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J.F. LEHMANNS VERLAG MÜNCHEN

Modern Trends in Human Leukemia

Biological, Biochemical, and Virological Aspects

MODERN TRENDS IN HUMAN LEUKEMIA

BIOLOGICAL, BIOCHEMICAL AND VIROLOGICAL ASPECTS

Edited by

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Workshop: Modern Trends in Human Leukemia
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On behalf of the Deutsche Gesellschaft für Hämatologie
and the National Cancer Institute
the Workshop was organized by

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Under the protection: Niedersächsischer Sozialminister
Kurt Partzsch

„Glauben Sie, daß Peter von Amiens den ersten Kreuzzug zusammengetrommelt hätte, wenn er so etwa beim Erdbeerpflücken einem Freunde mitgeteilt hätte, das Grab Christi sei vernachlässigt und es müsse für ein Gitter gesorgt werden? "
(Theodor Fontane; Stechlin, Vol. 3, p. 346)

Cited in a letter Karl Thomas wrote asking for financial aid for his institute.
(Karl Thomas, 1883–1969, former director of the Medizinische Forschungsanstalt, Max-Planck Gesellschaft, Göttingen)

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Preface

Nearly every month there are workshops, conferences or congresses devoted to the problems of human leukemia, for our knowledge is quite limited. In large measure, these conferences have been concerned with special aspects of leukemia. In this workshop we have brought together scientists from different research areas in human leukemia. Therefore the title "Modern Trends in Human Leukemia" does not only apply to the discussion of the importance of molecular biology, but also includes the 100 year old history of the leukemic cell as the basis of biological and immunological aspects in human leukemia. Modern trends in human leukemia need to be discussed based on the past, present and possible future information gathered from all different, but related topics.

The idea to bring together highly qualified biochemists, medical doctors, and virologists to learn, like students, about each other's fields has been very unusual. But to understand human leukemia, the virologists and biochemists have to learn more about the properties of the human blood cell, and the medical doctors have to learn where and how leukemic misinformation can influence the normal regulation of the molecular control mechanism in a blood cell.

To start such a workshop, therefore, was to start an experiment. In this experiment, the hope was that these scientists would learn about each other's research fields and also teach others about their own specialized fields as well. The final aim was that those in the workshop would discuss the whole problem of human leukemia, and cooperative research programs among the different specialized groups would be stimulated.

We tried the experiment for three long days and nights in a 350 year old farmhouse. I would like to thank all people who made this possible. Our hope is now that you can be stimulated and encouraged to try similar experiments.

Rolf Neth

Round table discussion
"Flett" and "Grotdeel" of "De Emhoff"

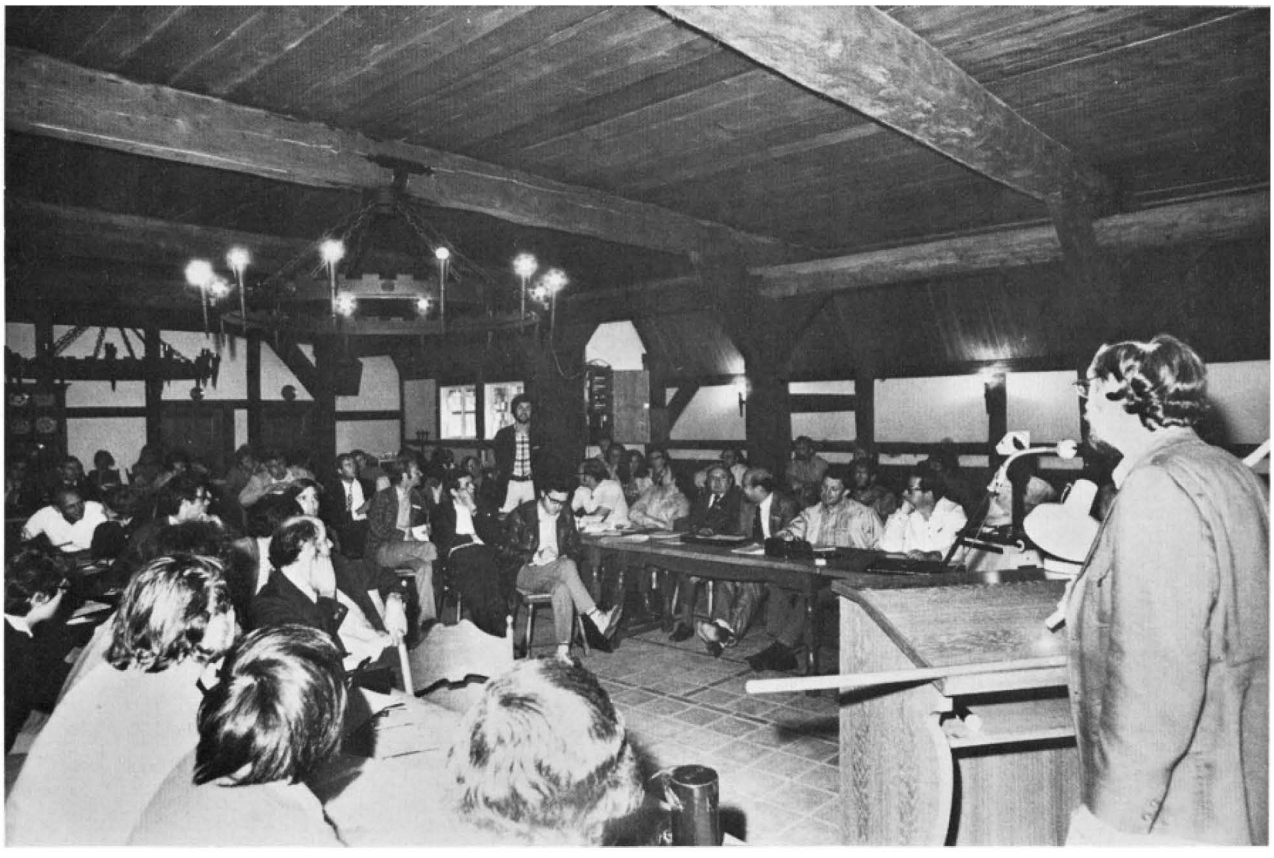

Foto: Moldvay (STERN Magazin)

HUMAN LEUKEMIA – AN OVERVIEW

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Prior to twenty-five years ago, there was no specific therapy for acute leukemia and survival of individuals with these diseases was usually no more than 3 or 4 months. There was no useful specific therapy, treatment consisting largely of blood transfusions and other supportive measures. X-irradiation, radioactive phosphorous, benzene, potassium arsenite, and nitrogen mustard, although of some use in chronic leukemia, were of little value in acute leukemia. Then, in 1948, Farber and his colleagues (1) reported that folic acid antagonists could induce complete remission in acute lymphocytic leukemia of children.

Subsequent work demonstrated that these agents, particularly aminopterin, would induce remissions in approximately 30 % of children with acute leukemia

EFFECTIVE DRUGS
IN CHILDHOOD ACUTE LYMPHOCYTIC LEUKEMIA

	NO. PATIENTS	CR (%)
6 MP	43	27
MTX	48	22
VCR	103	57
CYTOXAN	45	40
ASPARAGINASE	32	44
DAUNOMYCIN	82	15
ADRIAMYCIN	144	25
PREDNISONE	337	63
ARA-C	122	7
BCNU		
TG		
HYDROXYUREA		

CR= COMPLETE REMISSION

MODIFIED, HENDERSON, 1969

Fig. 1: Useful drugs in the treatment of childhood acute lymphocytic leukemia. Modified from Henderson, E. S.: Treatment of Acute Leukemia. Seminars in Hematology 6: 271–319, 1969.

but in far fewer adults with acute leukemia. Unfortunately, remissions were temporary, the patients soon became refractory to the agents and survival was affected little, if at all. Important as these observations were, there followed little systematic fundamental work aimed at the control of cancer and specifically, leukemia. However, the observations with aminopterin and amethopterin gave rise to a good deal of optimism that curative treatment could soon be achieved and there followed a gradually intensifying effort to discover other drugs which could induce remissions. During the last two decades approximately one dozen agents (Figs. 1 & 2) have been found which are effective in acute leukemia. Some of these were discovered empirically and others were developed as an outgrowth of biochemical or other rationale. As a consequence, the incidence and duration of remissions have increased greatly and survival has gradually been extended so that median survival is now 36 months or more for childhood acute lymphocytic leukemia (Fig. 3). In some studies, this is now at approximately 5 years. Unfortunately, in adult acute leukemia, progress has been far slower. Remission rates of 50 % are not unusual but survival has been lengthened only relatively little. These results in childhood and adult leukemia have not been achieved with any single agent but are due to the use of combinations of drugs, a better understanding of the importance of drug scheduling, supportive care, and patient protection.

EFFECTIVE DRUGS IN AML (ADULT)

	CR (%)
PREDNISONE	15
6 MP	10
MTX	3, 16
ARA-C	21, 37, 44
DAUNORUBICIN	38, 50
ADRIAMYCIN	27
METHYL GAG	45
VINCRISTINE	20
BCNU	
TG	
HYDROXYUREA	

CR= COMPLETE REMISSION

MODIFIED, HENDERSON, 1969

Fig. 2 Drugs for the treatment of adult acute myelocytic leukemia. For many of these, the number of patients treated with the individual drugs, is too few to make complete remission rates meaningful.

CHANGING PROGNOSIS IN ACUTE LYMPHOCYTIC LEUKEMIA

YEARS	GROUP OR INVESTIGATOR	TYPE OF THERAPY	MEDIAN SURVIVAL (MO. FROM DIAGNOSIS)
1937-53	Tivey	Supportive	3.5
1946-47	Burchenal	Supportive	3.5
1944-60	Boggs	Supportive → Pred., MTX, 6-MP	7.0
1956-57	Hendersen	Pred., MTX, 6-MP	5.0
1961	Boggs	Pred., MTX, 6-MP	12.0
1954-62	Cutler	Pred., 6-MP, MTX	12.0
1958-62	ALGB	Pred., MTX, 6-MP, Cytoxan	12.0
1959-63	Burchenal	Pred., MTX, 6-MP, Cytoxan	13.0
1955-63	Zuelzer	Pred., MTX, 6-MP	16.0
1963-64	NCI	VAMP	24.0
1963-65	ALGB	Combination of Pred., 6-MP, VCR, MTX, Cytoxan	24.0
1965-66	NCI	POMP	33.0
1966-68	ALGB	Combination of Pred., VCR, MTX, 6-MP, daunomycin	> 36.0
1966-71	St. Jude's	Combination of VCR, Pred., 6-MP, MTX, Cytoxan	> 36.0

Fig. 3: Progressive improvement in survival in patients with acute lymphocytic leukemia. Carter, S. K.: The Chemotherapeutic Approach to Cancer Therapy: A Quick Overview. In Year Book of Cancer, 1972. Clark, R. L. & Cumley, R. W. (eds). Year Book Medical Publishers, Chicago. pgs. 475—498.

A patient with acute leukemia dies because leukemic cells have compromised the function of an organ or normal tissue to the extent that some vital function is no longer possible. Suppression of marrow function is frequent either as a consequence of the disease itself or due to the use of myelotoxic drugs. Bleeding due to thrombocytopenia was until relatively recently the most common cause of death but at present, infections, particularly gram negative infections, are the most serious problem (2). The generous use of platelet transfusions has been responsible for the diminution of fatal thrombocytopenic hemorrhage but granulocyte transfusions have not been widely accepted probably due to the fact that until the last few years the procurement of normal granulocytes in large quantities has not been possible. In addition, there were problems in designing controlled studies to evaluate their effectiveness. However, it has recently been shown that histocompatible granulocyte transfusions are useful in the management of serious infections when given repeatedly to granulocytopenic patients (3). Another approach to the control of infection has been the use of protected environments and although their ultimate role in cancer therapy is yet to be defined, there is strong evidence that the incidence of infection is greatly reduced (4).

The strategy in the management of patients with acute leukemia has been to attempt to achieve rapid reduction of the leukemic cell population and restoration of normal bone marrow function followed by therapy designed to eradicate the neoplastic cells. Subsequently, maintenance therapy is instituted to keep the patient in remission and prevent overt appearance of the disease.

Although with the years more agents with activity in leukemia have been discovered, the most important factor in the improved prognosis in acute leukemia has

been the employment of drug combinations based on the underlying principle of using agents with different dose-limiting toxicities and with different mechanisms of action in order to minimize the development of drug resistance. There is abundant evidence that combinations of drugs can achieve remission rates as great or greater than predicted for additive effects of the single drugs employed (Fig. 4).

The role of immunotherapy in the management of patients with acute leukemia remains to be determined. There is evidence for tumor associated or tumor specific antigens on the surface of acute leukemia blast cells and prognosis appears to be related to immune reactivity. There have been many attempts to manipulate the immune mechanism to therapeutic advantage using immunization with syngeneic, allogeneic or isogenic cells, BCG and other immune enhancers, transfusion of immune sera, and syngeneic or allogeneic bone marrow transplants. Unfortunately, in spite of all these efforts, the role of immunotherapy in acute leukemia remains uncertain.

The success of chemotherapy in acute leukemia is undoubtedly dependent on exploitation of differences in cell uptake, biochemical control mechanisms, and cell kinetics and other factors which are not completely understood. Most of the advances in the treatment of leukemia have been achieved through the empirical search for anti-tumor drugs. Contributing factors include: 1. Synthesis or isolation of drugs from natural products and their evaluation for anti-tumor activity in animal

SINGLE VS. DRUG COMBINATIONS IN A.L.L.

	MARROW REMISSION (%)
PREDNISONE	67
VINCRISTINE	57
6 MP	27
MTX	22
PREDNISONE + MTX	80
PREDNISONE + VINCRISTINE	87
PREDNISONE + 6 MP	82
VAMP	88
POMP	94

Fig. 4: Examples of superiority of drug combinations compared to individual drugs.

systems. 2. Elucidation of their effects at the biochemical level. 3. Pharmacological and toxicological studies in animals in order to anticipate better pharmacologic disposition and toxicity in man and to provide guidance as to the route, dose, and schedule to be employed in man. 4. Pharmacologic studies in man. 5. Experimental trials in cancer patients to determine optimal dosage and schedules.

Undoubtedly, one of the major factors contributing to the success of chemotherapy, particularly against the rapidly growing tumors has been an understanding of the importance of drug scheduling concentrations at the target site and duration of effect. There are now numerous examples, both experimental and clinical, where a drug may be relatively ineffective on one schedule of administration yet result in a total remission with prolongation of survival on another schedule. The toxicity of an agent against both normal and neoplastic cells is directly related to its concentration (C) at the target and the duration of time (T) that this level is maintained. This so-called C x T concept is markedly affected by dose and schedules and optimally, the maximum number of tumor cells will be destroyed with minimal effect on the normal cells. It follows, of course, that different drugs are metabolized at different rates and their distribution in the body may vary.

Unfortunately, for many drugs, there appears to be little correlation between schedule dependency studies in L1210 or other experimental systems and clinical results. One of the difficulties lies in the fact that the cellular growth characteristics of L1210 leukemia and normal mouse marrow and the relationship between the two does not resemble any of the cancers in man including acute leukemia. More data are needed, not only of pharmacologic characteristics of drugs but also of the cell kinetics of both normal and tumor tissues at any given moment.

6. Supportive care, as already indicated, has allowed the clinician to treat more aggressively resulting in a greater cell kill. 7. Appropriate therapy to eradicate sequestered leukemic cells (as in the central nervous system). 8. The appreciation of the fact that acute leukemia is not a single entity and that the response to a given treatment varies according to the type of leukemia. The traditional classification of leukemia is based on morphologic description and clinical course and recently, cytogenetic analysis has been added to help in identifying certain subclasses and as a guide in prognosis. Many characteristics of leukemic cell populations – biochemical, kinetic, colony forming, cytochemical and ultrastructural – have been studied but most new classification proposals have been based on the use of finer cytological characteristics than those presently employed. Unfortunately, these are generally too difficult and controversial for general adoption. Nevertheless, it is obvious that the current classification is inadequate and a better scheme is needed in order to predict the course of leukemia and response to therapy.

In a broad sense, it can be stated that the vast amount of knowledge of leukemia including cell kinetics, biochemistry, molecular biology, cytogenetics, virology, and immunology has had relatively little impact on the management of patients with these diseases. This is true in spite of many optimistic opinions often expressed by investigators involved in these studies. The literature abounds with presumably logical concepts of leukemia cell growth and with sequences of macromolecular synthesis but who can say with real conviction that these reports have as yet had any impact in changing the prognosis of even a single patient with leukemia?

It is true that within the last decade, the relevance of cell kinetics of leukemic and normal leukocytes to successful chemotherapy of cancer has come to be recognized. An integral part of the anti-tumor development effort has been the constant search for drugs with "selective toxicity", i. e., drugs which could selectively destroy cancer cells without undue damage to normal cells. Unfortunately, this goal has never really been achieved and most clinically useful agents have significant and usually serious effects on normal tissue, particularly those with relatively rapid turnover times, the bone marrow and the gastrointestinal tract.

As is well known, under normal circumstances granulocytopoiesis is a cell renewal system so that cell production equals cell death. In patients with leukemia in relapse, granulocytopoiesis usually exceeds cell loss and an expanding cell population is the result (5). Granulocytes in the adult are produced in the bone marrow where there is an orderly division and maturation from the earliest cell, the stem cell, successively through the various cell types to the mature polymorphonuclear leukocytes so that fairly distinct morphologic compartments are identifiable (Fig. 5). In leukemia, in contrast to the normal situation, there is evidence from cell kinetic studies and from histological examination that leukemic cells may be produced in a variety of sites in addition to the bone marrow, i. e., lymph nodes, liver, spleen, testes, etc. The process of maturation and differentiation is disturbed and morphologic classification based on maturation is usually not possible.

Available evidence suggests that a cell perhaps similar to a small lymphocyte may be the common stem cell and that the erythroid, myeloid and megakaryocytic cell lines are probably derived from this pluri-potential cell. The stem cell compartment must be able to maintain itself against continued removal of cells for differentiation, reconstitute itself if depletion occurs, and be capable of increasing its rate of cell production upon demand. There is now good evidence in man that there is a single compartment which gives rise to these various cell lines. Support for this concept is provided from the observations by Whang *et al.* that the Ph^1 chromosome is present not only in granulocyte precursors but also in erythrocytic and megakaryocytic precursors (6). This suggests that the chromosomal defect arises in a cell

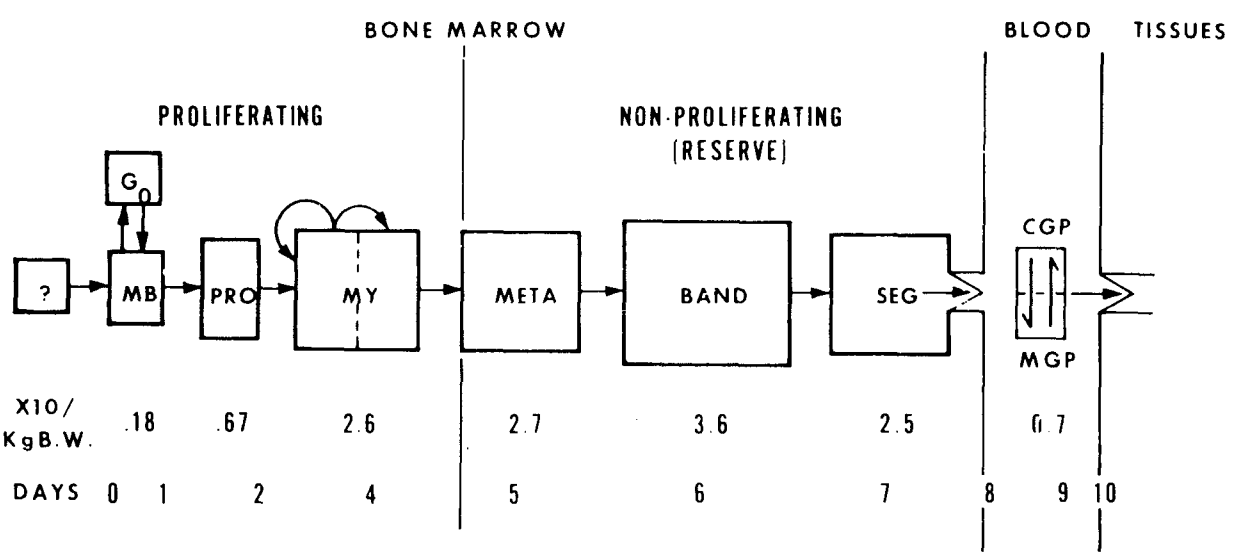

Fig. 5: Model for normal leukocyte kinetics.

which is a common stem cell for the three cell lines. Similarly, studies of the hematopoietic system of the mouse utilizing the spleen colony technique have also provided data suggesting that there is a single pluripotential stem cell.

In addition to the stem cell compartment there is also a large differential proliferating pool consisting of myeloblasts and promyelocytes. The next compartment in the sequence is the myelocyte pool composed of large and small myelocytes; the large cells representing a dividing pool supplying cells to the small cell maturation pool.

The proportion of proliferative cells in the bone marrow of patients with acute leukemia is relatively low compared to normal marrow (7–10). In normal bone marrow, approximately one-third of the myeloid cells are in proliferation with an average labeling index of about 30 % (11). Generation times for the myeloblasts, promyelocyte, and myelocyte have been estimated at 24, 60, and 54 hours respectively (12) with a maximum DNA synthesis time of 24 hours. It is now known that there may be a wide distribution of intervals for each of the phases. The variability in length of the G_1 phase has the most relevance to the chemotherapy of patients with leukemia since most of the presently available anti-leukemic agents do not affect cells in the long G_1 or so-called G_0 phase. This will be considered at greater length below.

With the completion of maturation, the granulocyte enters the so-called "mature granulocyte reserve" of the bone marrow. Estimates vary, but there are approximately $2–3 \times 10^{11}$ granulocytes in this compartment (13), and there are thus 10–20 times as many bands and segmented granulocytes in reserve as there are circulating in the blood.

The release of granulocytes into the blood is an interesting phenomenon which unfortunately is not well understood. Recent work suggests that changes in the biophysical properties of the cytoplasm as differentiation and maturation occur may be important factors (14).

It is important at this point to mention, if only briefly, some of the observations which have been made in recent years concerning granulocyte production *in vitro* (15). With both mouse and human bone marrow cells, colonies grown *in vitro* and arising from the colony-forming cell (CFC) require the continuous presence of a stimulatory substance, colony stimulating factor (CSF), which is found in sera and urine from normal and leukemic individuals and from mice. In the absence of this material, colony growth is not sustained and the cells rapidly die. It has been suggested that CSF is specific for neutrophils and that its major source are mature granulocytes. If this were the case, there would be no stimulus if an individual were rendered neutropenic and increasing myelopoiesis would result in the presence of granulocytosis. To confuse the issue further, there is good evidence that mature granulocytes are inhibitory (16) and that monocytes may be the source of material controlling granulocytosis (17).

CSF is a glycoprotein with a molecular weight of approximately 190,000 and is considered by many to be a growth regulator or granulopoietin for the granulocytic series analogous to erythropoietin for the red cell series. The function of CSF *in vivo* has not yet been elucidated; however, patients with acute lymphocytic or stem cell leukemia generally have elevated levels while those with acute myelocytic

leukemia have depressed levels (18). During remission, the levels in patients with acute myelocytic leukemia rise to normal or high values.

Diffusible granulocytopoietic stimulator (DGS) has been reported to be present *in vivo* in mice following the injection of endotoxin or after irradiation and has been shown to stimulate granulocyte production in Millipore filters implanted intraperitoneally (19). Preliminary data suggest that this material is different from CSF.

The relationship of CSF, DGS, chalones and other inhibitors, antichalone and leukocyte inducing factor is at present unclear and certainly somewhat bewildering. If there is a defect in this system in leukemia, its precise location is difficult to ascertain from reports in the literature. Finally, the significance, if any, of these observations for the treatment of patients with leukemia remains to be determined.

In contrast to the orderly unidirectional progression of division, maturation and release from the bone marrow of leukocytes in the hematologically normal individual, the picture in leukemia is largely one of confusion with marked deviation from the steady state (Fig. 6). In acute leukemia, normal leukocytes are replaced by large numbers of blasts both in the bone marrow and in the peripheral blood where the count may or may not be elevated. The spleen, liver and lymph nodes may be infiltrated with these cells and enlarged.

Years ago, it was assumed that in leukemia the orderly process of normal myelopoiesis was greatly disturbed owing to some unidentified influence and the myeloid precursors were rapidly and excessively proliferating. This hypothesis was never substantiated and was replaced by the current concept, first suggested by Astaldi and Mauri (7) that leukemic cells do not proliferate wildly, but that there is

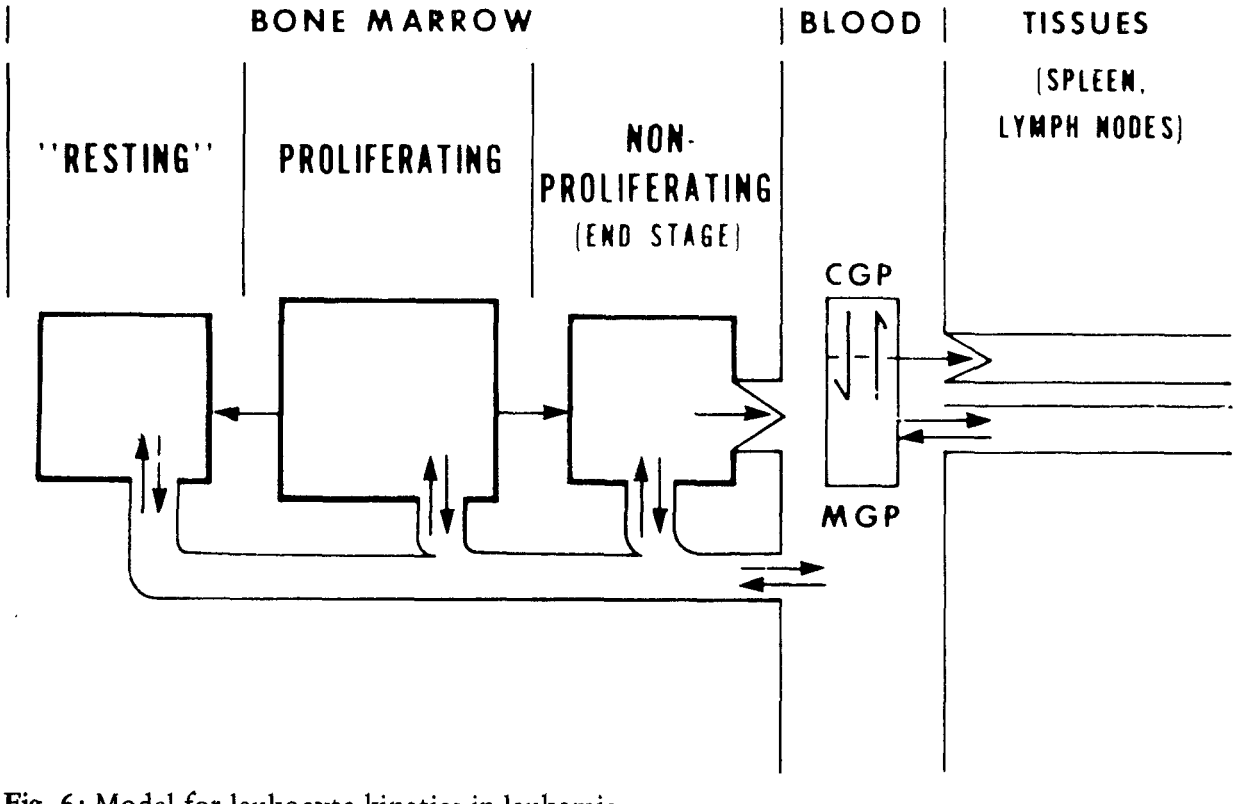

Fig. 6: Model for leukocyte kinetics in leukemia.

some maturation defect accompanied by the accumulation of large numbers of immature myeloid cells. Based on stathmokinetic and *in vitro* labeling studies with ^{3}HTdR, Gavosto et al (8, 9), suggested that the proliferative capacity in acute leukemia was very low compared to normal bone marrow and that the labeling index of blast cells in acute leukemia was in proportion to the size of the cells, the larger cells being considered the younger ones. These cells, in both AML and ALL, comprised a relatively small percentage of leukemia cells in the bone marrow and had a high labeling index (range 24–52) both after *in vitro* labeling with ^{3}HTdR and after a pulse label *in vivo* (20). In contrast, the labeling index of the small cells was quite low. It is now generally accepted that the large cells are the dividing or cycling population and that the small cells are the "resting" (G_0) or non-proliferating population. However, this population is obviously not "resting" in the strict sense and most likely is comprised of cells in a very prolonged G_1 phase. It is hypothesized, based on the interpretation of data obtained in patients with acute leukemia using ^{3}HTdR labeling (21), that the small "non-dividing" leukemia cells are capable of re-entering the proliferative cycle.

Studies in the spontaneous AKR mouse leukemia employing a cell separation technique conclusively demonstrate that the small cells have a normal component of DNA and even after labeling with ^{3}HTdR for a period equivalent to 5 cell cycle times, unlabeled cells are still present. These small cells are heterogeneous consisting of both non-clonogenic cells and clonogenic cells residing in either a G_0 or a long G_1 phase of the cell cycle (22–24). Upon transplantation to young normal AKR mice, the small cells are capable of proliferating and causing death due to leukemia.

There have been many cell kinetic studies in acute leukemia and although some of the data on cell cycle characteristics of leukemic leukocytes may be suspect it appears that (1) the majority of leukemic cells are capable of DNA synthesis but that most of the blasts are not in active proliferation (2) cell cycle times vary greatly (25–28), ranging from 60 to 200 hours and are generally somewhat longer than those for the early normal myeloid precursors and (3) the intravascular life of leukemic leukocytes is prolonged.

In contrast to the simple exponential intravascular disappearance pattern of normal granulocytes, leukocyte disappearance curves in patients with acute leukemia are often complex and prolonged (29–30). This may be present even when the patients are in remission and suggests that morphologically normal appearing granulocytes in these patients are still defective. On the other hand, extra-corpuscular factors cannot be ruled out since prolonged intravascular curves have been reported in patients with non-leukemic malignancies (31).

In hematologically normal individuals, granulocytes once having left the vascular tree, do not return but in AML (32, 33) as in CML (34) leukemic cells may enter the spleen and then recycle to the blood and the bone marrow. Leukemic cells are rarely seen dividing in the peripheral blood and the proportion able to incorporate ^{3}HTdR is less than that in the bone marrow.

The foregoing is a brief review of the current status of information concerning leukocyte kinetics in acute leukemia. The precise defect in acute leukemia specifically acute myelocytic leukemia, is not known but as has been postulated by Gallo (35) and others, the findings are consistent with a block in the normal process of

maturation of myeloid elements. Until the *in vitro* colony work discussed above this was considered irreversible but it now appears that leukemic cells can be made to mature under appropriate circumstances in the presence of a certain protein factor(s).

The cause of this disturbance in maturation is also not clear at the present time but in the last two or three years, a great deal of evidence has been accumulated strongly suggesting that RNA tumor viruses are involved. It is beyond the scope of this paper to review this evidence but regardless of whether one accepts the oncogene theory or the protovirus theory, the finding of the enzyme, reverse transcriptase, may be a most important development as far as the potential for controlling or curing acute leukemia. This enzyme appears to be distinct from RNA dependent DNA polymerase activities which have been reported in normal cells (36, 37). If reverse transcriptase is unique to leukemic cells it represents a prime target for therapeutic attack providing its presence is required for maintenance of the neoplastic state. Other DNA polymerases in leukemic cells, if qualitatively different from their counterparts may also be important targets. In any case, the reports of selective toxicity of rifamycin derivatives for leukemic cells are exciting (38) even though the precise mechanism for this toxicity is still unclear (37). Undoubtedly, other compounds will be found with similar or better selectivity.

The accumulating evidence suggesting that a virus may be the etiologic agent in leukemia and that reverse transcriptase plays an important role in the initiation of the desease and perhaps, in its maintenance raise important questions particularly in relation to relapses in patients after long apparently disease free intervals. Such relapses have been postulated to be due to 1) persistance of resting cells and their re-entry into cycle 2) a failure of the immune mechanism in preventing the appearance of clinically detectable leukemic cells arising from a small cluster of cycling cells 3) re-induction by the agent responsible for the initial event. The latter possibility gains some support from the experience with normal marrow transplants into leukemic patients in which leukemic transformation of donor cells were observed. However, other explanations for this phenomenon are possible. In addition, specific cytogenetic abnormalities when present in acute leukemia tend to disappear when the patient is in remission but the same abnormalities recur in late relapses. It would be most unlikely that a virus would cause precisely the same abnormality upon re-infection. However, it is conceivable that a sub-virus moiety might bind at the same site and produce the same karyotypic defect.

How has this knowledge I have reviewed been utilized in the management of patients and has it been useful? Based on data from animal studies and certain kinetic considerations it is possible to conceptualize (Fig. 7) neoplastic cell populations, including leukemia (20). Populations with a high proportion of cells in active proliferation and with a high clonogenic potential are classified into compartment A; cells temporarily non-dividing but capable of re-entering the growth cycle (cells in G_0 or in with a prolonged G_1 phase) are in compartment B; cells which are incapable of reverting to proliferation and are end-stage or mature are in compartment C; and finally, dying cells and cells undergoing lysis and resorption belong to compartment D.

In leukemia and in other neoplastic populations, growth occurs when the input

from compartment A exceeds the loss in compartment D (or with A constant, the loss in D decreases, a very unusual situation). At an early stage, the proportion of cells in active cycle (i. e. in A) is high and the proportion in a resting phase (i. e. in B) is low. As the disease progresses, the proportion in B increases and the doubling time of the whole population lengthens. This change in proliferative characteristics from early exponential growth is best described by a Gompertzian function (39). Obviously, the deviation from exponential growth may also occur from an increase in cell loss, a lengthening of Tc, or a combination of these factors. It is important to note, however, that growth fraction and cell loss are probably the prime determinants governing the rate of tumor growth although growth characteristics may be changed as a consequence of therapy. It has been shown, in fact, that regrowth of L1210 following treatment with BCNU is accompanied by cells dividing with a longer Tc (40) and similar observations have also been reported in acute leukemia (41).

Remissions occur when the loss in compartment D exceeds the input from A. Most clinical by useful anti-tumor drugs affect compartment A cells. These are the so-called cycle active drugs and include the anti-metabolites and the mitotic inhibitors. Alkylating agents and functionally related compounds probably have their major effect on compartment A cells but do also exert an effect on cells in compartment B. Unfortunately, the effects on these cells are not well understood and as will be seen, the persistence of these cells after treatment represents one of the serious problems in the management of patients with leukemia.

Kinetic data on normal and leukemic animal and human leukocyte populations have been examined with relation to response to chemotherapy (20). A number of observations emerge including 1) there is a direct relationship between the labeling index and the response to chemotherapy; 2) there is an inverse relationship between doubling time and response and 3) alkylating agents are more effective against

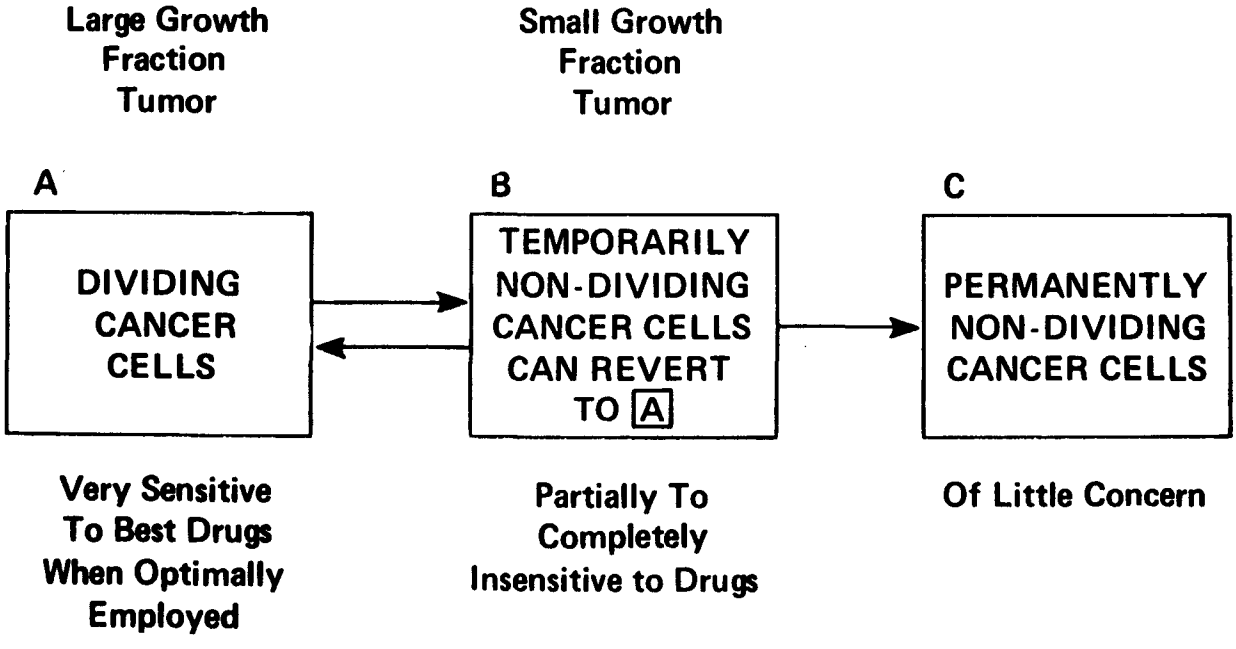

Fig. 7: Relationship between tumor growth characteristics and response to therapy (modified from ref. 20).

tumors with long doubling times and low growth fractions (compartment B) compared to anti-metabolites. Responsiveness appears to be related to the size of the growth fraction since as the growth fraction decreases with advancing disease, the likelihood of obtaining a tumor regression or cure declines.

At diagnosis, in an adult with acute leukemia, there are approximately 10^{12} leukemic cells and the labeling index is quite low. A "remission" by current criteria is achieved when a 3 log reduction in cells is obtained with chemotherapy and although there may be 10^9 leukemic cells in the body, they are not detectable by the presently available techniques. Experience has shown, however, that continued aggressive therapy is necessary or the patient will quickly relapse. In any case, even though normal myeloid precursors are also affected by the agents employed, the normal elements regenerate more rapidly (shorter cell cycle time and higher growth fraction) and the leukemic cells are no longer detectable on blood or bone marrow examination.

If the body burden in acute leukemia at diagnosis or relapse totals approximately 10^{12}, theoretically a 12 or 13 log reduction should affect a cure. The word "theoretically" needs to be emphasized since it may not be necessary to achieve at 12 or 13 log reduction for a cure if the immune mechanism is invoked to eradicate the last 2 or 3 logs of tumor cells.

In most cases of acute lymphocytic leukemia, the most responsive of the acute leukemias, the body burden of leukemic cells is reduced to 10^3 or 10^4 cells following vigorous combination chemotherapy. With prolonged therapy there is evidence that residual leukemic cells may number 100 or fewer and there are data in both man (25) and animals (23) that these remaining cells may be predominantly resting cells. Following continuous infusion of ^{3}HTdR in patients with acute leukemia for as long as 20 days, a small but significant proportion of leukemic cells remain unlabeled (25). In spontaneous AKR leukemia, as discussed above, these small cells upon transplantation to young normal AKR mice, are capable of proliferating and causing death due to leukemia (22). There is good reason to believe that the kinetic behavior of leukemic cells in the advanced disease in man is similar to that of the leukemic population in spontaneous AKR leukemia and it appears quite likely that resting small cells in the human disease are also capable of resuming proliferation. Since resting cells are relatively insensitive to current chemotherapeutic agents, it would appear to be appropriate to use some form of immunotherapy in an attempt to eradicate them completely. However, thus far, as discussed above, this has not be achieved.

Both advanced L1210 leukemia and spontaneous AKR leukemia are relatively insensitive to cycle active agents, presumably due to the low growth fraction in both situations. However, if the total leukemic cell population is reduced by treating with a non-cycle active drug, the residual cells are stimulated to resume proliferation and are then susceptible to a cycle active agent such as arabinosylcytosine (42, 43). This concept underlies some of the attempts to gain a therapeutic advantage in human leukemia. For example, extracorporeal irradiation (44), intensive leukapheresis (45) and attempts at cell synchronization (46) have been employed in an effort to recruit resting cells to enter proliferation in AML. Unfortunately, these procedures have not lead to a higher remission rate or to a prolongation of survival

following treatment. It is obvious that elucidation of the control mechanisms governing both the entry of cells into prolonged G_1 or G_0 is urgently needed.

A great deal of consideration in this paper has been given to attempts to achieve selective toxicity for tumor cells by trying to take advantage of a variety of differences between normal and neoplastic cells such as growth characteristics. Although these have not been totally successful, important progress has been achieved in controlling cancer in man. However, there are other avenues which deserve important emphasis and some of these, particularly following on the recent developments in molecular biology, have already been mentioned. Another approach which deserves attention lies in studies of the cell membrane. There is growing evidence that neoplastic cell surfaces may have therapeutically exploitable differences. The work with concanavalin A and wheat germ agglutinin has helped to elucidate cell membrane structure (47, 48). The agglutination of viral and chemically transformed cells is of great interest although some normal cells are also affected (49). These observations appear to deserve further work for potential application to treatment of patients with leukemia and other neoplastic disorders.

In summary, in this paper, I have attempted to review some of the concepts in acute leukemia and the status of treatment of patients with these diseases. Recent developments in several areas directly and indirectly related to leukemia add greatly to our knowledge of these disturbances and appear to have important implications for their control or cure.

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THE MOLECULAR BASIS OF GENE EXPRESSION

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Our present understanding of gene expression and transcription is based on experiments performed and models proposed only little more than 10 years ago.(1)

According to this understanding the genetic information is contained in and arranged linearly on nucleic acid molecules (DNA and RNA) in form of a specific base sequence (adenine = A, guanine = G, cytosine = C, thymine = T, and in the case of RNA uracil = U instead of thymine).

Three bases form one triplet which specifically codes (Nirenberg and Matthaei, 1961) for either an amino acid, a starting or a termination signal in protein synthesis. The genetic code summarizes the coding properties of all 64 possible triplets (F. H. C. Crick, 1966,(2)).

In general, DNA is the genetic material. Exceptions are some bacterial-, plant-, and animal viruses, e. g. the RNA-tumor-viruses. The genetic material of cells consists of one or more comparatively long DNA strands of high molecular weight, which are mainly localized in a nucleus.

For proteinsynthesis the genetic information of the DNA must 1) be available in defined units, 2) transcribed into m-RNA, and 3) a regulation mechanism is required which controls the species and amount of proteins to be synthesized at a given time.

The organization of DNA

The operon model by Jacob and Monod (developed for bacterial genes in 1961) proved highly useful for organizing genetic information in defined units. In this model the functional unit of gene expression is the operon, which consists of several structural genes (one gene codes for one protein) and some regulatory sites controlling the same biochemical pathway. The model specifies that the genes of one operon are usually transcribed together to produce one single messenger RNA (m-RNA synthesis or transcription). The enzyme synthesizing the m-RNA is the DNA dependent RNA polymerase or transcriptase. It appears to bind to a „promotor" site of the operon to initiate m-RNA synthesis unless negative control is exerted by a „repressor" molecule which can bind to the „operator" site localized between promotor region and structural genes. In this case, m-RNA synthesis would be initiated by depression of the operon i. e. inactivation of the repressor by an „inducer", which leads to lower affinity of the repressor for the operator site.

THE GENETIC CODE

1st position	2nd position U	2nd position C	2nd position A	2nd position G	3rd position
U	PHE	SER	TYR	CYS	U
	PHE	SER	RYR	CYS	C
	LEU	SER	Ochre (Chain-termination)	(Chain-termination)	A
	LEU	SER	Amber (Chain-termination)	TRP	G
C	LEU	PRO	HIS	ARG	U
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLUN	ARG	A
	LEU	PRO	GLUN	ARG	G
A	ILEU	THR	ASPN	SER	U
	ILEU	THR	ASPN	SER	C
	ILEU	THR	LYS	ARG	A
	METH	THR	LYS	LRG	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

Transcription

The synthesis of m-RNA (= transcription) provides a mediator molecule between the „immobile" DNA in the nucleus and the sites of protein synthesis at the ribosomes in the cytoplasm. m-RNA, which was proven to be complementary in its base sequence to the template DNA (Hall and Spiegelman, 1961), thus represents a true copy of the DNA and contains all genetic information of the corresponding stretch of DNA on a comparatively small and mobile molecule which can be degraded easily after it has fulfilled its function.

Transcription, in addition, is a step in cell metabolism at which the kind and amount of proteins to be synthesized can be regulated.

