered that an ALL-associated antigen circulates in leukemic patients. It seems that this glycoprotein antigen (molecular weight of 135 000) is shed from leukemic cells and reaches the circulation. As the characteristics of this antigen are very similar to those of the differentiation antigen on leukemic cells described by Greaves, it may be useful if these groups would exchange reagents in order to ascertain this point.

It may be of interest to determine if the ALL-associated antigen is immunogenic in humans. In view of its presence in the circulation it may not be easy

to detect the corresponding circulating antibodies. To answer this question it will probably be necessary to dissociate putative circulating immune complexes.

2. Antibodies Reactive With C-Type Viruses

Two attempts to find circulating antibodies reactive with C-type viral antigens in the normal human population were presented. Snyder's results, in conformity with those presented this morning by Kurth, indicated the presence of antibodies directed against exogeneous animal C-type viral antigens. Hogg and her colleagues, on the other hand, in a carefully executed study, were unable to detect such antibodies in human beings. Neither did they detect viral components on fresh peripheral leukocytes from normal individuals or from leukemia patients. The reason for this discrepancy is not clear.

Several points of technical or methodological nature are probably relevant in connection with these and similar findings.

Cross-reactivity and specificity: Investigators detecting serological reactivity against viral antigens must rule out the possibility that this reactivity stems from the exposure to heterophile cross-reacting immunogens present in human-associated bacteria, in ingested food, drugs, etc. This was indeed done by the present authors.

On the other hand, it should be remembered that a wide spectrum of cross-reactivity does not mean that the reaction is non-specific. In this connection it may be useful to consider any immune reactivity involving the active-site of the antibody molecule as a specific reaction. Thus, a more extensive use of $F(ab')_2$ fragments in serological assays would be encouraged.

Absorption: The studies summarized here and many of those reported elsewhere involve absorptions as an essential step in defining the specificity and cross reactivity patterns of antiserum reagents. Absorptions are valuable only if a full depletion of reactivity against the absorbing antigen was achieved. Any study involving absorptions should therefore include an assay to ascertain a complete and exhaustive absorption.

The Moroni group has previously shown that expression of endogenous C-type viruses in lymphocytes is increased as a result of a response to B cell mitogens. In another study they asked the question whether or not viral gene expression is physiologically required for an immune response. They could show that an antiserum directed against an endogenous xenotropic BALB/c virus suppressed the immune response of mice against sheep erythrocytes in-vivo as well as in-vitro. A new finding given in the poster presented by DeLamarter is that an antiserum directed against Friend leukemia virus acted as a B cell mitogen. The antibody performing this function was, however, not sufficiently characterized and its specificity not defined.

This work, as well as the studies of others showing that C-type viral expression on lymphocytes increases after an immune stimulation, may pos-

sibly explain antibody formation against such viruses in normal individuals. It is thus not unlikely that virus-associated antigens may reach immunogenic doses in immunized or mitogen-stimulated animals. Hence, an immune response against these antigens may actually be a result of a proliferative response of lymphocytes, either to unrelated antigens or to mitogens.

3. Tumor-Host Relations

The next two presentations deal with cellular immune functions of leukemic patients.

In the work of Knight and his colleagues, it was indicated that a certain proportion of patients with untreated acute phase myelogenous leukemia are non reactive towards allogeneic cells. Fractionation of leukocytes from non reactive patients on nylon wool columns yielded, in some cases, allorespon-siveness either in the adherent or in the non-adherent fractions. No explanation was offered for this phenomenon. Reconstitution experiments could show whether a suppressor cell population was separated or inactivated during the fractionation procedure.

Culturing of peripheral blood lymphocytes from leukemia patients in the presence of a factor present in conditioned medium brings about increased T cell functions of the cultured cells. This finding is of particular interest since human T-cell cultures are usually not easy to maintain.

The increased reactivity of the cultured T-cells from patients is in line with results of others showing that functions of lymphocytes from tumor-bearing individuals may be increased following culturing. This phenomenon may be due to the removal of blocking molecules from the membrane of the lymphocyte.

The purpose of the study of Oliver was to augment specific cellular reactivity of acute myeloid leukemic patients toward their malignant cells. To achieve this aim Oliver employed the principle of "Pool-priming" described this morning by Bach, namely, that generation of cytotoxic T-cells requires two types of cellular antigens, the LD and the CD determinants, which could even be present on different cells. It was found that Daudi cells, a lymphoblastoid cell line provided HLA D (LD) determinants which were apparently missing from the autologous blasts. Thus, the addition of Daudi cells to mixed cultures composed of remission lymphocytes and of autologous blasts augmented considerably the cytotoxic activity of the lymphocytes which were identified as T cells. Cold-target inhibition assays suggested that the target antigen was not present on the allogeneic Daudi cells.

Oliver also demonstrated that addition of lymphocyte interferon to the 3-cell mixed culture augmented cytotoxicity. The mechanism for this phenomenon is not known. It is of interest to mention that interferon seems also to augment the cytotoxic activity mediated by natural killer cells.

The work of Keisari, although not involving human leukemia, is of relevance to this Meeting as will be pointed out.

Some malignancies, including leukemias, evoke an immune response

against antigens associated with the malignant cell. It can be shown that as a result of this response certain immune effector mechanisms such as immunocytes or antibodies make contact with tumor cells in-vivo. For example, several authors have found that certain leukemia cells are coated in-vivo with Ig, possibly antibody.

In-vivo coated tumor cells seem to lose their surface-bound Ig upon transfer to culture conditions. The disappearance of the coating Ig molecules might be a result of three nonmutually exclusive mechanisms:

1. Endocytosis; 2. shedding; 3. degradation of the cell-bound Ig molecules by cellular proteases.

Keisari's findings indicated that tumors contain proteolytic enzymes capable of degrading anti-tumor antibodies as well as other IgG molecules in cell-free systems, and that the degradation products of the anti-tumor antibodies blocked lymphocyte-mediated and complement-dependent cytotoxicity at the target cell level. These experiments were carried out in cell-free systems and at a low pH. Further experiments showed that antibody-coated viable lymphoma cells under physiological conditions in culture were capable of degrading their antibody coat into low molecular weight degradation products. Lack of degradation of unrelated antibodies present in the culture medium suggested that under the experimental conditions employed, binding of antibodies to their target cells is an essential prerequisite for their degradation, and that degradation took place in the close vicinity of, or inside, the cells.

It is not unlikely that antibodies localized on the tumor cell, may be affected considerably by proteases originating in tumor cells. The results of Cotropia et al., showing that Ig molecules coating human leukemic cells are partially degraded, indicate that degradation of Ig by malignant cells can occur in-vivo. The most obvious consequence of such a degradation would be a continuous consumption of anti-tumor antibodies, resulting in their selective depletion.

The last paper to be reviewed is that of Joshua dealing with the definition of certain cell surface antigens of human leukocytes. He used Milstein's approach of producing hybridomas by fusing non-secreting murine plasmacytoma cells with cells producing antibodies to such antigens. This approach is being very rapidly introduced to all areas of immunology including, of course, tumor immunology.

In the present study the authors produced several cell-hybrids recognizing individual membrane antigens. They were also able to assign the expression of several surface antigens of leukocytes to particular chromosomes.

Introduction to Oncogenesis by RNA Tumor Viruses

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A. RNA Tumor Viruses

RNA tumor viruses, or retroviruses as they are now called, are known to induce a variety of neoplasms in their natural host species. Thus the lymphoid leukosis prevalent in cattle, cats and chickens, and the thymic lymphomas and mammary carcinomas of mice are typically caused by retroviruses. Rare, extremely acute neoplasms, such as sarcomas, and erythroid and myeloid leukemias, are also recognized to result from retrovirus infection, as well as non-malignant diseases, such as osteopetrosis in chickens, anemia in cats, autoimmune and paralytic diseases in mice. The problem of identifying retroviruses with neoplastic potential in humans remains equivocal, although tantalising items of evidence continue to be thrown up, as described by several contributors to this volume. While much of the impetus and funding for research in viral oncology is based on the search for human tumor viruses, retrovirus research today is proving most useful for providing conceptual models of oncogenesis and experimental systems for probing the molecular and cell biology of neoplasia.

B. Transmission of Retroviruses

Although the genetic information of retrovirus particles is contained in RNA molecules, upon infection of the host cell this information is transcribed by the viral enzyme, RNA-directed DNA polymerase (reverse transcriptase) into a double-stranded DNA provirus. This provirus, like the genomes of DNA tumor viruses, becomes inserted into host chromosomal DNA, so that the "integrated" viral genes become adopted by the host as extra genetic information. Integration is probably not the oncogenic event itself, although the insertion of new DNA sequences at inappropriate sites could very conceivably cause disruption of cellular regulatory mechanisms. Nevertheless, integration is the means by which viral genes may be heritably transmitted to daughter cells. Furthermore, during the evolution of retrovirus-host relations, retrovirus genomes have on occasion become integrated into cells of the host germ line, with the result that the viral genes are now inherited from one generation to the next as host Mendelian factors. Such stable, inherited viral genomes (called endogenous viruses), with the exception of certain inbred strains of mice, are not known to be oncogenic, but may give rise to oncogenic agents on reactivation to viral form, and may become recombined with other DNA sequences to form new genetic elements that are potentially oncogenic.

Thus retroviruses can exist in latent form by masquerading as host genetic information. These endogenous viral genes may be unexpressed for many host generations, or some viral antigens may be synthesized in certain types of host cell. On occasion, complete virus may be activated, either spontaneously, or by treatment of the host cell with ionizing radiation or chemical carcinogens and mutagens. The reactivation phenomenon led to a hypothesis that all cases of oncogenesis by diverse agents might be accounted for by activation of endogenous viruses. This now seems unlikely, and the most efficient virus inducing agents, such as halogenated pyrimidines, have little carcinogenetic potential. The latency and inheritance of retrovirus genomes considerably complicates any analysis of epidemiology, not least because some of the newly activated viruses frequently cannot reinfect cells of the species in which they are inherited, but may be infectious for foreign species, a phenomenon called xenotropism.

Leukemogenic retroviruses, with the important exception of murine leukemia viruses, are typically transmitted as infectious agents. Thus leukosis is a contagious disease in cats and cattle which is spread horizontally by close contact with infected individuals. Horizontal infection of chickens results most frequently in effective viral immunity, but 'vertical' infection of eggs leads to immunological tolerance and as a consequence of, perhaps, a persistently high viral load, such congenital infection typically causes lymphoid leukemia. Congenital retrovirus infection also occurs in several mammalian species via either the placenta or milk, and activation of endogenous virus in mouse embryos can even lead to a 'reverse vertical' infection of a non-viremic mother.

The evolutionary origin of retroviruses is often obscure. The exogenous retrovirus causing bovine leukemia appeared as a new enzootic agent in Danish cattle some years ago and might have been transmitted from another species. The endogenous virus of cats has been acquired from primates related to baboons, whereas the exogenous viruses of the gibbon-woolly monkey group are related to endogenous rodent viruses. Clearly there has been much hopping in and out of host genomes of different species in the evolution and spread of retroviruses.

C. Retrovirus Genes

The proteins of retroviruses are, of course, antigenic, and the preparation of specific antisera for radioimmunoassays and other immunological techniques has been of great use for studying viral gene expression and virus relationships. The other major analytical tool in molecular virology is nucleic acid hybridisation. With the preparation of specific radioactive probes, the presence and expression of retroviruses and of single retrovirus genes can be accurately monitored, and the evolutionary relationships between retroviruses in different host species can be precisely assessed. Many of the papers presented in this volume therefore, describe studies employing refined immunological and molecular hybridization techniques.

Non-defective retroviruses have a simple unit genome comprising three well-defined genes coding for virion proteins. The *gag* gene encodes a large, precursor polypeptide which becomes proteolytically cleaved to generate the internal or core antigens of the virion. These proteins are named according to their estimated molecular weight, e.g. murine p30 denotes the major core protein of 30000 daltons of murine leukemia virus (MuLV). Precursor polypeptides are similarly labeled pr65, or pr90, etc. The *env* gene encodes the proteins located in the envelope of the virion which is derived by budding from the plasma membrane of the host cell; thus murine gp70 denotes the glycosylated envelope protein of MuLV of approximately 70000 daltons. The *pol* gene encodes the polymerase (reverse transcriptase).

The three genes are ordered in the genomic RNA molecular in the sequence 5′-gag-pol-env-3′; apart from some nucleotide sequences at the 3′ end of the molecule, they appear to account for the entire genome. The genomic RNA can act as a messenger RNA for translation of *gag* and *pol* proteins, but the *env* proteins appear to be translated from a separately transcribed mRNA species.

D. Oncogenesis

Retroviruses can be roughly divided into three groups on the basis of oncogenicity. Those that cause acute neoplasms with short latent periods between infection and the appearance of the tumor are called 'strongly transforming' viruses. In most cases these viruses will also transform appropriate cells in culture. Those that cause tumors only after long latent periods (months rather than days in mice and chickens, the most closely studied host species) are called 'weakly transforming' viruses, and in vitro transformation systems have not to date been devized for these viruses. Some endogenous retroviruses such as those of cats and chickens, may be regarded as 'non-transforming' viruses, but this view may have to be modified when they are studied in more detail.

I. Strongly Transforming Viruses

These viruses occur only very rarely in nature, but their recognition, isolation and experimental use has led to major advances in our understanding of viral oncogenesis. The best known and most venerable example of a strongly transforming virus is the Rous sarcoma virus (RSV) of chickens; others are avian myeloblastosis and erythroblastosis viruses, and murine Friend erythroleukemia, Abelson lymphoma and Moloney, Kirsten and Harvey sarcoma viruses. RSV has a gene, designated *src* for sarcoma induction, in addition to the three genes essential for viral replication. Studies of deletion mutants and temperature-sensitive mutants have shown that the *src* gene is essential for fibroblast transformation and for sarcomagenesis, but is not required for viral replication. Recent data from Erikson and his colleagues (reported in this volume) indicate that the *src* gene product is a cytoplasmic protein of

60000 daltons that possesses protein kinase activity. Precisely how this protein effects transformation and what are the crucial targets in the cell for phosphorylation remains to be determined. Nevertheless it is a remarkable advance in experimental oncogenesis that an enzyme has been identified with an oncogene.

In most strains of RSV, the *src* gene is carried as an extra gene to the viral genes, in the order 5′-gag-pol-env-src-3′. All other well studied strongly transforming viruses are defective for replication, that is, new genetic information specifying neoplastic transformation (*onc* genes) appears to be inserted in the viral genome in place of essential genes for replication. A part of the *gag* gene and the 3′ end of the viral genome are maintained, giving a typical structure 5′-*ga*-onc-3′ (see Duesberg's paper, this volume). The infectivity of such defective viruses relies on the presence of replication-competent 'helper' viruses, and the disease spectrum caused by such viruses depends on the properties of this complex virus population. Transformed cells can be obtained in culture by cloning cells infected with the infectious virus complex at dilutions that prevent infection by the helper virus in addition to the defective virus. Such transformed, 'nonproducer' cells have been analysed for expression of virus related proteins, and recently several new proteins possibly carrying oncogenic functions have been detected, as reported in this volume. The polypeptide coded by the supposed *onc* region of the viral genome actually starts in what remains of the *gag* gene. Since the polypeptide therefore bears some *gag* antigens, it can be identified by immunoprecipitation with anti-gag antisera from lysates of transformed cells. Such *gag-onc* 'polyproteins' have been detected in cells transformed by avian myelocytoma and erythroleukemia viruses (see Graf's paper in this volume) and murine Abelson leukemia cells. These proteins are not related to the *src* protein or to each other, and each may have individual functions resulting in neoplastic transformation. This would account for the high degree of specificity of the target cell for transformation, as each virus causes a specific type of cancer or leukemia.

Oncogenes appear to originate from the host, as genetic elements related by molecular hybridization to viral oncogenes are found in the host genome, though not linked to endogenous viral elements. Possibly the natural host sequences code for normal proteins important in the function or differentiation of particular cell types. When picked up and modified in viral genomes, and then re-inserted into appropriate target cells, they may cause disruption to regulatory cell functions blocking or even reversing the normal pathway of differentiation. One popular view is that some oncogene products may represent or mimic mitogenic hormones specific for the target tissues. Further analysis of the oncogenes of strongly transforming viruses should tell us much, both about differentiation and neoplasia, especially in hemopoietic cells.

II. Weakly Transforming Viruses

These viruses do not appear to carry oncogenes distinguishable from the three viral genes, *gag*, *pol* and *env*. Commonly occurring weakly transforming viruses are the murine mammary carcinoma virus, murine thymic lymphoma viruses, avian bursal leukosis viruses, and the leukemia viruses of cats and cattle. In contrast to the strongly transforming viruses, the tumors they cause appear after long latent periods, and only very few of the cells that become infected subsequently give rise to tumors. The tumors are probably clonal in origin, whereas with strongly transforming viruses such as RSV the tumors grow as quickly by infection and transformation of new target cells, as by mitosis of the originally transformed cell. There is growing evidence for the murine viruses inducing thymic lymphomas that genetic recombination involving the *env* gene takes place, often between xenotropic and mouse-tropic endogenous viruses, giving rise to new virus variants which may interact with and transform different cell types than those recognized by the parental viruses. The recombinant *env*-coded glycoproteins may play a dual role, both in allowing the virus to recognize and infect specific target cells bearing appropriate receptors for the glycoproteins and acting as a perpetual mitogenic stimulus to such cells.

E. Human Retroviruses

Homo sapiens is the only intensively studied vertebrate species from which C-type viruses cannot regularly be isolated. However, there are now a sufficient number of virus isolates and reports of virus footprints (see Gallo's contribution to this volume) to take the presence of retroviruses quite seriously. If that is so, the viruses must normally remain latent. Thus two basic questions really remain open: whether retroviruses are natural infections of man and whether they play a role in leukemogenesis. In the search for human retroviruses, more use might be made of marker-rescue techniques in using animal helper viruses for complementation or rescue of possible defective or fragmented viral genomes in human cells. Even if viral genomes do not operate in human leukemogenesis, the studies of viral leukemia in animals, and of the mechanism of action of oncogenes acquired by retroviruses, will prove to be of great importance in our understanding the nature of leukemia.

Mechanism of Leukemogenesis and of Target Cell Specificity by Defective Avian Leukemia Viruses: A Hypothesis

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Introduction

Avian leukemia viruses comprise a class of retroviruses isolated in their majority from field cases of the domestic chicken. A group of these agents, which are defective for replication and are therefore referred to as "defective leukemia viruses" (DLV), cause a variety of types of acute leukemia and other neoplasms of nonhematopoietic origin within weeks or months after infection. All DLV-strains isolated so far are capable of in vitro transformation of hematopoietic tissues (for review see Graf and Beug, 1978). In our recent studies, we have mainly concentrated on two model virus strains: avian erythroblastosis virus (AEV) and myelocytomatosis virus strain MC29. Clone-purified AEV causes an acute erythroleukemia but also sarcomas (Graf et al., 1977). MC29 causes myelocytomatosis and tumors of predominantly epitheloid origin (for references, see Graf and Beug, 1978).

The aim of this article is to summarize the present state of our research on AEV and MC29 with regard to their interaction with hematopoietic cells and to present a new hypothesis about their target cell specificity and mechanism of leukemogenesis.

Results and Discussion

Characterization of Hematopoietic Cells Transformed by AEV and MC29

The first question we asked was: do hematopoietic cells transformed by AEV and MC29 in vitro express erythroid and myeloid differentiation parameters, respectively, as do leukemic cells from virus-infected birds on the basis of morphological and staining criteria?

To study this question, chick bone marrow cells were infected with the respective viruses and colonies of transformed cells isolated and grown into mass cultures as described before (Graf, 1973; Graf, 1975; Graf et al., 1978b). These cultures were then examined with regard to the expression of a series of erythroid and myeloid differentiation parameters. The results obtained, some of which have been published in a preliminary form (Graf et al., 1976a, b; Graf et al., 1977; Graf et al., 1978b) are summarized in Table 1. As can be seen, AEV-transformed cells are erythroid in nature, whereas MC29-transformed cells have myeloid properties and in several respects resemble

Table 1. Differentiation parameters in AEV- and MC29-transformed chick bone marrow cells

Differentiation parameters		Virus used for infection	
		AEV	MC29
Erythroid	Hemoglobin	+	–
	Histone H5	+	–
	Erythrocyte cell surface antigen	+	–
Myeloid	Phagocytic capacity	–	+
	Fc receptors	–	+
	Macrophage/granulocyte cell surface antigen	–	+

macrophages. With both viruses, in particular with AEV, the transformed cells express differentiated functions but at a lesser degree than terminally differentiated cells of the corresponding lineage. For example, AEV-transformed cells exhibit levels of hemoglobin which are about 100 times lower than those of adult erythrocytes (Graf et al., 1978b).

Leukemia cells from AEV-infected birds, when brought into culture, were indistinguishable from the in vitro transformed cells for all differentiation parameters mentioned in Table 1.

Characterization of the Hematopoietic Target Cells for Transformation by AEV and MC29

The second question asked was: do both our model leukemia viruses affect pluripotent hematopoietic stem cells, inducing them to differentiate exclusively into one or the other differentiation lineages, or do AEV and MC29 transform erythroid and myeloid progenitor cells, respectively?

That the latter alternative is correct could be shown by the separation of the target cells prior to infection and by the demonstration that they already express lineage specific membrane antigens. The corresponding body of evidence (Graf et al., 1978b; Graf and Beug, 1978, and unpublished results of Beug, v. Kirchbach, Meyer-Glauner, Royer-Pokora and Graf) cannot be described here in detail and is only briefly summarized in Table 2.

Taken together, our results suggest that the hematopoietic cells transformed by AEV and MC29 as well as the normal hematopoietic target cells for transformation by these viruses represent immature erythroid and myeloid cells, respectively.

Table 2. Characterization of the hematopoietic target cells of AEV and MC29

Properties of the target cells of	
AEV	MC29
Nonadherent	Adherent + nonadherent
Not phagocytic	A fraction is phagocytic
Erythroid cell surface antigens	Macrophage/granulocyte cell surface antigen(s)

A Viral Gene Product is Required for the Maintenance of the Undifferentiated Stage of DLV-transformed Hematopoietic Cells

The above results, in conjunction with the finding that AEV transformed erythroblasts could be induced to differentiate by addition of butyric acid (Graf et al., 1978b), raised the possibility that AEV and MC29 transform hematopoietic cells by blocking their capacity to differentiate.

The third question, therefore, was: is a viral gene product required for the maintenance of the "transformed" state of the leukemic cells? And if so, can the leukemic cells be induced to differentiate further by inactivating this gene product?

If both of the above assumptions were true, it should be possible to isolate mutants of AEV or MC29 temperature sensitive (ts) for the maintenance of the undifferentiated state in the respective transformed cells. Attempts to isolate such mutants were made in the AEV system, mainly because simple assays were available for the qualitative and quantitative detection of hemoglobin in chicken hematopoietic cells.

It was indeed possible to isolate a mutant of the desired type (Graf et al., 1978a). At 35°, cells transformed by this mutant (designated *ts* 34 AEV) exhibited low amounts of hemoglobin as detected by benzidine staining or by radioimmunoassay. After 3 days of shift to 41°, with both tests, a drastic increase in hemoglobin levels was observed (Fig. 1 and 2; and Graf et al., 1978a). Control experiments showed that the ts-lesion was located in the defective, transforming ts34 AEV virus and not in its helper virus (Graf et al., 1978a). As shown in Fig. 2, the shift-induced increase in hemoglobin was reversible

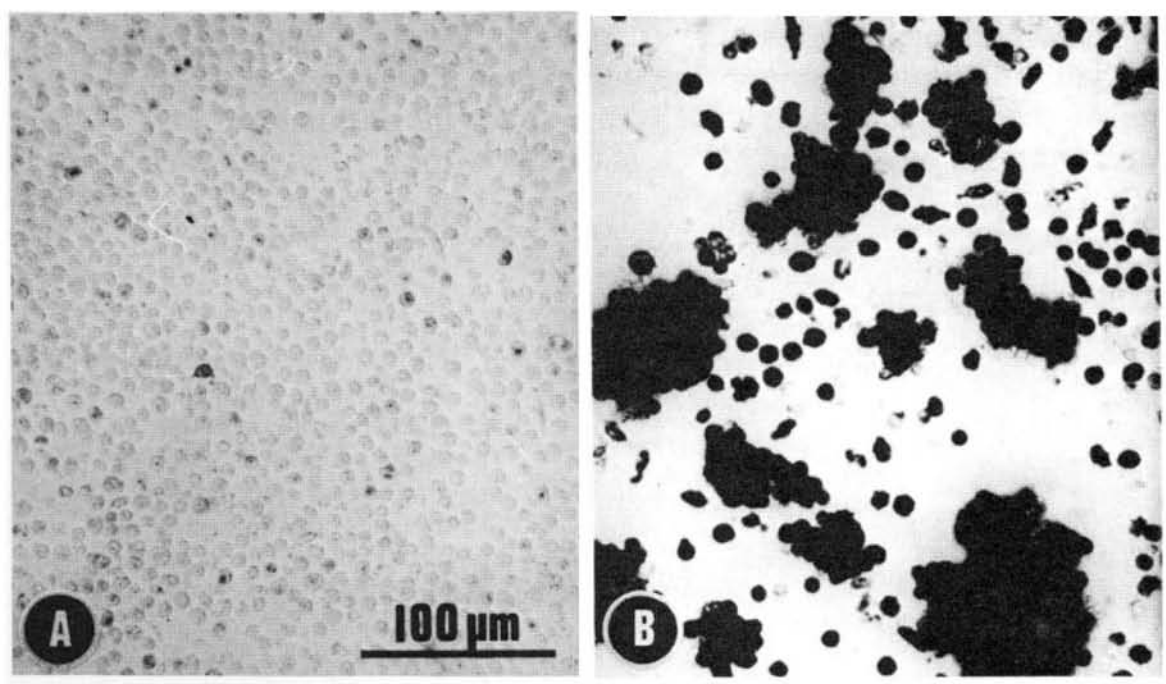

Fig. 1. Effect of temperature on the expression of hemoglobin in ts 34 AEV-infected chick bone marrow cells. Erythroblasts from a single transformed colony were grown up at 35° and aliquots were seeded at 35° (A) and 41° (B). Three days later the cells were stained with benzidine by the method of Orkin et al. (1975)

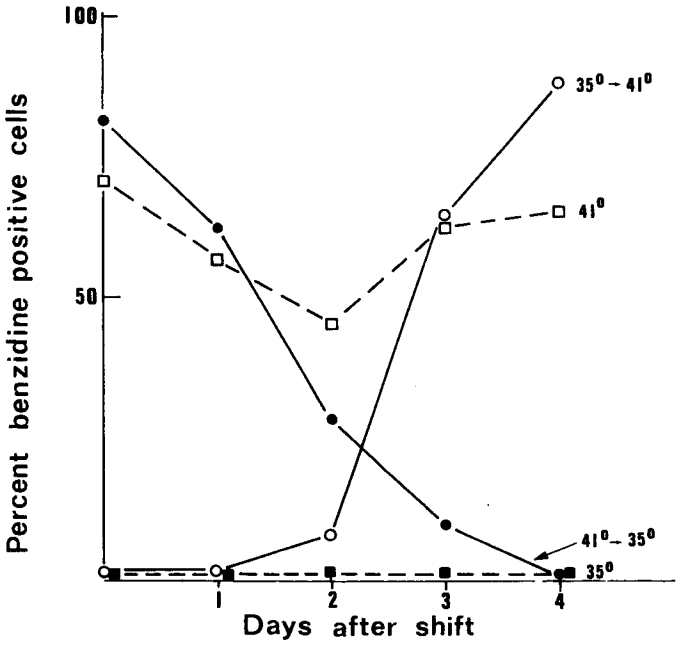

Fig. 2. Kinetics of hemoglobin expression in ts34 AEV-erythroblasts as determined by benzidine staining

after shift back of the cells to 35°. Because of the high body temperature of the chicken (41,8°), the mutant could also be tested for its leukemogenicity under "non-permissive" conditions. As shown in Fig. 3, *ts* 34 AEV is in fact significantly less leukemogenic than the parental wild type AEV.

The data discussed so far support the notion that AEV and MC29 specifically transform immature hematopoietic cells already exhibiting lineage specific differentiation markers, and that these cells are blocked in their ability to terminally differentiate by the action of a viral gene product. This concept is schematically depicted in Fig. 4. Similar ideas have been proposed in earlier communications (Graf et al., 1976a, b; Graf and Beug, 1978b).

The model shown, contains no information about the molecular basis of the observed target cell specificity nor about the mechanism of leukemic transformation by AEV and MC29. In the following, a few hypotheses relevant to these topics will be discussed.

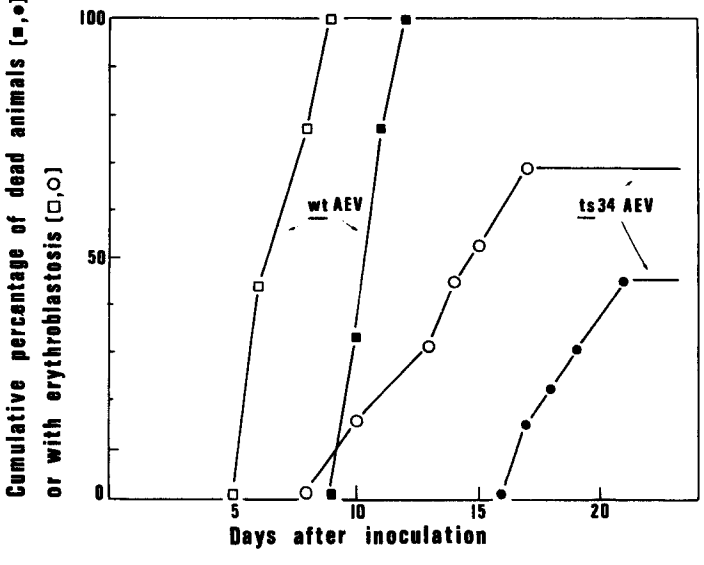

Fig. 3. Leukemogenicity of ts34 AEV. Three groups of 8-day old chicks were injected i.v. with 0,1 ml of growth medium containing either 1000 colony forming units of cell-free ts34 AEV (13 chicks) or 10 colony forming units of cell-free wild type AEV (9 chicks). All animals were observed at 2–3 day intervals for the appearance of erythroblastosis by preparing blood smears and for their death over a period of 3 months. Chicks with ts34 AEV, circles; with AEV, squares

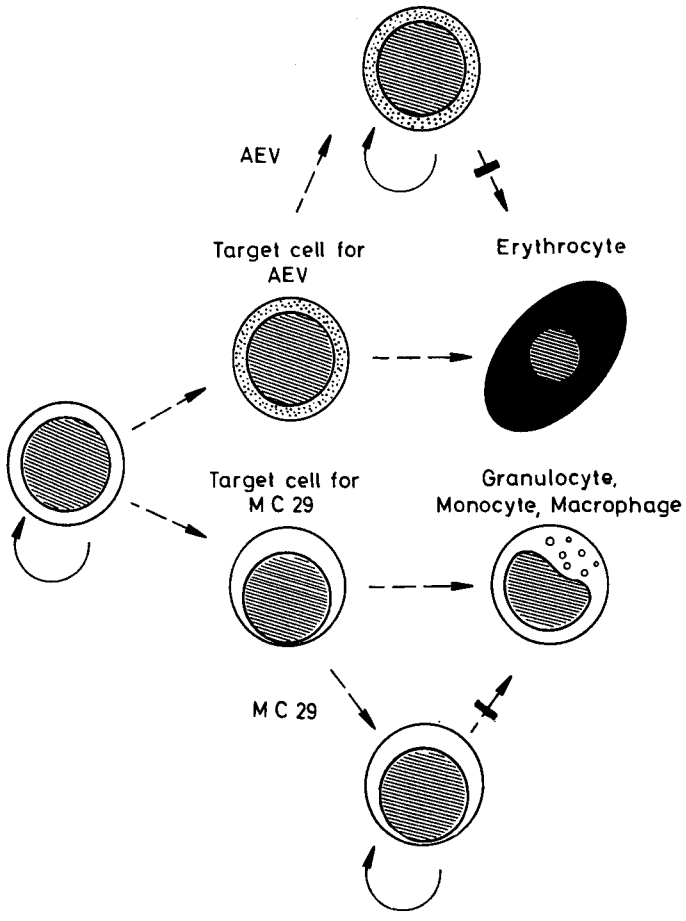

Fig. 4. Diagram illustrating the lineage specific leukemic transformation of hematopoietic cells by AEV and MC29. Curved arrows: proliferation; broken arrows: differentiation, bars: block of differentiation

Basic Possibilities to Explain the Target Cell Specificity of AEV and MC29

The simplest explanation for the transformation target cell specificity of AEV and MC29 would be that these viruses are unable to replicate in cells which they cannot transform. We have tested this model using chick macrophage cultures free of erythroid cells and fibroblasts. They were shown earlier to be resistant to transformation by AEV but not to MC29 (Graf et al., 1976a, b). Macrophages were infected with AEV and tested for the amount and type of virus produced at various days after infection. The results in Fig. 5 show that after an eclipse period of about two days both transforming AEV and its helper virus are synthesized by the infected macrophages. In a similar experiment, MC29 was found to be synthesized by AEV-transformed erythroblasts after superinfection with MC29 virus (unpublished results).

That DLV's can replicate (in the presence of a helper virus) in hematopoietic cells not susceptible to transformation rules out the possibility that they are restricted in nontarget cells at the level of penetration. In addition, if it is assumed that integration is a prerequisite for replication (Bishop, 1978) these results, together with those obtained with the ts mutant of AEV discussed above, indicate that integration of the viral genome alone cannot be sufficient to induce a leukemic cell transformation. They are neither compatible with any simple model in which an integration specificity accounts for the target cell specificity of DLV's. Instead, it appears as if the continuous

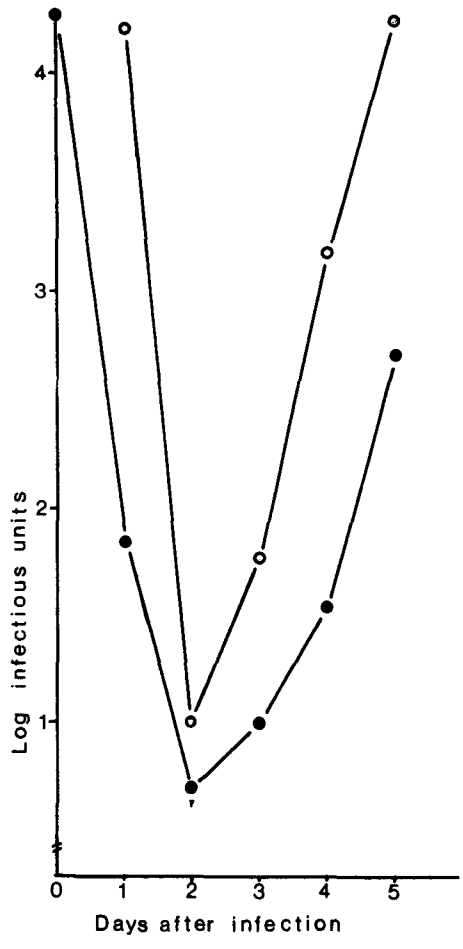

Fig. 5. Replication of AEV in macrophages. Tertiary macrophage cultures derived from the bone marrow of a 3 week old chick were infected with a RAV-2 pseudotype of AEV. Virus infectivity was determined at daily intervals thereafter and the medium was changed after each harvest. Closed circles: focus forming units/ml; open circles: plaque forming units per ml (the number of plaques is a measure of the infectivity of the helper virus)

expression of a viral gene product (probably a protein) is required for the maintenance of leukemic transformation and the restriction is therefore likely to occur either at the level of protein expression or protein recognition. In the first case, viral transformation protein would be synthesized in target cells only. In the second case it would be synthesized in hematopoietic cells from all lineages but since it would not be recognized in nontarget cells, it would induce a transformation in specific target cells only.

We favor the latter possibility and have constructed a new hypothesis which will be outlined in the following.

A Hypothesis Explaining Leukemogenesis and Target Cell Specificity of DLV's (Fig. 6)

Recent findings have shown that the *src* gene of avian sarcoma viruses is present in the genome of normal cells (Stéhelin et al., 1976). Similar data are emerging for AEV and MC29-specific sequences (the putative transforming sequences or *"onc"* genes).

We now propose that certain genes involved in hematopoietic differentiation, arbitrarily termed b, have been picked up (and modified) by DLV's during evolution, now constituting the transforming sequences of the virus (b*). Consequently, transforming proteins of DLV's (B*) represent modified hematopoietic differentiation proteins which differ for different virus strains.

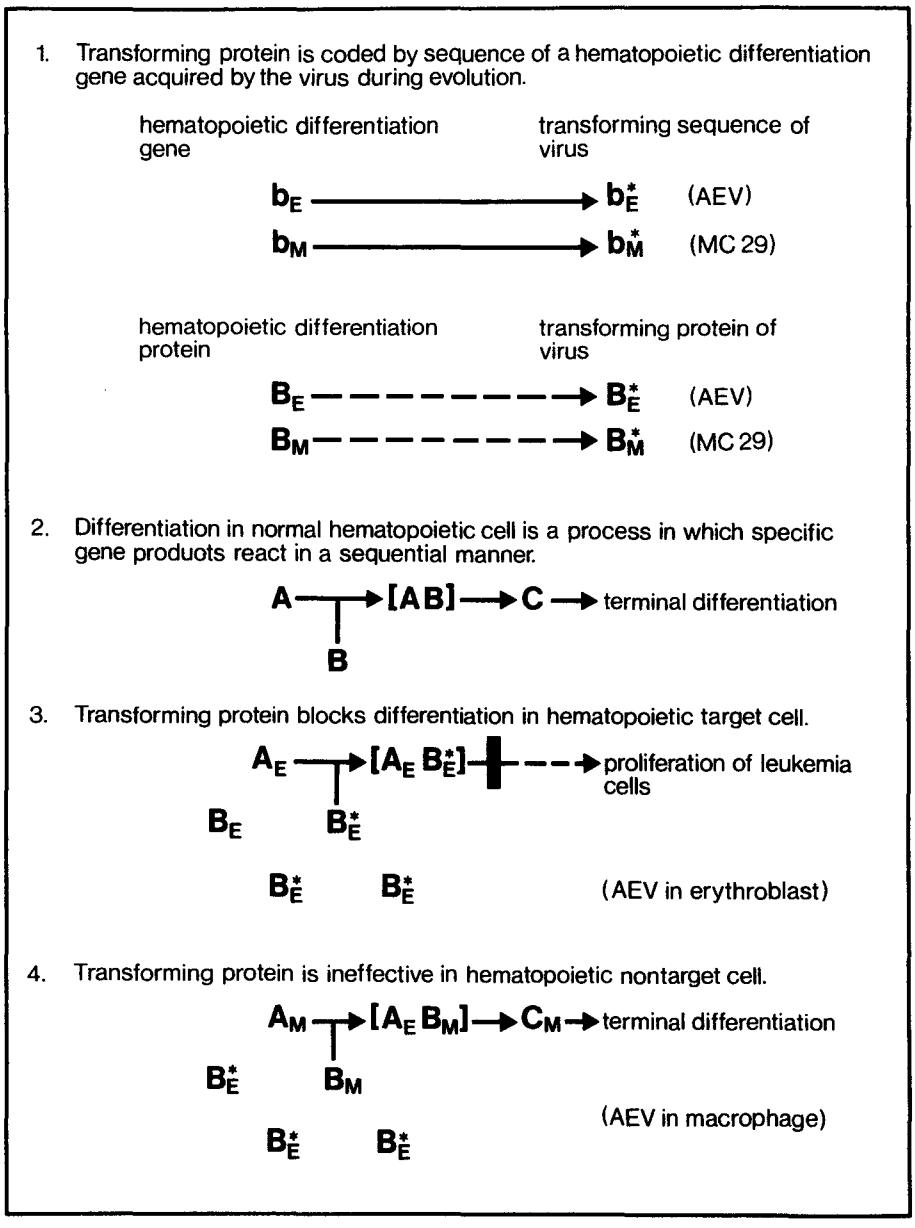

Fig. 6. Schematic representation of a hypothesis explaining the target cell specificity of DLV's and the mechanism of leukemogenesis as consequences of a differentiation block

Thus, AEV would code for a modified erythroid differentiation protein (B^*_E) and MC29 for a modified myeloid protein (B^*_M). It should be emphasized here that the designations used serve only formal purposes and cellular proteins such as B_E and B_M are not meant to be homologous in a functional way.

The second assumption is that a necessary step in hematopoietic differentiation is a process in which specific gene products react in a sequential manner. For example, a differentiation protein B interacts with a receptor A forming a complex [AB] which in turn can be converted to or induces another differentiation factor C etc., finally leading to terminal differentiation.

In the DLV-infected hematopoietic target cell (exemplified in Fig. 6, point 3 by an erythroblast infected with AEV), viral transforming protein (B^*_E) is synthesized in large amounts, thus displacing by competition its normal cel-

lular counterpart (B_E) from its corresponding receptor (A_E). We postulate that because of the modification in B^*_E the complex formed is nonfunctional and therefore causes a block in differentiation. In the infected hematopoietic nontarget cell (exemplified in Fig. 6, point 4 by an immature macrophage infected with AEV), there is no receptor of the A_E-type for B_E to react with and terminal differentiation can therefore proceed undisturbed.

Our hypothesis allows several predictions to be made which are experimentally testable.

First, sequences related to viral *onc* sequences should be present in the DNA of normal cells. This seems to be indeed the case for both AEV (Saule, Roussell and Stéhelin, personal communication), and for MC29 (Sheiness and Bishop, personal communication). In addition, these sequences appear to be highly conserved during evolution as might be expected from genes which play a role during differentiation.

Second, RNA homologous to the *onc* sequences of AEV should be expressed in normal erythroid target cells; and those of MC29 should be expressed in normal myeloid cells. This possibility is currently being explored in collaboration with the group of D. Stéhelin.

Third, DLV's which differ in their target cell specificity should have different *onc* genes and those with a similar specificity should have related ones. Preliminary experiments have shown that MC29-specific cDNA (probably homologous to the onc_{MC29} sequences) does not hybridize to AEV RNA but to MH2 RNA (Sheiness and Bishop, personal communication). MH2 is a virus which has similar biological properties as MC29 (Vogt et al., 1977; for discussion, see also Graf and Beug, 1978). Conversely, AEV-specific cDNA does not hybridize with RNA from MC29 (Saule, Roussell and Stéhelin, personal communication).

Fourth, viral transforming proteins should not only be expressed in target cells but also in virus-infected nontarget cells.

Fifth, transforming protein of AEV should crossreact with a cellular protein synthesized in erythroid cells but not in other hematopoietic cells while that of MC29 should crossreact with a protein present in normal myeloid cells. Candidates for such transforming proteins are a 75000 d protein synthesized in AEV-transformed cells (Hayman et al., 1978) and a 110000 d protein found in MC29-transformed cells (Bister et al., 1977). We are currently trying to develop antibodies against these polypeptides which would be of much help in testing predictions 4 and 5, the perhaps most crucial predictions of our hypothesis.

Our hypothesis can also be extended to explain leukemogenesis by nondefective leukemia viruses with a long period of latency. In this case we postulate that copies of their DNA provirus may integrate in or adjacent to genes of the b-type, thus causing a modification of the corresponding gene product. The production of aberrant B-type proteins would then lead to an arrest of differentiation and to the proliferation of immature "leukemic" cells. The long period of latency of nondefective leukemia viruses would be explained by the assumption that this process occurs only rarely.

Concluding Remarks

What is the significance of the studies described with defective avian leukemia virus for the problem of human leukemia?

Earlier hopes that infectious leukemia viruses play a role in the etiology of the human disease could not be substantiated. In addition, most if not all human leukemias are monoclonal disorders (for review, see Fialkow, 1976). Despite these obvious differences a basic similarity to virus-induced avian leukemias remains: it is becoming increasingly evident that cells from different types of human lymphoid and myeloid leukemia express differentiation parameters similar to those of normal hematopoietic cells at different stages of differentiation (for review, see Greaves and Janossy, 1978; Greaves et al., this volume). These cells can be induced in tissue culture to further differentiate by the addition of chemicals such as dimethylsulfoxide (Collins et al., 1978), in a similar way as Friend leukemia virus- or AEV-transformed erythroleukemia cells (Friend et al., 1971; Graf et al., 1978b) or myeloid cells transformed by Rauscher leukemia virus (Fibach et al., 1972). This suggests that in both virus-induced leukemias and spontaneous leukemias, the differentiation of early hematopoietic cells is blocked during leukemogenesis. In the light of our hypothesis for the mechanism of transformation by DLV's, that specific transformation proteins representing modified hematopoietic differentiation proteins of the cell cause a differentiation block in the leukemia cells, analogous proteins might be altered in human leukemia cells such as by somatic mutations in the respective genes.

Elucidation of the mechanism of leukemic transformation by defective avian leukemia viruses may thus lead to a better understanding of human leukemia as a disease of differentiation.

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Infectious Leukemias in Domestic Animals

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Introduction

Viruses have been regarded as substantial candidates for the aetiology of cancer in man since the demonstration that the prime factors in the pathogenesis of naturally occurring leukemias in certain domestic animals were viruses of what is now known as the retrovirus group. Early in this century leukosis in domestic poultry was shown experimentally to be infectious and enzootic bovine leukosis was long considered by many veterinarians in continental Europe to be transmissible on epidemiological grounds. Later the discovery of feline leukemia virus added further substance to the suggestion that leukemia in most, if not all, animal species might be caused by viruses. Since then viruses have been isolated from spontaneous leukemia in captive gibbons but no further leukemogenic viruses have been isolated from any other domestic animal and the evidence for viral aetiology of leukemia in man is still equivocal.

Obviously it is naive to consider that the natural history of leukemia in all species might be the same: That virus might be isolated from each case of neoplasia as easily as it is found in leukemia in chickens or that the thread of transmission might be followed as easily as in cats. There is, however, a great deal to be learnt from the epidemiological studies which have been carried out in these species which is relevant to studies of leukemia in man. In this brief review I will discuss some of the evidence from which we derive our interpretation of the natural occurrence of virus-induced leukemias in animals and will outline similarities and differences between the disease in each species. Where appropriate the relevance to human leukemia will be discussed.

Avian leukosis

Avian leukosis was the first malignant disease shown to be infectious. The disease has always occurred among laying birds in poultry flocks throughout the world with an incidence of about 4–10% of all deaths. The most common form is lymphoid leukosis in which the cells involved are lymphoblasts derived from the bursa of Fabricius in which the initial transformation event occurs. During the first years of this century leukosis was transmitted to chickens using cell-free material derived from spontaneous tumours [1]. Much later as methods for demonstrating the avian leukosis viruses (ALV) and anti-viral

antibodies were developed, the extent of infection in chicken flocks became obvious [2,3]. The virus is present in all commercial chicken flocks and almost all members of the flock become infected before they reach sexual maturity. ALV is maintained in chickens by a cycle of transmission in which virus is transmitted epigenetically through the egg. If the egg is free of maternal antibody the growth of virus is unrestricted in the developing embryo and the newly hatched chicken becomes persistently infected. No antiviral immune responses are detected in such birds, which develop into hens producing eggs of which, again, a high proportion will contain virus. Thus the cycle is complete. It is in these viraemic, non-immune birds that leukosis occurs.

Immunity is, indeed, the major factor determining the proportion of hens which are at high risk of developing disease. Some viraemic hens in a flock have in addition to the virus, virus-neutralising antibodies which are also transmitted in the egg. The proportion of eggs from these birds which contain virus is much lower than in non-immune birds. Further, maternal antibody appears to restrict early replication of virus in congenitally infected chicks so that they are not rendered tolerant to viral antigens and, like their dams, develop antibodies.

It is considered that contact transmission is of little relevance in the natural history of ALV or in the pathogenesis of leukosis. Undoubtedly horizontal transmission does occur but present evidence suggests that this results mainly in immunity [4].

Bovine Leukosis

A form of leukemia known as enzootic bovine leukosis has been known for many years to be prevalent in cattle in Europe. Leukosis in cattle is a lymphosarcoma which occurs in four forms: adult multicentric, adolescent thymic, calf multicentric and a skin type. The adult multicentric form predominates in herds affected by enzootic leukosis. Typically in these herds multiple cases of the disease occur so that time-space clusters are evident; the incidence of the disease may be as high as 10% of the population at risk per annum [5].

During the early part of the century it became clear that enzootic leukosis was spreading westwards from eastern Europe. By the time the disease reached Denmark and Sweden more detailed prospective epidemiological observations were made from which it was concluded that the disease spread from hight to low incidence areas following the importation of cattle [6,7]. German and Danish veterinarians showed that haematological changes occurred in cattle in multiple case herds [6,8]. In particular a persistent lymphocytosis was established in many cows which was considered to be a pre-leukemic sign.

Enzootic leukosis is now found throughout eastern Europe, in Sweden, Germany, France and Italy, and North America. The British Isles were free of the disease until very recently when it was diagnosed in cattle imported into Scotland from Canada. In countries and certain areas within countries in which the enzootic disease has not been found, a sporadic form of lymphosarcoma is also seen which is discussed below.

The major factor in the occurrence of enzootic leukosis is undoubtedly the presence in members of the herd of bovine leukemia virus (BLV). Early experiments on transmission of leukosis by cell-free extracts were unsuccessful although transplantation of cells reproduced the disease. Attempts to visualise a virus in tumours by electron microscopy met with little success. The virus was eventually found when short term cultures of leukocytes from cattle with leukaemia were examined in the electronmicroscope [9]. Subsequently permanent virus producing cell cultures were established and antibodies were detected which reacted with viral antigens in the cells using immunofluorescence.

The epidemiological studies which followed the development of these tests revealed that BLV infection is common among cattle in multiple case herds with a prevalence of between 30 and 95% but does not occur in leukaemia-free herds [10]. In this system the presence of antiviral antibodies is strongly correlated with the isolation of virus. That virus is spread horizontally between cattle is suggested by the finding that newborn calves have no serological evidence of BLV but the age of 48 months most have developed antibodies.

Feline Leukemia

Leukemia is the most frequently diagnosed neoplasm in domestic cats [11]. Involvement of lymphoid cells is most common but myeloid and erythroid leukemias are seen quite frequently. The lymphoid malignancies occur in four main clinico-pathological forms: thymic, multicentric and alimentary lymphosarcoma and lymphatic leukemia. Feline leukemia virus (FeLV) is isolated from 90% of the thymic, 70% of the multicentric but only 33% of the alimentary cases. Investigation of communities with a high prevalence of active infection (see below) as well as experimental studies have revealed that FeLV also causes other diseases: aplastic and haemolytic anaemia, immunosuppression and early foetal death [12].

FeLV is transmitted by contact and congenitally [12]. Infection of young kittens by inoculation of FeLV experimentally or by contact with cats excreting the virus in the saliva, often results in a persistent viraemia which is prodromal to the development of leukemia often after a latent period of several years. Congenital transmission of FeLV is also common. All of the kittens born of viraemic dams are persistently infected. These, and kittens experimentally infected within the first 10–12 weeks of life, never show a detectable immune response to the virus.

The incidence of feline leukemia is related to epidemiological situation. In populations of free range urban and suburban cats, exposure to FeLV is widespread based on the prevalence of antibody to FOCMA (feline oncornavirus-associated cell membrane antigen) which is present on the surface of FeLV transformed leukemic cells. In urban Glasgow, about 50% of all adult cats have antibody [12]. The prevalence of these antibodies is related to the degree of roaming, young cats having the lowest prevalence and older stray

cats the highest. In spite of the frequency of exposure very few cats (less than 5%) have an active persistent viraemia [14] and consequently the incidence of lymphosarcoma is low (estimated at 0,05%). Whether the virus which induces persistent viraemia in these cats is obtained by horizontal or congenital transmission is not known owing to problems of tracing contacts and even parents.

A very different situation is observed in cats in closed multicat households in which FeLV is enzootic [12]. There the prevalence of anti-FOCMA antibodies is high but in contrast to free range cats there is a large proportion of viraemic cats (30–40%). Virus neutralising antibodies are also common among these cats (40%). As might be expected the incidence of lymphosarcoma is high, the disease occurring most frequently in cats which have been viraemic, often for long periods.

It is believed that the differences between these patterns is a reflection of the dose of virus to which the cats were exposed: free range cats have frequent intermittent contact with other cats, a few of which will be excreting FeLV so that the virus dose is low and will tend to immunise. Susceptible cats in closed households, on the other hand, are exposed to large, frequent doses of virus from carrier cats and tend to develop persistent viraemia with greater frequency.

Immunity is again a major determinant of the outcome of FeLV infection in individual cats. There is a marked correlation between the presence of virus neutralising antibodies and the absence of viraemia suggesting that these antibodies are important to the abrogation of FeLV infection [12]; and also between the possession of high titres of anti-FOCMA antibodies and protection from leukemia [15].

Virus-Negative Leukemias

Most investigations of leukemia in poultry, cattle and cats have not unnaturally been concerned with those cases in which a virus is involved. However, from many cases of leukemia in cattle and cats no virus may be isolated and recently virus-free cases of avian leukosis have been described.

In areas in which enzootic bovine leukosis is not encountered the occurrence of lymphosarcoma in cattle is sporadic and many more cases of the adolescent thymic and calfhood multicentric types are seen. These do not typically occur in multiple case herds and BLV has not been isolated from any such case.

As mentioned above, virus-negative leukemias are common in cats: in our series half are virus-free and these are mainly from the free range population. This reflects the proportion of alimentary lymphosarcomas only one third of which yields virus. As far as is known the clinico-pathological findings in virus-negative and virus-positive cases are identical although detailed analysis of the cell types and their surface markers have not yet been made.

The importance of the virus-negative leukemias, especially in cats, is that they may be analogous to leukemia in man: And because it is possible to study the relationship with a known leukemogenic virus. The evidence for

the involvement of FeLV in the pathogenesis of virus-negative lymphosarcoma in cats is equivocal. There is some serological evidence of FeLV exposure in a proportion of these cases. We have evidence that cases of FeLV-negative leukemia occur in households where FeLV is not present; M. Essex and his colleagues have, however, found clusters of virus-free cases in the same households where virus-positive cases also occur. Further evidence that FeLV may be involved is that FOCMA, which is present on the surface of FeLV-positive leukemic cells, is also found in virus-negative tumours [16]. However, FeLV-specific proviral DNA in excess of the level found in normal, uninfected cat cells has not been found in virus-free tumours [17].

Conclusions

The features which are common to avian, bovine and feline leukemia are: many cases are associated with virus which is isolated from the blood; the viruses are contagious; a persistent virus infection may be established; the latent period from infection to clinical disease is long and the disease characteristically occurs after sexual maturity; and animals are often healthy during the latent period and may continue to excrete virus.

There are, however, sufficient differences between the host responses in these species to introduce a note of caution in extrapolating findings from any one to leukaemia in another species. For example, the presence of virus in individual cats, hens or cattle indicates a certain risk of developing leukemia; but the interpretation of the presence of antibody is different in each species: in cattle it means that the animal has an active virus infection; in cats virus neutralising antibody is related to the abrogation of virus infection and resistance to challenge, and anti-FOCMA antibodies protect against leukemia; while in chickens, virus and neutralising antibodies often occur together.

A question which concerns epidemiologists studying human leukemia is whether epidemiological investigations of the distribution of cases can determine whether leukemia in man is likely to be an infectious disease. In cats, chickens and cattle, time-space clusters of the disease were found, the nature of which strongly suggested that leukemia was infectious. In man the evidence for clustering is not strong. However, it is important to note that even in cats, epidemiological studies based on cancer registry returns failed to produce evidence of contagious spread [18]; some clustering was found but it was concluded that this might have been due to chance. Of course subsequent serological and virological studies established the infectious nature of the disease. This was most apparent in closed communities of cats. It would not be possible to demonstrate that leukemia was infectious in free range cats despite the observation that FeLV is widespread in this population.

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Biochemical and Epidemiological Studies on Bovine Leukemia Virus (BLV)

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Abbreviations Used

ID gp 60:	Immunodiffusion test based on BLV glycoprotein (MW = 60000) as antigen
RIA gp 60:	Radioimmunoassay test based on BLV glycoprotein (MW = 60000) as antigen
RIA p 24:	Radioimmunoassay test based on BLV internal protein p 24 (MW = 24000) as antigen
BLV:	Bovine Leukemia Virus
PL:	Persistent Lymphocytosis
EBL:	Enzootic Bovine Leucosis
SBL:	Sporadic Bovine Leucosis

1. Enzootic Bovine Leucosis: The Disease

One generally distinguishes two types of bovine leucoses: an enzootic type and a sporadic type [3].

Here, we will be solely dealing with the enzootic type, the so-called Enzootic Bovine Leucosis (EBL) The basic features of this lymphoproliferative disease are:

- it is contagious; it spreads within a herd through contacts, saliva, milk, ... and from herd to herd mainly through commercial exchanges;
- it is induced by Bovine Leukemia Virus (BLV) a retrovirus *exogenous* to the bovine species [10];
- it involves the B lymphocytes [15, 17]
- it can be easily transmitted by the virus to cattle or sheep. Experimental BLV infection (but no clinical disease, sofar) has been obtained in goats and chimpanzees. No natural transmission of BLV to man seems to occur [3,6];
- BLV infected animals develop a humoral response directed against the viral antigens.

Enzootic bovine leucosis is a chronic disease that develops over a long period of time (several years generally). In natural conditions, very few cattle less than 2 years of age harbor antibodies to BLV antigens [11]. The same is true where BLV is searched for by its biological property of inducing syncytia [7] or early polykaryocytosis. If, however, a search is made *among the off-*

spring of BLV infected parents, it appears that as much as *14% of calves are infected at birth.* As discussed in [3], this situation reflects congenital infection by BLV and not true vertical transmission. BLV infection always induces a humoral antibody response and sometimes induces an hematological disorder called "Persistent Lymphocytosis" (PL). In such cases, the lymphocyte population is made of normal cells and a variable percentage of tumor cells as proven by molecular hybridization studies [3, 10]. PL has a genetic background [1] being much more frequent in some families within a breed than in other families of the same breed. With time, tumor development may occur, a phenomenon very poorly understood at the present time. Tumors may appear practically everywhere, in the digestive tract, the respiratory tract, muscles, ... but they are always lymphoïd. Most lymph nodes are enlarged, sometimes some of them only [2, 14, 19].

2. BLV: The Causative Agent of EBL

BLV is a retrovirus [3] produced in large quantities by essentially two cell culture systems, the Fetal Lamb Kidney cell-line [18] and the Tb_1Lu, a bat cell-line [13]. Morphologically the virus can be considered as a C-type although it displays some unusual peculiarities [3].

2.1. BLV Genome

It is a 60–70 S RNA molecule associated with reverse transcriptase. The number of genes and their order along the RNA molecule are not precisely known so far. DNA complementary to the RNA genome and representative of it, has been synthesized and extensively used in molecular hybridization experiments. The results of these studies are illustrated in Fig. 1 A and 1 B and call for the following comments:

If we take salmon sperm DNA as a control (histogram 11) it appears that normal bovine DNA hybridizes some 4% better than the control. We now know that this is due to contamination of 70S RNA used as template by 28S ribosomal RNA. DNAs from FLK-BLV producing cells (histogram 2), from buffy coat cells of an animal in persistent lymphocytosis with tumor (histogram 4) and from bovine enzootic tumor (histogram 5) hybridize with a maximum of 45% of the probe at a Cot value of 30000. This result is compatible with one proviral DNA copy per haploïd genome, if every cell contains the viral information. DNAs from tissues infiltrated with tumorous lymphocytes, hybridize to BLV c DNA to an extent that is roughly proportional to the degree of infiltration (histograms 3, 6 and 7). Sheep infected by BLV (histograms 9 and 10) show the same pattern of hybridization as cattle do. DNAs from human leukemic cells do not anneal to BLV c DNA.

That BLV is exogenous to the bovine genus was definitely established by recycling the ^3H BLV c DNA probe on normal bovine DNA. Results are illustrated in Fig. 1 B. They prove 1°) that BLV is largely if not totally exogenous to the bovine genus, 2°) that EBL is an infections disease, thus amenable to eradication.

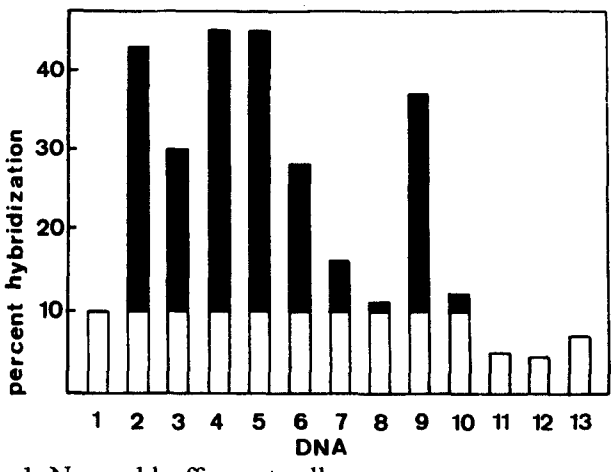

Fig. 1A. Hybridization of BLV ^3H cDNA to various bovine, ovine and human cellular DNAs. Hybridizations between 2400 cpm of ^3H cDNA (specific activity: $1{,}8 \times 10^7$ cpm/μg) and 250 μg of cellular DNA were performed in 0,4 M phosphate buffer (pH = 6,8) and 0,05% SDS in a final volume of 85 μl at 68°C. At a Cot value of 30000. samples were assayed for S_1 resistance. Source of DNA:

1. Normal buffy coat cells.
2. FLK cell line.
3. Buffy coat cells from a cow in persistent lymphocytosis without tumors.
4. Buffy coat cells from a cow in persistent lymphocytosis with tumors.
5. EBL tumor.
6. Liver moderately infiltrated with lymphocytes (EBL).
7. Kidney slightly infiltrated with lymphocytes (EBL).
8. Tumorous lymphnode from an SBL case.
9. Cutaneous tumor from a sheep infected with BLV.
10. Liver from the same leukemic sheep.
11. Salmon sperm.
12. Human chronic lymphatic leukemia.
13. Human chronic lymphatic leukemia.

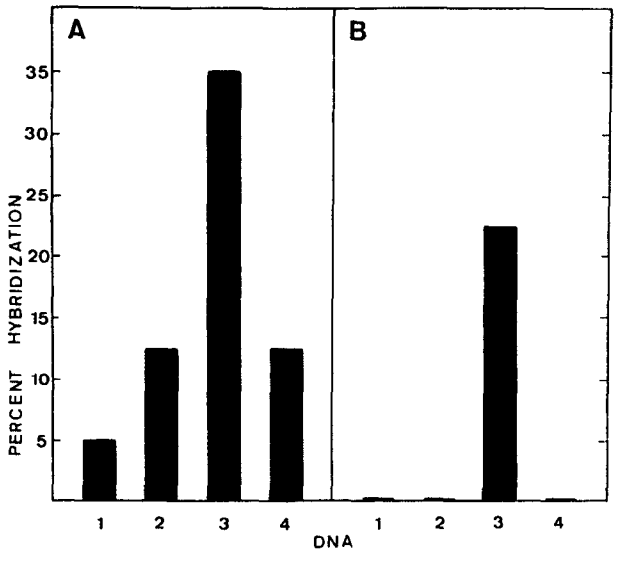

Fig. 1B. Hybridization of BLV ^3H cDNA (panel A) and recycled BLV ^3H cDNA (panel B) to the following cellular DNAs: 1. Salmon sperm; 2. normal bovine buffy coat cells; 3. EBL tumor; 4. SL tumorous lymph node. 2400 cpm of BLV ^3H cDNA (or recycled cDNA) and 250 μg of cellular DNA were hybridized in 0,4 M phosphate buffer (pH = 6,8) and 0,05% SDS. At a cellular Cot value of 30000 samples were assayed for S_1 resistance

2.2. BLV Proteins

BLV virions, at least, contain:
2 glycoproteins: gp60 and gp30 linked together within the virus envelope
4 non-glycosylated polypeptides p24, p15, p12 and p10
one reverse transcriptase, MW = 58000–70000 ([4] and Drescher et al., in preparation)

That the above mentioned proteins are indeed viral antigens rests upon two lines of evidence:

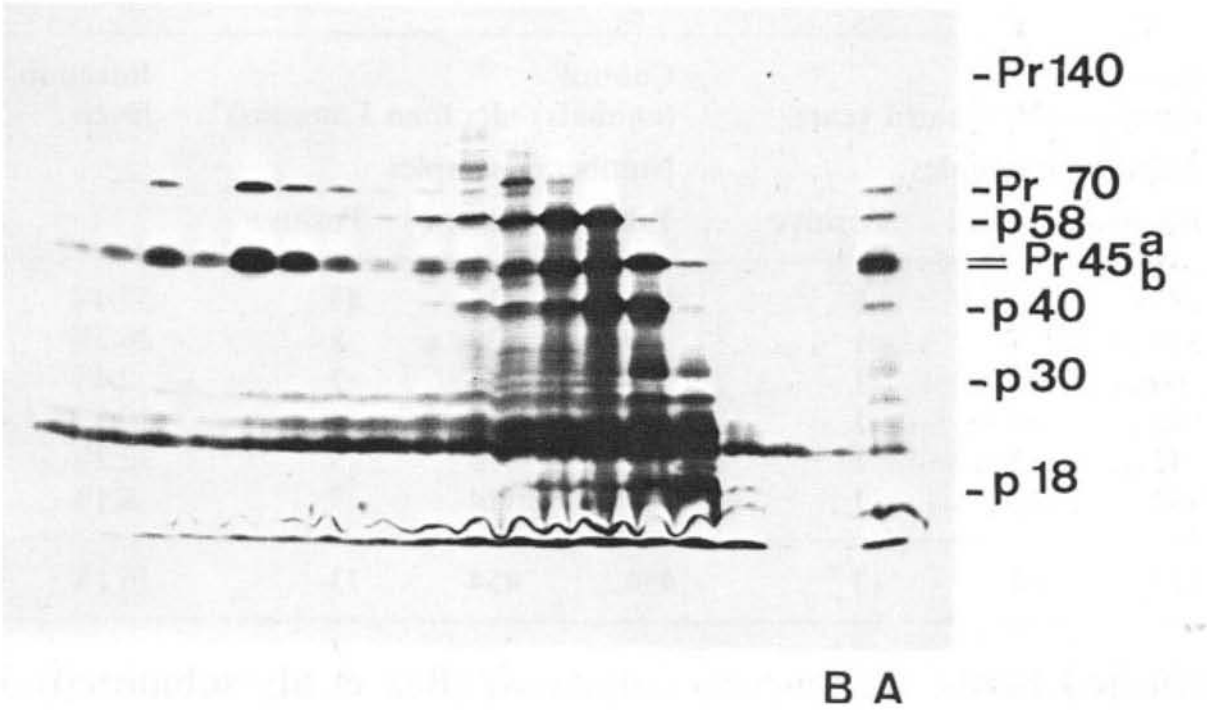

Fig. 2. Fluorograph of SDS-polyacrylamide gel of immune precipitates. Oocytes microinjected with a 1 mg/ml solution of 30–40S BLV RNA were labeled for 20 hours in Barth medium with 2 mCi/ml of ^{3}H leucine, lysed immediately (D) or chased in culture medium containing excess unlabeled leucine for 100 hours (E) and 300 hours (F) and then lysed. Non injected control oocytes were incubated in parallel (A–C) and indirect immune precipitation carried out on the same amount of homogenate with 4 µl of polyvalent anti-BLV serum. After 2 hours at 37°C and overnight incubation at 4°C, 100 µl of a 10% W/V Staphylococcus aureus suspension was added and incubation continued for another 4 hours at 4°C. Bacterial suspension was collected by centrifugation and washed. Immune complexes were separated by boiling 2 minutes in SDS containing buffer and analyzed by electrophoresis on a 15% polyacrylamide slab gel in the presence of SDS. G: ^{3}H amino acids labeled BLV marker

1. BLV infected animals synthesize antibodies directed against at least 4 of them (gp60, gp30, p24, p15).
2. BLV infected cells synthesize protein precursors to the gag group (p24, p15), a presumed gag-pol precursor and a precursor to BLV glycoproteins. In vitro protein synthesizing systems programmed with BLV 30–40S viral RNA synthesize gag precursors and the putative gag-pol precursor (Fig. 2). In adequate systems, these precursors mature into viral structural antigens [8]. Subgenomic fractions of BLV 35S RNA code for a number of polypeptides with molecular weights as 58000, 45000, 40000, 35000, 18000, ... (Fig. 3).

The 58000, 45000 and 40000 Molecular weight polypeptides are coded by m RNAs sedimenting in sucrose gradients in the 16S to 18S region and do not seem to be related one to the other by fingerprint analysis. Apparently, they are not of viral origin as preannealing of RNAs with BLV 35S-c DNA does not block their synthesis in reticulocyte cell-free systems.

On the other hand, polypeptide 18000 is undoubtedly of viral origin. Its biosynthesis in reticulocyte lysates is blocked if 16S to 18S m RNA is preannealed with BLV 35S-c DNA. Our present efforts are attempting to identify the region of the BLV genome coding for polypeptide 18000.

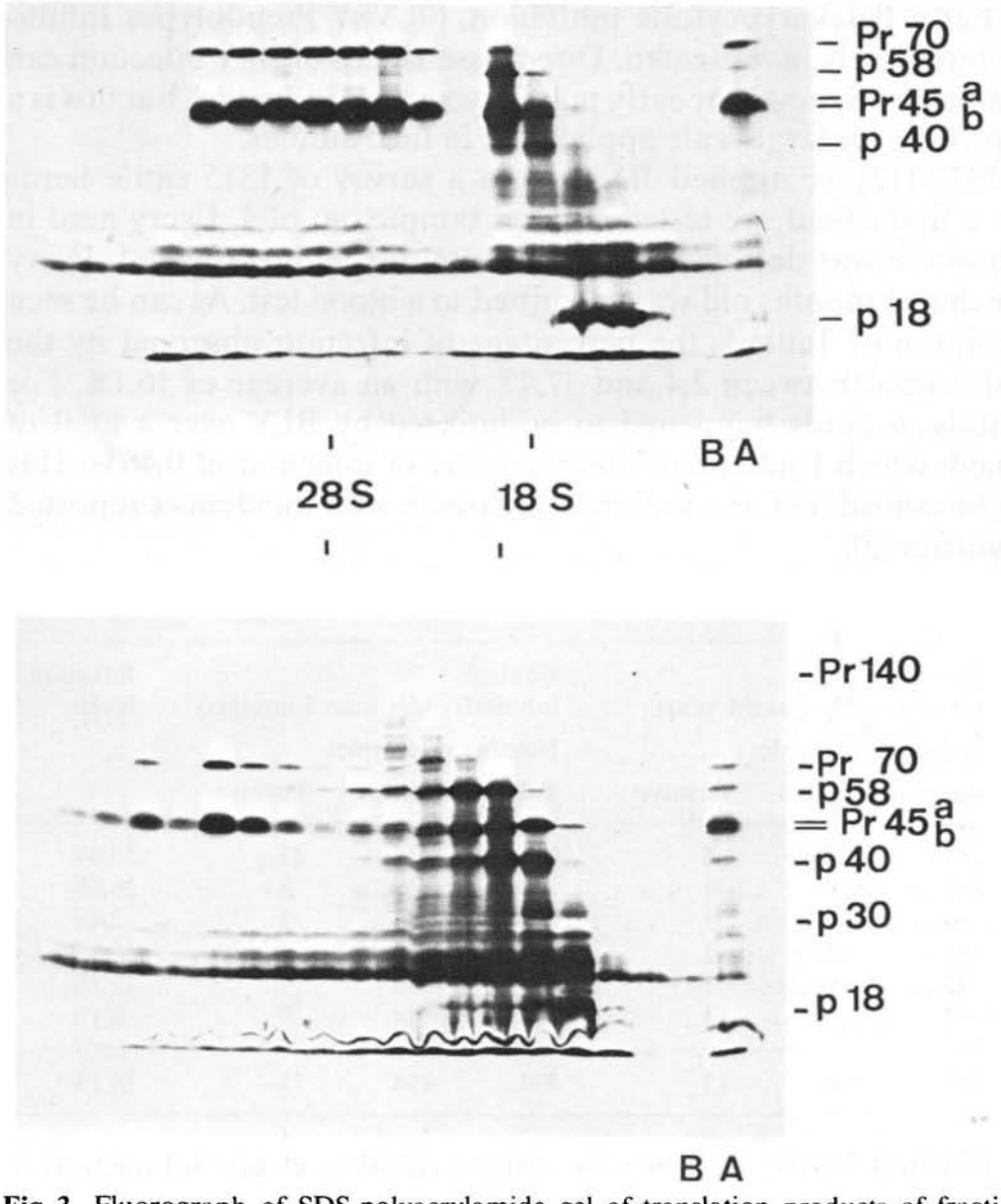

Fig. 3. Fluorograph of SDS-polyacrylamide gel of translation products of fractionated BLV virion RNA. Heat denaturated (95°C for 1 minute in Tris-HCl, 10^{-2} M, pH = 7,4, EDTA 10^{-3} M) BLV 60–70S RNA (80 μg) was fractionated by oligo dT cellulose chromatography and the poly-A-containing fraction (lower panel) or poly-A-deficient fraction (upper panel) were sedimented in a linear 15–30% glycerol gradient in Tris-HCl 10^{-2} M, pH = 7,4, NaCl 0,1 M, EDTA 0,01 M in a SW 41 rotor at 40000 rpm for 4 hours at 20°C. The RNA of each fraction was precipitated twice with ethanol, calf liver t-RNA being added as a carrier. One fourth of the RNA of each fraction was used to program protein synthesis in a messenger-dependent reticulocyte cell-free lysate. Analysis of translation products is made on a 15% SDS-polyacrylamide slab gel.
Track A: complete translation product of poly A-containing (lower panel) or poly A-deficient (upper panel) BLV RNA.
Track B: control, no RNA added

3. Epidemiology of BLV

Search for antibodies to BLV structural antigens is the present basis of all epidemiological investigations and eradication campaigns [3]. The presently most popular serological method is agar gel immunodiffusion based on BLV gp60, but other techniques such as radioimmunoassays ([5] and Bex et al.,

submitted), Early Polykaryocytosis Inhibition, [9] VSV Pseudotypes Inhibition [20] are intensively investigated. Direct assessment of BLV infection can even be obtained by Syncytia or early polykaryocytosis induction but this is a too tedious process for large scale application in field studies.

In 1976–1977 [12] we applied JD gp60 in a survey of 1315 cattle herds (Table 1). In a first round, we tested 1 blood sample out of 4. Every herd in which one positive was detected was then more deeply investigated. Every animal more than 3 months old was submitted to a blood test. As can be seen in the last column of Table 1, the percentage of infection observed by the method used, varied between 2,4 and 37,4% with an average of 16,1%. For the whole study, 6 herds happened to be infected by BLV over a total of 1315 examined, which leads to an observed rate of infection of 0,46%. This number can be considered as small in comparison with incidences reported for other countries [3].

Table 1. Survey of 1315 herds by ID gp60 and Control of this survey

Herd n°	Survey (animals older than 1 year) Number of samples			Control (animals older than 3 months) Number of samples			Infection levels
	Received	Tested	Positive	Taken	Tested	Positive	
1	80	22	8	115	115	43	37,4%
2	20	5	1	30	30	8	26,7%
3	60	15	1	83	83	2	2,4%
4	33	9	1	38	38	4	10,5%
5	42	12	1	74	74	9	12,2%
6	84	21	1	114	114	7	6,1%
Total	323	84	13	454	454	73	16,1%

Within infected herds, we recently compared (Bex et al., submitted) 3 serological tests, namely ID gp60, RIA gp60 and RIA p24. Over 345 animals tested, 104 were positive in RIA gp60, 101 in ID gp60 and 99 in RIA p24. The investigated herds were most probably foci of old BLV infections in which most "susceptible" animals had reached such antibody levels that ID gp60 was almost as sensitive as RIA gp60 and, indeed, more sensitive than RIA p24.

It should perhaps be mentioned here that the Commission of European Communities recently recommended to eradicate Enzootic Bovine Leucosis. The strict exogenous character of BLV and its apparent low progression power make the European recommendation quite feasible.

4. Host-Virus Relationship

The incoming of BLV into a recipient immediately elicits an antibody response to BLV structural antigens. The intensity of this response probably depends on age of the host and its genetic make-up, virus dose, health status, environ-

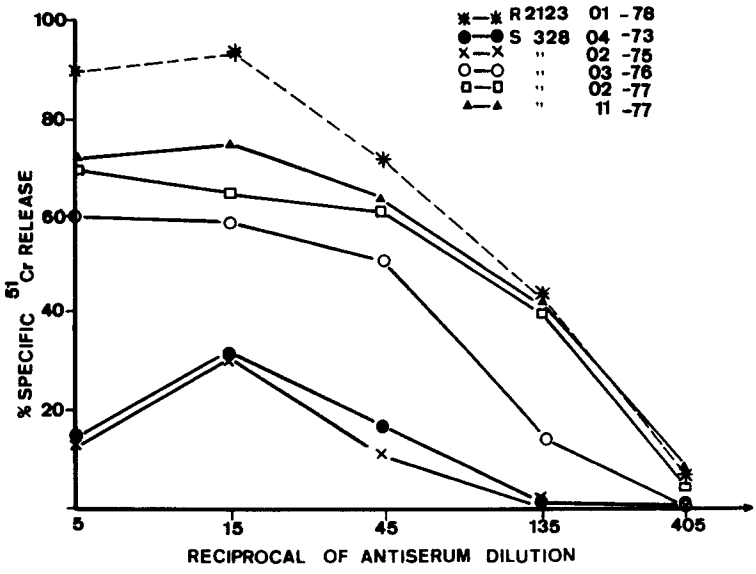

Fig. 4. Evolution of cytotoxic activity of a sheep serum (animal n° 328) during the last two years of the animal's life. Target cells were Fetal Lamb Kidney cells infected by and producing BLV (These cells are most probably not transformed by BLV). One of our best cytotoxic bovine sera (R2123) was used here as a reference

ment, ... In a recent study (Bex et al., submitted) we followed six sheep inoculated at birth, by the oral route, by whole leukemic bovine blood. As a rule, antibody levels to BLV gp60 and p24 rised steadily until the animal's death. In parallel we followed the complement dependent cytotoxicity of these sheep sera toward a BLV-producer cell line Fetal Lamb Kidney cells [16]. Results examplified in Fig. 4 clearly show that serum cytotoxicity increased with time, reaching a maximum level in the tumor phase of the disease at the animal's death. Immunoglobulins active in the cytotoxic reaction belong to the Ig G_1 sub-class.

5. Conclusions

The lymphoproliferative disease, Enzootic Bovine Leucosis, is an infectious disease caused by a retrovirus called BLV (Bovine Leukemia Virus).

The nature and mode of action of virus gene products and the mechanisms involved in the host-virus interplay are presently under intense investigation.

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Leukemia Specific Antigens: FOCMA and Immune Surveillance*

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A. Introduction

The domestic cat is one of few species where most cases of naturally occurring leukemia and lymphoma are known to be caused by viruses [14]. The RNA retroviruses that cause these diseases are well characterized, and they are related to the viruses that cause similar tumors in laboratory mice [13,15]. The malignancies associated with feline leukemia virus (FeLV) infection include T and B cell lymphomas, lymphoblastic leukemias, and myeloid leukemias [7,44,48,56,63]. Feline sarcoma viruses (FeSV), which are defective for replication, induce multicentric fibrosarcomas and melanomas in vivo [36,62,82], and transform fibroblasts in vitro [5,73].

Many studies on the biology and natural history of feline leukemia have been directed to issues that seem appropriate for a further understanding of leukemia of man. Among these, we have addressed the following questions: a) is leukemia transmitted in a horizontal (infectious) manner or in a vertical (genetic) manner? b) does a specific immunosurveillance response to the tumor cells serve to protect infected cats from leukemia development? c) do tumor cells have tumor specific antigen markers that are expressed in the absence of virus structural proteins? and finally, d) is it possible to establish whether the feline leukemia virus (FeLV) causes lymphoid tumors that neither make virus particles nor express virus structural proteins, nor contain full copies of the viral genome. The latter question appears important to our understanding of any possible role that retroviruses may play in human malignancies since they have generally not been found to be associated with these tumors in man. Recent information gathered in the feline model which relates to these questions will be discussed below.

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B. Virus-Induced Tumors of Cats

I. Etiologic Agents

The genetic map of the feline retroviruses is apparently the same as the map for the murine agents [59,83]. From the 5′ end of the mRNA, the first gene (gag) codes for a polyprotein of approximately 75000 daltons which is subsequently cleaved after translation to form 4 smaller peptides which all become located in the core of the mature virion. The peptides, designated p15, p12, p30, and p10, occur in the latter order starting from the 5′ end of the genome. The major capsid protein is p30, a protein which is immunogenic in cats [6,84]. The second gene from the 5′ end is pol, which codes for the reverse transcriptase-RNase H complex.

The third gene, env, codes for a polyprotein which is cleaved to form two components situated at the surface of the virion. The first, usually designated gp70, becomes the virion envelope knobs or spikes. It is also immunogenic, and the target for virus neutralizing antibody. The feline viruses have been divided into 3 subgroups (A–C) based on type specific antigenic differences manifested by the gp70 molecule [74]. The subgroups which are designated on the basis of virus neutralization serology show a comparable distinction in interference concerning host-cell attachment [74]. Differences in sensitivity of infection of cells from heterologous species are also partially related to the virus subgroup and some evidence suggests a partial correlation between subgroup designation and pathogenicity [55]. The second peptide associated with the env gene polyprotein is designated p15e. It is highly conserved among viruses that infect various species and it appears to be associated with immune suppression by the virus [3,65].

II. Pathobiology

FeLV is associated with lymphoma as well as lymphoid and myeloid leukemia. Although one highly selected strain of virus has been regularly associated with the induction of the thymic form of lymphoma [50], most isolates produce various forms of hematopoietic tumors when inoculated into neonatal kittens [64]. The reported relative ratio of lymphoma incidence to leukemia incidence in the natural population has varied widely according to geographical location and/or institution. In the British Isles, for example, cases of true leukemia appeared to be very rare [18,56]. Conversely, in Boston the relative incidence of true leukemia was about the same as the incidence of lymphoma [18]. Similarly, geographical variations occurred concerning the form of lymphoma observed. In Glasgow, the alimentary form was the most common type of lymphoma reported [18]. In Boston, the alimentary form accounted for less than 10% of all lymphoma cases while the thymic form was most common [18]. A pathologic breakdown of the cases observed at the Angell Memorial Animal Hospital from 1972 to 1976 is presented in Table 1.

Both myeloid and lymphoid leukemias appear to be caused by FeLV [8,44]. The clinicopathologic parameters observed in feline lymphoid leukemia

Table 1. Classification by pathologic form of cases of lymphoid malignancies observed at the Angell Memorial Animal Hospital in Boston for 1972 through 1976

Pathologic form	Number of cases	Proportion of total	Number virus positive	Proportion virus positive
Lymphoid leukemia	77	42%	52	68%
Lymphoma (total)	107	58%	71	66%
Thymic	49	27%	37	76%
Multicentric	20	11%	11	55%
Alimentary	14	8%	7	50%
Other	24	13%	16	67%

have been compared to the parameters observed in acute lymphoblastic leukemia of children [8].

Most cases of spontaneous feline lymphoid malignancies appears to be T cell tumors [7,48]. Essentially all cases that have a primary location in the thymus are T cell tumors, although many that arise at a different site are also of T cell origin. Some B cell tumors occur, primarily originating in the gut wall as the alimentary form of lymphoma [63]. Null cell tumors have also been reported [48].

When inoculated into newborn kittens, the Rickard strain of FeLV causes thymic involution as the first apparent pathologic alteration [57]. Following this involution, the first cells observed in the thymic site are malignant T cells which contain the tumor specific antigen designated the feline oncornavirus associated cell membrane antigen (FOCMA) [25]. See Table 2.

Table 2. Expression of FOCMA and FeLV proteins on freshly biopsied lymphoid cells from cats inoculated with FeLV[a]

		Nonthymic lymphoid cells			*Thymic lymphoid cells*		
Time after inoculation (weeks)	Disease status	FeLV (infectious centers)	Presence of FOCMA	Membrane FeLV gp70 and p30	FeLV (infectious centers)	Presence of FOCMA	Membrane FeLV gp70 and p30
4	normal	4/4[b]	0/4	4/4	4/4	0/4	4/4
8	normal	4/4	0/3	3/3	3/3	0/3	3/3
12	normal	2/3	0/3	3/3	2/3	1/3	3/3
16	normal	1/1	0/1	1/1	1/1	1/1	1/1
	leukemic	2/2	0/2	2/2	2/2	2/2	2/2
20–24	leukemic	6/6	2/6	6/6	6/6	6/6	6/6

[a] Cells from bone marrow, spleen, mesentric lymph nodes, and buffy coat were tested from each cat. All preps from different organs of the same animal (other than thymus) gave the same result. The only exception was the presence of a significant population of FOCMA positive cells in the spleens of 2 of 6 leukemic animals even though less than 10% of the cells in the bone marrow, buffy coat, or mesentric lymph nodes of the same animals were FOCMA positive.

[b] Number positive over total number tested

The proportion of spontaneous cases of feline lymphoma and leukemia which contain detectable FeLV varies with both geographical location and pathologic form [18]. Both virus isolation procedures and serologic tests have established that from 50% to 90% of the spontaneous cases of leukemia and lymphoma contain readily detectable FeLV. Although the proportion of virus negative cases appears to be highest for the B cell alimentary form of the disease, all pathologic forms include a significant minority that appear to be "virus-negative" by all criteria [32].

FeLV is regularly excreted in saliva from essentially all healthy and leukemic cats that are viremic [34]. Infectious virus is not ordinarily present in significant levels in feces, urine, or fleas taken from infected cats but the levels of infectious virus in saliva are as high or higher than the levels found in blood plasma [34]. FeLV is inactivated rapidly within a few minutes at 56°C, but stable for several days at 25–35°C [33].

C. Epidemiology of Feline Leukemia and Lymphoma

That FeLV is transmitted as a contagious agent in cats is now well established [19,46,49,58]. Initially this conclusion was doubted, largely because it had been previous established that many related retroviruses were genetically transmitted in inbred strains of mice [52]. The first suggestion that FeLV and leukemia might be horizontally transmitted in the cat came with reports that leukemia [9,10] and lymphoma [22,46,56,76] occasionally occurred in clusters. Since these reports were sometimes anecdotal and difficult to evaluate due to a lack of data on the base cat population, their significance was questioned [75].

We became convinced that the clusters were not due to chance alone when the high incidence of leukemia/lymphoma continued to occur, in a prospective sense, in the same cluster households [9,22]. Subsequently, extensive supporting seroepidemiologic evidence for FeLV transmission was obtained in some of the same households [9,12,19,28,31,37,38,42,43,45,46,84].

Another clue which suggested that FeLV-associated malignancies were horizontally transmitted was the finding that healthy uninoculated control cats developed leukemia/lymphoma more often than would be expected due to chance alone following exposure to leukemic cats [45,58,71]. This occurred under both laboratory and field conditions.

Using serologic techniques, healthy cats known to be exposed to either leukemic cats and/or cats viremic with FeLV were examined for the presence of antibodies to FOCMA [9,19,22,26,28,37,38], complement dependent cytotoxic antibodies [37,38,66], FeLV neutralizing antibodies [19,42,45,72], radioimmunoprecipitating antibodies to FeLV p30 [19,31,84], radioimmunoprecipitating antibodies to FeLV gp70 [19,31,84], and antibodies which neutralized the activity of FeLV reverse transcriptase [53]. The healthy cats examined included animals exposed to FeLV under both experimental and natural conditions. In each case populations of cats known to be exposed to FeLV had much higher frequencies of detectable levels of antibody than cats

of comparable genetic backgrounds with no history of FeLV exposure. Similarly, the geometric mean antibody titers were regularly higher for cats that were known to have been exposed to FeLV when compared to cats that were not known to be exposed to FeLV. The latter category included both "specific pathogen free" laboratory cats, and conventional cats from laboratory and household pet backgrounds.

Along with the examination of such FeLV-exposed cat populations for various antibody activities, numerous individuals from the same populations were checked for the presence of circulating infectious virus [19,42,46,55], the presence of major viral antigens by fixed cell immunofluorescence [6,22, 42,46,47,84], and the presence of FeLV p30 and gp70 antigens by radioimmunoprecipitation [31,84]. Again, healthy cats from populations known to be contact exposed to either known FeLV-infected animals and/or known cases of leukemia/lymphoma were examined. Up to 50% of the healthy cats in such populations were found to be actively infected with FeLV [9,22,41, 42,46]. Conversely, no more than 1–2% of the healthy cats with a history of no known exposure to FeLV were found to be actively infected [17,41,46].

Along with the serologic examination of existing cat populations for the presence or absence of FeLV-related antigens and antibodies, tracer specific-pathogen-free cats were placed in natural environments where FeLV was known to be present (leukemia cluster households) and subsequently examined for prospective seroconversion [19]. The cats placed in this new environment were previously confirmed to be negative by all of the serologic tests. Essentially all developed evidence of either transient or persistent FeLV infections within a few months after being introduced to the new environment [19].

As a final measure, a study was undertaken to determine if subsequent infections could be prevented in known high-risk FeLV exposure environments by the elimination of FeLV-excretor cats [43,45]. This approach was also successful, demonstrating that the numbers of subsequent FeLV infections and cases of FeLV-associated diseases could be either drastically reduced or completely eliminated by these procedures [43,45].

D. Role of the Immune Response

All of the FeLV structural proteins appear to be immunogenic in adult cats that become exposed to virus. These include each of the gag gene peptides p15, p12, p30, and p10 [20,84,85], reverse transcriptase [53], and the env gene proteins gp70 [20,84,85], and p15e [86]. At least in the case of the major core protein (p30) and the major envelope protein (gp70), several antigenic determinants are present on the same molecules, including those with group, subgroup, or type, and interspecies specific determinants. In cats the major immunogenic determinants of the p30 is group specific while the major immunogen on the gp70 is subgroup specific [6,55]. Conversely, different primary antigenic determinants on the same molecules may be recognized as significant immunogens when non-feline species such as goats or rabbits are inocu-

lated with FeLV [20]. This might appear to be inconsequential, except for the possibility that different antigenic determinants might be expressed on the surface of FeLV-infected cells when compared to reactivities seen with either intact virus or purified immunoprecipitating virion components. For example, it is conceivable that anti-gp70 serum from the homologous species (cat) might effectively neutralize circulating cell-free FeLV but not attack FeLV-infected cells while goat or rabbit antiserum to the same molecule might cause cytolysis of infected cells. A major consequence of this spectrum of reactivity might be a more beneficial anti-tumor therapeutic effect in the presence of antiserum from a heterologous species which would otherwise react similarly to cat antiserum when tested by a radioimmunoassay with the purified molecule.

An effective antibody response by cats to the gp70 is associated with the reduction and/or elimination of circulating FeLV [19,42,84]. The clinical significance of the antibody responses to the other virion structural antigens has not been determined. Antibodies to other FeLV antigens are frequently present, but they are not always concordant with the presence of antibodies to gp70 [31,84]. Cats with persistent viremia appear to lack free antibodies to all of the FeLV structural proteins [20,84,85].

Aside from the virus structural antigens, an important antigen in both FeLV and FeSV induced tumors is FOCMA [23,24,30]. As opposed to the situation with the viral structural antigens, the antibody response to FOCMA is closely correlated with protection from development of leukemia, lymphoma, and fibrosarcoma, whether laboratory induced or naturally occurring [12,14,20, 23,24,26,28,30,31,37,38]. Cats that become naturally infected with FeLV that remain healthy and viremic for long periods, for example, usually also maintain readily detectable levels of antibody to FOCMA [14,26]. Cats with either naturally occurring leukemia or lymphoma as well as those cats bearing tumors induced by FeLV and FeSV contain either no detectable antibody to FOCMA or very low levels of such antibodies [14,18,26,28]. Additionally, a poor antibody response to FOCMA serves, in a prognostic sense, as a risk factor for subsequent tumor development [14,26]. FOCMA antibody titers determined for cats in several populations are summarized in Table 3.

Table 3. FOCMA antibody titers for healthy and neoplastic cats from laboratory and field environments

	Number tested	Number and percent of cats with antibody titers of:		
		<4	4–16	>16
[illegible][b]	131	116 (89)	15 (11)	0
[illegible]	76	6 (8)	48 (63)	22 (29)
[illegible]exposed to virus[c]	221	218 (99)	3 (1)	0

[illegible]kemia, lymphoma, and fibrosarcoma
[illegible]nd/or FeSV under either laboratory or field conditions
[illegible]nd minimal disease free laboratory cats

Feline serum samples which contain detectable levels of antibodies to FOCMA usually also contain antibodies which are lytic for cultured feline lymphoma cells using the ^{51}Cr release test [37,38]. The complement-dependent antibodies (CDA) function best in the presence of cat complement, but the lysis requires up to 20 hours to reach maximum effect. A 90–95% overall correlation was found between CDA as detected with cat complement and FOCMA antibodies as detected by indirect membrane immunosurveillance [37.38]. When guinea pig complement or rabbit complement rather than cat complement was used in the CDA assay, the correlation was reduced to 79% and 65%, respectively [37,38].

Cats that become infected with FeLV manifest a generalized syndrome of immunosuppression [3,21,42,57,70]. As a result, viremic healthy cats have a significantly increased risk for development of various infectious diseases [11.21,42]. Many possible explanations can be considered to explain the observed immunosuppression. FeLV causes thymic atrophy and wasting disease. following either laboratory injections or natural infections [1,46,57]. Persistent viremia with FeLV has been associated with depressed numbers of peripheral blood lymphoid cells [21], depressed counts of peripheral T cells [16], variations in complement levels [39], and increased suppressor cell activity [3,4]. One of the viral structural proteins, p15e, has been demonstrated to have a profound effect on various lymphocyte functions [3,65]. Persistently viremic cats also have a high risk for development of immune complex glomerulonephritis [54].

E. Tumor Specific Antigens

FOCMA was first described on cultured feline lymphoma cells taken from a cat inoculated with FeLV [23,24]. The antisera which reacted with the lymphoma cell surface were taken from cats previously inoculated with FeSV [23.24,30]. Subsequent studies indicated antisera taken from cats either inoculated with FeLV or horizontally exposed to FeLV gave the same reaction [12,17.19,22,26,49]. Thus, it was clear that FeLV was capable of inducing the antigen. Whether or not FeSV alone could also induce the antigen was unclear because the FeSV preparations inoculated into cats always contained an excess of FeLV helper virus.

To determine if FeSV alone could induce FOCMA it was necessary to study FeSV transformed nonproducer cells, which were not superinfected with FeLV [27,80,81]. The initial approaches involved the examination of mink cells transformed by either FeSV or murine sarcoma viruses. FOCMA was regularly expressed on cells transformed by FeSV, but not expressed on cells that were morphologically transformed by other viruses. The antigen was not expressed on nontransformed cells infected by FeLV, and no change in the level of expression was seen on nonproducer transformed cells following superinfection with FeLV. No relationship was found between expression of FOCMA and either activation of the endogenous mink virus or expression of the mink virus structural proteins [27,80,81,85]. Additionally,

FOCMA was found to be specifically induced by FeSV in cells from various other species including subhuman primates, dogs, and rats [81]. Conversely, FOCMA was not found in feline cells carrying the murine sarcoma virus genome, even when these cells were superinfected with FeLV [27,80]. The observation that FOCMA induction could be transmitted across species barriers with FeSV strongly suggested that this transformation-specific antigen was encoded for by FeSV. The correlation between FOCMA expression and FeSV transformation is summarized in Table 4.

FeSV-transformed nonproducer mink cells were examined by radio-immunoprecipitation for all of the major FeLV-related virion proteins [77,85]. The cells were found to be free of the major env gene product gp70 and free of the major gag gene product p30. The cells were also free of p10, but significant levels of the gag 5′ end peptides p15 and p12 were present in the FeSV-transformed nonproducer cells. Using antisera to FOCMA and/or p15 and p12, several classes of molecules were detected in the transformed cells. The first was an 85 000 dalton molecule which could be precipitated with antisera to each of the three antigens. The second species, which could be precipitated with antisera to FOCMA but not with antisera to p15 or p12, was about 65 000 daltons. The 65 000 dalton species was found in largest amounts after apparent cleavage from the 85 000 dalton species. Along with the 65 000 species, the cells also contained free p15 and p12, and a precursor of approximately 25 000 daltons which had both p15 and p12 activity [85].

The 85 000 dalton species was also identified in pseudotyped feline sarcoma virions rescued from the mink cells using various serologically distinguishable helper viruses such as the endogenous baboon retrovirus and the xeno-

Table 4. FOCMA expression in malignant and non-malignant cells of feline and mink origin

Species	Description of cells	Malignant phenotype	Antigens present on cell surface		
			FOCMA	FeLV gp70	FeLV p30
cat	cultured FeLV producer lymphoma cells	+	+	+	+
cat	freshly biopsied FeLV producer lymphoma or leukemia cells	+	+	+	+
cat	freshly biopsied nonproducer lymphoma or leukemia cells	+	+	–	–
cat	freshly biopsied or cultured normal cells infected with FeLV	–	–	+	+
cat	fibroblasts transformed with FeSV, superinfected with FeLV	+	+	+	+
cat	fibroblasts transformed with murine sarcoma virus, superinfected with FeLV	+	–	+	+
mink	fibroblasts infected with FeLV	–	–	+	+
mink	fibroblasts transformed with FeSV, nonproducer	+	+	–	–
mink	fibroblasts transformed with FeSV, superinfected with FeLV	+	+	+	+
mink	fibroblasts transformed with murine sarcoma virus	+	–	–	–

tropic murine retroviruses [77,78]. As in the case of cell extracts, the pseudotyped FeSV's contain FOCMA, FeLV p15, and FeLV p12. All the p30, p10, and gp70 found in the pseudotyped virions was specific for the helper virus [77]. FOCMA is not found in either FeLV or the helper viruses used to rescue FeSV. A radioimmunoassay has now been developed for the 85000 dalton species of FOCMA [78].

Since FOCMA activity was originally detected using a cultured FeLV-producer feline lymphoma line, studies were undertaken to determine the association between FOCMA, FeLV production, and the malignant phenotype in various biopsied lymphoid cell preparations [25,27,29,47,48]. FOCMA was found to be present on biopsied malignant feline lymphoid cells, irregardless of whether they were taken from T or B cell tumors, or whether or not detectable FeLV was also present (see following section), or whether the cells were from cases of lymphoblastic leukemia or lymphoma, or whether the malignant cells were from natural cases of tumors or laboratory induced cases following inoculation of the animals with FeLV [25,27,29,47,48]. FeLV-producer lymphoid cells that were morphologically normal were negative for FOCMA, even when the normal cells were taken from cats with lymphoma.