According to present thinking (4), transcription is initiated by attachment of transcriptase to the promotor site and subsequent binding of two ribonucleotides to the enzyme. Chain elongation occurs by sequential addition of nucleoside monophosphates to the 3′-terminus of the nascent RNA-chain. The mechanism of termination of transcription at the end of the operon possibly involves certain DNA termination sequences but is not yet completely understood. After release from its template DNA and transport to the ribosomes, the m-RNA is then thought to be available for translation and protein synthesis.

Reverse transcriptase

In the context of this workshop it appears useful to briefly define an enzyme which is of importance for the understanding of a number of papers presented here and which, because of its name, caused some confusion as to its relatedness to transcription.

Until three years ago it was believed that the flow of genetic information was only in the direction DNA to RNA and RNA to RNA. However, Temin and Baltimore in 1970, found an enzyme in RNA-tumor viruses, which can synthesize DNA on a single stranded RNA template, and thus reversed this conception.

Because of its effect opposite to that of the DNA directed RNA synthesizing transcriptase, this enzyme was called „reverse transcriptase". Its operational definition includes four parameters. The DNA-synthesis mediated by a reverse transcriptase 1) must accept single stranded natural RNA as template, 2) must be sensitive to ribonuclease, 3) must depend on the presence of all four deoxyribonucleotides, and 4) the DNA product of the reaction must be complementary in its base sequence to its RNA template. The latter is tested by so called back-hybridization.

At present, there is no indication what so ever that reverse transcriptase has anything to do with transcription of DNA into m-RNA.

Translation

Proteins are linear polymers of amino acids, and the problem in their synthesis is how to put the correct amino acid into the correct position in the chain. There are 20 amino acids which can serve as precursors of proteins; some amino acids – the formation of hydroxyproline from proline in collagen is an example of this. The basic elements of the mechanism of protein synthesis are fairly clear, though detailed understanding of many aspects is still lacking (3). This account starts with a description of the important elements, continues with an account of the cycle of events leading to the synthesis of a protein, and concludes with a description of how and under what circumstances the synthesis of proteins can be regulated.

Ribosomes

Proteins are synthesized on ribosomes; at some point during the process all the elements to be described interact with these particles. They are roughly spherical particles, about 200 Å in diameter with a molecular weight of about 4×10^6. About half the mass is protein, and half RNA. At low Mg^{++} concentrations, or in the presence

of certain proteins, the ribosome dissociates into two unequal subunits which are known by their sedimentation rates, 60S and 40S. The 60S subunit contains two species of RNA with molecular weights of 1.6×10^6 and 1×10^5 (28S and 5S RNA) while the 40S particle contains one RNA molecule with a molecular weight of 8×10^5 (18S RNA). There are probably few, if any overlaps between the protein content of these particles – that is, one can assign every protein of the intact, 80S ribosomes to either the 40S or 60S subunit. There are 50–60 different proteins in ribosomes from mammalian tissues.

Transfer RNA

For every ribosome in the cell there are between 10 and 20 molecules of transfer RNA. This is a heterogeneous class of RNA with an average molecular weight of about 25.000 sedimenting at about 4S; they contain between 75 and 95 nucleotide residues. Despite the heterogenity, these molecules possess several features in common. They all terminate in the sequence –CCA, and each molecule can accept an amino acid bound by an ester linkage to the ribosome of the terminal adenosine residue. The combination of the amino acid with its correct tRNA is catalysed by a series of 20 or perhaps more enzymes which require ATP for activity, performing the overall reaction:

Amino acid + ATP + tRNA = Aminoacyl-tRNA + AMP + PPI

The combination of each amino acid with its cognate tRNA is absolutely specific.

Messenger RNA

The sequence of amino acids in proteins is specified by messenger RNA. Ribosomes attach to mRNA at one end, at a strictly specified site, and proceed along the mRNA until they reach a termination signal, and release the protein they have been assembling. The translation of the sequence of nucleotides in the mRNA is accomplished by aminoacyl tRNA, which binds specifically to codons in the mRNA by classical base-pairing. This automatically brings the amino acid at the other end of the tRNA into the correct register so that a peptide bond can form between it and the growing polypeptide chain. Messenger RNA itself has proved an elusive entity, mainly because there is very little of it compared to the tRNA and ribosomal RNA, and also because it is exceedingly heterogeneous, both as regards size (which must be roughly proportional to the size of the protein it specifies) and composition, which is also a function of the protein it codes for. However, it is now possible to prepare specific mRNA – examples are the mRNA for globin, immunoglobins, viral proteins of several species, silk fibroin, and so on – the list lengthens almost daily at the time of writing.

„Factors"

Besides the proteins of the ribosome, several enzymes which are only loosely or transiently associated with the ribosomes catalyse the process of protein synthesis. There seem to be at least 6 or 7 identifiable activities, though some would extend the list. There appear to be at least three, and possibly 6 factors involved in initiation, two in the assembly, and two for termination of the chains. Some of these proteins are well-characterized and highly purified; others are not.

The Ribosome Cycle

Immediately after a ribosome has finished making a protein, it leaves the mRNA, drops the finished protein, and dissociates into its subunits. The first identifiable step in the next cycle of synthesis is the binding of methionyl-tRNA$_f$ to the 40S subunit, catalysed by an initiation factor (or two) and GTP. This tRNA is unique in several respects, and is the only known tRNA which can bind to ribosomes in the absence of mRNA. It is used only for starting new protein chains; another methionyl-tRNA exists to put methionine into internal positions in the chain. Having bound the initiator tRNA, the mRNA is bound to the 40S/met-tRNA$_f$ complex. This probably requires a protein or proteins, and following the correct binding of the message, the 60S subunit joins on. The next amino acid specified by the mRNA is now brought into position, attached to its tRNA, and catalysed by a protein whose function is to carry tRNA from the synthetases to the ribosomes; this process appears to involve GTP binding and probably hydrolysis also. Having bound correctly, the first amino acid forms a peptide bond to the second, and the ester linkage between the methionine and the tRNA is also broken. The enzyme which catalyses the peptide bond formation is an integral part of the 60S subunit, and cannot be removed. The situation is now that a dipeptide, bound to tRNA, is located in the same place as that tRNA was located when it first entered the ribosome; it is now necessary to move the peptidyl-tRNA into the location that was first occupied by the met-tRNA$_f$, so that there is a space for the next aminoacyl-tRNA to enter the ribosome, and so that the next triplet codon on the mRNA is properly positioned. The movement, called translocation, is catalysed by another enzyme, called EF II; GTP hydrolysis is again involved. After translocation, the scene is set for another round of the cycle to occur, and the process continues with EF I catalysed binding of aminoacyl-tRNA, peptide bond formation and translocation until a termination signal in the mRNA is reached, at which point of protein termination factor enters the ribosome instead of a tRNA, and translocation probably results the release of the completed protein.

The Ribosome Cycle in Eukaryotes

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1. Native 40S subunit + Met-tRNA$_f$ + GTP → 40S/met-tRNA$_f$/GTP
2. 40S/met-tRNA$_f$/GTP + mRNA → 40S/mRNA/met-tRNA$_f$(GTP?)
3. 40S/mRNA/met-tRNA$_f$ + native 60S subunit → 80S/mRNA/met-tRNA$_f$
4. 80S/mRNA/met-tRNA$_f$ + val-tRNA $\xrightarrow{EFI\ GTP}$ 80S/mRNA/met. val-tRNA
5. Translocation of met. val-tRNA from A site to P site, catalysed by EFII and GTP
6. Repeat steps 4 and 5 until termination codon is reached, when R factor binds, and combined peptide bond formation to ? water and translocation release nascent chain from the ribosomes and the ribosomes from the mRNA. At this point, the ribosomes exist as subunits, and have a choice of either repeating the cycle or becoming relatively inactive 80S ribosomes.

The details of these reactions are in most cases quite obscure, particularly the movement of the tRNA and mRNA with respect to the ribosome. Many of the ideas about the mechanism have come from taking the components apart by means of washing ribosomes with strong salt solutions, and adding back wash fractions to see what is needed to restore activity. Also important, however, are a range of inhibitors which affect various aspects of the cycle; for example, aurinetricarboxylic acid, pactamycin, and edeine all inhibit various phases of the initiation process; sparsomycin, and anisomycin inhibit peptide bond formation; diphtheria toxin (+ NAD) and cycloheximide inhibit translocation; and puromycin mimics the end of aminoacyl-tRNA closely enough that it causes the premature release of nascent peptides from the ribosome. This list is not exhaustive; the importance of these inhibitors is that they may cause the ribosomes to accumulate at one point in the cycle, which reduces their heterogeneity and makes the study of intermediates in the cycle a possibility.

The Control of Protein Synthesis

There are very few well-understood examples of control of protein synthesis. It is an article of faith that ribosomes are indiscriminate translators of whatever mRNA happens to come their way, so that most of the control of the type of protein synthesized in cells is made at the level of transcription of DNA into RNA, or the subsequent processing of the RNA or its transport to the cytoplasm. In the case of infection by viruses like Vaccinia, VSV, polio, and other viruses which replicate in the cytoplasm, it seems that viral RNA is poured into the cytoplasm and takes over; some believe that they also subvert the ribosomes, so that they can only read viral messages; but there is little evidence that this is in fact the case. There is one situation in which it seems that protein synthesis can be turned on and off during the cell-cycle, in the synthesis of histones. Histone synthesis occurs only during the S-phase of the cell-cycle, and is strongly inhibited by inhibitors of DNA synthesis. The basis for this control is unknown. Other case of control of protein synthesis seem to be more general – a kind of overall quantity control. This occurs during amino-acid starvation, at elevated temperatures, during mitosis, in serum deprivation and various other conditions which are sub-optimal for cell-growth. In these conditions, protein synthesis is inhibited non specifically at the level of initiation. The basis of this control is not understood.

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CYTOLOGY AND CYTOCHEMISTRY OF THE LEUKEMIC CELL

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In contrast to the modern science of molecular biology, cytology and cytochemistry of human blood cells have a long history. Some of the principal mechanisms of protein biosynthesis were already discovered with cytological methods in the 19th century. In 1899, a young French anatomist, GANIER (8), demonstrated in his thesis the relationship between specific morphological changes in the cytoplasm of pancreatic glands and the secret production of these glands. During the synthesis of this specific pancreatic secret, the cytoplasm becomes basophilic. GANIER named this active cytoplasm: ergastoplasm, meaning that this cytoplasm produces something, however, we know today that this "something" is actually protein. With the lightmicroscope GARNIER has demonstrated in his experiments a relationship between acid groups in the cytoplasm and protein synthesis.

Forty years later BRACHET (3) and CASPERSON (4) demonstrated by cytochemical methods that this acid groups consisted of RNA and they found the dependent relationship between RNA synthesis and protein synthesis. Today we know that the cause of GANIER's basophilia is the high RNA content of the ribosomes, which are the centre of protein synthesis (5)

This short historical introduction has shown us that lightmicroscopy is necessary and not a hopeless method in modern biological research.

Studying the cytology and cytochemistry of the human leukemic cell, we can prove how valuable the lightmicroscope is. Therefore, we must ask ourselves the following questions:

1. What do we know about cytological and cytochemical markers in normal and leukemic cell differentiation?
2. Do we have specific markers which show us the leukemic transformation of a single blood cell?
3. Which of these markers can give us information for our diagnostic and therapeutic behavior with the leukemic patient?

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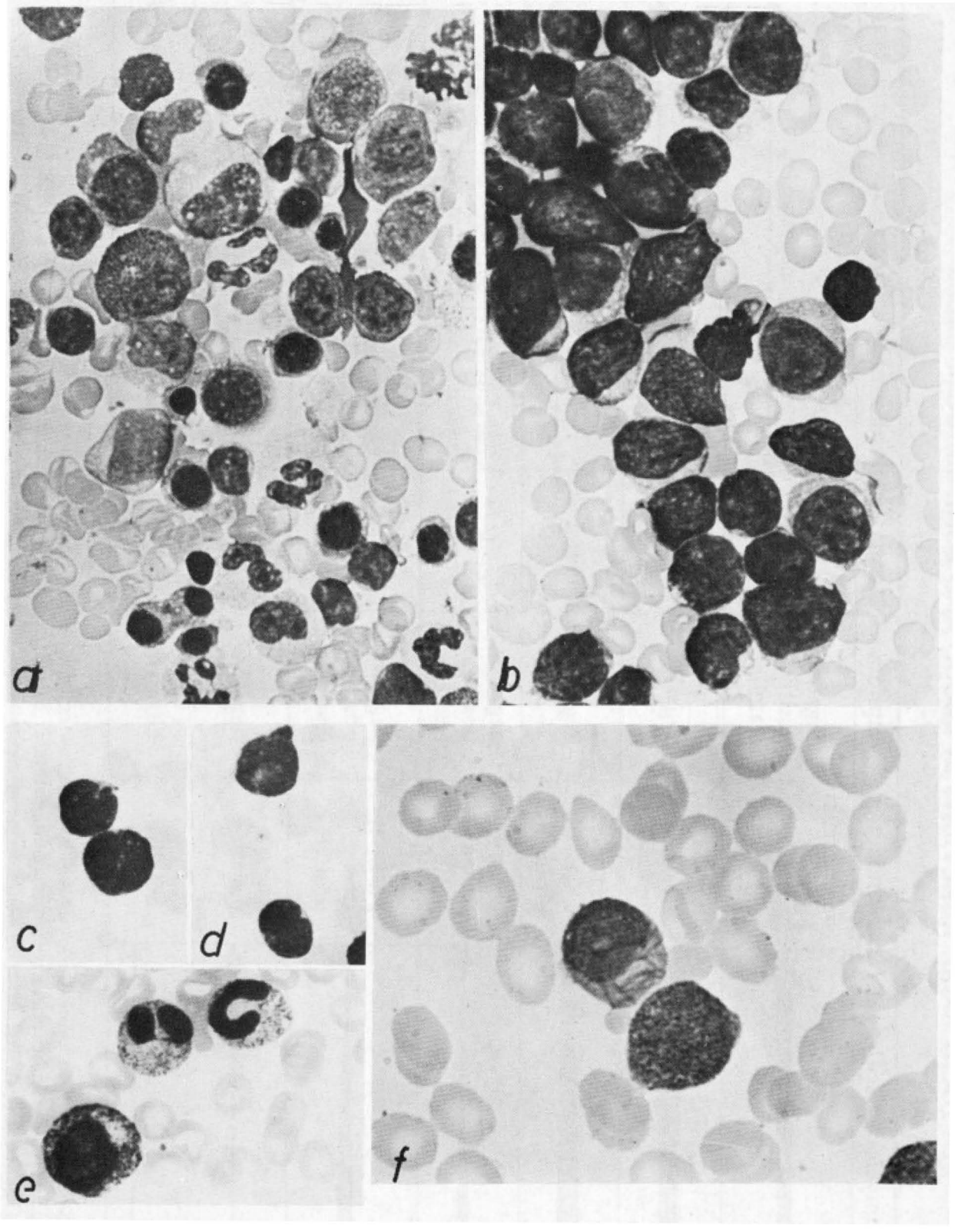


Fig. 1

a) normal bone marrow, various stages of maturation of erythropoietic and leukopoietic cells,
b) leukemic bone marrow, blast cells, showing the same differentiation,
c+d) blasts with nuclear cleavage of the Rieder cell type,
e) pseudo pelger cell between a promyelocyte and a metamyelocyte,
f) Auer rods in the cytoplasm of a parapromyelocyte.
a–e) 700 x; f): 1,500 x

CYTOCHEMICAL REACTIONS IN BLOOD AND BONE MARROW CELLS

Reaction	Promyeloc.—Neut. P. M. N.	Monocyte	Lymphocyte	Plasma Cell	Pronormbl.	Normobl.	Erythrocyte
PAS	▬▬▬▶	weak diffus positive peripheral granules	negative, positive granules	negative	negative		
PEROXY-DASE	▬▬▬▶	negative, weak positive	negative	negative	negative		
N-AS-D-CL-ESTERASE	▬▬▬▶	negative, weak positive	negative	negative	negative		
ALPHA-N-ESTERASE	▬- - -▶	positive, strong positive	negative, positive granules	weak positive	paranuclear positive	perinuclear positive	negative, positive granules
ACID PHOSPHA-TASE	▬▬▬▶	positive, strong positive	negative, positive granules	diffus positive, positive granules	paranuclear positive	paranuclear positive, negative	negative
ALKALINE PHOSPHA-TASE	- - -▬▶	negative	negative	negative	negative		
SULFIDE SILVER	◀▬ negative ▬▶	negative	negative	negative	▬▬▬▶		

Tab. 1 methods for cytochemical reactions in blood and bone marrow cells: PAS: Mc MANUS and HOTCHKISS mod. (30), peroxydase: GRAHAM-KNOLL (30), naphthol-AS-D-chloroacetate-esterase: LEDER (17), alpha-naphthyl-acetate-esterase: LEDER (17), acid phosphatase: GOLDBERG and BARKA mod. (17), alkaline phosphatase: KAPLOW mod. (23), sulfide-silver: NETH et al. (24).

1. What do we know about cytological and cytochemical markers in normal and leukemic cell differentiation?

Besides the well known cytology of the different blood cell lines of granulopoiesis, erythropoiesis, lymphopoiesis and thrombopoiesis (Fig. 1 a), the specific cytochemical markers in each cell line are also well known (Tab. 1). Leukemic cells show abnormalities in the differentiation between the nucleus and cytoplasm (Fig. 1 b). In addition, these cells may show deformities of the nucleus, such as nuclear cleavage of the Rieder cell type (Fig. 1 c–d) or such as the acquired Pelger-Huet anomaly of neutrophils (Fig. 1 e). Abnormalities are also shown by azurophil granules, called Auer rods (Fig. 1 f) or by defective granularity in the eosinophils.

A cytological classification of the undifferentiated leukemic blast cells is mostly impossible (Fig. 2 a–h), however, leukemic cells can be classified with the aid of specific cytochemical markers based on the reactions in normal blood cells (19, 7, 10, 22, 1, 20, 27, 12, 18, 21). HAYHOE and his coworkers (10) used periodic acid Schiff (PAS)-, peroxidase- and sudan black reaction to classify the leukemic blast cells in lymphoblastic (Fig. 2 i), myeloblastic (Fig. 2 j), monocytic (Fig. 2 k) and erythroblastic cells. In addition to the above mentioned reactions we used: Naphtol-AS-D-chloroacetate-esterase (N-AS-D-Cl-esterase), alphanaphthylacetate-esterase (alpha-N-Esterase), acid and alkaline phosphatase and sulfide-silver reaction (Tab. 2).

To know how far cytochemical markers in leukemic blast cells might give genetic parameters we need, in addition to the cytochemical reactions, the biochemical analysis of the specific products in the blood cell lines. We already know that hemoglobin is a specific cell product in the erythropoietic cell line, therefore, in this cell line we could have a good chance to compare cytochemical markers with biochemical analysis.

LEDER (15, 16) and DRESCHER (6) have demonstrated with cytochemical methods that in some undifferentiated leukemic cells there is an erythropoietic specific acid phosphatase reaction. We found, in addition to the specific acid phosphatase reaction non-hem iron with the sulfide-silver-reaction (SSR) (29) in this undifferentiated leukemic cells (2) (Fig. 4 a–c). Until now we could not demonstrate the presence of hemoglobin in these leukemic cells, but we could demonstrate that the polysomes in these leukemic cells have messenger RNA with the information for a protein which coeluates with the added carrier Alpha- and Beta-globin chains (Fig. 4 d). The messenger RNA of these leukemic cells was tested in the Xenopus oocyte system (14) and the synthesized labelled product was analysed on carboxy-methylcellulose-urea columns. We are now hypothesizing that the coeluated activities are Alpha- and Beta-globin chains. Experiments to prove this by fingerprints are in progress. We have to look for similar experiments to find out how far more cytochemical markers (proved by biochemical methods) can be used to demonstrate specific steps in normal and leukemic cell differentiation.

2. Do we have specific markers which show us the leukemic transformation of a single blood cell?

Until now, most of the cytochemical markers have not been specific for the detection of a single leukemic cell in normal appearing blood and bone marrow smears. In a few cases some markers, such as PAS-positive-blocks or peroxidase-positive Auer rods give us evidence of leukemic transformation. It is very difficult,

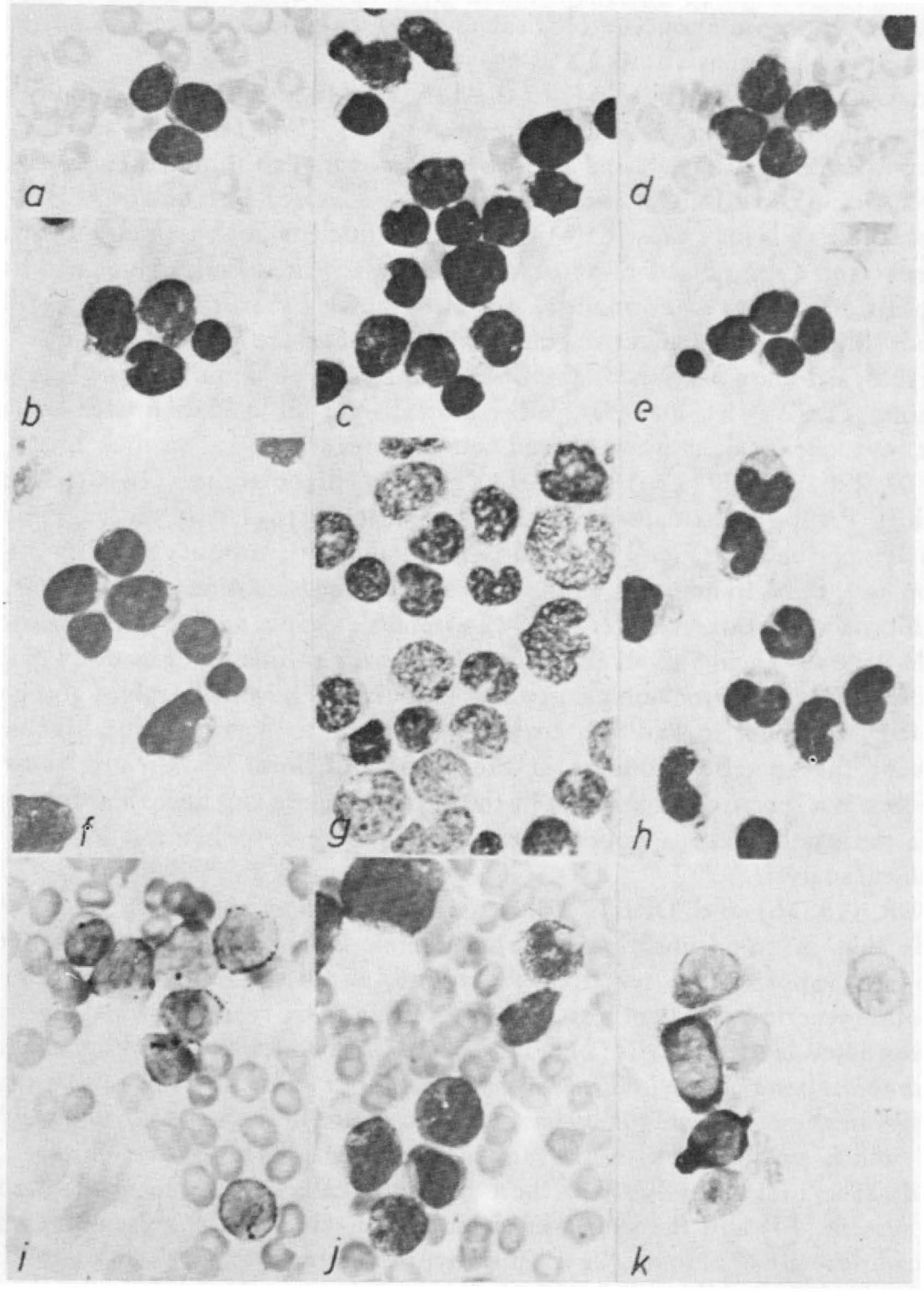


Fig. 2

a–h) Various morphological aspects of leukemic cells differentiated by cytochemical reactions, May-Grünwald-Giemsa-stain.
a) undifferentiated blasts, b) and c) paralymphoblasts, d) and e) paramyeloblasts, f) and g) paraproerythroblasts, h) paramonocytes.

i–k) cytochemical differentiation of leukemic cells: i) PAS positive blocks and granules in the cytoplasm of blast cells, paralymphoblastic leukemia, j) peroxidase: faint positivity or negative reaction in the cytoplasm of paramyeloblasts and parapromyelocytes, k) alpha-naphthylacetate-esterase: varying intensity of reaction in the cytoplasm of paramonocytes.

a–k) 700 x

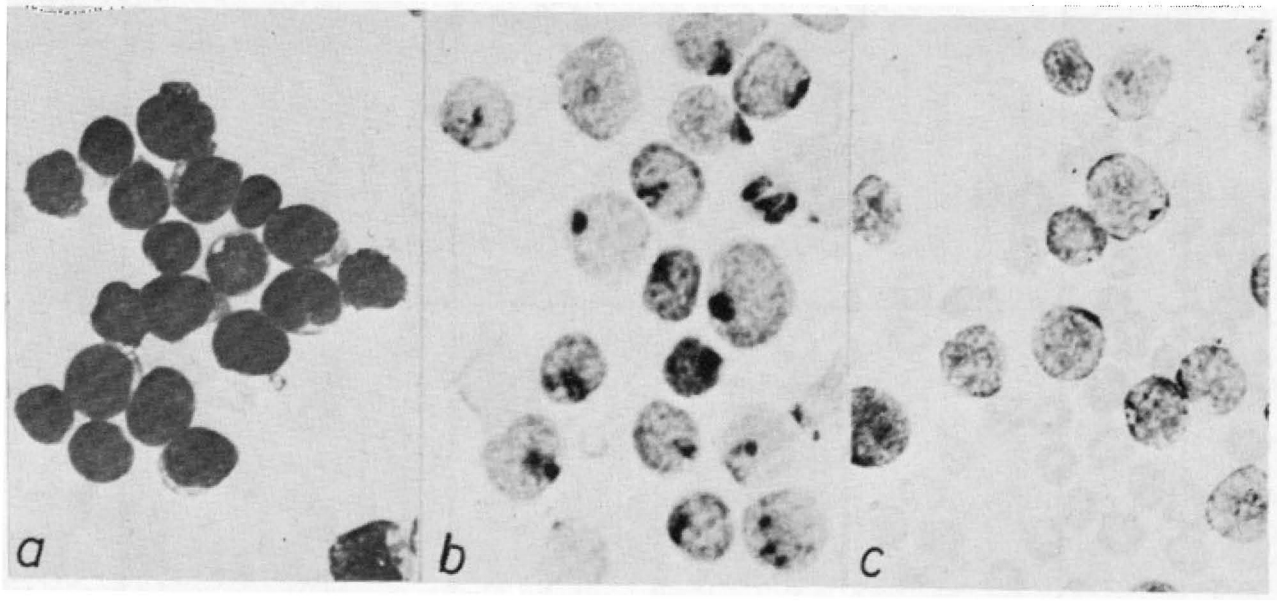
CYTOCHEMICAL DIFFERENTIATION OF STEM CELL LEUKEMIAS (N=259)

TYPE	PAS	PEROXYDASE	N-AS-D-CL ESTERASE	α-N-ESTERASE	ACID PHOSPHATASE
1. NON-DIFFERENTIATED LEUKEMIA N=47	negative (positive fine granules in a few cells)	negative	negative	negative	negative (pos. diffuse tinge, pos. fine granules in a few cells)
2. PARALYMPHOBLASTIC LEUKEMIA*) N=149	positive coarse granules blocks	negative	negative	negative (positive granules in a few cells)	negative (pos. diffuse tinge in a few cells)
3. PARAMYELOBLASTIC LEUKEMIA N=46	positive diffuse tinge, fine granules	positive up to 3 %	positive up to 1 %	negative (positive granules in a few cells)	negative
4. STEM CELL**) LEUKEMIA WITH RED CELL LIKE CYTOCHEMICAL PATTERN N=17	negative (positive granules and blocks in a few cells)	negative (weak positive in a few cells)	negative (weak positive in a few cells)	positive paranuclear up to 61 %	positive paranuclear up to 100 %

*) further investigations have shown that this group is not only of one genetic origin (18)

**) further investigations have shown that in this group leukemias with Thymus Tumors

Tab. 2



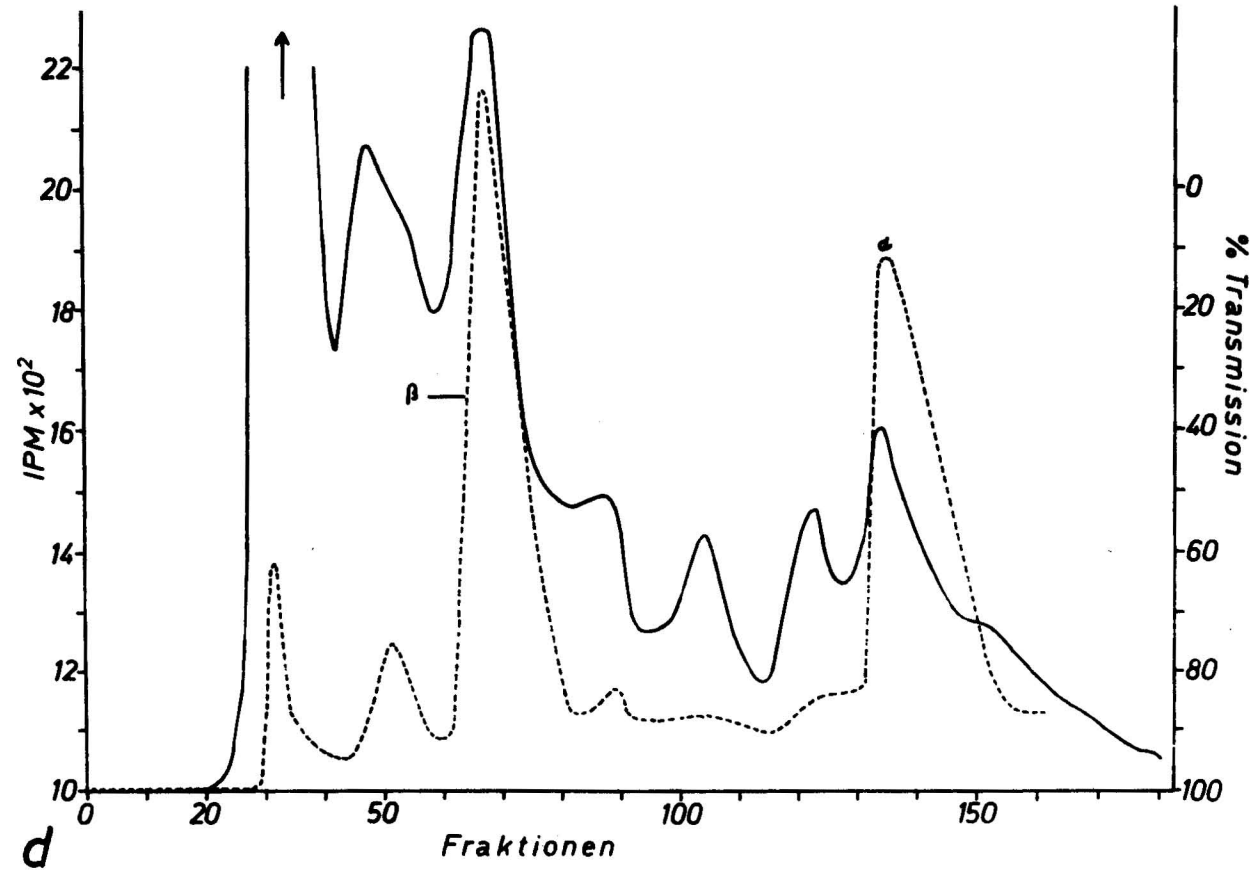


Fig. 4
Blasts of acute leukemia with red cell like cytochemical pattern.
a) May-Grünwald-Giemsa stain, b) acid phosphatase: paranuclear localized red reaction product, c) staining for non-hem-iron: black granules and brown diffuse tinge in the cytoplasm.
a–c) 700 x
d) Globin synthesized in frog oocytes in response to '8–16 S' RNA extracted from polyribosomes of the above leukemic blood cells.

however, to demonstrate single leukemic cells between normal blood and bone marrow cells with PAS or peroxidase. Because of this difficulty, we need more specific methods for recognizing a single leukemic cell.

As we know, the transformation of a normal cell to a malignant cell is generally accompanied by varying alterations in the cell membrane. One of the specific alterations on the cell surface, that can be made visible by morphological methods, is the appearance of new antigens. These antigens can be seen on DNA virus as well as on RNA virus transformed cells. BAUER and his group, using different techniques, demonstrated tumor specific surface antigens on cells of chicken, mice and hamsters which were transformed by avian tumor virus (9, 13). There is increasing evidence that human leukemic cells could possibly have tumor associated antigens similar to those shown in studies with virus induced leukemia in animals (11, 13). To visualize human leukemic cells tumor specific surface antigens are the most important markers. One of the possibilities to make the tumor specific antigens visible on the cell surface is to use the immunoferritin method (9). Until now the immunoferritin method has been most successful only with an electronmicroscope. Because the electronmicroscope is difficult to operate, this method has proven too complicated for marking a single leukemic cell.

Therefore, we applied the immunoferritin method to the lightmicroscope, using the sulfide silver reaction (SSR) (Fig. 3 a) (29). With the SSR one can make a ferritin solution visible in the lightmicroscope, because iron particles can be magnified to more than 100.000 : 1 (24). We showed with the SSR a specific antigen binding on the surface of rabbit lymphocytes immunized with apoferritin (Fig. 3 b–d) (25). Using the same method we also demonstrated a specific binding of ferritin labelled antigens on the cell surface after immunization with human γ-globulin.

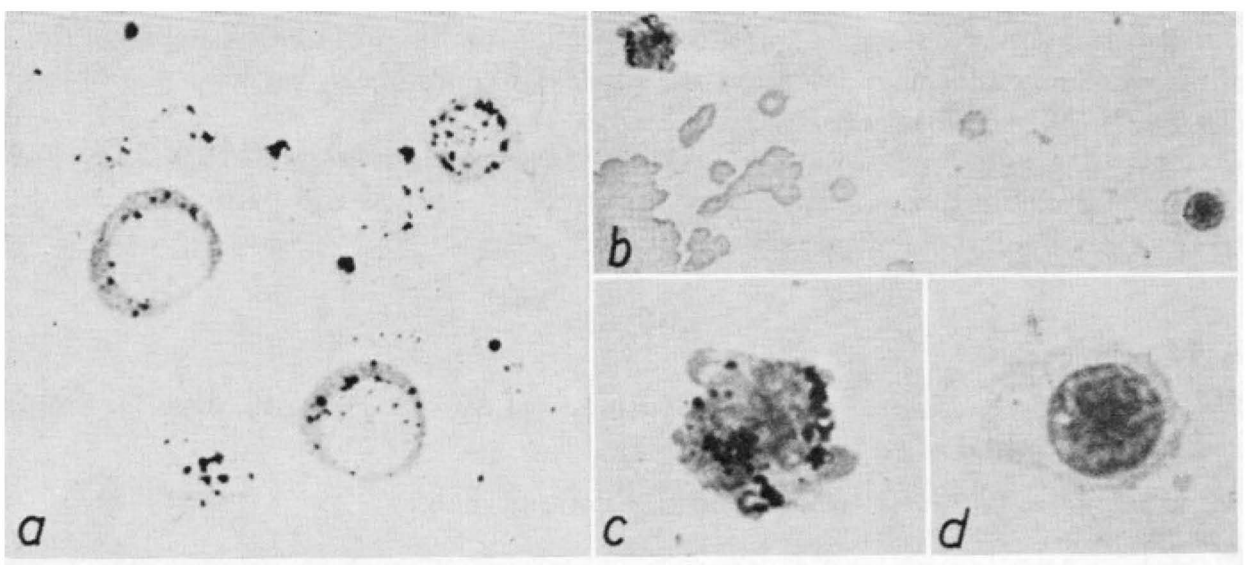


Fig. 3

a) staining for non-hem-iron : diffuse brown tinge and black granules in the cytoplasm of erythroblasts and erythrocytes of a child with rh-erythroblastosis: 1.000 x

b–d) demonstrating specific binding of ferritin on blood lymphocytes after immunisation with apoferritin. Normal lymphocyte with negative reaction right and left a stimulated lymphocyte with basophilic cytoplasm and positive reaction.
b): 648 x; c) and d): 1,200 x (25)

If one could find tumor specific surface antigens in human leukemic cells, then it might be possible to mark antibodies against these antigens with ferritin, peroxidase or immunofluorescence to make the specific binding of these antibodies visible. Then we might have the opportunity to detect single leukemic cells in the blood and bone marrow smears at a very early stage of leukemia.

3. Which of these markers can give us information for our diagnostic and therapeutic behavior with the leukemic patient?

HAYHOE and his coworkers demonstrated nearly ten years ago a relationship between cytological and cytochemical markers in leukemic cells and therapeutic results (10). All further investigations in the past few years have been concerned with proving HAYHOE's results. We found in 300 acute leukemia cases in children a much better prognosis for the undifferentiated or paralymphoblastic types of leukemias than for the myeloblastic types. The therapeutic concept of PINKEL and his group (28) contributed much to therapy in leukemic children. According to the therapeutic results of this group it must be proven again how far cytological and cytochemical methods may be helpful as prognostic factors in leukemia.

Summary

Cytological and cytochemical methods offer additional items concerning the classification of leukemia with regard to prognosis and therapeutic consequences.

The morphologic demonstration of a single leukemic cell is impossible until now. But there is increasing evidence that human leukemic cells could have tumor associated antigens. Based on the assumption that tumor specific surface antigens exist in human leukemia, one can speculate about an early diagnosis by using the sulfide-silver immunoferritin method or other techniques for visualizing these antigens. Such an early detection of single leukemic cells can have therapeutic consequences to the patient.

Comparing cytochemical and biochemical investigations stemcell leukemias show that it is possible to demonstrate specific steps of normal and pathologic cell differentiation by cytochemical methods.

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ELECTRON MICROSCOPIC AND CYTOCHEMIC FINDINGS IN Di-GUGLIELMO'S SYNDROME AND IN OTHER FORMS OF LEUKEMIA

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The leukemic cell shows many peculiarities in contrast to normal ones. In particular, these are not specific for leukemia; by their quantity and combination they improve classification and understanding of this disease.

In order to avoid an exhaustive list of morphologic findings, a certain order of our results we shall try to achieve. In doing this we accept the risk of possibly oversimplifying or speculating.

Morphologic findings in leukemic cells can be divided into 3 groups according to increasing "specificity" (tab. 1).

I. "Degenerative" changes

These are found in cells, which have been disturbed in some way in their environment. Typical for these changes are vacuolization, formation of myelin-figures, rupture of the membranes of cells and of cell organelles. The following reasons for these findings in leukemic cells could be considered: disturbed interaction of cell organelles; impaired supply of high-energy-compounds, for instance by damage of mitochondria; superannuating of the cells.

II. Changes that are often observed in "malignant" cells

To this group primarily the peculiarities of the nucleus of a malignant cell may be mentioned: large and irregular nuclei; "nuclear pockets", conspicious nucleoli; evenly distributed and lose chromatin. It is only partially possible to explain these findings as an expression of a special activity of certain function: enlargement of the nuclear surface by "nuclear pockets", lose chromatin, large nucleoli for the purpose of an increased synthesis of DNA or of proteins. Other changes would rather indicate an impaired interaction of organelles or a compensatory hyperthrophy of the organelle-systems caused by disturbed partial functions: augmentation and enlargement of mitochondria, abnormally large Golgi-fields, extended bundles of fibrilles.

[1] Study Ass. Haematol-EUROTOM-GSF No. 031-64 BIAD

Table 1. Electron microscopic findings in leukemic cells.

I.	"degenerative" changes	1. vacuolization of cytoplasm and of cell organelles 2. myelin figures 3. breakage of membranes
II.	changes common to all kinds of "malignant" cells	1. polymorphism of cells 2. dissociation in maturation of nucleus and cytoplasm (immature nuclei; high nuclear-cytoplasmic ratio) 3. large, irregular nuclei; prominent nucleoli; "nuclear pockets" 4. large, irregular of augmented cell organelles, esp. mitochondria 5. intracytoplasmatic fibrills
III.	changes specific to leukemic cells	1. abnormal granules in myeloid cells 2. abnormal activities of enzymes 3. ferritin and ferruginous micelles in erythroblasts 4. transformation of mitochondria into cytosomes 5. abnormal deposits of PAS-positive material

III. Few changes are characteristic for certain forms of leukemia

As a rule, these are based upon the impairment of organelle-systems of correspondingly differentiated cells. So we find in structure and enzyme activity abnormal granules in all forms of myeloic leukemias; morphologic equivalents of a disturbed hemoglobin synthesis and an abnormal expulsion of nuclei in malignant erythroblasts; conspicious depositis of PAS-positive materials in cells containing glycogen.
Especially this last group of morphologic changes will be dealt with in the following.

Anomalies of Granules

The *primary granules* of myeloic cells are equipped with acid phosphatase and with peroxidase under the assistance of perinuclear space, rough surfaced endoplasmic reticulum and Golgi-cisterns [BAINTON e. a. 1968, 71]. This procedure can be made visible electron microscopically [methods: HUHN e. a. 1971]. Hereby poorly differentiated leukemic cells may be classified into a certain myeloic cell line before larger quantities of enzyme are synthesized, packed into granules and become visible by light microscopy. The enzymes, listed in table 2, were localized in the primary granules by means of biochemistry, cytochemistry or electron microscopy. In myeloic leukemias the following defects of enzyme activity could be demonstrated in neutrophils [SCHMALZL e. a. 1973].

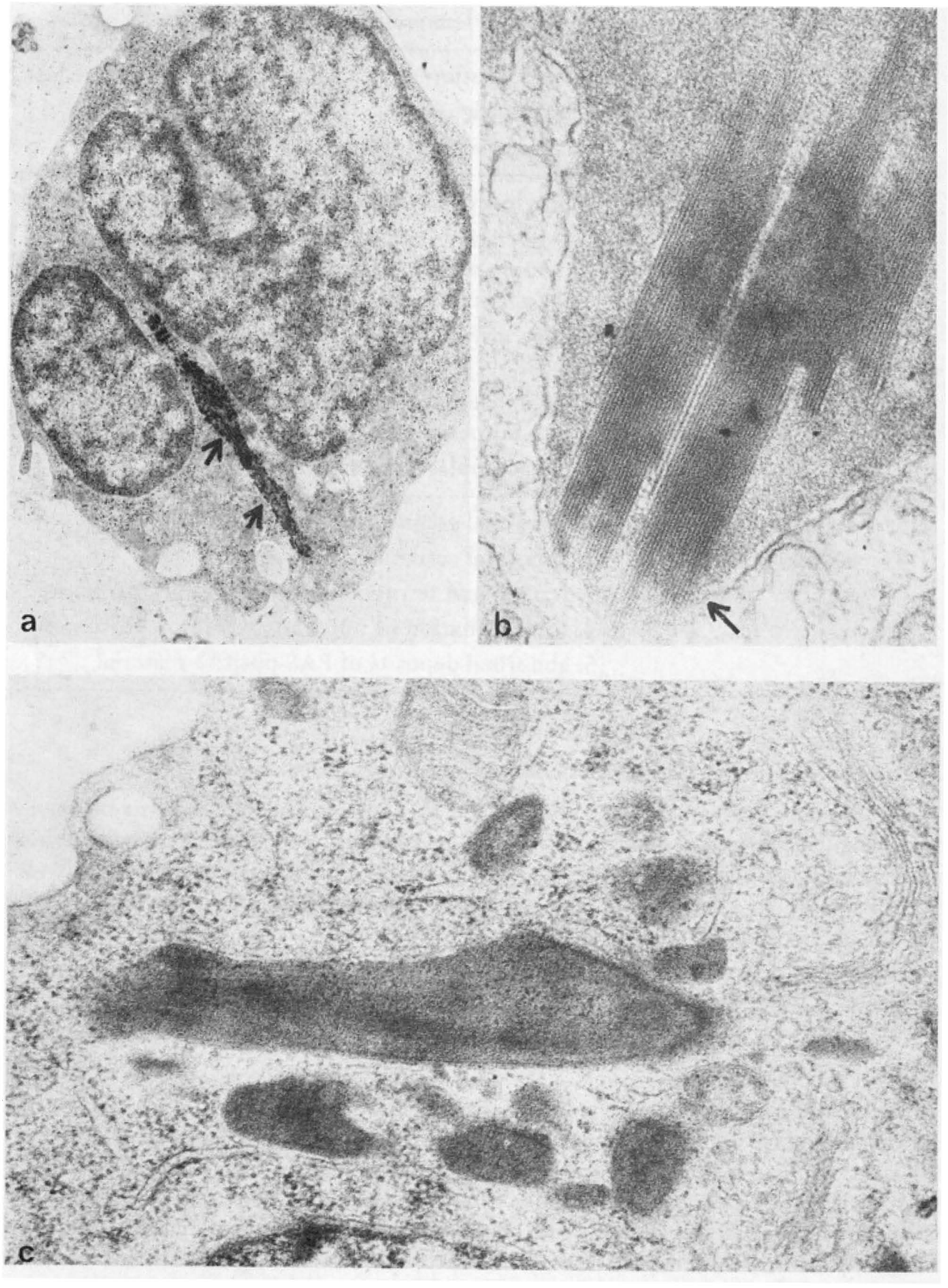


Fig. 1: Leukemic changes of primary granules a) myeloblast; activity of acid phosph. in Auer-rod b) cristalloid structures in prim. granules c) promyelocyte; Auer-rod and prim. granules.