Taken together, these results indicate that FOCMA is encoded for by FeSV, but they do not reveal whether or not the same antigen (or a closely related cross-reactive antigen) is also encoded for by FeLV. Several possible explanations can be considered. For example, FOCMA might represent the src gene of FeSV, which was originally acquired from normal fibroblasts by non-transforming FeLV. In this case, the gene would presumably be somewhat related to those expressed during normal differentiation processes. FeLV might then act by derepressing the gene when the virion genome interacts with the cell in such a way as to cause leukemia. If FeLV was uniquely qualified to do this, because of some evolutionary process of co-development between the virus and that specific region of the cell genome, we might expect that tumors of mesodermal cell origin caused by factors other than FeLV would not express FOCMA. Alternatively, if FOCMA was a common requirement to the process of de-differentiation associated with malignancy, we might expect that tumors induced in lymphoid and/or fibroblastoid cells with chemicals, irradiation, or other viruses might also express FOCMA. Since "FeLV-negative" lymphoid tumors normally express FOCMA, we would obviously have to postulate that the later tumors were initiated by FeLV if FOCMA is either FeLV encoded or induced in the cell only by FeLV.

The possibility that FOCMA is also encoded for by FeLV can also be considered. Although it does not appear to be gag, pol, or env, it is possible for example that feline retroviruses which actually cause leukemia might possess a "leuk" or "onc" gene analogous to "src" which codes for FOCMA. An FeLV carrying such a gene has not been detected as yet, but classical nucleic acid hybridization experiments would not detect such an agent anyway if it existed as a small minority in an excess of non-leukemogenic helper virus. The latter situation is of course present for FeSV, as well as for the defective acute leukemia viruses of chickens [2]. Still another possibility is that FOCMA

might be encoded for by sequences normally found in replication competent FeLV. In the case of Rauscher virus a protein "by-product" of unknown function has been identified which arises from the gag-pol precursor polyprotein. This protein does not become part of the subsequent gag or pol virion proteins [78]. Although perhaps even more unlikely, information to make FOCMA might be generated from standard FeLV gag, pol, or env nucleotides in the form of a "spliced" mRNA made from integrated DNA provirus.

F. "Virus Negative" Tumors

About 20–30% of the spontaneous lymphoid tumors found in cats from the northeastern USA lack detectable FeLV [18]. In Glasgow, up to 50% of the feline lymphomas are negative for FeLV [18,57]. Although the proportion of B cell tumors that are "virus negative" is highest (up to 65%) a significant minority of the T cell tumors also lack detectable FeLV [48]. Additionally, all of the major gross pathologic forms of lymphoid malignancies may occur in the "virus-negative" state. The examination of tumor cell homogenates using a sensitive radioimmunoprecipitation technique failed to yield detectable levels of any of the virus proteins [84,85]. As in the case of FeSV transformed nonproducer mink cells, the "virus-negative" tumors lacked detectable p30, p10, or gp70. Unlike the FeSV transformed nonproducer cells, the "virus-negative" tumors also lacked detectable p15 or p12. Thus, the question obviously arises concerning the etiology of the "virus-negative" tumors. If, for example, it could be established that the "virus-negative" tumors were originally caused by FeLV, as were the morphologically indistinguishable "virus-positive" tumors, one might expect that the "virus-negative" feline tumors would be an excellent model for attempting to understand "virus-negative" leukemia and lymphomas of man.

One obvious approach to study the possible role of exogenously acquired FeLV in the etiology of "virus-negative" tumors is the approach of nucleic acid hybridization. Several studies have been reported. These include the use of DNA probes, made from FeLV with reverse transcriptase, to test for hybridization with lymphoma cell RNA [35,61]. The DNA probes used represented all 3 of the subgroups of FeLV, as well as two independently distinguishable FeSV's. The results failed to reveal hybridization with the "virus-negative" lymphoma cells which was above background levels detected in normal feline tissue. A second approach employed iodinated virion RNA as a probe to hybridize with lymphoma cell DNA [60]. Again, "virus-negative" lymphoid cells lacked evidence of FeLV-related provirus sequences above the background levels found in normal cells. Levels of hybridization obtained with virus negative tissues varied from about 15% to more than 60%, but tumor tissues did not necessarily have values that were any higher than normal tissues.

Other approaches can be used to study the possible association between FeLV and the "virus-negative" tumors. Feline lymphoid tumors often occur

in clusters within households where cats are exposed to large doses of FeLV [10,22,46,76]. We recently sought to determine if "virus-negative" leukemia occurred in a large FeLV exposure leukemia cluster household at a higher rate that it would be expected to occur in the population at large. This was found to be the case.

Under experimental conditions, serum immunotherapy directed to circulating FeLV and/or FOCMA has been employed [40,67,69]. Both classes of antiserum appear to have a beneficial effect under appropriate conditions. Antiserum to virion antigens could presumably function by eliminating FeLV-producer cells, as well as by eliminating free virus. We recently postulated that immunoselection might be one possible mechanism by which "virus-negative" tumors might be caused by FeLV and/or FeSV [20]. If fo example, an anti-FeLV response was to eliminate virus producer cells in vivo, allowing nonproducer transformed cells to "sneak-through", the result might be a "virus-negative" tumor. We have observed this apparent course of events in a few fibrosarcoma-bearing cats treated with anti-FeLV [68]. The absence of a parallel anti-FOCMA response apparently allowed the development of "virus-negative" tumors following the initial regression of "virus-positive" tumors. Whether or not the "virus-negative" secondary tumors were also negative for the provirus genome by hybridization remains to be determined. Additionally, a few cats have been observed to develop "virus-negative" leukemia, under natural conditions after previous infections with FeLV [40]. Direct evidence that naturally occurring "virus-negative" feline lymphomas are caused by FeLV is lacking. However, the regular presence of FOCMA, an antigen known to be encoded by FeSV, on "virus-negative" tumor cells, as well as the curious epidemiologic association between exposure to FeLV and the existence of "virus-negative" tumors cannot be ignored. Further investigations on the identity of FeLV-related partial provirus sequences in the "virus-negative" tumor cells seems warranted. If such tumors can be clearly linked to FeLV, the rationale for an intensified search for analogous defective viruses in human tumor tissues becomes obvious.

G. Summary

In cats, horizontally transmitted viruses cause leukemia and lymphoma under natural conditions. As with other retroviruses, feline leukemia virus (FeLV) contains products of 3 major genes; the virus core gag gene products, the polymerase, and the virus envelope glycoprotein. When cells are transformed in vitro by the related feline sarcoma virus (FeSV), an additional protein, FOCMA is expressed at the cell membrane. FOCMA, which is FeSV-coded, is transformation and/or tumor specific and expressed regardless of whether or not the cells make virus or contain virus structural antigens. Lymphoid leukemia cells also express FOCMA, both when FeLV is used to induce the disease in laboratory cats and when the tumors occur under natural conditions. FOCMA is expressed on both T and B lymphoid leukemia cells, but not expressed on non-malignant lymphoid cells, even when they are infected

with FeLV. About one-third of the naturally occurring lymphoid tumors of cats lack detectable FeLV proteins and varying portions of the FeLV provirus. Despite this, they regularly express FOCMA, which is the target of an immunosurveillance response that functions effectively under most conditions. FOCMA thus provides a useful model for antigens that might be expressed in "virus-negative" leukemias of man.

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A Single Genetic Locus Determines the Efficacy of Serum Therapy Against Murine Adenocarcinoma 755a

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Introduction

We have recently described a unique model system for immunotherapy where immunological management of a highly lethal tumor, the ascites form of the Adenocarcinoma 755 (AD755a), could be carried out reproducibly and with remarkable efficiency [7]. Some of the basic features of this model are summarized in Table 1. The studies to be described in this report will concern themselves primarily with a more in depth analysis of the phenomenon of strain variation in relation to the protective capacity of the serum, and were done in an effort to gain some insight into the mechanism by which the serum mediates its powerful effect.

Preparation of Hyperimmune Anti-AD755a Antiserum

Ad755a was uniformly lethal in B6 mice after intraperitoneal (ip) inoculation of as few as 50 cells per mouse (Table 1). In contrast, when AD755a cells were injected subcutaneously (sc) into B6 mice in a dose range between 1×10^5 and 5×10^5 cells, a transient nodule appeared that was resorbed completely by week 2–3 after injection. Mice that had rejected AD755a cells inoculated sc were resistant to a later ip challenge with these cells. On this basis, B6 mice

Table 1. Characteristics of AD755a tumor system

AD755a is a "universal" tumor and is lethal in all mouse strains tested. Inoculation of fewer than 50 cells intraperitoneally gives 100% lethality and fewer than ten cells results in approximately 80% lethality. The tumor grows equally well in all strains tested.
Immunization in "syngeneic" C57B1/6J mice by subcutaneous inoculation of AD755a provides protection against an intraperitoneal challenge of 10^4 LD_{100} and this protection persists for greater than 90 days.
Serum or immune cells from mice hyperimmunized by multiple intraperitoneal injections after an initial subcutaneous immunization can transfer protection to a normal animal in quantities of 5–10 μl against a challenge of 1×10^5 AD755a cells.
Preliminary study of the protective factor(s) shows it is contained in the IgG fraction. It has an effective half-life in vivo of greater than 4.5 days and less then 9.0 days.
Studies in several mouse strains have revealed that the protective capacity of the serum is strain dependent.

were hyperimmunized against the AD755a cells by an initial sc inoculation of 10^4 AD755a cells followed by sc inoculations 2–3 weeks later of 1×10^5 and 5×10^5 cells respectively. Subsequently, increasing doses of AD755a cells from 1×10^4 to 1×10^6 were given ip over a period of seven weeks. After four additional ip inoculations with 1×10^6 cells, serum was taken. Animals were boosted and bled monthly thereafter.

Normal B6 mice were inoculated ip with various volumes of the immune sera at the same time that they were challenged with AD755a cells. Protection against 10^5 tumor cells was obtained with as little as 5–10 µl of B6 anti-AD755a serum. Subsequent experiments demonstrated that similar titration end points could also be obtained after sc or intravenous injection of the serum; this indicated that the serum and tumor cells did not need to be administered together for successful passive immunotherapy to be achieved. Finally, serum administered as late as 3 days after tumor inoculation was also protective, although larger quantities were required.

Protective Capacity of Immune Serum in Various Mouse Strains

Titration of B6 anti-AD755a immune serum against AD755a tumor cells given ip to other strains of mice revealed a significant strain specificity of the immune protective capacity (Table 2).

The results emphasize the variation observed earlier [7] where only a few strains were tested. The mice are arbitrarily subdivided into three groups based on their titration end point (see legend of Table 2). All standard C57B1 strains, as well as strains 129/J, CBA/J and A/J, were protected by low quantities of serum (10 µl) including strains congenic at H-2 (*) and Fv-2 (**).

At the opposite end of the spectrum were mouse strains which were not protected by at least ten (AKR) or more than 30 times (BALB/cJ) the quantity of serum used to protect C57B1/6J. Both Fv-1^n (NIH) and Fv-1^b (BALB) mice were members of this group, indicating no relationship to the Fv-1 gene. A variety of strains, however, could be protected by intermediate quantities of serum (group II).

Some conclusions can be drawn from this analysis which indicates that protection did not seem to correlate with the H-2 type or with two well-known viral markers. Moreover, the variations observed cannot be explained by a difference in growth rate of the tumor since at the dosage given (10^5 tumor cells), the tumor was uniformly lethal within very nearly the same time period after administration in all strains tested.

Genetic Analysis of the AD755a System

The strain variation observed could be due to a number of factors including several immunological and virological parameters. Since some of the loci controlling these factors are known, a genetic analysis of the system might

Table 2. Strain analysis for protection against the AD755a tumor

Mouse strain	*Minimum protective volume (μl)*	
C57B1/6J	10	
C57B1/6J (male)	10	
C57B1/6By	10	
B6.C-H-2^{d}/By [a]	10	
B6.C-H-7^{b}/By [b]	10	
C57B1/10J	10	
B10.A/SgSn [a]	10	
B10-H-2^{a}-H-7^{b}Wts[a]	10	I
B10.129(21M)/SnJ	10	
B10.D2/nSn[a]	10	
C57B1/KsJ	10	
129/J	10	
CBA/J	10	
A/J	10	
B10.Br/SgSn [a]	35	
C3H/HeJ	35	
C3H/HeJ (male)	35	
C3H.SW/Sn [a]	50	
DBA/2J	50	
SJL/J	50	
C58J	50+	II
RF/J	50+	
NZB/BINJ	50+	
STU	50+	
P/J	75	
SEC/1ReJ	75	
DBA/1J	>75	
NIH/Sw	>75	
NIH/Sw (Nu/Nu)	>75	
PL/J	>75	
RII/2J	>75	
SM/J	>75	III
BUB/BnJ	>75	
BALB/c By	>75	
CD-1/Cr	>100	
AKR	>100	
BALB/cJ	>300	
BALB/cJ (male)	>300	

[a] H-2 congenics
[b] Fv-2 congenics

1. Unless indicated, females were tested
2. Same serum pool used in all strains tested
3. Strains titrated at 0, 10, 20, 35, 50, 75 or 100 and 300 μl where indicated. Four mice tested at each level

provide some clues as to the significance of this variation, and even illuminate the nature of the mechanism of protection itself. Using C57B1/6J (B6) (protected [P] at 10 μ) and BALB/cJ (C) (not protected [–] at 300 μl) mice, we carried out classical mating crosses F_1, F_2, F_1 backcrosses) to establish the number of genes involved in the phenomenon and their penetrance (dominant or recessive).

F_1 Generation

Both F_1 breeding combinations (C × B6), and the reverse (B6 × C) indicated that an intermediate quantity of serum (~75 µl) was required for protection of the F_1 hybrids and that there was no sex-linked effect (Table 3). The fact that these animals could be protected excludes the presence of a single dominant locus from BALB/cJ mice which blocks protection.

Table 3. Protection of parental and F_1 mice by immune serum

Mouse strain		*Minimum quantity of protective serum*
C57Bl/b	Parental	15 µl
BALB/cJ		>300 µl
(B6 × C) F_1		75 µl
(C × B6) F_1		75 µl

F_2 and F_1 Backcross Progeny Testing

The results of the second generation cross (C × B6) involving 313 mice, where protection was assayed at 15 µl of serum, are shown in Table 4. Since 22,4%, or one quarter of the mice resembled the B6 parent, this strongly suggests that a single locus is involved in the process. Studies to determine the percentage resembling the BALB/cJ parent confirm this notion (Table 4). For this study, 100 µl of serum was used; a quantity capable of protecting all F_1 hybrid mice and animals resembling the B6 parent, but not capable of protecting animals resembling the BALB/cJ parent. The observed values for the individual F_1 backcross generations are also in close agreement with the expected values for a single operative locus. A diagrammatic representation of the various crosses is presented in Fig. 1.

Table 4. Protection of F_2 and F_1 backcross (BC) progeny A: By 15 µl of immune serum

Cross	*Number of mice*	*Percent protected*	*Percent expected for one locus*
(C × B6) F_2	313	22,4	25,0
(B6 × F_1) BC	50	42,0	50,0
(F_1 × C) BC	52	0	0
B: By 100 µl of immune serum			
(C × B6) F_2	51	68,6	75,0

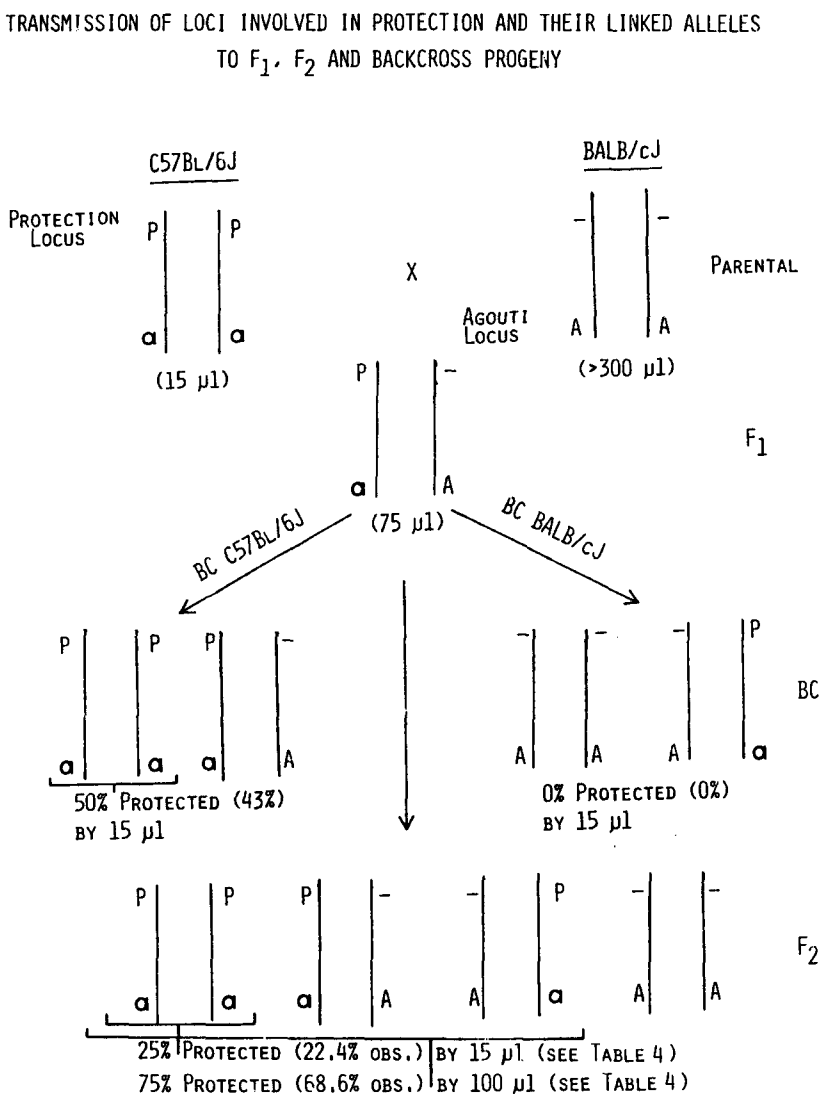

Fig. 1. Diagrammatic representation of genetic crosses between C57Bl/6J and BALB/cJ mice and their offspring demonstrating the putative relationship between loci associated with protection and that of the agouti coat color

Linkage Studies to Determine the Position of the Locus Controlling Protection

During the course of these experiments, we noticed that certain alleles (agouti) controlling coat color might be linked to the protection locus. In crosses between BALB/cJ and C57Bl/6J mice, multiple genes located on separate chromosomes determine coat color [11]. The a locus confers the agouti characteristic which is represented by hair with a yellow banding. The locus is dominant except in albino mice where melanin is not produced. The C57Bl/6J parents are black mice, homozygous nonagouti (aa), while BALB/cJ mice are white (no melanin) but homozygous at agouti for the wild-type allele (AA).

Analysis of the (C × B6) F_1 to the B6 parental type backcross generation at the 15 µl protection end point in relation to coat color yields the results

outlined in Table 5. Assuming no linkage with any color marker, one would expect that the percentage of mice protected in the agouti and nonagouti categories would be the same as the overall percentage protected (ideally 50%, 43,4% experimentally). On the other hand, a close linkage to agouti should result in no protection for agouti mice since these would resemble the BALB/cJ parent; and complete protection of nonagouti animals (like B6 parents). The observed percentages represent significant deviations from either idealized situation suggesting a definitive, but distant, linkage of the protective function to the agouti allele. Similar analyses of the F_2 generation support the linkage to the agouti allele (data not shown).

Table 5. Linkage of protective function to agouti in the backcross of (C × B6) F_1 to B6 parental type

	Agouti	*Nonagouti*	*Total*
Number tested	70	36	106
Number protected	16	30	46
Percent protected	22.9	83.3	43.4
Percent expected without linkage	50.0	50.0	50.0
Percent recombination	$\frac{16 + (36 - 30)}{106} \times 100 = 20.8$		

Both agouti mice which are protected and nonagouti animals not protected represent recombinants and from the frequency of recombination (20,8%, Table 5), one can tentatively place the allele associated with protection about 21 map units from the agouti locus. A similar analysis of the F_2 generation is in close agreement with this conclusion (data not shown). Since the agouti locus has been mapped, the protective function is located in chromosome 2, linkage group V. Our preliminary data employing recombinant inbred strains suggests that the function maps toward the centromere from agouti (unpublished observations).

The Specificity of Protection is Dictated by Virus Associated Antigens

The mechanism by which AD755a tumor challenge is rejected and the identity of the antigens involved in the induction of immune transfer capacity remain to be defined. Possibly relevant to these questions were the observations of Brandes and Groth [1] that virus particles were present in both the solid and ascites form of Adenocarcinoma 755. We have recently demonstrated that this agent, termed ADV (Adenocarcinoma-755a virus) is a type-C virus closely related to the Friend and Rauscher murine leukemia agents [3]. Moreover, the B6 anti-AD755a serum could neutralize ADV and viruses of the FMR (Friend-Moloney-Rauscher) group, possessed a high antibody titer in radioimmunoassays with the major glycoprotein of Friend virus (gp71) and effectively lysed AD755a tumor cells or murine cells infected with Friend virus.

Indeed protection by this antiserum seems to correlate with an antigen associated with FMR viruses. Some of this information is summarized in Table 6. We have observed complete cross protection with universal tumors which likewise express FMR antigen, but none with tumors expressing unrelated viral antigens or no known viral antigens. Of considerable interest is that introduction of FMR viral antigens on a non virus-producing (NP) tumor (the Harvey sarcoma virus-induced C57B1 sarcoma [C57B1 (MSV HA)]) now renders this tumor ([C57B1 (MSV HA) FLV]) susceptible to rejection by an AD755a immune mouse.

Moreover, it has been possible to immunize mice with intact or disrupted Friend virus against challenge with AD755a. The viral specificity extends even to this parameter since AKR virus was not able to immunize against the tumor (Table 6).

Table 6. Specificity of protection seems to correlate with oncornavirus associated antigens

	Associated virus type
1. *Complete* cross-protection seen between AD755a and:	
S-180a	FMR
EAC	FMR
C57B1 (MSV HA) FLV	FMR
2. *No* cross-protection seen between AD755a and:	
6C3HED	Gross – AKR
EL-4	Gross – AKR
C57B1 (MSV HA) NP	None
3. Immunization against AD755a was possible with:	
Intact FLV	
Disrupted FLV	
But not with:	
Intact AKR	
Disrupted AKR	

Attempts to Achieve Protection Against AD755a Tumor Challenge with Anti-Viral Antisera

The concordance between protection and the presence of FMR viruses suggested that a viral component might represent the target antigen on the tumor surface. The most likely candidate for this was the major surface glycoprotein of the virus, not only because of its strategic location but also because the anti-AD755a serum possessed a high antibody titer against this virion component. Thus, it was reasonable to attempt protection with hyperimmune antisera to gp71 or whole virus. These antisera had anti-gp71 titers equal to or in some cases, much greater than those of anti-AD755a antisera.

The results of these studies (Table 7) were disappointing, however, since *none* of these antisera were able to mediate protection. Moreover, absorption of the protective anti-AD755a antiserum with Friend virus or purified gp71 under conditions where all of the anti-gp71 activity is removed, had no effect on its ability to reject AD755a tumor challenge.

Table 7. Effectiveness of anti-tumor vs. anti-viral antibodies in abrogation of AD755a tumor growth

Antiserum	*Specificities*	*Prevention of tumor growth*
Anti-AD755a	Anti-Tumor, Anti-Virus	+
Anti-AD755a abs. with FLV	Anti-Tumor	+
Anti-AD755a abs. with AD755a cells	?	+
Anti-FLV, anti-FLV gp71	Anti-Virus	–

Attempts to Identify a Non-Structural Viral-Induced Antigen Associated with AD755a Tumor Cells

The results presented in the previous section could be interpreted to signify the presence of a non-structural virus-associated antigen which was responsible for induction of the transferable protective antibody population, as well as serving as a target antigen. Such virus-induced non-structural proteins have been identified on MLV-induced YAC lymphoma [5,6,8] and on feline leukemia and sarcoma cells [9]. A direct attempt to immune precipitate an analogous component from surface labeled AD755a cells with various antisera was carried out. As is shown in Fig. 2, the major component precipitated from surface iodinated AD755a cells with both mouse anti-FLV (Fig. 2A) and mouse anti-AD755a (Fig. 2B) antisera is represented by gp71. In fact, except for a minor component of about 45000 MW, no other distinct molecular species are evident in the anti-AD755a antiserum immune precipitates. That the major component represents gp71 is substantiated by its nearly quantitative removal subsequent to absorption of the antiserum with purified Friend virus (Fig. 2C). Although a few minor components remain after this absorption, the results indicate that most of the reactivity of the anti-AD755a serum against iodinated species on the tumor cell surface is directed toward gp71. Similar results were obtained when the cell surface glycoproteins were labeled with galactose oxidase (data not shown). Thus these results indicate that antigens other than gp71 cannot be identified using these procedures. However, a second antigen could be either inaccessible to external labeling or inactivated following disruption of the cells prior to immune precipitation. Alternatively, gp71 may indeed represent the only relevant antigen involved in production and binding of protective antibody, as discussed below.

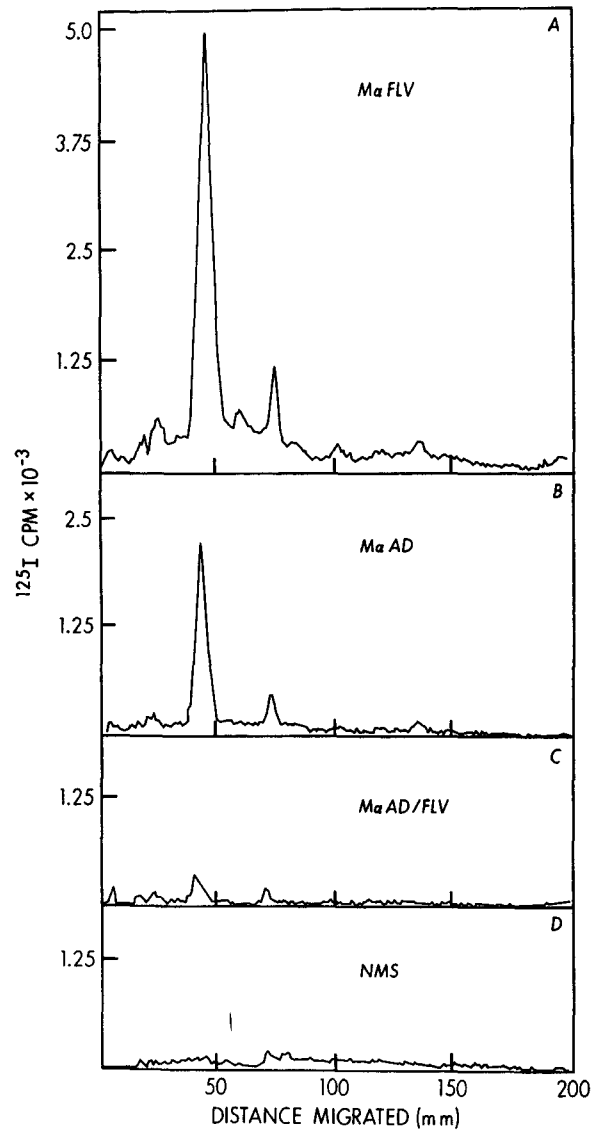

Fig. 2. Analyses of ^{125}I-labeled AD755a cells after immune precipitation with various mouse sera followed by electrophoresis on SDS polyacrylamide gels. (A) MαFLV (B6 serum raised against purified Friend leukemia virus); (B) MαAD (B6 anti-AD755a serum); (C) MαAD/FLV (B6 anti-AD755a serum absorbed with FLV); and (D) NMS (normal mouse serum)

Protection Appears to Require a Cytophilic Antibody

An observation which was made early in this study which bears heavily on the mechanism of protection is that sequential absorption of the hyperimmune anti-AD antiserum ten times with fresh AD755a cells *at its titer end point* had no effect on the protective capacity of the serum (see Table 7). Moreover, although antibodies absorbed to the cell surface under conditions of great excess could yield lysis in the presence of added complement; they were unable to provide protection when these antibody coated cells were inoculated as tumor challenge. Thus, the protective function was not easily absorbed by the target cells.

Along with the inability to directly absorb the protective serum with the target cell, the potent protective capacity of small volumes of the immune serum, as well as its apparent strain specificity, may be indicative of a cellular component in the passive serum transfer process. Phase contrast microscopic analysis of AD755a cells after interaction with the B6 immune serum and

normal B6 peritoneal exudate cells, both in vivo and in vitro, demonstrated the induction of large mononuclear cell attachment to the tumor cells (Fig. 3). This response was not observed when normal B6 mouse serum was substituted and suggests that the B6 anti-AD755a serum may be capable of activating macrophages or other mononuclear cells for tumor cell destruction. It is of interest in this regard that antisera directed against FLV or FLV-producing cells which are not protective, also do not have the capacity to form rosettes in vivo. It is thus possible that the protective factor is a cytophilic antibody with affinity for a host effector cell, presumably a macrophage. Because of the previously described viral specificity of the tumor rejection process, we tentatively postulate that a viral component remains the principal candidate for the target antigen, but that its function in this regard can only be demonstrated through a cooperative action between the antibody and the appropriate effector cell. Studies are planned to identify this component and the corresponding antibody population through in vitro and in vivo assays involving effector cells.

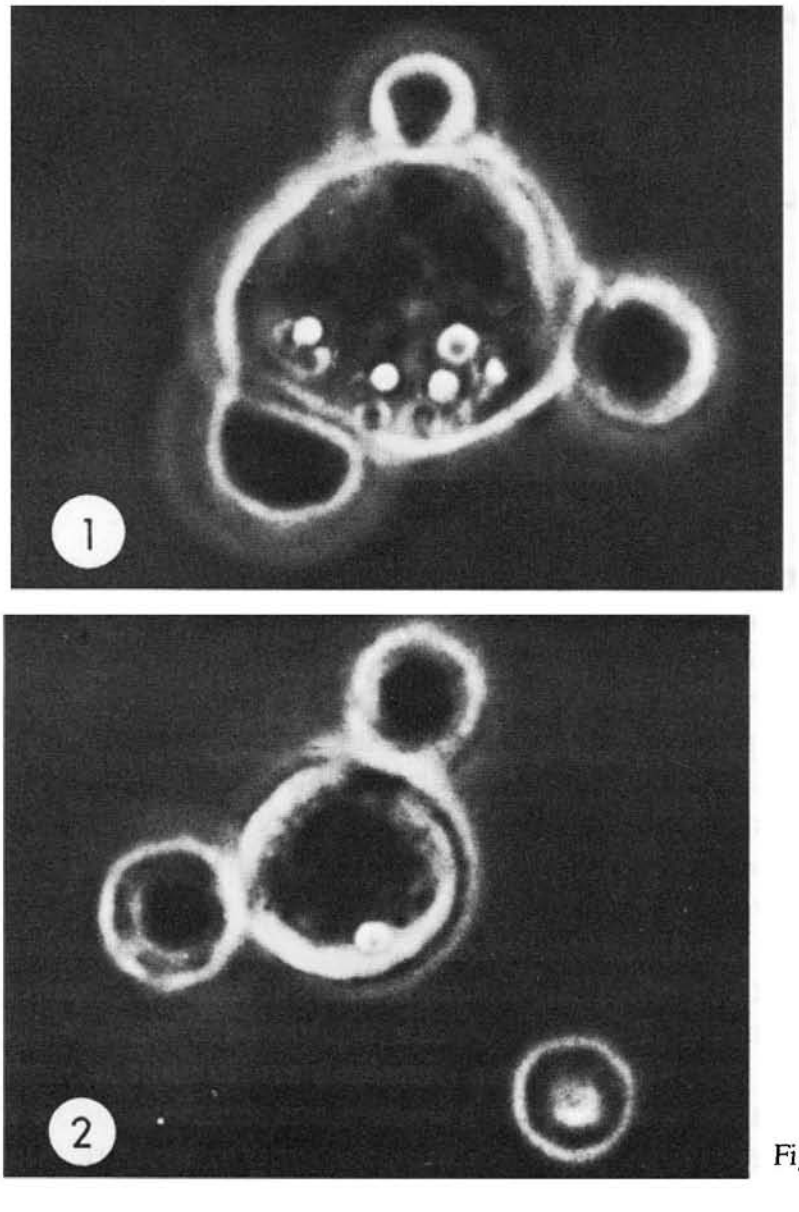

Fig. 3

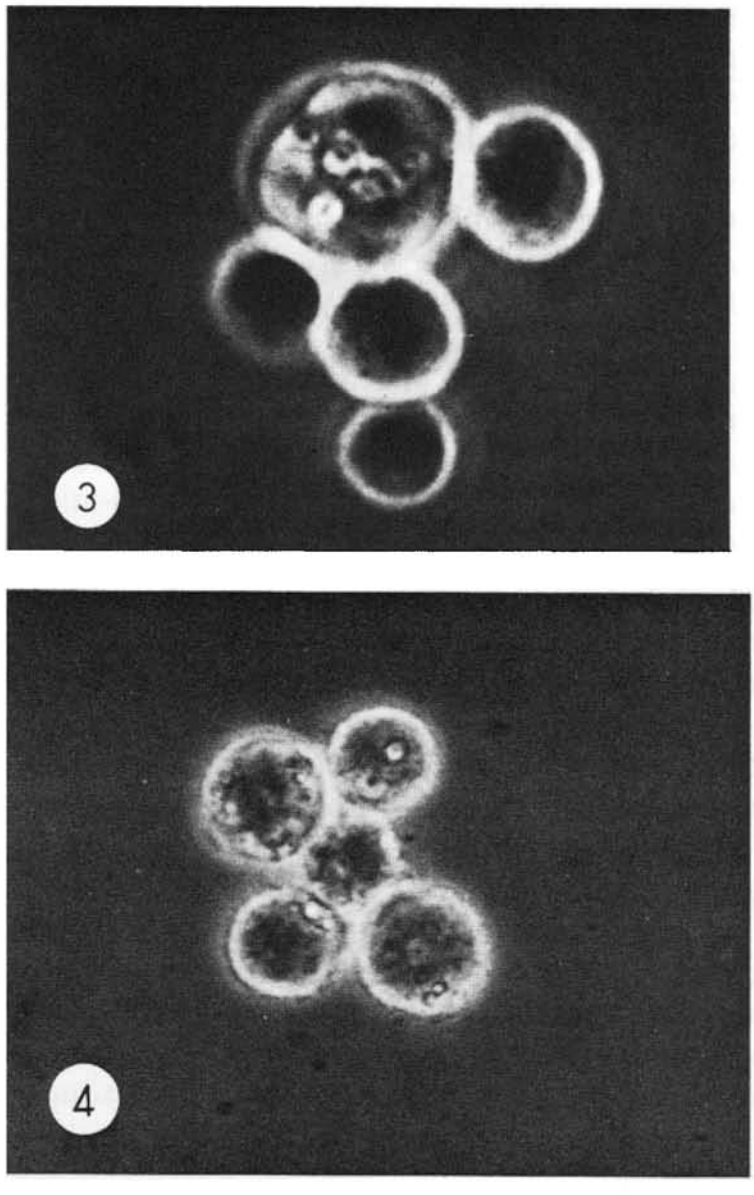

Fig. 3.1–4 Phase-contrast microscopic analysis of mouse peritoneal aspirates collected 2 hr (1). 3 hr (2) or 6 hr (3) after ip inoculation of AD755a cells and B6 mouse anti-AD755a serum. Note that tumor cells (large granular cells) are bound to large mononuclear cells. Similar analyses were carried out in vitro by mixing AD755a cells. normal B6 mouse peritoneal exudate cells and B6 mouse anti-AD755a serum for 1 hr (4). Note binding of tumor cells to a large mononuclear cell in a rosette formation. reverse of binding pattern seen in vivo (see 1–3). This rosette reaction was not observed when normal B6 mouse serum was substituted for immune serum. ×400

Concluding Remarks

In summary, AD755a can be used as an animal model system of tumor rejection that involves lymphoid cell and serum factors. This system can serve in the examination of the immune recognition and immune response mechanisms participating in tumor rejection, as well as in the study of mouse strain-specific interactions between serum factors and lymphoid cells, which may possibly mediate the observed transfer of tumor immunity.

The strain dependence of serum transfer protection was found to be controlled by a locus linked to agouti which itself is situated on linkage group V, chromosome 2. No function has been mapped in this region which could account for the phenomenon observed suggesting that the locus controlling protection by serum transfer is a new discovery. Extensive fine mapping using appropriate congenic and recombinant inbred strains is in progress to better establish its location.

Several parameters thus far noted in this system may reflect the function of this locus. These include

1. genes controlling various activities related to the virus associated with the AD755a tumors, particularly those which might affect the immune system of the recipient; or 2. immune response functions which regulate the cooperative effects between the protective antibody and the host effector cells, which might include factors such as Fc receptor specificity for the protective antibody.

The question of antigenic specificity remains elusive. The data obtained thus far strongly suggest a viral related component as the antigen involved in both the generation of protective antibody and as a target for tumor rejection. The best candidate for the antigen at present is the gp71 surface glycoprotein of ADV. However, we would have to postulate that two forms of antibody to this antigen are produced during our immunization procedure. One is a classical antibody and can be measured in virus neutralization, cytotoxicity and radioimmunoassay analyses and can be efficiently absorbed with virus or target cells. The other is an antibody with little or no affinity for the target cell. In the presence of both lymphoid cells and target cells, however, this antibody induces rosette formation, linking the target and effector cells very efficiently. Antibodies with similar functions have also been described by Haskill and colleagues [12, 13]. Studies are now in progress to determine the nature of this antibody subclass and the mechanism by which it induces rosette formation.

An alternative explanation is that our immunization protocol results in the formation of an anti-idiotype antibody. Such antibodies can actively stimulate the host immune response in a very specific fashion [4], particularly if administered in the presence of antigen [2, 10]. Thus, their existence would explain both the potency and specificity of the protective function, as well as the relative inability of the protective factor to bind to the target cell. Studies are in progress to determine if such antibodies are present and their relationship to the viral gp71 antigen.

Having noted the powerful protective function of antiserum prepared as described against AD755a tumor challenge, we have prepared similar antisera against other murine tumors. Such sera also demonstrate strong antitumor effects, particularly against virus associated sarcomas (see Table 6). Application of this principle to sarcomas in cats induced by the Snyder-Theilen strain of feline sarcoma virus caused a dramatic regression of lethal tumors in 8 of 9 cats at a relatively late stage of tumor growth, where the primary tumors were more than 7 cm in diameter and the animals were near death. Studies to determine whether similar factors are involved in this form of tumor rejection are also in progress (de Noronha and Bolognesi, in preparation).

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The Search for Infectious Viral DNA in Human Leukemic Cells

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That human leukemia has a viral origin has been suggested many times (see [6] for references). Leukemic cells have been shown to bear intracellular virus-like particles [1,4], to share nucleotide sequences with murine [1] or primate [4] viruses, and to synthesize proteins that cross-react with antibodies to virus-specific proteins [18]. Some authors reported the release of C-type particles from leukemic cells subjected to in vitro culture conditions [11,12,13,16], and Gallagher and Gallo [3] have shown budding C-type virus in leukemic cells sustained proliferating in vitro with medium conditioned by a particular human embryonic cell culture. This virus, now called HL23V, was further studied and found to be infectious for a wide variety of cells. Viruses growing in these new hosts were referred to as "secondary viruses" [20].

During horizontal transmission animal C-type viruses are known to synthesize a provirus composed of double-stranded DNA of about 6×10^6 daltons which, because of its infectivity, can be conveniently detected in a transfection assay (see [9] for review). Viral genetic information, endogenously carried in the cellular chromosome, is usually not infectious ([2], for exceptions see [17]). In human leukemia an infectious provirus should be found if the disease arises from a horizontal spread of a leukemia virus. We report here on our search for such a provirus.

In initial experiments [7] the DNA was extracted from secondary HL23V-1 virus-infected bat B88 cells, and used to transfect both human rhabdomyosarcoma A204 and bat B88 cell cultures. Transfection assays were carried out using calcium phosphate [5] or DEAE-dextran [8] techniques. In these assays 20 to 50 μg DNA was delivered per about 10^7 recipient cells. The cells were then kept growing for about 1 month and assayed for reverse transcriptase activity in the culture medium. Four out of ten B88 and none out of five A204 cultures were found to produce C-type virus. Reverse transcriptase neutralization tests performed by Robert E. Gallagher have shown that viruses recovered in these transfections resemble simian sarcoma virus and baboon endogenous virus to approximately the same extent as does the parent HL23V-1 virus. We concluded that after horizontal transmission, the HL23V isolate synthesizes an infectious provirus. This was later confirmed by others [14].

In order to detect such a provirus before the rounds of virus replication in vitro, the DNA was extracted from fresh-frozen uncultured leukemic leukocytes from the HL-23 patient, and also from patients HL-7 with chronic myelogenous leukemia and HL-11 with chronic monomyelocytic leukemia.

Wong-Staal et al. [21] showed by molecular hybridization that leukemic tissues in these patients, unlike normal human tissues, contain baboon endogenous virus-specific DNA sequences suggesting that horizontal transmission of a primate virus had occurred among humans.

The results of transfection assays performed with these DNAs are shown in Table 1. It is clear that no infectious virus could be isolated. Some DNA-treated cultures, however, exhibited a transient burst of DNA polymerase activity in the culture medium. This is demonstrated in Fig. 1. To reproduce the burst, about 107 days after the first DNA treatment the cultures N° 38 and 39 and N° 44 and 45 were treated again with HL-7 and HL-11 DNA, respectively. Two control cultures received normal human foreskin DNA. This time the DNA treatment was repeated 17 times during 20 cell passages and 20 μg DNA was delivered per culture of 10^7 cells at each treatment. Reverse transcriptase activity was assayed weekly as in Fig. 1 and found to be within the background limits.

In further experiments HL-23 DNA (20 μg/10^7 cells) was assayed on feline embryo cells and also in a mixture of these cells and rabbit cornea cells. Transfection assay was carried out using the calcium phosphate coprecipitation technique, combined with the boost of DMSO [19] 4 h or 4 h and 10 days after transfection. Reverse transcriptase assays were carried out in 10 ml of the culture medium weekly for up to 2 months and provided only background counts.

The burst of DNA polymerase activity after transfection is reminiscent of the transient virus replication observed with HL23V-1 virus in various animal and human cells [20]. The burst shown in Fig. 1 may be due to the release of C-type particles. It is not known if these particles were specified by the transfecting DNA. We suspect that leukemic cells carry a defective provirus

Table 1. Transfection assays using DNA from fresh-frozen, uncultured human leukemic leukocytes

Experiment N°	Culture N°	Source of DNA (patient)	Recipient cells	μg DNA per culture	Reverse transcriptase assay	Number of passages after transfection
I	31–35	HL-7	B88, bat lung	50	negative	7
IIa	36–40	HL-7	SIRC, rabbit cornea	40	burst in N° 39	17
	41–45	HL-11	SIRC, rabbit cornea	40	burst in N° 45	17
	46–50	HL-23	SIRC, rabbit cornea	18	burst in N° 47	17
III	53–55	HL-23	A7573, dog thymus	20	negative	16
	56–58	HL-23	FEF-1, feline embryo	20	negative	lost at passage 5
IV	59–63	HL-23	FEF-1, feline embryo	40	negative	25

DNA was extracted from cells according to a modified Marmur's procedure ([8, 15], and unpublished data) and then administered to about 10^7 recipient cells using the Ca phosphate transfection technique [5]. The cells were passaged once weekly. Before each passage 10 ml of the culture medium were withdrawn, clarified at 10,400 g for 30 min, and the particulate fraction spun down in a Spinco Ti50 rotor at 38,000 rpm and 4°C for 60 min. The pellet was assayed for reverse transcriptase activity as described [10].

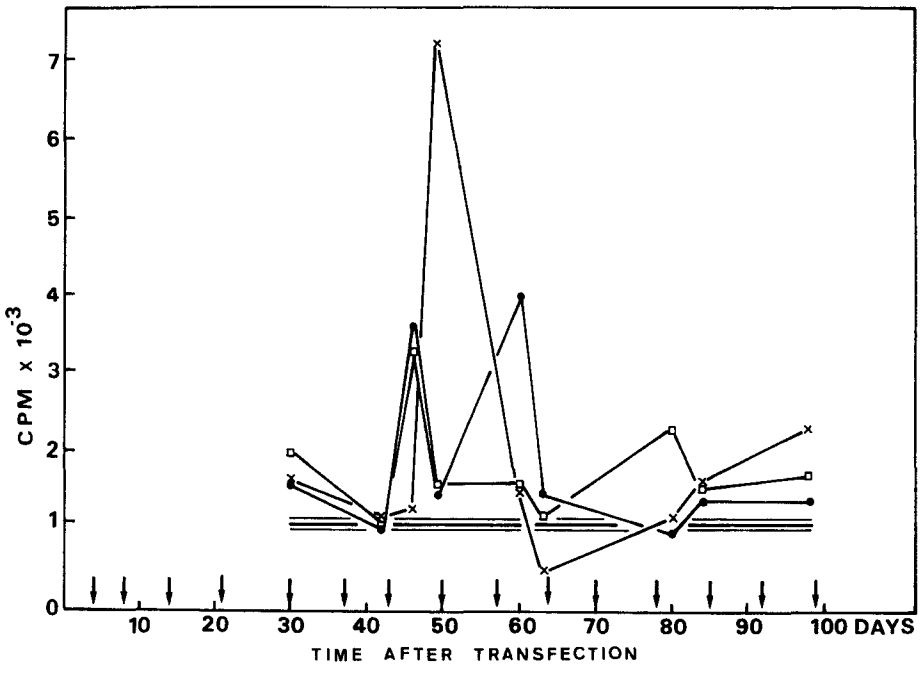

Fig. 1. Reverse transcriptase assay (given in cpm of incorporated ^{3}H-TMP) of the culture medium of DNA-treated cultures N° 39 (×), 45 (□), and 47 (●) at different times after transfection. Mean background counts and the standard error of the mean are given by a thick and two thin straight lines, respectively. Cell passages are indicated by arrows. Other conditions are described in Table 1

which gives rise to a poorly replicating virus in transfected cells. In this context the results of the last experiments would show that different procedures such as repeated transfections, co-culture with different animal cells, or DMSO boosts are all unable to render this virus fully infectious.

Other possibilities could not be eliminated. For instance, (i) no virus is recovered after transfection if the DNA used contains too small an amount of infectious provirus. In infections initiated by avian sarcoma virus in virus-resistant chicken cells no infectious provirus is detected when the virus replicates in less than one per 10^4 cells. (ii) The fact that endogenous proviruses are usually noninfectious [2] suggests that genetic elements of the endogenous provirus are located on different chromosomes or separated by spacers large enough to render the provirus inefficient in transfection. If this is also true of the human leukemia provirus, C-type particles may be produced after splicing in leukemic cells, but not after transfection of leukemic cell DNA.

In conclusion, transfection assays so far performed failed to detect infectious provirus in human leukemic cells. A possibility remains, however, that these cells carry a defective provirus or a provirus composed of more than one genetic element unable to generate, upon transfection, an infectious virus.

Acknowledgments

HL23V-1-infected bat cells as well as fresh-frozen leukemic leukocytes from HL-23, HL-7, and HL-11 patients were kindly provided by R.C. Gallo. Rabbit cornea, dog thymus, and feline embryo cell lines were obtained from P.D. Markham. This work was supported in part by D.G.R.S.T., Contract N° 76.7.1656.01.

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Infection of Mouse Bone Marrow Cells with Abelson Murine Leukemia Virus and Establishment of Producer Cell Lines

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We have recently described a technique for the establishment of long-term cultures of mouse bone marrow cells (Dexter et al., 1977). In that communication we reported the successful infection of the cells in vitro with the Friend leukemia virus complex and the subsequent development of producer cultures of the Friend spleen focus-forming virus. Here we describe the infection of the mouse bone marrow cultures with Abelson murine leukemia virus (MuLV-A) (Abelson and Rabstein, 1970); establishment of lines of primitive "blast" cells which chronically produce MuLV-A in vitro has been accomplished and a proportion of these cells contain intracytoplasmic immunoglobulin M (IgM). Thus these transformed cells can be classified as early B lymphocytes.

MuLV-A causes a thymus-independent rapidly progressing lymphosarcoma in adult mice (Abelson and Rabstein, 1970). Viral infection in vitro and in vivo leads to the production of tumours which can be classified as null (non-B, non-T) cell tumours. If mice are pre-treated with the mineral oil pristane, there appears to be a high incidence of immunoglobulin-producing (B cell) plasmacytomas (Potter et al., 1973; Sklar et al., 1974; Rosenberg et al., 1975). Previous reports of in vitro transformation of splenocytes (Sklar et al., 1974), foetal liver (Rosenberg et al., 1975) or bone marrow (Rosenberg and Baltimore, 1976), documented a requirement for a reducing agent such as 2-mercaptoethanol for the establishment of virus-producing cell lines; in our system, this requirement is obviated.