1. decrease or loss of the activity of peroxidase, of naphthol-ASD-chloracetate-esterase and/or of acid phosphatase;
2. isolated decrease or loss of peroxidase;
3. isolated decrease or loss of naphthol-ASD-chloracetate-esterase.

Primary granules of leukemic cells frequently show pecularities of their *fine structure*: they may contain cristalloid inclusions which penetrate their membrane transforming into Auer-bodies (fig. 1). Auerbodies as a rule exhibit activity in acid phosphatase, usually in peroxidase and naphthol-ASD-chloracetate-esterase [ACKERMANN 1950; FISCHER e. a. 1966; HUHN e. a. 1968]. Further on, primary granules are abnormally enlarged, may conglomerate or may appear as small, commalike structures (fig. 2).

The *secondary neutrophil granules* differ from the primary ones by their structure and enzyme activities (table 2). The neutrophils of a patient suffering from monocytic leukemia exhibited an excessive augmentation of alkaline phosphatase-activity extending to granulocyte precursors. In some cases of myeloblastic leukemia activity of alkaline phosphatase was demonstrated in myeloblasts [MALASKOWA e. a. 1968; SCHUBERT e. a. 1968]. Concerning their *fine structure*, secundary granules contain conspicious amounts of laminar systems or cristalloid structures (fig. 2b).

Monocytic leukemia can be considered as a special form of malignancy of the myeloic cells [HUHN e. a. 1971; SCHMALZL e. a. 1968]. According to the degree of differentiation the leukemic monocyte obtains, we may differentiate between a *promonocytic* and a *monocytic* form. *Promonocytic leukemia* is characterized by undifferentiated blasts containing few granules and low enzyme-activity restricted to the perinuclear space and the rough surfaced endoplasmic reticulum. In *monocytic leukemia* the leukemic cell equals the normal monocyte according to contents in organelles, enzymes and to the ability of phagocytosis.

A third form of monocytic leukemia may be delimitated [SCHMALZL e. a. 1972]. The leukemic cells in this disease can be identified as monocytes, but in addition concerning their fine structure and enzyme-activities they show characteristics of granulocytic cells. In one case like this we found Auer-bodies. This special form of monocytic leukemia was termed *monomyelocytic leukemia.*

Summarizing the hitherto existing findings, we can make the following statements:

1. The formation of primary and secondary granules represents a specific activity of myeloic cells, reflecting the degree of maturation. The content in granules and in enzymes in leukemic cells determines the cytoplasmic differentiation of the leukemic cell population.
2. Different disturbances of granule-formation frequently occur in leukemic cells:
   a) reduction of granules in a cell;
   b) elevation or reduction of enzyme-activity in morphological (qual. and quan.) normal granules;
   c) morphologic (qual.) abnormal granules with normal enzyme activity.
3. In acute (myeloic) leukemias mature granulocytes may show defects of their granules.

Table 2. Activity of different enzymes in granules of neutrophils and monocytes (Schmalzl e. al. 1973).

Granules in Neutrophils				Granules in Monocytes	
	Primary Granules		Secondary Granules		
bce	acid phosphatase	bce	alkaline phosphatase	bce	acid phosphatase
b	acid ribonuclease	bc	lysozyme (muramidase)	b	acid ribonuclease
b	acid desoxyribonuclease	bc	lactoferrine	b	cathepsin
b	cathepsin	bc	naphthylamidase	bc	ß-glucuronidase
bc	ß-glucuronidase			bc	lysozyme (muramidase)
bce	peroxidase			bce	peroxidase
bc	NaF-resistant NASE			bc	NaF-sensitive NASE
bce	arylsulfatase			c	NASDCE
(bce)	neutral proteases (NASDCE)				
b	bactericidal proteins				

b: localized to granules by biochemical methods
c: cytochemical or immunological demonstration
e: localized to granules by electron microscopic methods

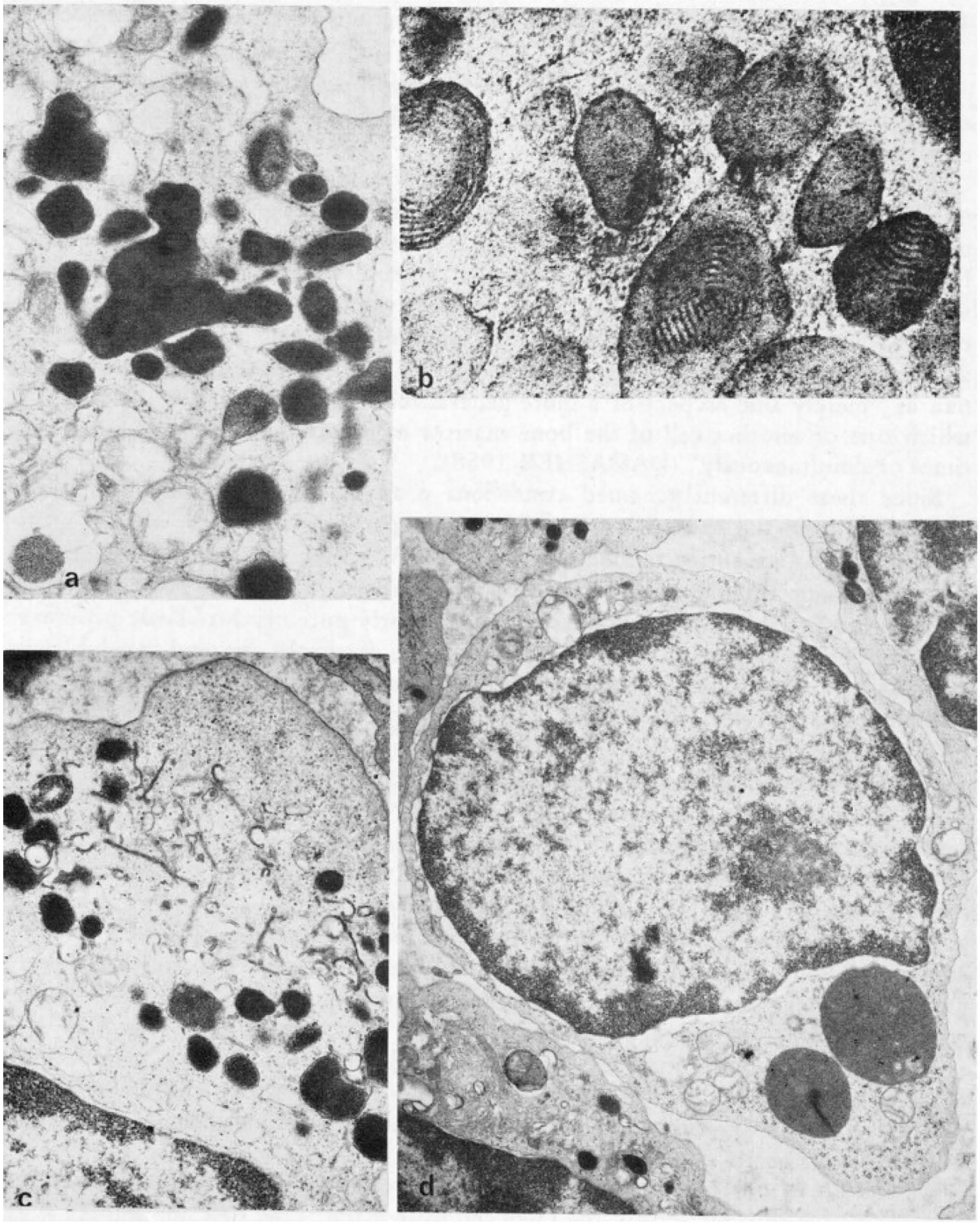


Fig. 2: Leukemic changes of prim. and sec. granules a) promyelocyte; confluent primary granules b) laminar structures in secondary granules c) comma-like primary graunles d) enlarged primary granules.

4. Transitional forms between leukemias of the granulocytic and the monocytic series can be observed.

We now will consider the *malignancies of erythropoietic cells*. Here we expect characteristic morphologic findings depending on hemoglobin-synthesis and nuclear expulsion.

A condition characterized by a generalized malignant proliferation of the nucleated red blood cells resembling leukemia, was first recognized as a clinical and pathological entity by Di GUGLIELMO in 1923; he termed it "*acute erythremia*". In 1917 this author had described a mixed erythroblastic-leukocytic proliferation, which he called "*erythroleukemia*". As the years went on, an increasing number of cases of apparently "pure" erythroblastic disorder gave way to erythroleukemia, and finally to myeloblastic leukemias. Thus, DAMASHEK thought of erythroleukemia as "merely one ascpect of a more generalized myelo-proliferative disorder, in which one or another cell of the bone marrow might participate either at various times or simultaneously" (DAMASHEK 1958).

Since these differently named conditions probably represent only transistory clinical stages in the natural history of the same pathological entity, we have grouped them into one entity termed "*DI GUGLIELMO'S-SYNDROME*". The clinical and cytochemic findings of 7 patients suffering from Di Guglielmo's-Syndrome are summarized in table3. In 4 patients we saw a nearly pure erythroblastic proliferation, in 3 patients mixed proliferation of both, erythroblastic and myeloblastic elements. In all patients anemia and thrombopenia; in only 3 patients more than 10.000 nucleated cells [HUHN e. a. 1973]. The type of erythroblasts predominating in one patient reached a very *different degree of differentiation*: in one patient there were quite undifferentiated blasts, and only cytochemic and electron microscopic methods made sure that they belonged to the erythropoietic series. In a second patient, the malignant cells appeared quite polymorphous, extending from undifferentiated blasts to atypical normoblasts. In the remaining patients atypical normoblasts predominated in blood and bone marrow.

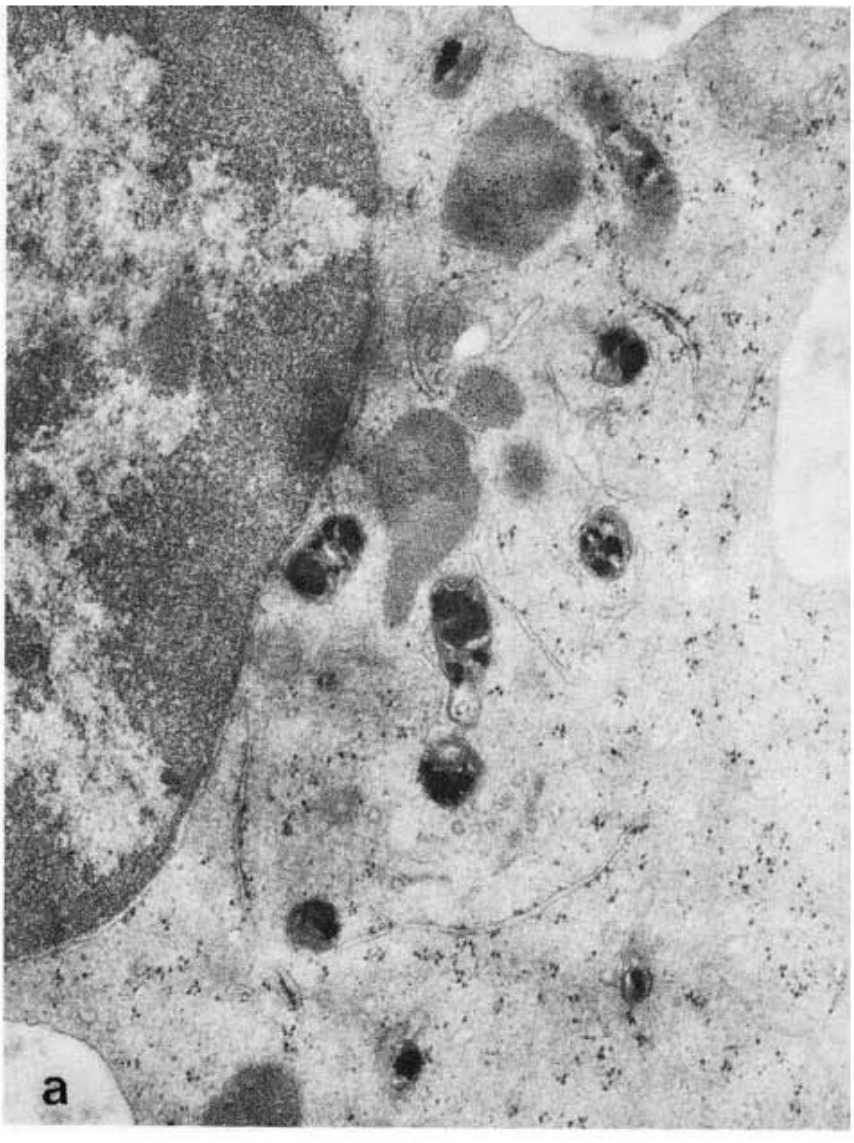
Only occasionally, *deposits of Prussian blue-positive material* were clearly augmented and numerous "ringed sideroblasts" could be seen. By electron microscopy, cytosomes and especially mitochondria containing ferritin and hemosiderin could be demonstrated in all patients, and even so in proerythroblasts (fig. 3).

PAS-positive material in proerythroblasts was deposited in the form of spots and granules, in normoblasts diffusely. PAS-positive deposits do not prove the diagnosis of Di Guglielmo's-Syndrome. They can be demonstrated in sideroblastic anemias as well as in thalassemia.

The *nature of the PAS-positive material* was investigated by electron microscopy: two successive thin sections of the same cell were made – the first one stained with PAS and checked by light microscopy, the second one stained with lead hydroxide or silver methenamine and checked by electron microscopy. In most cases, the PAS-positive material consisted of typical glycogen particles, either localized or diffusely distributed. In some cells, however, the PAS-positive granules appeared empty in lead-stained electron microscopic sections and stained weakly with the silver methenamine (fig. 4). We also got very similar results in lymphoblastic leukemia, but never in normal blood cells. Summarized, the PAS-positive material de-

Table 3. Findings (blood, bone marrow) and course in 7 patients with Di Guglielmo-Syndrome.

		findings at diagnosis							course		
		blood					bone marrow		months		
	patients age sex	hb (g%)	platelets (/mm^3)	nucleated (/mm^3)	erythrobl. (/mm^3)	myelobl. (/mm^3)	erythrobl. (% of nucleated)	myelobl.	diagnosis - death	therapy	cause of
"acute erythremia"	K.S. 43 a ♂	4.9	7.000	2.300	69	0	96 %	0	1.5	ARA-C Predn.	intest. hemorrh.
	H.S. 30 a ♀	8.6	69.000	3.600	0	0	75 %	1 %	6	ARA-C Predn.	intest. hemorrh.
	K.F. 66 a ♂	9.6	28.000	18.400	770	0	60 %	10 %	4	Busul. fan	anemia
	J.W. 68 a ♂	6.7	12.000	4.600	2.800	92	90 %	2 %	2	ARA-C Predn.	intest. hemorrh.
"erythroleukemia"	A.R. 52 a ♂	1.8	13.000	47.700	7.600	32.900	30 %	66 %	1	ARA-C Predn.	intest. hemorrh.
	K.C. 40 a ♂	3.8	89.000	4.100	1.940	820	20 %	75 %	4.5	ARA-C DNR VCR 6-MP Predn.	pneumonia (Soor)
	A.S. 48 a ♀	8.6	88.000	15.000	6.000	4.500	40 %	58 %	0.5		Sepsis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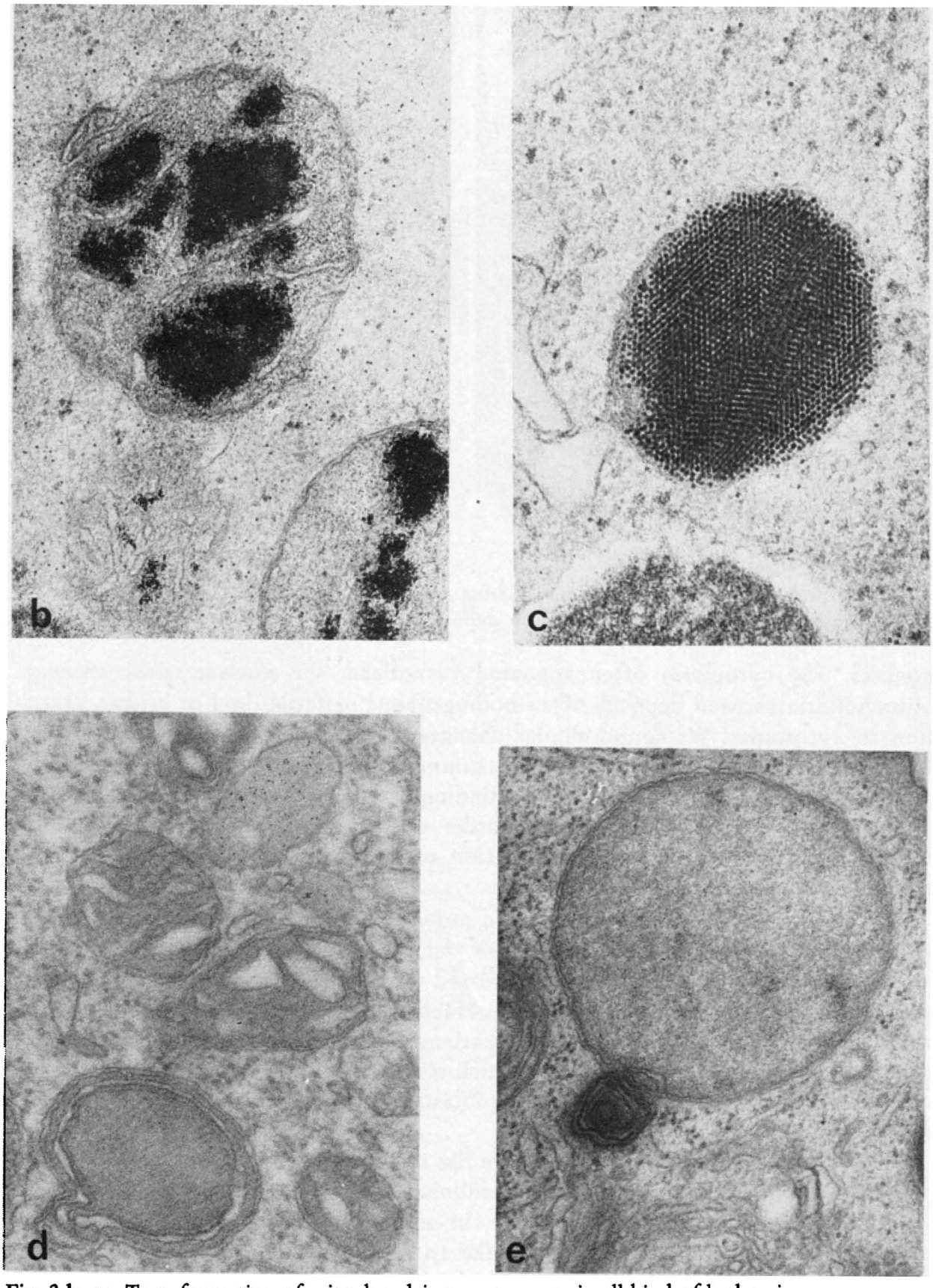
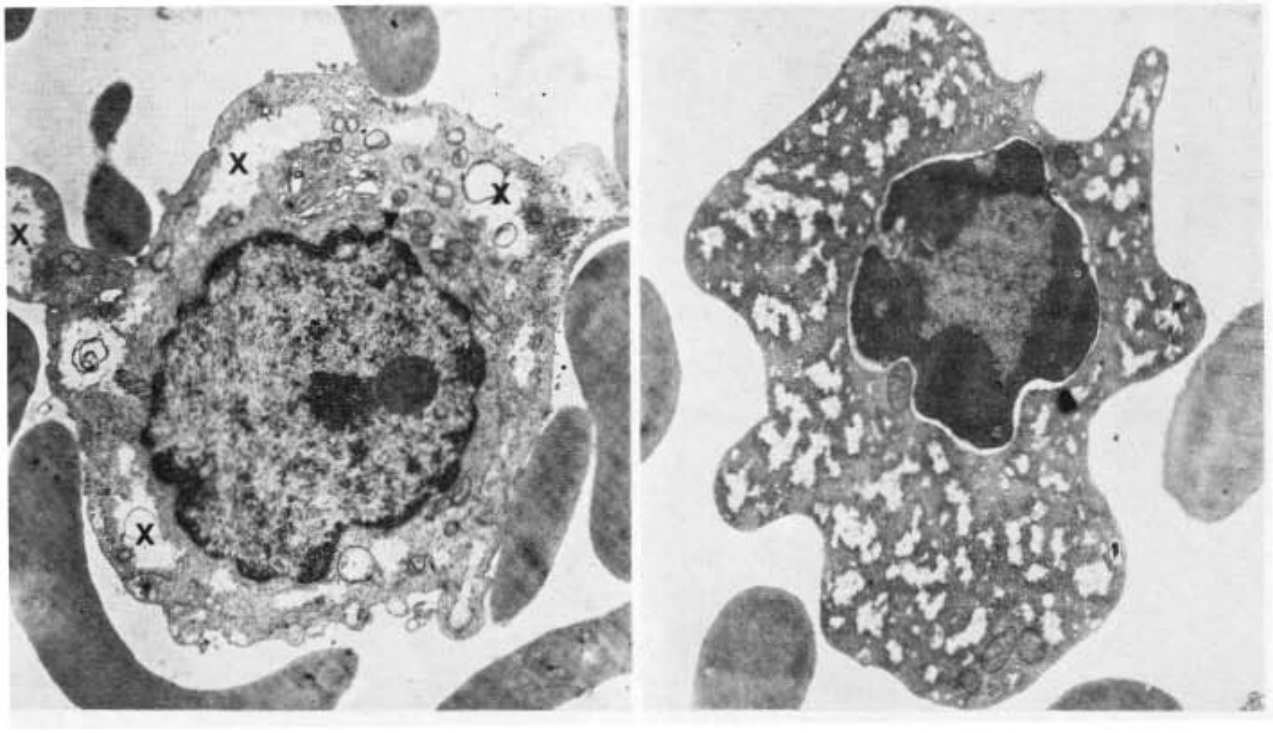


Fig. 3 b–e: Transformation of mitochondria to cytosomes in all kind of leukemia

b–c: Di Guglielmo's syndrome; deposits of homogenous material, of ferruginous micelles, of ferritin in mitochondria

d–e: lymphoblastic leukemia, deposits of homogenous material in mitochondria.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quence of the disturbed mitochondrial function. Vacuolisation with widening of perinuclear space may be of special importance in erythroblasts: The normal performance of nuclear expulsion will be impossible in such a cell.

In the preceding part we tried to demonstrate the predominance of characteristic abnormalities of the granules in myeloic leukemias and of hemoglobin synthesis and nuclear expulsion in malignancies of red cell precursors. In *all forms of leukemias*, especially in lymphoblastic leukemia, we may observe more or less frequently:

1. Deposits of a PAS-positive material, which may appear electron microscopically as 200–300 Å large glycogen particles or as "empty" irregular spots not stainable with lead-hydroxide.
2. Furthermore we see characteristic abnormalities of mitochondria (fig. 3): depositis of a homogeneous or finely granular material, loss of cristae and transition to cytosomes.

For the better diagnosis and classification of leukemias of the *lymphoreticular system* the light and electron microscopic demonstration of membrane-antigens and membrane-immunoglobulin-receptors by combined *immuno-histochemical methods* will be of increasing importance.

At the end of my explanation this shall be demonstrated with the example of *hairy-cell-leukemia* (investigations in cooperation with Dr. H. Asamer, Innsbruck).

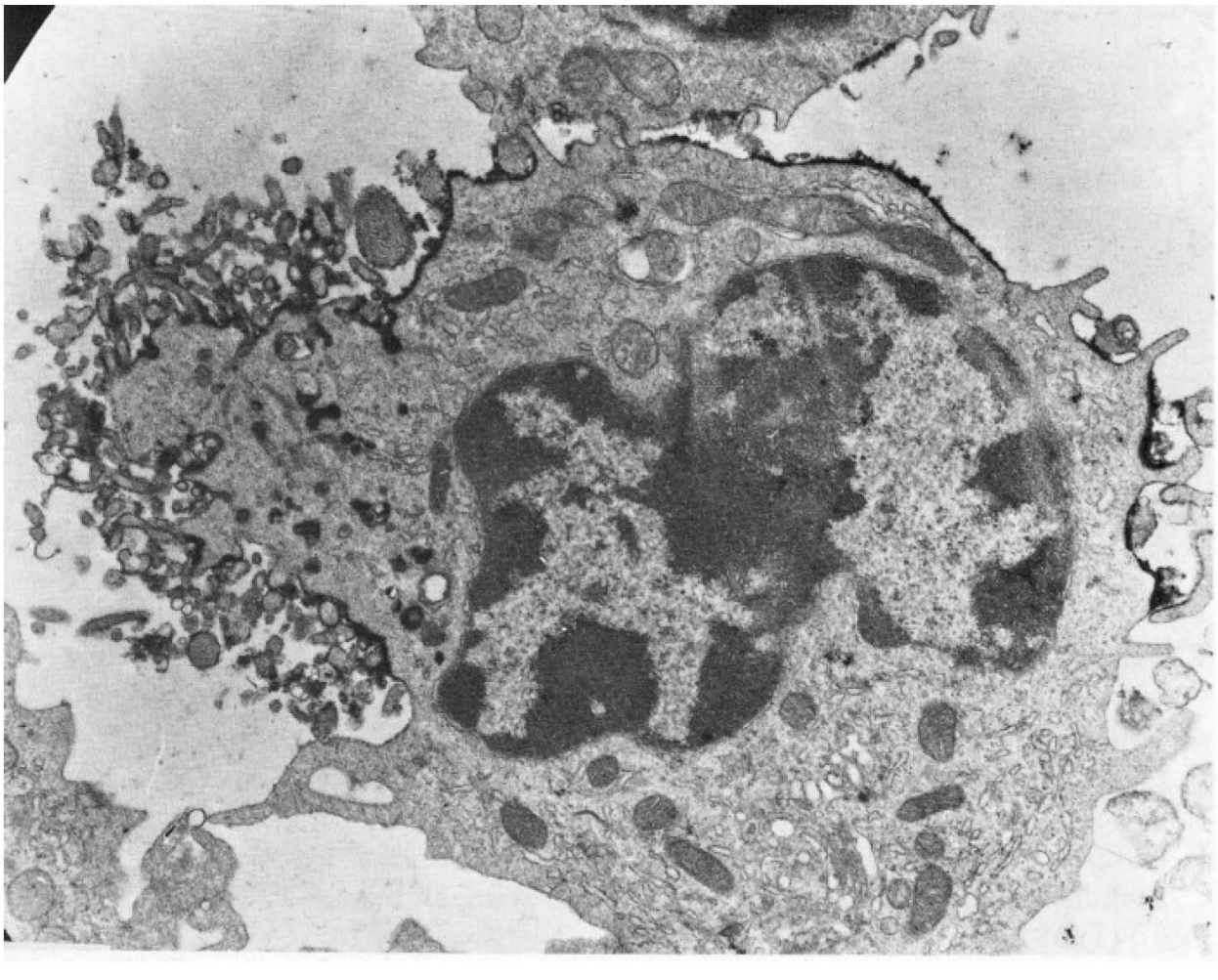

Fig. 5: Hairy cell; demonstation of K-immunoglobulinreceptors by perocidase-labelled antibody.

The leukemic cells of this rare malignancy are characterized by numerous microvilli-like protrusions of the cell membrane. In addition, the cells contain very many small vesicles, few ribosomes or ergastoplasm, small granules and prominent bundles of fibrills. Perinuclear space, endoplasmic reticulum and granules exhibit distinct activity of acid phosphatase, but not of peroxidase. After incubation with ferritin only small quantities are phagocytized and deposited in vacuols or cytosomes. All these findings indicate the lymphocytic but not the monocytic or histiocytic nature of the "hairy cell". This was confirmed by the demonstration of immunoglobulin-receptors of the cell membrane. By fluorescence microscopy (Dr. Asamer) and by an indirect method using peroxidase-coupled immunoglobulins, immunoglobulin receptors of the IgG-K-type could be demonstrated (fig. 5). After these findings, the "hairy cell" seems to be a special form of B-lymphocyte.

Summarizing, we conclude: electron microscopic and cytochemic investigations can facilitate diagnosis and exact classification of leukemias in certain cases. In addition, by these means the understanding of the normal and the pathologically altered functions of the cell and of their organelles is improved. Conclusions concerning the pathogenesis or even aetiology of leukemia are speculative up to now.

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THE LEUKEMIC CELL IN VIVO AND IN VITRO

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Before considering the problem of leukemia it seems appropriate to briefly review the normal regulation of hemopoiesis. I shall confine my remarks to normal myelopoiesis and those leukemias associated with disorders of myelopoiesis i. e. acute myelocytic, progranulocytic and undifferentiated stem cell leukemias as well as chronic myelocytic leukemias.

A schematic outline of normal myelopoiesis (1) is shown in figure 1. It is to be noted that the myeloid system shares a common precursor cell with the erythroid and megakaryocytic series. This common precursor cell is referred to as the pluripotent hemopoietic stem cell. Intermediate between the pluripotent stem cell and those recognizable myeloid elements, which may be identified under the light microscope, is a compartment which is committed to myelopoiesis. Similar compartments exist for the erythroid and megakaryocytic series. There is evidence, which has recently been reviewed (1), that suggests that monocytes and myeloid elements derive from a common committed precursor cell in which case the committed myeloid stem cell compartment can not be considered truly unipotent. Further the myeloblast and progranulocyte differentiates along one of three lines the neutrophil, eosinophil or the basophil. We do not know at what stage this commitment is made i. e. committed stem cell, myeloblast or progranulocyte, nor what is the decision making mechnism.

In animals there is a technique to evaluate the pluripotent stem cell compartment (2) but in man it cannot be assayed and inferences must be drawn from animal experimentation. Briefly the assay for the pluripotent stem cell or CFU consists of transplanting marrow or spleen from the animals in question into heavily irradiated syngeneic mice. The animals are sacrificed after 8–10 days by which time discrete colonies are formed in the spleen. These colonies arise from a single cell and morphologically may be erythroid, myeloid, megakaryocytic, differentiated or

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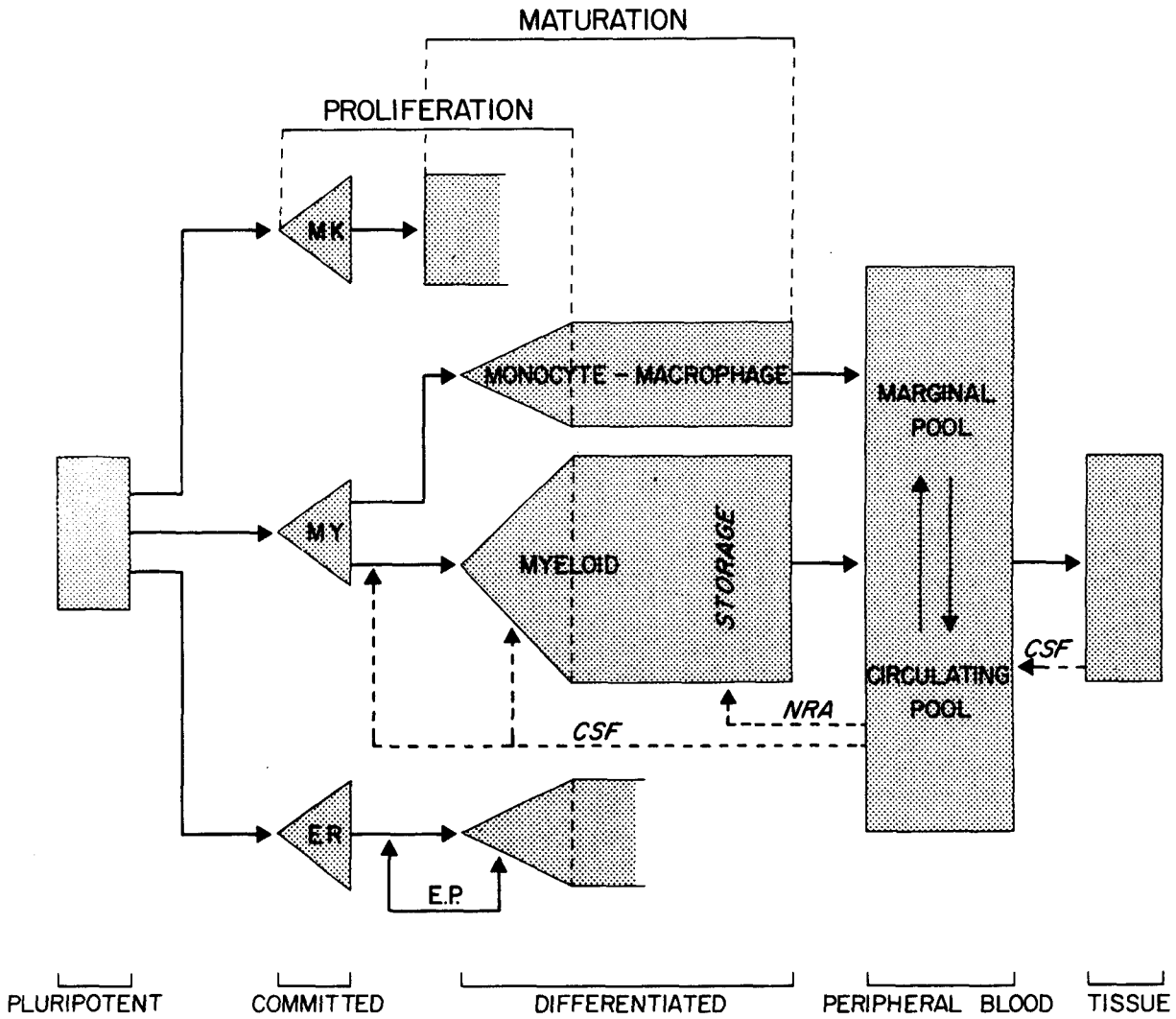

Fig. 1: Schematic representation of hemopoiesis: MK-Megakaryocytes; MY-Myeloid; ER-Erythroid; EP-Erythropoietin; CSF-Colony Stimulating Factor; NRA-Neutrophil Releasing Activity; solid line (EP) indicates established in vivo activity; dashed line (CSF) indicates putative activity. (From Progress in Hematology).

mixed. When a colony is dissected out and transplanted into a secondary recipient, the colonies formed from this innoculum will have the same spectrum of morphologic types as seen in the primary transplant, indicating the pluripotent characteristics of the cells giving rise to colonies as well as the capacity for self renewal.

The committed myeloid stem cell (CFC) may be evaluated in vitro by culturing bone marrow in soft agar (3, 4). The growth of cells from murine marrow is dependent upon the presence of a glycoprotein-colony stimulating factor. In man, however, autonomous growth is observed when the cell concentration in the dish is sufficiently high. The latter presumably reflects the presence in human marrow of a cell capable of producing the colony stimulating factor. This cell is most likely the monocyte (5, 6).

The hormone erythropoietin is responsible for the differentiation of erythroid committed cells into pronormoblasts and prolonged administration produces a sustained erythrocytosis. It is tempting to suggest that colony stimulating factor

serves a similar function for myelopoiesis but as yet firm evidence in the intact animal to establish a physiologic role of CSF has not been forthcoming. There are, however, indirect data from which it has been infered that CSF differentiates the myeloid committed stem cells into myeloblasts in the intact animal (1).

After differentiation, the myeloid cells undergo a series of divisions while at the same time maturing. The capacity for further DNA synthesis ceases at the myelocytic stage but there is further maturation of the granulocytic elements within the bone marrow prior to release into the peripheral blood. Thus the bone marrow contains a large storage pool of nonproliferating granulocytes, metamyelocytes, bands and mature polymorphonuclear cells. It has been shown experimentally that certain experimental manipulations (7, 8), e. g. the administration of endotoxin, effect the release of a protein, which is capable of releasing granulocytes from the storage compartment into the peripheral blood; this plasma factor has been referred to as neutrophil releasing activity (1).

The mature granulocytes have a relatively short intravascular sojourn, the half-time being of the order of 6.7 hours (9). During this time the granulocytes are distributed between the central circulating blood and the margins of capillaries (9). Thus there is a marginal and circulating pool of granulocytes, which are normally in equilibrium. Granulocytosis may result from a shift of cells from the marginal to the circulating pool, as is seen after epinephrine.

There are several lines of evidence to suggest that myelopoiesis normally is governed by a feedback loop from the peripheral blood which is regulated by a function of the circulating neutrophils. A feedback loop requires a time delay between measurement of a particular function in the peripheral blood and correction by changing the production rate. This time delay would include an interval for the production of a regulatory hormone, its effect on the committed stem cell and the time required for maturation of the differentiated myeloblast and its progeny. From this consideration one would expect oscillations of the peripheral neutrophil count and further that the period of these oscillations would be twice the time delay for differentiation, maturation and release. Normally oscillations in the peripheral granulocyte values are not seen. This is thought to reflect the dampening effect of the intramedullary storage pool (10). If so, then removal of the storage compartment should lead to detectable oscillations in the peripheral granulocyte count. Morley and I (11) observed that when animals were given cyclophosphamide, thus reducing the stem cell compartment and in consequence the storage compartment, a cyclical neutropenia developed and the period of the oscillations was twice the projected time delay for bone marrow transit.

Further evidence for feedback control of granulopoiesis was provided by studies in which one hind leg of an animal was shielded while the remainder of the body was irradiated (12, 13). The shielded limb permitted us to measure the myeloid response to neutropenia. Different levels of neutropenia were produced by varying the dose of irradiation. As may be seen in figures 2 and 3 the concentration of both the committed myeloid compartment, as assessed by the CFC technique, and the differentiated proliferating myeloid cells (myeloblasts and promyelocytes) varied as a log-linear function of the peripheral neutrophil concentration. These data therefore provide firm evidence that myelopoiesis is regulated by a feedback loop from the peripheral blood.

It seems most likely that the mechanism regulating myelopoiesis is not the neutrophil concentration but rather a function thereof. We have observed, as neutropenia developed, an increase in colony stimulating factor (14) and a decrease in lipoprotein inhibitor of in vitro colony formation in irradiated animals (15). The time course suggested that the increased generation of CSF might be due to bacteremia and endotoxemia. This notion was supported by the observation that germfree animals did not develop colony stimulating factor after irradiation as did conventional animals (16). When treated with endotoxin, however, germfree animals did produce CSF (15). This led to the concept that endotoxemia was important in CSF generation and in further experiments in normal animals a dose response relationship was observed between the dose of endotoxin and the amount of colony stimulating factor generated (17). It is attractive to suggest therefore that the regulation of myelopoiesis in a significant measure is related to intermittent bacteremia or endotoxemia and perhaps also the breakdown products of normal tissues which result in the release of pyrogens or endotoxin. Thus in the face of local infection or tissue necrosis one would

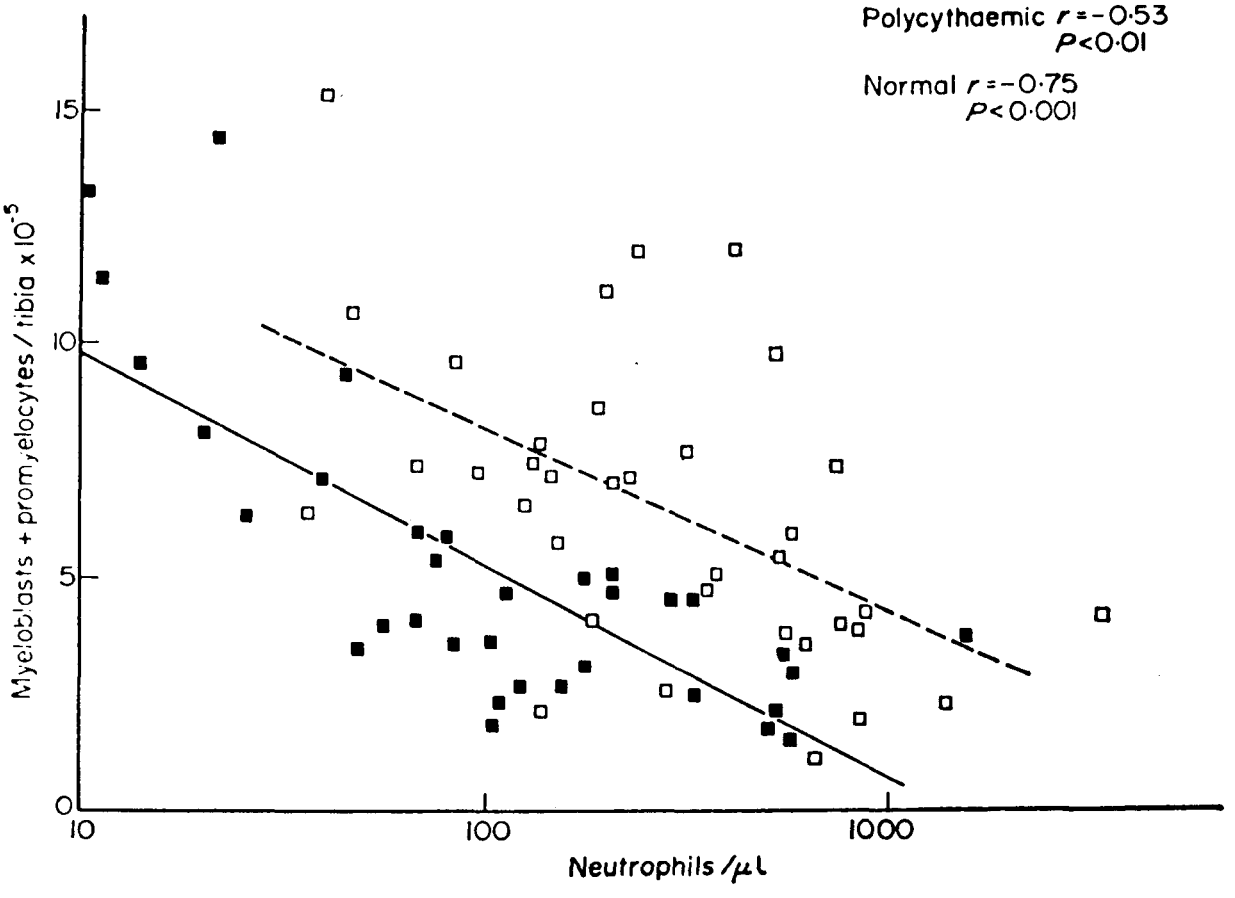

Fig. 2: Effect of polycythaemia on the size of the myeloblast-promyelocyte compartment on day 4 after various doses of irradiation. Each point shows the myeloblast-promyelocyte compartment compared with the neutrophil count from a polycythaemic (□) or normal (■) mouse. The regression lines were calculated by the method of least squares. In each case the size of the myeloblast-promyelocyte compartment is significantly ($P > 0.01$) related to the logarithm of the neutrophil count but the regression line for the polycythaemic animals lies significantly ($P > 0.01$) above that for the normal animals. (From Morley et al. 1970, reprinted from Br. J. Haematol. (24)).