Bone marrow cultures from 6–8 week old female BALB/c mice were established from femoral marrow aspirates suspended in Fischer's medium with 20% horse serum. After 3 weeks in culture, with weekly withdrawal of and replacement of one-half of the medium, a second aspirate of syngeneic cells was added to each culture. Approximately 2 hours later, 7×10^4 plaque forming units (pfu) of the Moloney MuLV-M helper virus and 2×10^3 focus forming units (ffu) of the MuLV-A were added to each experimental culture. All cultures were then incubated at 37° with weekly demi-depopulation and addition of fresh medium. The cells and media removed each week were assayed by several techniques to ascertain the virus status and the nature of the cell population. Two assays were used to determine virus production: 1. the XC syncytial plaque assay (Rowe et al., 1970) for quantitation of the MuLV-M helper virus population and 2. the focus assay (Hartley and Rowe, 1966) on NIH/3T3 cells for measurement of the transforming capacity of the defective

Abelson virus component (Scher and Siegler, 1975). After the first week in culture, the titre rose considerably (to $> 10^5$ pfu/ml) and thereafter remained constant. As expected, the MuLV-M was consistently found to be in excess (3–400 fold) of the MuLV-A.

The numbers and types of cells maintained in the suspension fluids were analysed by several criteria (Table 1). The cultures showed an initial decrease in total cell number after one week post-infection. In uninfected cultures, the cell number remained relatively constant throughout the course of the experiment. On the other hand, the MuLV-A infected cultures showed a dramatic increase in total cell number at 5 weeks. At about the same time, the cell population in the infected cultures showed a predominant shift toward undifferentiated blast cells and a decline in the proportion of granulocytes (of all stages) and mononuclear macrophages. This is in marked contrast to the control cells in which the predominant population was composed of mononuclear cells by week 7.

Table 1. Differential analysis of bone marrow cell cultures

Weeks in culture	Culture	Morphology (%)		
		Blast	Granulocyte	Mononuclear
3	Control	36	34	30
	Infected	48	12	37
5	Control	41	5	54
	Infected	96	0	4
7	Control	0	0	100
	Infected	99	0	1
9	Control	0	0	100
	Infected	100	0	0

Two assays for analysis of stem cell differentiation were also utilized. The agar colony assay for granulocytic cell precursors (CFU-C) (Bradley and Metcalf, 1966) was performed according to the technique of Dexter and Testa (1976). By 4 weeks, neither control nor infected cells formed CFU-C. However, by the time that the infected cells demonstrated feeder-independent autonomy (see below) at week 15, several thousand cells per culture were capable of growing as agar colonies. These differed from normal CFU-C in forming tightly clustered colonies composed of undifferentiated blasts with no evidence of granulopoiesis. In addition, there was little or no decrease in the number of "CFU-C" in the absence of granulocyte colony stimulating factor (CSF). Furthermore, such CSF-independent colony forming cells could be found in infected cell cultures by week 6 if mercaptoethanol (5×10^{-5} M) was present in the agar medium. These data indicate that the cells are not true CFU-C and represent blast cells which have been "transformed" by viral infection, thus acquiring the ability to replicate in an agar medium.

Pluripotent haematopoietic stem cells (CFU-S) (Till and McCulloch, 1969) were lost early during the culture period.

At the earliest period checked (one week post-infection), the MuLV-A infected cells were capable of inducing leukemia within 2–4 weeks in adult syngeneic BALB/c mice and in heterogeneic BDF_1 mice as well. The leukemias were characterised by massive enlargement of the spleen and of most lymph nodes (cervical, inguinal, brachial, axillary and mesenteric). The leukemic cells were identified morphologically as large, undifferentiated blast cells. Of particular interest was the finding that these cells lacked both θ and surface Ig antigens by immunofluorescent staining techniques.

At 15 weeks, the cultured bone marrow cells appeared to be autonomous and were capable of forming agar colonies in the absence of 2-mercaptoethanol. Thereafter the cells exhibited an increased ability to grow in suspension without feeder cells. The cell lines thus established were uniform in morphology and appeared to be large vacuolated, undifferentiated blast cells.

Although the cells appear to be of the lymphoid series, they do not show the typical identifying markers of T (θ antigen) or B (surface Ig) cells and are perhaps best classified as null cells. As cells infected with Abelson are thought to be pre-B lymphocytes, we attempted to characterise the nature of the established cell line. It was found that 2–5% of the cells contained intracytoplasmic IgM as identified by immunofluorescence techniques. We attempted to induce lymphoblastoid differentiation by a variety of compounds. As shown in Table 2, the proportion of cells with intracytoplasmic IgM was greatly stimulated by treatment with dimethyl sulphoxide (DMSO) or lipopolysaccharide (LPS). No treatment has yet been found to stimulate the production of surface Ig.

Table 2. Stimulation of intracytoplasmic IgM production

Treatment	% Cells staining for	
	Intracytoplasmic IgM	Surface Ig
None	1–4	0
Lipopolysaccharide		
5 μg/ml	23	0
20 μg/ml	39	0
Dimethyl sulphoxide, 1%	23	0
Dextran sulphate, 20 μg/ml	4	0
Tuberculin PPD, 10 μg/ml	2	0

We thus conclude that Abelson virus infection of bone marrow cells has led to the transformation of cells in the early B-lymphocyte lineage. In the uncloned population, there are two cell populations: One is IgM-negative and another which constitutively produces IgM. The experiments using DMSO and LPS suggest that such treatment can either cause preferential proliferation of the IgM-positive cells or induce some of the IgM-negative cells to synthesize IgM. Attempts are now being made to select clones from each of

these cell types. However, this provides the first proof that Abelson virus can transform cells of the B-lymphocyte series in vitro and suggests that such cells may be the target for leukemogenesis in vivo.

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Cocultivation as a Tool for the Detection of Oncoviruses in Childhood Leukemia*

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I. Introduction

In our study on the possible role of type-C oncoviruses in human leukemia we applied the technique of cocultivation of human bone marrow cells with an animal indicator cell line. The cocultivation technique proved to be very useful, for instance, for the isolation of endogenous primate viruses (Todaro et al., 1978). When bone marrow of a leukemic child was cocultivated with the rat XC cell line, a type-C virus was readily detected which was related to the simian sarcoma-virus (SiSV) (Nooter et al., 1975). The use of the XC cell line has been abandoned by us because of the pronounced cytopathic effect of primate viruses in this line and the danger of activation of the endogenous rat virus.

We changed to the dog thymus cell line A7573, which is known to be permissive for SiSV-related viruses (Teich et al., 1975), without a cytopathic effect. Till now, no retrovirus has been induced in this and any other canine cell line. We already reported detection of type-C viruses in bone marrow samples from two leukemic children in this assay system (Nooter et al., 1977). We here describe our results on a relative large scale study of cocultivation of cells and human BM samples. SiSV cross-reacting antigens could be detected in some of these cocultures by means of the indirect cytoplasmic immunofluorescence assay (IFA).

II. Characterization of Antisera

Two rabbit antisera were used in this study: one directed against the p30 of SiSV and one the p30 of Rauscher murine leukemia virus (R-MuLV).

In cytoplasmic IFA antiviral antisera, which have not been absorbed for anti-FCS activity, react with every cell line tested. After FCS absorption, the antiserum to SiSV-p30 gives a strong reaction (endpoint titer 1:320–640) with cultures infected with SiSV and a weakly positive reaction with cultures releasing R-MuLV (endpoint titer 1:20–40) (Table 1). No reaction was found with cultures producing bovine leukemia virus. The RA-MuLV-p30 antiserum gave a considerably stronger reaction with cells producing R-MuLV

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Table 1. Immunofluorescence endpoint titers of control cell cultures

Control cell cultures	Antisera	
	RA-SiSV-p30	RA-R-MuLV-p30
Virus positive:		
REF + SiSV[1]	320–640	20– 40
BALB/3T3 + R-MuLV[2]	40– 80	160–320
BoEF + BoLV[3] (N = 3)	10	10
Virus negative:		
animal:		
BALB/3T3. REF. R1–69[4]. SIRC[5]. A7573. FRhL[6]. BoEF (N = 7)	10	10
human:		
skin fibrobl. (7). lung fibrobl. (1) R970[7]. WHE[8]. A204[9]. NC37[10] (N = 12)	10	10

1 primary culture of rat embryonic fibroblasts (REF) productively infected with SiSV.
2 mouse BALB/3T3 cells productively infected with R-MuLV.
3 bovine embryonic fibroblasts (BoEF) productively infected with bovine leukemia virus (BoLV).
4 rat osteosarcoma cell line.
5 rabbit cornea cell line.
6 rhesus monkey fetal lung cell line (Flow Laboratories. Irvine. Scotland).
7 human osteosarcoma cells nonproductively infected with the Kirsten strain of the mouse sarcoma virus
8 primary culture of whole human embryo cells.
9 human rhabdomyosarcoma cell line.
10 human lymphoblastoid cell line.

than with SiSV-positive cells. Neither antiserum reacted with a variety of animal (n = 7) and human (n = 12) cell lines, which do not release a retrovirus. The virus negative lines of human origin included the NC37 cell line, in which SiSV was grown, used for p30 isolation and subsequent antiserum preparation.

III. Detection of SiSV-p30 Related Antigens in Human Bone Marrow Cocultures

After approximately one to two months of culture, in some cocultures of human bone marrow and canine A7573 cells, antigens appear which can be detected with the RA-SiSV-p30 antiserum but not with the RA-MuLV-p30 antiserum. The cytoplasmic fluorescence was of a granular nature. Maximally 60% of the cells stained positive. A few positive cocultures were followed at two-week intervals and it appeared that the positive IFA staining persisted for about 2 months but then gradually declined during the following passages and disappeared completely. The endpoint titer on the positive cocultures never exceeded 1:80.

Altogether in 8 out of 38 cocultures SiSV-p30-related antigens appeared (Table 2). Most positive cases were found in the group of leukemic donors

Table 2. Immunofluorescence endpoint titers of antisera to type-C retroviruses in cocultures of human bone marrow with dog cells

Coculture	Diagnosis	Sex	Age in years	Antisera[a]	
				RA-SiSV-p30	RA-R-MuLV-p30
	Leukemia patients:				
A1	ALL	♂	3	+ (40)	–
A2	ALL	♂	5	–	–
A3	ALL	♂	6	+ (80)	–
A4	AML	♂	8	+ (40)	–
A5	ALL	♀	5	+ (80)	–
A8	AML	♂	66	–	–
A24	CML	♂	31	–	–
A25	AML	♂	70	–	–
A26	CML	♂	76	–	–
A28	ALL	♀	5	+ (40)	–
A30	AML	♂	74	–	–
A31	AML	♂	52	+ (40)	–
A32	AML	♀	73	–	–
A35	AML	♀	46	–	–
A36	AML	♀	9	–	–
A37	AMML	♀	cong.	–	–
A38	ALL	♂	1	–	–
	Non-leukemic patients:				
A6	lymphosarcoma	♀	8	–	–
A7	renal transplantation	♀	44	–	–
A9	aplastic anemia	♂	8	–	–
A15	pyruvate kinase deficiency	♂	39	–	N.T.
A16	pure red cell aplasia	♂	18	–	N.T.
A18	myelofibrosis	♂	68	–	–
A19	aplastic anemia	♀	12	–	N.T.
A20	non-Hodgkin lymphoma	♂	8	+ (40)	–
A23	non-Hodgkin lymphoma	♂	30	–	N.T.
A27	bronchus carcinoma	♂	56	–	–
A29	myelofibrosis	♂	75	–	–
A33	myelofibrosis	♂	72	–	–
	Normal individuals:				
A10	normal	♀	14	–	–
A11	normal	♀	11	–	N.T.
A12	normal	♂	9	–	–
A13	normal	♀	62	–	–
A14	normal	♂	24	–	N.T.
A17	normal	♀	51	–	N.T.
A21	normal	♂	32	–	–
A22	normal	♂	8	+ (40)	N.T.
A34	normal	♀	14	–	–

[a] Fluorescence staining was scored as positive when the reciprocal of the endpoint titer of the antiviral antiserum was 40 or higher. The numbers in parentheses indicate endpoint titers which are expressed as the reciprocal of the highest dilution showing virus specific staining.

(6 of 17). Of these 6 positive cocultures 5 were derived from leukemic children. In the group of nonleukemic patients only 1 of 12 cocultures scored positive. The positive sample came from a child with a non-Hodgkin lymphoma. Only 1 of 9 cocultures, derived from normal bone marrow donors, contained SiSV-p30 related antigens. This sample was derived from an 8-year-old child.

Reproducibility of detection of virus-related antigens in such cocultures was demonstrated in two separate instances. Of two leukemic donors (numbers 1 and 5), which were positive in our assay, additional bone marrow samples which had been stored in liquid nitrogen, were also tested with the cocultivation technique at a later time and found to be positive as well.

IV. Blocking Tests

Specificity of the detection of SiSV-p30 related antigens in the cocultures was demonstrated by the absorption of the reactivity with purified SiSV grown in rat cells and not with purified mouse mammary tumor virus (MuMTV). The positive staining of both SiSV-producing cells and a positive culture was completely blocked after absorption of the RA-SiSV-p30 antiserum with SiSV, while absorption with MuMTV resulted in only a slight decrease in the titer of the antiserum.

V. Reverse Transcriptase Assays

In addition to IFA, 5 of 8 positive cocultures were screened for extracellular reverse transcriptase. These cocultures have been followed for eight months (Table 3). In coculture A1 a small burst of reverse transcriptase was found during the first month. No significant activity was found in the seven following months. When a second coculture was started from the same bone marrow, a similar result was obtained.

Only in coculture A4, which was reinitiated from stored bone marrow, high polymerase activity was found, which persists already for two months.

Table 3. Reverse transcriptase activity of cocultures positive in the IFA

	RT activity after month in culture (cpm × 10^{-3})					
Coculture months	1	2	3	4	6	8
A1 I	*14,7*	0.4	0,9	1.6	1.1	1.7
A1 II	*12,1*	0,3	0.6	1,2	0,8	0,5
A3	0,9	1.8	2.3	0,4	1.7	N.T.
A4 I	1.7	0,4	0.8	1,9	2,3	N.T.
A4 II	N.T.	*116,4*	*236*			
A5	2,5	0.5	1.3	0,5	0,6	N.T.
A22	0,7	0,9	0.1	3,0	N.T.	N.T.

This isolate in which only one cell line is involved, is highly promising for further studies like bioassay as done with SKA21-3, a virus isolated from a leukemic child after a complicated procedure, involving several cell lines (Nooter et al., 1978). This SKA21-3 virus proved to be leukemogenic to rats.

VI. Discussion

In the experiments presented here we detected SiSV-p30 related antigen in 8 of 38 cocultures of human bone marrow samples and the canine cell line, A7573. Our recent results obtained by cocultivation can be explained by intimate contact between the bone marrow cells and the indicator cells, which allow transfer and subsequent propagation of the few virus particles, present in the original bone marrow samples.

Crucial for the detection of viral footprints in these cocultures is the specificity of antisera and immunofluorescence technique. After absorption of immunoglobulin fractions of the sera, no reaction was seen with any animal or human cell line, which do not produce a retrovirus. Sera of bovine origin may be a source of unwarranted contamination with bovine viruses. However. no reactions were found with the p30-antisera on bovine leukemia virus producing fibroblasts. As the SiSV has been grown in a human lymphoid cell line (NC37), it is possible that the purified SiSV-p30 preparation is contaminated with human lymphoid cell antigens. However, absorption of the RA-SiSV-p30 antiserum with SiSV grown in rat cells fully blocked the reaction with the positive cocultures.

All in all, 7 of the 8 cocultures which were positive for viral antigens are derived from children. Five of the 6 positive donors in the leukemic group are children.

From these results emerges an association between childhood leukemia and this virus-related antigen. Coincidentally the two positive cases in the control groups are also children. These results and our earlier studies suggest childhood leukemia to be a highly suitable disease entity for further virological studies.

Our results suggest a transmissible agent to be involved in childhood leukemia. Despite the lack of epidemiological evidence for the infectious nature of childhood leukemia, this virus seems to be horizontally transmitted as man does not have SiSV-related sequences in its normal cellular DNA. The possible sources of virus with regard to transmission are rather restricted: saliva, milk. urine, feces, sperm and placenta. In the cat, the saliva proves to be sole source of leukemia virus (Francis et al., 1977). In gibbons apes, however, the urine was the main source of virus excretion. Milk is the main route of transmission of exogenous leukemia viruses in mice (Law and Moloney, 1961). Obviously. the route of transmission in man is unpredictable.

So far, retroviruses have never been found by means of electron microscopy in human urine and feces. The so-called simultaneous detection test gave repeatedly positive results with regard to retroviruses in human milk (Schlom et al.. 1972). In view of the presence of exogenous viruses in a few

human embryonic cell lines (Panem et al., 1977), it is tempting to speculate that either placenta or sperm are vectors for a putative human leukemia virus. Since SiSV-sequences seem difficult to detect in tumor tissues, it must be assumed that only a selected population contains complete infectious virus.

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Evidence for the Presence of an Oncornaviral Reverse Transcriptase in an Orbital Tumor Associated to Acute Myelomonocytic Leukemia in Children: Biochemical and Immunological Characterization of the Enzyme

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Several reports suggest that human tumors contain a DNA polymerase with biochemical properties resembling reverse transcriptases, associated to RNA tumor viruses [1–6]. However, only in a few cases, these enzymes have been characterized serologically to make it certain, that they really are of viral origin [1–4]. Such studies, in particular, the immunological characterization of reverse transcriptases from human tumors are of great importance in considering the possibility that humans may harbor viruses related to oncornaviruses which induce tumors in non-human primates, or other animal species. For the last few years, it has been our objective to purify the cellular DNA polymerases (α, β and γ) and reverse transcriptases from human tumor biopsies, and characterize them biochemically as well as immunologically [2–4,7,8].

This communication describes the purification and characterization of a reverse transcriptase from an orbital tumor of a child with acute myelomonocytic leukemia (AMML). This tumor has been reported to be associated to AMML in children in some regions of Turkey [9, 10] (see R. Miller elsewhere in this volume). The tumor consists of primitive white blood cells with supportive connective tissue, stroma, and vessels. Since these tumors were devoid of the characteristic green color, and no periosteal or other bone changes were present, Cavdar et al. [9] suggest the term granulocytic sarcoma to be more appropriate than the chloroma. The striking feature is the occurrence of ocular lesions before the onset of AMML varying from 20 days to 8 months [9].

The methods employed in the isolation and purification of reverse transcriptase from the orbital tumor are reported elsewhere [2,3]. The activities of orbital tumor reverse transcriptase at various steps of purification, using different template-primers are documented in Table 1. The single peak of activity eluted from the phosphocellulose column at 0,21 M salt concentration (Fraction V, Table 1), represents a 1340-fold purification over the enzyme activity of the crude homogenate using poly rC. $(dG)_{12-18}$ as the template-primer, and 2781-fold purification using poly rA. $(dT)_{12}$. The optimum reaction conditions for the orbital reverse transcriptase activity with poly rA. $(dT)_{12}$ as template-primer were as follows: Ionic concentrations for Mn^{++}, 0.4 mM; Mg^{++}, 0.8 mM; and KCl, 60 mM; and the pH-optimum of the reaction was found to be around 7.8.

Table 1. DNA-polymerase activities at various purification steps

Source of protein and protein content	*Template-primer: divalent cation* Activity and Purification	$(dT)_{12-18} \cdot (A)_n$		$(dG)_{12-18} \cdot (C)_n$		$(dT)_{10} \cdot (dA)_n$	
		Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}	Mn^{++}	Mg^{++}
Crude Tumor Homogenate (I) 6710 mg/90 ml	Total[2]	9248.73	7339.43	2307.07	1398.83	11827.17	3145.97
	Specific[3]	1.38 (0.12)	1.09 (0.02)	0.35 (0.08)	0.21 (0.09)	1.76	0.47
	Purification fold	1.0	1.0	1.0	1.0	1.0	1.0
Disrupted Microsomal Pellet (II) 38.0 mg/25 ml	Total	1443.17	985.72	1248.91	1061.03	1328.34	272.48
	Specific	37.98 (0.66)	25.94 (0.03)	32.87 (0.79)	27.92 (1.71)	34.56	7.17
	Purification fold	27.52	23.80	93.91	132.95	19.28	15.29
0.35 M KCl Eluate off DEAE 23 Cellulose (III) 15.84 mg/36 ml	Total	1122.46	465.24	281.49	883.58	892.64	213.0
	Specific	70.86	29.37	17.77	55.78	56.35	13.45
	Purification fold	51.35	26.94	50.77	265.62	31.96	28.67
0.07 M KCl Eluate off DEAE 52 Cellulose (IV) 0.525 mg/21 ml	Total	573.84	204.36	255.43	80.28	264.03	57.22
	Specific	1093.03	389.03	486.53	152.91	502.92	108.99
	Purification fold	792.05	357.12	1390.09	728.14	285.26	232.39
0.21 M KCl Eluate off Phospho-cellulose (V) 0.01 mg/2 ml[1]	Total	38.38	6.46	4.69	1.56	0.01	<0.01
	Specific	3838.07	646.16	469.16	156.38	1.14	<0.01
	Purification fold	2781.21	592.81	1340.46	744.67	0.64	<0.5

DNA polymerase assays were carried out at 30°C for 60 min. in a reaction mixture of 0.05 ml. which contained: 50 mM Tris/HCl pH 7.8. 60 mM KCl. 0.4 mM $MnCl_2$. or 8 mM $MgCl_2$. and 1 mM DTT. The primer-template concentration used was 50 µg/ml; other conditions are the same as described by Chandra and Steel [2]. Numbers in brackets give the endogenous incorporation.

The purified orbital tumor reverse transcriptase showed a strong preference for the template-primers poly rA. $(dT)_{12}$, poly rC. $(dG)_{12-18}$ and poly-rC (OMe). $(dG)_{12-18}$, as seen in Table 2; whereas poly dA. $(dT)_{10}$ was clearly ineffective. These results agree with those reported for other mammalian C-type oncornavirus DNA polymerases [11]. The inability to utilize the primers $(dT)_{12-18}$ or $(dG)_{12-18}$ indicates no terminal transferase activity was present in the purified enzyme preparation. Transcription of heteropolymeric regions of a 70S RNA from R(Mu)LV, and stimulation of its utilization by addition of the primer $(dT)_{12-18}$ further support the oncogenic nature of the orbital enzyme and its similarity to other known RNA tumor virus reverse transcriptases.

Table 3 summarizes the responses of the orbital tumor and SiSV-1 reverse transcriptases when challenged with antibodies to various type-C virus DNA polymerases. The serological cross-reactivity of SiSV-1 DNA polymerase with antibodies to gibbon-ape leukemia virus (GaLV) reverse transcriptase was previously demonstrated by Sarin and Gallo [12]. As shown in Table 3, both the human orbital tumor and SiSV-1 enzymes demonstrated marked

Table 2. Primer-Template activities of the orbital tumor RNA-dependent DNA polymerase

Primer-Template	pmol ^{3}H-dNMP incorporated in 60 min. per mg protein	
Divalent cation	Mn^{2+}	Mg^{2+}
activated DNA	0.31	0.68
$(dT)_{12-18} \cdot (A)_n$	3476.11	603.07
$(dT)_{10} \cdot (dA)_n$	1.14	<0.01
$(dG)_{12-18} \cdot (C)_n$	461.01	174.92
$(dG)_{12-18} \cdot (OMeC)_n$	53.33	3.95
$(dG)_{12-18}$	<0.1	NT
RLV 70S RNA	138.22	NT
RLV 70S RNA + $(dT)_{12-18}$	262.61	NT
$(dT)_{12-18}$	<0.01	NT

Assays were carried out for 60 min. in a reaction mixture described under Table 1. The primer-template concentration used was 50 µg per ml: R(Mu)LV-70S RNA was used at a concentration of 20 µg/ml. ^{3}H-dTTP was used as the labeled substrate for activated DNA. poly rA · $(dT)_{12}$. poly dA · $(dT)_{10}$. R(Mu)LV-RNA and oligo dT. ^{3}H-dGTP was used as the labeled substrate for $(dG)_{12-18}$. poly rC · oligo dG. and Poly rC(OMe) · oligo dG. NT = not tested.

Table 3. Preliminary results of the effect of various type-C virus DNA polymerase antibodies on the reverse-transcriptase activity of the orbital tumor

Source of reverse transcriptase	IgG (µg)	Specific anti-reverse transcriptase (IgG) added: non-immune (control) IgG	anti-RLV RT IgG	anti-AMV RT IgG	anti-GaLV RT IgG
		^{3}H-dTMP incorporation (% of enzyme activity[a])			
SSV-1	8	93.4	91.2	94.5	17.4
	13	101	NT	NT	23.0
	32	101	79.0	NT	21.0
Orbital tumor from AMML patient	8	98.2	94.0	97.8	NT
	13	103	NT	NT	44.3
	32	114	93.2	NT	40.2

Orbital tumor DNA polymerase (10 µl) was incubated with 10 µl of non-immune rabbit sera IgG. or the indicated immune sera IgG at 4°C for 4 hr. before assaying for the enzyme activity. For comparison. SiSV-1 reverse transcriptase was similarly challenged with immune and non-immune sera. NT = not tested.

[a] The % of ^{3}H-TMP incorporation expresses activity. compared to that of enzyme without IgG.

inhibition with antibodies to GaLV-polymerase. The orbital tumor reverse transcriptase was not inhibited by antibodies to the DNA polymerase from two non-primate sources, avian myeloblastosis virus and Rauscher leukemia virus, at the concentrations challenged thus far. Owing to the low amounts of purified enzyme, repetition and further elaboration of the immunological data await the availability of more tumor material.

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Inactivation and Lysis of Oncornaviruses by Human and Primate Complement

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Oncornavirus genomes, viral antigens and infectious viruses have been shown to be present in malignant and normal tissues and sera of many animal species including avian, murine, feline, bovine, ovine and recently primates by molecular hybridization, immunologically and by virus isolations. To date, there is no clear evidence of isolation or the presence of oncornaviruses in man. The long history of failures to isolate oncornaviruses from human leukemias and sarcomas, indicates that these viruses do not play a prominent role in these malignancies. Although oncornaviruses are antigenic in man, at least Rauscher murine leukemia virus, antibodies to viral antigens, have only infrequently been found in serum of patients with leukemias and in normal individuals. However, as many negative results have been reported, using same techniques for antibodies detection i.e., radio immuno assays.

As previously reported (Welsh, 1975; Cooper, 1976; Gallagher, 1978; Sherwin, 1978) human and primate sera, inactivates murine, feline, simian, and avian oncornaviruses, heated human (56° for 30′) or fresh guinea pig, rabbit, rat, bovine or murine sera had no effect.

Heated human or primate sera plus guinea pig complement were equally ineffective. The inactivation of oncornaviruses was antibodies-independent and the mechanism of inactivation was by viral lysis, since the internal enzyme, RNA dependent DNA polymerase and viral RNA were released after incubation.

Inactivation was complement-dependent, as heated human or heated primate sera, or C2, C4 or C8-deficient human sera did not produce viral lysis. When purified C2, C4, or C8 were added to deficient human sera, virolytic activity has been restored.

The C1Q subunit of the first human C component attaches directly to the oncornavirus envelope in the absence of immunoglobulin binding of C1. CIQ leads to activation of C1 and thus of the classical C pathway, accompanied by deposition of C components on the viral envelope and lysis on completion of complement sequence.

Recently Gallagher et al. and Sherwin et al. reported and confirmed our previous observations of complement mediated lysis of oncornaviruses by human and primate sera. Although the gibbons are unusual among the primates that they are so susceptible to horizontal infection with oncornaviruses in captivity. As shown by Gallagher et al., only the viremic gibbon had no detectable serum lytic activity. A normal adult gibbon had serum lytic activ-

ity, before and after deliberate inoculation with gibbon ape leukemia virus. Post inoculation, this gibbon was not viremic, developed antibodies within three weeks post-inoculation and has remained healthy. It is possible, that during the early period of captivity, the gibbons are exposed to many viral, bacterial and mycotic infections. Such infections could affect the compliment levels and make them susceptible to infection with oncornaviruses of Mus caroli or Mus cervicolor as reported by Todaro et al.

Leukemias and lymphomas among primates are rare and there are few reported cases in the literature. In an outbreak of spontaneous malignant lymphoma in rhesus monkeys (45/450) at the California Primate Research Center at the University of California in Davis, oncornaviruses were not isolated and not seen by electron microscopic examination of malignant cells (Takemoto, H., Pers. Communication).

In collaboration with Drs. Russell and Vanderlip, we tried to isolate oncornaviruses from a male adult baboon (Papio papio) who developed an osteosarcoma of the mandibule. Cultures were established from tumor and from normal tissues. Oncornaviruses were not isolated from the tumor nor from long term cultures of normal tissues and are still free of C type viruses 24 months in continuous cultivation. Electron microscopic examination of the osteosarcoma and of kidneys, lungs, brain, epidydimus, testes, and lymphnodes were negative for C type viruses. Although as shown by Benveniste and Todaro the baboons have multiple oncornavirus gene copies in their DNA. However, the virus is under strict cellular control and only seldom expressed. There is no evidence to date that oncornaviruses of baboons are horizontally transmitted in nature. It is of interest that majority of the isolates from human leukemias are identical to C type virus isolate M-7 from Papio cynocephalus, which has been distributed to several laboratories. In laboratories where M-7 was not used experimentally, cell cultures from other laboratories were obtained, which could have been infected with M-7.

The fact that all the so called human isolates from leukemic and normal human embryonic tissues are identical to M-7 isolate, deserves great caution. As shown by Benveniste and Todaro the three different isolates from baboon subspecies: Papio cynocephalus, P. hamadryas and P. papio differ from each other by molecular hybridization. There were other excellent markers in the 27000 Dalton protein which we have established in our laboratory (Gautsch et al., 1978) which confirm the identity of the so-called "human" isolates. For the record, I would like to state that in species where C type viruses are horizontally spread, such as gibbons, feline, bovine, and equine, antibodies to all viral peptides are readily demonstrated by several immunological tests and these results are in full agreement between the various laboratories around the world.

Lysis of oncornaviruses by human and primate complement is of major immunological importance as a defense mechanism against horizontal infection. Since humans are in close contact with viremic cats the virolytic activity of complement might be responsible as to why infection with these viruses doesn't occur.

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The Role of Gene Rearrangement in Evolution

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Chromosomes are commonly regarded as conservative structures, in which an exact amount of genetic information is arranged in a definite sequential order. This order is normally preserved when information is exchanged between chromosomes, and guaranteed by a set of recombination enzymes that function only with paired sectors of homologous DNA. But processes such as inversion, deletion, duplication and translocation, often involving recombination between apparently non-homologous chromosomal regions, can alter this sequential order. Chromosomal rearrangements resulting from such events have been observed, in some cases with a disturbingly high frequency, thus inviting speculation about their biological significance. The processes involved have been termed collectively "illegitimate recombination", reflecting our bias for conventional pathways based on sequence homology, but evidence is accumulating which may legitimatize them as important aspects of evolution or even differentiation.

The presence of IS elements in the chromosome of E. coli was originally revealed by the transposition of these DNA elements form their natural positions into indicator systems, resulting in a recognizable mutant phenotype. If, for example, the gal operon is used as an indicator system, mutations can be isolated which are caused by the integration of an IS element into one of the three structural genes of the operon. Not only is the thus interrupted structural gene inactivated, but the expression of the promoter distal genes is also abolished. These mutations therefore are strongly polar. The analysis of the nature of such mutations has been facilitated by the isolation of gal transducing phage and the development of techniques for examining heteroduplex DNA in the electron microscope. With help of these tools it is possible to inspect hybrid DNA molecules consisting of one DNA strand carrying the strongly polar mutation paired with the complementary strand of λ dgal in the electron microscope. The strongly polar mutation is seen as a single stranded DNA loop emerging from a position in the double stranded heteroduplex molecule which corresponds to the map position of the mutation. Analysis of various independently isolated strongly polar mutations with the above technique revealed the existence of different categories of IS elements. The elements were numbered according to the order of their detection. IS1 is about 800 nucleotide pairs long, while IS2, IS3, IS4 and IS5 are each approximately 1400 basepairs long. (For review see Starlinger and Saedler, 1976.) In the following paragraphs we will concentrate on the topics listed below.

A. Chromosomal rearrangements mediated by IS1 (Reif and Saedler, 1975, 1977; Nevers, Reif and Saedler, 1977; Nevers and Saedler, 1978)
B. IS elements found in strategical positions on certain plasmids (Hu et al., 1975)
C. Detection of mini-insertion elements (D. Ghosal and H. Saedler, 1977)

A. Chromosomal Rearrangements Mediated by IS1

IS1 a known to occur in multiple copies in the chromosome of E. coli K12 (Saedler and Heiss, 1973). They also seem to be integral parts of at least some bacterial plasmids like F^+ and R (Hu et al., 1975).

The formation of new chromosomal sequences can result from translocation, duplication, inversion or deletion of genetic material. All these events seem to play a role in the evolution of plasmids as well as chromosomes. IS-elements appear to be responsible for some such chromosomal rearrangements.

Non-adjacent chromosomal regions can be brought together by deletion of the intermittent genetic material, resulting in a new chromosomal order. This reaction has been studied extensively in IS1 induced deletion formation (Reif and Saedler, 1975, 1977). The termini of the integrated IS1 elements are most important in this process. IS1 is retained in the deletion, thus allowing further rounds of rearrangements. IS1 can be considered as a generator for deletions, sometimes fusing the structural genes of the gal operon to other promoters and thus creating a new control circuit (Reif and Saedler, 1977). It is not yet clear, however, which enzymes are involved in this rather unusual type of recombination. Apparently the normal recombination pathways of E. coli are not involved. However, recently mutants were isolated which are deficient in IS1 induced deletion formation. Such mutants may be helpful in the analysis of the enzymes involved in illegitimate recombinational events (Nevers and Saedler, 1977).

B. IS Elements Are Also Found in Strategical Positions on Certain Plasmids

The R-factors of the fi^+ class are composed of two units, each capable of replicating autonomously if dissociated from each other. The RTF unit codes all functions necessary for cell to cell contact, thus allowing the transfer of the plasmid. The r-determinant carries most of the antibiotic resistance genes. An IS1-element separates the RTF unit from the r-determinant at each junction. Both IS1 elements are oriented in the same direction (Hu et al., 1975; Ptashne and Cohen, 1975). This finding suggests a model to explain the formation and dissociation of R-factors as well as the amplification of the antibiotic resistance genes. Rownd and Mickel (1971) showed that R-factors can dissociate into the RTF and the r-determinant in Proteus mirabilis. Dissociation may occur by recombination between the two homologues IS1 substrates of the co-integrate plasmid generating two units, each containing an IS1.

Fusion results from the reverse reaction. Amplification of antibiotic resistance genes could be due to recombination between the homologous IS1 elements of different r-determinant molecules, leading to co-integrate plasmids containing multiple copies of the r-determinant units.

In addition to IS1 other IS-elements are also observed on R-factors, either as mutations or as integral parts of the molecule. For example in R6 of R100-1 IS2 is found at a position within the transfer genes at which it does not cause a transfer defective mutation but rather contributes to the transfer positive character of the plasmids (Hu et al., 1975). Many of the antibiotic resistance genes can transpose to the various other DNA molecules (Cohen and Kopecko, 1976). At least one of the transposons is flanked by a known IS element (Mac Hattie and Jackowski, 1977).

In the evolution of R-plasmids, IS-elements therefore seem to play an important role.

C. Detection of Mini-Insertion DNA Elements

The detection of IS elements using the heteroduplex technique is limited by the size of the integrated DNA element. If, for example, an IS element is an order of magnitude smaller than IS1 to IS5, it cannot be readily recognized as a single stranded loop using the heteroduplex technique. To analyse very small insertions another technique is more adequate. If suitable restriction fragments are available, one containing an integrated mini-insertion and the same fragment without, they will band at different molecular weight positions when subjected to electrophoresis in agarose or polyacrylamide gels. In this manner two mutants were shown to be due to the integration of a very small piece of additional DNA. Fig. 1 gives the pattern of a Hind II, Hind III double digest of various plasmid DNAs. Slot 3 shows the pattern of the parental plasmid pDG1, which is Gal positive. The pattern of pDG12, in which an IS2 is integrated in the control region of the gal operon, eliminating expression of the gal genes, is presented in slot 4. Note the appearance of the new bands (e and f) and the shift in molecular weight of one band (from a to b), due to the integration of IS2. Slots 1 and 5 show the pattern of two independent Gal positive revertants obtained from plasmid pDG12. Note the increase in molecular weight of only band e in both mutants. This can only be explained by assuming that a small insertion is present in band e. Using appropriate markers (slot 2) as references, the increase in molecular weight can be calculated. Mutation 1 (slot 5) is due to the integration of about a 115 base-pair long piece of DNA, while the other mutation (slot 1) is about a 60 base-pair insertion. The former has been called IS6 and the latter IS7.

Both insertions confer a Gal positive phenotype to the cell carrying the plasmid. Since they seem to have integrated into IS2, they either destroy the polar signal on IS2 or, more likely, each carries its own turn-on signal.

Recently we sequenced both IS6 and IS7 and compared their DNA sequence of IS2 in the region of integration of these mini-insertions.

It is quite obvious that both IS6 and IS7 can be derived from IS2 sequences

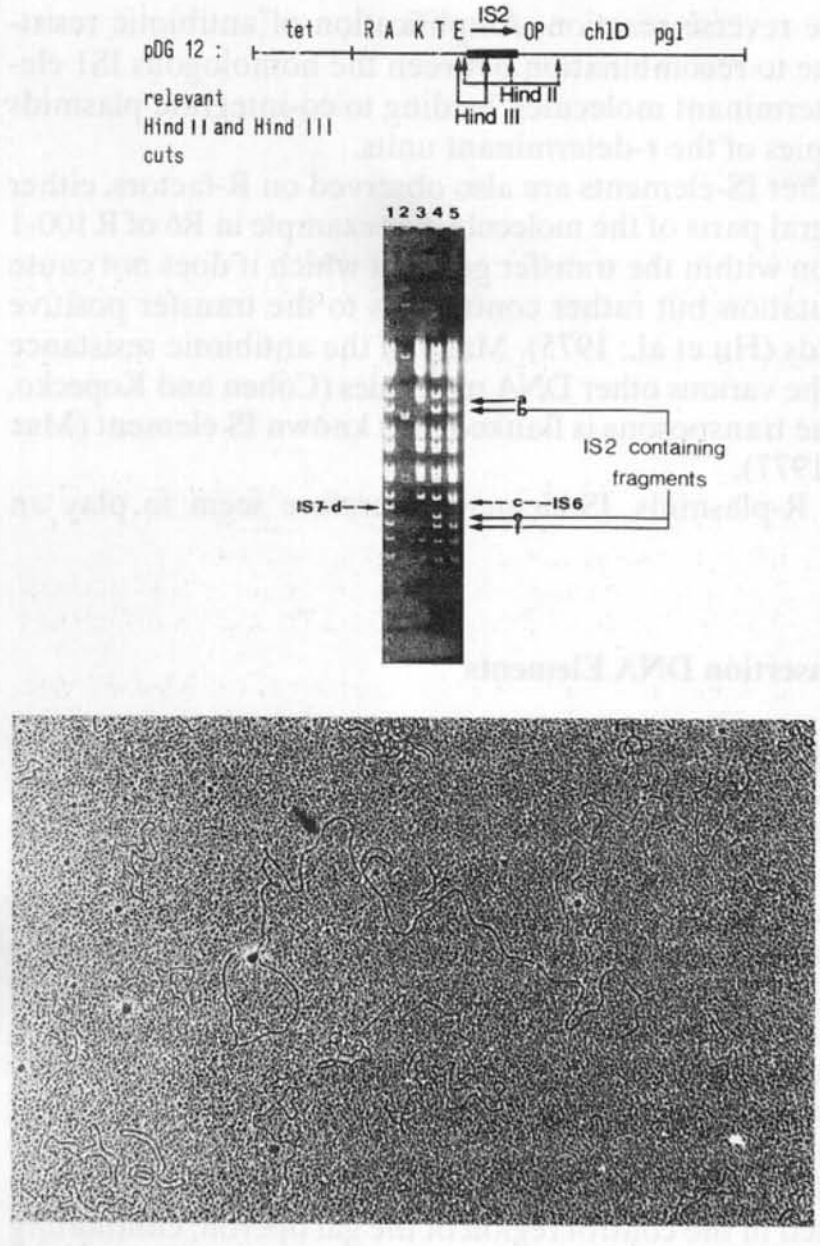

Fig. 1. Identification of the mini insertions IS6 and IS7

in a complicated manner. That is, genetic information from both DNA strands of IS2 seems to have multiplied and re-integrated in a rearranged form, resulting in the formation of a turn-on signal. (For detailed discussion see Ghosal and Saedler, 1978.)

Conclusions

IS elements are natural components of the E. coli chromosome. They can translocate from one position in the chromosome to another. Besides stimulating a number of illegitimate recombinational events, like deletion and transposition of other genes, which is thought to be of evolutionary importance, they also carry signals necessary for gene expression (Saedler et al.,

1974; Ghosal and Saedler, 1977, 1978). Similar events are also known to occur in higher organism (Nevers and Saedler, 1977).

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Bacterial Transposons

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A. Bacterial Plasmids

Bacterial plasmids have been known for more than twenty years and, during this period, one witnessed an extraordinary spread of those extra-chromosomal DNA elements.

Resistance plasmids were first identified in *Enterobacteriaceae* but, later on, have been found in almost every pathogenic bacterial species, including more recently *Hemophilus influenzae* and *Neisseria gonorrheae.*

Plasmids, however, do not only contribute to antibiotic resistance. They can code for many other properties, including heavy metal resistance, production of a cholera-like enterotoxin, tumorigenicity in plants and the capacity to catabolize various substrates. Some plasmids have been found to code for lactose fermentation and this type of plasmid also happens to be of medical concern. Their presence can indeed obscure the detection of pathogens such as *Salmonella* and *Yersinia* since the capacity to ferment lactose is the major key in the sorting of the *Enterobacteriaceae.*

Plasmids consist of covalently closed circular DNA and their essential feature is a replication region that ensures the propagation of the entire structure.

Some plasmids also contain an operon promoting the transfer of the plasmid itself from one bacteria to another one.

B. Translocation of Plasmid Genes

In 1974, Hedges and Jacob observed that the ampicillin resistance determinant of a plasmid called RP4 could be translocated to the bacterial chromosome and various other plasmids genetically unrelated. From one of these derivative plasmids, they could subsequently translocate the β-lactamase gene to a third plasmid. After transposition, the increase in molecular weight was similar in every instance. These authors concluded that a DNA sequence, including the β-lactamase gene, had a specific transposition mechanism and they called it a transposon (now Tn1).

Later on, Berg et al. (1975) observed the transposition of the kanamycin resistance genes from two different resistance plasmids to bacteriophage λ (Tn5 and Tn6).

Gottesman and Rosner (1975) made similar observations with a determinant for chloramphenicol resistance: initially detected on a resistance plasmid

and, later, transferred to phage P1, it could subsequently be translocated from P1 to bacteriophage λ (Tn9).

Still in the same year, Kleckner et al. (1975) reported that a genetic element. 8.3 Kb long, carrying the tetracycline resistance gene was also capable of translocation (Tn10).

Recently, we showed that genes coding for lactose fermentation can jump from pGC1, a plasmid that originated in *Yersinia enterocolitica*, to RPI, a resistance plasmid that originated in *Pseudomonas aeruginosa* (Cornelis et al., 1978) (Fig. 1). This lactose transposon that alters the biochemical phenotype of its host is 16,6 Kb long and has been given the number 951.

Some transposons are listed in Table 1.

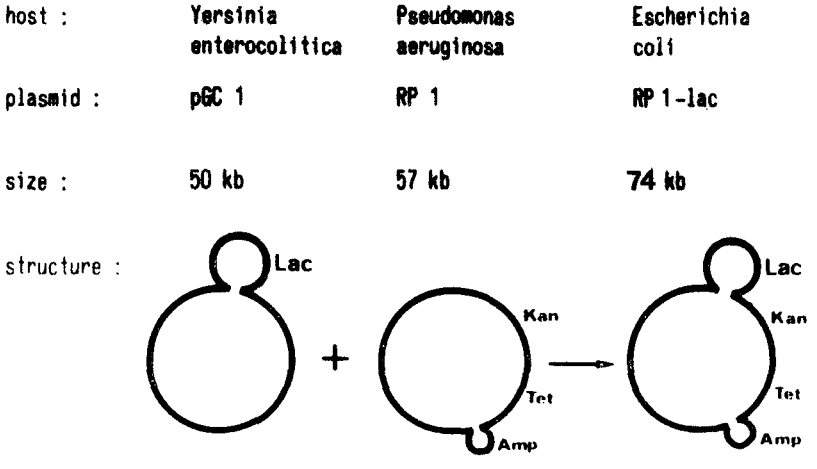

Fig. 1. Schematic representation of the transposition of Tn951. carrying a lactose operon. from pGC1 to RP1. RP1 confers resistance to kanamycine (Km). tetracycline (Tc) and ampicillin (Amp). The latter gene is part of another transposon (Tn1)

Table 1. Some transposable elements

Transposon	Genetical information	Size	References
Tn1, Tn2	β-lactam antibiotics resistance	4,5 Kb	Hedges et al., M.G.G. **132,** 31 (1974) Heffron et al., J. Bact. **122,** 250 (1975)
Tn5, Tn6	Kanamycin resistance	3,8 Kb	Berg et al., P.N.A.S. **72,** 3628 (1975)
Tn7	Trimethoprim and Streptomycin resistance	13.5 Kb	Barth et al., J. Bact. **125,** 800 (1976)
Tn9	Chloramphenicol resistance	2,6 Kb	Gottesman et al., P.N.A.S. **72,** 504 (1975)
Tn10	Tetracycline resistance	8,3 Kb	Kleckner et al., J.M.B. **97,** 561 (1975) Foster et al., J. Bact. **124,** 1153 (1975)
Tn402	Trimethoprim resistance	7.5 Kb	Shapiro et al., J. Bact. **129,** 1632 (1977)
Tn501	Mercuric ions resistance	7,8 Kb	Bennett et al., M.G.G. **159,** 101 (1978)
Tn951	Lactose fermentation	16,6 Kb	Cornelis et al., M.G.G. **160,** 215 (1978)

C. General Features of Transposition

As suggested by Hedges and Jacob (1974), transposons are specific DNA sequences of a precise length.

The size of any replicon suffering transposition does increase and the increment is constant for a given transposon.

Transposition does not require extended homology between the two replicons exchanging the transposon. This is shown in Table 2 for pGCI (*lac*$^+$, *tra* ts, *inc* unknown) and RPI (Amp, kan, tet, *tra*, *inc* P), the two plasmids exchanging Tn951. Thus, transposition cannot involve ordinary recombination occuring after physical breaking and reciprocal exchange of DNA. Accordingly, in some instances at least, transposition turns out to be independant of the *rec* A gene product, the bacterial function involved in such recombination process (Rubens et al., 1976).

Table 2. DNA-DNA hybridization between pGCI and RPI

DNA bound to filter	^{3}H-pGC1 DNA hybridized	
	Exp I	Exp II
pDG1	64	101
pGC1	2300	2303
RP1	74	112
RP1::Tn951	1490	—

Filters were loaded with 2 mcg of DNA. The values given are normalized to 10000 cpm input. pDGI is an unrelated plasmid taken as a negative control. For details, see Cornelis et al., 1978

Transposition is therefore considered as some kind of "illegitimate" recombination involving the termini.

The known transposons can jump on a great number of targets such as different plasmids, bacteriophages and bacterial chromosomes. There seems however to be some kind of specificity: Tn1 for instance was found by Hedges and Jacob (1974) to transpose from RP4 to three different plasmids but not on a fourth one. Bennett and Richmond (1976) also observed that the transposition frequencies of Tn1 could vary 10000 times according to the chosen target. Kretschmer et al. (1977) observed the same phenomenon with Tn3.

Inserted transposons can readily be detected on small replicons such as plasmids and bacteriophages by electron microscopy. When a mixture of two different DNAs sharing homologous regions is denatured and allowed to reanneal slowly, a number of hybrid molecules are generated. If such "heteroduplex" molecules are constructed between a given plasmid and its own derivative carrying a transposon, the structure observed in electron microscopy will be a circular double stranded plasmid carrying a single stranded loop corresponding to the transposon. This kind of analysis clearly demonstrates that transposons are inserted without any loss of DNA from the recipient plasmid.