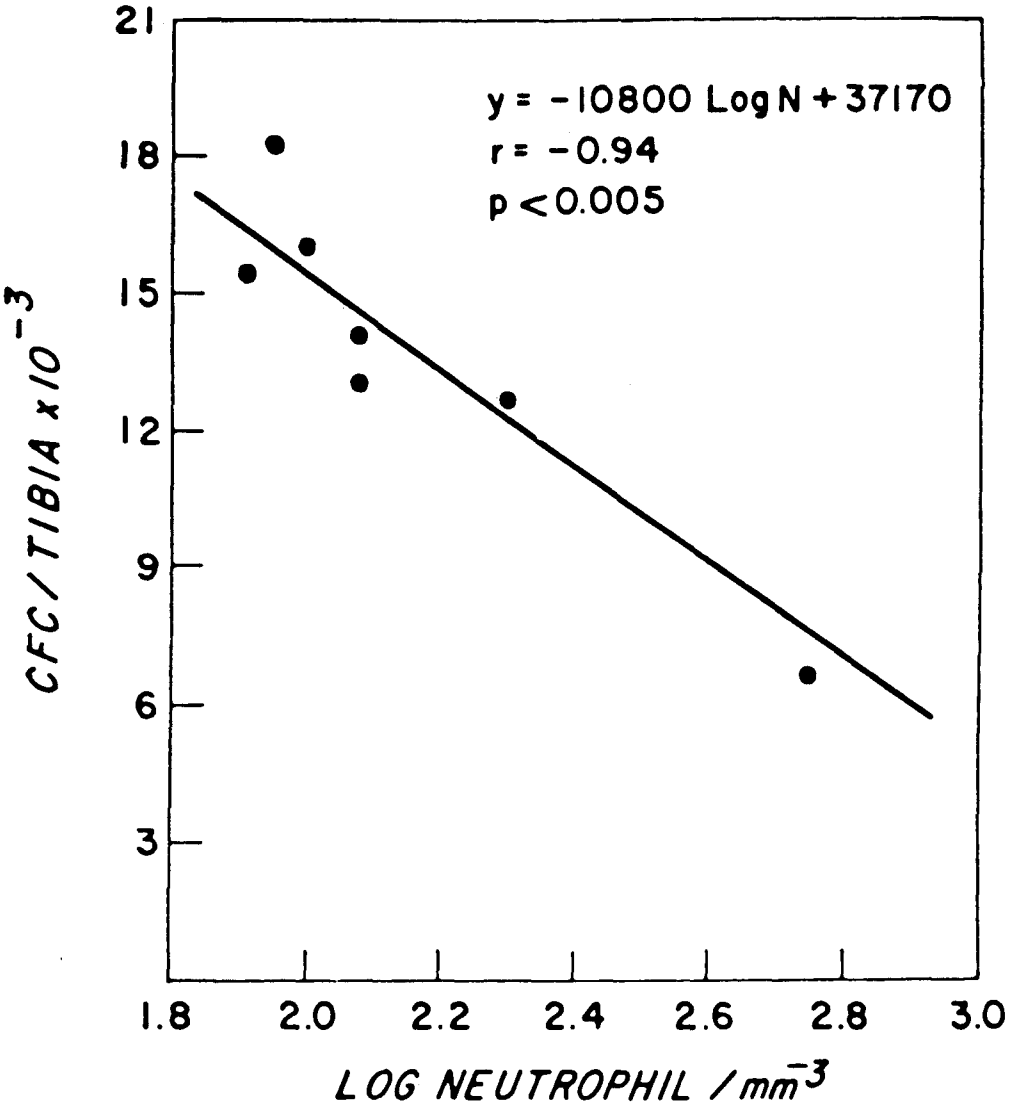

Fig. 3: The relationship between the size of the CFC compartment and the blood neutrophil count in ILS mice for days 2–8 after irradiation. CFC are the average of mean values for composite results of at least three experiments per point. Correlation coefficient r and the regression line are calculated from these values and are significant when $P > 0.005$. (From Rickard et al. 1971. reprinted from Blood (13).)

anticipate increased CSF production which would produce the granulocytosis, which is seen under such circumstances. Neutropenia of course is associated with infection and increased CSF generation has been reported (18).

This hypothesis is appealing but there are still a number of questions to be resolved, principally the problem of tolerance. It is well known that an animal repeatedly injected with endotoxin will become tolerant to its pyrogenic effects and this has been observed in respect to colony stimulating factor (15). Moreover, there appears to be cross-tolerance between various endotoxins.

A major problem in assessing the granulopoietic response to a variety of stimuli has been the storage pool. This has made it difficult to separate granulocytosis due to a

true increase in proliferation from release of granulocytes from the storage pool. Further, the possibility exists that the depletion of the storage pool triggers an intramedullary feedback loop which may affect myelopoiesis. The use of the millipore diffusion chamber has been introduced to circumvent this problem (19, 20). Normal bone marrow is implanted into these chambers which are then placed intraperitoneally into host animals. Manipulation of the hosts prior to implantation of the chambers provides an opportunity for the study of growth regulation within the in vivo culture system. Using this technique Tyler and his associate (21) have demonstrated that the proliferation of pluripotent stem cells as well as the growth of myeloid elements is enhanced in neutropenic animals. The best explanation for these results is that a humoral factor is produced in the neutropenic host which diffuses into the chambers. The nature of this humoral factor and its relation to the colony stimulating factor have not been established.

From the above we may conclude that myelopoiesis is governed by a feedback regulatory mechanism from the peripheral blood and tissues. There is indirect evidence to suggest that this regulation may be accomplished by the glycoprotein, colony stimulating factor, which is responsible for the differentiation of myeloid elements in the soft agar culture system. There is, however, no direct in vivo evidence that CSF plays this central role. Finally the mechanisms governing the production of CSF have not been completely resolved. It is clear that many tissues have the capacity to produce this protein and that endotoxin plays an important role in its generation. Further studies, however, will be necessary before final definitive conclusions can be drawn about its physiologic role in the regulation of myelopoiesis.

The Leukemic Cell

The two principal types of myelocytic leukemia, are the acute leukemias, including acute myelocytic, progranulocytic and myelomonocytic leukemia, and chronic myelocytic leukemia. Patients with chronic myelocytic leukemia after a variable period of time may enter what is termed a blast cell crisis which in all respects appears to be similar to acute myelocytic leukemia. This is most likely a reflection of the natural history of the disease although the role of chemotherapeutic agents and irradiation in effecting the transformation from CML to blast cell crisis has not been fully evaluated.

It is clear from cytogenetic studies that chronic myelocytic leukemia is basically a disease of the pluripotent stem cell compartment. The vast majority of patients have an abnormal chromosome, the Philadelphis chromosome (PH_1) which is seen in erythroid and megakaryocytic cells as well as in the myelocytic cells. Differentiation in many respects is normal and growth by and large is confined to the bone marrow and spleen, which might be considered a normal hemopoietic organ. The lack of the enzyme alkaline phosphatase in the mature granulocytes of CML indicates that differentiation is not entirely normal. Further, the intravascular life of the granulocyte in CML is prolonged. Immature cells, myelocytes, promyelocytes and myeloblasts are released into the peripheral blood suggesting some change in the transit pattern from bone marrow to peripheral blood. It has been suggested that the release of granulocytes is normally regulated by membrane properties (22). If so, then the premature release of immature cells suggest a change in the membrane and is further

evidence of abnormal differentiation. Although there are these demonstrable abnormalities of the granulocytes, it is clear that in some measure normal regulatory mechanism persists. Oscillation of the peripheral white count and also the platelet count has been documented in a number of patients with CML (23), indicating the existence of a feedback mechanism. In most instances, however, the period of oscillation is longer than normal.

The prolonged life span of the granulocytes together with the retention of a measure of feedback control suggests that during its initial phase chronic myelocytic leukemia in part is an accumulative disease. Undoubtedly there is additional input from the stem cell compartment, because arithmetically the prolonged intravascular life span could not account for the level of granulocytosis seen in some patients nor for the oscillation seen in those where oscillatory phenomenon have been documented.

The acute myelocytic leukemias may show varying patterns. In some, examination of the bone marrow reveals only abnormal myeloid elements; in others erythropoiesis and megakaryocytopoiesis may be present. Even during the preleukemic phase there may be identifiable morphologic abnormalities in megakaryocytes and erythroid elements. When cytogenetic abnormalities exist, these may be present in the megakaryocytes and erythroid elements as well as the myeloid. These observations suggest that acute myeloblastic leukemia at least in some instances originates from the stem cell. It seems likely to me that this is true for all patients with acute myeloblastic leukemias. Of paramount importance in considering the pathophysiology of acute myelocytic leukemia is the fact that complete remissions can be achieved with chemotherapy and normal hemopoiesis re-established. Thus, normal pluri-potent stem cells must be present in the bone marrow together with the leukemic stem cells. This suggests the existence of clones of pluripotent cells some of which are leukemic the other normal. Thus, if leukemia is of viral origin the abnormal information transmitted by the virus is present in some but not all pluripotent stem cell lines. One might suggest as a corollary that in some instances the viral infection of a stem cell will result in a lethal transformation and death of what might otherwise have become a leukemic clone.

In others non-lethal information develops and an abnormal line of differentiation results producing leukemia. In still others the viral infection may not be manifest or affects DNA in such a way that normal myelopoiesis is unaffected. The alternative explanation of course would be that some stem cells become infected with virus but others do not.

Since the introduction of the soft agar technique for the culture of myeloid precursors there has been considerable interest in a number of laboratories as to the role of the myeloid committed stem cell and the differentiating colony stimulating factor in leukemic patients. It has been reported that normal differentiation may be achieved when bone marrow from a leukemic patient is cultured in vitro. In a rather large survey Moore (24) found that the marrow from about 60 % of patients with acute leukemia developed small clusters in culture rather than normal colonies, 20 % developed colonies and 20 % failed to grow. The central issue is whether in cultures of bone marrow from leukemic patients normal colonies develop from a normal pluripotent stem cell or under these cultural conditions are normal myeloid elements differentiated from a leukemic stem cell line? Moore and Metcalf (25) have reported

that cytogenetic markers present in leukemic cells were found in all metaphases of cultured leukemic marrow. Unfortunately pooled rather than single colonies were examined. These data indicate that leukemic cells are proliferating in culture but do not provide the final answer that normal granulocytes develop in culture from leukemic progenitors. To be candid there is relatively little data published on the morphologic aspects of the differentiated colonies from culture of leukemic cells and in a recent conference the photomicrographs which were shown of leukemic cultures did not persuade me that anyone had succeeded in differentiating leukemic cells into normal myeloid elements. It has also been suggested by Mintz and Sachs (26) that an inhibitor may be involved in the failure of leukemic myeloblast to differentiate.

Clearly, further information is needed on the characteristics of leukemic cells in the soft agar system. Are normal stem cells differentiating? Are leukemic cells capable of normal differentiation and the production of normal differentiated polymorphonuclear cells? What, if any, is the role of colony stimulating factor in the failure of differentiation? What, if any, is the role of inhibitors in this failure of differentiation?

The concept that myeloid leukemia represents merely a failure of differentiation from the myeloblast stage due to an impaired interaction between colony stimulating factor and the target cells seems to oversimplify the issue. This would be somewhat analogous to pernicious anemia in which the morphologic abnormalities may be as striking as those seen in acute leukemia. This disease has been shown to be the result of abnormal differentiation due to a deficiency of B_{12}. Administration of the vitamin immediately corrects the abnormal proliferation. It seems to me that those who consider acute leukemia in this light overlook one major and important characteristic of the leukemic cell. In pernicious anemia the abnormal cellular proliferation is confined to the normal areas of hemopoiesis – the bone marrow. In acute myelocytic leukemia as in other neoplastic diseases proliferation occurs not only in the site of normal cell production but elsewhere in the body. Thus in acute myelocytic leukemia leukemic proliferation may be seen virtually anywhere, the skin, the heart, the liver, brain, etc. Thus the normal mechanisms which restrict cell production to a specific anatomic area are no longer operative. It seems most likely that the normal mechanism controlling the site of cell production is an interaction between the cells and their immediate environment or microenvironment. Thus in acute myelocytic leukemia the growth pattern reflects not only a failure of differentiation but other cellular abnormalities, most likely in the membrane structure, which permit metastatic growth. In this respect acute myeloblastic leukemia appears to be more than a simple failure of differentiation of the myeloblast. For this reason it seems unlikely that the resolution of acute leukemia will result from efforts directed solely at differentiation of myeloblasts.

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HEMOPOIETIC STEM CELLS AND LEUKEMIA

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Summary

Normal function of hemopoietic tissues depends on a continuous supply of functional mature cells through maturation of progenitor cells and stem cells. Leukemia is believed to be resulted from a disturbance in this maturation process. In mouse, the stem cells are measured by spleen colony technique (CFU-S measurement) and the progenitor cells by *in vitro* culture technique (CFU-C measurement). Sachs and his colleagues reported that human leukemic cells can be induced to mature in the *in vitro* culture system in the presence of colony-stimulating activity (CSA). These findings were partially confirmed and extented to many leukemic cases. Our attempt to establish a relationship between the type of leukemia and nature of leukemic cell maturation in culture was not successful. Using RLV induced leukemia in mice as a model system, the effect of RLV infection on the number of CFU-C and CFU-S was studied. Our results showed that there was an early transitory depression of CFU-S and a long-term stimulation of CFU-S following RLV infection. These results suggest that the earliest event following RLV infection is the committment of CFU-S to CFU-C and therefore CFU-S are target cells of RLV. However, these conclusions do not exclude the possibility that the other cell types could also be target cells.

The mature granulocytes derived from leukemic cells show budding virus particles. The budding virus particles were also found in mature granulocytes and anucleated erythrocytes obtained from RLV infected mice.

I. Concept of a Stem Cell

The normal function of hemopoiesis is carried out by various kinds of mature blood cells, such as erythrocytes, granulocytes, megakaryocytes and lymphoid cells. The life span of these mature cells are relatively short. Therefore, a continuous supply of these mature cells is essential for the normal function of the hemopoietic tissue. These mature cells are derived from a class of early undifferentiated cells through a sequence of differentiation and proliferation. This class of early undifferentiated cells are termed "stem cells" (1, 2). It is important to emphasize that the stem cells are defined according to their functions. The morphology of this class of cell is not known.

A stem cell should have the following properties. It should be capable of 1) proliferation, 2) differentiation, 3) self-renewal upon proliferation and 4) response to physiological demands. If a stem cell is capable of giving rise to more than one type of differentiated cells, it is termed a *multipotent stem cell* whereas if it is capable of giving rise only to one type of differentiated cell it is termed *unipotent stem cell*. A multipotent stem cell by definition is a precursor of a unipotent stem cell (progenitor cell). Their relationship is diagrammatically illustrated in Fig. 1. This figure depicts the transition from stem cell to progenitor cell and the transition from progenitor cell to differentiated cell which are dependent on the presence of specific factors. The production of these regulatory factors presumably are under physiological control.

Any cause which disturbs the process of maturation shown in Fig. 1 will naturally impair the normal function of hemopoietic tissue. Leukemia is believed to result from a disturbance of this maturation process (3–8). There are three possible outcomes that could result from this disturbance (see Fig. 2). These are: (A) blockage of differentiation (4–8), (B) aberration of differentiation and (C) reversal of differentiation. In (A) and (C), the leukemic cells should represent some cell types present during the course of normal differentiation and in (B), the leukemic cells should be different from any of the known cell types. The models in this figure also indicate that the target cells of leukemogenesis (with respect to their stage of differentiation) are not well defined. So far there is no conclusive evidence to support or rule out any of these possibilities or to implicate a particular stage of cell differentiation as the target cell for the initiation of leukemogenesis.

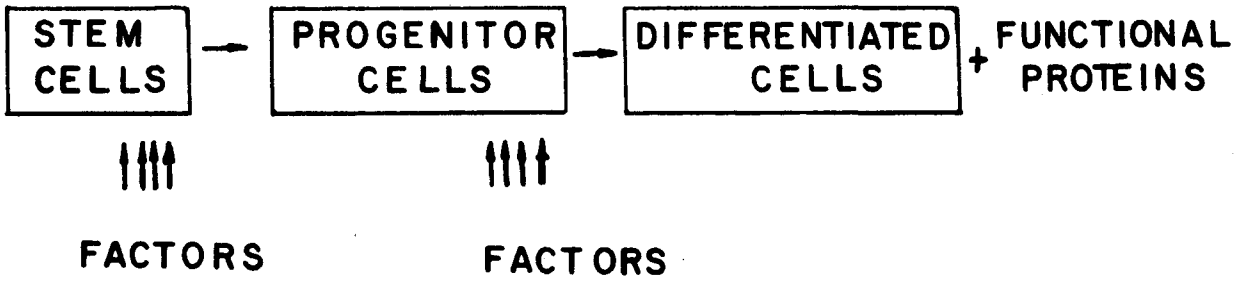


Fig. 1: A diagrammatical representation of maturation of hemopietic stem cells. Abbreviations: S, multipotent stem cells; P, progenitor cells, unipotent stem cells; D, differentiated cells and elements, such as granulocytes, erythrocytes, and platelets. Functional (mature cell) proteins refer to proteins such as hemoglobin of erythrocytes, hydrolytic enzymes from granules of granulocytes, and globulin of antibody-producing cells.

II. Measurement of Stem Cells

Currently there are two functional assays available, one for stem cells, the other for progenitor cells. Both of them are based on clonal methods. They are summarized in Table 1. In mice, stem cells can be assayed by the spleen colony technique (2). The assay is performed by injecting an adequate number of nucleated cells into a lethally irradiated recipient. After a period of 8–10 days, the spleen is removed. The macro-nodules which develop on the surface of the spleen are then counted. These nodules are called spleen colonies. Each colony was shown to derive from a multipotent stem cell as judged by a chromosomal analysis using radiation induced chromosomal aberrations as markers (9) and by studying the cellular composition of each colony (10, 11). Apparently, the development of a spleen colony represents a response of a stem cell to a physiological demand after irradiation and is regulated by the microenvironment (12) which contain cells that produce short-term mediated humoral factors (13).

The progenitor cell for granulocytic cells can be assayed by an in vitro method originally developed by Bradley and Metacalf (14) and by Pluznik and Sachs (15). A single cell suspension is prepared in a semi-soft medium (either 0.3 % agar [14, 15] or 0.8 % methyl cellulose [16]) in the presence of colony stimulating activity (CSA). The CSA is supplied either in the same layer as the cell suspension (16) or in a separate layer of 0.5 % agar containing conditioned medium or feeder cells (14, 15). The conditioned medium was generally prepared by growing cell population containing factor producing cells in culture medium (either in the presence or in the absence of

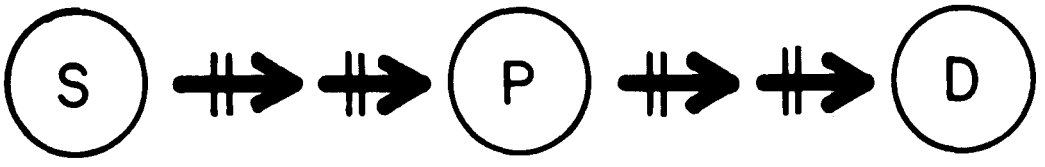
Table 1. Colony Methods for the Assay of Haemopoietic Stem Cells and Progenitor Cells

Term	Intrepretation	Detected By	References
CFU-S	Pluripotent Stem Cells	Spleen Colony Assay	2
CFU-C	Unipotent Progenitors of granulopoiesis	CSA-Dependent Colony Formation in culture	14&15
CFU-E	Unipotent Progenitors granulopoiesis	Erythropoietin Dependent Colony Formation in Culture	17

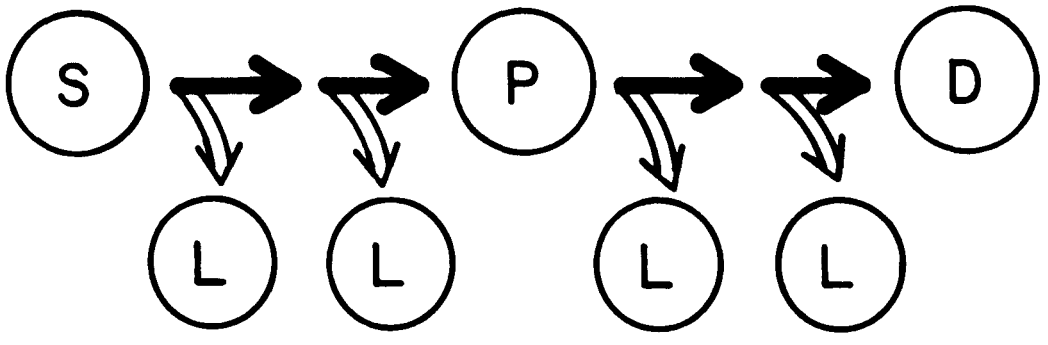
CSA = colony-stimulating activity for granulocytic colonies.
CFU-S = Colony forming unit on spleen.
CFU-C = Colony forming unit of granulocytic cells in culture.
CFU-E = Colony forming unit of erythropoietic cells in culture.

MODELS OF LEUKEMIA AS A DISEASE OF DIFFERENTIATION

1. BLOCKAGE OF DIFFERENTIATION



2. ABERRATION OF DIFFERENTIATION



3. REVERSAL OF DIFFERENTIATION

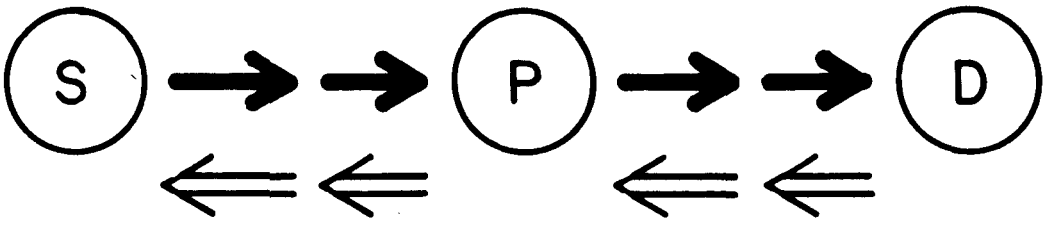


Fig. 2: Abbreviations:
S, P, and D are the same as those of Figure 1; L: leukemic cells; solid arrow, direction of normal differentiation; open arrow, mode of disturbance of normal differentiation.

fetal calf serum) for 4 to 6 days (15). The number of colonies is generally recorded 8 to 10 days after plating. At this stage, the colony size may be as large as 10^4 cells. The colonies are classified as granulocytic or macrophage colonies depending on the composition of cells in the colonies. Both of these colony types contain cells at various stages of maturation. If erythropoietin is present in this culture system the second day after plating, some very small colonies containing erythropoietic cells (8–100 cells) may develop (17, 18). In mice, colony formation is strictly dependent on the addition of CSA. CSA for assaying mouse colony forming cells can be obtained from mouse sera (19), human urine (20), medium from cultured fibroblasts (21), and from embryonic primary culture cells (22). CSA are glycoproteins which can be purified by use of concanavalin A sepharose chromatography. The molecular weight estimates of this activity have indicated heterogeneity ranging from 10,000 to 190,000 (23). It is not known whether CSA is a population of heterogenous glycoproteins or a complex protein containing many subunits.

This *in vitro* culture assay for progenitor cells has been successfully applied to human blood cells (24–27). Similar to colonies derived from mouse cells, the colonies derived from human blood cells (CFU-C) also contain granulocytic cells at various stages of maturation, but in general, the colony sizes of human origin are much smaller (50–1,000 cells) than those of mouse origin. The specificity of CSA required for the *in vitro* colonies of human origin also differs from that of mouse origin. In most cases, CSA prepared from human cells can stimulate the growth of colonies of *both* human and murine origin, but the CSA prepared from mouse cells stimulates *only* mouse colonies. *One difficulty in obtaining a quantitative measurement of human colony forming cells is the lack of an absolute requirement for exogenous CSA for colony formation.* This is due to a contamination of factor-producing cells in the human blood cell population. A successful attempt to separate the factor producing cells from the colony forming cells has recently been reported (28). Obviously, separation of these two cell populations is essential for a successful study of the maturation process.

III. Maturation of Human Leukemic Cells in Culture

One of the most important observations made during the course of studies of human progenitor cells in culture is that apparently *some* leukemic blast cells can be induced to mature in culture in the presence of appropriate CSA to apparently mature normal *appearing* granulocytes (25, 29). The evidence that these mature cells are derived from leukemic cells is based on some chromosomal analyses (30, 31). But the argument is not conclusive, because one still cannot rule out the possibility that the mature cells are derived from normal cells which contains the same chromosomal aberration. However, since the disclosure of maturation of human leukemic cells in culture, many studies have been carried out to establish a pattern as to which type of leukemic cells seems to be "inducible" to mature. The result of these studies are also inconclusive. A summary of such studies carried out in our laboratory in the past year is shown in Table 2. The capacity of colony formation varies with various types of leukemia and also varies within the same type of leukemia. This variation might be due to either difficulty in assuring reproducibly standardized *in vitro* colonies assay

Table 2. Differentiation of Leukemic Cells in Culture

Cell Source[1]	Colony Forming Ability	Differentiation of Colony Cells	Factor Production Ability
Normal	++	yes	+
AML	0 – +++	yes, no[2]	–
AML (Remission)	++	yes	N.T.[3]
AMML	++	yes	–
CML	+	yes	N.T.[3]
CML (Blast Crisis)	0 – +	no, yes[2]	±
ALL	–	–	–

1. All of cell sources are bone marrow.
2. Yes: presence of mature granulocytic cells in culture.
   No: absence of mature granulocytic cells in culture.
3. N.T.: Not Tested

systems for human blood cells or a lack of precise standards for classification of leukemia (classifications presently being based almost entirely on morphology). For example, leukemic cells from some patients with acute myelocytic leukemia (AML) are not able to form any colonies while some are able to produce many more colonies than cells derived from the blood or bone marrow of normal individuals. In general, the number of colony forming cells from AML blood is significantly lower than that from normal individuals. Moreover, colonies derived from AML cells sometimes do not contain mature granulocytes. In remission, the efficiency of colony formation and maturation of colony cells returns to normal. The remission is defined as the return of blood cell count to a normal level and disappearance of blast type leukemic cells. In acute monomyelocytic leukemia (AMML), the leukemia cells behave similarly to normal cells with respect to colony formation. In chronic myelocytic leukemia, the leukemia blood cells contain less colony forming cells than that of normal blood cells, and they contain even less colony forming cells when blast crisis occurs. At this stage, most cells in colonies are not mature (however, there are exceptions), patients with acute lymphocytic leukemia do not form any colonies. This is not surprising if the *in vitro* CSA dependent colonies are, in fact, derived from granulocytic cells. These findings are more or less similar to those reported from other laboratories, e. g., Robinson (32).

We have examined human leukemic cells not only for their ability to respond to factor (CSA) but also for their ability to produce CSA. These results are also shown in Table 2. Leukemic cells are poor producers of CSA. Since this information in the table is still incomplete and since one cannot rule out the possibility of inhibitors of CSA released by leukemic cells, this conclusion is tentative.

It would seem that both colony forming (factor responding) cells (CFC) and factor producing cells (FPC) are required to achieve maturation *in vitro* and probably *in vivo*. At the cellular level the defect in leukemia may be of two general types: one, a defect

in factor production, and the other, a defect in response to factor stimulation. Leukemias might be classified in this manner and further, according to the degree of responsiveness to CSA. Reclassification of leukemia based on detectable physiological functions may be helpful in obtaining meaningful conclusions from these *in vitro* cell maturation systems.

IV. RNA Oncogenic Viruses and Human Leukemogenesis

The *apparent* inducement of human leukemic cells to develop into seemingly normal mature cells supports the idea that leukemia is due to a disturbance in the maturation process. The cause of this disturbance is not known. However, there are now strong reasons for suspecting a role for type-C RNA tumor viruses or information derived from these viruses in the pathogenesis of the disease. Expression of viral genomes in human leukemic cells either from endogenous genetic information or from an exogenous infection has been recently supported from results of extensive studies in searching for the footprints of viral information in human leukemic cells by Gallo and colleagues and Spiegelman and his colleagues. The evidence for the existence of viral information in human cells is as follows: 1) Virus-like reverse transcriptases have been isolated from some human leukemic cells but not from normal proliferative cells. These enzymes have the biochemical characteristics like most of known mammalian viral reverse transcriptases (33–35). For example, this enzyme activity is sensitive to RNAse. The enzymes are able to transcribe the heterogenous part of 70S RNA isolated from primate and mammalian viruses. They prefer $(dT)_{12-18} \cdot (rA)n$ as template-primer over $(dT)_{12-18} \cdot (dA)n$ and are able to use $(dG)_{12-18} \cdot (rC)n$. These are the characteristics of *viral* reverse transcriptase (35, 36); 2) The leukemic reverse transcriptase is immunologically related to reverse transcriptase of primate type-C RNA tumor viruses (37, 38). (They were inhibited by antibodies against viral reverse transcriptase expecially of primate, but also of murine RNA tumor viruses [37, 38]. This suggests that the human leukemic enzyme shares a common antigenic determinant with primate and murine viral enzymes.); 3) Human leukemic cells contain an RNA sequence homologous to DNA products prepared from an endogenous reaction of mammalian and primate RNA oncogenic viruses (39, 40, 41); 4) The size of the RNA template-primer for the leukemic reverse transcriptase appears to be the same as viral RNA (42, 43); 5) The reverse transcriptase and associated RNA are present in a post-mitochondrial cytoplasmic particle which retains morphological integrity on repeated high speed centrifugation and has a density of 1.15 to 1.18 (43), characteristics typical of type-C RNA tumor viruses. It now appears unequivocal that these particles are viral-related. This information has been recently reviewed in detail elsewhere (44).

V. RNA Oncogenic Viruses and Murine Stem Cells

With the above background in mind, it is evident that studies designed to determine the affect of animal type-C viruses on normal hemopoiesis are of immediate interest. The data described below was derived from our beginning attempts in this direction in mice. One advantage of an animal system is that one can follow a defined course of leukemic development, especially the early events following viral infection. Informa-

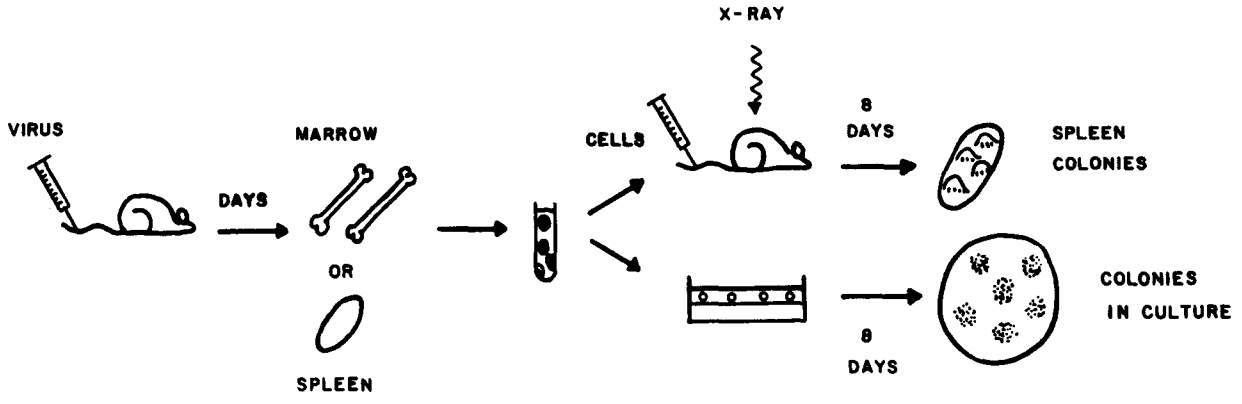


Fig. 3: Experimental design for the effect of RLV infection on CFU-S and CFU-C compartments. Eight-week old NIH Swiss mice were used for this experiment. RLV was injected intravenously through the tail vein. To prepare cell suspensions, at least five uninfected and infected mice were used, respectively, Spleen colony technique was performed as described previously (2). The CFU-C was measured according to the procedures described by Pluznik and Sachs (15), except that CSA was prepared from primary culture of BALB/C embryonic cells.

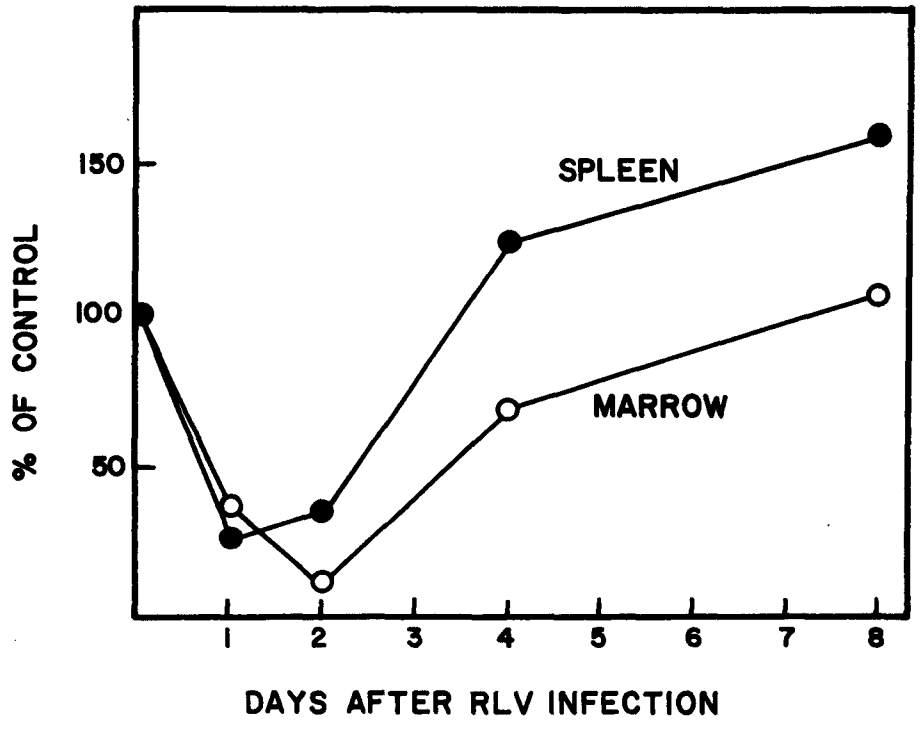


Fig. 4: Effect of RLV infection on CFU-S from marrow and spleens.
The spleen colony assay was carried out as described previously (2). The spleens were fixed in Bouin solution. Each point is an average of 7 spleens. Day 0 started with injection of RLV.

of this experiment for spleen colony formation is shown in Figure 4. There is an initial dip in the number of colony forming units (CFU-S) per unit cell number injected both from spleen and marrow cells on the second and third days after injection of RLV. However, the CFU-S per unit cell number then gradually recovered to a normal level. A short period of "overshoot" was observed on the fourth and fifth day after RLV injection. Since the cell number increased during the course of leukemia development, the total CFU-S, in fact, increased more than 10 fold by one month following the development of leukemia.

The effect of RLV infection on the *progenitor* compartment of marrow is shown in Figure 5. Soon after viral infection, the number of CFU-C (granulocytic progenitor cells) per 10^5 nucleated cells increased about 10 fold. This level was maintained for a few weeks and then gradually decreased by the fifth week after infection. We did not determine whether CFU-C eventually decreases to a normal or a subnormal level since infected animals started to die by the eighth week after inoculation with RLV. This rise and decline of CFU-C following leukemic development might provide an explanation for the variation in measuring CFU-C in human leukemic cell populations.

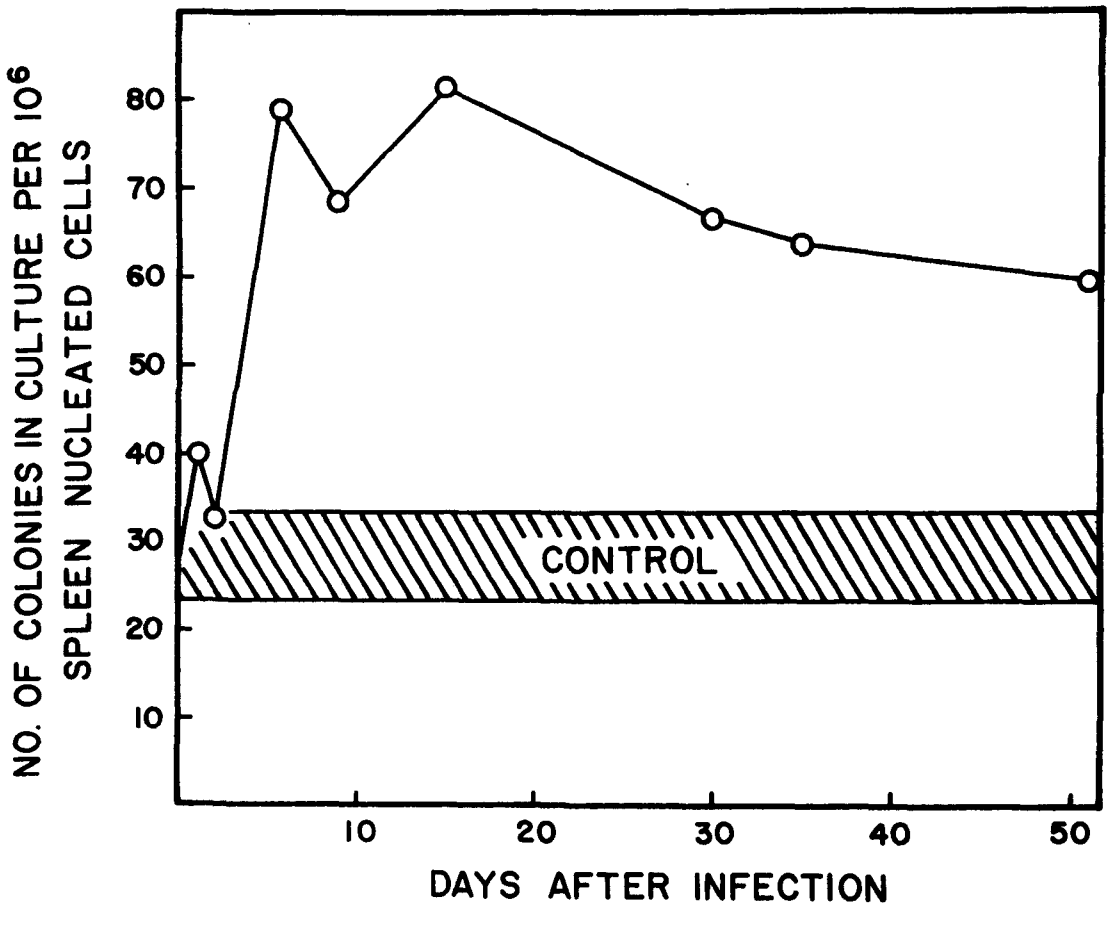


Fig. 6: Effect of RLV infection on CFU-C from spleens of uninfected and infected mice. The procedures are the same as those described in Figure 5.

The number of CFU-C may be a function of the stage of leukemic development. Similar results were obtained when spleen cells were used to study the effect of CFU-C upon viral infection. The result of this experiment is shown in Figure 6. This graph was plotted as CFU-C per unit cell number as a function of time. Since spleen cell number increased at least 20 fold during the development of leukemia, the total CFU-C increased at least 120 fold. From a practical point of view, we wonder whether this increase of CFU-C in early infection might be useful as a diagnostic tool for pre-leukemia or an early stage of leukemia.

The effect on the number of CFU-C and CFU-S by RLV were not simply due to an antigenic stimulation since heat-inactivated viruses did not cause such an effect. Some agents such as Freund's complete adjuvant (45), pertussis vaccine (46), phenylhydrazine (47), and endotoxin (48) are able to enhance the number of CFU-C and CFU-S *in vivo*. However, these effects are transitory and, therefore, are different from the long-term effect of RLV.

Our observation on the initial decrease of CFU-S by RLV infection is in agreement with that reported by Seidel (49) as is our finding on the late effect of CFU-S by RLV infection (49) and this late effect is also similar to that reported by Okunewick, et al. (50). Many interpretations can account for the initial decrease of CFU-S accompanying the increase of CFU-C. One interpretation is that the CFU-S is a target cell of RLV. Upon infection, the stem cells are affected such that their capabilities for spleen colony formation are lost. Now, these infected stem cells are still capable of forming colonies in culture at evidenced by an increase of CFU-C. Perhaps this occurs by a commitment of the stem cell to granulocytic progenitors. However, our results did not rule out the possibilities that the other cell compartment could also be a target for RLV infection.

VI. Properties of Granulocytic Colonies Derived from Murine Leukemic Cells

1) The colonies contain apparent mature granulocytes and colony formation is absolutely dependent on addition of exogenous CSA. We found type-C RNA tumor viruses bud from the membrane of the *mature granulocytes*. In addition, on examining the other cell types from infected mice, we found that besides early immature cells, both mature granulocytes and *anucleated erythrocytes* show budding type virus particles. The phenomenon of the budding of virus particles from mature erythrocytes is interesting and puzzling.

2) With the H^3-thymidine suicide technique (51, 52), we found that progenitor cells from uninfected and leukemic mice have the same fraction of cells in cycle, — 50 % of CFU-C. Thus, the leukemic cell population apparently does not contain a larger fraction of cells in cell cycle. This is in agreement with the notion that leukemia results from a block in leukocyte maturation. This observation could be important not only from a physiological point of view, but also from a chemotherapeutical point of view.

3) Similar to normal granulocytic colonies, the granulocytic colonies which originated from leukemic populations can only be transferred in culture 2 to 3 times. Our attempts to establish a clonal line of colony forming cells has not been successful.

4) An injection of mature colony cells originating from a leukemic cell population induced leukemia in the recipient mice. However, it is not known whether this transplantability of leukemia is due to an infection by viruses associated with the cells or due to the leukemic nature of the transplanted cells. It is worthwhile to emphasize that we noted that several transplantable murine tumor cells all contain type-C virus particles. These cells are L1210 cells (53), Ml cells (54), and leukemic cells from Rauscher leukemia virus infected mouse (data not shown). These viruses may play an important role in the transplantability of these cells.

VII. Conclusion

Leukemia appears to result from a disturbance of the normal differentiation of hemopoietic stem cells. This disturbance might be a blockage, aberration or reversal of differentiation. In some cases, the blockage process of some cells can apparently be removed in culture as evidenced by apparent induction of leukemic cells to produce seemingly normal mature granulocytes in culture in the presence of CSA. This dependence of CSA on the maturation of leukocytes may cast some light on the process of leukemogenesis. The phenomenon of CSA stimulation may provide avenues for studying the process of leukocyte maturation at the molecular level and a potential new approach for the therapy of human leukemia and other blood illnesses. In this respect, however, it is surprising and puzzling that so far there are no reports evaluating *in vivo* results of treatment of leukemic mice with CSA.

Acknoweledgements

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ELECTROPHORETIC DISTRIBUTION PROFILES OF BONE MARROW STEM CELLS

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The preparative electrophoretic separation technique has found successful application in the separation of hemopoietic cells. Within human bone marrow cells – in mice we received similar datas – different distribution profiles could be observed. Granulopoiesis (fig. 1) shows a decreasing electrophoretic mobility (EPM) correlated with increasing maturation whereby the myeloblasts possess the highest and the segments the lowest EPM. Fraction 0 is characterized by the mass of erythrocytes. No differences exist between normal and leukemic granulopoiesis concerning the EPM. Antibody producing cells as normal plasma cells and lymphoid cells in M. WALDENSTRÖM show a low EPM.

The lymphoid cell series have intensively been investigated by ZEILLER et al. (1) by means of this method. Two subpopulations of lymphocytes in lymphoid tissues of rodents can be distinguished. The lymphocytes with high EPM mediated the graft versus host reactivity whereas the lymphocytes of low EPM provided antibody forming cells. Both cell classes have been classified as T- and B-cells, respectively. In cell cooperation experiments these cell functions have been confirmed (2).

Undergoing the total cell distribution of murine bone marrow the TILL and McCULLOCH-technique (3) for the demonstration of Colony Forming Units (CFU) different stem cell properties can be detected (fig. 2) (4). Each electrophoretically separated fraction was i. v. injected into lethally irradiated 8–12 weeks old mice (F_1 hybrids: DBA/2J/Bom x C3H/Tif/Bom). Isogenic grafts and hosts were used. Most of the colonies were found in the region of relatively higher EPM. As the CFU obtained of all fractions gave rise to differentiated cells and to new colonies in regrafting experiments the criteria of pluripotent stem cells were fulfilled.

According to the electrophoretic mobility different properties of the CFUs achieved could be demonstrated.

1) The colonies originated from bone marrow cells of high EPM showed another cellular composition than those ones of low EPM. The first ones are characterized by mostly erythropoietic cells while the other ones are more frequent mixed colonies of two or three hemopoietic cell systems.

2) Pretreatment of donor mice with lethal doses of vinblastine 24 or 48 hours before cell separation resulted in a complete loss of colonies derived of bone marrow cells of high EPM.
3) Reelectrophoresis of pooled colonies derived of either the slow or the fast region showed colony growth after the second electrophoresis *only* with cells of high EPM. Apparently the CFU of low EPM have transformed into CFU of high EPM after colony growth.

These results with rodent bone marrow have encouraged us to investigate human bone marrow stem cells with the preparative cell electrophoresis. A modified agar-colony-technique as developed by BRADLEY and METCALF (5) and the millipore chamber system were used as test for stem cell properties.