Moreover, heteroduplex analysis will indicate whether the insertions on two independantly isolated molecules occur at the same site or at two different sites. If the transposon maps at two different loci on the target plasmid,

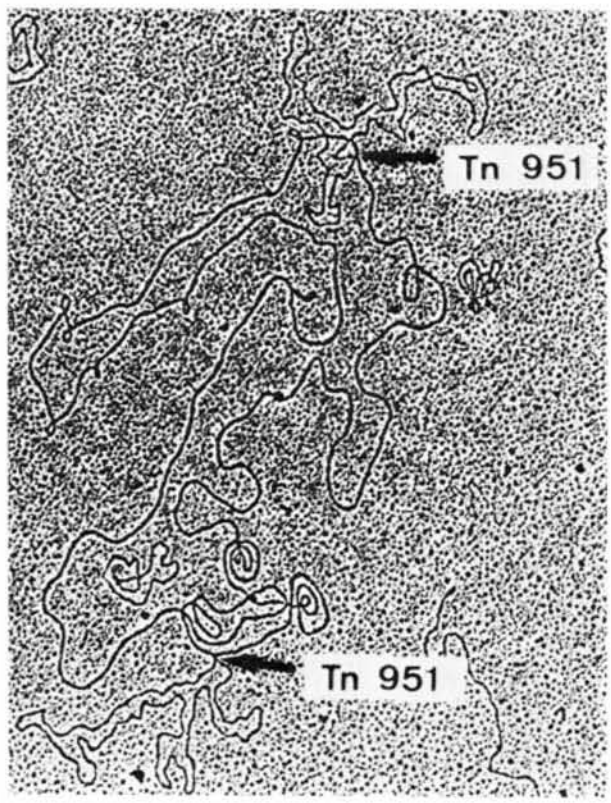

Fig. 2. Heteroduplex molecules formed between two different RP1 :: Tn951 molecules. The arrows indicate the positions where the single stranded loops (16.6 Kb) emerges from the 57 Kb long double stranded circular DNA

heteroduplex molecules will consist of a double stranded circular molecule having the length of the parent plasmid and two single stranded loops corresponding to the transposon (Fig. 2). The distance between the two insertion sites can be measured, but this type of analysis however doesn't allow to localize the insertion sites on the plasmid map, at least without any refinements.

This kind of information can be inferred from experiments using restriction endonucleases. These nucleases cleave DNA at very specific sites and thus generate defined fragments from a given replicon. These fragments are subsequently resolved by agarose gel electrophoresis. Partial digestions and double digestions using simultaneously two different nucleases allow to align these fragments and to draw a map, referred to as the physical map. Insertion of a transposon into a plasmid will either introduce new cleavage sites, generating new bands, or specifically increase the length of a given fragment. If one knows the physical map of the target plasmid, it is possible to map the transposition sites and even to determine the orientation of the transposon, making use of the restriction nucleases.

Electron microscopy and restriction nucleases have been used to map the insertion sites of various transposons on a number of targets. Clearly, the number of insertion sites is usually very high: we mapped eight insertions of Tn951 on RP1 and found eight different loci (Fig. 3). Heffron and co-workers (1975a) could detect at least 19 sites for Tn2 on an 8 Kb long target plasmid!

Considering that transposition consists of the insertion of DNA sequences of a certain length into a genome, it is not surprising that it sometimes leads to the inactivation of genes. Plasmid pGC530 is an RP1: Tn951 derivative where the insertion was found to map at coordinate 13 Kb, i.e. within the

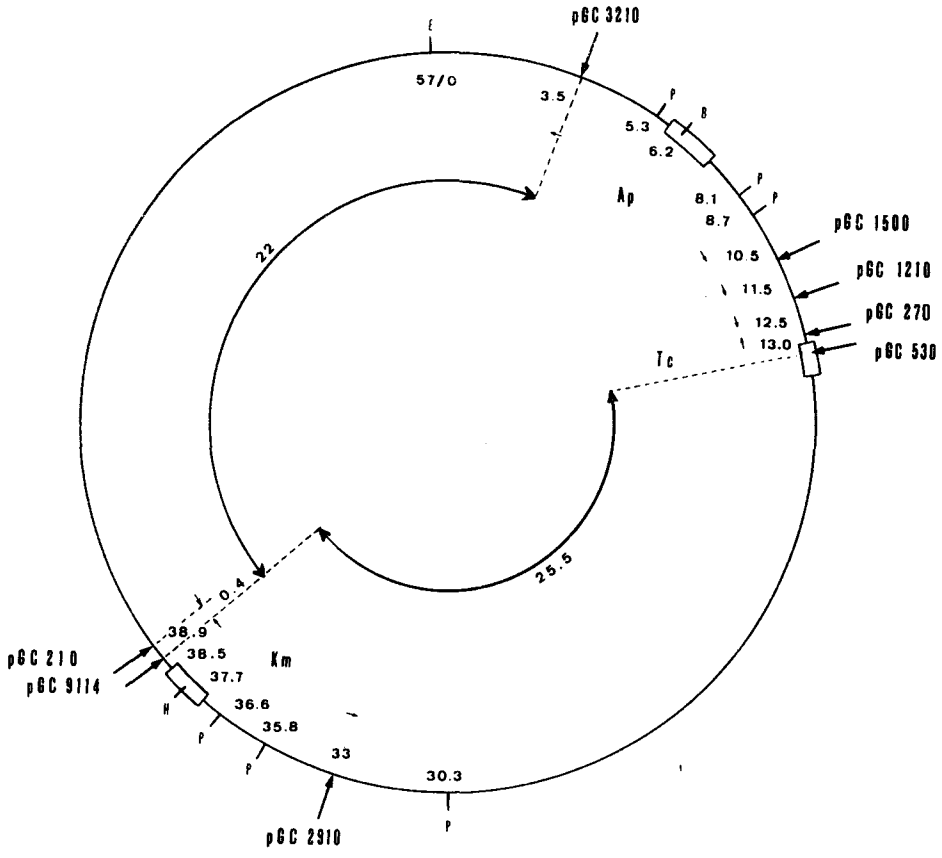

Fig. 3. Physical map of RP1 showing the 8 insertion sites of Tn951. pGC3210. pGC1500. pGC1210. pGC270. pGC530. pGC2910. pGC9114 and pGC210 are the 8 RP1 :: Tn951 derivatives. E = Eco R1 endonuclease cleavage site; B = Bam H1 endonuclease cleavage site; P = Pst 1 endonuclease cleavage site; H = Hind III endonuclease cleavage site; Ap = ampicillin resistance gene; Tc = tetracycline resistance gene; Km = kanamycin resistance gene. Coordinates are in kilobases. The inner lines refer to distances measured in heteroduplex analysis

tetracycline resistance gene (see Fig. 3). In accordance with our physical mapping, pGC530 does no more confer resistance to tetracycline. Insertion of a transposon not only abolishes the function of the gene into which the element lands but it can also be polar, affecting the expression of the genes located downstream on the transcriptional wave (Kleckner et al., 1975). For instance, Tn2 can land in the sulfonamide resistance gene of a plasmid and this inhibits the expression of the neighbouring streptomycin resistance gene.

Since transposons behave like mutagens and since their insertion can readily be physically mapped, they appear as valuable tools for the genetical analysis of plasmids. Tn7 has recently been used to analyse the transfer genes of the plasmid RP4 (Barth et al., 1978).

Transposons can sometimes be excised from a replicon where they have been inserted but the excision frequency is usually lower than the insertion frequency. Moreover, the process is often not precise. In our hands, a strain carrying pGC530 does not revert to tetracycline resistance, indicating that Tn951 does not excise accurately at a detectable frequency.

D. Structure of the Transposons

Five years ago, Sharp and co-workers (1973) discovered inverted repeats on plasmid DNA. These inverted repeats consist of identical DNA segments occuring twice in a genome but in opposite orientation. When a DNA fragment containing such inverted repeats or palindromes is submitted to heteroduplex analysis, intrastrand annealing of these fragments takes place giving a mushroom like structure (Fig. 4), consisting of a double stranded stem and a single stranded loop.

Later on, the mushroom like structure observed by Sharp et al. turned out to contain tetracycline resistance genes, in the loop. Moreover, the tetracycline transposon of Kleckner et al. (1975) gave the same structure in heteroduplex analysis. Other transposons were also found to consist of a DNA sequence flanked by two inverted repeats. For instance, Tn1 is flanked by a repeated sequence about 140 base pairs long (Rubens et al., 1976) and Tn5 is flanked by a repeated sequence ten times longer (Berg et al., 1975). These two inverted repeats do not correspond to any known Insertion Sequence. Tn9, on the other hand, is bordered by the well known sequence IS1 (Starlinger and Saedler, 1976) but repeated in tandem. Transposons are thus, often, if not always, flanked by repeated sequences.

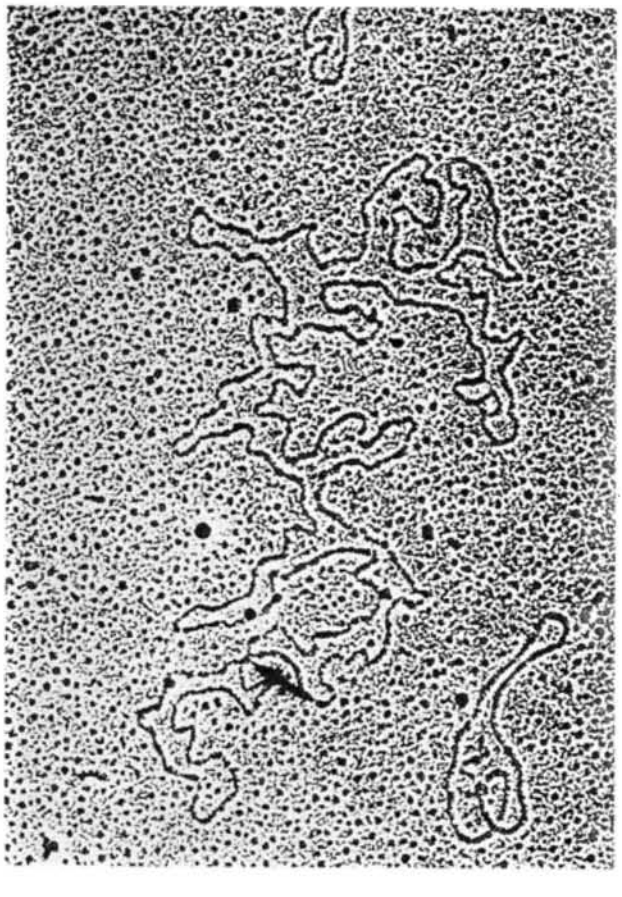

Fig. 4. Homoduplex formation of pGC1 DNA. The arrow indicates a small double stranded DNA region due to reannealing of small inverted repeats

E. Mechanism of Transposition

Although a number of models have been proposed, we have, so far, no clear understanding of the transposition mechanism. Experimental data from Heffron et al. (1977) suggest that a central region and the termini of Tn2 are required for transposition. The most attractive interpretation of their data is that the terminal sequence of the transposon serve a structural role while the central region encodes an enzyme required for transposition. Apart from that, it is not yet clear whether the process requires the recognition of a special sequence on the target.

F. Role of the Transposons in the Evolution of the Bacterial Plasmids

The discovery of transposable elements seems to account significantly for the structural and genetic diversity of plasmids. It has been known for long indeed that there is no relation between the genes carried by a plasmid and the type of plasmid. For instance, the TEM β-lactamase can be coded by plasmids that belong to, at least, 14 different incompatibility groups (Matthew and Hedges, 1976). It is very tempting to believe that the spread of Tn1 and Tn2 accounts for this situation. In accordance with this hypothesis, Heffron et al. (1975b) showed that, indeed, a great variety of different plasmids encoding the TEM lactamase contain a 4,5 Kb long sequence in common, while the TEM gene itself is much smaller.

The evolution of plasmids would thus essentially be due to terminus-site-specific recombination. Plasmids themselves seem to consist of two parts: one is essential for replication and transfer of the plasmid, while the other is a succession of transposons (and defective transposons?) acquired and lost during the history of the plasmid. In other words, plasmids would be nothing else than genetic carriers whose function is to replicate and propagate transposons.

G. Origin of the Transposons Carried Genes

So far, little is known about the origin of the transposons carried genes. For instance, the TEM β-lactamase coded by Tn1 and Tn2 appears different from all the known chromosomal β-lactamases.

For the lactose fermentation genes, however, we observed that the Tn951 coded operon is identical to the E. coli chromosomal lactose operon. In heteroduplex analysis, we detected 5,6 Kb homology and this length accounts for the genes encoding the repressor, the β-galactosidase and the permease (Fig. 5) (Cornelis et al., 1978). This indicates that, at least in some case, a transposon coded and a chromosomal (to date non transposable) operon may evolve from a common ancestor.

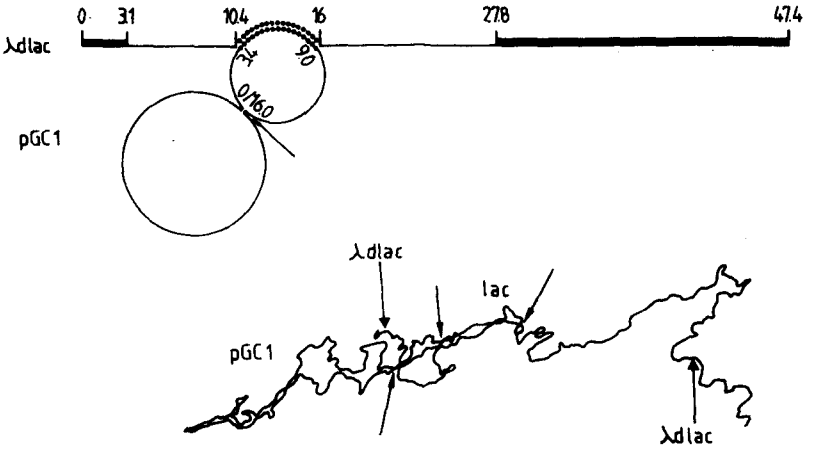

Fig. 5. Diagram of an heteroduplex molecule showing the 5.6 Kb homology between the E. coli chromosomal lactose operon (here carried by λ hdlac) and the Tn951 lac operon, here carried by pGC1

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The Virus-Cell Gene Balance Model of Cancerogenesis

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In recent years it became increasingly clear that infection of the human host by a number of viruses results in a probably life-long persistence of these agents within specific cells (reviewed e.g. zur Hausen, 1977a). Reactivation of persisting genomes is a well-known phenomenon in individuals infected by herpes group viruses (herpes simplex virus, varicella zoster virus) where neural cells have been identified as site of virus persistence. Infection with Epstein-Barr virus, the causative agent of infectious mononucleosis, leads to viral genome persistence in certain B-lymphocytes. They can be isolated and are readily propagated in tissue culture even years or decades after primary infection (reviewed by zur Hausen, 1975). Transmission of cytomegalovirus infections by blood transfusions has been frequently recorded, even from donors who acquired the virus several years earlier.

Besides herpes group viruses, however, it became more and more obvious that a number of additional agents may persist continuously within the human host. Adeno-viruses seem to be able to persist for long periods within adenoids and specific cells of the tonsils (reviewed by Green, 1970), hepatitis B virus seems to be a candidate for extremely long periods of persistence (Zuckerman, 1975) and the 2 human polyoma-like viruses, BK and JC, apparently remain latent for life-time. Reactivation of the latter appears to be mediated by immunosuppression and may result in excretion of large quantities of viral particles in the urine of affected patients. The papilloma- or wart viruses represent additional candidates for longtime persistence (zur Hausen, 1977b).

The observations cited above permit the statement that every individual, increasing with life-time, is exposed correspondingly to viral infections which result in an increasing "burden" of persisting genomes within certain cells. Although it is presently impossible to predict the actual percentage of cells being affected by viral genome persistence at a given age, it is probably justified to assume a substantial number in every cell compartment of human adults. This can be considered as an epigenetic potential which may remain genetically silent in most instances.

It is a remarkable feature of a substantial number of these persisting agents that many of them are oncogenic when inoculated into non-natural hosts:

Human BK and JC viruses induce malignant tumors after infection into newborn rodents. Brain tumors have been induced in owl monkeys after inoculation of JC virus (London et al., 1978).

Epstein-Barr virus induces malignant lymphomas and lymphoproliferative disease in cottontop marmosets (Shope et al., 1973, Epstein et al., 1973, Wenner et al., 1975).

Certain serotypes of human adenoviruses exert oncogenic potential after inoculation into newborn rodents.

Herpes simplex and cytomegaloviruses are considered as potential tumor viruses, since both of them seem to mediate malignant transformation of rodent cells in vitro (Duff and Rapp, 1971; Albrecht and Rapp, 1973).

It would be easy to prolong this series by including persisting animal viruses, like SV40, polyoma virus, the herpesviruses saimiri, ateles, papio and bovine papillomaviruses. All of these agents represent more or less harmless pathogens within their natural hosts but are effective oncogens in certain heterologous species.

Since tissue culture studies reveal that most of these viruses are able to transform specific cells of their native hosts in vitro, thus exhibiting their proliferation-stimulating capacity also in certain natural host cells, we have to postulate an in vivo mechanism which shields the host against the oncogenic potential of his usually ubiquitous tumor viruses. Such mechanism seems to be mandatory in evolution in order to prevent extinction of the host and guarantee optimal spreading for the viruses.

This protective control could be visualized by immunosurveillance (Burnet, 1970) or by intercellular or intracellular interference factors (zur Hausen, 1977a). Although immunosurveillance appears to play some role in the regulation of viral particle synthesis as evidenced by BK and (or) JC virus excretion in immunosuppressed patients, the rare occurrence of progressive multifocal leucencephalopathy (PML) due to JC virus replication within the brain of such patients and frequent zoster eruptions in patients with Hodgkin's disease, there exists little evidence for a role of immunosurveillance in the prevention of oncogenic properties of persisting natural tumor viruses. Experimental data do not support a role of the immune system in the suppression of oncogenic expression of such agents. Moreover, the efficient induction of tumors by such agents in immunocompetent heterologous species lends little support for this model.

An attractive alternative is the postulation of an intra- or intercellular regulatory system which controls the expression of viral "oncogens" (zur Hausen, 1977a). Cellular interference factor(s) (CIF) interfer with synthesis or function of virus-specified gene products which mediate and maintain the transformed phenotype of a cell. The essential features of such model are outlined in Fig. 1. A viral transforming factor (VTF) is controlled by a cellular set of regulatory genes which mediate this control by a cellular interference factor (CIF). The intracellular control would represent a direct interaction, intercellular control could be mediated by an indirect interaction requiring the existence of a diploid set of "response" genes in the respective target cells. They would need activation by factors of different cell compartments in order to respond with CIF synthesis.

According to this model carcinogenesis depends on the presence of an "effector" mediating the synthesis of a transforming protein and the failure of

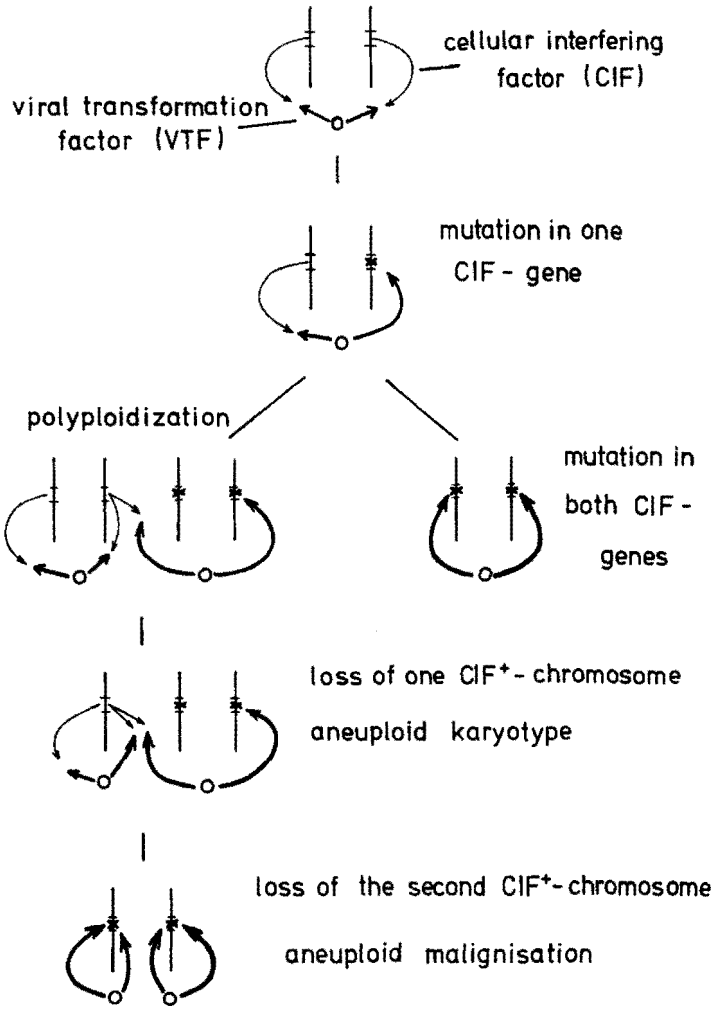

Fig. 1

the controlling CIF alleles. The postulation of a balanced control by CIF of the effector gene(s) (zur Hausen, 1977a) predicts an enhanced growth potential of effector-positive cells carrying a mutation in one CIF gene of the allelic set. This would result in the outgrowth of monoclonal, phenotypically normal cells and could explain observations by Fialkow (see this volume) in patients with chronic myelogenous leukemia and erythrocythemia vera, revealing not only monoclonality in the respective tumor cells but also in normal cells derived from the same stemline.

Mutations in both sets of CIF genes of an effector-containing cell should be an extremely rare event if we consider the spontaneous mutation rate for a specific gene (Spandidos and Siminovitch, 1978; Barret and Ts'o, 1978). They would result in immediate "malignisation" of a diploid tumor cell. Such events, however, would be somewhat facilitated by selective growth advantage of cells carrying a mutation in one CIF allele or by specific integration of viral DNA into these genes or specific mutagenisation due to viral or other agents (zur Hausen, 1967).

Polyploidization should enhance the progression to malignancy in some subsequent steps. Polyploid cells show an increased tendency to loss of individual chromosomes and it should be statistically predictable how many cell divisions are required before the loss of both functioning alleles results in an aneuploid tumor cell.

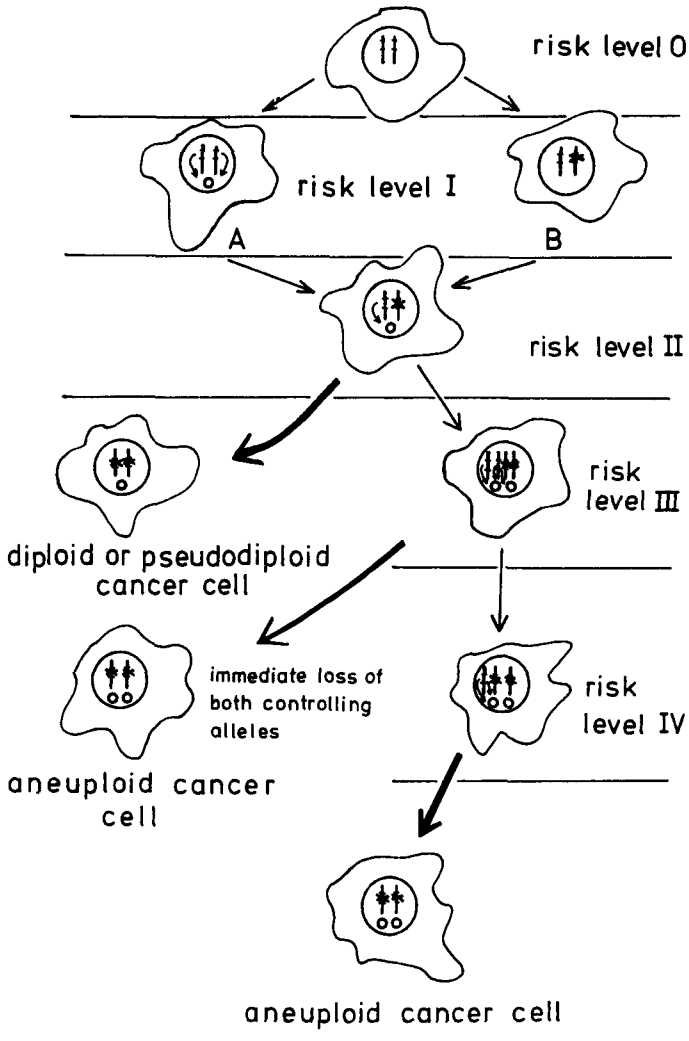

Fig. 2

The model permits the staging of risk levels for malignisation of individual cells. This is attempted in Fig. 2. An effector-free normal cell would be at risk level 0. It is questionable whether such cells exist in reality since genes of endogenous (vertically transmitted) viruses and possibly also genes of cellular origin may possess effector properties. It is possible, however, that the difficulties in transforming human cells in tissue culture by chemical and physical carcinogens (initiators) when compared to rodent cells, may result from the lack of effectors or their reduced number in comparison to e.g. mouse cells.

The model of carcinogenesis described here conveniently explains some of the prevalent features of cancer cells:

the monoclonality,

the stepwise tumor progression, going along with long latency periods,

the prevalent aneuploid karyotype often associated with specific chromosomal aberrations and the commonly observed recessive character of malignancy by intraspecies fusion of malignant with normal cells (Stanbridge and Wilkinson, 1978).

Tumor initiators would act by irreversibly mutagenizing CIF genes. This is in line with the mutagenic potential of the vast majority of chemical carcinogens and of X- and UV-irradiation.

The role of tumor promoters which appear to be non-mutagenic would fit into the scheme according to recent observations (zur Hausen et al., 1978a and b): At least promoters of the diterpene type are effective inducers of persisting genomes of herpesviruses and probably also of some other types of viruses. Their role could thus be envisaged in a transient amplification of effector molecules which would increase the target cell pool for malignant transformation. In addition, an intracellular effector amplification in a CIF-balanced system could shift the balance towards proliferation.

The reconciliation of a model with most well established observations in cancerogenesis does not prove its correctness. It would fulfill, however, its purpose by stimulating experiments which prove or disprove its substance, if this provides further insight in the complex development of human cancer and in effective means of its control.

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Comparative Analysis of RNA Tumor Virus Genomes

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Introduction

Comparative structural analysis of RNA tumor virus genomes is relevant to several questions regarding the biology of these viruses and their role in neoplastic disease. We have recently developed and applied procedures that facilitate such studies. Using these methods we have studied murine, primate, feline and avian C-type viruses. In some cases our studies permit direct comparison of the nucleotide sequence.

The questions investigated that will be discussed here include:

1. The relationship amongst viruses isolated from the AKR mouse. Viruses that differ in oncogenic potency and in host range have been isolated from the tissues of AKR mice. A comparison of the structure of the Akv virus, an ecotropic N-tropic virus that forms plaques in the XC test (XC^+) with that of a xenotropic AKR virus is presented. The structure of an ecotropic, N-tropic XC^- virus produced by lymphoid cell lines established from spontaneous AKR thymomas is also described. These studies structurally define a new class of AKR virus.
2. The sequence relationship amongst primate viruses of the woolly-gibbon group. Analysis of the structure of four independent isolates of gibbon ape C-type viruses and an isolate from a woolly monkey are presented.

The structure of a virus isolated from a patient with acute myelogenous leukemia (HL23) that has previously been shown to be related to the woolly monkey isolate is also investigated.

3. The sequence relationships among viruses of the feline leukosis group (FeLV): This work shows that these isolates are very closely related; the sequence of at least half the genome is identical.
4. The relationships between the transformation defective and non-defective Rous Sarcoma virus PrB is investigated. Sequence information of portions of the genome that code for the transformation function and for the structural genes is presented.

Results

A Micromethod for Detailed Characterization of High Molecular Weight RNA

We have developed a method for detailed studies of high molecular weight RNA available in small quantities. The procedures (Detailed methods are giv-

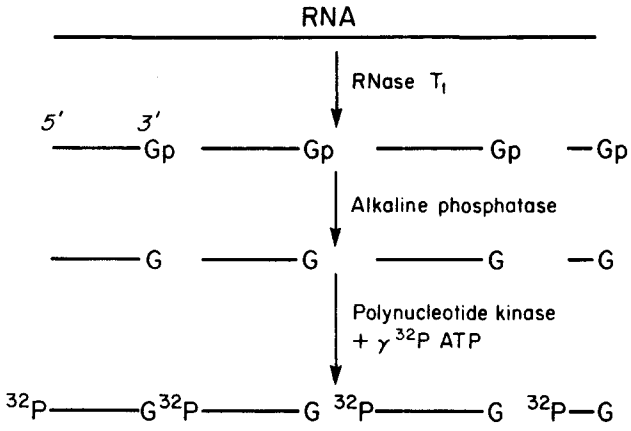


Fig. 1. Diagram showing the enzymatic reactions used for characterization of viral RNA

en in Pedersen and Haseltine, 1978 and diagrammed in Fig. 1) involve specific cleavage of a small amount of *non-radioactive* RNA after guanosine residues by ribonuclease T_1 followed by 5′-^{32}P-labelling of the T_1 resistant oligonucleotides. The mixture of 5′-^{32}P-labelled oligonucleotides is then fractionated by two-dimensional gel electrophoresis. By autoradiography of the gel these oligonucleotides will show a pattern (termed a RNase T_1 fingerprint) which is characteristic for each RNA sample. For further analysis the unique oligonucleotides can be eluted and their nucleotide sequence determined using recently developed methods for RNA sequencing that depend upon partial digestion of 5′ end-labelled RNA with ribonucleases that cleave the RNA at specific nucleotides (Donis-Keller et al., 1977).

When these procedures are applied to 200–400 ng of an RNA species about 10,000 nucleotides long, each T_1 resistant oligonucleotide will be labelled with ^{32}P at about 100,000 dpm. Our high resolution gel electrophoresis system makes it possible to isolate 50–80 pure unique T_1 resistant oligonucleotides from viral RNA. The amount of radioactivity in each oligonucleotide is sufficient for complete nucleotide sequence determination. The total sequence information thus obtained from less than 1 μg of RNA corresponds to 10–15% of the entire RNA molecule.

The AKR Viruses

The AKR strain of mouse was bred for high incidence of leukemia. Between 6 to 12 months of age AKR mice develop thymic leukemia. Genetic, virological and biochemical studies have demonstrated that a number of genetic factors are involved. These include two genetic loci that encode the information for the Akv virus (for a review see Tooze, 1973, and Rowe, 1973). The viruses (Akv-1, Akv-2) produced by these two loci are identical or very closely related (Rommelaere et al., 1977). Early in the life of the mouse, this virus appears in the tissues of the developing embryo. The mice remain viremic throughout their life. This virus, the Akv virus, is an ecotropic, N-tropic, XC^+ virus. It does not induce disease when injected into newborn C3Hf (N-tropic mice) nor does it accelerate the time of onset of thymic leukemia when injected into newborn AKR mice (Nowinski and Hays, 1978).

The relationship of this virus to the ultimate event of transformation of the thymic lymphocytes remains unclear. Although the virus itself is not oncogenic, its expression is correlated with the induction of the disease since strains of AKR that do not produce the AKR virus do not develop spontaneous thymic leukemias at a high frequency (Rowe, 1973).

A further complexity is presented by the observation that the virus is present from very early times in the mouse life but that leukemia developes only later.

Recently several viruses with biologic properties different from those of the Akv virus have been isolated from the tissues of leukemic or pre-leukemic AKR mice. These include xenotropic viruses, viruses that do not grow on cells of murine origin, and polytropic viruses, viruses that grow on both cells of heterologous species and murine origin (Kawashima et al., 1976; Hartley et al., 1977). The polytropic viruses (also called MCF viruses because they have been observed to cause cytopathic effects on mink cells) have been isolated by growth of extracts of AKR leukemic and pre-leukemic tissues on mink cells. The biologic and biochemical properties of these viruses resemble those of recombinants between a xenotropic virus and the Akv virus. The glycoprotein of these viruses have tryptic peptides that are characteristic of both the ecotropic and xenotropic murine viruses (Elder et al., 1977). Moreover, analysis of the genome by mapping of the T_1 resistant oligonucleotides and by heteroduplex analysis shows that these polytropic viruses differ from the ecotropic viruses by a substitution of genetic information in the 3′ region of the genome, the region in which is suspected to encode the glycoprotein. However, a simple model for the genesis of these viruses via a recombination between two parental virus appears to be excluded by the observation that the sequence of the substituted region differs for independent isolates (Rommelaere et al., 1978, and see below).

The appearance of these viruses in extracts of leukemic tissues has prompted speculation that they might be the transforming agents. However, whereas some of these viruses accelerate the appearance of thymic leukemia when injected into newborn AKR mice, others do not (Nowinski and Hays, 1978).

Recently another family of viruses have been isolated from the tissues of AKR mice. These are the viruses produced by lymphoid cell lines established in culture from the thymus of leukemic mice (Nowinski et al., 1978). The virus produced by 5 of 8 of these spontaneous leukemia cell lines (SL1, SL2, SL3, SL7, SL8) is ecotropic, N-tropic XC^-. Two other lines produce either a polytropic (SL4) or xenotropic (SL5) virus in addition to the ecotropic N-tropic XC negative virus and one produces an ecotropic, NB-tropic virus in addition to a polytropic virus (SL6). Some of these viruses accelerate the appearance of thymic disease when injected into the newborn AKR mice (SL1, SL2, SL3, SL4, SL6) and others do not (SL5, SL7, SL8) (Nowinski and Hays, 1978).

In order to understand the relationships among this complex family of virus with the eventual goal of understanding leukemogenesis in terms of the structure of the viruses, we have begun a detailed characterization of the genomes of those viruses using the techniques of T_1 oligonucleotide mapping and sequence analysis described above.

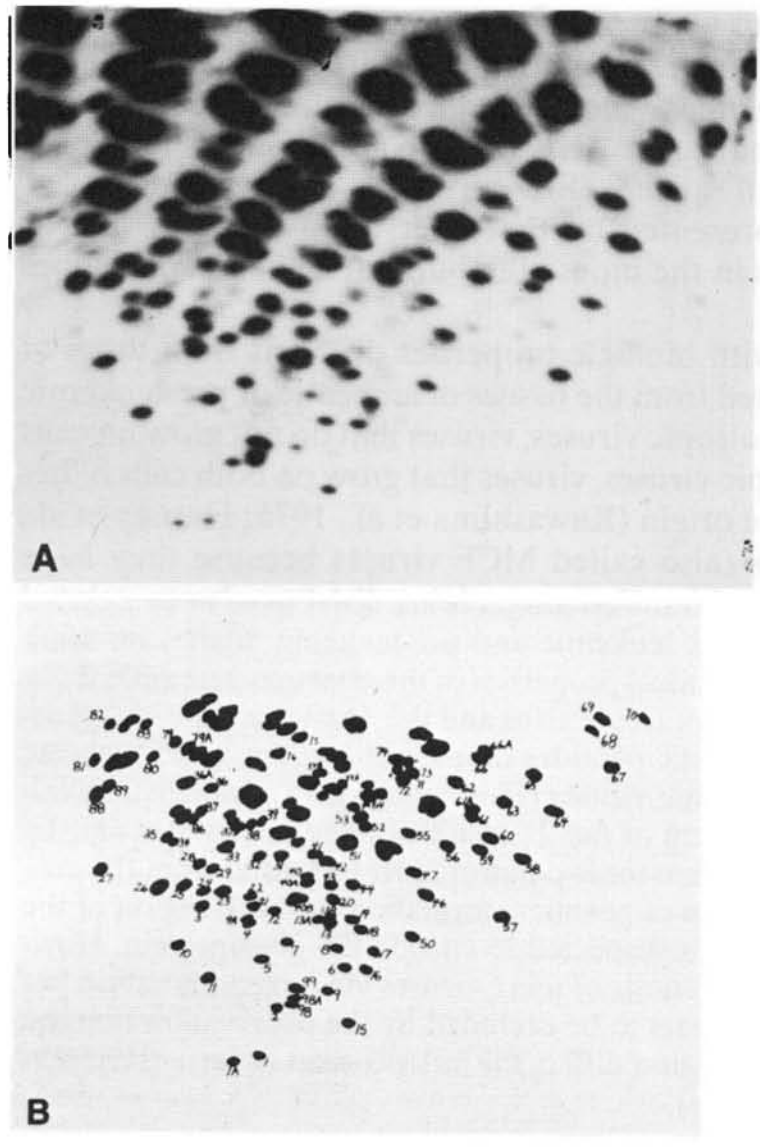


Fig. 2. *A.* RNase T_1 fingerprint of RNA from Akv virus. *B.* Schematic diagram of this fingerprint

Our point of departure has been to characterize the structure of the Akv virus genome in detail. The fingerprint of the RNA loci of this virus is presented in Fig. 2 together with a schematic representation in which each unique oligonucleotide has been assigned a number. This virus produced by an AKR embryo fibroblast cell line may be a mixture of two viruses that differ very slightly in the sequence. By sequence analysis the two oligonucleotides 1 and 1A are found to differ by only a single nucleotide. The method used to determine the sequence of the oligonucleotides is illustrated in Fig. 3. The 5′ labelled oligonucleotide is divided into aliquots which are partially digested with different base specific ribonucleases. The ribonuclease cleavage sites are then mapped by electrophoreses on a polyacrylamide gel under denaturing conditions (Donis-Keller et al., 1977).

The order of the T_1 resistant oligonucleotides can be determined by analysis of the fingerprints of poly A selected fraction of genomic RNA of different length. The oligonucleotides present in short poly A containing RNA are clos-

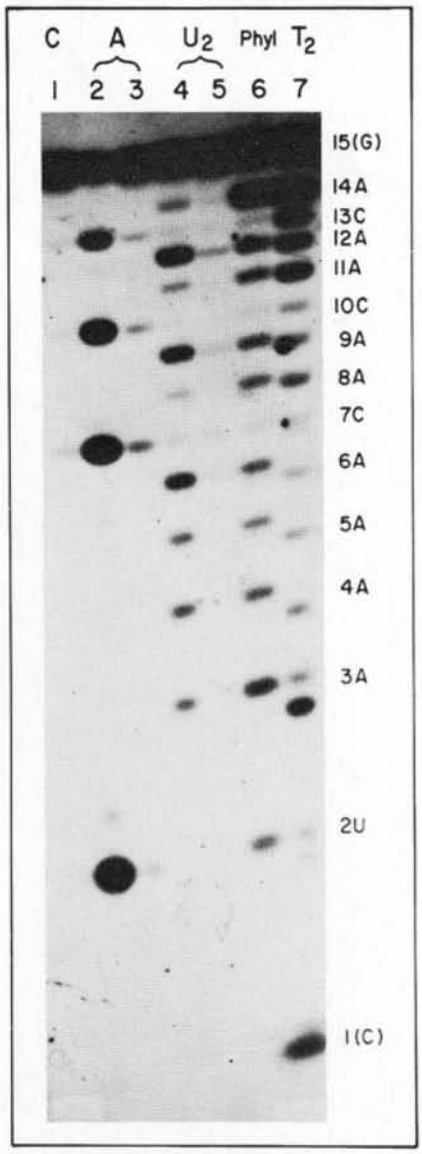


Fig. 3. Autoradiogram of polyacrylamide gel used for nucleotide sequence determination of a RNase T_1 resistant oligonucleotide. The following samples were loaded onto the gel.
No. 1: Unfragmented oligonucleotide; nos. 2 and 3: Oligonucleotide partially digested with RNase A (cleaves after U and C residues); nos. 4 and 5: Oligonucleotide partially digested with RNase U_2 (cleaves after A residues); no. 6: Oligonucleotide digested with Phy RNases (cleave after A and U); no. 7: Oligonucleotide partially digested with RNase T_2 (cleaves after all residues)

est to the 3′ end of the genome. We have fingerprinted several size classes of Akv-RNA that were selected to contain poly A by passage over an oligo dT-cellulose column. The order of the T_1 resistant oligonucleotides along the genome is given in Table 1. No order of the oligonucleotides within the brackets

Table 1. Ordering of T_1 resistant oligonucleotides on the Akv genome

Group No.	Nucleotide No.
I 5′ region	(1, 1A, 2, 3, 5, 12, 15, 16, 21, 35, 38, 40A, 41, 43, 49, 54A, 59, 60, 81, 98)
II	(4, 6, 7, 13, 13B, 26, 28, 29, 37, 37A, 50)
III	(8, 9, 13A, 17, 20, 22, 24, 31A, 33, 39, 46, 64, 80)
IV	(10, 11, 18, 31, 40B, 40C, 44, 47, 56, 98A)
V 3′ region	(68, 65, 69, 57, 42, 99, 55B, 63, 55A, 34, 25)

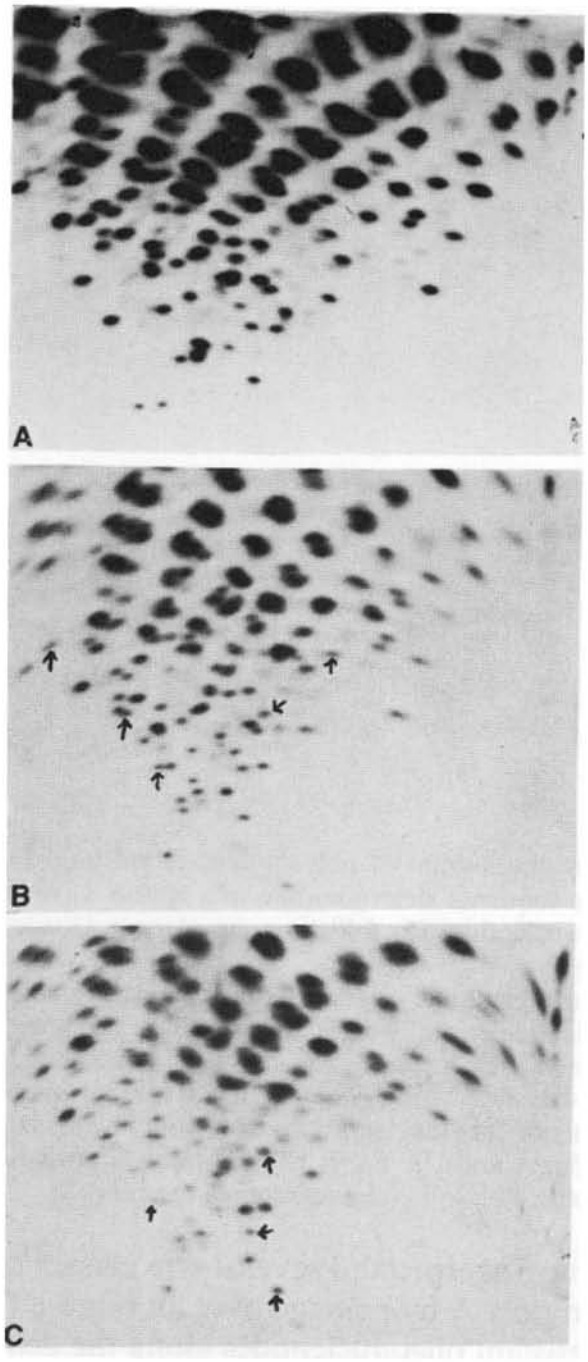


Fig. 4. RNase T_1 fingerprint of RNA from A. Akv B. MCF 247 C. MB34

is implied. The oligonucleotides numbered in Fig. 5 have been identified by complete or partial sequence analysis.

The fingerprints of two polytropic MCF viruses (MCF247 and MB34) are presented in Fig. 4. Comparison of these fingerprints with that of the Akv virus shows that these viruses are structurally related.

The majority (60%) of the oligonucleotides are shared by all three isolates. However, there are several oligonucleotides that are unique to each polytropic isolate, and some of these are indicated by the arrows in Fig. 6. Moreover, a common set of oligonucleotides characteristic for the Akv virus is missing in each of the MCF viruses. These include oligonucleotides 8, 9, 13A, 17, 20, 31A, 46, 10, 11, 31, 56, 98A, 21. These are located near the 3′ end of the gen-

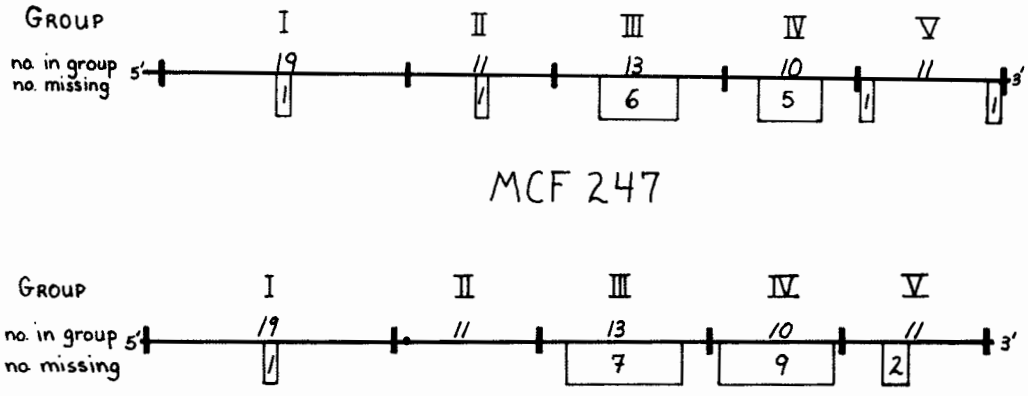


Fig. 5. Diagrams showing the location on the Akv virus genome of RNase T_1 resistant oligonucleotides absent in the two MCF viruses MCF247 and MB 34

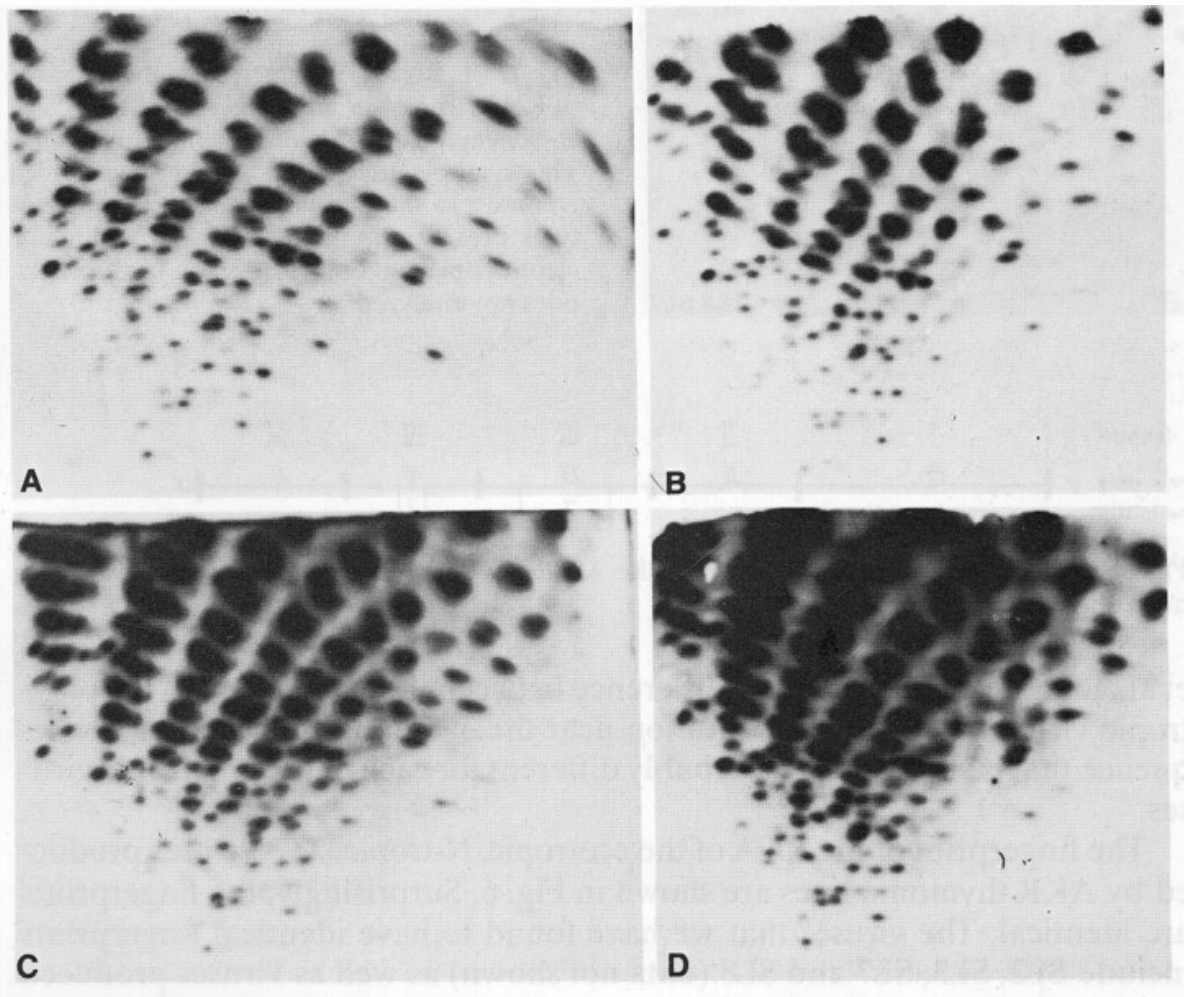


Fig. 6. RNase T_1 fingerprints of RNA from A. AKR SL2 B. AKR SL3 C. AKR SL7 D. AKR SL3 Cl 1

ome. Several other Akv oligonucleotides are missing from the fingerprint of each polytropic virus. Some but not all, are located near the 3′ end of the genome. A schematic diagram that portrays the location along the genome of Akv oligonucleotides missing from the fingerprints of each polytropic virus is given in Fig. 5. This data is consistant with the observation of Rommelaere

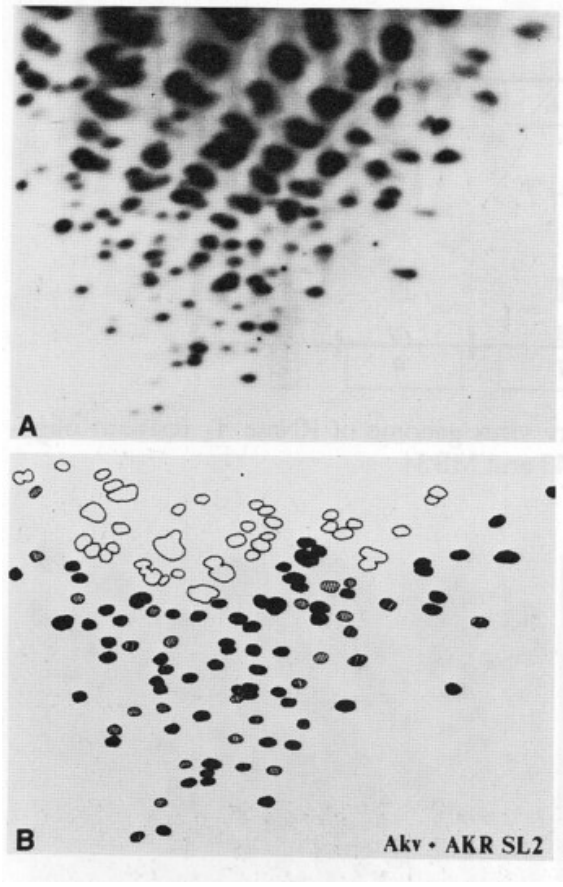


Fig. 7. *A.* RNase T_1 fingerprint of a mixture of RNAs from Akv virus and AKR SL2.
B. Schematic diagram of the same fingerprint. The symbols used are: ● oligonucleotides common to the two viruses; ○ oligonucleotides present only in AKR SL2; ○ oligonucleotides present only in Akv; ○ oligonucleotides not analyzed

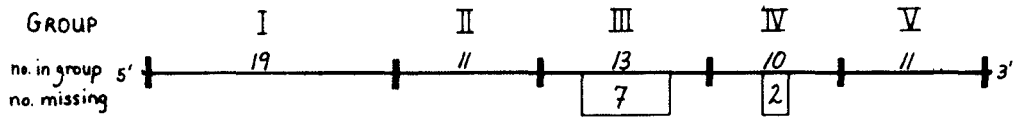


Fig. 8. Diagram showing the location on the Akv virus genome of RNase T_1 resistant oligonucleotides absent in AKR SL2

et al., that the major structural difference between the Akv virus and the polytropic virus consists of a substitution near the 3′ end of the genome. The sequence that is substituted is porbably different for each of the polytropic viruses.

The fingerprint of the RNA of the ecotropic, N-tropic, XC^- viruses produced by AKR thymoma lines are shown in Fig. 6. Surprisingly, the fingerprints are identical. The viruses that we have found to have identical fingerprints include SL2, SL3, SL7 and SL8 (data not shown) as well as viruses produced by cloned cells derived from SL2 (not shown) and SL3. We shall call this virus the AKR-SL (spontaneous lymphoma).

The AKR-SL virus is structurally related to the Akv virus. The majority of the oligonucleotides have the same electrophoretic mobility as shown in Fig. 7. However, oligonucleotides are unique to the AKR-SL virus. Moreover, a set of oligonucleotides present in the Akv fingerprint is missing from the AKR-SL fingerprint. The missing oligonucleotides are all located near the 3′ end of the AKR genome as shown in Fig. 8. The set of oligonucleotides that is missing from the AKR-SL fingerprint includes some but not all of the oligo-

nucleotides located near the 3′ of the AKR genome that are missing from all the polytropic viruses.

These studies identify structurally a new class of AKR virus. This virus, the ecotropic, N-tropic XC^- is the major species produced by AKR thymoma cells lines that have been established in culture. It probably differs from the Akv virus by a substitution in the 3′ region of the genome. Some of the SL lines also produce detectable levels of either xenotropic or polytropic viruses. The amount of these viruses is not sufficient to appear in the fingerprints. However, passage of this virus on mink cells would favor enrichment of the virus for xenotropic and polytropic components.

The relationship of the AKR-SL virus to the induction of the disease is unclear. Whereas, SL2 and SL3 accelerate thymic disease in AKR mice, SL7 and SL8 do not (Nowinski and Hays, 1978). It is possible that either defective genomes or viruses produced at lower titers that the AKR-SL virus induce the disease.

We have begun a detailed characterization of the genome of a xenotropic virus AKR-6, a virus that was derived from a thymus of a two month old AKR mouse by Janet Hartley. The fingerprint of this virus (Fig. 9) is very different

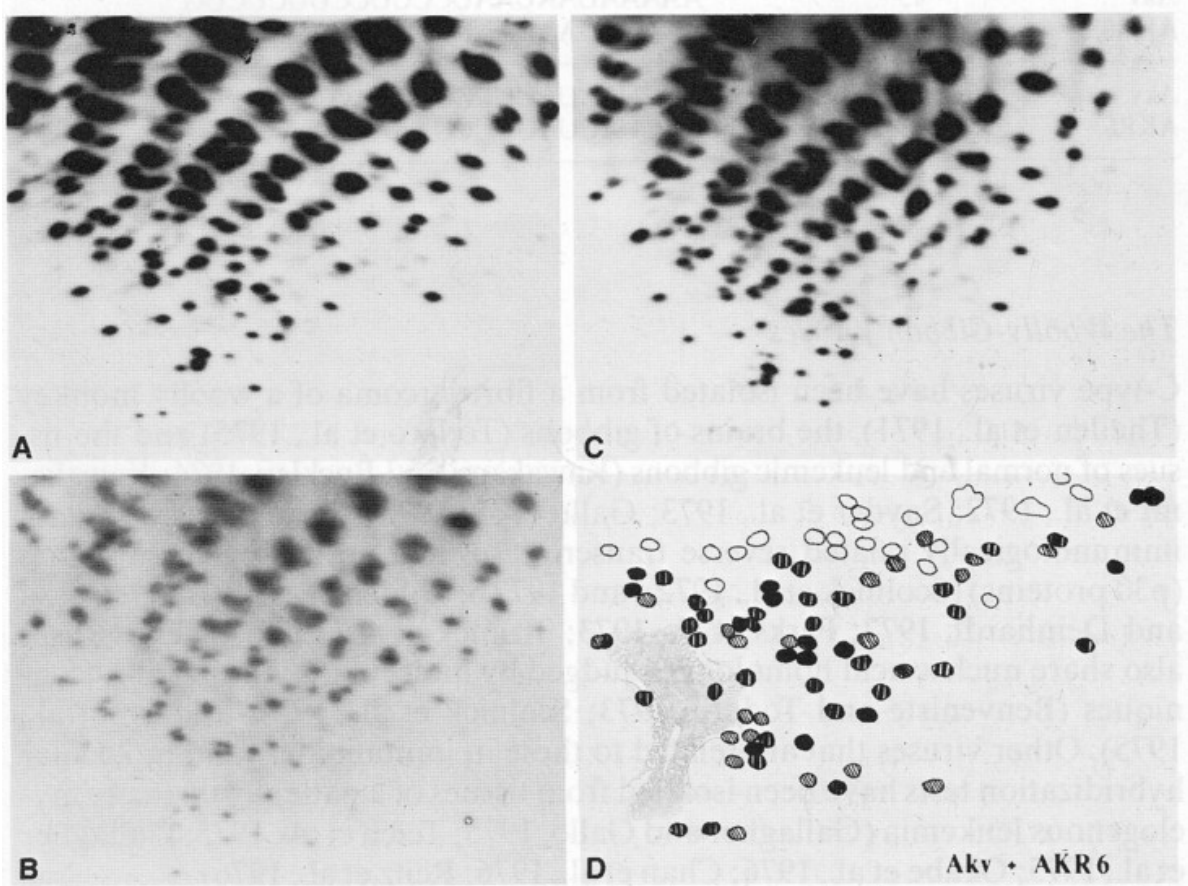


Fig. 9. Comparison of the RNase T_1 fingerprint of RNA from Akv virus (A) with the fingerprint of RNA from the xenotropic virus AKR6 (B) Panel C shows a RNase T_1 fingerprint of a mixture of RNA from the two viruses. (D) Schematic diagram of the picture shown in C. The symbols used are: ● oligonucleotides present in both viruses; ○ oligonucleotides present only in AKR6; ○ oligonucleotides only in Akv; ○ oligonucleotides not analyzed

from that of the Akv virus. Only a minority of the oligonucleotides have the same electrophoretic mobility. However, sequence analysis of some of the unique oligonucleotides shows that these viruses share many common sequences. The sequence of three oligonucleotides is identical in both viruses. Of ten large AKR-6 specific oligonucleotides that have been sequenced, five were found to be related by one or two base changes to oligonucleotides present in the Akv fingerprint (Table 2). It is noteworthy that a majority of the oligonucleotides present in the AKR-SL and AKR polytropic virus that are not present in the Akv virus are not derived from AKR-6, at least as judged by their electrophoretic mobility.

Table 2. Examples of related sequence in Akv and AKR6 RNA

Virus	Oligonucleotide No.	Nucleotide sequence
Akv	1	UAUCUCCCAAACUCUCCCCCUCUCCAACG
AKR 6	101	UCUCUCCCAAACUCUCCCUCUCUCCAACG
Akv	15	AAAAAUAAUAAUCCUUCCUUCUCUG
AKR6	108	AAAAAUAAUAACCCUUCCUUCUCUG
Akv	18	AUCUACUAUUCCUAAAAG
AKR6	113	AUCUACUAUUUCUAAAAG

The Woolly-Gibbon Viruses

C-type viruses have been isolated from a fibrosarcoma of a woolly monkey (Theilen et al., 1971), the brains of gibbons (Todaro et al., 1975) and the tissues of normal and leukemic gibbons (Kawakami and Buckley, 1974; Kawakami et al., 1972; Snyder et al., 1973; Gallo et al., 1978). These viruses contain immunologically related reverse transcriptases and group-specific antigens (p30 proteins) (Scolnick et al., 1972a and 1972b; Gilden et al., 1974; Hoekstra and Deinhardt, 1973; Parks et al., 1973; Tronick et al., 1975). These viruses also share nucleic acid homology as judged by molecular hybridization techniques (Benveniste and Todaro, 1973; Scolnick et al., 1974; Todaro et al., 1975). Other viruses that are related to these in immunologic and molecular hybridization tests have been isolated from tissues of a patient with acute myelogenous leukemia (Gallagher and Gallo, 1975; Teich et al., 1975; Gallagher et al., 1975; Okabe et al., 1976; Chan et al., 1976; Reitz et al., 1976).

To elucidate the structural relationships among these viruses, we have fingerprinted the genomes of the woolly monkey virus (SSAV or WLV) and several independently isolated gibbon ape viruses (GaLV) as well as virus isolated from the cultured cells of a patient with acute myelogenous leukemia (HL23V).

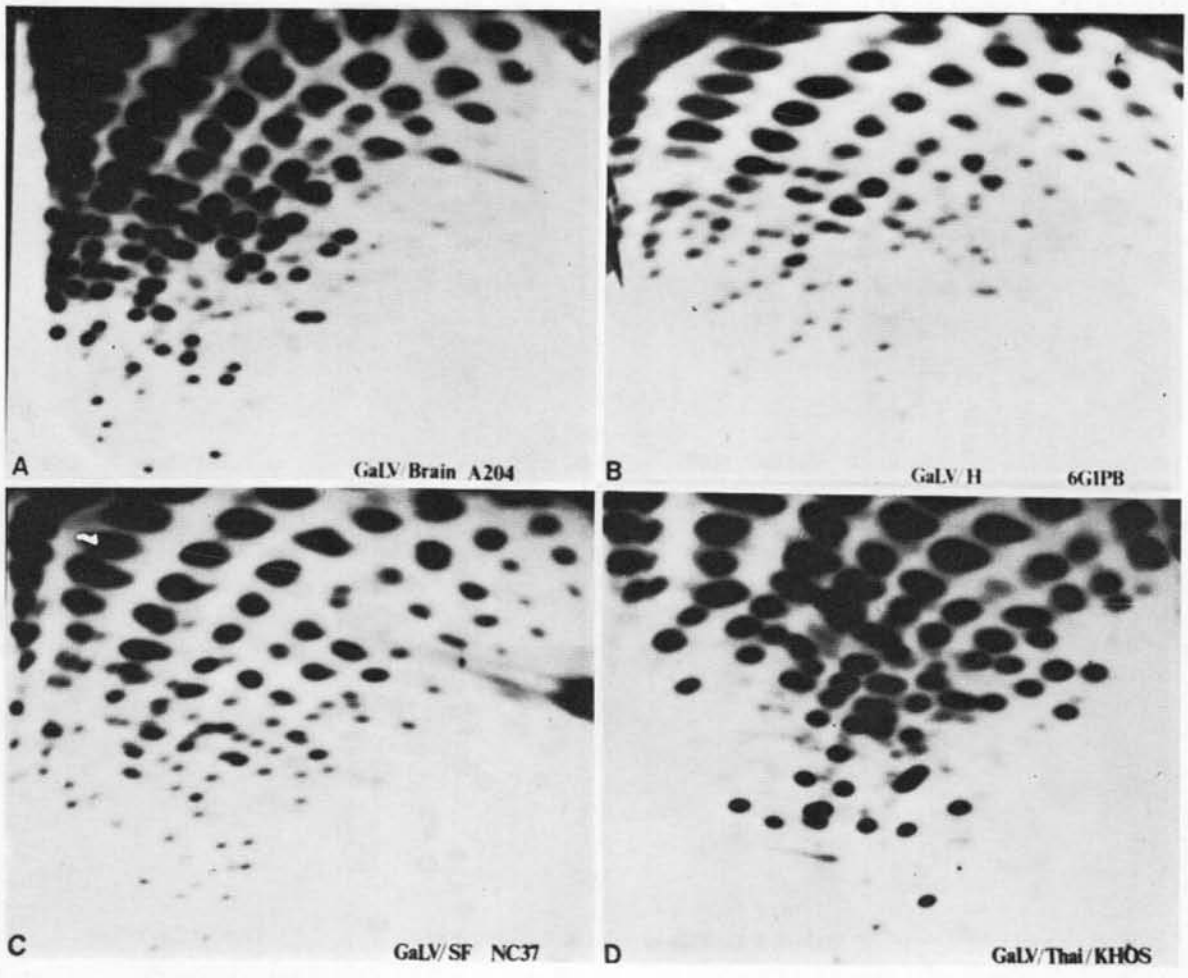


Fig. 10. RNase T_1 fingerprints of four isolates of gibbon ape viruses. A. $GaLV_{Brain}$, isolated by cocultivation of normal gibbon brain tissue with human rhabdomyosarcoma cells (A204) (Todaro et al., 1975). Virus was a gift from G. Todaro. B. $GaLV_H$, isolated from the blood of a gibbon with acute lymphatic leukemia (6 GIPB) (Gallo et al., 1978). Virus was obtained from R. Gallagher. C. $GaLV_{SF}$, isolated from a lymphosarcoma and grown in human lymphoblastoid cells (NC37) (Kawakami et al., 1972; Snyder et al., 1973). D. $GaLV_{Thai}$, isolated from a gibbon with granulocytic leukemia and grown in human osteogenic sarcoma cells transformed by Kirsten sarcoma virus (KHOS) (Kawakami and Buckley, 1974). Both $GaLV_{SF}$ and $GaLV_{Thai}$ were given to us by M. Reitz

The RNase T_1 fingerprints of the genomes of four independent isolates of gibbon virus and of SSAV are shown in Figs. 10 and 13. The genomes of two of the gibbon viruses, $GaLV_H$ and $GaLV_{Brain}$, are closely related to one another as judged by the similarities of their RNase T_1 oligonucleotide patterns (Fig. 11). By studying the oligonucleotides which comigrated on polyacrylamide gels when RNase T_1 digests of $GaLV_H$ and $GaLV_{Brain}$ RNAs were mixed, we estimate that a minimum of 12% of the genomes are identical. The sequence of more than a quarter of these oligonucleotides was studied. In most cases, oligonucleotides which comigrated had the same sequence. The two oligonucleotides whose sequences were not identical appeared to be a mixture of more than one RNase T_1 product. In contrast, only 2,5% of the genome of each virus is distinct. Assuming that the oligonucleotides are randomly positioned as much as 70% of the genomes could be identical.

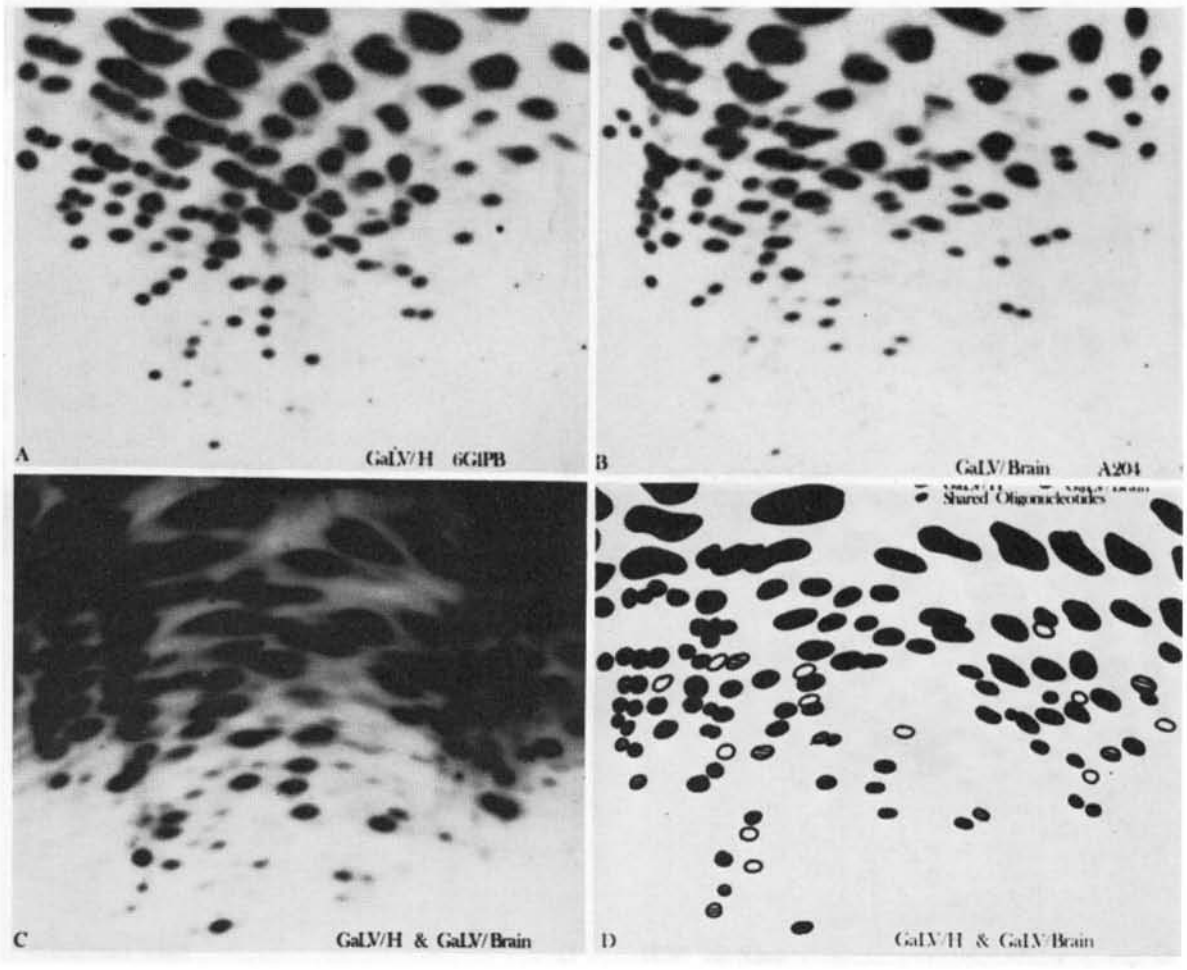


Fig. 11. Comparison of the genomes of two gibbon ape viruses, $GaLV_H$ and $GaLV_{Brain}$. The RNase T_1 fingerprints of A. $GaLV_H$, B. $GaLV_{Brain}$ and C. a mixture of $GaLV_H$ and $GaLV_{Brain}$ are shown. D. A schematic illustration of the mixture shown in panel C. The oligonucleotides shared by these two viruses as well as those unique to one or the other of the viruses are diagrammed. The assignment of two oligonucleotides as a shared sequence was also confirmed for twenty of the large oligonucleotides by the position of adenosine residues within each oligonucleotide by RNase U_2 digestion

The other gibbon viruses and SSAV show a smaler degree of similarity by fingerprint analysis of their genomes. For example, fewer of the longer characteristic oligonucleotides of SSAV RNA and $GaLV_{SF}$ RNA have the same electrophoretic mobility (Fig. 12). The fingerprints of the viruses shown in Figs. 10, 11 and 12 demonstrate that this method provides a means of identifying viruses and of distinguishing between closely related isolates of primate viruses.

We have also fingerprinted the genomes of SSAV viruses with different passage histories (Fig. 13). The RNase T_1 fingerprints of these viral RNA isolates are very similar but not identical to one another. WLV RNA has only two oligonucleotides not present in the SSV-1 genome whereas SSV-1 RNA has four oligonucleotides not found in the WLV genome. Because SSAV has only been isolated from a single woolly monkey, the nucleotide sequence of the viral RNA changed during passage.

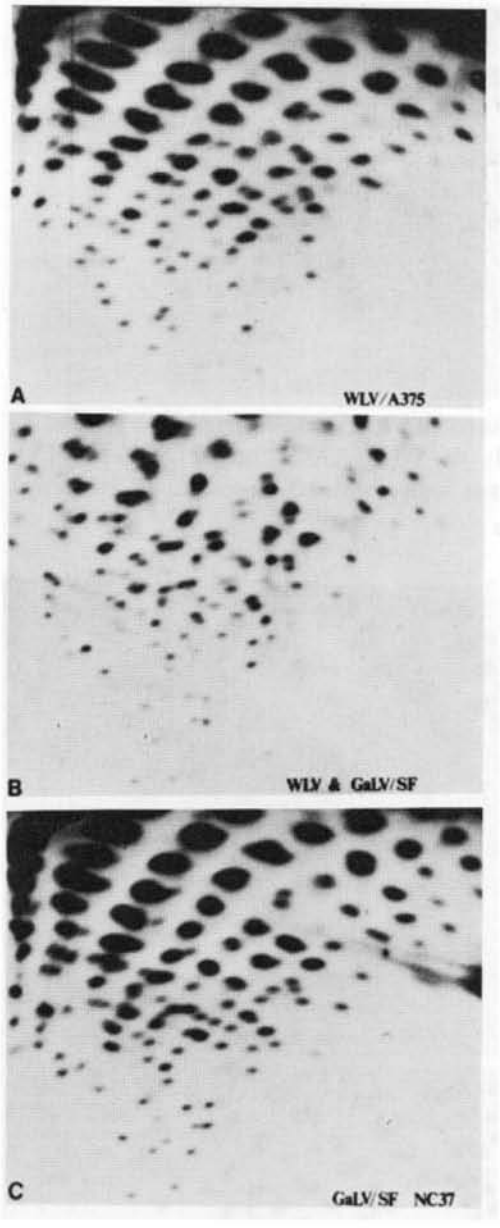


Fig. 12. Comparison of the genomes of Simian sarcoma associated virus, WLV, and gibbon ape virus, $GaLV_{SF}$. The RNase T_1 fingerprints of A. WLV/A375, isolated from a fibrosarcoma of a woolly monkey (Theilen et al., 1971) and grown in human cells given to us by S. Aaronson; B. a mixture of WLV/A375 and $GaLV_{SF}$ and C. $GaLV_{SF}$ are shown

The RNase T_1 fingerprints of the genomes of viruses released by cultivation of leukemic tissues from a patient with acute myelogenous leukemia (HL23V) are presented in Figs. 14 and 15. These viral isolates are closely related to SSAV. The majority of the oligonucleotides of HL23V RNA have the same electrophoretic mobility as those of WLV RNA (Fig. 14C). Studying the oligonucleotides which are present only once in the genome and shared by WLV RNA and HL23V RNA, there appears to be a minimum of 11% of the genomes which are identical. We determined the position of the adenosine residues in one-half of the oligonucleotides which comigrated on polyacrylamide gels when digests of the two RNAs are mixed. In all cases, oligonucleotides which comigrated had the same sequence. However, a minority of the genome is unique to the HL23V isolate (3%) or to WLV (1,5%). Assuming that these oligonucleotides are present throughout the genome, we estimate that as much as 75% of these viral RNAs are identical.

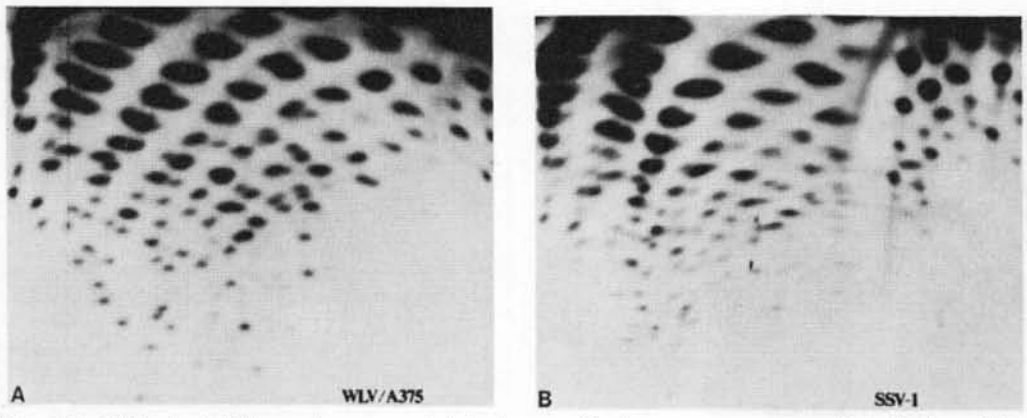


Fig. 13. Effect of different passage histories on Simian sarcoma associated virus. The RNase T_1 fingerprints of two SSAV virus isolates are compared: A. WLV/A375 and B. SSV-1, SSAV grown in human lymphoblastoid cells (NC37). This virus was obtained from the FCRC Viral Resources Laboratory

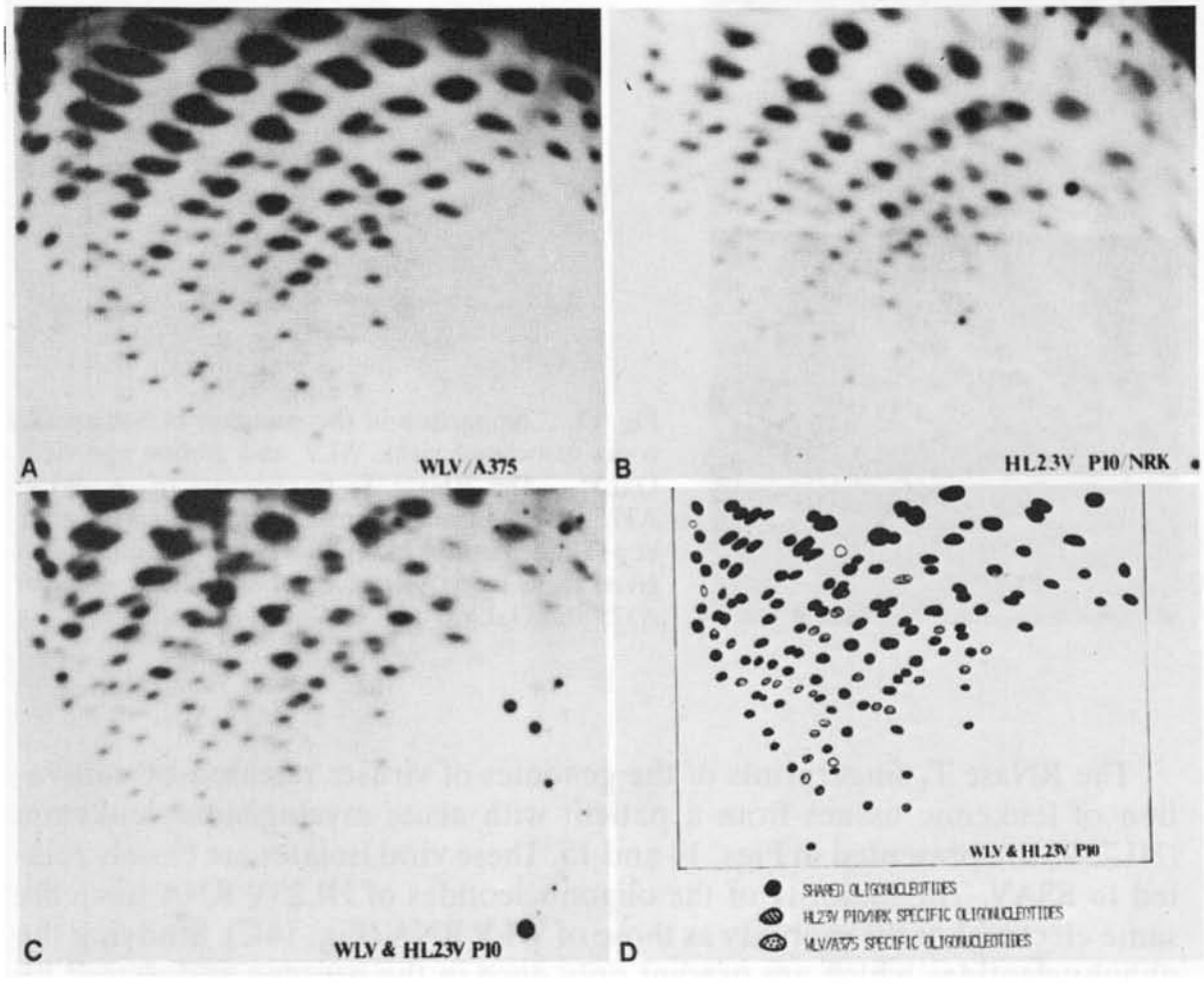


Fig. 14. Comparison of the genomes of Simian sarcoma associated virus, WLV, and a virus, HL23V, released by cultured human myeloid leukemia cells. The RNase T_1 fingerprints of A. WLV/A375; B. HL23V p10/NRK, isolated by cultivation of leukocytes from the first peripheral blood sample that had been passaged ten times before the culture fluid was used to infect normal rat kidney fibroblasts (Teich et al., 1975; Gallagher and Gallo, 1975); and C. a mixture of WLV/A375 and HL23V p10/NRK. D. a schematic illustration of the mixture shown in panel C. The oligonucleotides shared by these two viruses as well as those unique to one or the other of the viruses are diagrammed. The assignment of two oligonucleotides as a shared sequence was also confirmed for many of the large oligonucleotides by the position of adenosine residues within each oligonucleotide by RNase U_2 digestion. HL23V p10/NRK RNA was obtained from M. Reitz

when injected into newborn kittens (Jarrett et al., 1973; Essex, 1975). Moreover, epidemiological evidence demonstrates that these viruses are the causative agents of feline leukemia in domestic cat populations (Hardy et al., 1973; Cotter et al., 1975; Hardy et al., 1976; Essex et al., 1977).

The feline viruses have been shown to be structurally related by tests of molecular hybridization (Levin et al., 1976). However, they are not identical. These viruses have been divided into three groups, FeLV A, B, and C, on the basis of neutralization and interference tests (Sarma and Log, 1973). These viruses also differ in their host range (Jarrett et al., 1973; Sarma et al., 1975).

To determine the degree of structural similarity of these viruses at the level of nucleic acid sequence, fingerprint analysis of viruses of the three subgroups was done. The viruses analyzed include the cloned isolates FeLV A/Glasgow 1, FeLV B/Sarma (ST-FeSV), and FeLV C/Sarma (FL74) that were grown in feline embryo fibroblasts, and FeLV A/Rickard that was produced by an established lymphoid tumor line F422 (Rickard et al., 1969).

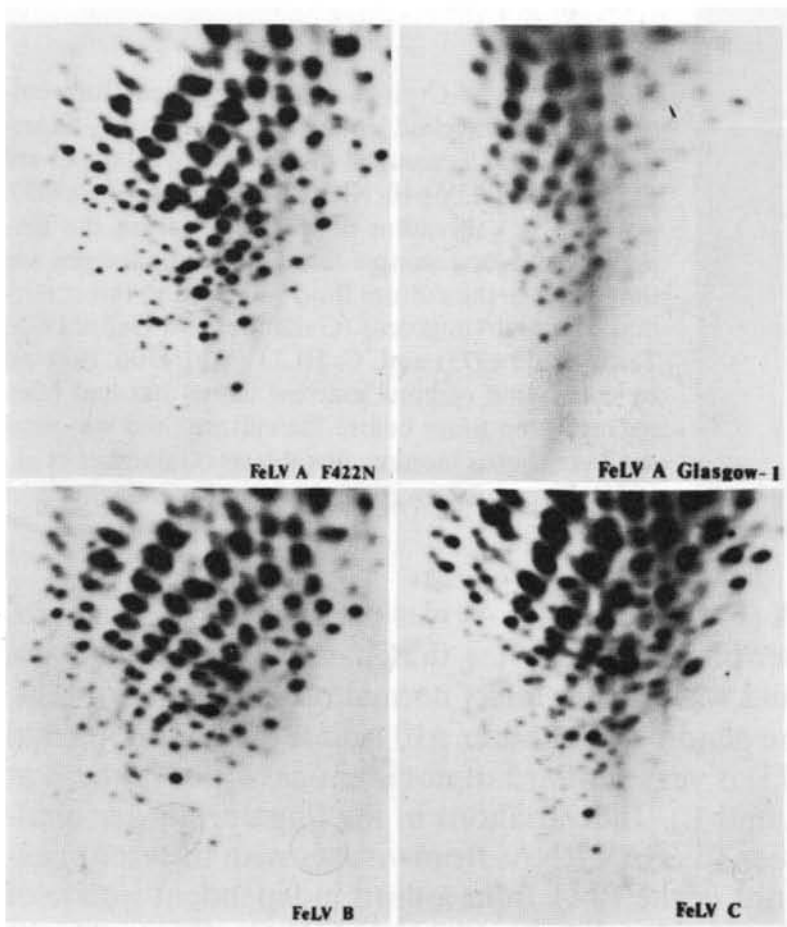


Fig. 16. RNase T_1 fingerprints of the subgroups of FeLV. *Upper left:* FeLV A/Rickard isolated and grown in F422 cells, a spontaneous lymphoid cell line, provided by M. Essex. *Upper right:* FeLV A/Glasgow 1 isolated from a cat with alimentary lymphosarcoma and grown in feline embryo fibroblasts. *Lower left:* FeLV B/Sarma (ST-FeSV), the purified subgroup B helper virus from a stock of Snyder-Theilen FeSV, grown in feline embryo fibroblasts. *Lower right:* FeLV C/Sarma (FL74) purified from a mixture of subgroups A, B, and C isolated from a spontaneous lymphoid cell line, FL 74, and grown in feline embryo fibroblasts. FeLV A/Glasgow 1, FeLV B and FeLV C were provided by O. Jarrett

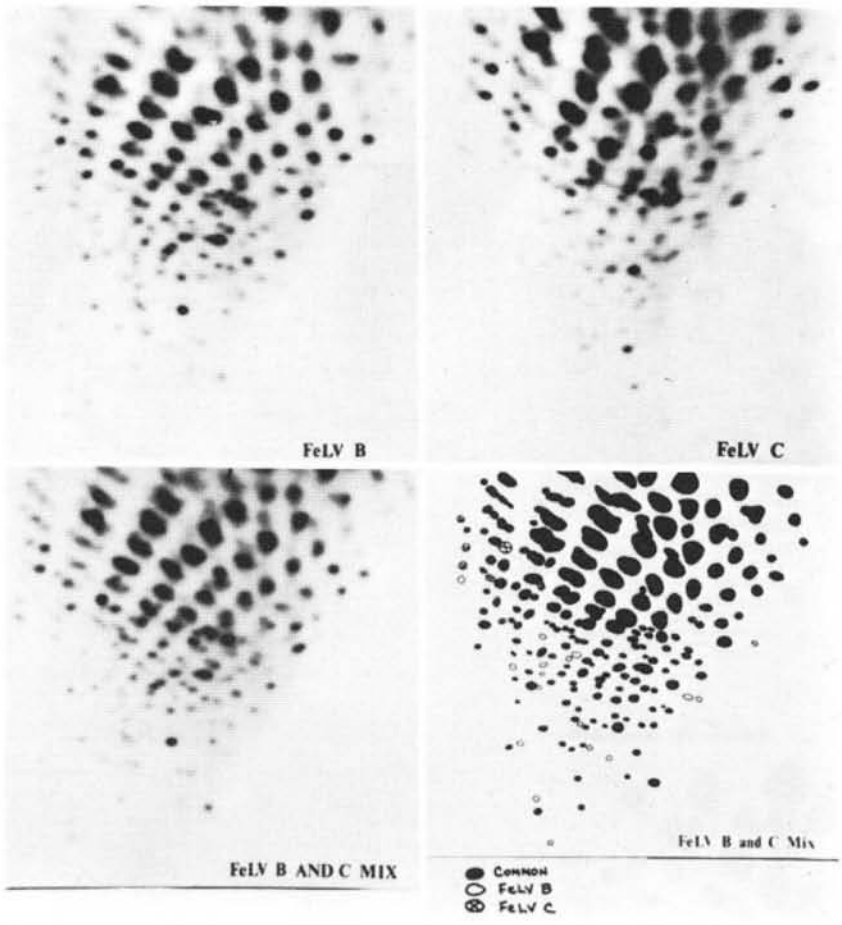


Fig. 17. Comparison of the genomes of FeLV: RNase T_1 fingerprints of isolates from subgroups Band C. *Upper left:* FeLV B/Sarma (ST-FeSV). *Upper right:* FeLV C/Sarma (FL74). *Lower left:* Mixture of FeLV B and FeLV C. *Lower right:* Schematic drawing of a mixture of subgroups B and C

The fingerprints of these viruses are presented in Fig. 16. To determine the degree of structural similarity, RNase T_1 oligonucleotides from the subgroups were mixed together and the resulting fingerprints are shown in Fig. 17. Experiments done but not pictured include mixes of FeLV A/Rickard and FeLV A/Glasgow 1, FeLV A/Rickard and FeLV B/Sarma (ST-FeSV), and FeLV A/Rickard and FeLV C/Sarma (FL74).

These experiments demonstrated that the feline leukosis viruses are structurally closely related. Over half the unique oligonucleotides of each isolate are found in all the isolates studied as judged by their electrophoretic mobility in the mixing experiments. This set of "common" FeLV oligonucleotides is diagrammed in Fig. 18. However, a set of oligonucleotides unique to each isolate was also observed. The fingerprints of the two subgroup A viruses studied were not identical.

The similarity in the fingerprints of the feline leukosis viruses suggests that approximately half of the genome is identical.

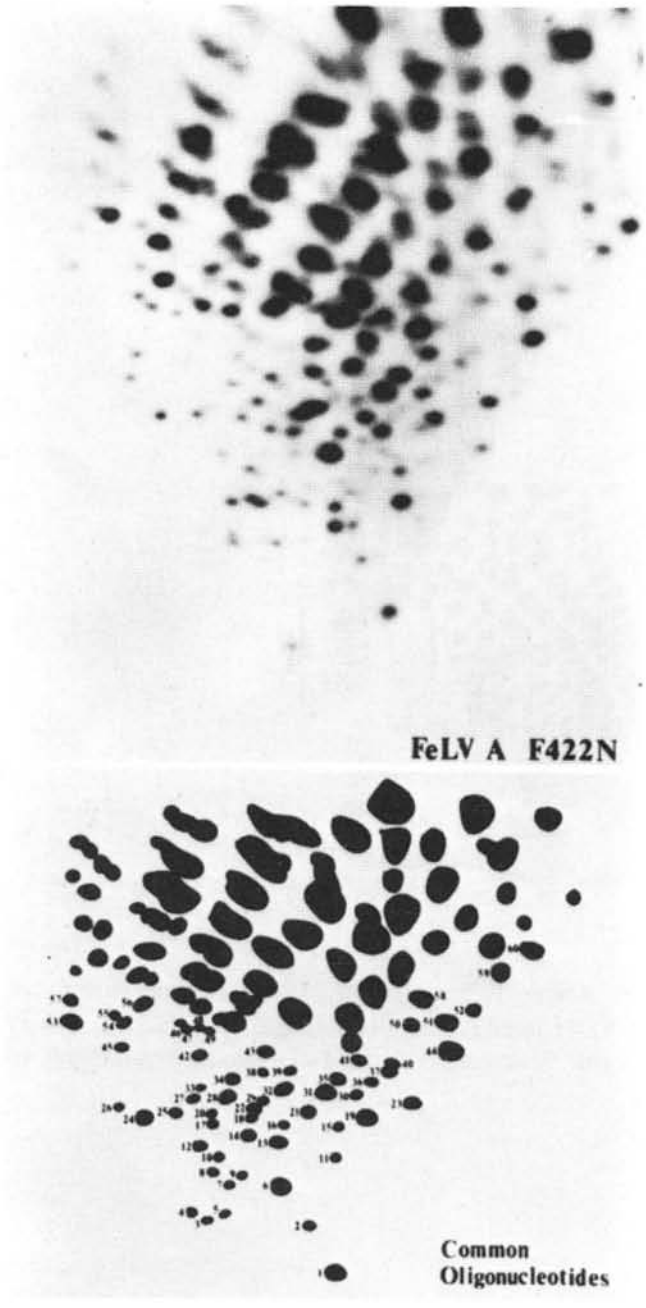
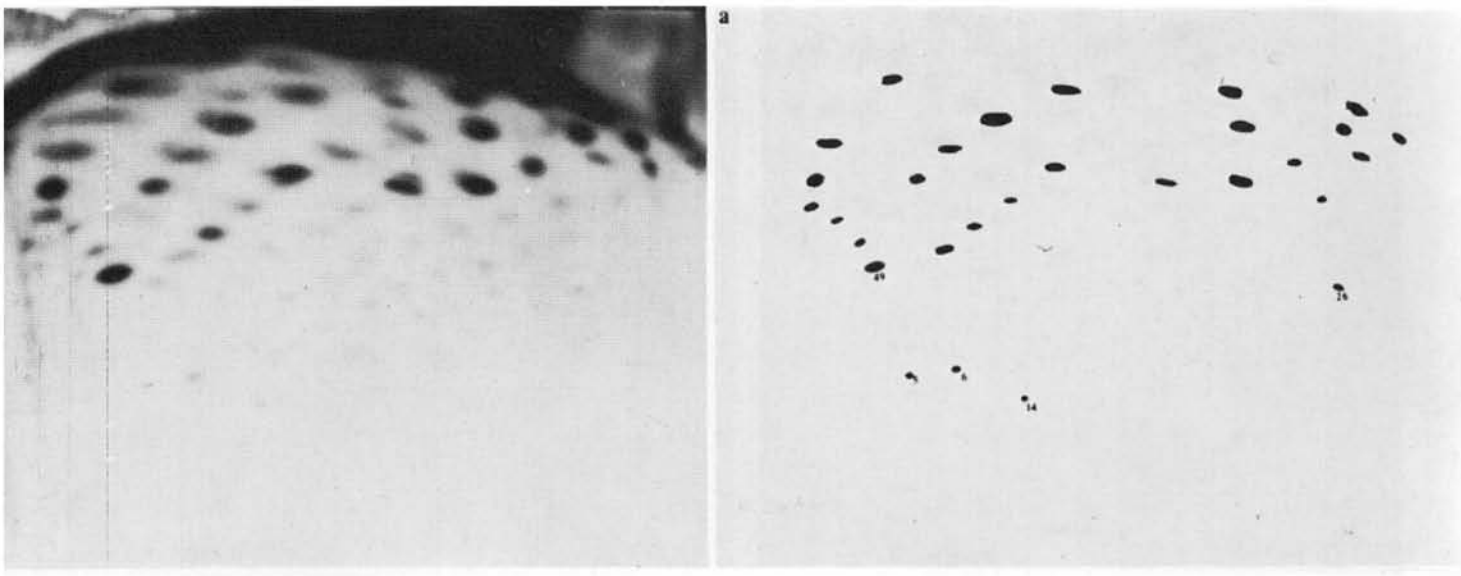


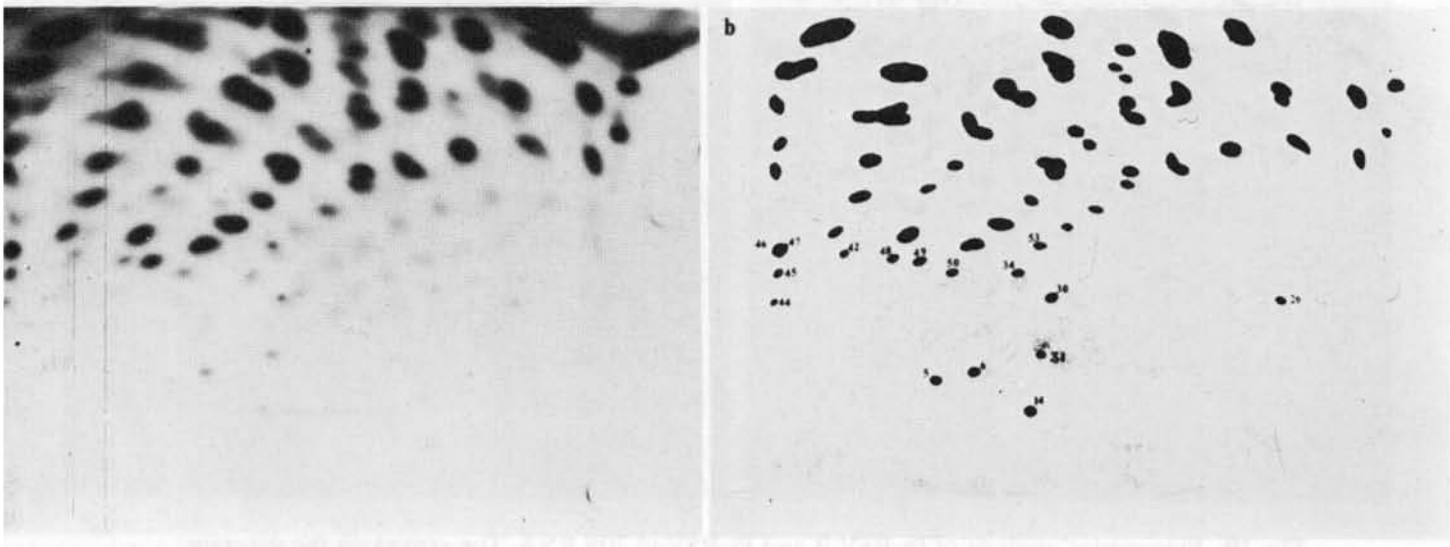
Fig. 18. RNase T_1 oligonucleotides shared between FeLV A/Rickard, FeLV A/Glasgow 1, FeLV B/Sarma (ST-FeSV), and FeLV C/Sarma (FL74). *Top:* Representative fingerprint of FeLV, FeLV A/Rickard. *Bottom:* Schematic drawing of the "common" oligonucleotides

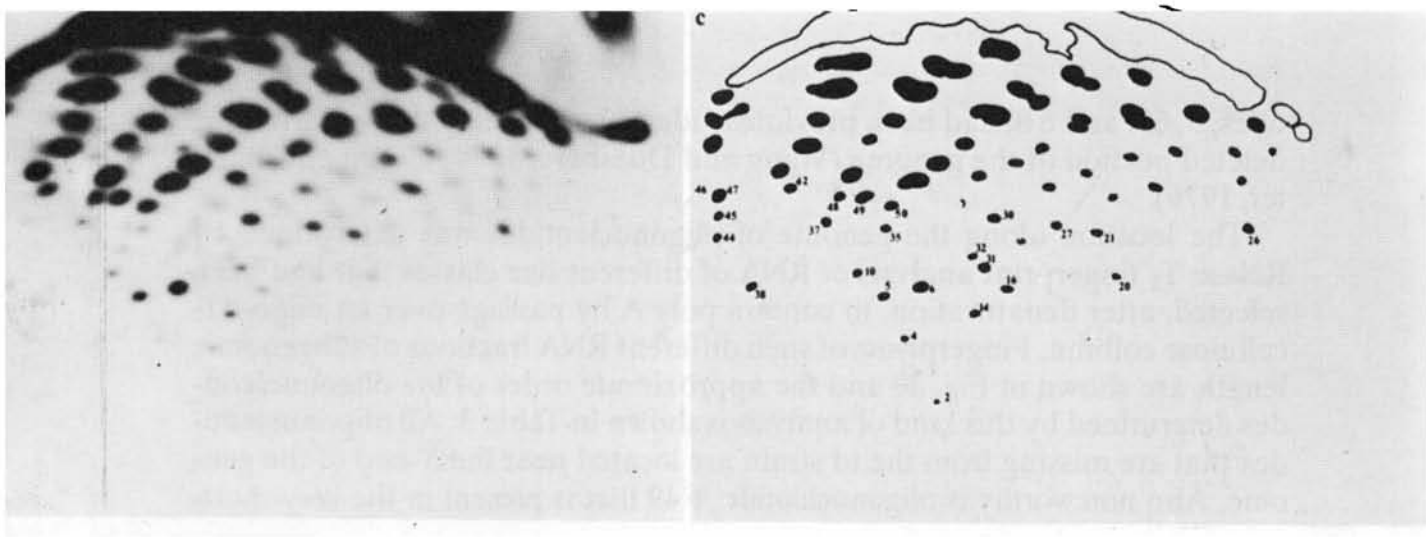
Rous Sarcoma Virus

We have applied the improved fingerprinting technique to Rous sarcoma virus Prague B in order to obtain more detailed information regarding the sequences of the sarcoma gene. A variant of this strain of virus has been isolated that lacks the capacity to transform fibroblasts in culture (Coffin and Billeter, 1976). This transformation defective strain is deleted for a portion of the genome, about 1500 nucleotides near the 3′ end of the genome (Junghans et al., 1977).