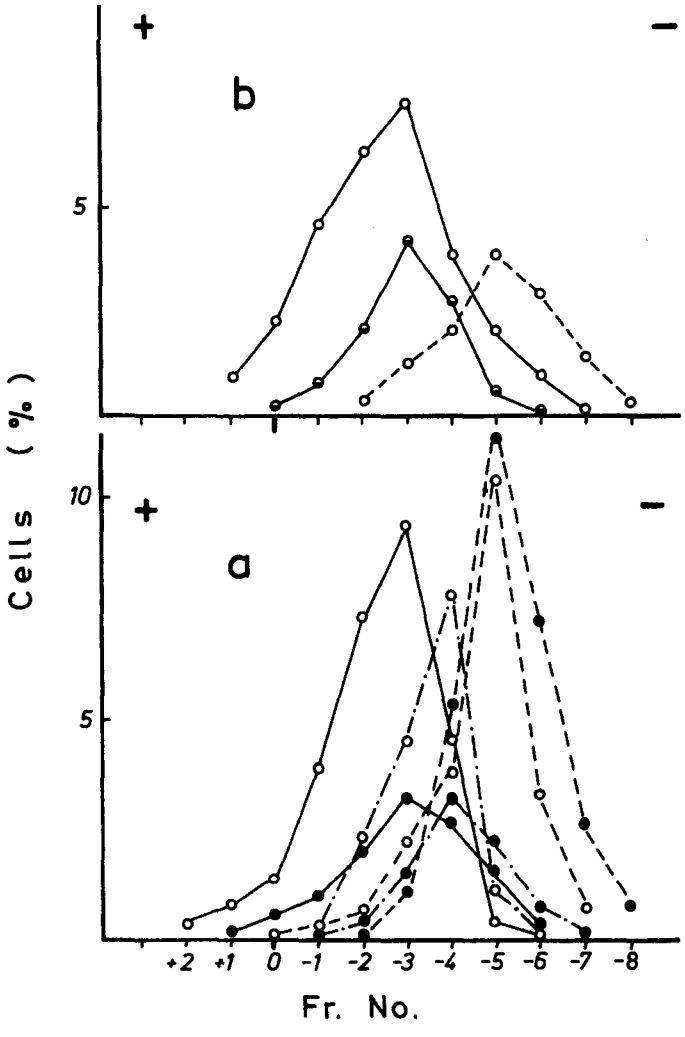

Fig. 1: Electrophoretic Distribution Profiles of Granulopoiesis.

a) —— promyelocytes and myelocytes
—.— metamyelocytes and rods
– – – segments
● normal, O chronic myeloid leukemia

b) acute myeloblastic leukemia
O—O myeloblasts
O—O promyelocytes and myelocytes
O- -O segments

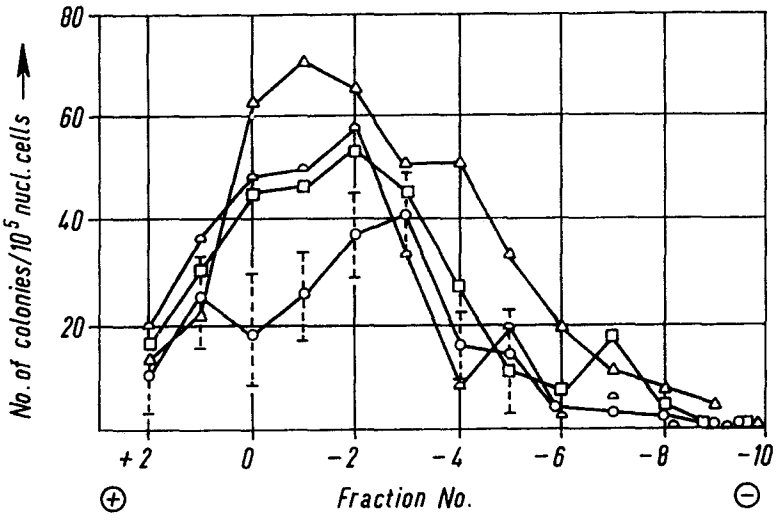

Fig. 2: Relative distribution of CFU in electrophoretically separated normal bone marrow. Four separate experiments are shown. Each point represents the mean of 10 animals. Vertical bars indicate 90 % confidence limits (4).

Patient	Region of EPM / Diagnosis	High	Medium	Low
		Number of colonies per 3 x 10^5 implanted nucleated BM cells		
		$\bar{X}$	$\bar{X}$	$\bar{X}$
S	Normal	160		7
Wi	Normal	127		12
We	Normal	63		35
K	Chronic grannlocytic leukemia	98		5
M	Acute leukemia (blast cells POX +++)	18	11	6
H	Acute leukemia (blast cells non specific esterase +++)	31	31	18
B	Acute leukemia (blast cells glycogen staining+++)	14		10
		73 ±		13 ±

Fig. 3: Colony Formation by Human Nucleated Bone Marrow Cells in Agar-gel after Separation in an Electric Field.

The agar in vitro system based on a CO_2-free medium buffered by carrier ampholytes. The optimum of colony growth was found at pH 6.7. The results obtained by this method were comparable to those reported in literature (6, 7).

After cell separation of human bone marrow the nucleated cells were devided into two fractions of low and high EPM respectively. The slow migrating fractions consisted predominantly of rods, segments, mature monocytes, normoblasts, plas-

ma cells and lymphoid cells, whereas the high EPM region showed mostly erythrocytes, myeloblasts, immature granulocytes and in addition lymphoid cells. In two cases a third region with medium EPM was distinguished. The table (fig. 3) shows the distribution of agar colonies of 7 cases related to the different regions of EPM. Definitely higher colony counts were found with cells of high EPM. In the cases of normal and chronic myelocytic leukemia bone marrow ten to twenty times as much colonies were counted in comparison to the slow EPM region. The other cases showed the same tendency but in a lesser degree.

In parallel the nucleated cells of the two separated fractions were implanted into millipore chambers which were repeatedly grafted into sublethally irradiated mice. After two weeks all the chambers were colonized. No differences were detected in cell composition and number of cells in the chambers regardless wether the chamber content was derived from bone marrow cells with high or low EPM.

Our results may allow the following tentative conclusions:

There are existing two murine and probably two human bone marrow cells with stem cell properties. One is characterized by a higher EPM, a sensitivity against antimitotic drugs like vinblastine and a limited capacity of differentiation. The other one shows a lower EPM, vinblastine resistence and a possible higher capacity of differentiation. It seems probably that under certain conditions this slow migrating cell transforms into a faster migrating one.

We feel that the assumption of an electrophoretically slow migrating omnipotent hemic stem cell is allowed. This fact might be the key for isolating these cells from the faster migrating, graft versus host reactivity mediating T-cells. Thus the different electrophoretic behavior of bone marrow cells might gain importance in bone marrow transplantation.

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DIFFUSION CHAMBER TECHNIQUE APPLIED IN HUMAN ACUTE LEUKAEMIA

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Introduction

The diffusion chamber technique was originally developed by Algire (1) for investigation of immunological problems and was then used by other workers for the isolated growth of various tissues under in vivo conditions. Later the technique was applied to the growth of haematological cells (2) and developed as a quantitative test system for haematological stem cells by Benestad, Bϕyum and Breivik (3, 4, 5). Since most assays for haematological stem cells are limited to laboratory animals, the importance of the diffusion chamber method is that it can be applied to test for human stem cells. With the in vitro agar colony technique, the myelopoietic committed stem cell can be tested (6) and with the plasma clot cultures also the erythropoietic committed stem cell (7). The diffusion chamber technique provides a milieu in which the pluripotent stem cell can proliferate (5, 8) and differentiate since all three cell series – myelopoiesis, erythropoiesis and megakaryocytopoiesis are found in cultures of human bone marrow cells (9) and of peripheral blood cells (10) in diffusion chambers.

Methods

The principle of the method is as follows. The chamber is usually built from a plexiglas ring, whose sides are closed by two MF-Millipore filters, pore size 0.22 μ or 0.45 μ through which nutrients can diffuse. After sterilisation, the chambers are filled through a hole in the ring with the cell suspension to be studied, sealed and implanted into the peritoneal cavity of normal or pre-treated animals. The chambers can be

harvested at different intervals, and are shaken in medium containing 0,5 % pronase to disperse the clot cells which has formed (unless histological sections are desired). The cell suspension is then removed from the chamber by puncture with a fine Pasteur pipette and, after washing out the chamber, transferred to a small centrifuge tube where the total volume is found by weighing. The total cell number can then be determined and smears made from the cell pellet after centrifugation.

We are using this system to study the growth of peripheral blood cells from normal subjects or leukaemic patient*. Human peripheral blood was chosen in preference to bone marrow, since it is easily available and especially because the cell population to be implanted can be confined to about 99 % mononuclear cells (lymphocytes and monocytes) by removal of granulocytes and erythrocytes on an Isopaque-Ficoll gradient (11). The subsequent appearance of immature precursor cells in the chambers can then be taken as evidence of their development from stem cells and there is no confusion with the possible survival of immature cells present in the original inoculum. Furthermore, a possible stimulatory (12) or inhibitory (13) effect of granulocytes on cell growth is excluded so that a cell inoculum of more constant composition is implanted which is subject only to stimulation by factors in the host animal which can be maintained constant or varied at will.

Growth of Normal Human Blood Cells

The growth of cells when normal mononuclear blood cells are implanted into mice pre-irradiated with 650 R has the following course (Figure 1). There is an initial decrease in cell number to 70 % to 80 %, owing to the technique and to cell death, followed by an increase after the 6th day, reaching a maximum on the 13th day of at least twice the previous minimum. As soon as 24 hours after implantation, immature blast cells can be observed. An increase in these blast cells follows and myelopoietic precursors appear after the sixth day. Erythropoietic precursors are very few in comparison and megakaryocytes of different maturation stages appear from the ninth day onwards.

Growth of Cells from Human Acute Leukaemias

The following questions in human acute leukaemia are presently being investigated:

1. What is the proliferative capacity of cells from patients with acute leukaemia? Is there any relationship between the capacity for proliferation of leukaemic cells and the type and/or stage of the leukaemia?

Up to know, the proliferation and differentiation of leukaemic cells has been investigated by several groups using the agar colony technique. However, the agar colony technique is a test for the stem cell committed to myelopoiesis. Since the leukaemic lesion probably lies at an earlier stage, that is at the level of the pluripotent stem cell, it is desirable to use in addition a method such as the diffusion chamber technique which permits the proliferation and differentiation of cells at this level of development. In all patients, therefore, mononuclear blood cells separated on an

* For the possibility of investigating patients under their care, we thank Prof. H. Heimpel, Dept. of Haematology, Ulm University, and the members of the Süddeutsche Hämoblastosegruppe.

Isopaque-Ficoll gradient were tested for their growth potential in both the in vivo diffusion chamber technique and the in vitro agar technique.

In the diffusion chamber experiments, the growth pattern of cells from patients with acute leukaemia shows wide variations compared with the normal pattern (Fig. 1), as is illustrated by the examples given in Fig. 2. In some cases, the increase in cell number may be more than twice that from normal cells, in some cases it is similar to normal and in other cases there may be little or no growth at all. The relevance of these data to the type and stage of the disease, or to the prognosis cannot be assessed at present since too few cases have been investigated. However, the varying growth of leukaemic cells when brought into a "normal" milieu shows that they have different proliferative capacities.

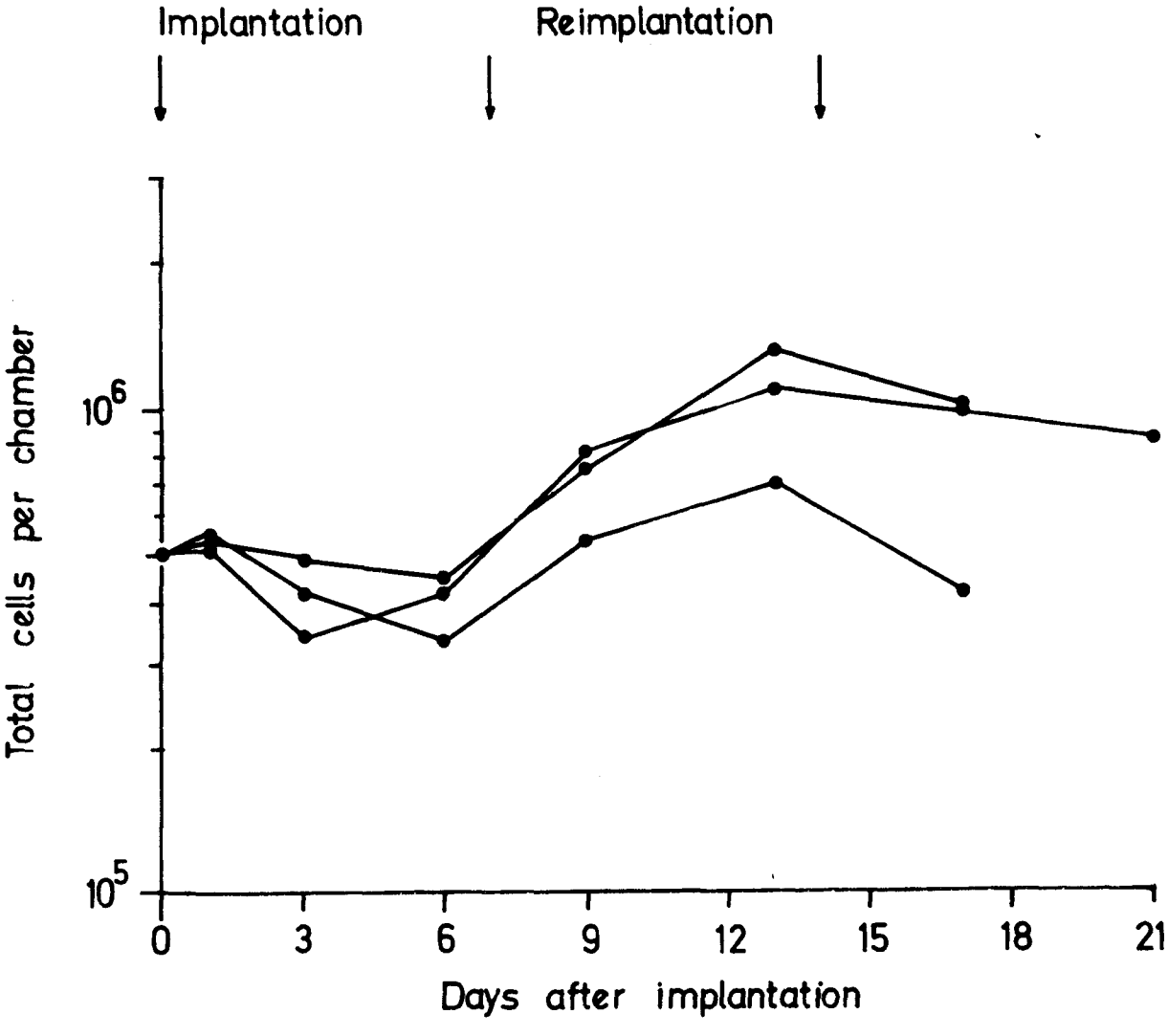

Fig. 1: Total cell number per chamber as a function of time after implantation of mononuclear cells from the peripheral blood of three normal subjects. Chambers were filled with 5 x 10^5 nucleated cells and implanted into CBA mice pre-irradiated with 650 R, two chambers per host. Chambers were harvested routinely at 1, 3, 6, 9, 13, 17 and 21 days after implantation. At 7 days and 14 days the chambers were re-implanted into new pre-irradiated hosts. The curves show mean values for 6 chambers at each interval.

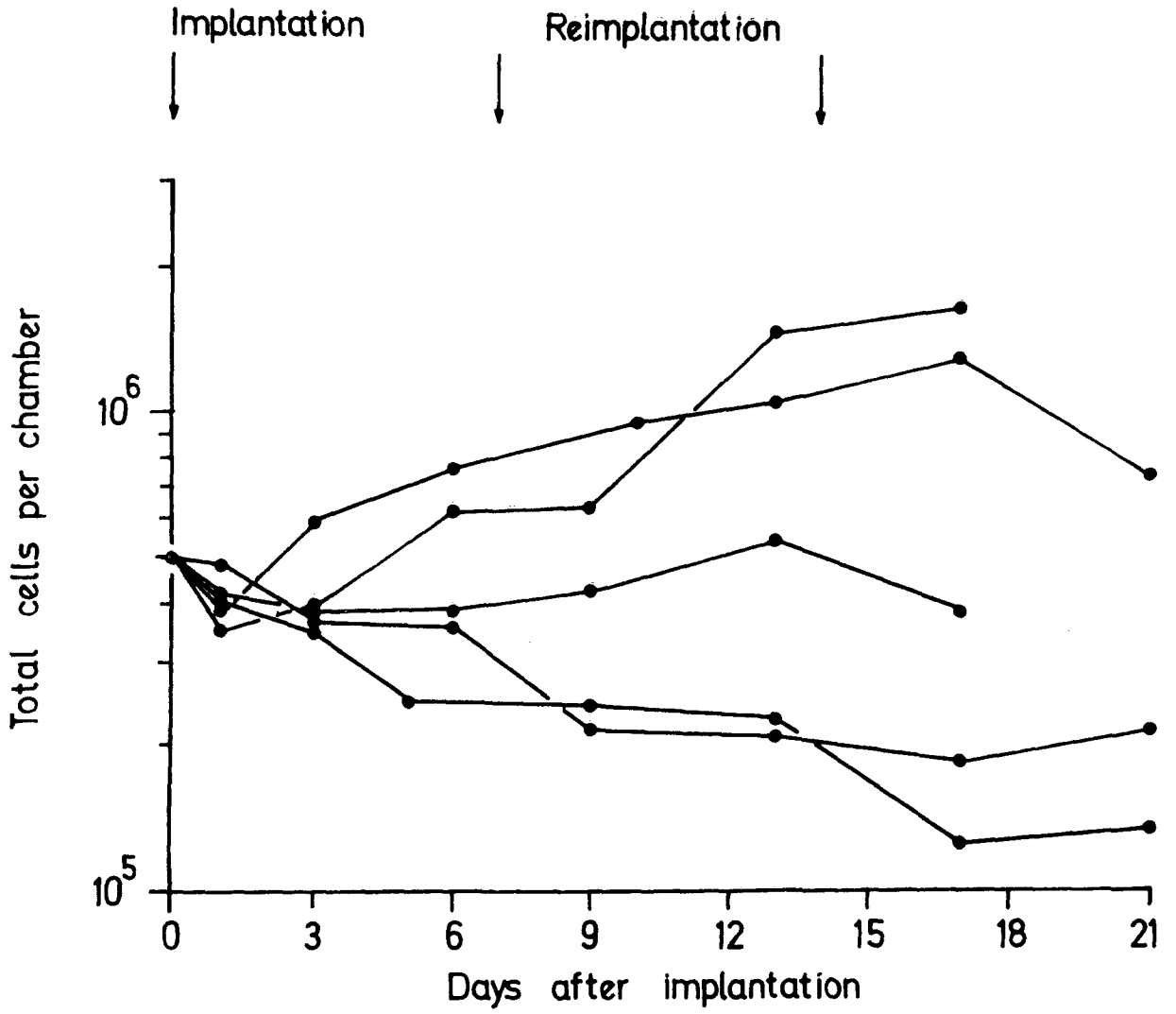

Fig. 2: As Figure 1. Results for cells from five patients with varying types of acute leukaemia.

In comparing the growth of cells in the two systems, there are again wide variations. In some patients the leukaemic cells show neither growth in diffusion chambers nor colony formation in agar culture, whereas in others exceeding growth and some maturation in diffusion chambers is associyted with an abundant but diffuse growth in agar culture with only small cluster formation. An important finding is that in some cases growth in the in vivo and in vitro systems is quite different, with poor growth in diffusion chambers but good colony formation in agar culture. Such a difference in growth could arise because different stimulatory or inhibitory factors are provided in the in vivo and the in vitro milieu thereby probably supporting the growth of different types of cells.

The second question we are interested in is:

2. Do leukaemic blast cells possess a capacity to differentiate similar to that of normal pluripotent stem cells, or of different unipotent committed stem cells, i. e. can they in a "normal" milieu produce recognisable precursor cells of the myelopoietic, erythropoietic and megakaryocytic series?

That cells from some leukaemic patients can mature along the myelopoietic pathway is suggested by the observation of Auer bodies in mature granulocytes (14). Maturation of leukaemic cells has also been found in agar culture (15, 16) and of rat leukaemic cells in diffusion chambers (17). We have also found that myelopoiesis and mature granulocytes develop in diffusion chambers in growth of cells from some patients studied. In two cases mature megakaryocytes were observed. An underlying problem here, however, is the distinction between growth from leukaemic cells and from remaining normal cells, which necessitates separation of leukaemic and normal cells. Since a reliable method for this does not yet exist, the problem can perhaps be circumvented in various ways: 1) by choosing for investigation patients with very high numbers of leukaemic blast cells in the blodd, 2) by cytogenetic analysis for common chromosome abnormalities or 3) by pre-labelling of the cell suspension. Pre-labelling with ^{3}H-thymidine in vitro seems profitable since very few normal blood cells incorporate ^{3}H-thymidine in vitro and the labelled cells are almost exclusively leukaemic blast cells, so that the occurrence of labelled maturing cells in diffusion chambers would be strong evidence for their leukaemic blast cell origin. Autoradiographic studies of cells from 12 leukaemic patients investigated are in progress and have already shown that in one patient, labelled granulocytes could be observed when blast cells from peripheral blood were pre-labelled with ^{3}H-thymidine. Further results should show whether or not this method is a practicable mean of deciding the "normal" or "leukaemic" origin of the differentiated cells.

The diffusion chamber technique provides further the possibility of investigating cell growth and maturation under conditions where the levels of humoral regulatory factors are changed, for instance after intraperitoneal administration of erythropoietin or factors which stimulate (18) or inhibit (19) colony growth in agar culture. The host animals can also be treated to alter the endogenous levels of such factors, for instance by hypertransfusion of bleeding (6).

In conclusion, the comparison of growth of leukaemic cells in diffusion chambers and in agar culture should reveal what proliferative and differentiating capacity the leukaemic cells have. Furthermore, it is hoped that such studies could lead to a functional classification with respect to the different types and stages of human acute leukaemia.

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DIFFERENTIATION AND TRANSFORMATION OF HEMATOPOIETIC CELLS IN CULTURE

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Recent findings of viral specific RNA in human tumors such as human breast cancer (1), leukemias (2), sarcomas (3) and lymphomas (4) which are related to RNA of RNA tumor viruses of animals support the idea of a virus aetiology in human cancer. The establishing of cell cultures of human origin transformed in vitro should provide a powerful aid in the search for human cancer. Diseases which are related to leukemia and which can be considered as being preleukemic stages seem to be ideal systems to study the malignant transformation in man. In addition the disease to be studied should be reasonably frequent and the disease entity well characterized. Furthermore, during the course of the disease some similarities should occur between the disease and experimental RNA tumor virus induced diseases in animals. This seems to be true for Polycythaemia vera (PV).

PV is a proliferative disease of the hematopoietic system, mainly of the erythropoietic compartment. Often PV changes, expecially after irradiation and chemotherapeutical treatment, to acute leukemia, chronic myelogenic leukemia or myeloid metaplasia (5). In the development of the Friend virus induced leukemia in mice a similar polycythemic stage is observed (6). In the Friend polycythemia as well as in PV, an unregulated multiplication, but normal differentiation of the cells of the red compartment is observed. In both diseases the abnormal erythropoietic precursor cells do not respond to the proteohormone erythropoietin. We have shown that Friend virus transformed erythropoietic tissue culture cells also do not respond to erythropoietin (7). For these reasons bone marrow cells of patients with Polycythaemia vera seem to be an appropriate material for establishing cultures to study processes leading to leukemic transformation in man.

Cell Culture:

Bone marrow of a 69-year old male patient with PV has been cultivated. After a proliferative phase of two weeks the cells successively became fibroblastoid and formed monolayers. After 8 weeks the contact inhibited monolayer cultures did not contain cells in suspension. During the ninth week a "piling up" has been observed on different loci of the monolayers of two parallel cultures. The spontaneously transformed cells grow in suspension as single cells or in clusters. They have a generation time of about 24 hours. Figure 1 shows the possible correlation between the time course of the cell culture and the clinical course of the disease.

Cytologically there was no difference between the cell types of the culture and normal bone marrow during the first 3 weeks. After the transformation, however, 90 % of the cells are blasts, 8 % orthochromatic erythroblasts and a small portion lymphoid cells (see Table 1).

Globin synthesis

The cytological observations of orthochromatic erythroblasts led to biochemical investigations for globin synthesis, although the cells gave a benzidine negative reaction. Cells were labelled with ^{3}H- and ^{14}C-leucine and globin chains were isolated (8, 9). Radioactively labelled tissue culture protein eluted as defined peaks together with the α and β carrier globin. Tryptic fingerprint analysis of α- and β-chains were carried out as described (10). Preliminary results show that α- and

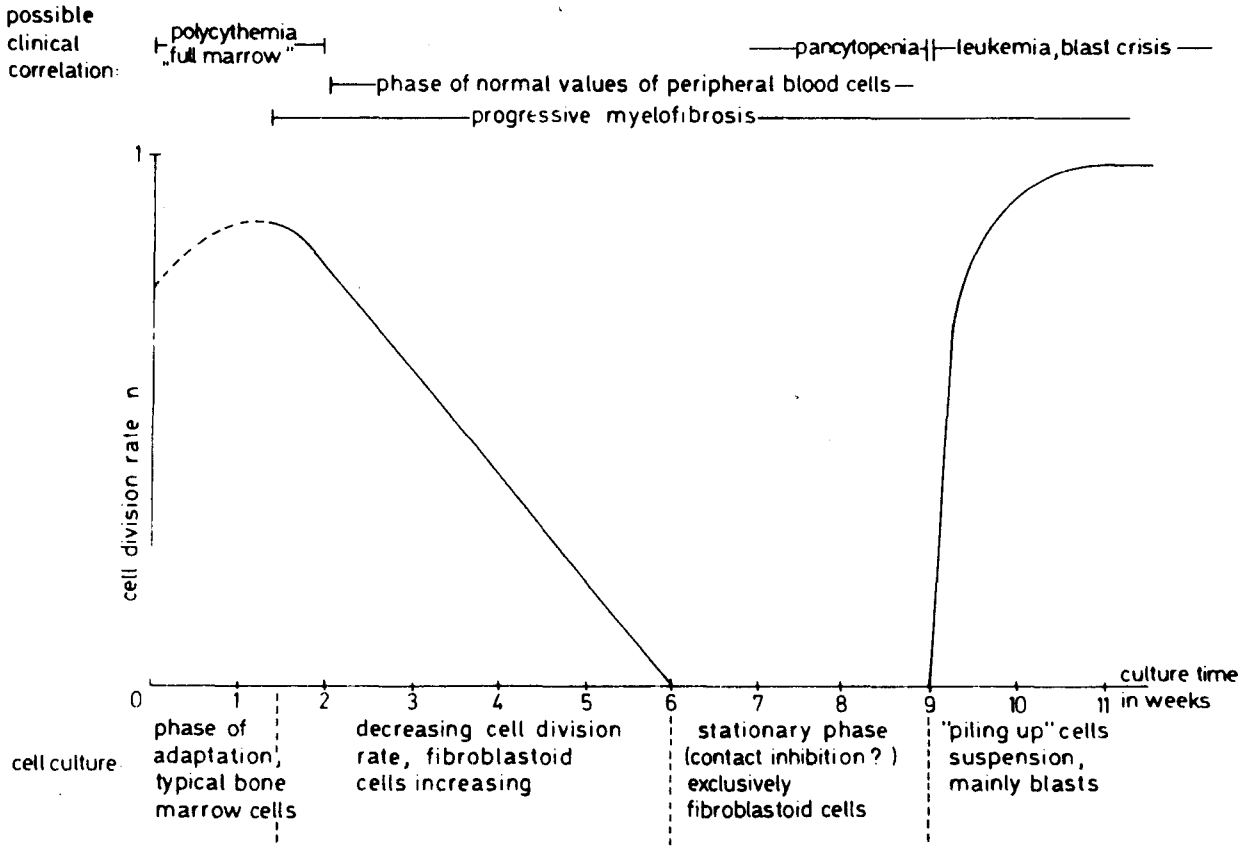


Fig. 1: Schematic representation of changes leading to PV tissue culture and possible clinical correlation.

Table 1: Cytological and cytochemical classification of the cells during the process of establishing the cell culture

culture time in weeks	granulopoiesis			erythropoiesis			
	segm	eos	precursor cells	poly chromatic	ortho	ery	monocytes
3	+	+++	+++	++	++	–	+
9	+	–	–	–	+	+	–
12	–	–	–	–	+	–	–
16	–	–	–	–	+	–	–
22	–	–	–	–	++	–	–

	lymphocytes	blasts	lymphoid cells
3	+	–	–
9	–(+)	(+)	+
12	–	+++	++
16	–	++++	+
22	–	++++	(+)

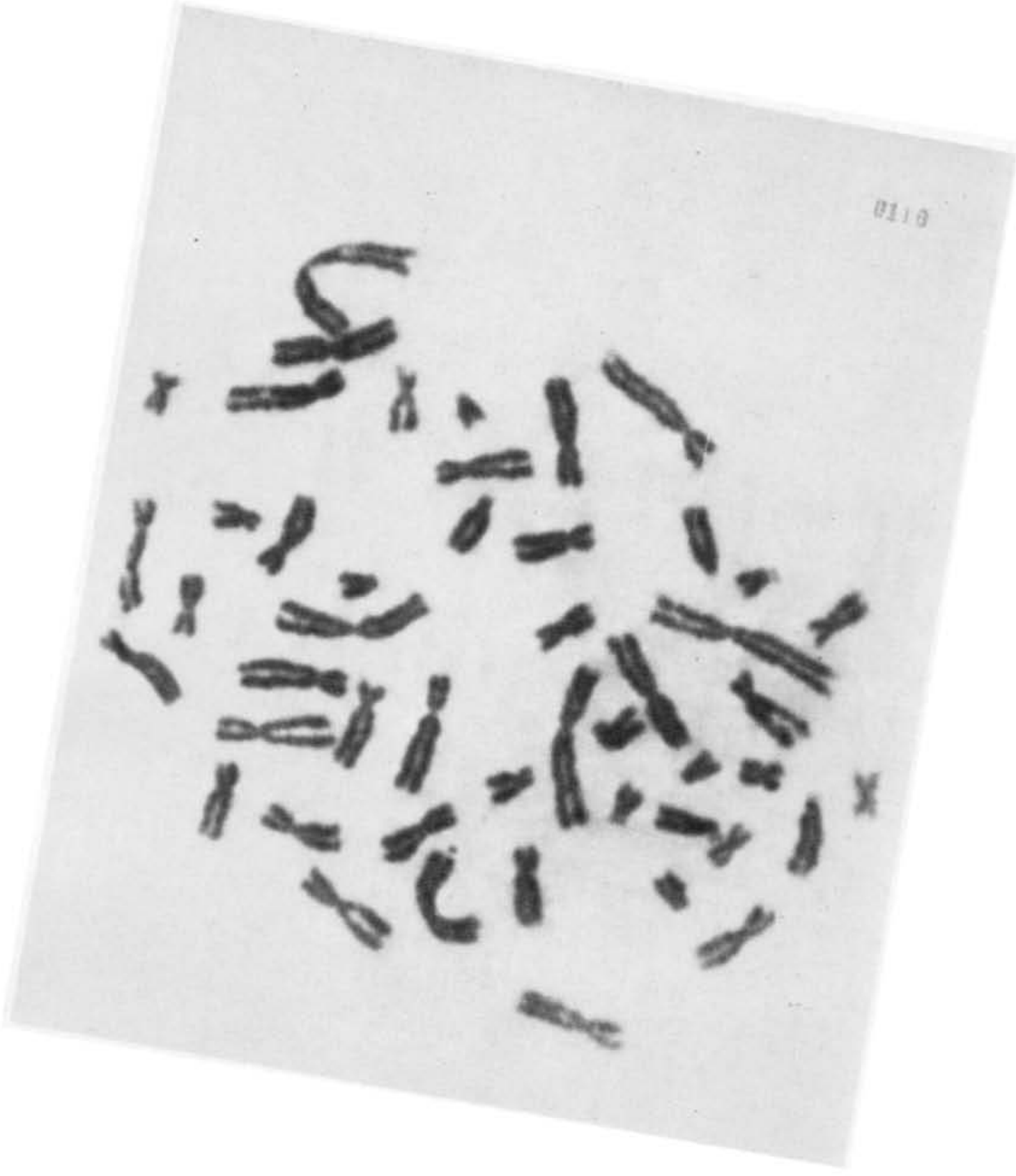
β-type peptides are present. Stimulation with 1 % DMSO for 5 days had no effect on globin synthesis contrary to the Friend virus induced erythroleukemic mouse cells in culture (10).

Karotype

The karyotype is hyperdiploid. All cells (n = 20) contained 48 chromosomes. The 2 additional chromosomes appear as one large metacentric and one large submetacentric chromosome (see Figures 2 and 3). Furthermore, a change in one chromosome of the F-group (deletion or inversion) can be observed. This anomaly is considered to be a specific aberration for some cells in several patients with PV (11).

High molecular weight RNA and reverse transcriptase

The possible role of the RNA dependent DNA polymerase as a key enzyme in neoplastic transformation of human cells has guided several investigations since the discovery of the enzyme in RNA tumor viruses (12, 13). For the simultaneous detection of high molecular weight RNA and reverse transcriptase in PV cells the method of SCHLOM and SPIEGELMAN (14) was applied. It was found earlier that the initial DNA product of the reaction of reverse transcriptase on 70S RNA is complexed via hydrogen bonds to the template. This was demonstrated by the unusual position of DNA product in glycerol velocity and Cs_2SO_4 equilibrium gradients. After mild heat denaturation the DNA product was detected in the expected positions in Cs_2SO_4 and glycerol gradients (15, 16).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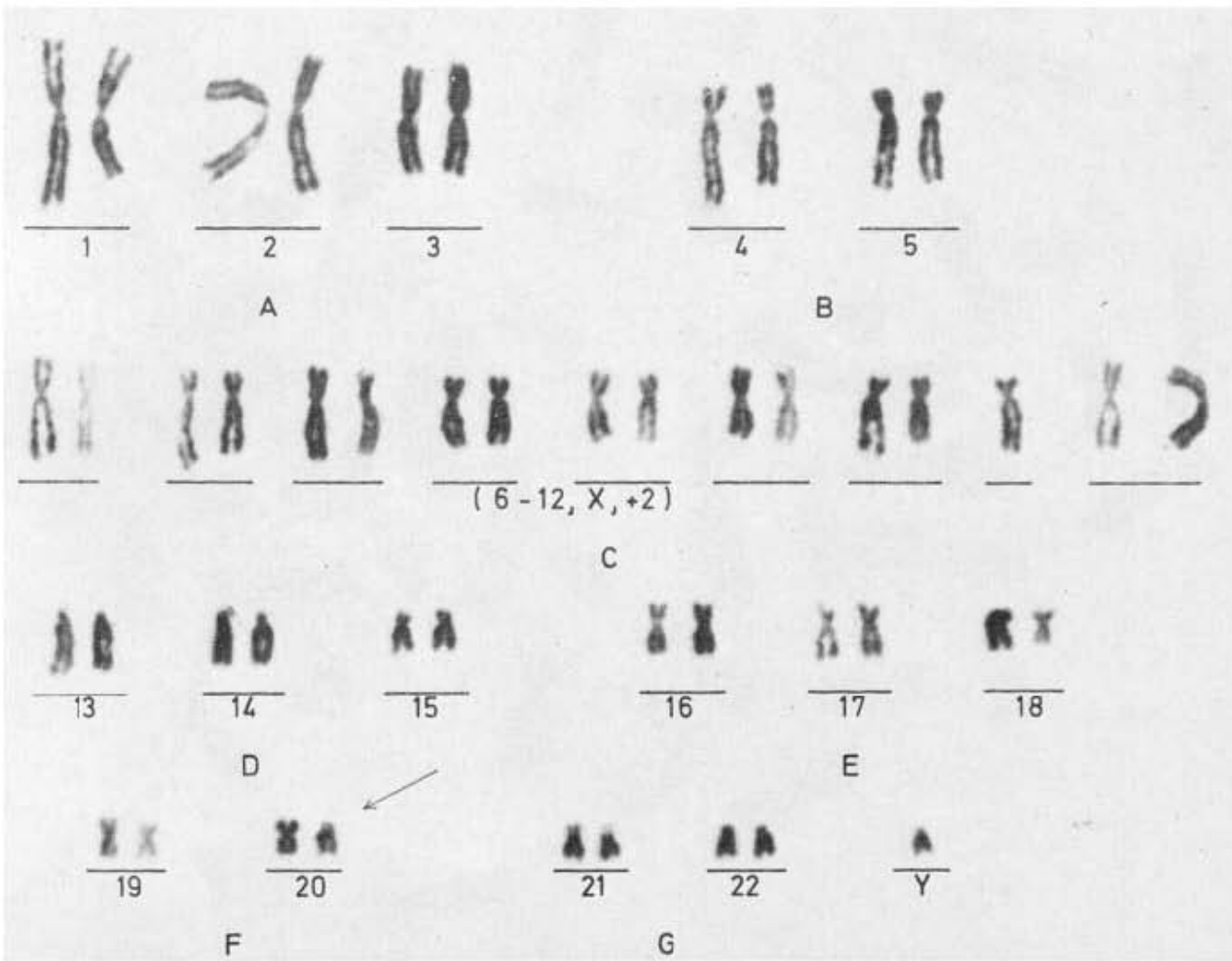
1
2
3
4
5
A
B
(6 - 12, X, +2)
C
13
14
15
16
17
18
D
E
19
20
21
22
Y
F
G

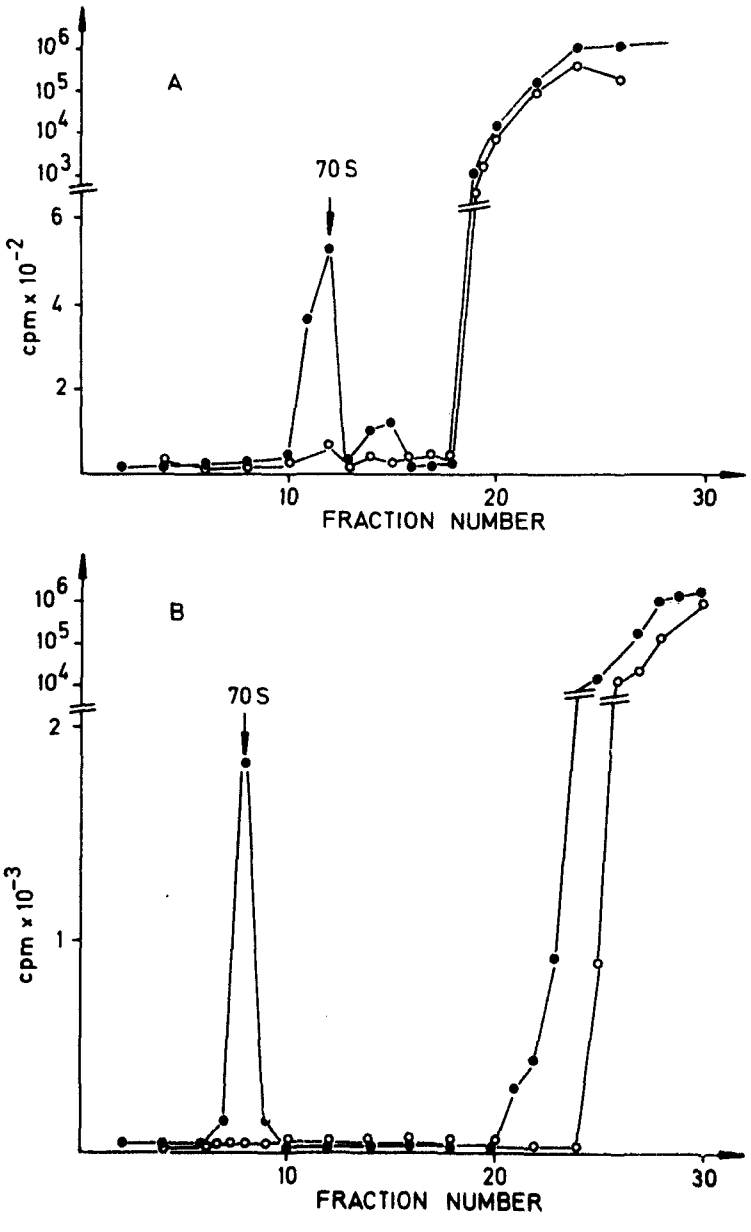

Fig. 4: Detection of high molecular weight ^{3}H-DNA/RNA complex in PV cells. Material with a density of about 1.16–1.19 g/cm^3 was collected from a 20–60 % sucrose gradient and pelleted by high speed centrifugation. The pellet was incubated in 0.5 ml of 0.3 % Nonidet P40, 0.005 M DTT, 0.01 M Tris HCl. pH 8.3 for 15 min. at 0 °C. DNA was synthesized in a RNA dependent DNA synthesizing mixture (1 ml) containing 0.01 M Tris-HCl, pH 8.3, 0.02 M NaCl, 0.005 M $MgCl_2$, 5 x 10^{-4} M of each dATP, dCTP, dGTP and 5 x 10^{-5} M ^{3}H-dTTP (3500 cpm/pmol). 50 µg/ml actinomycin D was added to inhibit DNA dependent DNA synthesis. After synthesis for 15 min. at 37 °C the reaction was stopped by addition of 1 % SDS and 0.2 M NaCl final concentration. The mixture was deproteinized by phenol-cresol extraction. The aqueous phase was layered on a 15–30 % linear glycerol gradient and centrifuged in a Spinco SW 40 rotor at 38,000 rpm for 3 hours at 4 °C. Fractions were collected through the bottom and assayed for radioactivity. A: one aliquot was preincubated with RNase (20 µg/ml) for 30 min. at 37 °C (o), the other aliquot was directly applied to the gradient (·). B: reaction mixture contained ^{3}H-dCTP and ^{3}H-dGTP, (o) preincubation with RNase, (·) no RNase.

A high speed pellet fraction was isolated from the cytoplasm of PV cells, resuspended and centrifuged through a 20–60 % linear sucrose gradient. Single fractions of the gradient have been tested for incorporation of ^{3}H-TMP on the synthetic template poly rA/oligo dT. Material of a density of 1.16–1.19 g/ml was used for the simultaneous detection of high molecular weight RNA and reverse transcriptase and for the isolation of the enzyme. After incubation of the material for 15 min. at 0 °C in a buffer containing 0.3 % of the detergent Nonidet P40 a reverse transcriptase reaction was carried out in the presence of actinomycin D to

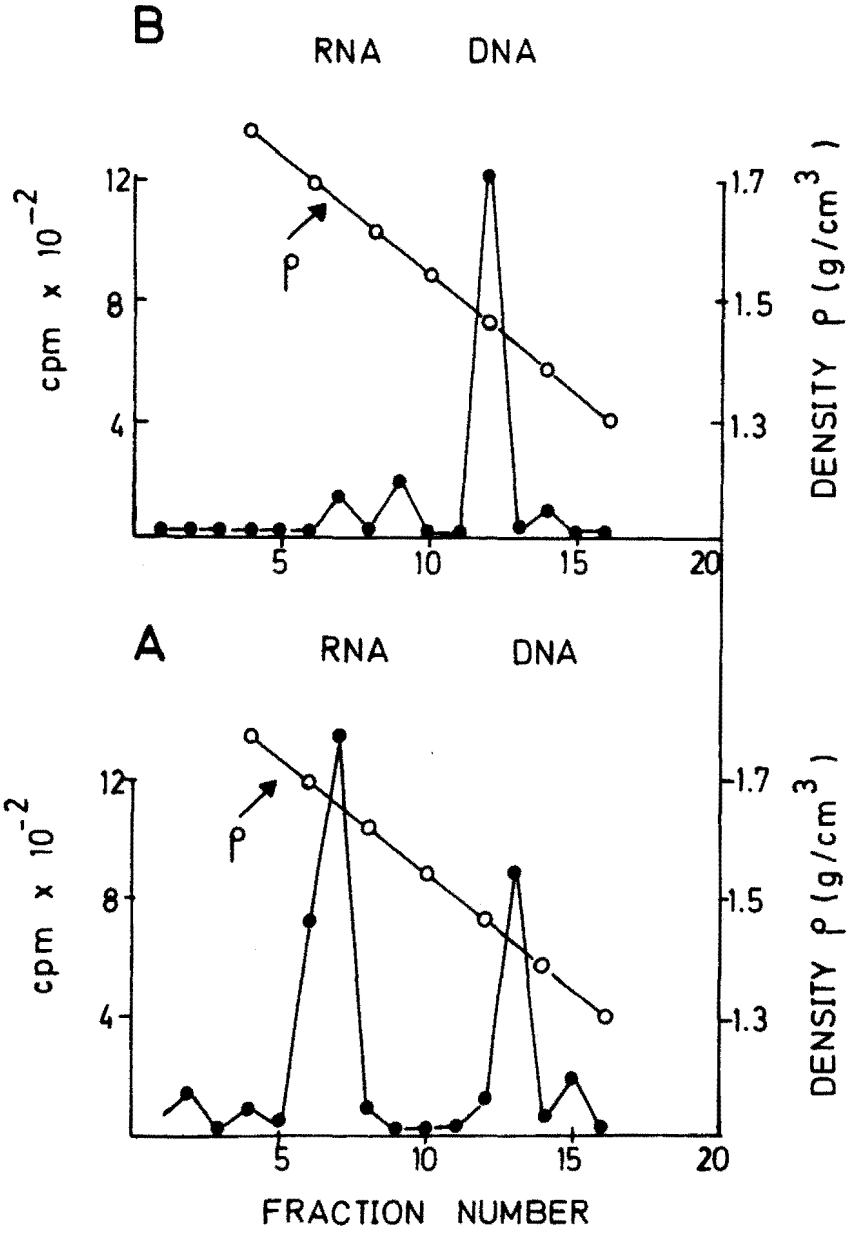

Fig. 5: Cs_2SO_4 equilibrium density centrifugation of the ^{3}H-DNA product. Appropriate fractions from the glycerol gradient were pooled and the radioactive material was precipitated with ethanol in the presence of yeast RNA carrier. The precipitate was dissolved in 11 ml half-saturated caesium sulphate (ρ = 1.51 g/ml) and centrifuged in a Spinco 75 Ti rotor at 44,000 rpm for 48 hours at 20 °C (Fig. 5a). The radioactive material, which bands at a density of 1.62–1.66 g/ml, was subjected to alkaline hydrolysis (0.4 N NaOH, 24 hours, 43 °C), neutralized with HCl and re-centrifuged in Cs_2SO_4 under the same conditions as described above (Fig. 5b).

inhibit DNA dependent DNA synthesis. The reaction was stopped by SDS treatment, deproteinized with phenol-cresol and centrifuged in a 15–30 % glycerol gradient with a size marker (Figure 4). The radioactive moiety of the 70S region was subjected to Cs_2SO_4 equilibrium density centrifugation (Fig. 5). Most of the radioactivity is found in the RNA region (1.64–1.68 g/ml), although some material is found in the DNA region, probably due to a breakdown during the isolation procedure (Fig. 5a). After alkaline hydrolysis the same material bands in the DNA region (Fig. 5b).