RNase T_1 fingerprints of the non-defective (nd) and transformation defective (td) strains of the virus are shown in Fig. 19. The arrows indicate those oligonucleotides that are missing from the td strain. The missing oligonucleotide include numbers: 5, 6 (A + B), 48, 42, 45 and 44. Three of the oligonucleo-


a


b


c

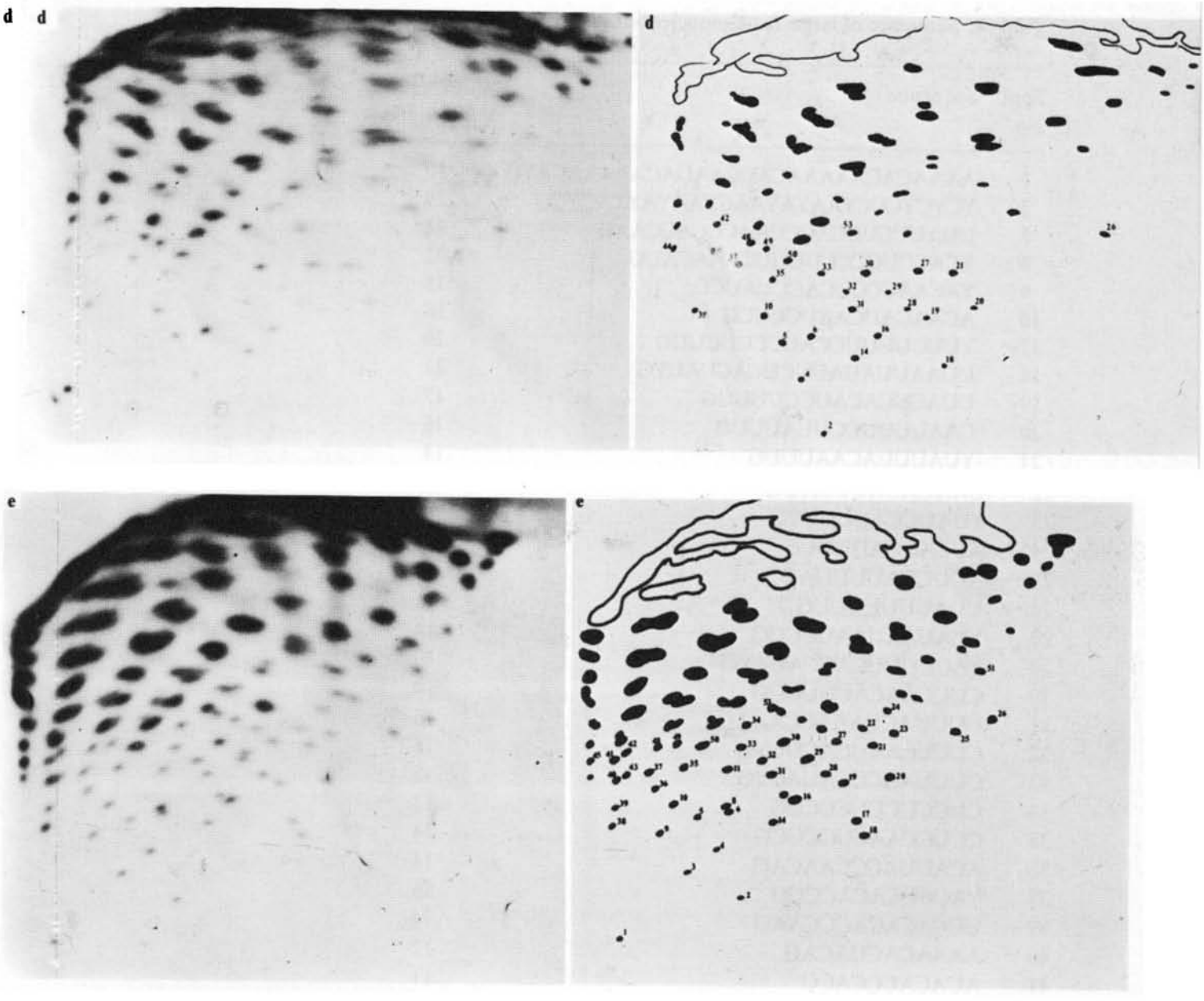


Fig. 20. Mapping of RNase T_1 oligonucleotides derived from different sizes of Pr-RSV-B RNA fragments. RNA fragments obtained by heating 35S poly-A-containing RNA, were separated by sedimentation in sucrose density gradient, followed by poly-A-selection on oligo-T-cellulose column. The size of RNA fragments increase in order of a, b, c, d and e. Panel E shows the fingerprint of 35S RNA

Table 3. Order of large T_1 Oligonucleotides on Pr-B RSV RNA

5′ end (11, 8, 16B, 15, 24, 23, 25, 3, 17, 39) (18, 1, 36, 22, 41, 20, 40, 43, 9, 33, 29, 28, 12, 9) (50B, 4, 35, 16A, 2, 32, 20, 10, 37, 38, 27) (21, 53, 50A, 30, 31, 48, 42, 47, 46, 45, 44) (26, 14, 6, 5, 49)-poly A

[a] Interferred by visual examination of the fingerprints of fractionated Pr-B RNA, as described in Fig. 20. The order of oligonucleotides within parentheses is undetermined

Table 4. Sequence of large T_1 oligonucleotides from Pr-B 70S RNA

Spot No.	Sequence	Chain length
1	AAAACACAAAAACACCAAUACAAAAACAYG	30
2	YCYCYCCCYAAYAYAACYAAYAYYACYCG	29
3	UUAUUCUCCACCCAACCCACCAAG	24
4	YCACCUCCCCUUUUCAAACAUG	22
9	YACAACCCUCACCUAUCG	18
10	ACAACAUCACUCCUCG	16
17	YUUCUUUUCCAUCCCUCUUG	20
18	UUAAUUAUAUUCUCACUAUYG	21
19	UUACUCACAUCCUUUUG	17
20	CAAUUUUCCUUAUUUG	16
21	YUAUUUACAAUUUG	14
22	UAAUAUAUCUAUG	13
23	YUAUCCAUUUUYG	13
24	AUCACUAUUUUG	12
25	AUUUCAUUUUAG	12
26	UUAUUUUAUUG	11
27	YUAUAAUAAUCCUG	14
29	YACUUUUCACCAUAYG	15
30	CUUCAACACUUCUG	14
31	UUUUACUAAACCAAAG	16
32	CUCUCAAUCACUUYG	15
33	CUUUACCCCAUACYG	15
34	CUCCUCUUCCCCG	13
35	CCUCCAACUCCUCG	14
36	ACAUUACCCAACAG	14
37	YACCUCACACCUG	13
39	UCCACACACCCAAG	14
40	AAAACACUACAG	12
41	AUACACCCACG	11
42	ACAACAAUACG	11
43	AYAUAAAAACAG	12
44	CACCCACCACG	11
45	YAAACCACAG	10
46	CAACACCAG	9
47	AACACCAAG	9
48	AUACAUCCCCAG	12
49	AUACAAUAAACG	12
52	AUAUUUYG	8
53	AUAAUAUACAUG	12

est poly A containing RNA fragment. This oligonucleotide is probably the one identified by Wang et al. (1975) as the C or constant oligonucleotide, present in both RSVtd and nd strains. This oligonucleotide is located between the td deletion and the 3′ end of the genome.

To further characterize the structure of the RSV genome the sequence of most of the unique oligonucleotides was determined as described above. The sequences are presented in Table 4. The table includes the sequence of oligonucleotides derived from the region deleted in the td strain.

The spots numbered 6, 16, 38 and 50 are not sequenced because they contain a mixture of oligonucleotides.

Discussion

The improved techniques of oligonucleotide fingerprinting using small unlabelled RNA have permitted us to extend the range of problems that can be approached using this method. Viruses that cannot be efficiently labelled in cell culture, either because of their low titer or because of the cell type (for example the lymphoid lines) can be studied by this method. Moreover, sequence determination of oligonucleotides permits very detailed comparative analysis of viral genomes. An example of the utility of this approach is the unexpected similarity in sequence of the unique oligonucleotides of Akv virus and the AKR-6.

This approach is not limited to the C-type viruses. The method is suitable for the study on any high molecular RNA species. We have, in collaboration with others, successfully applied the method to problems of human and avian influenza viruses, measles virus, vesicular stomatitis virus and silk worm fibroin message.

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Detection of Integrated Type-C Viral DNA Fragments in Two Primates (Human and Gibbon) by the Restriction Enzyme Blotting Technique

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There have been reports of sporadic findings of type-C markers in human cells. Most commonly these markers were related to the two classes of primate type-C viruses: The simian sarcoma virus-simian sarcoma associated virus complex [SiSV (SiSAV)] and gibbon ape leukemia virus (GaLV) group and the baboon endogenous virus, BaEV (see Gallo elsewhere in this book). However, detection of proviral sequences related to either of these virus groups was rare, and a preliminary survey indicated that DNA from leukemic tissues, although hybridizing more viral probe (SiSAV and BaEV) than DNA from normal tissues, displayed a broad range of hybridization values which never approached the level of DNA from virus-infected tissue culture cells (Gallo, elsewhere in this book). One possible explanation for the low but possibly significant hybridization values found in fresh leukemic cells is that subgenomic fragments could have been integrated in the DNA of these tissues rather than complete provirus. However, unequivocal evidence for partial provirus integration of type-C RNA viruses is still lacking in the literature. In this report we show by restriction enzyme-blotting analysis, that (i) a few tissues from a gibbon ape exposed to GaLV contained an incomplete provirus; (ii) DNA from *all* human DNA contained sequences that hybridize specifically to SiSV-SiSAV genomes, suggesting a recombination event between these viruses and human DNA via infection; (iii) DNA from two leukemia DNA samples showed *extra*, presumably acquired, viral fragments related to BaEV. One of the samples was from the uncultured leukocytes of patient HL23 (Gallo, elsewhere in this book). In addition one other human DNA sample showed *extra* viral fragments related to SiSAV.

Partial Provirus in a Gibbon Exposed to GaLV

We obtained tissues from two gibbons from a colony in Hall's Island, Bermuda, G-1 was leukemic (acute T-cell leukemia) and viremic, and a distinct isolate of GaLV (designed $GaLV_H$) was isolated from its leukocytes after culture (Gallo et al., 1978; Reitz et al., 1979). G-4 although terminally ill with emaciation (cause unknown), was not frankly leukemic or viremic, but its serum contained high titer of antibody against GaLV antigens (Gallagher et al., 1978). When DNA from tissues of G-4 were examined for GaLV proviral DNA sequences by hybridization, all tissues were negative with the exception

of the spleen, liver and kidney. DNA from these three tissues hybridized 30 to 50% of $GaLV_H$ or SiSAV probes relative to DNA from G-1 tissues (Wong-Staal, Reitz, and Gallo, 1979) suggesting that these tissues may harbor an incomplete provirus, although other possibilities exist. We therefore decided to analyze the samples by restriction enzymes followed by the blotting technique described by Southern (1975) to determine whether proviral fragments may be present. A schema of the procedure used is presented in Fig. 1. DNA digested with the site specific endonuclease is fractionated on agarose gels and then the DNA is "blotted" onto a sheet of nitrocellulose filter paper. The DNA positioned on these filters is hybridized to excess ^{125}I viral RNA and autoradiographs are prepared. Discrete fragments containing DNA sequences that hybridize the labeled probes can be visualized. As shown in Fig. 2, after digestion of the cellular DNA's with Bam HI, two viral fragments were detected in tissues of G-1, corresponding to sizes of 2,0, and 1,5 $\times 10^6$ daltons. However, DNA from the kidney and liver of G-4 contained only the $1{,}5 \times 10^6$ dalton fragment (Fig. 2). Since this fragment is subgenomic in size, the result clearly indicates that an incomplete provirus is integrated in

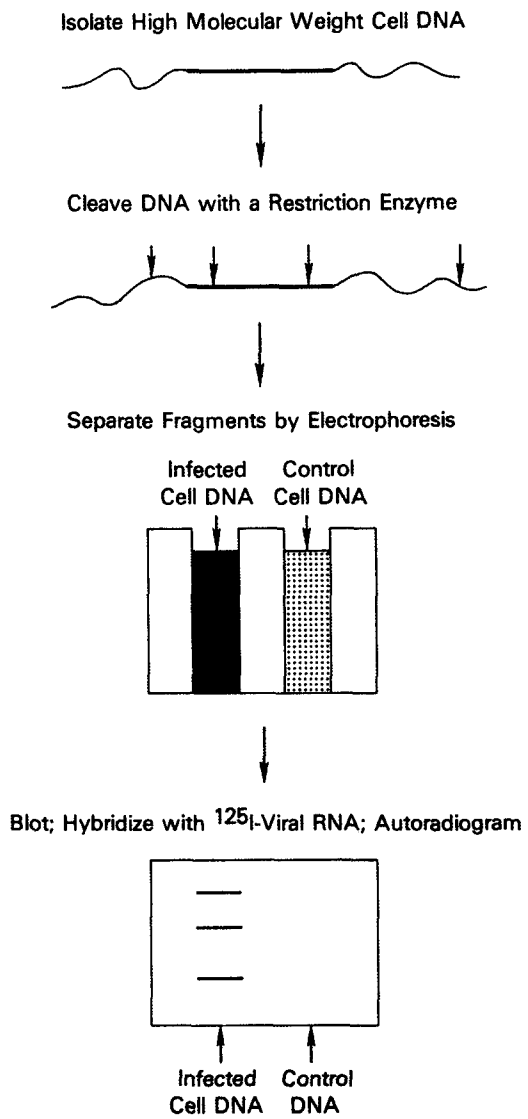

Fig. 1. Scheme for detection of integrated viral sequences by restriction enzyme-blotting. High molecular weight DNA is purified from cells or tissues and cleaved with a given restriction enzyme. Cleavage sites for the enzyme are distributed all over the DNA, including the provirus (indicated here by the dark straight line). The resultant heterogeneous collection of DNA fragments is then separated by electrophoresis on agarose gels for fractionation on the basis of size. After electrophoresis, staining with ethidium bromide reveals a broad distribution of DNA. However, after transfer of the DNA to nitrocellulose filter paper, only the fragments containing part or all of the provirus will hybridize to ^{125}I-viral RNA. In the hypothetical case presented in the schema, there are three fragments. These are visualized by autoratiography

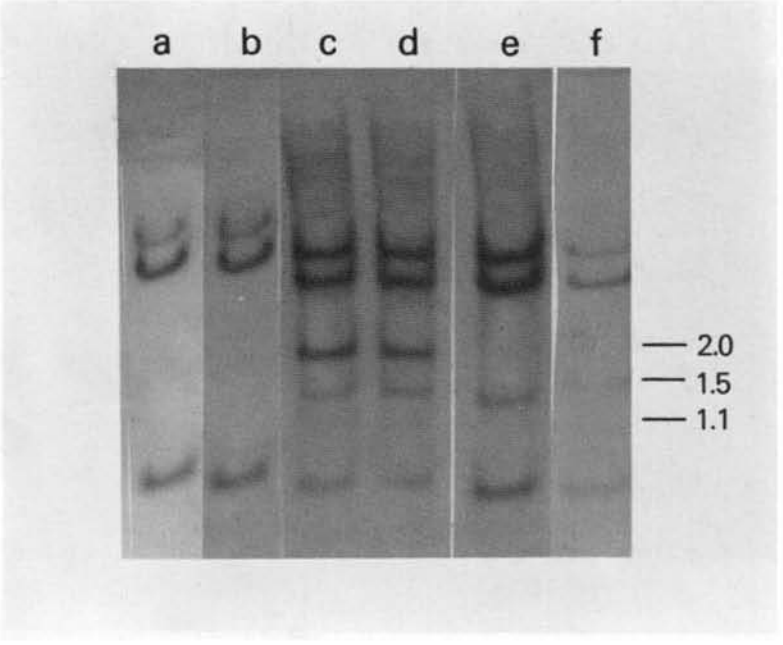

Fig. 2. Cleavage pattern of integrated viral sequences in DNA from tissues of G-1 and G-4. 25 μg of each cellular DNA was digested with Bam HI and electrophoresed in 0,8% agarose. Transfer of the DNA to nitrocellulose filters was carried out according to Southern (1975). Hybridization was carried out with ^{125}I-ribosomal RNA as control or ^{125}I-SiSAV RNA in 50% formamide, 3XSSC, 0,5% sodium dodecyl sulfate, 5 mg/ml tRNA, 0,02% each of ficoll, polyvinylpyrrolidone and BSA at 37°. Molecular weights are given in daltons $\times 10^{-6}$ *a)* Normal gibbon DNA hybridized to ^{125}I-rRNA as control. *b)* Normal gibbon spleen DNA × ^{125}I-SiSAV RNA. *c)* G-1 heart DNA × ^{125}I-SiSAV RNA. *d)* G-1 lymph node DNA × ^{125}I-SiSAV RNA. *e)* G-4 liver DNA × ^{125}I-SiSAV RNA. *f)* G-4 kidney DNA × ^{125}I-SiSAV RNA

the DNA from these tissues. DNA from a normal gibbon spleen did not contain any viral specific fragments. (The three bands shown in normal gibbon spleen DNA are ribosomal DNA bands due to small amounts of RNA in the viral RNA preparation. See ^{125}I-rRNA control.) Thus the $1{,}5 \times 10^6$ dalton detected in DNA from kidney and liver of G-4 represents an acquired partial provirus by the animal.

Detection of an Endogenous Human DNA Fragment That Hybridized Specifically to SiSAV-GaLV

When DNA from human cells (including fresh tissues of spleen, liver, kidney, placenta, leukocytes or tissue culture cells) was digested with Bam HI, and hybridized to ^{125}I-RNA from different type-C viruses, RNA from SiSV-SiSAV detected a fragment at $8{,}0 \times 10^6$ daltons in size in the DNA of all tissues tested. These results cannot be explained by human cellular nucleic acids contaminating the viral ^{125}I-RNA probe because the hybridizations were performed with three different strains of SiSV-SiSAV grown in different hosts, marmoset, human and rat and probes from each of these viruses gave the same result.

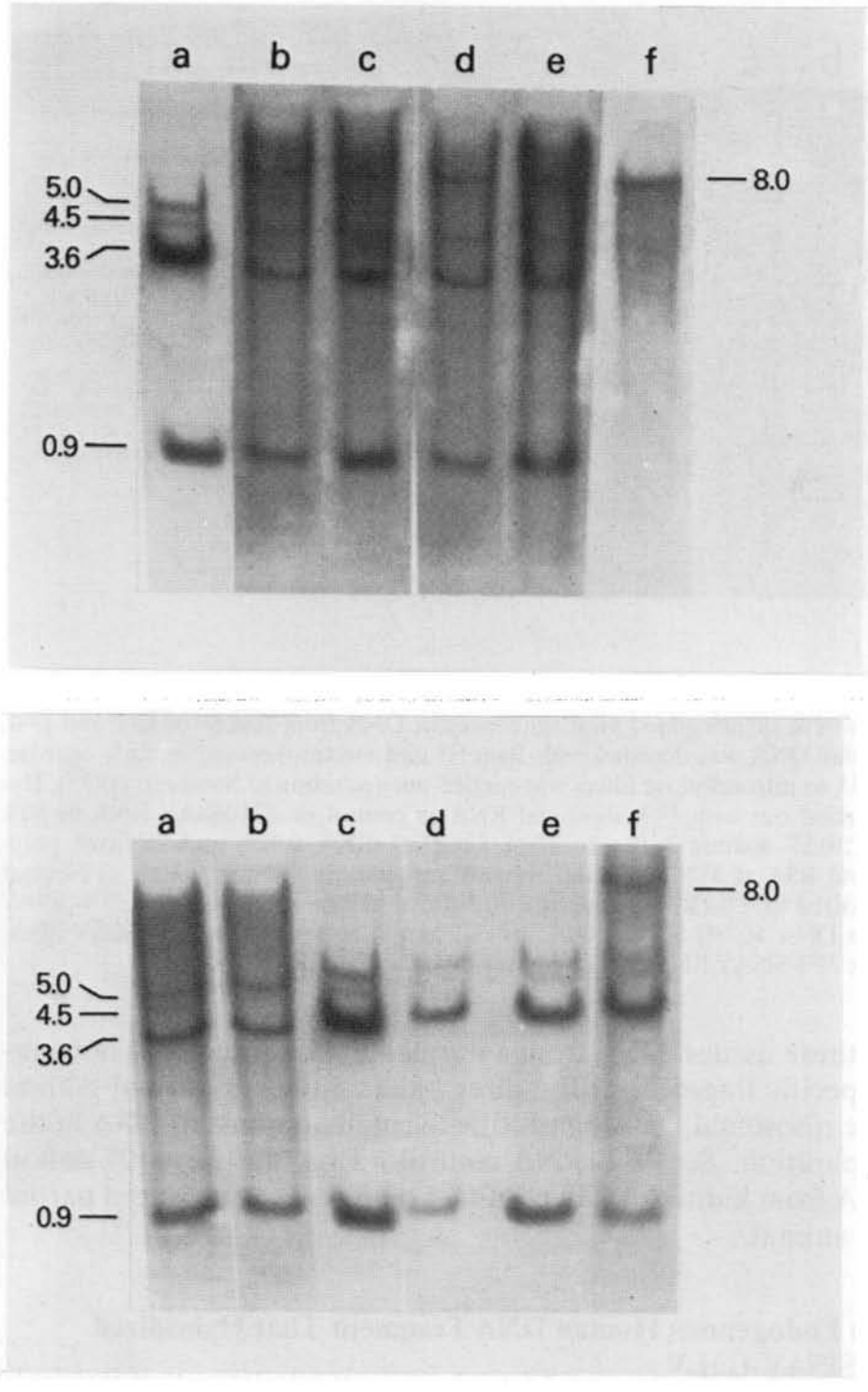

Fig. 3. Detection of an endogenous human DNA fragment that hybridized to genomic RNA of SiSAV-GaLV. Experiments were carried out as described in legend to Fig. 2. Forty different human DNA samples, including fresh tissues of spleen, liver, kidney placenta as well as various tissue culture cell lines gave identical results, therefore only a typical pattern is shown. Human DNA digested with Bam HI was hybridized to ^{125}I-RNA of various viruses. *A.* Viruses of the SiSV-SiSAV group: *a)* rRNA control; *b)* SiSAV (A204), grown in human rhabdomyosarcoma cells; *c)* 705 RNA of SiSV (SiSAV) (71API), produced by a marmoset tumor cell line; *d)* 355 RNA of SiSV (SiSAV) (71API); *e)* SiSAV (M55), grown in normal rat kidney cells; *f)* same as e), but selected on oligo-dT cellulose column for poly (A). *B.* Other viruses: *a)* GaLVH; *b)* the endogenous cat virus RD114; *c)* Feline leukemia virus, strain Rickard, $FeLV_R$; *d)* Murine leukemia virus, strain Rauscher, $MuLV_R$; *e)* Baboon endogenous virus, BaEV (M7); *f)* Filter from e) rehybridized to SiSAV (M55)

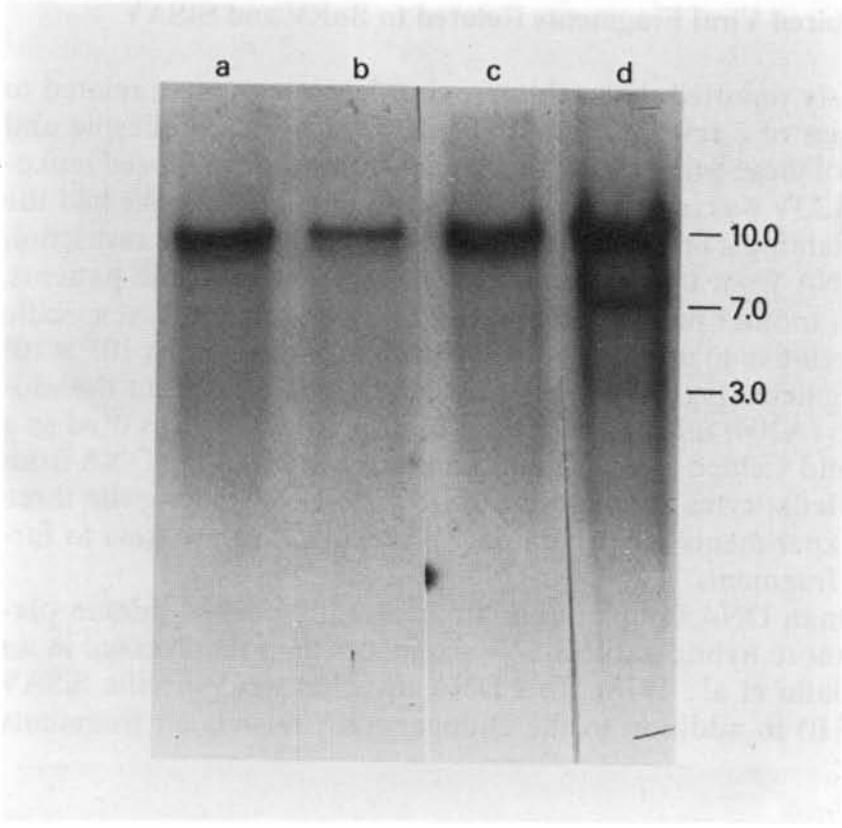

Fig. 4. Digestion of the endogenous SiSAV-related human DNA with Hind III human DNA digested with Hind III was hybridized to: *a)* ^{125}I ribosomal RNA: *b)* ^{125}I BaEV RNA: *c)* ^{125}I FeLV RNA: *d)* ^{125}I SiSAV (M55) RNA

All other viruses, including $GaLV_H$ and BaEV, did not detect any viral specific DNA fragments in DNA from normal human tissues. Representative results using these viral RNA probes are depicted in Fig. 3. To further verify the results with Bam HI, another enzymes, Hind III, was used to analyze the human DNA sequences that interact with SiSV-SSAV, and the results are shown in Fig. 4. Hind III yielded two fragments of 7,0 and $3{,}5 \times 10^6$ daltons. These sequences were not derived from a conserved set of mammalian DNA sequences, since DNA from two primates, gibbon and baboon, as well as two lower species, cat and dog, did not reveal any specific fragments hybridizing SiSV-SSAV (see earlier results for normal gibbons, other data not shown). The negative data with DNA from these animals also indicates that the positive results with human DNA are not from cellular nucleic acids contaminating the viral probe.

Since two of the three probes used SiSV (71API), SiSV (NRK) were never passaged through human cells in the laboratory, a plausible explanation is that viruses of the SiSV-SiSAV group have infected humans in the past, and have recombined with human cellular sequences. We plan to study the nature of these sequences and their mode of expression in human cells after they are purified and amplified by cloning them in bacteria.

Detection of Acquired Viral Fragments Related to BaEV and SiSAV

We have previously reported detectable proviral DNA sequences related to the BaEV in tissues of a few leukemic patients (Wong-Staal, Gillespie and Gallo, 1976) one of these being HL23, the patient from whose cultured leukocytes the virus HL23V was isolated (Gallagher and Gallo, 1975). We had the opportunity to examine a few of these samples by "blotting" after restriction with Hind III. DNA from uncultured leukocytes of two leukemic patients, HL23 and HL49 (another patient with AML), revealed several viral specific DNA fragments relative to normal controls (which only displayed a $10{,}0 \times 10^6$ dalton rDNA fragment (Fig. 5A). DNA from human tissue culture rhabdomyosarcoma cells (A204) infected with the M7 strain of BaEV, was used as a positive control and yielded a very similar banding pattern as the DNA from HL23 and HL49 leukocytes although there are differences among the three samples. These experiments are preliminary and work is in progress to further define these fragments.

One other human DNA sample, from an apparently normal human placenta contained more hybridizable SiSAV sequences than the average in an earlier survey (Gallo et al., 1978). This DNA revealed viral specific SiSAV fragments (Fig. 5B) in addition to the endogenous viral-related fragments described earlier.

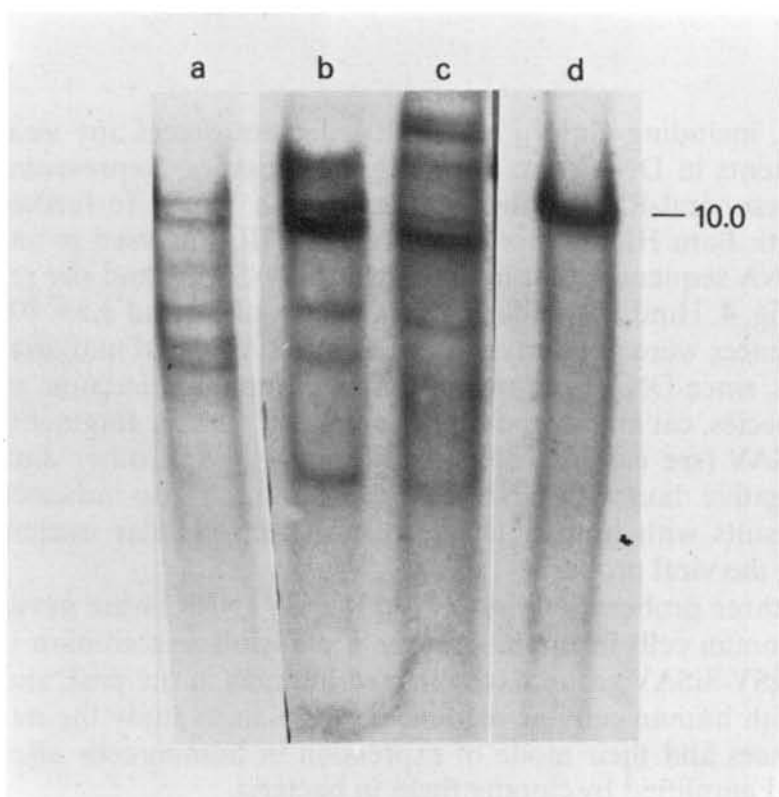

Fig. 5A

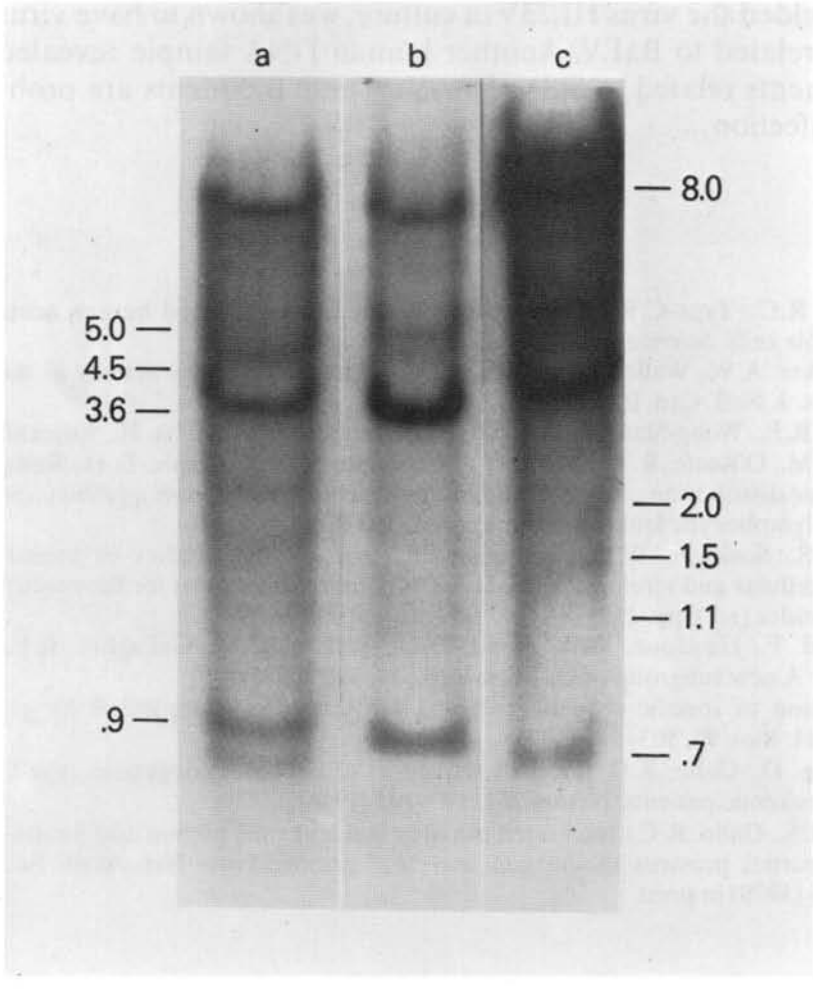

Fig. 5B

Fig. 5. Detection of "acquired" DNA fragments related to BaEV and SiSAV in several human DNA samples. *A.* DNA from uncultured leukocytes of two patients. HL23 and HL49. was treated with the restriction enzyme Hind III and processed as described in Fig. 2. A204 (M7) a human rhabdomyosarcoma cell line infected with BaEV (P. cynocephalus). and normal human spleen were used as positive and negative controls respectively. Hybridization was carried out with ^{125}I-BaEV RNA: *a)* A204 (M7); *b)* HL49; *c)* HL23; *d)* normal human spleen. *B.* DNA from 2 normal placentas and from NC37 (SiSAV). a human lymphoid cell line infected with SiSAV. were digested with Bam HI and hybridized to ^{125}I SiSAV RNA; *1.* NC37 (SiSV); *2.* normal placenta # 6; *3.* normal placenta called NP3. (NP3 was previously shown to hybridize significantly higher levels of SiSV probes than found with other DNA from normal human tissues)

Summary

We have shown that 1. partial provirus integration can be a possible result of a natural infection. and may serve as a model in animal systems where a viral etiology is implicated but detection of a major fraction of the virus genome is rare; 2. All human DNA contains some sequences that hybridize specifically with genomes of SiSV-SiSAV. suggesting that viruses of this group have infected humans in the past and recombined with human cellular DNA. 3. Finally. DNA from uncultured leukocytes of two leukemic patients. one be-

ing HL23, which yielded the virus HL23V in culture, was shown to have virus specific fragments related to BaEV. Another human DNA sample revealed virus specific fragments related to SiSV (SiSAV). These fragments are probably acquired by infection.

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Genetic Transmission of Moloney Leukemia Virus: Mapping of the Chromosomal Integration Site

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Abstract

Mice genetically transmitting the exogenous Moloney leukemia virus (Balb/Mo) have been previously derived. These animals carried one copy of Moloney virus DNA (M-MuLV) in their germ line and transmitted the virus as a single Mendelian gene to the next generation.

Homozygous BALB/Mo mice were used to genetically map the M-MuLV locus. Embryo fibroblasts were fused to established Chinese hamster cells and somatic cell hybrids were selected. Segregation of mouse chromosomal markers in the hybrids was correlated to the loss of M-MuLV-specific sequences as detected by molecular hybridization. Of 15 isozymes located on different mouse chromosomes only triosephosphate isomerase segregated syntenic with the M-MuLV gene, suggesting that the virus was integrated on chromosome No. 6. This was confirmed by sexual genetic experiments analyzing segregation of Moloney viremia and two markers on chromosome 6 and 15, respectively. The results show that M-MuLV expression is linked to wa-1 on chromosome 6 at a distance of about 30 map units. These data define a new genetic locus, *Mov-1*, representing the structural gene of M-MuLV in BALB/Mo mice.

Introduction

Endogenous C-type viruses are transmitted genetically from one generation to the next [1,2,3] and thus are present in every cell of the animal. In contrast, exogenous C-type viruses, which are transmitted by horizontal infection, are not part of the germ line of an animal and thus are not transmitted genetically. The apparent resistance of the germ line to infection with leukemia virus may be explained by the "organtropism" of leukemia viruses; only certain "target" tissues such as spleen or thymus cells are susceptible to infection, whereas most other non-target tissues cannot be infected by leukemia virus [4,5].

It has been possible to overcome the "organtropism" restriction of the exogenous Moloney leukemia virus (M-MuLV) by infecting mice at the 4–8 cell preimplantation stage before differentiation into "target" or "non-target" cells has taken place. These embryos developed into normal adult animals, which carried M-MuLV-specific sequences in all organs including cells of the germ line [5]. Thus the exogenous virus, M-MuLV, was established as an

endogenous virus in these mice (= BALB/Mo). Genetic and biochemical evidence indicated that these mice were heterozygous for a single Mendelian locus with one proviral copy integrated at this site. Mice, homozygous at this locus, were derived and molecular hybridization studies have demonstrated a somatic amplification of these virus sequences in target cells during the process of leukemogenesis [5,6].

BALB/Mo mice develop a specific thymus-dependent leukemia similar to the disease observed in AKR mice. A number of genetic factors appear to control the development of leukemia by endogenous C-type viruses and/or the susceptibility to leukemic transformation by exogenous C-type viruses. Attempts to elucidate genetic factors involved in virus replication and transformation include the use of sexual genetic experiments and the use of interspecies somatic cell hybrids preferentially segregating the chromosomes of one species. The following genes related to replication or regulation of MuLV have been assigned to individual mouse chromosomes: Fv-1 to chromosome No. 4 [7], Rec-1 to No. 5 and Ram-1 to No. 8 [8], Fv-2 to No. 9 [9] and Rgv-1 to No. 17 [10]. In human cells a locus controlling the replication of Baboon endogenous type-C virus has been assigned to chromosome No. 6 [11]. So far, Akv-1 represents the only genetically defined locus representing the proviral genome of an endogenous virus. The Akv-1 locus has been mapped by sexual genetics on chromosome 7 near Gpl-1 [12,13]. Another proviral genome in the AKR strain, Akv-2, has not been assigned to a particular mouse chromosome.

In this paper we summarize our experiments to genetically map the integration site of M-MuLV in BALB/Mo mice.

Methods

All methods used to quantitate virus-specific sequences by molecular hybridization and to measure virus expression in animals have been described previously [4–6]. The M-MuLV cDNA used was selected against uninfected mouse DNA to remove all sequences which cross-hybridize with endogenous mouse viruses [6]. Cell fusion experiments and isoenzyme tests were performed essentially as described elsewhere [14–17]. Mice were bred in our laboratory or purchased from the Jackson Laboratory.

Results

We have used both sexual and somatic cell genetic approaches to map the M-MuLV locus in the mouse genome. First sexual genetic experiments utilizing crosses between BALB/Mo and C57BL mice excluded five chromosomes as potential integration sites. We then utilized interspecies somatic cell hybrids, which segregated specifically mouse chromosomes to identify the chromosome syntenic with the M-MuLV locus. Evidence described in the following section suggested chromosome No. 6 as integration site for M-MuLV. This was confirmed in subsequent sexual genetic experiments using appropriate linkage test strains of mice.

A. Linkage Studies Using Somatic Cell Hybridization

Fibroblast cultures were established from BALB/Mo embryos homozygous for the M-MuLV locus. Fibroblasts are non-target cells for M-MuLV and therefore spontaneous virus expression is repressed [4]. As expected, molecular hybridization experiments showed the presence of 2 M-MuLV copies per diploid mouse genome. However, when these cultures are superinfected with M-MuLV, they produce high titers of virus and virus production is accompanied by an amplification of M-MuLV-specific DNA sequences (unpublished).

These mouse fibroblasts were fused to established Wg 3-h-O Chinese hamster cells [17] in the presence of polyethylene glycol. It has been shown previously that somatic cell hybrids isolated after fusion of secondary mouse fibroblasts and established Chinese hamster cells segregate mouse chromosomes [18]. Hybrid clones were selected in HAT medium and analyzed: (i) for the presence of mouse chromosomes by isozyme determinations, (ii) for the presence of M-MuLV-specific DNA sequences by molecular hybridization, and (iii) for virus production by assaying for infectious virus by the XC assay. The latter test was important since it has been observed that fusion of mouse macrophages to human cells readily activates endogenous mouse viruses [19]. Activation and production of the endogenous M-MuLV could possibly lead to reintegration of the virus in either mouse or hamster chromosomes and thus prevent identification of the original integration site by segregation analysis. Therefore, all hybrid clones were tested for the presence of infectious virus. None of 30 clones tested produced virus, indicating that expression of integrated M-MuLV in BALB/Mo fibroblasts was under tight control and that M-MuLV was not activated upon fusion of BALB/Mo fibroblasts to hamster cells.

So far, we have analyzed 30 independent mouse-Chinese hamster clones segregating mouse chromosomes. Table 1 is a summary of our observations correlating M-MuLV-specific sequences with the presence of isozyme markers for 15 mouse chromosomes enzyme markers were assayed for chromosomes 3, 13, 15, 16 and 17. The expression of mouse triosephosphate isomerase, whose gene has been recently assigned to chromosome No. 6 [20], was concordant in 93% of the clones with the presence of M-MuLV specific sequences. Only one out of 30 hybrid clones had lost the enzyme but retained M-MuLV-specific sequences, and one clone had retained the enzyme but lost the virus sequences. Karyotype analyses have been performed on these two exceptions: no chromosome No. 6 could be identified by morphological criteria. This suggests that chromosome No. 6 was fragmented and transposed to another chromosome in these clones. All other enzyme markers showed a highly asyntenic segregation with the M-MuLV gene. It should be emphasized here that our experiments using molecular hybridization with a specific cDNA probe identify the structural gene of M-MuLV and not some regulatory locus for virus expression. These data therefore suggest, but do not prove, that M-MuLV in BALB/Mo mice is integrated on chromosome No. 6. So far, we cannot rule out that chromosomes 3, 13, 15, 16 or 17 were retained

Table 1. Correlation between mouse isozymes and M-MuLV-specific DNA sequences in mouse-chinese hamster hybrid clones

Mouse isozyme	Mouse chromosome	Isozyme retention/ M-MuLV DNA present (% asyntenic segregation)
Dipeptidase-1	1	27
Adenylate kinase 1	2	40
Phosphoglucomutase-2	4	53
Phosphoglucomutase-1	5	50
Trisephosphate isomerase	6	7
Glucosephosphate isomerase	7	22
Adenine phosphoribosyl-transferase	8	30
Mannosephosphate isomerase	9	40
Tripeptidase-1	10	27
Galactokinase	11	50
Acid phosphatase	12	43
Esterase-10	14	30
Dipeptidase-2	18	43
Glutamate-oxalacetate transaminase	19	57
Hypoxanthine phosphoribosyl-transferase	X	37

Thirty independent somatic cell hybrid clones were analyzed for the presence of 15 mouse chromosomes by isozyme determinations and for the presence of M-MuLV-specific sequences by molecular hybridization.

in many hybrids and thus could possibly carry the M-MuLV gene. However, results obtained in sexual genetic experiments described in the next section strongly support the notion that the M-MuLV gene is integrated on chromosome No. 6.

B. Assignment of the M-MuLV Locus by Sexual Genetic Experiments

In our first genetic experiments to map the M-MuLV locus we mated BALB/Mo mice with C57BL mice and backcrossed the F-1 animals to C57BL. The resulting BC-1 (= backcross 1) animals were tested for correlation or lack of correlation between the presence of M-MuLV sequences and inheritance of 6 markers on 5 different chromosomes. Table 2 summarizes these data. It is evident that the M-MuLV gene was not linked to any of the markers tested. This included chromosome 7 on which the Akv-1 locus was mapped near the Gpl-1 marker [12]. These results are in agreement with the segregation data using somatic cell hybrids described above.

Since the somatic cell hybrid experiments suggested chromosome No. 6 as a possible integration site for M-MuLV, we crossed BALB/Mo with ABP/J mice. This strain is a linkage test strain carrying the morphological marker wa-1 (curled whiskers) on chromosome No. 6 and the coat colour marker bt (white belt) on chromosome 15. Since both markers are recessive, the viremic F-1 mice show straight whiskers and no belt (F-1 genotype: $Mo^+ + +/Mo^-$ bt wa-1). F-1 males were backcrossed to ABP females and the presence of M-MuLV in the serum was correlated to the expression of both recessive mark-

Table 2. Segregation analysis of cross between BALB/Mo × C57BL

Marker tested	Mouse chromosome	Total No. of BC-1 animals	Segregation of M-MuLV-specific sequences and markers in BC-1 generation	
			Parental	Recombinant
Agouti	2	94	53	41
MUP I	4	94	46	48
GPI-1	7	90	38	52
Hbb	7	94	45	49
Es-1	8	94	54	40
Mod-1	9	64	33	31

BALB/Mo animals were mated with C57BL mice. F-1 mice were backcrossed to C57BL and the resulting BC-1 animals analyzed for M-MuLV-specific DNA sequences in their liver DNA and for the indicated markers [16].

ers. A study of the segregation of the Mo^+ and wa-1 markers shows that 69% of the BC-1 animals were of parental genotype and 31% recombinants (Table 3A). This suggests strongly that the M-MuLV locus segregates together with the wa-1 locus. The frequency of recombinants assigns the M-MuLV locus 31 map units from wa-1 on chromosome No. 6.

As controls the segregation of the M-MuLV locus and the bt locus as well as the segregation of bt and wa-1 are given in Table 3B and C. About equal proportions of recombinant and parental genotypes are found, indicating independent segregation of these loci. These results confirm the assignment of the M-MuLV locus to chromosome 6 and argue against chromosome 15 as the Moloney virus carrying chromosome (see discussion).

Discussion

Integration of exogenous RNA tumor virus sequences into the chromosomal DNA of infected cells has been demonstrated in many systems [1,30,31]. The exogenous Moloney leukemia virus has been established as an endogenous virus in mice by infection at the 4–8 cell preimplantation stage and integration of viral specific information into single blastomeres [5]. The subline of mice derived from such an infection transmitted the M-MuLV gene in only one chromosomal locus, and this virus gene was maintained in the colony by paternal transmission from heterozygous males mated with normal females. The integration of M-MuLV into the paternal chromosome complement had no detectable effect on normal embryonal and postnatal development of the animals. Further experiments were performed to determine whether homozygosity at the M-MuLV locus was compatible with normal development. Molecular hybridization and genetic experiments were used to identify homozygous offspring derived from matings of heterozygous parents [6]. The results indicated that homozygosity at the M-MuLV locus has no effect on

Table 3. Segregation analysis of cross between BALB/Mo × ABP

P-1 Mo$^+$ + +/Mo$^+$ + + × Mo$^-$wa-1 bt/Mo$^-$wa-1 bt P-2

F-1 Mo$^+$ + +/Mo$^-$wa-1 bt × Mo$^-$wa-1 bt/Mo$^-$wa-1 bt P-2

BC-1

A. Segregation of Mo$^+$ and wa-1

BC-1	Mo$^+$ +	Mo$^-$ wa-1	Mo$^+$ wa-1	Mo$^-$ +
No. of animals	74	80	37	33
	69% parental		31% recombinant	

B. Segregation of Mo$^+$ and bt

BC-1	Mo$^+$ +	Mo$^-$ bt	Mo$^+$ bt	Mo$^-$ +
No. of animals	52	65	56	46
	53% parental		47% recombinant	

C. Segregation of bt and wa-1

BC-1	+ +	bt wa-1	+ wa-1	bt +
No. of animals	54	68	49	54
	54% parental		46% recombinant	

Homozygous BALB/Mo were mated with ABP mice. The resulting F-1 animals were backcrossed to ABP mice and the segregation of the markers wa-1, bt and Mo$^+$ was determined.

the normal development of the mice. Furthermore, it was observed that the development of leukemia is not influenced by the genotype of the mice because mice homozygous or heterozygous for the M-MuLV locus or normal animals infected at birth with virus developed disease at similar rates. The latter animals carry M-MuLV-specific sequences only in their target tissue and never transmit the virus genetically [5,6].

We have shown recently that BALB/Mo mice express virus-specific RNA sequences or virus-specific proteins in target tissues only, whereas the virus gene remains repressed in non-target tissues. Similarly, a somatic virus gene amplification during the process of leukemogenesis is observed in target tissues only [5,21,32]. It has been suggested that the observed tissue specificity

of expression and transformation of different RNA tumor viruses may be due to different but virus-specific integration sites into the host chromosomes [22]. As a first attempt to approach this question, we performed experiments to identify the mouse chromosome carrying the M-MuLV locus. It was of special interest to investigate whether M-MuLV was integrated into chromosome 15, since a trisomy of this chromosome has been shown to occur frequently in M-MuLV or AKR virus-induced lymphomas [23,24].

Somatic cell hybrids between BALB/Mo fibroblasts and hamster cells were analyzed for segregation of specific mouse chromosomes and M-MuLV-specific DNA sequences. Our preliminary experiments analyzing isozyme markers on 15 mouse chromosomes showed that only triosephosphate isomerase segregates syntenic with M-MuLV-specific sequences. The gene coding for this enzyme has been assigned recently to mouse chromosome No. 6 [20], and thus our experiments suggested that M-MuLV in BALB/Mo mice is located on this chromosome. This preliminary assignment was confirmed by sexual genetic experiments. We used two independently segregating markers, wa-1 on chromosome 6 and bt on chromosome 15, to study possible linkage of virus induction in BC-1 animals. The results in Table 3 show that M-MuLV was linked to the wa-1 marker and unlinked to the bt marker. The frequency of recombinants suggested that the M-MuLV locus is approximately 31 map units away from the wa-1 locus on chromosome No. 6. On the basis of these data we propose to call this gene *Mov-1* denoting Moloney virus in BALB/Mo mice.