After ultrasonication of the high speed pellet the DNA polymerase activity was extracted with a buffer containing a nonionic detergent and purified on DEAE- and phosphocellulose columns. The activity which elutes from the phosphocellulose column represents a 220-fold purification. Table 2 shows the response of the human PV DNA polymerase to various templates.

The complete reaction mixture consisted of the following in 0.05 ml: 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM manganese acetate, 20 μg/ml actionmycin D, 0.5 mM of each dATP, dCTP, dGTP, 0.1 mM ^{3}H-dTTP (35 cpm/pmol), 0.5–0.8 μg of PV DNA polymerase, 1 μg$(dT)_{12-18}$, 2 μg 9–12 S globin mRNA, 1 μg globin mRNA, (purified by gel electrophoresis), 2 μg R17 RNA. Reactions were carried out at 37 °C for 30 min.

Table 2. Template response of PV DNA polymerase

Template	pmol ^{3}H-dTTP incorporated in 30 min. at 37 °C	pmol ^{3}H-dCTP incorporated in 30 min. at 37 °C
poly rA $(dT)_{10}$	72	–
p-12S Globin mRNA (purified by sucrose gradient centrifugation)	–	3.8
Globin mRNA (purified by gel electrophoresis)	51	10.4
R 17 RNA	0.2	0.2

Electronmicroscopy

Electronmicroscopic studies on cells and homogenates reveal particles with diameters ranging from 900 to 1200 Å. By the study of numerous cells we found these particles in clusters with an average of 8 particles per cluster. The investigation did not include the examination of serial sections, so that a true statistical analysis was not possible. Figure 6 shows a section through a cell with a group of particles. In Fig. 7 a single isolated particle with a diameter of 1200 Å is shown. The electron-dense core and triple-lyered structure of the envelope are discernible.

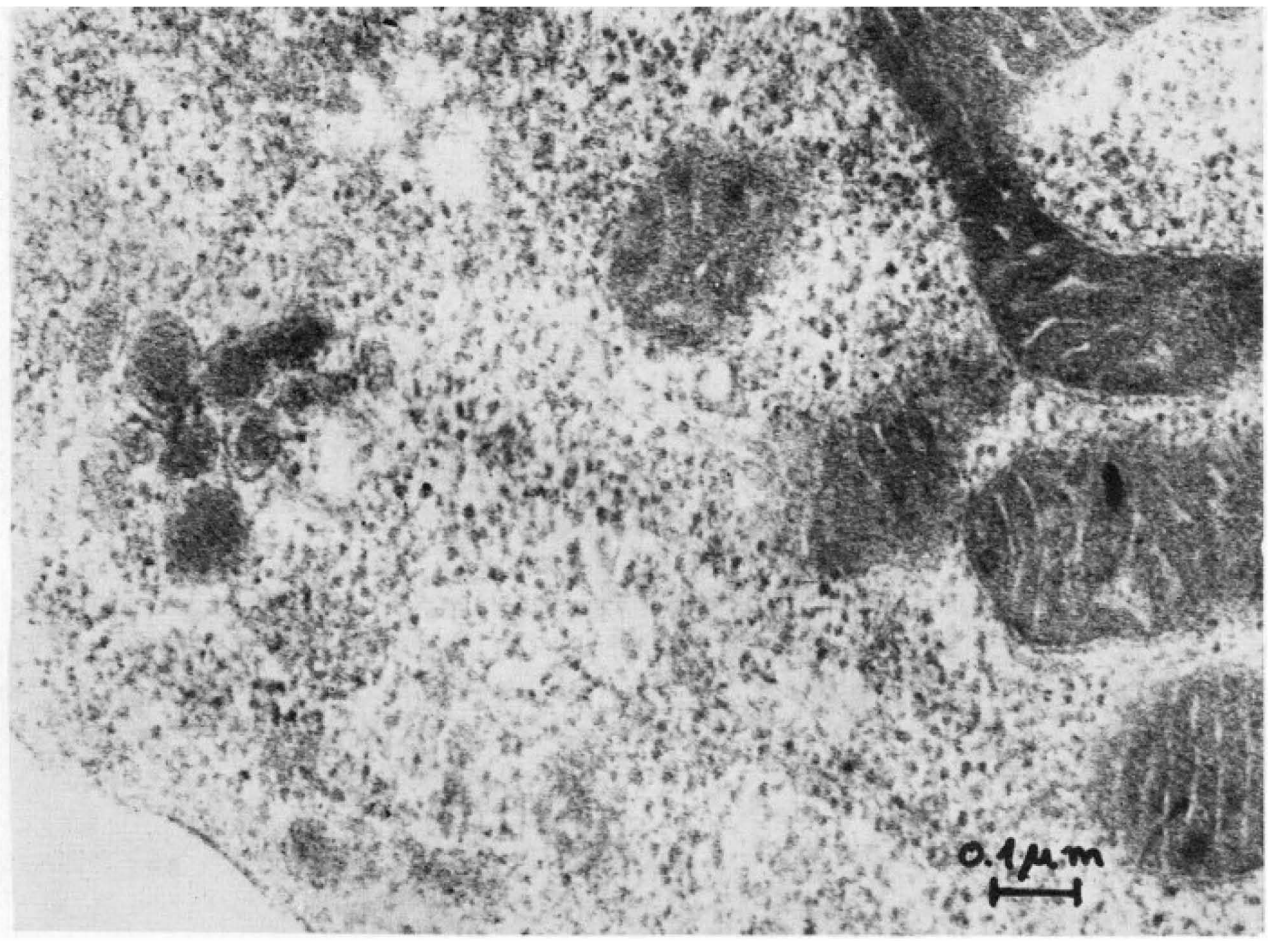

Fig. 6: Ultrathin section of a PV-cell. The cell was fixed with 2.5 % glutaraldehyde in 0.1 M potassium-sodium-phosphate-buffer, pH 7.2, followed by postfixation with 1 % osmiumtetroxide in isotonic sodium-veronal-acetate buffer, pH 7.2 and with 0.5 % uranylacetate in 0.1 M acetate buffer, pH 3.9. The specimens were dehydrated with ethanol and embedded in epon. Sections were stained with 3 % aqueous uranyl-acetate and 0.3 % lead-citrate. Photographs were taken in a JEOL Jem 100 B electron-microscope at 80 KV. Mag.: 80,000 x

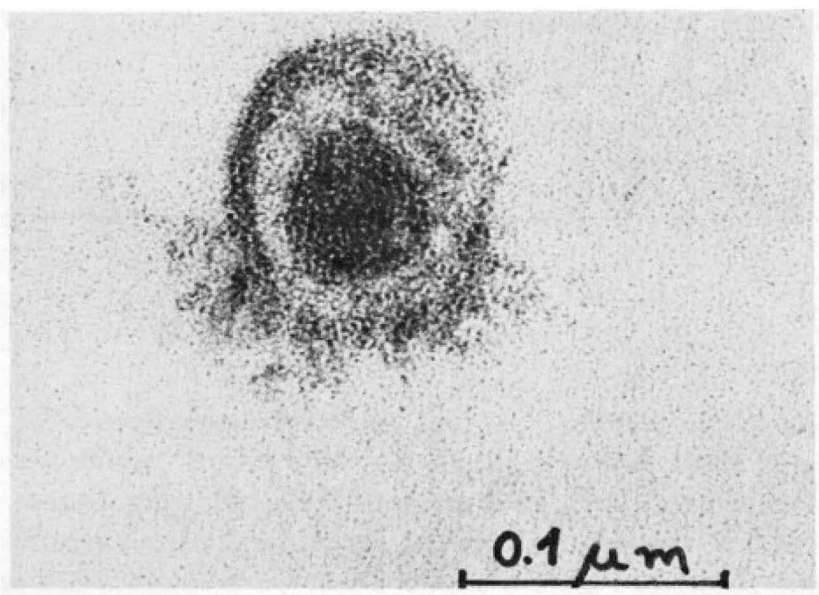

Fig. 7: Ultrathin section through a cytoplasmic pellet. Fixation and embedding as in Fig. 6. Mag.: 250,000 x

Summary

We have established a cell culture line from bone marrow of a patient with a Polycythaemia vera (PV) disease. During the time course of the establishing of the culture we observed a spontaneous transformation in vitro. Cytologically no difference was found between cell types of the culture and normal bone marrow during the first 3 weeks. After the transformation, however, 90 % of the cells are blasts, 8 % orthochromatic erythroblasts and a small portion lymphoid cells. The karyotype of the cells in culture is hyperdiploid and contains 48 chromosomes. Furthermore, a change in one chromosome of the F-group (inversion or deletion) is observed. The cells produce adult human globin and contain particles with high molecular weight RNA and reverse transcriptase activity. Investigations by electron microscopy revealed virus like particles.

Acknowledgments

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RELATIONSHIP BETWEEN CHROMOSOMAL INSTABILITY AND LEUKEMIA

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Since Th. Boveri put forth his famous hypothesis in 1914 that malignant cells are mutant clones with a genetically unbalanced chromosome complement, many scientists were able to show examples for such origin of tumors.

Tumor cells usually contain marker chromosomes which point to chromosomal rearrangements in the very beginning of malignant growth. Numerical alterations of the karyotype are very often observed in tumor cells. Both, the markers and the additional chromosomes characterize each individual tumor independent of type or site of the neoplasia.

There are two exceptions: the Philadelphia chromosome which is a delted chromosome No. 22, in the majority of cases with chronic myelogenous leukemia, and the other constant, interindividual chromosome aberration is the missing G-chromosome in meningioma.

These cytogenetic findings, the marker chromosomes and the two constant aberrations in chronic myelogenous leukemia and meningioma clearly indicate a clonal origin of the neoplasias. Biochemical studies of tumor tissues offer another argument for the clonal origin of many different tumors. One of the two X-chromosomes is genetically inactive in female cells, which occurs randomly and early in embryogenesis and is fixed from then on through life. This leads to two types of somatic cells: one population with the paternal, the other with the maternal X-chromosome active. Tumors always exhibit only one type of X-chromosomal enzyme in cases where the femal is heterozygote for different types of an X-chromosomal enzyme. This is also true for leukemic cells.

There are, however, tumors and leukemic cells with an apparently normal karyotype. One explanation is that subtile aberrations may not be detectable by the present techniques. On the other hand it is possible that there are changes like point mutations which are responsible for the unrestrained growth or other properties of cancer cells.

A certain sequence of events which finally cause the transformation into a cancer cell has not yet been determined. However, the fact, that cancer cells derive very often from a single cell with a visible chromosome rearrangement draws the attention to a few inherited diseases with high incidence of leukemia and cancer. These diseases also have an increased spontaneous chromosomal instability in common: Fanconi anemia (FA), Bloom's snydrome (BS) and Ataxia telangiectasia (AT). Clinically they are very different syndromes. FA is known as a pernicious-like

aplastic anemia in childhood, BS is characterized by stunted growth, sun-sensitivity of the skin and telangiectasia in exposed areas. AT is a neurological disease combined with telangiectasia.

A fourth recessive trait, Xeroderma pigmentosum, belongs to this group of diseases. The genetic defect is known: the UV specific endonuclease necessary for the first step in dark repair is missing so that UV induced damages in the DNA are not being repaired. The UV exposed skin of the patients is covered with lesions and later in life with multicentric cancers.

The spontaneous chromosome instability in FA, BS and AT is present in cultures from peripheral blood cells and from skin biopsies, in direct preparations of bone marrow cells or even in bone marrow smears. Cultured cells from patients with XP show increased breakage after UV irradiation. Although the genetic defects in FA, BS or AT are unknown, they are responsible for the chromosomal instability in each disease. James German and I were able to show that the genetic defects causing FA and BS act differently at the cellular level, i. e. the different genes express themselves very different cytogenetically. For instance, FA-cells show almost exclusively chromatid interchanges between non-homologous chromosomes. BS-cells, in contrast, have almost exclusively chromatid interchanges composed of homologous chromosomes, and in addition, the breaks occur seemingly at the same loci. Studies of the sites of breakage demonstrated a non-random distribution over the entire karyotype in both, FA and BS. In BS there are distinct sites of certain chromosomes which are far more involved in breakage and rearrangement than others. FA does not show such a clear pattern of breakage and reunion as BS does, however, there are also preferences for breakage at certain chromosome regions. Differences like that might indicate that the damage due to the genetic defect occurs at different times in the cell cycle in FA and BS. No study comparable with this has been made with AT-cells or XP-cells after UV irradiation. Both cell types grow very poorly in vitro.

The consequence of such aberrations are unbalanced karyotypes in the daughter cells after mitosis. This certainly leads to cell death in many instances. It also can lead to a surviving single cell which gives rise to a clonal cell population and eventually becomes malignant.

Hence, in these diseases the steps from chromosome mutation, the survival of a mutant cell, the development of a clonal cell population into the established tumor cells or leukemic cells can be observed.

Actually, in all four diseases, cell clones with rearrangements have been found:

In FA in bone marrow and lymphocyte cultures, in BS and XP in fibroblast cultures and in AT in lymphocyte cultures.

These findings are comparable with what is known about the effects of ionizing irradiation in man, the only situation where cell clones with abnormal chromosomal complements have been found in vivo. X-ray induces chromosomal breakage and predisposes to cancer and leukemia. A-bomb survivors in Japan were found with mutant cell clones in their peripheral blood cells showing stable chromosome rearrangements. The oncogenic implication of X-ray induced chromosomal breakage, of UV-light induced damage in the DNA of XP patients and of the chromosomal instability found spontaneously occuring in FA, BS and AT is one and the same.

Chromosomal instability appears characteristic of cell damage caused by irradiation or by genetic defects – from which cancer is likely to emerge. In the diseases discussed here, the incidence of cancer or leukemia is about 10 % in FA and BS, maybe less in AT, however, it is 100 % in XP patients. For FA it can be predicted that hormone therapy for treatment of the aplastic amenia will help the patient to survive longer in future, long enough to most probably experience leukemia.

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IMMUNOGLOBULIN-PRODUCTION AND SURFACE-BOUND IMMUNOGLOBULINS IN CHRONIC LYMPHOCYTIC LEUKEMIA, LYMPHOSARCOMA, AND SO-CALLED RETICULUM CELL SARCOMA

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Conventional light and electron microscopic methods do not allow a differentiation of T- and B-cells. A classification of neoplastic lymphatic cells into one of these subpopulations is therefore impossible with common morphological methods. It was not until specific cell funktions, i. e. immunoglobulin(IG)-production and the formation of surface-Ig(S-Ig)-receptors, were demonstrable that one could identify B-cells and thereby distinguish them from T-cells. The demonstration of Ig-production therefore represents a method which reveals more information about the nature of malignant lymphoma cells.

Results

We have studied the Ig-content of the tumor tissue homogenates from 18 cases of chronic lymphocytic leukemia (CLL), 11 cases of lymphocytic lymphosarcoma, and 18 cases of reticulum cell sarcoma (RCS). These Ig-determinations were performed with radial immunodiffusion. Fig. 1 shows the results. Ten of the 18 CLL, 7 of the 11 lymphocytic lymphosarcomas, and 14 of the 18 RCS revealed an increased IgM-content. In contrast, the serum-IgM-level was above the normal range only once in CLL, twice in lymphocytic lymphosarcoma, and 3 times in RCS. There was no correlation between the tissue-IgM-content and the serum-IgM-level. These findings indicate that the produced IgM was in most cases not released but instead retained in the tumor tissues. Other Ig-classes were seldom detected in the tumor tissues: IgG was increased in 2 CLL and IgA in one RCS.

In order to compare the results of our tissue-Ig-determinations with the data from S-Ig-labeling reported by other authors we have started parallel investigations of S-Ig and tissue homogenate-Ig. We used horse-raddish-peroxidase-labeled antibodies to demonstrate S-Ig, since this technique allows a relatively satisfactory cytological judgement of the labeled cells. Light microscopically the labeled antibodies are represented by a brown peroxidase reaction product, which due to its electron density also allows an electron microscopic demonstration of the antibodies.

* With support from the Deutsche Forschungsgemeinschaft.

Fig. 2 shows a representative of the most common type of lymphocytic lymphosarcoma in our material. This type is characterized by a uniform proliferation of small to mediumsized cells, whose nuclei are very irregular in shape and often notched.

With peroxidase-labeled antibodies 85–90 % of the cells of this lymphocytic lymphosarcoma reacted strongly positive for S-IgM/lambda (Fig. 3 and 4). Kappa-chains were not detectable. In the tissue homogenate the tumor revealed a slight, but highly significant increase in the amount of IgM. The serum-IgM-level, on the other hand, had decreased to below the norm, although the tumor had affected almost all of the lymphatic tissue.

A further case of this type of lymphocytic lymphosarcoma showed a very dense and strong labeling for S-IgM/kappa on nearly all of the cells. There was no increase in the tissue-IgM.

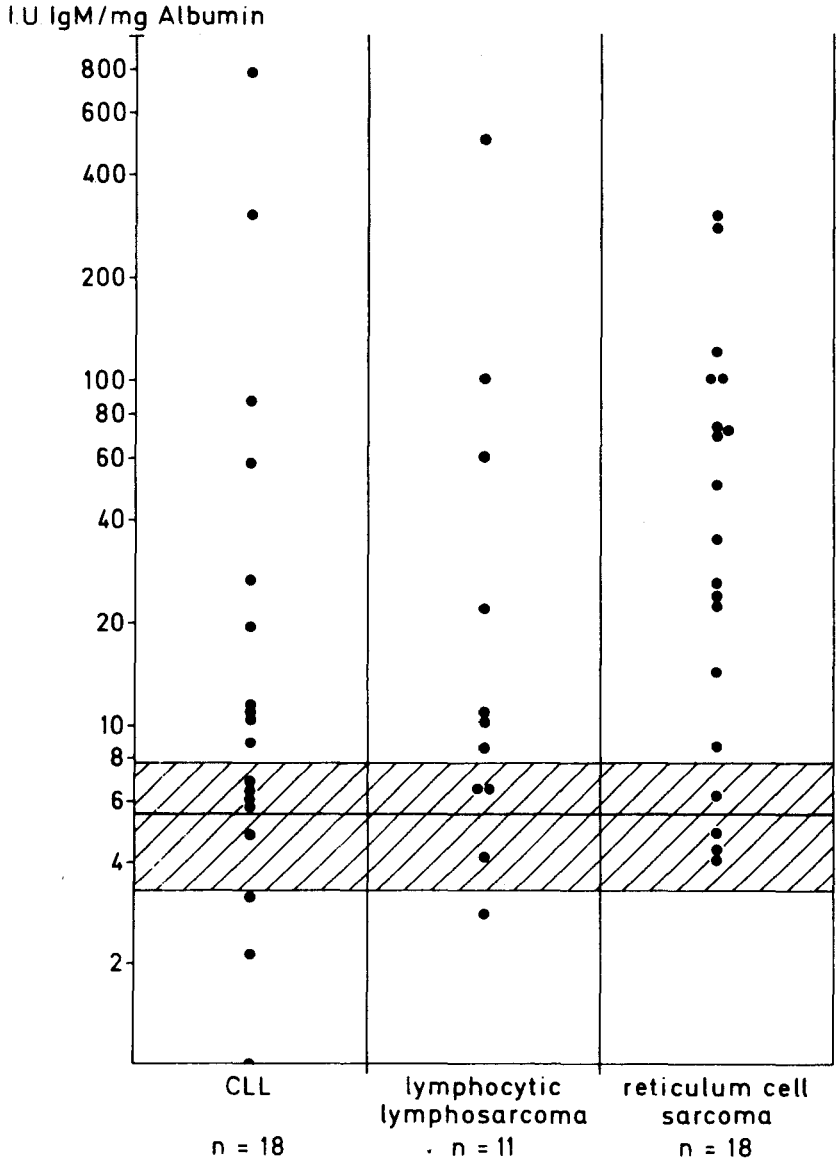

Fig. 1: Tissue homogenate-Ig-content of 47 lymphomas.

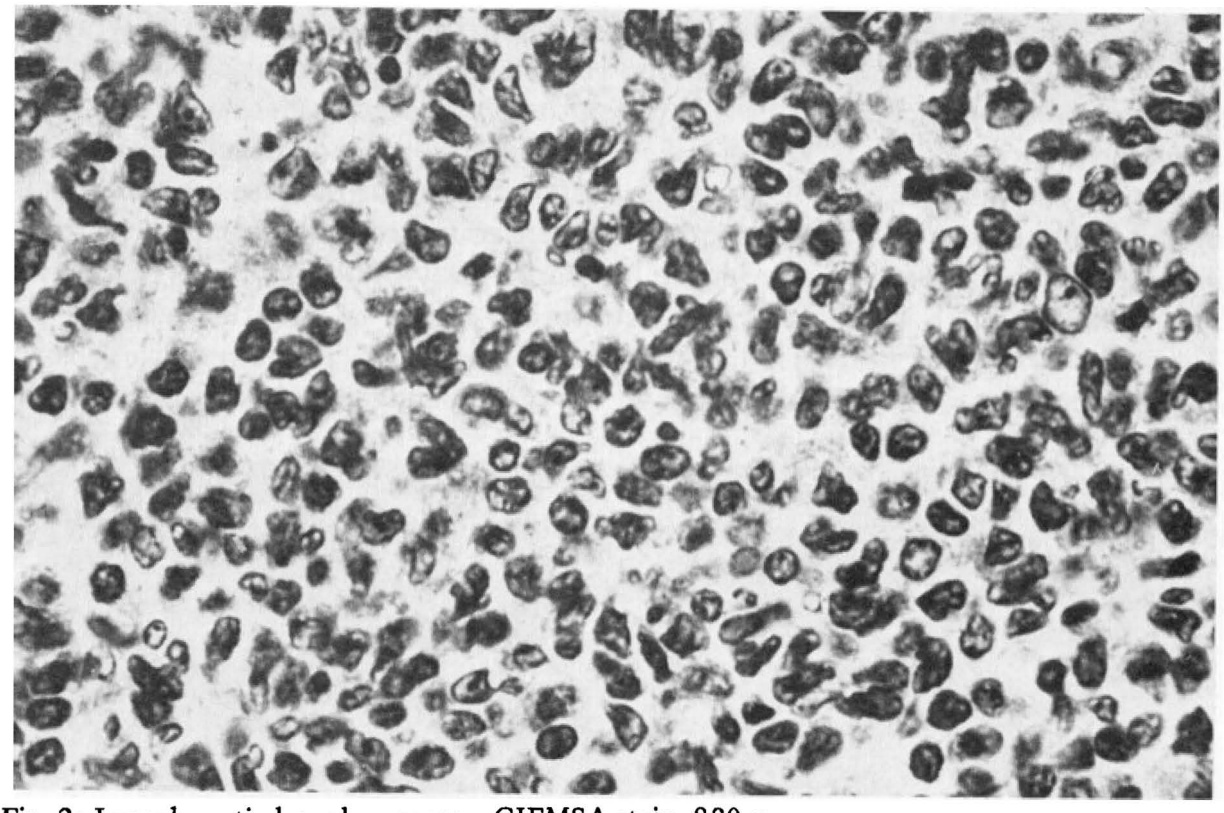

Fig. 2: Lymphocytic lymphosarcoma. GIEMSA-stain. 880 x

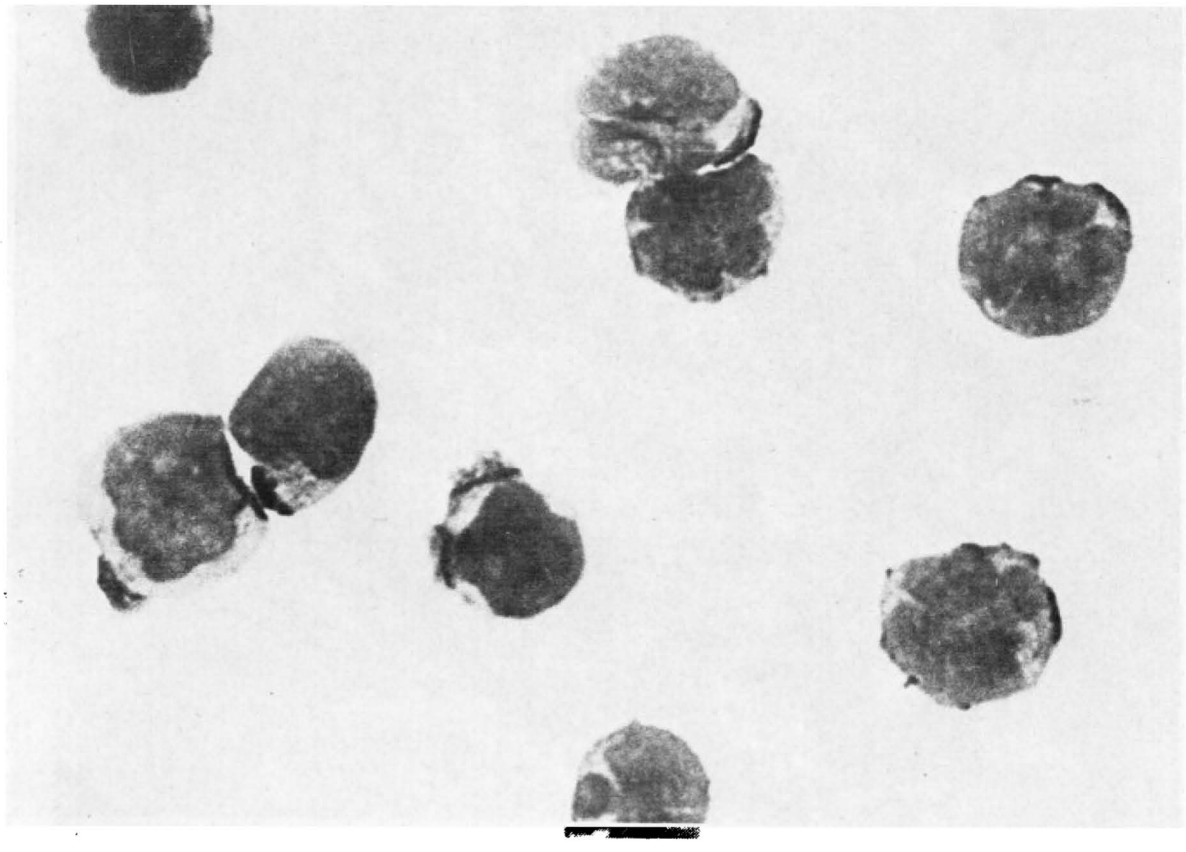

Fig. 3: Lymphosarcoma cells from the case shown in Fig. 2 labeled with horse-raddish-peroxidase-coupled antibodies. The labeled Ig is visible as black bands or spots on the cell surface. Nearly 85—90 % of the cells reacted positively for IgM/lambda. Note the distinct cap-formation. Haemalaun counterstain. 1,400 x

Histologically the CLL in our series was distinctly different from the lymphocytic lymphosarcoma. The CLL never showed a uniform proliferation, but instead a so-called pseudofollicular pattern: between the lymphocytes there were areas which appeared lighter in the GIEMSA-stain and which consisted of cells clearly larger than the lymphocytes. The most mitoses seemed to occur in these areas. The nuclei of the lymphocytes and of the larger cells were round to oval and never as irregular in shape as those of the cells in the lymphocytic lymphosarcoma type described above. In two CLL we found S-IgM and in one case S-IgG on the leukemic cells. The cells of one of the two S-IgM-positive CLL revealed a patchy pattern for the labeling product (Fig. 5). Although all of the labeling procedures were done at

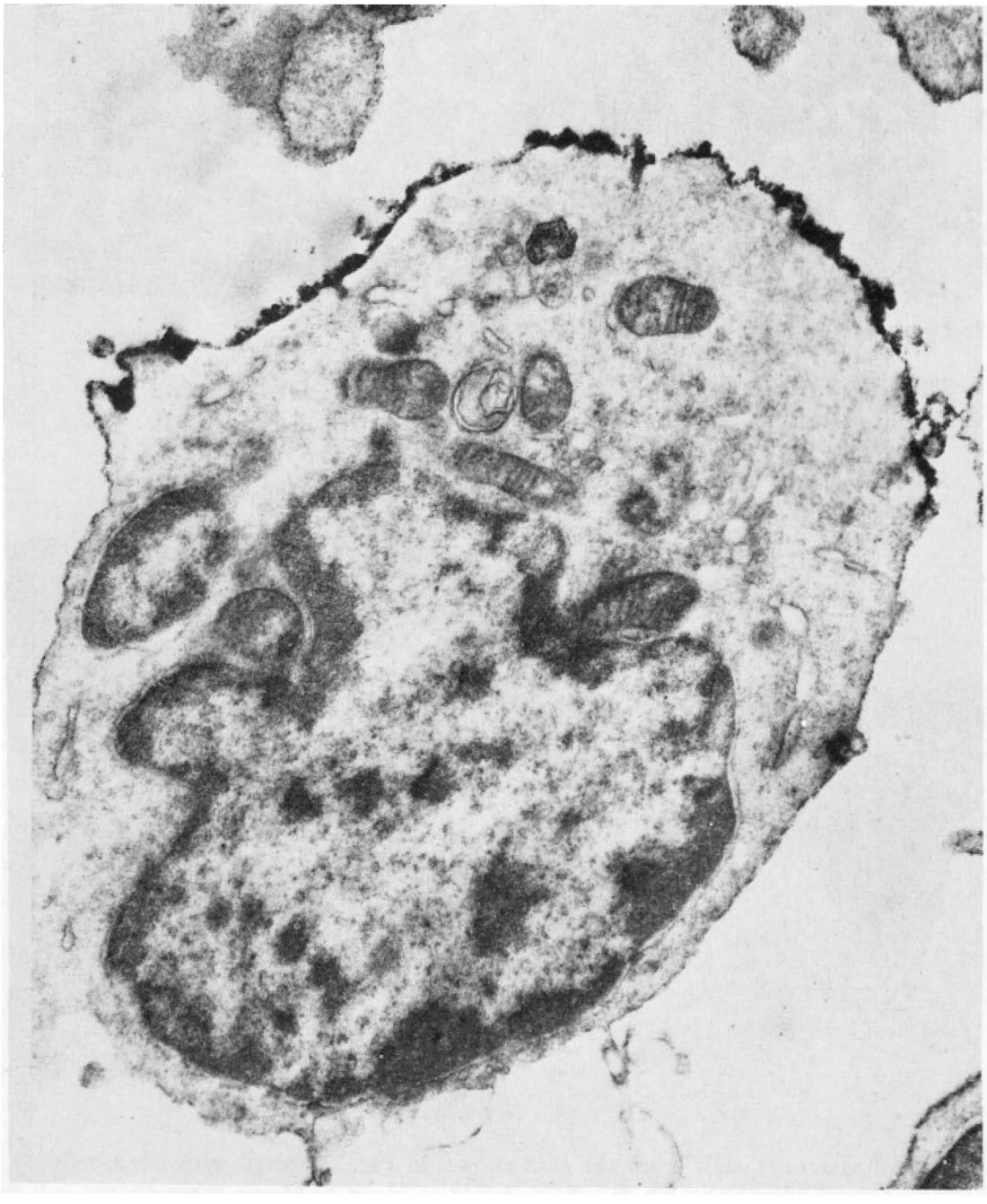

Fig. 4: Electron microscopic demonstration of S-IgM on the same lymphosarcoma cells with peroxidase-labeled antibodies. 17,000 x

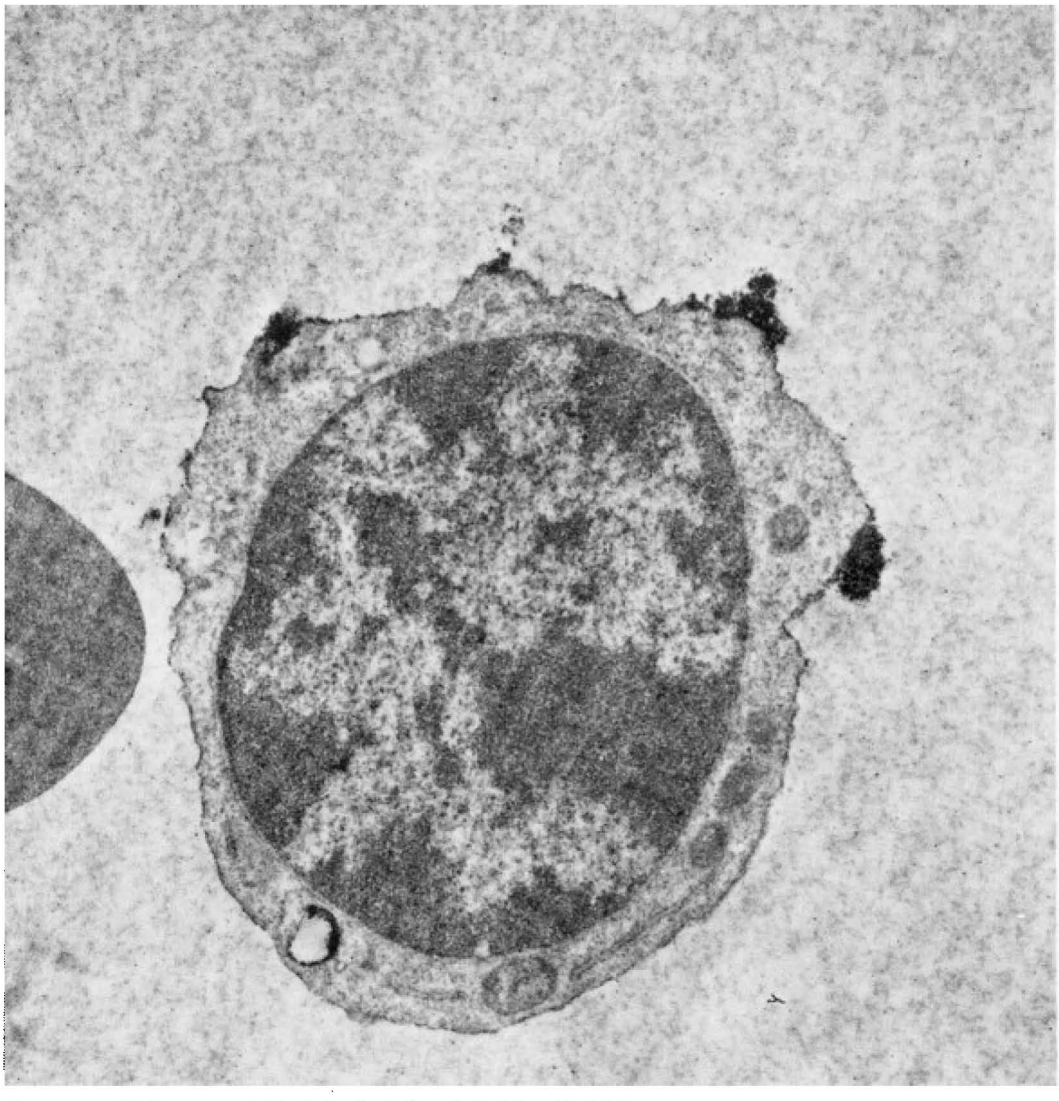

Fig. 5: Tumor cell from a CLL labeled for S-IgM. 15,000 x

25 °C, there was no cap-formation. Of two nearly equally densely labeled CLL one case revealed a highly increased and the other a slightly increased tissue-IgM-content. In the S-IgG-positive CLL we could demonstrate that the tissue-IgG was disc-electrophoretically monoclonal, whereas the serum-IgG was polyclonal (Fig. 6).

A strong S-Ig-labeling (Fig. 7 and 8) was revealed by a special type of chronic leukemia: leukemic reticuloendotheliosis or hairy cell leukemia, which is characterized by a chronic development, anemia, frequent leukopenia, extreme splenomegaly, absence of significant lymph node swelling, and the presence of so-called hairy cells in the blood and bone marrow, in the BILLROTH pulp cords of the spleen and in the intermediary sinus of the liver. The electron microscope showed that the S-Ig was often located at the hair-like projections. Under prolonged cap-formation conditions these hair-like projections bearing the S-Ig migrated to one pole of the cell (Fig. 8). The tissue homogenate of the spleen, which consisted almost only of hairy cells, showed an IgM-concentration within the normal range. However, despite this finding, we were able to extract significant amounts of IgM from a homogenate prepared from a cell suspension consisting of 98 % hairy cells isolated from the spleen and washed 4 times.

We were expecially struck by the results from the labeling of the RCS, or at least what has been collected under this term. We consider the following as the typical morphological characteristics of this lymphoma group. The RCS-cells have large nuclei with sparse nuclear chromatin and distinct nucleoli. The cytoplasm is clearly darker than the nucleus and hard to define (Fig. 9). Both of the RCS we had the chance to label reacted strongly for S-IgM. The tissue-IgM-content was also highly increased, whereas the serum-IgM-level lay within the normal range.

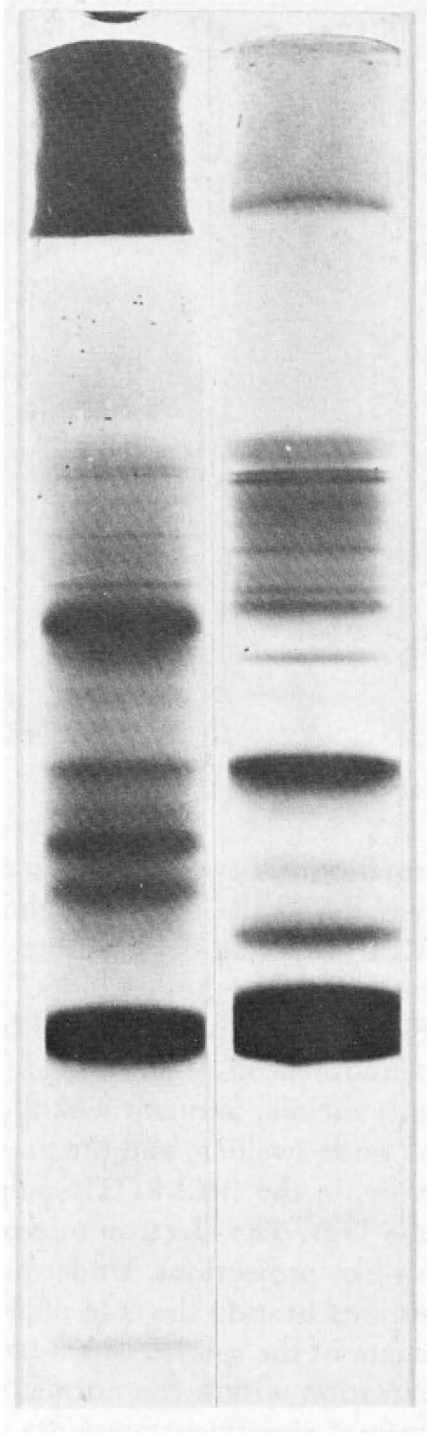

Fig. 6: Disc-electrophorogram of the lymph node homogenate (left) and the serum (right) of a CLL whose cells bear S-IgG. Note the monoclonal IgG-migration of the lymph node extract and the polyclonal IgG-migration of the corresponding serum.

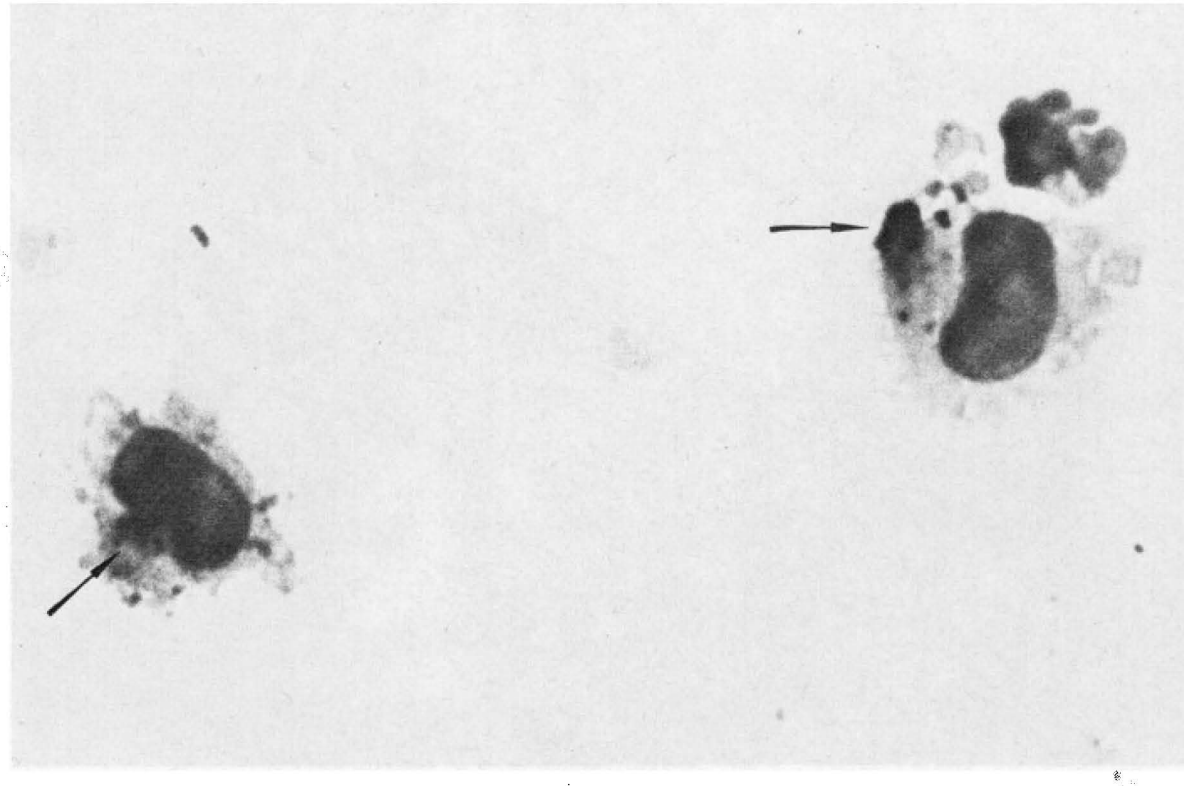

Fig. 7: "Hairy cells" labeled for S-IgM. The S-Ig-labeling (arrows) is collected at one pole of the cytoplasm. In the original slides the brown color of the S-Ig-labeling contrasted well with the blue-greyish counterstain of the cytoplasm. Haemalaun counterstain. 1,000 x

Discussion

2/3 of the lymphomas we investigated contained significantly increased amounts of IgM in the tumor tissue homogenates. However, the question as to the cytological localisation of the Ig could not be answered by these analyses alone. Besides a negligible blood fluid contamination there are only two possibilities for the origin of the tissue-Ig:

1. The tissue-Ig was mainly derived from the Ig bound to the cell membranes.
2. The tissue-Ig was mainly derived from the interior of the cells.

With surface-bound-Ig there remains the question as to whether it was merely passively taken up by the cell membranes or whether it was produced by the cells themselves. To answer these questions we needed the help of a direct visualization of the Ig with labeled antibodies at the cellular level. The data presented prove that IgM sat on the surface membrane of many tumor cells. In contrast, intracytoplasmic Ig was not detectable with labeled anti-Ig-antibodies. This finding is consistent with those of PREUD'HOMME and SELIGMANN (1972) and others. However, since we found no correlation between the density of S-Ig and the amount of extracted tissue-Ig, we believe that some of the tissue-Ig must have originated from the surface membranes of the homogenized cells, but that most of the tissue-Ig originated from the cell sap. This assumption is supported by the experimental work

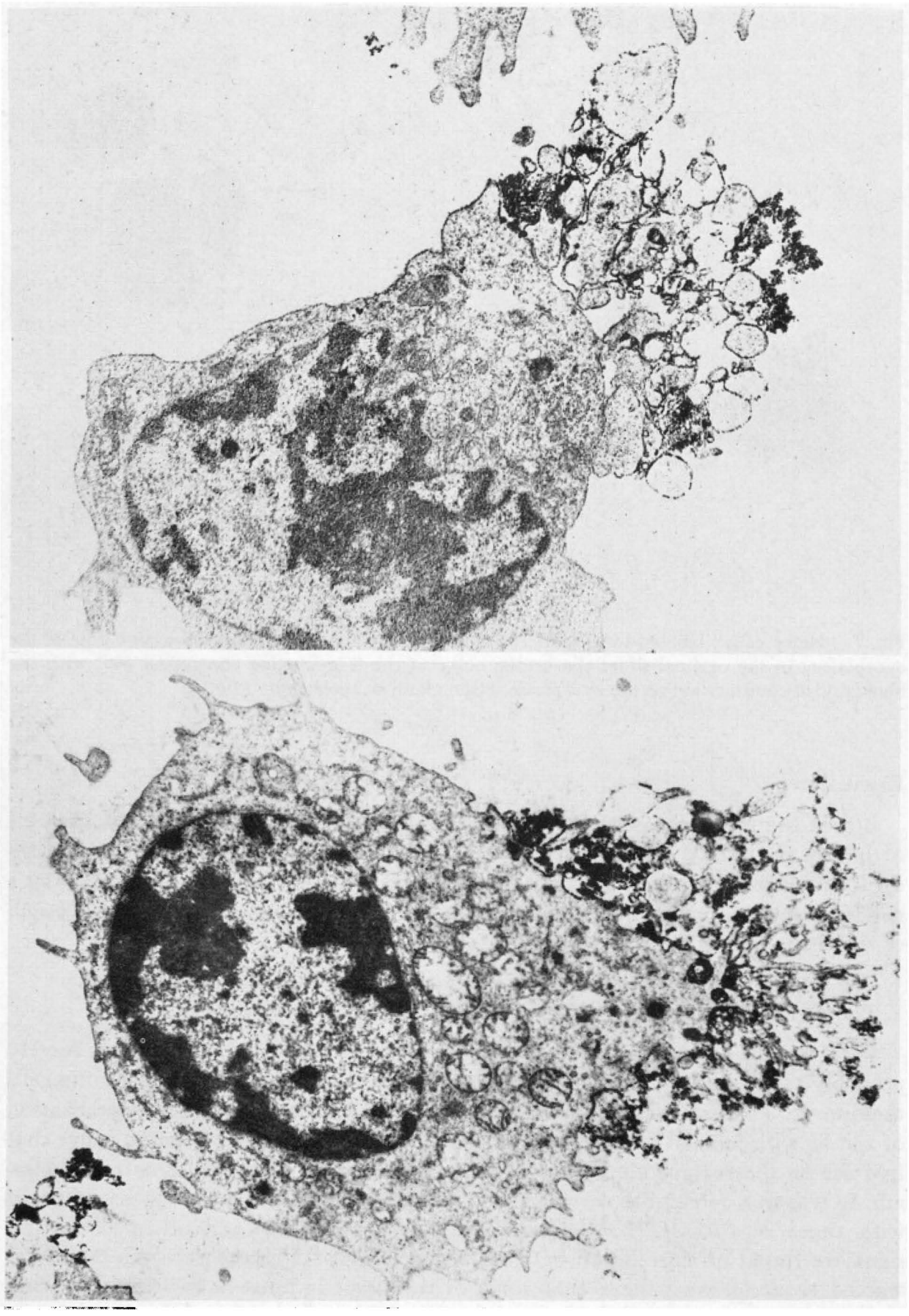

Fig. 8 a and b: Electron micrographs of two cells from a hairy cell leukemia which were labeled with peroxidase-coupled antibodies for S-Ig. a 11,000 x b 13,000 x

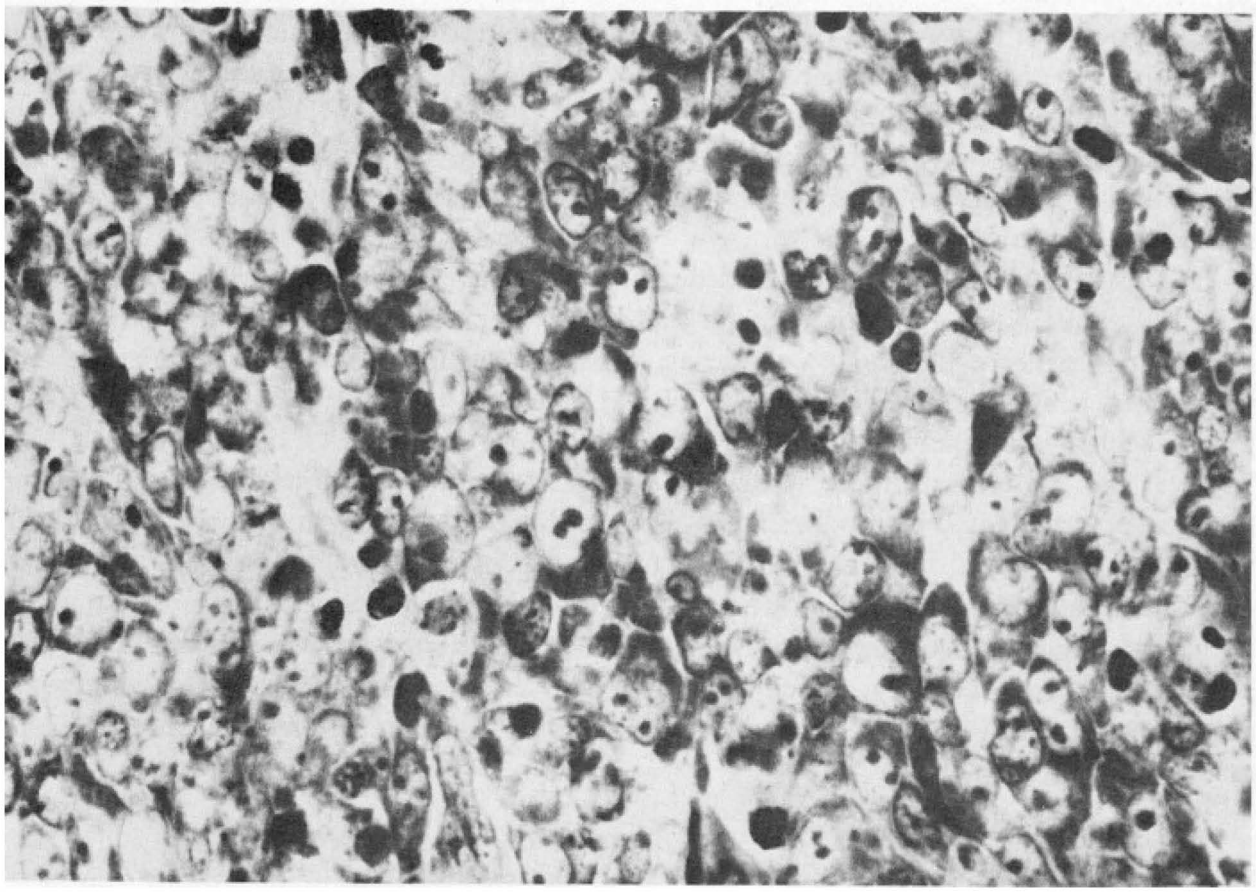

Fig. 9: Reticulum cell sarcoma. Note the contrast between the transparent nuclei and the basophilic cytoplasm. GIEMSA-stain. 880 x

of SHERR et al. (1972). They reported that the amount of intracytoplasmic Ig far exceeded that of S-Ig in a BURKITT-lymphoma-cell-line when the two Ig's were measured separately with the coprecipitation technique. These findings indicate that the tissue-Ig in most of our cases was derived from the cell sap and therefore represented a synthesis product of the tumor cells themselves.

The direct demonstration of intracytoplasmic Ig with fluorescin or peroxidase labeled antibodies failed probably because of a low affinity of the labeled antibodies to the intracytoplasmic Ig of the fixed or frozen cells and not because of a total absence of intracytoplasmic Ig.

We conclude that not only S-Ig, but also tissue-Ig can be used a reliable marker for B-cell lymphomas, even though the latter marker is not as sensitive as the former. The main advantage of the tissue-Ig-marker is that tissue-Ig-determination is not bound to viable cells. The tissue-Ig-content can also be determined in deep frozen or lyophilized material.

It is striking that the increased tissue-Ig belonged to the IgM-class in all but 3 of our cases: 2 CLL produced IgG and 1 RCS produced IgA. Also interesting were the results of the S-Ig-labeling and the homogenate-Ig-determination for the case of hairy cell leukemia. Until now it was not clear whether hairy cells are derived from reticular or from lymphatic cells. There have been many arguments in favor of both alternatives. However, the positive Ig-labeling results and the detection of IgM in

the homogenate of washed, purified hairy cells weight the arguments in favor of the lymphatic hypothesis. We therefore believe that hairy cell leukemia represents an irreversible proliferation of a special type of B-cells.

Ig-production was most constantly demonstrable in the so-called RCS-group. This finding is especially important since it sets the previously accepted nature of RCS-cells in doubt.

In 1930 ROULET described and defined „Retothelsarkom", which is now generally called RCS, as a tumor of cells of the reticulo-endothelial system with inclusion of the other phagocytic cell series. This conception was accepted all over the world and is still held valid today (BERNHARD and LEPLUS 1964; RAPPAPORT 1966; MORI and LENNERT 1969; MATHE et al. 1970; SCHNITZER and KASS 1973). The high accumulation of IgM in RCS and the demonstration of S-Ig on RCS-cells, however, speak decisively against a reticulocytogenous origin. Then, as far as we know, no one has been able to convincingly demonstrate Ig-synthesis in reticulum cells. We have also found no S-Ig-labeling of real reticulum cells, i. e. cells which are light microscopically characterized by strong non-specific esterase and acid phosphatase activity. Since Ig-production is a specific function of lymphatic cells, we believe that RCS, or at least those cases presented here, are of lymphatic origin.

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CELLULAR IMMUNE REACTIONS IN HUMAN ACUTE LEUKEMIA

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Cellular immune reactions are generally thought to play a major role in host resistance against tumor growth. Human acute leukemia cells have been found to contain tumor associated antigens, and it is possible to measure the cell-mediated immune response to these antigens. In addition to specific reactivity, it is quite important to evaluate the functional integrity of the cellular immune system in leukemia patients. The disease process itself or the chemotherapeutic agents could cause general depression in reactivity. The techniques currently being used for such studies are summarized in Table 1. In this paper, we will review the information available from each of these approaches, and discuss their possible relevance to the clinical state.

Table 1. Assays of Cellular Immunity in Human Acute Leukemia

Assays of competence of cellular immunity system
1) Delayed hypersensitivity skin reactions: standard recall antigens and ability to be sensitized to DNCB, KLH
2) Lymphocyte stimulation by mitogens (e. g. PHA), common antigens and allogeneic leukocytes
3) Enumeration of thymus-derived lymphocytes (T cells) and bone-marrow derived lymphocytes (B cells) in peripheral blood
4) Cytotoxic reactivity of lymphocytes against human lymphoid cell lines
Cell-mediated immunity against leukemia associated antigens
1) Delayed hypersensitivity to membrane and soluble extracts of blast cells
2) Lymphocyte stimulation by autologous blast cells and soluble extracts
3) Cytotoxic reactions against blast cells
4) Inhibition of leukocyte migration by extracts of blast cells

Competence of Cellular Immunity System

Three aspects of the competence of the cellular immunity system in acute leukemia patients are of interest: a) the effect of the tumor burden on reactivity; b) the effect of chemotherapy; and c) the inherent ability of patients to have cell-mediated immunological reactions against a variety of antigens. Unfortunately, it is difficult to clearly study each of these factors. The ideal time to obtain information on the patient's inherent reactivity would be prior to development of disease. Based on the theory of immunological surveillance (1, 2), one might anticipate that individuals developing leukemia have depressed immunological competence. However, it is not possible to obtain information at this time. It is practical to examine patients at the time of diagnosis, when tumor is present, and during clinical remission. Since chemotherapeutic agents are used to induce remission, their effects must also be considered.

Skin tests for delayed hypersensitivity have been extensively used to study cellular immunity in acute leukemia patients. In studies before thereapy (3) and shortly after the onset of therapy (4), decreased reactivity of some patients to standard recall skin test antigens was found. Hersh et al (4) found that depressed skin reactivity was associated with poor response to therapy for six months or longer had even less reactivity than that seen in initial studies (5). Chemotherapy may have had an important influence on these results. Borella and Webster (6), in a study of children with acute leukemia in remission, observed that long-term combination chemotherapy had immunosuppressive effects on skin test reactivity. Some treatment protocols appeared to be more immunosuppressive than others. Many of the patients of Hersh et al (4, 5) received COAP, which was noted to be very immunosuppressive.

Our laboratory also studied the response of ALL (acute lymphocytic leukemia) and AML (acute myelogenous leukemia) patients to a battery of standard recall antigens (7). We performed almost all of our tests after induction of remission, or at the time of bone marrow relapse. Patients at the National Cancer Institute are usually treated with combination hemotherapy, with repeated cycles of one week of treatment followed by two–three weeks off. To reduce the possible effects of treatment on the results, the patients were usually skin tested just prior to a course of therapy. Table 2 gives a summary of our data. Ninety-six percent of patients with ALL and all of the patients with AML reacted with at least one of the antigens, when tested in remission or in relapse. There were no significant correlations between the incidence of reactivity to any one of the particular antigens and clinical state, time of test in relation to chemotherapy, or length of survival. The reactivity of the ALL patients to PPD was high, due to immunotherapy with BCG. There are several possible explanations for the differences between our results and those of Hersh's group. Since they found that patients with intact skin reactivity were more likely to go into remission (4), it is possible that our patients, initially tested in remission, were a selected population. The type of chemotherapy used, and the timing of tests in relation to therapy, may also have been important differences.

Stimulation of patients' lymphocytes by mitogens, recall antigens and allogeneic leukocytes is another widely used technique for assessing immune competence.

Table 2. Skin Reactions to Recall Antigens

Patients	PPD+	% Positive Tests Mumps	Candida	Tricho.	SKSD++	% Anergic
ALL** remission	36	67	42	14	78	4
ALL, relapse	45	92	67	0	86	8
AML++ remission	13	83	33*	0*	72	0
AML, relapse	15	88	31*	0*	45	0
Normal	20	84	71	31	89	0

* Significantly less reactivity than in normal controls, $p < 0.05$.
\+ Purified protein derivative of tuberculin, intermediate strength.
++ Streptokinase, 40 units, streptodornase 10 units.
** Acute lymphocytic leukemia.
++ Acute myelogenous leukemia.

Hersh et al (4) found that one-third of leukemia patients studied had decreased responses to phytohemagglutinin and streptolysin O. As with the skin tests, most of these poorly reactive patients did not respond well to therapy. It has been found the time of testing, in relation to chemotherapy, has an important influence on the results (8, 9, 10). Reactivity was greatest 10–20 days after cessation of therapy, and in fact was sometimes higher than normal reactivity at this time.

Techniques have recently been developed which may allow enumeration of thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) in the peripheral blood. T cells ,appear to have receptors for sheep erythrocytes (E), while B cells have receptors for the third component of complement and can thereby bind E coated with antibody and complement (EAC). Rosette assays based on binding of E and EAC are easy to perform and may provide useful information in cancer patients. Many cancer patients have decreased percentages of circulating T cells (11, 12). Sen and Borella (13) have found that longterm chemotherapy caused depression in lymphocyte counts, and B cells were decreased more than T cells. After cessation of therapy, the B cells returned to normal levels within two to three months, whereas recovery of T cells took longer. No systematic serial study of these cell populations at different phases of disease have been reported as yet.

Rosenberg et al (14) have recently found that the lymphocytes of most normal individuals have cytotoxic reactivity against human lymphoid cell lines. McCoy et al (15) found that many patients with solid tumors and patients with immune deficiency diseases had significantly reduced activity. Fig. 1 shows the results with leukemia and lymphoma. Many of the leukemia patients had low reactivity. Reactivity was found to vary at different times in relation to chemotherapy (16). However, no consistent pattern was seen among different patients. Patients who were off therapy for four to eight weeks (points labelled Rx in Fig. 1) had normal or high reactivity.

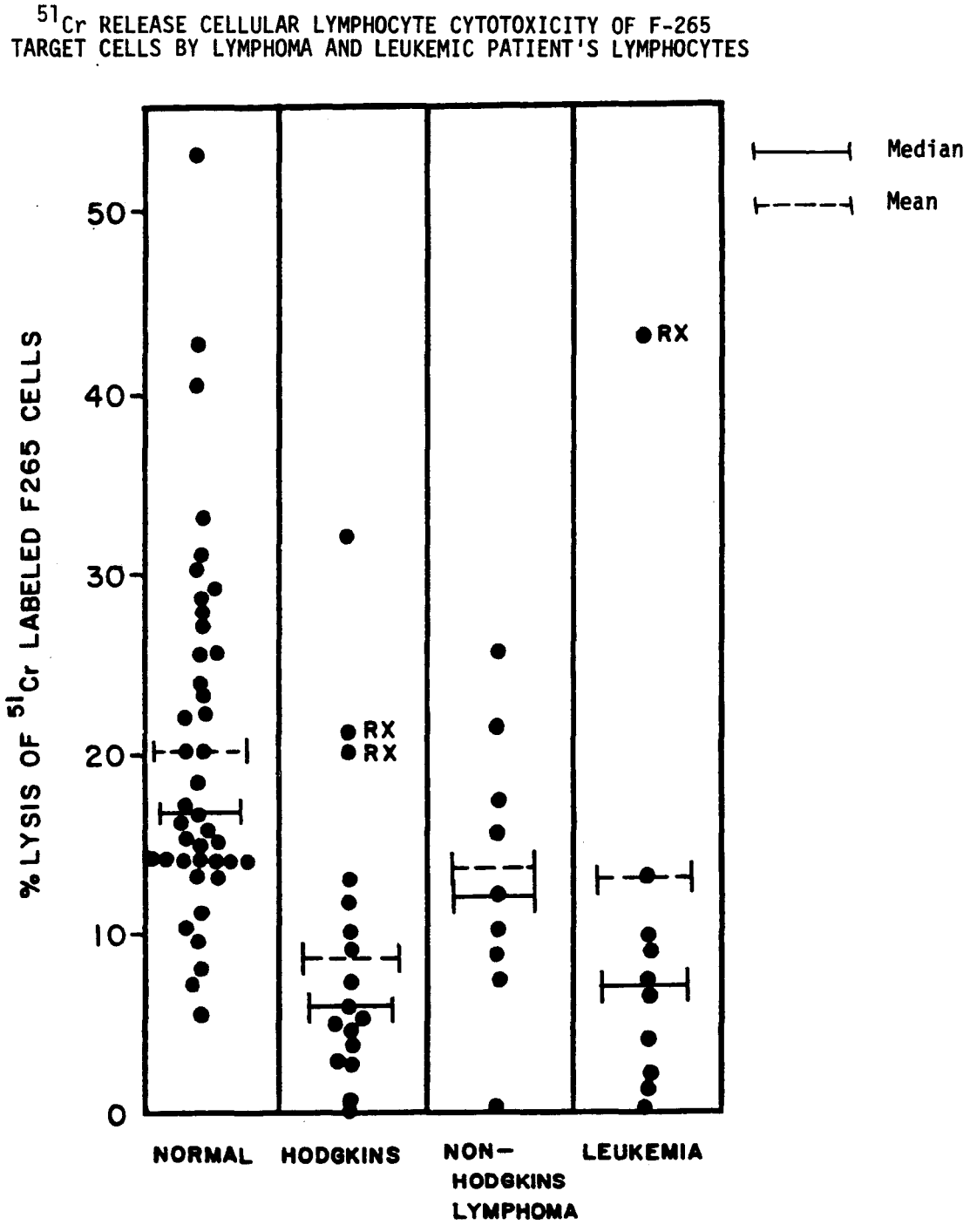

Fig. 1

Cell-Mediated Immunity Against Leukemia Associated Antigens

Skin testing with membrane or soluble extracts of blast cells has been used to measure delayed hypersensitivity reactions to leukemia associated antigens (7, 17). Table 3 is a summary of our tests with autologous membrane extracts of blast and remission cells (7). In patients with ALL who were in remission, positive reactions to autologous blast extracts were obtained in 20 of 44 tests. ALL patients tested in

relapse gave only one positive reaction. In AML, there was also a significant correlation of reactivity to blast extracts and clinical state. Serial skin tests were performed in 29 ALL and 8 AML patients (7). In most cases, reactivity to autologous blast cell extracts fluctuated in parallel with changes in clinical state. Patients who had positive reactions in remission generally became unreactive at the time of relapse. This represented specific depression, in that reactions to recall antigens did not vary significantly.

In contrast to our results with autologous membrane extracts, it has recently been reported that no positive reactions of acute leukemia patients were elicited by autologous viable cells (17) or mitomycin-C treated blast cells (3). Gutterman et al (17) did find, however, that 3M potassium chloride extracted soluble antigen produced positive skin reactions.

These studies indicated that the form of the antigen used for skin testing may influence the results. Our group has performed studies on antigens solubilized and separated from AML cells (18), as indicated in Figure 2. Skin reactive antigens could be solubilized by low frequency sonication, and then separated by Sephadex G-200 and DEAE-cellulose chromatography. Table 4 gives the representative results of testing a patient with autologous DEAE fractions. Skin reactions were elicited by two of the fractions from blast cells, and not by the comparable fractions from remission cells. It has been possible to further separate the AML skin reactive antigens, by gradient acrylamide gel electrophoresis. Tabel 5 shows a test, in which only one region of the gel containing the blast extract gave a positive reaction. No reactions were elicited by the comparable remission cell fractions.

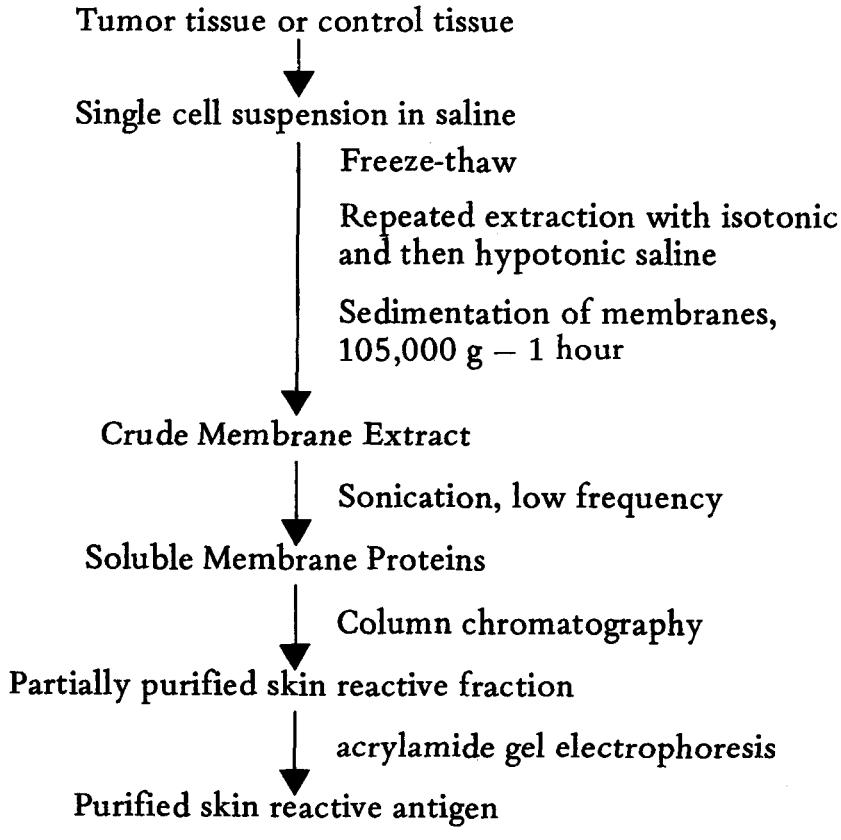

Fig. 2. Extraction procedure for solubilization and separation of skin reactive antigens on cells from patients with acute myelogenous leukemia.

Similar studies are now in progress with extracts of ALL cells and of fetal thymus cells. Some positive results have been obtained with the fetal thymus cells, which are of particular interest, since ALL blast cells appear to be T cells (12).

We have recently also tested patients with membrane extracts of cells from allogeneic leukemia patients (7). The results are summarized in Table 6. Positive reactions were observed in both AML and ALL patients, in response to extracts of blast cells from the same disease. Some of the extracts produced as much reactivity as the autologous extracts, while others were inactive. As with autologous extracts, reactivity to the allogeneic preparations correlated with disease status. The antigens detected on the allogeneic extracts appeared to be tumor associated, since extracts of remission cells or of normal leukocytes were unreactive.

Membrane extracts of human lymphoid cell lines have also given positive reactions in some patients with acute leukemia or lymphoma (19). Most of the cell lines were derived from Burkitt's lymphoma. As a control, patients were also tested with F-265, a cell line derived from normal lymphocytes. The results are summarized in Table 7. Patients with leukemia and lymphoma appear to be sensitized against an antigen on the tumor derived cell lines, whereas carcinoma

Table 3. Skin reactions to Autologous Membrane Extracts

	Number of Positive Reactions/Total Tests (%+)	
Patients	Blast Cell Extract	Remission Cell Extract
ALL,* remission	20/44 (45)	0/14 (0)
ALL, relapse	1/15 (7)	0/5 (0)
AML,+ remission	16/19 (84)	0/5 (0)
AML, relapse	9/18 (50)	0/4 (0)
Normal		1/20 (5)

* Acute lymphocytic leukemia.
+ Acute myelogenous leukemia.

Table 4. Skin Tests with DEAE-Cellulose Fractions of Soluble Autologous AML* Cells (Herberman, Char, and Hollinshead, 1973)

Material Tested	DEAE Fraction	Skin Test Results (mm. induration)
Blast Cells	b	– (2)
Sephadex fraction	c	+ (6. 9)
	d	+ (6. 0)
Remission cells,	b	– (0)
Sephadex fraction	c	– (0)
	d	– (0)

* Acute myelogenous leukemia

Table 5. Skin Tests with Acrylamide Gel Fractions (Herberman, Char, and Hollinshead, 1973)

Material Tested	Gel Region	Skin Test Results
Blast Cells –	1	–
DEAE fractions c + d	2	–
	3	+
Remission Cells –	1	–
DEAE fractions c + d	2	–
	3	–

Table 6. Skin Reactions to Allogeneic Membrane Extracts

Patients	Extract	Number Positive/ Total % Positive
ALL*, remission	ALL blasts	51/186 (27)
ALL, relapse	ALL blasts	1/33 (3)
ALL, remission	ALL remission cells	0/10 (0)
ALL, remission	AML blasts	0/8 (0)
ALL, remission	Normal leukocytes	0/20 (0)
AML+, remission	AML blasts	4/7 (57)
AML, remission	AML remission cells	0/5 (0)
AML, remission	ALL blasts	0/6 (0)
AML, remission	ALL remission cells	0/3 (0)
AML, remission	Normal leukocytes	0/5 (0)
Normal	Normal leukocytes	1/60 (2)

*Acute lymphocytic leukemia.
+Acute myelogenous leukemia.

patients were unreactive. Reactivity in the ALL patients has correlated well with clinical state. Thus far, only patients in remission have been reactive.

Several groups have found that remission lymphocytes of patients with acute leukemia could be stimulated by autologous blast cells (10, 20–23). As with mitogens and common antigens, stimulation by autologous blasts was found to correlate with the length of time after cessation of a course of chemotherapy (10, 16). Autologous blast cells generally produced maximum stimulation at 10–20 days after chemotherapy, but the peak response to blast cells did not always occur at the same time as the peak response to mitogens or allogeneic leukocytes (16). Positive stimulation has also been found to correlate with good prognosis (23, 24).

Soluble extracts, prepared by treatment of blast cells with 3M potassium chloride, have also been shown to stimulate the autologous lymphocytes (17). We plan to perform similar studies with the antigens solubilized by sonication.

Table 7. Skin Reactions to Extracts of Lymphoid Cell Lines

Patients	Tests Positive/Total Tests (%+)	
	Tumor Derived Cell Lines	F–265
ALL*	9/31 (29)	0/21 (0)
CLL+	2/2 (100)	0/1 (0)
AML ‡	2/2 (100)	0/2 (0)
CML**	0/1 (0)	0/1 (0)
CML, blasts crisis	4/4 (100)	0/4 (0)
Total leukemias	17/40 (43	0/29 (0)
Lymphomas	22/43 (51)	2/14 (14)
Carcinomas	0/12 (0)	0/11 (0)

* Acute lymphocytic leukemia.
+ Chronic lymphocytie leukemia.
‡ Acute myelogenous leukemia.
** Chronic myelogenous leukemia.

Table 8. Cell-Mediated ^{51}Cr Release Assay – Autologous Target Cells

Attacking Lymphocytes	Tests Positive/Total Tests (%+)	
	Target Cells	
	Blasts	Remission
Normal		0/30 (0)
ALL*	8/20 (40)	0/22 (0)
AML+	6/19 (32)	0/10 (0)

*Acute lymphocytic leukemia.
+Acute myelogenous leukemia.

The specificity of the antigens detected by lymphocyte stimulation is not completely clear. Some experiments have been performed with remission bone marrow as well as with relapse marrow (10). In some experiments, both blast cells and remission cells produced some stimulation. It remains to be determined whether the stimulation assay is detecting a differentiation antigen present on normal blast cells as well as on leukemic blasts.

In vitro cytotoxicity reactions have been particularly stressed as likely analogues of the cell-mediated defense against tumors (25). Our laboratory has tested the lymphocytes of patients with acute leukemia and of normal individuals against autologous and allogeneic target cells, by a ^{51}Cr release cytotoxicity assay (10, 26). Table 8 gives a summary of results obtained against autologous target cells. There was no reactivity of normals against their own lymphocytes. Both ALL and AML patients reacted against their blast cells, but not against their remission cells. The observed reactivity did not against their remission cells. The observed reactivity did

not correlate with clinical state. There was at least as much reactivity during bone marrow relapse as there was during remission. The results of tests against allogeneic target cells are given in Table 9. Only a low incidence of reactivity was observed against lymphocytes of normal individuals. In contrast, many positive reactions against blast cells were seen; lymphocytes from leukemia patients and also from normal controls had cytotoxic effects. Positive results were also seen against remission lymphocytes of the leukemia patients.

The cytotoxicity assay appears to be detecting leukemia associated antigens, but the specificity of the reactions may be different from that of the skin tests. In the studies with autologous cells, antigens were only detected on blast cells. In the allogeneic tests, some results with remission cells were also positive. It is likely that the remission cells contain antigens different from those on blast cells, and which are undetectable by skin tests.

There have been few studies of leukemia patients thus far with the leukocyte migration inhibition assay. However, using the assay of Rosenberg et al (27), Halterman et al (28) studied a pair of identical twins, one with leukemia. Leukemic antigen extract, which gave a positive skin reaction in the patient, also caused inhibition of the migration of her leukocytes. The same extract did not affect the migration of the normal twin's leukocytes.

To determine the possible relationships between skin testing, lymphocyte stimulation, and ^{51}Cr cytotoxicity assays, the three tests were performed on 20 patients (10). The results are summarized in Table 10. As noted above, results of skin tests correlated with clinical status. The in vitro assays did not correlate with stage of disease, nor did they correlate with each other. The reasons for the lack of correlations are not clear. It is quite possible that each assay is measuring different antigens. Leukemias in experimental animals have been shown to have a complex variety of antigens (29, 30). Studies with isolated, soluble antigens are now feasible and should help to decide this issue. The assays may also be measuring different phases of the immune response, and different subpopulations of lymphocytes may be responsible for the various effects. It is quite possible that the lymphocyte stimulation assay measures the primary recognition of foreign cell surface antigens.

Table 9. Cell-Mediated ^{51}Cr Release Assay – Allogeneic Target Cells

Target Cells	Tests Positive/Total Tests (%+)		
	Attacking Lymphocytes		
	Normal	ALL Patients	AML Patients
Normal lymphocytes	5/220 (2.3)	0/20 (0)	0/15 (0)
ALL* blasts	61/134 (46)	10/24 (42)	4/15 (27)
ALL remission cells	20/93 (22)	2/16 (13)	1/7 (14)
AML+ blasts	41/100 (41)	6/15 (40)	6/25 (24)
AML remission cells	15/50 (30)	0/4 (0)	0/5 (0)

*Acute lymphocytic leukemia.
+Acute myelogenous leukemia.

Table 10. Results of three assays of cellular immune reactivity in acute leukemia to autochthonous blasts cells

Patients	Tests positive/total number of tests		
	Skin Tests	Lymphocyte Cytotoxocity	Mixed Leukocyte Culture
ALL, relapse	1/3	1/2	2/3
ALL, remission	6/9	4/10	6/9
AML, relapse	2/5	2/3	3/6
AML, remission	8/9	2/10	9/14
Total	17/26 (65 %)	9/25 (36 %)	20/32 (63 %)

The lymphocyte cytotoxicity assays appear to detect the presence of presensitized cells, capable of rapidly reacting with the target cells. The skin tests are thought to depend on sensitized lymphocytes capable of releasing migration inhibitory factor and other soluble mediators, and also on the presence of adequate numbers of mononuclear cells to accumulate at the reaction site.

Use of Immunological Assays to Monitor Immunotherapy

There has been much recent interest in the use of immunotherapy in acute leukemia. Most of the studies already performed have been empirical, without assessment of the antigenicity of the immunizing cells and without immunological monitoring of the immune response to the therapy.

An immunotherapy study was performed on previously treated ALL patients, in which allogeneic ALL blast cells plus either BCG or methotrexate were given (24). Immune responses were serially determined, by skin testing and by lymphocyte stimulation. The most dramatic change was in the response of the BCG treated group to PPD. Skin tests with allogeneic blast extracts did not provide clear evidence for immunization, even against the HL-A antigens of the donor cells. There was, however, a correlation between skin reactivity against the extract of donor cells and the duration of remission. In the lymphocyte stimulation assays, there was also little evidence for immunization by the donor cells.

The use of these immunological assays could help in designing future immunotherapy trials. Allogeneic cells could be selected on the basis of their ability to elicit skin reactions, thereby documenting that the donor cells possess common antigens. In addition, monitoring with skin tests could be used to determine the immunogenicity of different immunizing preparations and schedules. It would be very helpful if autologous cells were available for skin testing and for in vitro tests, since they would permit a distinction between immunization to leukemia associated antigens and immunization to normal histocompatibility antigens. If an immunotherapy trial were clinically successful, it would provide important information on the predictive value of each of the assays.

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HUMAN IMMUNE RESPONSE TO RAUSCHER LEUKEMIA VIRUS[1]

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Abstract

Twenty patients were immunized with formalin-killed Rauscher leukemia virus. No untoward side effects were observed. Approximately three-fourths of the patients developed cell-mediated immunity as assessed by *in vitro* lymphocyte blastogenic responses. Approximately two-thirds of the patients developed antibody responses as measured by radio-immunoprecipitation, and one-half of the patients developed delayed hypersensitivity to the immunizing antigen.

The responses illicited were specific for the immunizing viral antigen because little or no response was illicited *in vitro* among the immunized patients' lymphocytes to virus-free tissue culture vehicle. The immune response to the viral antigens was also evaluated by lymphocyte stimulation with solubilized from transformed cells.

These data suggest that human subjects (patients with metastatic cancer and acute leukemia) can mount immune responses to oncogenic viruses of both humoral and cell-mediated immunity.

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Introduction

The fundamental premise on which this study is based is that murine leukemia viruses and a hypothesized viral agent associated with human leukemia may share antigens or may induce common antigens in transformed cells. The observations which tend to support this hypothesis include the following. First, the bone marrow cells of certain patients with leukemia react with fluorescent antibody made in other species against the Rauscher leukemia virus (1). Second, serum of some patients with acute leukemia has neutralizing activity against Rauscher virus (2). Third, antigen of the virus envelope has been found in the serum of some patients with erythroleukemia (3). Finally, when human embryonic kidney cells are transformed by the Rauscher leukemia virus they develop a cell membrane antigen also present in human leukemia cells and lymphoblastoid cell lines (4).

These data suggested that a study of the human immune response to the Rauscher leukemia virus would be of interest. First, it would tell us if human subjects could respond immunologically to an oncogenic virus. Second, it would generate data regarding the possible interrelationship of human and murine leukemia. Third, it would generate useful reagents for the study of oncogenic viruses and their relationship to transformed cells.

Therefore, the immune response of 20 patients with advanced malignant disease to a killed Rauscher leukemia virus preparation was studied. The majority of patients mounted a cell-mediated and humoral immune response both to virion and to murine antigens present in the virus envelope. The data indicate that this may be useful in approaching the objectives mentioned above.

Materials and Methods

Twenty patients with advanced malignancy were selected. There were 13 patients with metastatic solid tumors and seven patients with acute leukemia. The objectives of the study and the possible risks were carefully explained to each patient and informed consent was obtained. Each potential candidate for study was skin tested with a battery of five antigens to which most individuals are sensitive, including dermatophytin, dermatophytin-0, candida, varidase and mumps. For entry into the study, at least two of these skin tests were required to be positive. Each patient also had a set of lymphocyte cultures set up which were stimulated with phytohemagglutinin and streptolysin-0. To enter the study, the responses to these mitogens were required to be at least 20,000 counts per minute and 3,000 counts per minute, respectively.

After this preliminary evaluation, each patient received Rauscher leukemia virus intradermally on day 1, 14, 28 and 42 of the study. The development of delayed hypersensitivity to the immunizing antigens was determined on the subsequent two days. *In vitro* lymphocyte responses and serum antibody responses to the immunizations were measured on day 1, 7, 14, 21, 28, 35, 42 and 49.

Virus for immunization was grown in JLSV9 cells, harvested from the supernatant fluid and purified by double banding by density gradient centrifugation. It was inactivated by exposure to 0.05 percent formalin for two weeks at 4 °C. The final virus concentration was 1.7 mg/ml. Virus for *in vitro* lymphocyte cultures was

prepared in the same fashion but not inactivated with formalin. The control for the Rauscher leukemia virus used *in vitro* was doubly banded culture fluid from JLSV9 cultures which were not infected with the Rauscher virus (virus vehicle).

Antigen from transformed cells was prepared from JLSV9 cells by the hypertonic KCl extraction method of Reisfeld (5). Antigen from JLSV9 non-infected cells for use as a control was prepared in the same fashion.

Delayed hypersensitivity skin test responses to immunization were read as previously described (6). *In vitro* lymphocyte cultures were set up, stimulated and harvested as previously described (6). Each set of cultures consisted of a control and cultures stimulated with phytohemagglutinin, streptolysin-0, a dose range of Rauscher leukemia virus, a dose range of Rauscher leukemia virus control, a dose range of JLSV9 Rauscher infected cell antigen and a dose range of JLSV9 non-infected cell antigen.

Antibody titers were performed by a radioimmunoprecipitation technique[7]. Briefly, a Rauscher preparation labeled during proliferation of the virus with ^{3}H-leucine was mixed with an aliquot of the test serum, all immunoglobulin was then precipitated with goat anti-human IgG and goat antihuman IgM, and the dilution of the serum at which either 30 or 50 percent of the virus was precipitated was taken as the titer.

Results

No untoward side effects of any kind were noted during this study. All patients tolerated the immunizations, skin testing and blood drawing procedures very well. There was transient local discomfort at the injection sites, lasting about 10 seconds during immunization. The immunization procedure did not appear to alter the natural history of the patient's disease in any way.

Table 1 shows the lymphocyte blastogenic responses before and after immunization to the various mitogens and antigens. It can be seen that there were no

Table 1. Lymphocyte Blastogenic Responses Pre- and Post-Immunization

Lymphocyte Stimulant	Response in CPM*		Response in SI**	
	Pre-Imm.	Post-Imm.	Pre-Imm.	Post-Imm.
Control	0.3	0.3	1.0	1.0
PHA	46.9	49.7	180.5	221.2
SLO	6.1	3.6	18.2	15.4
RLV***	0.8	5.5	4.3	21.6
RLV Vehicle Control	0.0	0.3	1.1	1.4
JLSV9R***	0.5	1.8	2.6	6.9
JLSV9C	0.0	0.2	1.1	1.7

* Net counts per minute per 10^{6} lymphocytes x 10^{3} (median)
** Stimulation Index (median)
*** Data obtained from RLV and JLSV9R doses giving highest response and appropriate dose-associated controls.

Table 2. Relative Specificity of Response to Various Rauscher Leukemia Virus Related Materials

Parameter	Virus/Vehicle Ratio		JLSV9R/JLSV9C Ratio	
	Pre	Post	Pre	Post
Number	16/19*	16/18	18/19	16/18
Median	3.5	12.1	3.1	4.0
Mean	6.4	20.4	11.6	20.0

* No. with ratio >1/no. in whom ratio studied

Table 3. Development of In Vitro Lymphocyte Blastogenesis after Immunization with Formalinized RLV

Antigen	Number of Patients	Response to Immunization				
		In CPM		In SI		Overall
		Pre	Post	Pre	Post	
RLV	Positive	2	15	3	16	
	Responding		14		15	14
JLSV9R	Positive	7	13	14	16	
	Responding		9		9	7

Table 4. Serial Study of Lymphocyte Response to RLV

Parameter	Week of Study							
	1	2	3	4	5	6	7	8
CMP*								
Median	0.5	1.8	1.9	1.8	1.2	1.3	2.8	0.6
Mean	1.0	2.2	3.5	1.5	2.0	3.2	5.0	2.3
SI								
Median	1.9	8.0	2.5	5.5	7.3	6.6	9.4	4.6
Mean	1.6	9.7	18.3	7.0	14.4	7.8	19.2	18.7

* CPM = Counts per minute per 10^6 lymphs x 10^3

significant changes either in terms of counts per minute or stimulation index in the control, PHA and SLO responses. There was a striking increase in response to the Rauscher leukemia virus and a modest increase in response to the JLSV9 Rauscher infected cell antigen. Since there was only a slight increase in the response to the RLV vehicle control and the JLSV9 noninfected control cell antigen, the responses were presumably virus or virus induced antigen specific.

This was further investigated by calculating a specificity ratio by dividing the virus response by the vehicle response, or the JLSV9 Rauscher infected cell antigen response by its appropriate control. It can be seen in Table 2 that both pre- and post-immunization these ratios were greater than one, indicating specificity. The median ratio rose from 3.5 to 12.1 for the virus and 3.1 to 4.0 for the antigen. The mean ratios rose from 6.4 to 20.4 for the virus and from 11.6 to 20.0 for the antigen.

Table 3 shows the numbers of subjects who showed positive responses *in vitro* to the virus and the antigen before and after immunization, in terms of counts per

Table 5. Development of Delayed Hypersensitivity after Immunization with Formalinized RLV

No.	Pre-Immune	Post-Immune	Response to Immunization
1	3*	3	No
2	0	0	No
3	3	16	Yes
4	0	0	No
5	0	4	Yes
6	0	10	Yes
7	0	0	No
8	0	0	No
9	6	10.5	No
10	0	4.5	Yes
11	0	3	Yes
12	8	12	No
13	4	18	Yes
14	0	12.5	Yes
15	0	5	Yes
16	0	0	No
17	0	0	No
18	0	5	Yes
19	0	0	No
20	0	7.5	Yes
Mean	1.2	5.55	10/20
± SD	2.4	5.78	
SE	.5	1.29	
Median	0	4.25	
Range	0-6	0-18	

* Diameter induration in mm

minute and stimulation index and the numbers of patients who showed a true response to immunization comparing pre- and post-immunization values. It can be seen that approximately three-fourths of the 20 subjects showed a response to the Rauscher leukemia virus and that somewhat more than one-third of the subjects showed a response to the solubilized cell antigen.