The first structural gene comprised of the proviral DNA sequences of an endogenous virus, the AKR leukemia virus, was assigned to the *AKv-1* locus on chromosome No. 7 [12,13]. Another, unlinked proviral genome in AKR mice, *AKv-2*, has not been localized yet. The similarities between the AKR virus-induced and the M-MuLV-induced disease should be emphasized here. Both AKR and Moloney virus induce a thymus-derived leukemia with a frequent chromosome 15 trisomy. In both cases a somatic amplification of virus-specific sequences is observed during leukemogenesis, and in both cases virus gene expression seems to be restricted to the target organs [5,21,32]. The only two virus structural genes mapped so far, which induce thymus-dependent leukemia, *Mov-1* and *Akv-1*, are clearly non-allelic. We consider two alternatives to explain the molecular events during virus-induced thymus-dependent leukemogenesis. In the first hypothesis, leukemia viruses can integrate at a few virus-specific chromosomal sites to induce tissue-specific virus expression and virus gene amplification, which is followed by transformation of specific target cells [22]. Alternatively, the virus could integrate at one or a few out of a large number of possible integration sites. These original integration sites would not be specific for the virus, but the observed specificity of expression and transformation may depend on specific secondary integrations which occur during leukemogenesis. Only those lymphatic cells which carry additional virus sequences at transformation-specific sites might be selected for during the process of leukemogenesis.

In order to decide between these hypotheses, it will be necessary to develop new mouse lines which carry M-MuLV in their germ line and to map the

integration sites. It also would be interesting to study if *AKv-2* is integrated at a site homologous to *Mov-1*. These studies will contribute to our understanding of how a virus can transform a specific target cell.

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Integration Sites for Moloney Murine Leukemia Virus DNA in Infected Mouse Cells

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The integration of viral DNA in mouse cells infected with Moloney murine leukemia virus (M-MuLV) has been studied by the "blotting" technology introduced by Southern (1975). We were interested in determining if there is a single site in the chromosomal DNA at which M-MuLV viral DNA is integrated, a small number of sites, or a large number of possible integration sites. The technique used involved cleavage of infected cell DNA with a sequence-specific restriction endonuclease, resolution of the resulting DNA fragments by electrophoresis in 0,6% agarose gels, and blot transfer of the separated fragments to nitrocellulose filters. Detection of DNA fragments with sequence homology to M-MuLV was achieved by hybridization with ^{32}P-labeled M-MuLV complementary DNA (cDNA). The labeled cDNA (approximately 2×10^8 cpm/μg) was synthesized in an endogenous reaction using purified virus and added calf thymus oligo-deoxynucleotide primers in order to generate a uniformly representative probe (Fan and Verma, 1978).

The interpretation of the results of these experiments depends upon a knowledge of the cleavage pattern of viral DNA itself by each restriction endonuclease used. Fig. 1 shows cleavage of unintegrated M-MuLV linear proviral DNA by several restriction endonucleases. The molecular weight of the linear form of proviral DNA is $5{,}8 \times 10^6$ daltons. Most important for subsequent experiments is the fact that *Eco* RI endonuclease *does not* cleave unintegrated proviral DNA. Thus, cleavage of infected cell DNA should result in the appearance of virus-specific DNA fragments with molecular weights greater than $5{,}8 \times 10^6$ daltons, the exact size depending on the spacing of *Eco* RI cleavage sites in the host cell DNA surrounding the integrated M-MuLV DNA.

Fig. 2A shows the result when infected and uninfected cells DNAs are cleaved with RI and analyzed. It is immediately apparent that a large number of DNA fragments with sequence homology to M-MuLV are present in uninfected mouse cells. This is not entirely unexpected since mouse cells contain a family of sequences present in about 10 copies/haploid genome, which have sequence homology to about 50% of the genome of M-MuLV (Jaenisch et al., 1975). It is presumably these related sequences which are being detected in uninfected cell DNA. There are, however, additional bands present only in infected cell DNA, and these are tentatively identified as containing the M-MuLV DNA integrated during infection. Fig. 2B shows that the uninfected cell M-MuLV-related RI fragments are unique to mouse cells, and

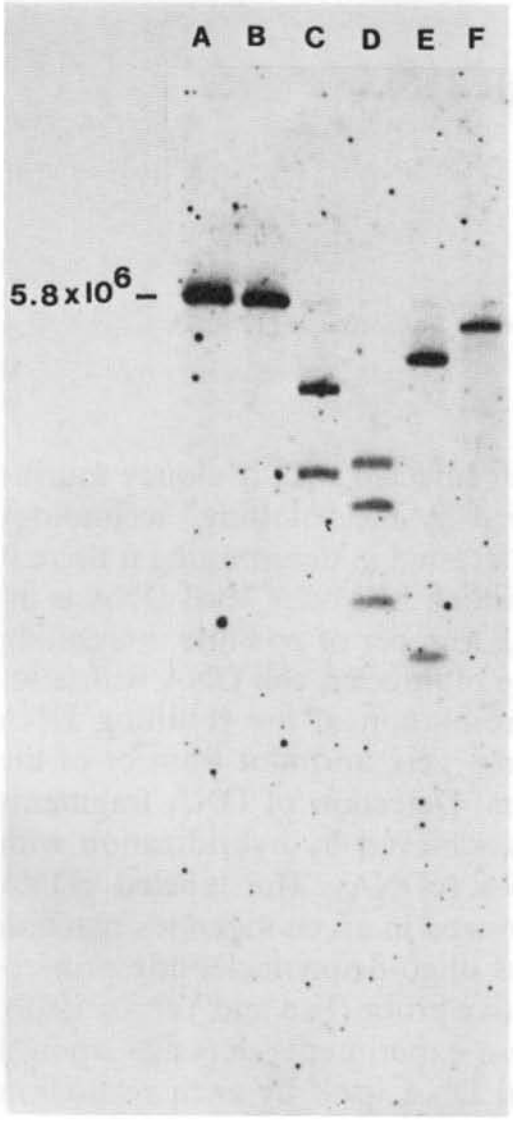

Fig. 1. Restriction enzyme cleavage pattern of unintegrated linear proviral DNA. Proviral DNA was isolated from 3T6 cells 20 hr after infection with Clone 1 M-MuLV. DNA was extracted from the cytoplasm and digested with various restriction enzymes. The DNA fragments were separated by electrophoresis in 0.6% agarose gels and transferred to nitrocellulose filters. The filters were hybridized with ^{32}P-labeled M-MuLV cDNA. An autoradiogram of the washed filter is shown. The molecular weight of linear proviral DNA is 5.8×10^6 daltons. *A* – Untreated proviral DNA. *B* – RI-treated. *C* – *Hind* III-treated. *D* – *Bam* HI-treated. *E* – *Hpa* I-treated. *F* – *Pst* I-treated

not detected in rat DNA. All hybridization can be competed by an excess of purified 38S genomic viral RNA (Fig. 2C). Thus, all of the fragments detected by hybridization to ^{32}P M-MuLV cDNA are related to viral DNA sequences, rather than related to possible nonviral sequences contaminating the ^{32}P cDNA probe.

Fig. 3 shows the results obtained with RI cleaved cellular DNA from uninfected and M-MuLV-infected NIH and BALB/c cells. The pattern of M-MuLV related bands in the two uninfected cell lines is similar, but some differences also exist. In each infected cell line, at least one additional DNA fragment not found in the uninfected parent can be identified and we tentatively conclude that these unique fragments contain the integrated M-MuLV DNA.

The molecular weights of the M-MuLV-specific RI fragments unique to infected cells are different in each different cell line examined (10 different lines examined). In addition, none of the M-MuLV-containing fragments from exogenously infected fibroblasts co-migrate with the unique M-MuLV-containing RI fragment from cell lines carrying an endogenous M-MuLV (Jaenisch, 1976; Bacheler and Fan, 1978). Thus, we conclude that many dif-

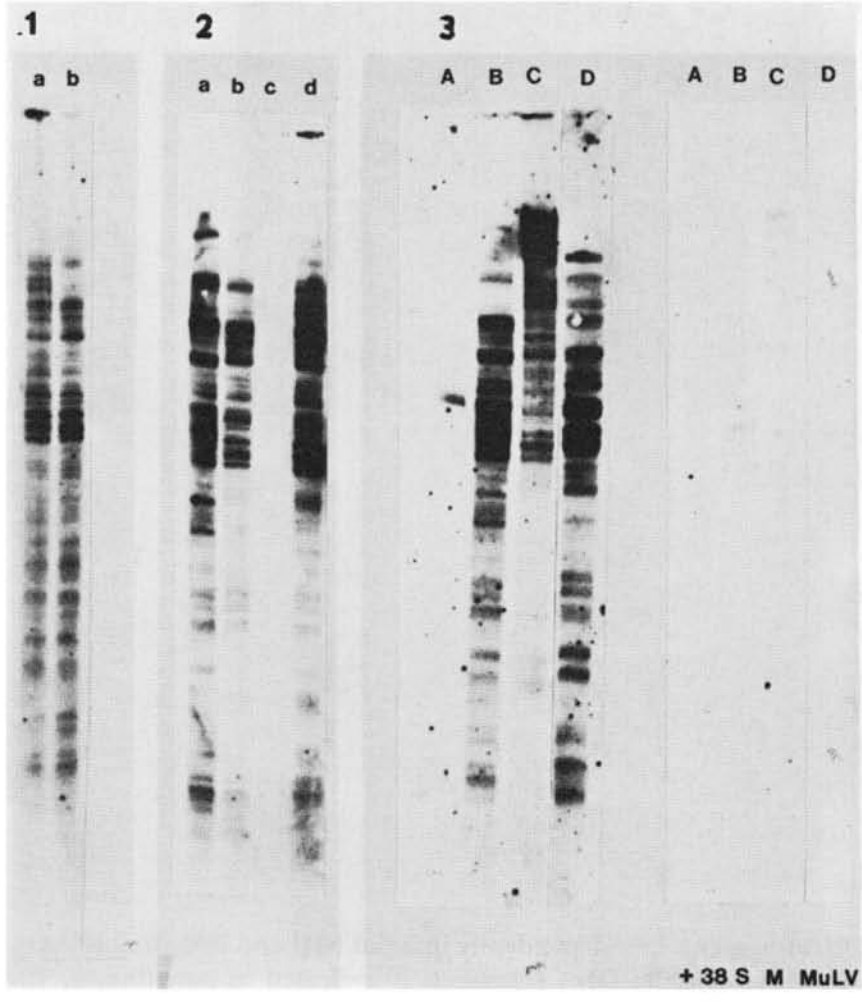

Fig. 2. M-MuLV related DNA fragments in infected and uninfected cell DNA. RI cleaved cell DNA from infected and uninfected lines was analyzed as in Fig. 1.
1. RI cleaved mouse cell DNA. *a* M-MuLV-infected NIH cell. *b* Uninfected NIH cell
2. RI cleaved mouse and rat cell DNA. *a* 3T6 uninfected NIH mouse cell line. *b* 3T3 uninfected NIH mouse cell line. *c* NRK uninfected rat cell line. *d* A9 M-MuLV infected NIH mouse cell line
3. RI cleaved mouse cell DNA. Replicate blots were hybridized in the presence or absence of an excess of unlabeled. purified 38S M-MuLV genomic RNA. *A* – Proviral DNA. *B* – G Clone 1 cell line from BALB/Mo mouse with an endogenous M-MuLV. *C* – A9 M-MuLV infected NIH cell line. *D* – 3T3 uninfected NIH cell line

ferent sites of integration for M-MuLV are possible upon exogenous infection of fibroblasts. This conclusion is based only upon the spacing of RI cleavage sites on either side of the integrated M-MuLV and it is possible that a small DNA region is specifically recognized during integration.

In each infected cell line examined, we have been able to detect at least one unique *Eco* RI fragment which presumably contains the integrated M-MuLV proviral DNA. Further evidence for the presence of an integrated M-MuLV DNA copy in each of these lines is provided by the results of digestions with *Bam* HI. *Bam* HI cleaves linear proviral DNA twice, resulting in three fragments of molecular weights of 2,5, 1,9 and $1{,}25 \times 10^6$ daltons. Verma and McKennett (1978) have shown that the $1{,}9 \times 10^6$ dalton B-fragment is an

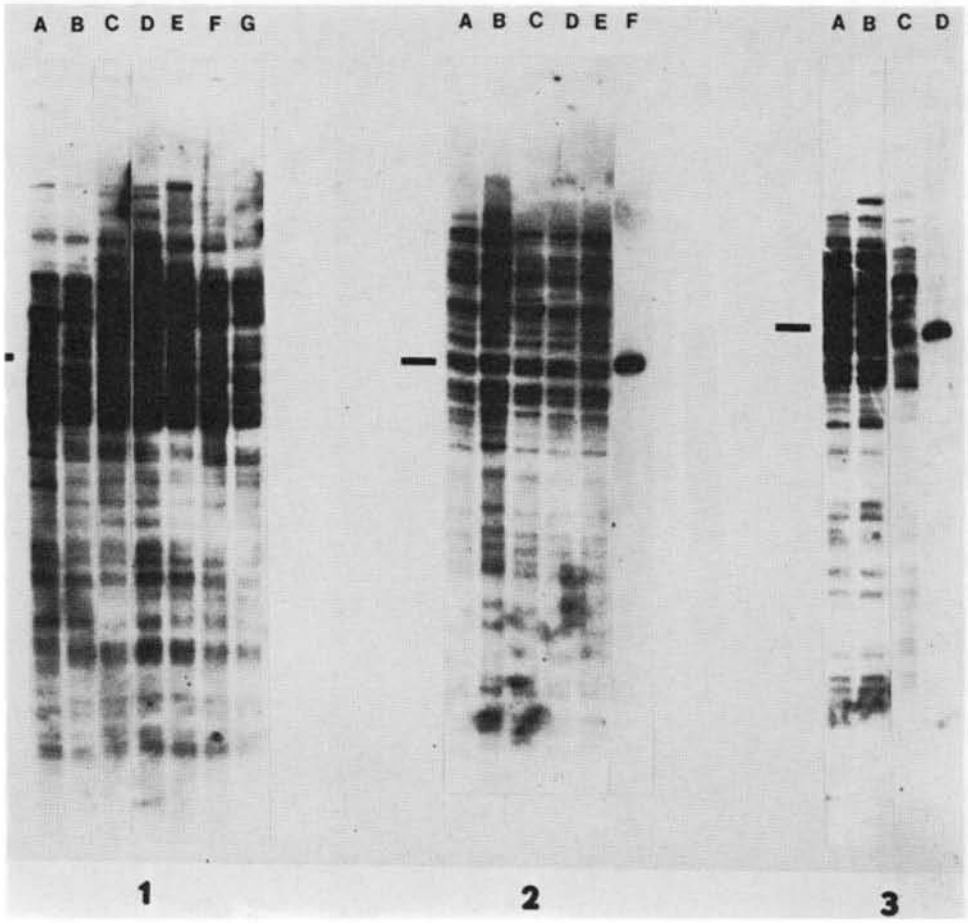

Fig. 3. M-MuLV related DNA fragments in independently infected NIH and BALB/c cell lines. The molecular weight of M-MuLV-specific DNA fragments is indicated in parentheses. The position of full length unintegrated proviral DNA is indicated by a bar.
1. *A* – 3T6 uninfected NIH. *B* – 3T3 uninfected NIH. *C* – A9 M-MuLV infected NIH (23–32. 21. 12.2. 8.7). *D* – B7 M-MuLV infected NIH (28–32. 21. 12.2. 8.7). *E* – E7 M-MuLV infected NIH (18.5). *F* – Clone 1 M-MuLV infected NIH (30. 21. 18). *G* – Clone 4A M-MuLV infected NIH (16.5).
2. *A* – M-MuLV infected SVT2 (17.6). *B* – M-MuLV infected SVT2 (18. 16.7). *C* – M-MuLV infected SVT2 (8.0). *D* – M-MuLV infected SVT2 (8.3). *E* – Uninfected SVT2. *F* – Proviral DNA (5.8).
3. *A* – Fibroblast line from BALB/c mice. *B–G* – Clone 1 fibroblast line from BALB/Mo mice carrying an endogenous M-MuLV (16.5–17). *C* – 2° MEF. from BALB/Mo mice (16.5–17). *D* – Proviral DNA (5.75)

internal fragment from the "middle" of viral DNA. Cleavage of each of the infected cell lines, but not uninfected cell DNA, results in the release of a 1.9×10^6 dalton fragment which co-migrates with the *Bam* HI-B fragment (Fig. 4). Fragments corresponding to the end fragments A and C are not seen. Thus, in each infected cell line, at least one M-MuLV viral DNA sequence arrangement retaining the internal cleavage sites for *Bam* HI is found. The sequence organization of the internal region of unintegrated proviral DNA is therefore retained upon integration.

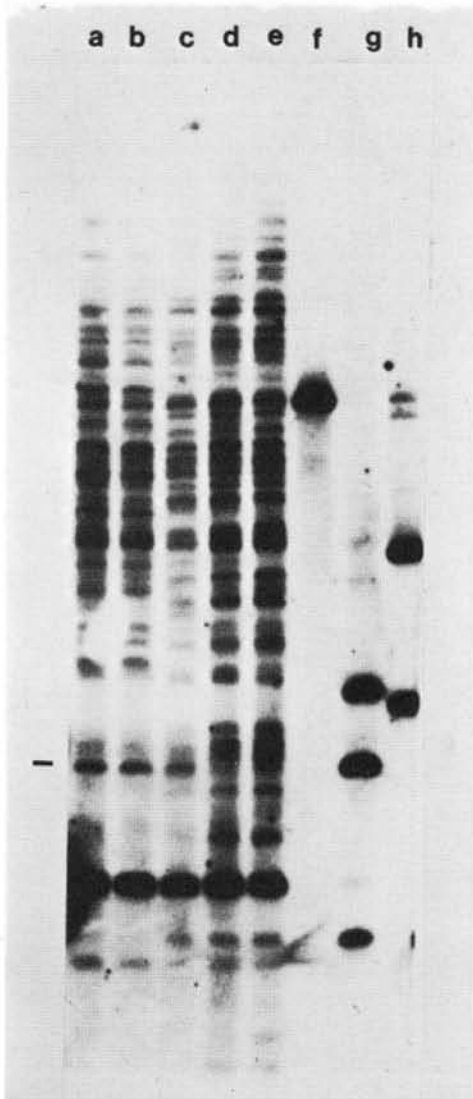

Fig. 4. M-MuLV related DNA fragments in infected and uninfected cell DNA cleaved with *Bam* HI. *A* – Clone 4A M-MuLV-infected NIH. *B* – Clone 3A M-MuLV-infected NIH. *C* – G Clone 1 BALB/Mo cell line carrying an endogenous M-MuLV. *D* – H uninfected BALB/c cell line. *E* – A31 uninfected BALB/c cell line. *F* – Proviral DNA. RI-treated. *G* – Proviral DNA. *Bam* HI-treated. *H* – Proviral DNA. *Hind* III-treated. The bar indicates the position of the 1.9×10^6 dalton *Bam* HI-B fragment

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Release of Particle Associated RNA Dependent DNA Polymerases by Primary Chicken and Quail Embryo Cells

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Introduction

Recently a new RNA dependent DNA polymerase has been described [1,2]. This enzyme is enclosed in sedimentable structures – "particles" –, and can be prepared from the allantoic fluid of embryonated virus free chicken eggs. The "particles" and the purified polymerase are different from the known avian RNA tumor viruses [1,2,3]. In this report we describe the appearance of this enzyme in the culture medium of primary chicken and quail embryo cells.

Results

Primary chicken and quail embryo cells release a particle associated RNA dependent DNA polymerase into the culture medium. The total amount of enzyme released is proportional to the number of cells plated. A maximum amount of enzyme is found in the medium when embryos of the age of six resp. eight days are used for culturing. The release after the onset of the cultures shows a well reproducible pattern with a peak around the third day and declines then within three to four days to background values. Secondary cultures release a significantly lower amount of enzyme. Tertiary or later cultures do not release detectable amounts of enzyme at all. In Fig. 1 the results obtained with primary chicken cells are shown. The experiments with primary quail cells led to almost identical results.

To characterize the enzyme, particles were purified from culture medium and the enzyme was tested for the ability to transcribe homo- and heteropolymeric RNA (Table 1). Table 1 clearly shows that the enzyme catalyzes RNA dependent DNA synthesis in the presence of an appropriate template primer complex. No endogenous DNA synthesis could be detected under the conditions used. When globin mRNA-oligo dT was used as a template primer complex the product was shown to consist of DNA with a base sequence complementary to globin mRNA.

The enzymes have further been characterized by determining the optimum conditions for reverse transcription of homopolymeric RNA. As can be seen from Table 2 both the enzymes have nearly identical optimum assay conditions and they are in this respect somewhat different from the respective enzyme of the Avian Myeloblastosis Virus (AMV). These differences be-

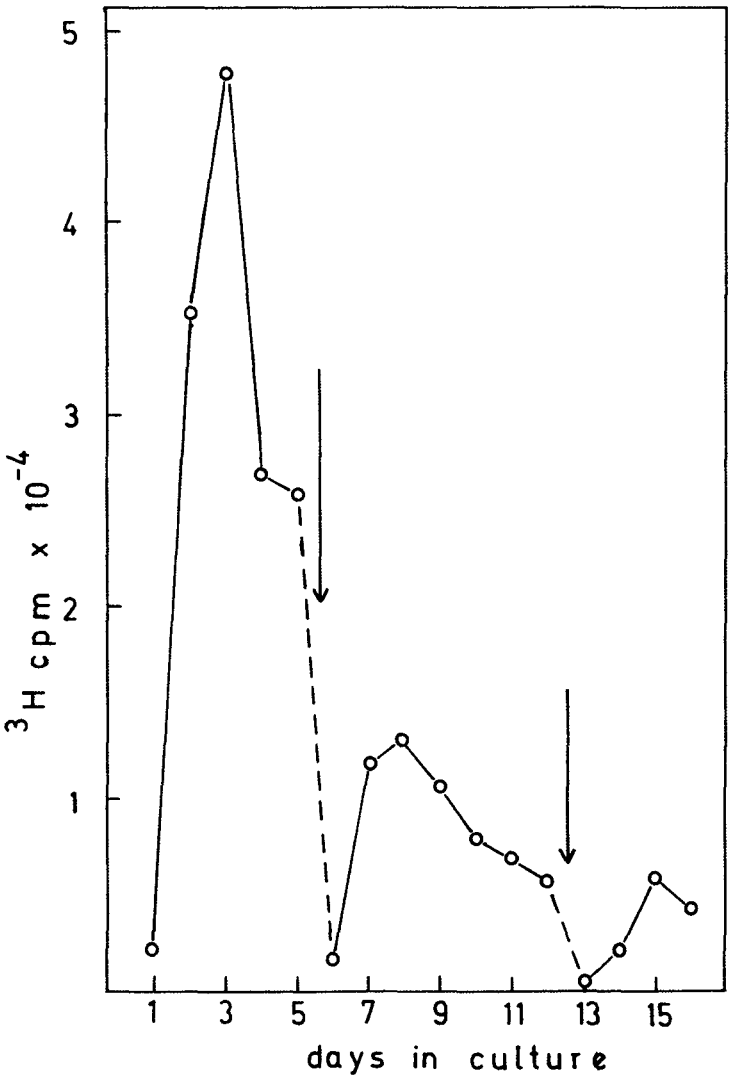

Fig. 1. Release of a particle bound DNA dependent DNA polymerase by primary. secondary and tertiary chicken cell cultures. Primary cells were plated at a density of 10×10^6 cells/plate; after 5 and after 12 days cultures were passaged (marked by arrows) and seeded at 5×10^6 cells per plate. Enzyme measurement as described in [4]

come more pronounced when the optimum conditions are studied in more details [2,5]. A distinct difference is found in inhibition experiments with IgG directed against the AMV-enzyme. In contrast to the viral enzyme the enzymes from primary quail and chicken cell cultures are not or only weakly inactivated under identical assay conditions. To obtain 50% inhibition of the "chicken enzyme" a 40–200 fold excess of IgG compared to the homologous AMV enzyme must be added [1,4], whereas in the case of the "quail enzyme" no inhibition could be observed (Fig. 2). In contrast to that, the polymerases from Rous Associated Virus O (RAV-O), an endogenous Avian Leucosis Virus (ALV) as well as polymerases from exogenous ALV/ASV are inhibited to the same extent as the AMV-enzyme [5]. This result shows that 1. all the polymerases within the ALV/ASV group behave identical in this assay and are 2. different from the enzymes described here.

The chicken- and quail-enzymes can also be distinguished from the AMV-enzyme when the enzymes are incubated at high temperatures (52°) with or

Table 1. Reverse transcription of heteropolymeric RNA by particle associated reverse transcriptase released by chicken and quail cells

Template	Primer	Conditions	cpm ^{3}H-dGMP incorporated	
			a	b
globin mRNA	$(dT)_{12}$	complete	5000	819
globin mRNA	$(dT)_{12}$	complete + RNAse	270	n.t.
globin mRNA	—	complete	190	93
70S AMV RNA	—	complete	220	n.t.
70S AMV RNA	$(dT)_{12}$	complete	2340	n.t.
poly (C)	$(dG)_{12-18}$	complete	113700	11300

The reaction mixture contained as described in the standard polymerase assay for reverse transcription of homopolymeric RNA (1): Magnesium acetate (10 mM). KCl (40–80 mM). Tris-HCl pH 8.3 (50 mM). dithioerythritol (10 mM). ATP (1 mM). BSA (50 µg/100 µl). template primer complex (1 µg). except that ^{3}H-dGTP (19 Ci/mMole) was present at a concentration of 26 µM (50 µCi per 100 µl assay).

The assays for reverse transcription of heteropolymeric RNA contained 3 µg RNA and 1 µ $(dT)_{12}$ respectively and unlabelled dATP. dCTP and dTTP at concentrations of 0.3 mM. When particle-bound polymerase was tested. the assay contained 0.1% NP40. The reaction volume was 100 µl. The incubation was at 37°C for 35 minutes for the chicken enzyme (a) and 45 minutes for the quail enzyme (b). Incorporation was determined as TCA precipitable counts in 30 µl (a) and 100 µl (b) resp.

Table 2. Different optimum conditions for AMV- and particle-polymerases

Enzyme from	Mg^{2+} (mM)	Mn^{2+} (mM)	K^+ (mM)	pH	Temp. (°C)	K_M-dGTP (µM)
AMV	10	0.8	80	8.5	40	3.3; 20[a]
Quail cells	5	0.5	60	8.5	45	4.0; 28[a]
Chicken cells	5	0.4	60	8.5	45	25

[a] biphasic Lineweaver-Burk plot

Standard polymerase assay conditions were as described in Table 1 with poly (C) $(dG)_{12}$ as template primer complex at a concentration of 1 µg per 100 µl assay and ^{3}H-dGTP (19 Ci/mMole) at a concentration of 2.6 µM (5 µCi per 100 µl assay).

without 70S AMV RNA (Table 3). They are significantly more stable at high temperatures compared to the AMV polymerase. In the presence of 70S RNA, AMV polymerase is markedly protected against heat denaturation; no such stabilizing effect of 70S RNA is detectable with the enzymes prepared from cell culture medium.

The chicken- and quail-enzymes are also different from the polymerase of the Reticuloendotheliosis virus (REV). As shown in Table 4 they prefer as cation Mg^{2+} instead of Mn^{2+} as does the REV enzyme. The ratios are 7,2, 4,6 and 0,4 resp.

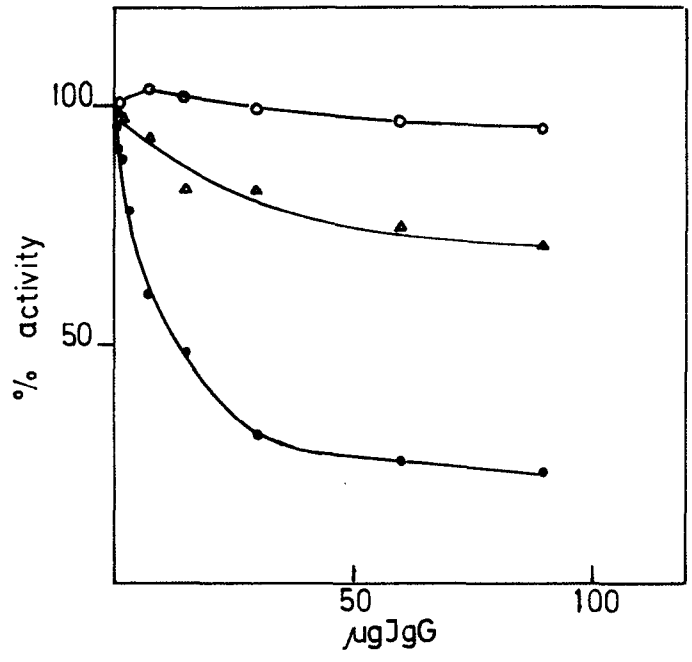

Fig. 2. IgG inhibition test of RNA dependent DNA polymerases from chick and quail embryo cell cultures. compared to AMV polymerase. Constant amounts of enzyme were preincubated with increasing amounts of IgG directed against AMV polymerase. Experimental details are as described in [4]. except that the incubation was carried out at 37°C for 15 min instead of 0°C for 15 h. This modification leads to a less rapid and less complete inactivation of the AMV enzyme than reported in [4]. but the activity of the quail-enzyme even in absence of IgG is better conserved

Table 3. Thermal inactivation of different reverse transcriptases with and without 70S AMV RNA at 52°C

Enzyme from	Time for 50% inhibition (min.) – 70S	Time for 50% inhibition (min.) + 70S
AMV[a]	0.8	4.7
Chicken cells	12	12
Quail cells	19	20

[a] Avian myeloblastosis virus

The enzymes were preincubated with and without 70S AMV RNA at 52°C for varying times and the residual activity determined in a standard polymerase assay with poly (C) $(dG)_{12}$ as template primer complex.

Table 4. Different cation preference of REV and particle polymerases

Enzyme from	Mg^{2+} ^3H cpm	Mn^{2+} ^3H cpm	Relation of activities Mg^{2+}/Mn^{2+}
REV[a]	478×10^3	1080×10^3	0.44
Quail cells	2.7×10^3	0.6×10^3	4.62
Chicken cells	13.0×10^3	1.8×10^3	7.22

[a] Reticulo endotheliosis virus

Standard polymerase assay conditions were. as described in Table 1. using 10 mM Mg^{2+} and 0.4 mM Mn^{2+} resp. Poly (C) $(dG)_{12}$ was used as template primer complex at a concentration of 1 µg per 100 µl assay and ^3H-dGTP (19 Ci/mMole) at a concentration of 2.6 µM (5 µCi per 100 µl assay).

No difference, however, is detected between the chicken-enzyme and the particle bound polymerase isolated from the allantoic fluid of embryonated chicken eggs [1]. For all properties tested [1,2,3,7] identical values were found.

Conclusions

These results show that primary chick embryo cells in culture release a particle bound RNA dependent DNA polymerase into the culture fluid. The enzyme is also found in the allantoic fluid of embryonated chicken eggs. This phenomenon is not restricted to the chicken system because primary quail embryo cells which are not known to express the so far known endogenous viruses release a particle bound RNA dependent DNA polymerase as well. In both cases this enzymes may be part of an hitherto unknown endogenous viral system. However, since the expression of the chicken- and quail-enzyme goes through a sharp peak during embryogenesis one may speculate on a physiological role for the newly described enzymes during development.

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Pluripotent Teratocarcinoma – Simian Virus 40 Transformed Mouse Fibroblasts Somatic Cell Hybrids

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Introduction

Mouse pluripotent embryonal carcinoma cells have been shown to be resistant to infection with polyoma virus and simian virus 40 but become susceptible to both viruses when allowed to differentiate in vitro [1,15]. The mechanism for this restriction is unknown but the block in the infectious cycle has been located after penetration of the virus particles and before synthesis of the early T antigen [2,16].

In this paper we describe the properties of hybrids between embryonal carcinoma cells and SV40 transformed mouse fibroblasts. This cross was undertaken in the hope to obtain hybrids with an embryonal carcinoma phenotype and containing an integrated SV40 genome. Such hybrids would be useful to study the expression of viral genes in embryonal carcinoma cells and during their differentiation. It is known from previous studies that different types of hybrid cells can result from crosses between embryonal carcinoma cells and differentiated cells. Whereas fusion with thymocytes [10] or Friend erythroleukemia cells [11] can give rise to hybrids with an embryonal carcinoma phenotype, fusion with fibroblasts have so far only given fibroblastic hybrids [3,7,9]. It has been proposed that the hybrid phenotype might at least in part depend on the chromosome constitution of the differentiated parent: the subtetraploidy of the fibroblasts used in these studies could favour the expression of the differentiated function [10].

In the experiments reported here, we have chosen as the fibroblastic parent cells from the SVT2 line of SV40 transformed fibroblasts, because of their near diploid chromosome constitution [8]. We have not yet succeeded in obtaining a stable embryonal carcinoma like hybrid and most hybrids appear to be fibroblastic. However we show that it is possible to obtain a new class of hybrids which retain some of the properties of the embryonal carcinoma parent.

Results

The cell lines used are: 1. PCC4azal, an azaguanine resistant clone of the embryonal carcinoma line PCC4 [6] which is near diploid [4]; 2. A clone of the SV40 transformed fibroblast line SVT2 which is resistant to 30 μg/ml of 5-bromodeoxyuridine (BUdR).

Table 1. Chromosome constitution of hybrid and parent cells[a,b]

Chromosomes no. mean (range)	PCC4 markers[c] 1/1	13/13	SVT2 markers[c] 1/3	14/14	19/19	19/M1
PCC4 39 (39–40)	1	1	–	–	–	–
SVT2 40 (35–45)	–	–	1	1	1 or 19/M1	1 or 19/19
F10 68 (63–80)	1 or 0	1	1 or 0	1	1 or 19/M1	1 or 19/19
D2 74 (71–78)	1	1	1	1	0	1

[a] Cells were grown in Dulbecco modified Eagle's medium containing 10% foetal calf serum in an atmosphere of 12% CO_2 and at 37°C. 3.10^5 cells from both parents were seeded in the same 100 mm dish and fused according to the Polyethyleneglycol procedure (12). 24 hours later, they were seeded at lower density (2.10^5 cells per 100 mm dish). After an other day hybrid cells were selected in HAT medium (supplemented with 68 mg/l hypoxanthine. 0.88 mg/l aminopterin and 19 mg/l thymidine) with medium changes every two days. After two weeks the morphology of the colonies was examined and clones were isolated.

[b] Chromosome preparations were made according to a standard air drying procedure. Individual chromosomes and markers were identified by their G-banding pattern using the trypsin-Giemsa method (14).

[c] PCC4 markers (4) and SVT2 markers (8) have been described previously

Hybrids between cells from the two lines have been selected in HAT medium as described in legend for Table 1. Colonies arise at a frequency of about 1 in 10^4 cells, most of which resemble the fibroblastic parent. However one in ten colonies appears to be composed of cells with a different morphology. Colonies were isolated and two of them were chosen for further studies: F10 which is fibroblastic and D2 which is made of round and loosely attached cells.

It is shown in Table 1 that both clones are subtetraploid and contain marker chromosomes from both SVT2 and PCC4. They therefore appear to contain most chromosomes from both parents. This is particularly striking for the D2 hybrid which contains all marker chromosomes as well as four copies of most autosomes as illustrated in Fig. 1.

Some properties related to the transformed phenotype of the SVT2 cells are shown in Table 2. F10 is as tumorigenic as the SVT2 parent and the tumors produced are typical fibrosarcomas. The SV40 T antigen is expressed in 100% of the cells. Saturation density in 10% fetal calf serum does not correlate here with tumorigenicity and is ten times lower for the hybrid than for SVT2 cells. N2 is less tumorigenic and the tumors obtained are sarcomas of a different type. The T antigen is heterogeneous with only 80% of positive cells, approximately a third of which are weakly stained.

We have also examined the distribution of cell surface antigens which have been studied in the mouse teratocarcinoma system [5]: 1. The early embryonic F9 antigen which is common to all embryonal carcinoma cells tested, cleavage embryos and spermatozoa, but is absent from differentiated cells. 2. The H-2 antigen, from the major histocompatibility locus, which is present on a variety of differentiated cells and also appears on embryonal carcinoma cells when they are allowed to differentiate. Results in Table 2 show that the F10 hybrid carries the $H\text{-}2^d$ haplotype from SVT2 cells (Balb/c

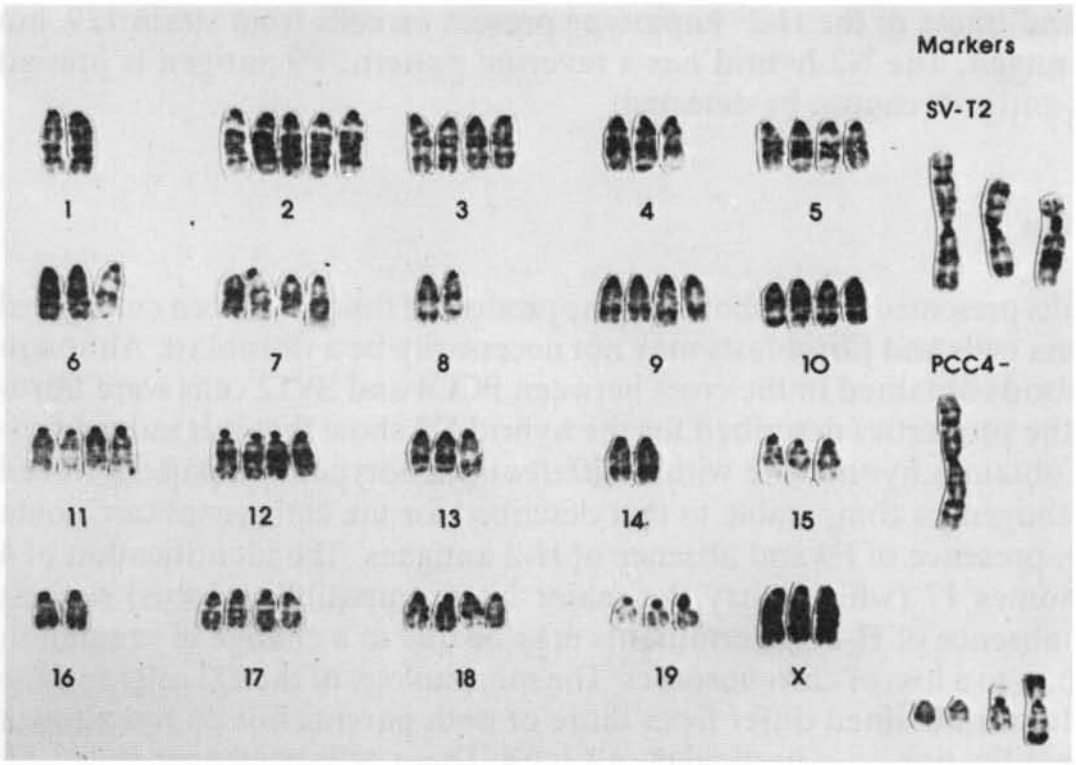

Fig. 1. Trypsin-Giemsa karyotype of one D2 hybrid cell

Table 2

Morphology		Tumorigenicity		SV40 T antigen	Saturation density	Cell surface antigens[d]		
		Effi-ciency[a]	Type of tumor[b]	% positive cells[c]	cells per $cm^2 \times 10^{-5}$	F9	H-2^d	H-2^b
PCC4azal	embryonal carinoma	high	embryonal carcinoma with multiple differentiations	0	ND	+	–	–
SVT2 BUdR^r	fibroblast	high	fibrosarcoma	100	10.6	–	+	–
F10	fibroblast	high	fibrosarcoma	100	1.1	–	+	traces
D2	round	low	polymorphic sarcoma	80	1.2	+	–	–

[a] Cells were injected subcutaneously and intraperitoneally in (129/SV × Balb/c) F1 hybrid mice irradiated with 600 rads from a cesium source. "High" refers to tumor formation in 100% of the mice after injection of 3.10^6 cells; "low" corresponds to tumor formation in 50% of the mice when injected with 10^7 cells.

[b] Histological preparations were made and examined by Dr. J. Gaillard (Institut Pasteur) as described previously (6).

[c] SV40 T antigen was detected by indirect immunofluorescence (1).

[d] Cell surface antigens were detected as described previously (5). In brief, the specific antisera were absorbed with the cells to be tested. Residual activity was examined in direct cytotoxicity tests with adsorbed rabbit complement on the appropriate cells: F9 cells for the anti-F9 serum, DBA/2 (H-2^d) and 129/Sv (H-2^{bc}) lymphocytes for the anti-H-2^d and anti-H-2^b sera respectively. Antisera were kindly given by P. Dubois (Institut Pasteur)

strain) and traces of the H-2^b haplotype present on cells from strain 129, but no F9 antigen. The N2 hybrid has a reversed pattern: F9 antigen is present but H-2 antigens cannot be detected.

Discussion

The results presented above show that the product of fusion between embryonal carcinoma cells and fibroblasts may not necessarily be a fibroblast. Although most hybrids obtained in the cross between PCC4 and SVT2 cells were fibroblastic, the properties described for the hybrid D2 show that it is indeed possible to obtain a hybrid line with a different phenotype. The pattern of cell surface antigens is comparable to that described for the embryonal carcinoma cells: i.e. presence of F9 and absence of H-2 antigens. The identification of 4 chromosomes 17 (which carry the major histocompatibility locus) suggest that the absence of H-2^d determinants may be due to a change in regulation rather than to a loss of chromosomes. The morphology of the D2 cells and the type of tumor obtained differ from those of both parents but do not allow a clear identification to a particular cell type. These cells might represent an intermediate state of differentiation. The use of a pseudo diploid fibroblast may have allowed the obtention of such cells.

The expression of the transformed phenotype of SVT2 cells is modified in the D2 hybrids as shown by decreased tumorigenicity and reduced expression of T antigen. The heterogeneous staining for T antigen resembles that described for SV40 intermediate transformed cells [13]. These results encourage us to think that such hybrids might be useful to study the effect of the host genome on the expression of viral genes. Further characterization of this and other independently isolated hybrids is in progress.

Acknowledgement

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Summary of Meeting on Modern Trends in Human Leukemia

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An international meeting concerned with various aspects of leukemia, including etiology, diagnosis, pathogenesis, and clinical treatment, represents a major challenge and a promise for interaction among scientists with various backgrounds and representing many disciplines. The organizers of the third meeting on human leukemia in Wilsede molded the framework for this interaction by assembling a distinguished group of scientists, thus providing both formal and informal opportunities to discuss problems pertaining to leukemia. All sessions included highlights of new and exciting findings that were well presented by the investigators. Regrettably, many found it difficult (and in some instances impossible) to communicate information to a mixed group of scientists in such a way that those not directly involved in a particular type of research were able to digest, interpret, and comment on the work. Despite this shortcoming, it became obvious during the meeting that major progress is being made in better diagnosis of different forms of leukemia and that the clinician is better able to respond to the challenge of curing the patient because of it.

The magnitude of the problem in the human population can best be summarized by noting that there will be an estimated 88300 cases of leukemias and lymphomas in the United States resulting in approximately 57900 deaths. Translated on a worldwide level, these estimates would suggest that1 766000 new cases will develop during 1978 and that approximately 1 160000 deaths will occur because of these diseases. Leukemia alone will claim 21500 new victims in the United States and 430000 victims globally, causing an estimated 15100 deaths in the United States and approximately 300000 deaths on an international basis. Thus, these diseases represent a significant impact on society and the number of cases is likely to increase as the population continues to rise unless measures are found to prevent new cases or to abort the disease early after its onset.

It is obvious that the etiology of leukemias and lymphomas in the human population still remains elusive despite large-scale efforts to demonstrate causation. While some epidemiologists continue to claim that the absence of clusters of childhood leukemia indicates that an infectious entity plays no role in the disease, it has become increasingly evident that no single environmental factor or group of factors can yet be singled out as the offending agent. This, coupled with the well-known ability of viruses to cause leukemia and lymphoma in a wide variety of animal species, continues to focus atten-

tion on this area. Recent information concerning feline leukemia suggests that clusters are absent in the natural population (except when cats are concentrated in large numbers in households) despite the fact that this disease clearly appears to be caused by a leukemia virus which generally spreads by horizontal transmission. In fact, cat registries did not reveal that the disease was infectious until households were examined and seroepidemiology was employed. Of great importance is the recent finding concerning the absence of additional copies of virus sequences in some leukemic cats, a situation that may be analogous to human leukemia and may explain the difficulty in finding specific sequences relatable to viruses in human leukemic cells.

It has not yet been possible to incriminate recent human oncornavirus isolates in the etiology of leukemia. Clouding these isolations of putative human viruses is the fact that after careful examination, most of the isolates consist of a mixture of viruses of simian and rodent origin. The explanation for such mixtures remains obscure. It is noteworthy, perhaps, that sequences that can be related to those found in the genome of Gibbon ape sarcoma virus have been detected in human cells. These results require confirmation and their significance is unclear at this time. Thus, the continued failure to come to grips with the etiology of leukemia focuses more attention on early diagnosis and better classification of the various neoplasias.

Improved methods of chromosome analysis continue to reveal specific changes in the karyotype of leukemic cells. For example, in 97% of chronic myelogenous leukemia, chromosomes 9 and 22 are involved in translocations. In 50–75% of acute promyelocytic leukemia, there is a translocation of chromosome 17 to chromosome 15 and in Burkitt lymphoma cells, a translocation of chromosome 8 to chromosome 14 is regularly observed. The origin of these translocations is not known, but they clearly serve as useful markers for diagnosis and it is beginning to appear that they will help clarify the regimen of therapy to be used in individual patients. The use of enzyme markers, such as glucose-6-phosphate dehydrogenase, is also proving useful in the continued study of pathogenesis. Certainly, these and other studies are valuable in understanding differentiation, but the hope that differentiation studies will further knowledge of the neoplastic process has not yet been definitively realized.

Even though progress in pathogenesis of human leukemias and lymphomas is agonizingly slow, major advances are being made in model systems designed to demonstrate how the addition of virus genetic information can cause transformation. Thus, one of the more exciting reports at the meeting involved the possible structure and function of the gene product coded by the sarcoma (src) gene of the avian sarcoma viruses. Identification of a protein with a molecular weight of 60000 daltons by immunoprecipitation using rabbit serum revealed that the purified product has protein kinase activity which may affect regulation of various cell characteristics. What remains unknown is how this kinase specifically affects regulation and leads to a malignant phenotype. Additional information concerning the gene products of other sarcoma and leukemia viruses and their roles in oncogenesis should be forthcoming soon.

The isolation of putative human leukemia viruses and the possible existence of some simian sarcoma virus sequences in human leukemic cells has also prompted examination of human sera for reactivity against a variety of known animal oncornaviruses. Regrettably, observations concerning antibody in the sera of some patients against the simian sarcoma virus p70 antigens are not matched by findings of those antigens in human tissues. Rather, human tissues contain a protein that appears to be similar to the p30 of these viruses and this discrepancy clearly needs to be resolved. Overall, evidence that human neoplastic tissues contain virus-specific antigens and that the sera contain antibodies against those antigens remains relatively weak and requires further clarification.

Luckily, progress in leukemia treatment has been substantial and represents a major achievement. Remission rates remain better than those observed in other common neoplastic diseases such as breast and lung carcinomas, although improvement is still required to reduce both morbidity and mortality. Treatment results still vary considerably from one center to another and more communication between clinicians and basic scientists would go far to facilitate new approaches. Unfortunately, the language of the research scientist and clinician often differ, and their views of the problems involved often diverge considerably. It is meetings of the kind that took place in Wilsede that hopefully will bring those divergent views to a common level. Among the major problems besetting continued progress is the variety of systems being developed by individual investigators despite their similarities to many existing systems. Perhaps it is time to remind those working in oncology that great progress was made in understanding the properties of bacterial viruses because most investigators agreed to work on only certain viruses in an effort to facilitate progress. In an era of diminished funding, this may well serve as a model for current studies in oncology.

The relative rarity of leukemia in the human population remains instrumental in its confusing etiology. However, there are numerous infectious diseases known to be caused by viruses that are equally uncommon (such as encephalitis due to herpes simplex virus or subacute sclerosing panencephalitis due to measles virus) and which fail to cluster in the population. Clinicians, with the help of virologists, immunologists, and pathologists, have been able to demonstrate etiology using sensitive virus isolation techniques and sensitive (and specific) methods involving serology. However, this requires the preparation of specific probes which, unfortunately, are not yet available for studies of human leukemias and most lymphomas. The failure to generate such specific molecular and immunologic probes has perpetuated confusion when looking for genetic sequences and antigens in human tissues, and the corresponding antibody in sera or other fluids in the human body. Hence, as brought out by the meeting, further refinement of technology and extremely conservative and careful interpretation of observations continue to be the leading requirements in this field.

In summary, it would appear that leukemia may be the first important human neoplastic disease to be controllable. Theoretical model systems are highly encouraging, and are leading to a better understanding of cell conver-

sion from a normal to a malignant phenotype. If etiologic studies concerning viruses are to be pursued, it would seem logical to look for agents early in life (in healthy children and during pregnancy), because if such viruses are to persist in the population, they clearly must replicate in most members of the population early in life, only to cause disease at some subsequent time. Although viruses may play a prominent role in cancer etiology it is plausible that other factors are involved in initiating disease. Techniques that have been developed in the infectious disease laboratory clearly should be applied more effectively to study the etiology of human leukemia and other neoplastic diseases with the long-range hope that isolation of such agents and identification of the disease may lead to prevention, rather than to the more expensive and more traumatic treatments that are now available. The field seems ripe for a breakthrough. No one will benefit more than future generations who will thank the dedicated efforts of investigators such as those attending the Wilsede meeting.

Subject Index

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