Table 6. Antibody Response after RLV Immunization by Radioimmunoprecipitation Assay

Patient Number	Pre-Immune		Post-Immune	
	30%	50%	30%	50%
1	0	0	1,024	379
2	0	0	512	48
3	0	0	0	0
4	0	0	256	96
5	0	0	256	128
6	0	0	64	0
7	0	0	1,024	512
8	0	0	198	64
9	0	0	1,500	512
10	0	0	0	0
11	0	0	512	256
12	0	0	2,000	1,024
13	0	0	0	0
14	0	0	0	0
15	0	0	16	0
16	0	0	16	0

Table 7. Immune Response after RLV Immunization Comparison of Various Absorption Methods

Serum Absorption	Reciprocal of Serum Dilution						
	16	32	64	128	256	512	1,024
None	84*	95	93	92	94	86	81
JLSV 6	87	86	84	82	75	64	48
3 T 3	95	95	95	94	87	84	66
In Vivo	95	92	90	87	82	72	42
None	87	87	85	82	71	63	48
In Vivo	20	17	16	16	16	14	12

* Percent precipitation at indicated dilution

Table 8. Correlation of Antibody Response to RLV and other Parameters of RLV Immunity

Immunoligical Parameter	Positive or Negative	Antibody Response	
		Positive or Negative	
Delayed Hypersensitivity	+	5	4
	–	4	3
Lymphocyte Response to RLV (CPM)	+	8	1
	–	5	2
Lymphocyte Response to JLSV9R (CPM)	+	7	1*
	–	2	6

* Significant Difference

Table 4 shows the kinetics of the response in terms of the median counts per minute and stimulation index. It can be seen that the vigorous responsiveness developed between one and two weeks after immunization or in the second and third week of the study that there was a decline in reactivity between the fourth and the sixth week with a return of vigorous activity subsequently and another decline at the end of the follow-up period.

The delayed hypersensitivity responses pre- and post-immunization are shown in Table 5. Only five subjects showed reactivity prior to immunization with a mean response of 1.2 mm in diameter of induration. After immunization, 13 subjects showed positive reactivity with a mean diameter of induration of 5.5 mm. Ten of the twenty gained activity after immunization or showing a greater than 100 percent increase in reactivity.

Antibody titers have been run on the sera of 16 of the 20 patients. Before immunization, some of the patients showed antibody titers to the virus but these were reduced to the background level by *in vitro* absorption of the serum on JLSV6 cells or by *in vivo* absorption of the sera in BALB-C mice. After immunization, as shown in Table 6, 12 of the patients showed a 50 percent precipitation titer. This was after *in vivo* absorption of the sera. It might be noted that these titers remained positive after double absorption of the serum both *in vivo* and *in vitro*, that there were no positive pre-immune sera with true anti-viral activity, that a series of control subjects not immunized with virus but given BCG by scarification did not develop antibody and that these sera had no antibody activity using the same assay against the RD 114 virus. Table 7 shows the effects of absorption on the antibody titer in immune (upper four lines) and non-immune (lower two lines) serum. It can be seen that in the non-immune serum precipitating antibody was completely absorbed *in vivo*.

Table 8 shows an attempt to correlate the antibody response to the RLV to the other parameters of RLV immunity. The antibody responses did not correlate with delayed hypersensitivity responses or *in vitro* lymphocyte responses to the RLV. However, they did correlate with *in vitro* lymphocyte responses to the JLSV9 Rauscher infected cell antigen in terms of counts per minute.

Discussion

While the evidence is very strong that many animal malignant tumors are caused by viruses, the evidence is still circumstantial in man that any human malignancy is virus induced. For instance, attempts to isolate human cancer viruses by inoculation of cell-free extracts from human cancer materials into animals have failed (7a). Also, attempts to isolate oncogenic viruses from human cancer materials in tissue culture have failed (8). In general, attempts to unmask oncogenic viruses from human cancer materials in tissue culture have also failed. While initial enthusiasm greeted the observation that virus-like particles were observed in various malignant tissues, it is felt today that these were, for the most part, various cell fragments or artifacts (9). Human adenoviruses, which clearly cause malignancies in animal species, are apparently not oncogenic in man (10), and the Herpes virus group is probably only associated etiologically with one human malignancy, namely Burkitt's lymphoma (11), altough nasopharyangeal carcinoma and carcinoma of the cervix may also be caused by this virus group.

The strongest evidence for a viral etiology in cancer involves the RNA tumor viruses, namely type-B viruses in human breast cancer and type-C viruses in human leukemia and lymphoma. This includes discovery of antigenic relationships, observations of particles by electronmicroscopy, detection of viral genomes in cancer tissues by hybridization experiments and viral enzymes in human cancer tissue (12). Because of the observations relating the Rauscher leukemia virus with human leukemia cited in the introduction, we thought it would be worthwhile to study the human immune responses to such a virus. The data generated in the study of the first 20 patients indicates that human subjects can indeed mount an immune response to formalinized Rauscher leukemia virus. Approximately two-thirds of the patients studied mounted such an immune response and this involved both humoral and cell-mediated immunity. Both patients with solid tumors and with acute leukemia responded, so that apparently there is no immunological tolerance to viral antigens in leukemia patients.

The study generated some evidence of specificity. Thus, the response to the virus was greater than to the appropriate non-virus containing vehicle and the response to the antigens solubilized from virus infected cells was greater than the response to the antigen solubilized from the same cells which were not virus-infected.

In terms of the antibody response, while there was antibody to mouse antigens in both non-immune and immune subjects, in the immunized individuals there was also antibody to the virus capsule antigens themselves which could not be absorbed out by mouse tissue *in vivo* or *in vitro*. This was demonstrated by absorption studies. That antibody from immune subjects as well as non-immune subjects had antibody which reacted with virus but which could be partially or completely

absorbed out by normal mouse tissues, suggesting that mouse antigen is an integral part of the virus envelope structure. This is not surprising since the virus buds from the cell membrane. The nature of this antigen would be extremely interesting and it would be important to know whether it is normal cell surface antigen, such as H-2 antigen, or whether it is virus induced non-virion antigen or a cell associated antigen such as embryonic antigen.

These studies indicate that further work along these lines is warranted, first to clearly define the spectrum of reactivity of immunized subjects, second to determine the best preparation, dose and schedule of vaccine administration, third to prove specificity of anti-viral immunity induced by these immunization procedures, and finally to determine the relationship between the various viral and non-viral antigens to which the immune response is induced. Studies along these lines may well lead to knowledge on which the development of anti-viral vaccines for use in human cancer immunotherapy could be developed.

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THE CLINICAL SIGNIFICANCE OF IMMUNOLOGICAL FINDINGS IN BURKITT'S LYMPHOMA

(EPSTEIN-BARR VIRUS-ASSOCIATED ANTIBODIES/ BCG TREATMENT)

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Abbreviations: BL = Burkitt's lymphoma; EA = EBV-induced early antigen complex, divided into D ("diffuse") and R ("restricted") subcomponents; EBV = Epstein-Barr virus; MA = EBV-associated cell membrane antigen complex; PDT = pertussis-diphtheria-tetanus triple vaccine; VCA = EB viral capsid antigen complex.

Abstract

All Burkitt's lymphoma patients studied have had antibodies against certain Epstein-Barr virus-associated antigens, in contrast to control persons who sometimes lack such detectable antibodies. High titers of one type of Epstein-Barr virus-associated antibody correlated to a greater risk for recurrences and death than low titers, and another virus associated antibody was sometimes noted to decrease significantly before late tumor recurrences. BCG inoculations regularly increased the latter antibody titers.

BL occurs with time and space clustering, suggesting epidemiological factors in its etiology. Long term survival after few and relatively low doses of cytostatics, and occasional spontaneous regressions have been noted. These circumstances made immunologists and virologists investigate the disease, resulting in the demonstration of EBV-associated antigens: MA on tumor cells and derived lines by KLEIN *et al.* (1), VCA in tumor-derived lines by HENLE and HENLE (2), and EA in recently EBV-superinfected lymphoblastoid lines (3). EA was further divided into R and D components (4).

Every BL patient studied has had antibodies to MA (5) and these antibodies decreased suddenly 1/2 year before a recurrence after 4 years tumor regression in one patient (6). Control persons did not have the same high incidence of high anti-MA levels as BL patients, though some control subjects had antibody levels comparable to those of most BL cases. Similar findings were obtained with regard to anti-VCA, but these antibodies were not clearly influenced by clinical events (7). High anti-EA titers correlated to a greater risk for recurrences and death than low titers (8) and the significant antibody usually seemed to be anti-R (9).

We have followed BL patients horizontally by antibody titrations during the course of their disease to see whether titer changes occurred in relation to clinical events.

Material and methods

BL patients' sera were tested for anti-MA by their blocking of the direct membrane immunofluorescence reaction between a BL-derived IgG and BL-derived cultured cells expressing MA (10). Blocking activity was measured by a blocking index, ranging from 0.00 to 1.00. The index fell after serum dilution and a titer could be calculated with titer endpoint = index 0.4 (11). Anti-VCA and anti-EA were tested by indirect immunofluorescence, using serum dilutions on fixed smears of antigen-positive cells (2, 7, 3, 4).

Histologically verified BL patients that became clinically tumor free after chemotherapy were given up to 10 intradermal applications of BCG (Glaxo Ltd) by a Heaf gun, usually with 3 weeks intervals. The procedure was described in detail (12). Some patients received one i. m. injection of 0.5 ml pertussis-diphteria-tetanus vaccine ("Trivax", Burroughs Wellcome and Co.) in exchange for one of the later BCG inoculations.

Results

A study of 5 BL patients (12) showed that anti-MA titers usually were stable during chemotherapy and tumor regression. A slight titer peak was noted in time relation to recurrences in 3 patients and larger peaks in all patients following BCG inoculations. The latter increases had a maximal size of 1–2 $\log_2$ units but usually faded during repeated BCG administrations. Prolonged tumor-free survival did not change these titers significantly, as a rule.

Anti-EA sometimes started to increase before clinical recognition of recurrent tumors. In most patients these titers decreased slowly during prolonged remission, even during BCG treatment.

Anti-VCA usually showed an increase during the first few months after admission and sometimes peaks in time relation to recurrent tumors. No significant changes were seen during BCG treatment and prolonged survival.

The observed fading of anti-MA titer peaks during repeated BCG administrations was speculated to follow an increasing host immunisation to BCG, that would limit its effect as a non-specific immune adjuvant. For this reason one of the later BCG inoculations was exchanged for PDT in some patients. The first one of these had widespread recurrences closely after the PDT vaccination. A serologic analysis showed that anti-MA titers decreased significantly well before PDT was given, accompanied by rising anti-EA (13). These findings indicated that the host-tumor balance had changed already before PDT vaccination.

Discussion

EBV-associated antibody titers were found to sometimes change significantly before recurrences and during non-specific immune stimulant therapy in BL patients. The mechanisms responsible for these changes remain largely unknown.

BL biopsy cells have MA but no detectable EA or VCA (14), and this may explain the unique behavior of anti-MA titers during BCG inoculation periods. VCA synthesis must occur somewhere, however, to maintain the high anti-VCA titers observed during prolonged tumorfree survival. The simultaneous decrease of anti-EA seen in some patients could be due to lack of antigenic stimulation during sustained remission.

The clinical value of BCG treatment in BL patients is still unknown in the absence of an adequate control group for the patients in this pilot study.

Sera were obatined from Mr. P. Clifford and Mr. S. Singh, Department of Head and Neck Surgery, Kenyatta National Hospital, Nairobi. Titration of anti-MA was done in collaboration with Professor G. Klein, Dept. of Tumor Biology, Karolinska Institute; anti-VCA and anti-EA titrations were performed by Drs. G. Henle and W. Henle, Div. of Virology, The Childrens' Hospital of Philadelphia, Philadelphia, Pa., USA. These investigations were conducted under Contracts No. NIH-NCI-E-69-2005 and PH-43-66-477 within the Special Virus-Cancer Program of the National Cancer Institute, National Institutes of Health, USPHS. Grants also received from the Swedish Cancer Society, the Cancer Society in Stockholm, and Åke Wiberg's Foundation.

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TUMOR VIRUS RNAs AND TUMOR VIRUS GENES

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Abstract

The RNAs of several classes of avian and murine RNA tumor viruses were compared. The 60–70S RNA complex of cloned nondefective avian sarcoma viruses contains only 30–40S RNA species of class a, which are larger than the 30–40S RNA species of class b found in avian transformation-defective or leukosis viruses. The RNA of a recombinant avian sarcoma virus, carrying a host range marker of a leukosis virus parent, also consists only of class a subunits. This implies that recombination among avian tumor viruses involves crossing-over rather than reassortment. The class a RNA subunits of nondefective avian sarcoma viruses and the class b RNAs of transformation-defective, leukosis viruses of the same subgroup and strain have very similar oligonucleotide-fingerprint patterns and are at least 60 % homologous if compared by RNA-DNA hybridization. It is suggested that the class b RNA of leukosis viruses is a deletion of class a RNA from corresponding nondefective sarcoma viruses and that their structural relationship may be expressed as $a = b + x$. We assume, that x represents genetic information directly or indirectly involved in transformation of fibroblasts.

Passage at high multiplicity of cloned sarcoma viruses, containing only 30–40S RNA of class a, led to the appearance of 30–40S RNA of class b in progeny virus.

Two replication-defective avian sarcoma viruses (Bryan RSV and MC 29) lack 30–40S RNA of class a. They probably contain distinct types af 30–40S RNA resembling class b RNA of leukosis viruses in size but differing in composition. The Kirsten murine sarcoma virus, which appears to be more defectice than Bryan RSV or MC 29, has a 30–40S RNA which is even smaller than of its leukosis helper virus.

These observations suggest that a correlation exists between the size of the viral RNA species and defects in transformation-and/or replication genes of the corresponding viruses. The greater the extent of the defectiveness, the smaller is the size of the viral RNA. Possible mechanisms generating deletions in tumor virus RNA are discussed.

Different classes of RNA tumor viruses:

Two classes of RNA tumor viruses can be readily distinguished on the basis of their pathological effects on cells (1–6): (i) Sarcoma viruses, which transform fibroblasts in tissue culture and cause solid tumors in the animal and (ii) leukosis or leukemia viruses, most of which fail to transform fibroblasts in tissue culture, but cause a lymphatic or myeloblastic leukemia in the animal. A possible exception appear to be

some endogenous leukosis-type viruses; for example, the endogenous avian leukosis virus RAV (O) has so far not been found to cause a disease in chicken (Vogt, P. K., G. Purchase and R. Weiss (1973) personal communication). Endogenous murine leukemia virus of AKR-mice has the same serotype and N-tropism as known leukemogenic virus strains, however it has not yet been tested whether it causes leukemia in mice (Rowe, W. P. (1973) personal communcation).

Since in the avian tumor virus group, sarcoma and leukosis viruses have been found which resemble each other in most biological and biochemical properties except transforming ability for fibroblasts (4, 5), many, and perhaps all avian leukosis viruses may be considered as defective sarcoma viruses which have lost their ability to transform fibroblasts. Consequently those avian leukosis viruses which have been derived in the laboratory (4, 5) from sarcoma viruses have been termed transformation-defective (td) viruses (7).

In addition replication-defective sarcoma viruses exist in the avian (8) and murine tumor virus group (9). These may be considered sarcoma viruses which have lost all or part of the genetic information required for virus-replication and/or virus-structure. All replication-defective RNA tumor viruses rely of necessity on associated helper viruses or helper cells to complement their defective structural or replicative functions (8, 10). Since the known helper viruses do not repair, by genetic recombination, the defectiveness of replication-defective sarcoma viruses, their defectiveness is genetically stable (8, 10).

If we were to set up a hierarchy of decreasing genetic potentials among different classes of RNA tumor viruses we could distinguish the following categories: Firstly, the nondefective sarcoma viruses, which carry all genes essential for replication and cell transformation (examples: Prague Rous sarcoma virus (PR RSV), Schmidt Ruppin (SR) RSV, B77 RSV). From these the category of transformation-defective (td) and leukosis viruses could be formally derived by deletion of genes required for cell transformation (examples: td PR RSV, Rous associated virus (RAV) and murine leukemia virus. Similarly the replication-defective sarcoma viruses may be considered as a viral category which has lost all or part of the genetic potential of nondefective sarcoma viruses required for virus replication (examples: Bryan RSV, Kirsten murine sarcoma virus (MSV), Moloney MSV). In addition sarcoma viruses may exist which are both replication- and transformation-defective and leukosis viruses may exist which are replication-defective (see Fig. 8).

It is the purpose of this report to summarize and extend correlations made (3, 5, 6, 7, 26, 31) between different genetic potentials of RNA tumor viruses and various physically defined classes of viral RNA.

The 60–70S RNA complex of RNA tumor viruses:

RNA tumor viruses contain a 60–70S RNA corresponding to an approximate molecular weight of 1×10^7 (11). This RNA probably represents part or all of the viral genome (11, 12). The 60–70S RNA can be dissociated by heat or organic solvents (formamide, dimethysulfoxide) to yield molecules which sediment at 30–40S and some smaller RNA species (11, 13) and was therefore proposed to have a subunit structure (11).

Some recent observations have lent biological credibility to the possible subunit structure of viral 60–70S RNA. It was found that avian sarcoma viruses harvested a 3 min intervals from cells contain 30–40S instead of 60–70S RNA. Upon incubation of such virus at 40° the 30–40S RNA species is converted to 60–70S RNA (14). Hence the 30–40S subunits appear to be precursors rather than fragments of 60–70S RNA (14).

The 30–40S RNA species of nondefective avian sarcoma viruses has been resolved electrophoretically into two size classes, a and b (3). Electrophoresis in formamide gels indicates that class b RNA is about 12 % smaller than class a RNA (13). Molecular weight estimates of class a RNA in formamide gels are between 2.9 and 3.4 x 10^6 depending on the reference RNA used as marker (13). The presence of class a RNA is well correlated with viral ability to induce focus formation in fibroblast cultures (7). Exceptions to this rule are replication-defective viruses such as the Bryan RSV and avian leukosis virus strain MC 29 which have focus forming ability but contain RNA which migrates with size class b (5, 6). Avian leukosis viruses and transformation-defective derivatives of sarcoma viruses contain only class b RNA (3, 5, 6, 7).

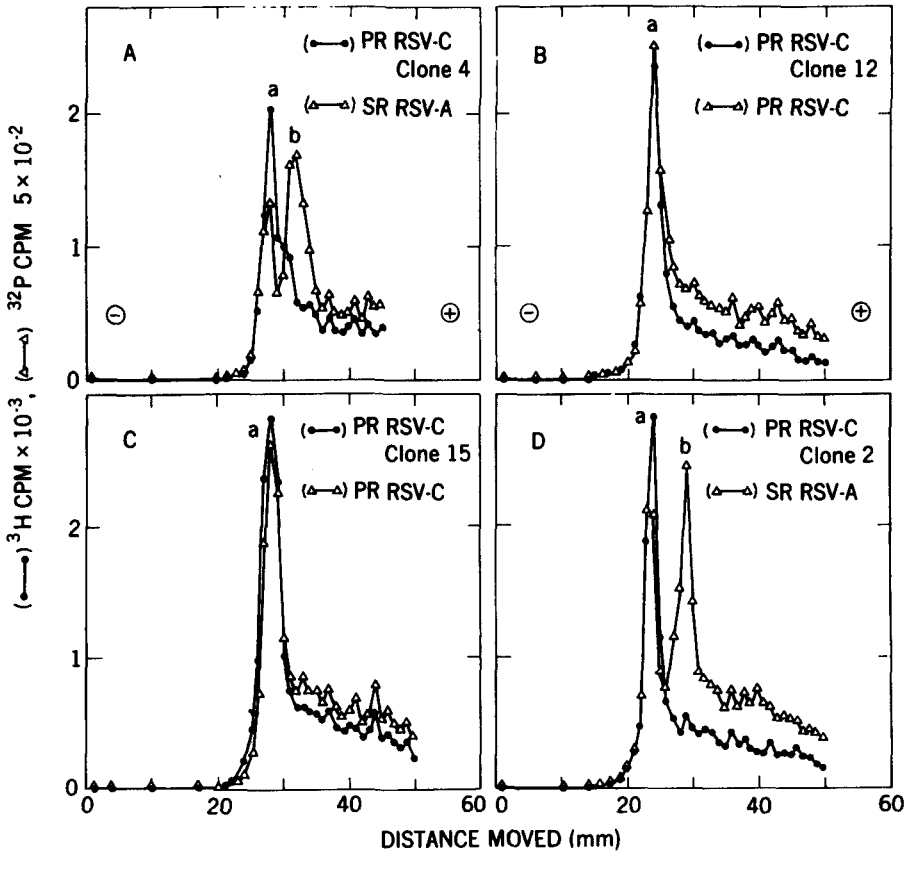


Fig. 1: The 60–70S RNA of 4 clones of PR RSV-C, propagated in cloned colonies of transformed cells, after heat-dissociation an electrophoresis in polyacrylamide. (A) Single colony-derived PR RSV-C clone 4 with a standard of uncloned SR RSV-A containing both class a and class b RNA. (B) Single colony-derived PR RSV-C clone 12 with cloned PR RSV-C as described for (A). (C) Single colony-derived PR RSV-C clone 15 with cloned PR RSV-C as for (A). (D) Single colony-derived PR RSV-C clone 2 with uncloned SR RSV-A as for (A). The data are from Duesberg and Vogt (7).

The 60–70S RNA of cloned avian leukosis and sarcoma viruses contains only one size class of 30–40S RNA.

The ratio of class a and b RNA in conventionally prepared stocks of avian sarcoma viruses is not constant; it varies with the passage history of the virus as well as with the virus strain (3, 5, 7). It has been shown that nondefective avian sarcoma viruses can segregate transformation-defective derivatives which no longer form foci in tissue culture but can replicate and are antigenically indistinguishable form the parental sarcoma virus (4, 5). Such transformation-defectives contain only class b RNA (3, 5, 6, 7). This observation suggested that class b RNA present in conventional stocks of nondefective sarcoma viruses might derive from transformation-defective segregants. Therefore, sarcoma viruses were cloned, both by single focus isolation and from colonies developing in agar after infection at low multiplicity (7). Figures 1. 5A,C and 6A,D show that rigorous cloning eliminates class b RNA from nondefective avian sarcoma viruses. The characteristic RNA pattern showing only class a RNA was seen in cultures derived from individual agar colonies for over two month, the lifetime of the cultures. We conclude that nondefective sarcoma viruses contain only class a RNA (7).

The 70S RNA of a recombinant: Support for crossing-over.

The 60–70S RNA of pure sarcoma viruses thus contains only the larger size class a subunits. The RNA subunits of leukosis viruses are exclusively of the smaller class b. The RNA of a recombinant between leukosis and sarcoma virus should then reveal the mechanism of recombination: if class a und b RNA are genome segments and recombinants arise by reassortment of these segments (15 ,16), the 60–70S RNA of the recombinant should contain both class a and class b subunits. However if recombinants are the result of crossing-over, their 60–70S RNA need not contain both size classes of subunits. Figure 2 shows an electropherogram of the heat-dissociated 60–70S RNA obtained from a recombinant of PR RSV-A and RAV-2. The gel pattern shows one major peak which co-migrates with class a RNA of a standard cloned sarcoma virus PR RSV-B. No distinct peak of class b RNA was discernible. This observation suggests the occurrence of crossing-over between RNA tumor virus genomes. However, the suggestion is based on an idealized interpretation of the electropherograms, which assumes that the heterogeneous material migrating faster than class a RNA conists of breakown products derived from class a RNA. Recently we have obtained direct support for this assumption. Comparing the RNA associated with the peak fractions of class a RNA of this recombinant to the minor heterogenous RNAs of the same virus by oligonucleotide fingerprinting (Duesberg, P. H, & Lai, M. M-C. unpublished, 1973), we found that their oligonucleotide fingerprints were identical. This indicates that the majority of the smaller RNA species are breakdown products of class a RNA rather than distinct subgenomic fragments.

High frequency recombination as observed between various strains of avian and murine RNA tumor viruses (15, 16, 17, 18) can be explained readily with a polyploid nonsegmented genome model (11, 12, 19, 20). The polyploid progeny from a mixed infection would be largely heterozygous. Such heterozygotes would contain different genomes united in the same 60–70S complex, thus increasing the chances for

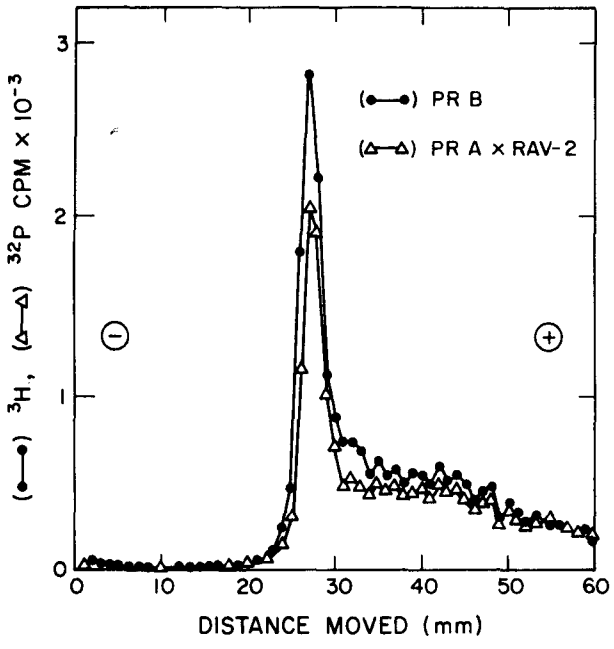


Fig. 2: The 60—70S RNA of cloned nondefective sarcoma virus PR RSV-B and of a recombinant between PR RSV-A and leukosis virus RAV-2 after heat-dissociation and electrophoresis in 2 % polyacrylamide. Conditions were as described for Fig. 1. The data are from Duesberg and Vogt (unpublished 1973).

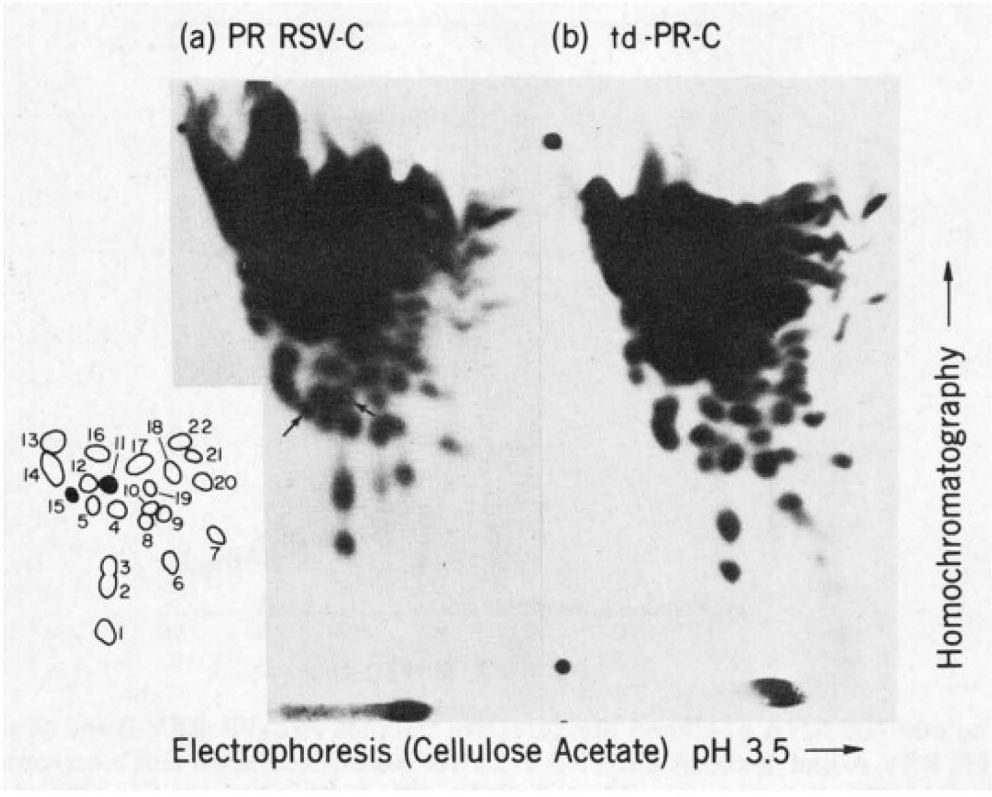
crossing-over in the next cycle of infection. DNA synthesis directed by the heterozygote 70S RNA template seems the most likely step for the occurrence of high-frequency crossing-over (20).

Sequence homology between class a and class b RNA of avian tumor viruses of the same subgroup and strain.

Given sarcoma viruses with only a subunits and transformation-defecitve (td) derivatives with only b subunits (4, 5, 6, 7, 26) we may predict that a subunits and b subunits of viruses of the same subgroup and strain must share homologous sequences. This follows because such corresponding virus strains probably share all replicative and many structural properties and therefore must have genetic information in common. We have tested this prediction by comparative oligonucleotide fingerprint analyses and RNA-DNA hybridizations of a and b subunits of corresponding viruses (21).

It was found by "fingerprinting" that all (about 20—25) large RNase TI-resistant oligonucleotides present in class b subunits of three transformation-defective viruses (td PR RSV-C, td B77 RSV-C and td SR RSV-A have homologous counterparts in the class a subunits of corresponding nondefective sarcoma viruses (PR RSV-C, B77 RSV-C and SR RSV-A (Fig. 3A, B, C)). In addition class a subunits contain a few (one or two) additional oligonucleotides which are not present in class b (Fig. 3 A, a; B, a; C, a, c). Thus the oligonucleotide fingerprints of transforming and td avian tumor viruses of the same subgroup and strain are very similar. By contrast the patterns of the avian tumor viruses of different strains and subgroups shown in Fig. 3 A, B and C were very different (21). This indicates that this method is well suited to detect similarities and differences among related virus strains.

A


(a) PR RSV-C
(b) td-PR-C
Homochromatography
Electrophoresis (Cellulose Acetate) pH 3.5

B

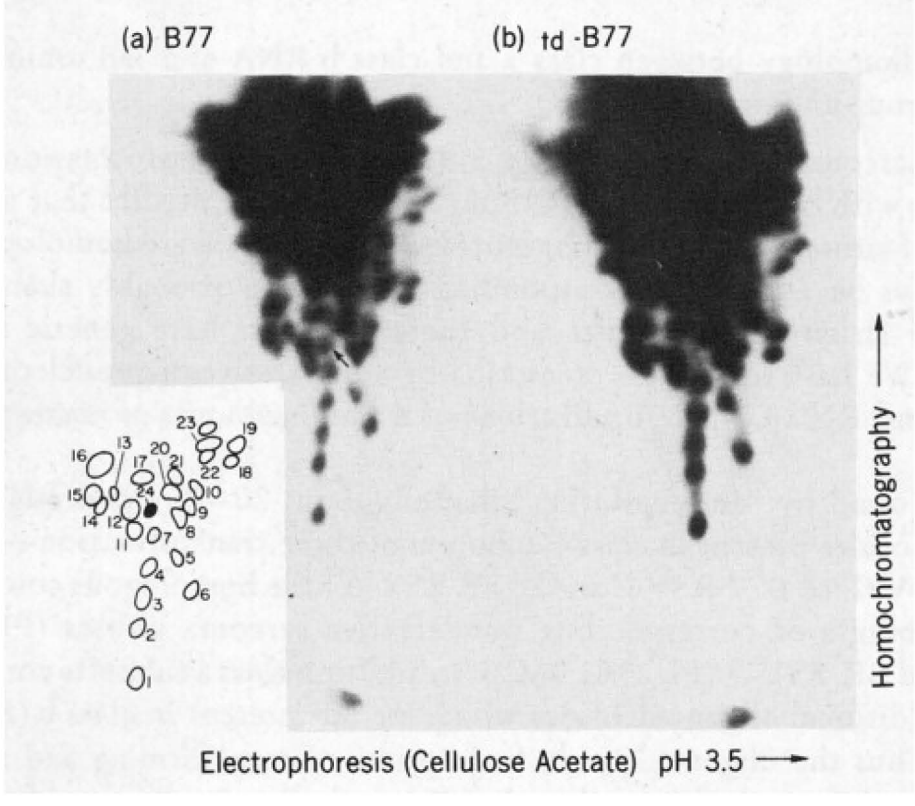
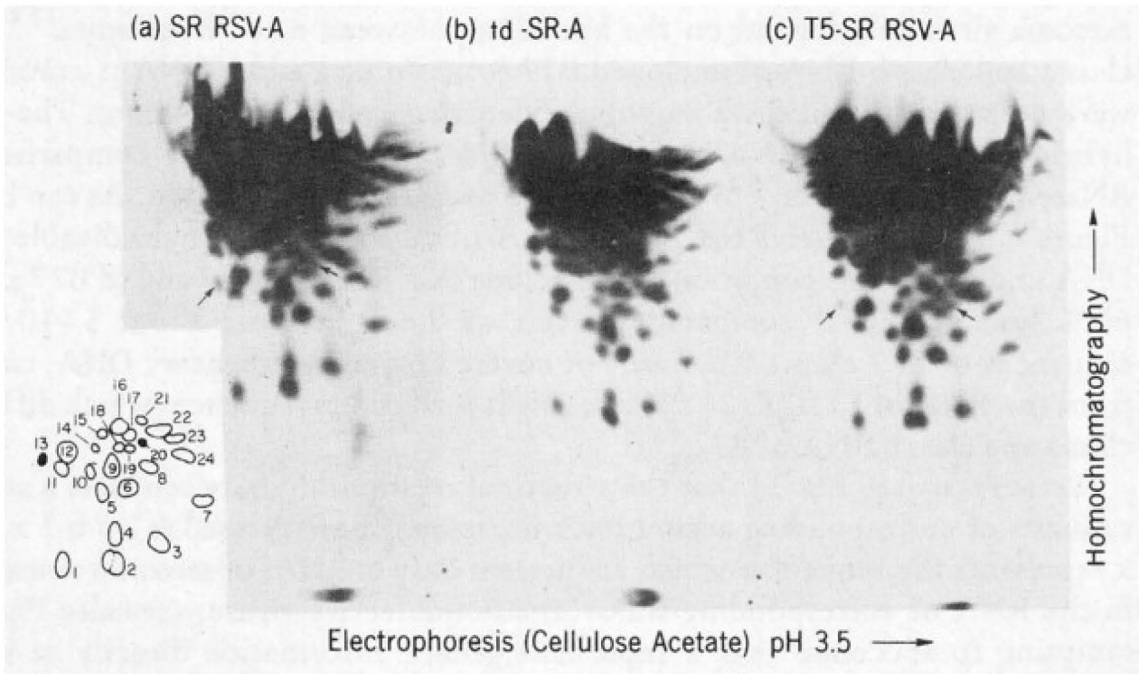

(a) B77
(b) td-B77
Homochromatography
Electrophoresis (Cellulose Acetate) pH 3.5

Fig. 3



C

Fig. 3: Two-dimensional separations of RNase T1 digests of 60–70S ^{32}P-RNAs of three nondefective sarcoma viruses (Aa, Ba, C,a,b) and of their transformation-defective counterparts (A, b, B,b, C,c). The patterns were recorded by autoradiography. The method was a modification of that described by Brownlee and Sanger (36). The oligonucleotide patterns of the following virus strains are shown: PR RSV-C (A,a), transformation-defective PR RSV-C (A,b), B77 RSV-C (B,a), transformation-defective B77 (B,b), SR RSV-A (C,a), a mutant of SR RSV-A which is temperature-sensitive for transforming ability T5 (37) (C,b) and transformation-defective SR RSV-A (C,c). The inserts are tracings of the major large oligonucleotides. The spots traced in black in the inserts and marked by arrows in the autoradiograms denote oligonucleotides found only in sarcoma viruses but not in corresponding transformation-defective viruses. The data are from Lai, Duesberg, Horst and Vogt (21).

However in comparing RNase T1-resistant oligonucleotides we are limited to only 2–3 % of the total viral RNA. The remainder of the RNA is degraded to fragments too small for analysis by "fingerprinting". Moreover, it cannot be said with certainty whether homologous oligonucleotides of a given sarcoma virus and its td derivative have the same sequence (21). Yet, it is plausible that most of the homologous oligonucleotides of a given sarcoma virus and its td derivative have the same or a very similar sequence for two reasons. First it is quite unlikely that two nonidentical RNAs with molecular weights of about 3×10^6 yield two homologous oligonucleotide patterns consisting of 20 large RNase T1-resistant oligonucleotides which have identical electrophoretic and chromatographic properties but have different sequences. Second it may be expected that biologically closely related viruses, such as sarcoma viruses and their td counterparts share many sequences. However we cannot fathom completely the relationship between class a subunits of a given sarcoma virus and the class b subunits of a corresponding td derivative by this method alone.

Therfore we have compared class a and class b RNA of B77 RSV-C by hybridization with the DNA transcribed from RNA of td B77 by viral polymerase. It is expected that this DNA will be about 70 % complementary to b subunit (22). However, the extent of hybridization between this "class b" DNA and class a RNA of a corresponding

sarcoma virus will depend on the homology between a and b subunits. ^{32}P-labeled class a and class b RNA of uncloned B77 (containing a and b RNA at a ratio of 1 : 2) were prepared by elution from polyacrylamidegel electropherograms. The extent of hybridization of ^{32}P-RNA with the ^{3}H-DNA was measured by comparison of the RNase-resistance of the ^{32}P-RNA before and after hybridization. As can be seen in Figure 4, 70 % of class b RNA and 60–65 % of class a RNA was hybridizable to td B77 DNA under the same condition. We conclude that RNAs of B77 and td B77 are at least 60 % homologous if compared by this method. Further, about 5–10 % of the sequences of B77 class a RNA are not covered by complementary DNA, transcribed from the RNA of td B77 (21). These might well be the sequences which differentiate class a and class b RNA of B77.

We suggest (see Fig. 5) that the structural relationship between class a and class b subunits of corresponding avian tumor viruses may be expressed as a = b + x, and that x represents the sequences which are present only in RNAs of sarcoma viruses but not in the RNA of corresponding transformationdefective viruses (see also Fig. 8). It is tempting to speculate that x represents genetic information directly or indirectly involved in transformation of fibroblasts.

Reappearance of class b RNA in cloned stocks of nondefective avian sarcoma viruses.

The close chemical relationship between a and b subunits made it likely that leukosis viruses may originate from sarcoma viruses by deletion of a sequence(s) x. This hypothesis was suggested by earlier studies in which we had observed that transformation-defective viruses segregate spontaneously from cloned nondefective sarcoma viruses (4, 5). We are asking now whether extensive or complete transition

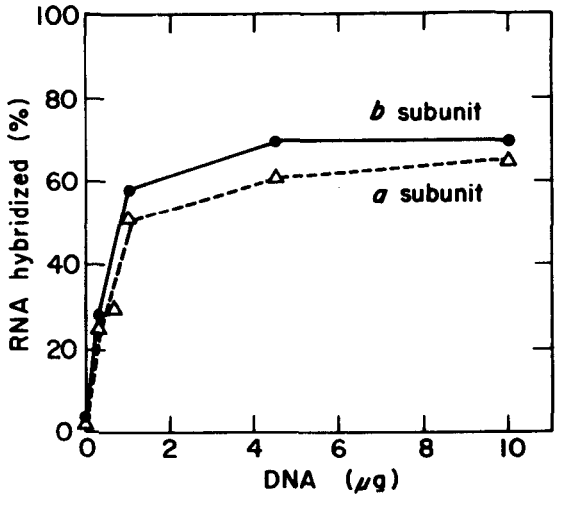


Fig. 4: Hybridization of ^{32}P-labeled class a (△---△) and class b (●——●) RNA of B77 RSV-C with DNA synthesized in vitro (22) from transformation-defective B77 virus. The percentage of RNA hybridized to DNA of transformation-defective B77 was measured by comparison of the RNase-resistance of ^{32}P-RNA before and after hybridization. The data are from Lai, Duesberg, Horst and Vogt (21).

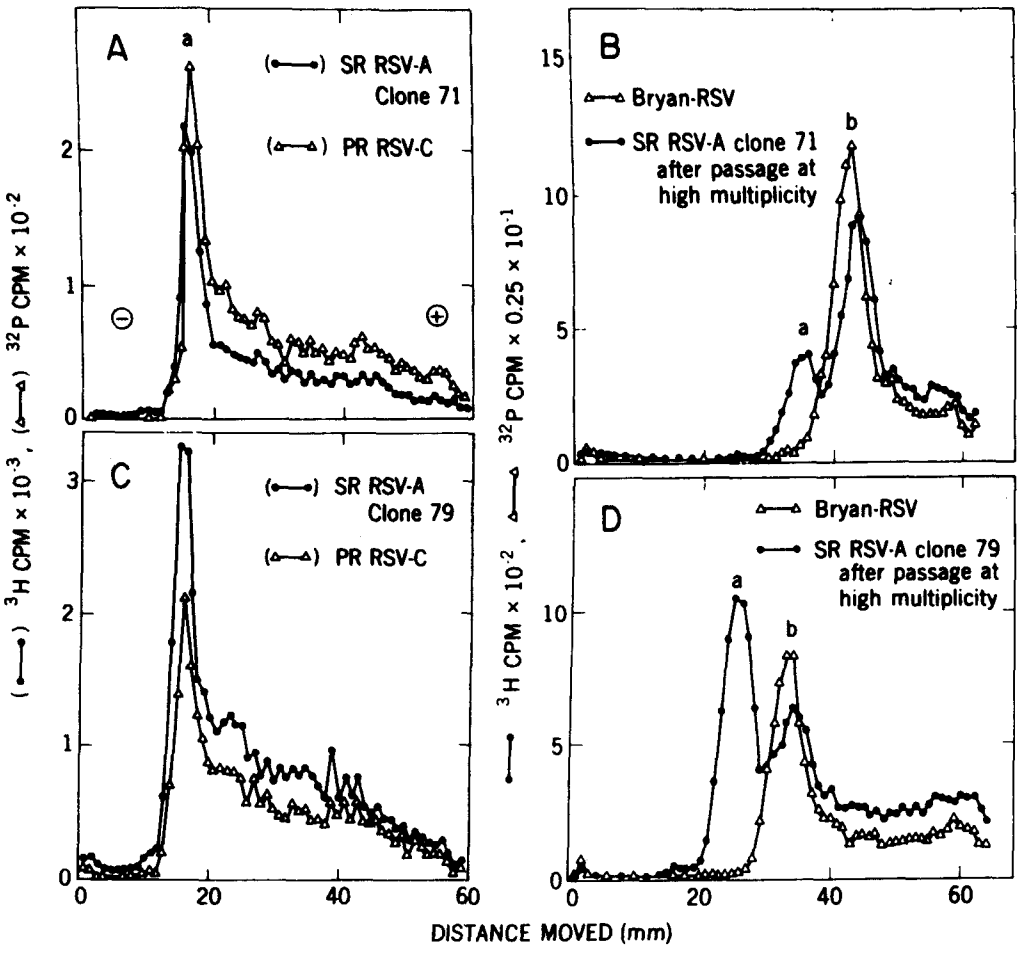


Fig. 5: The 60—70S RNA of 2 clones of SR RSV-A before (A, C) and after (B, D) five passages of the virus at high multiplicity of infection in chick embryo fibroblasts, after heat-dissociation and elektrophoresis in polyacrylamide. (A, C) show the RNA of clones 71 and clone 79 of SR RSV-A with a class A RNA standard of cloned PR RSV-C RNA as described for Fig. 1. (B, D) show the RNA of the two clones of SR RSV-A after passage at high multiplicity with a standard of Bryan RSV(RAV) RNA which contains only 30—40S RNA of class b (5, 6). The data are from Duesberg, Vogt, Maisel, Lai and Canaani (26).

from class a RNA of cloned nondefective sarcoma viruses to class b RNA of td derivatives can be demonstrated within a few viral life cycles. It was shown that the occurrence of deletions leading to incomplete or defective cytocidal viruses is favored by passage of viruses at high multiplicity of infection (23, 24, 25).

Several clonal preparations of nondefective avian sarcoma virus (SR RSV-A and B77) were propagated at high multiplicities (10 FFU per cell) for five passages with harvests at five day intervals (26). At the end of the fifth passage the viral RNA was labeled, extracted and analyzed by gel electrophoresis after heat-dissociation. Figures 5 und 6 show that the avian sarcoma viruses which, at the beginning of this experiment, contained only class a RNA (Figs. 5A, C, 6A, D) show significant amounts of class b RNA (Figs. 5B, D, 6B, C, E, F). In two cases class b even exceeded the level of class a (Figs. 5B, 6E, F) and in one, class a RNA was no longer detectable (Fig. 6B, C). Further the reappearance of class b RNA correlated roughly with a decrease (SR RSV) or an almost complete loss (B77) of the transformed morphology

of the host cell (26). It is likely that the reappearance of class b RNA represents segregation of transformation-defective viruses from the cloned sarcoma virus, and biological tests to examine this hypothesis are in progress. The results summarized in Figs. 5 and 6 suggest that infection at high multiplicity may lead to the appearance of class b RNA in nondefective avian sarcoma virus stocks similar to the von Magnus phenomenon in myxoviruses (23, 27). However, our experiments are still preliminary, and a causal relationship between high multiplicity of infection and reemergence of class b remains to be established (26). Nevertheless it appears that a complete transition from class a to class b RNA can be obtained within a few passages of nondefective avian sarcoma viruses at high multiplicity of infection.

The RNA of replication-defective RNA tumor viruses.

Most RNA sarcoma viruses are defective in replication and depend on a helper virus for the synthesis of infectious progeny. The extent of the defect varies with different virus strains: Bryan RSV is able to produce certain structural proteins which are

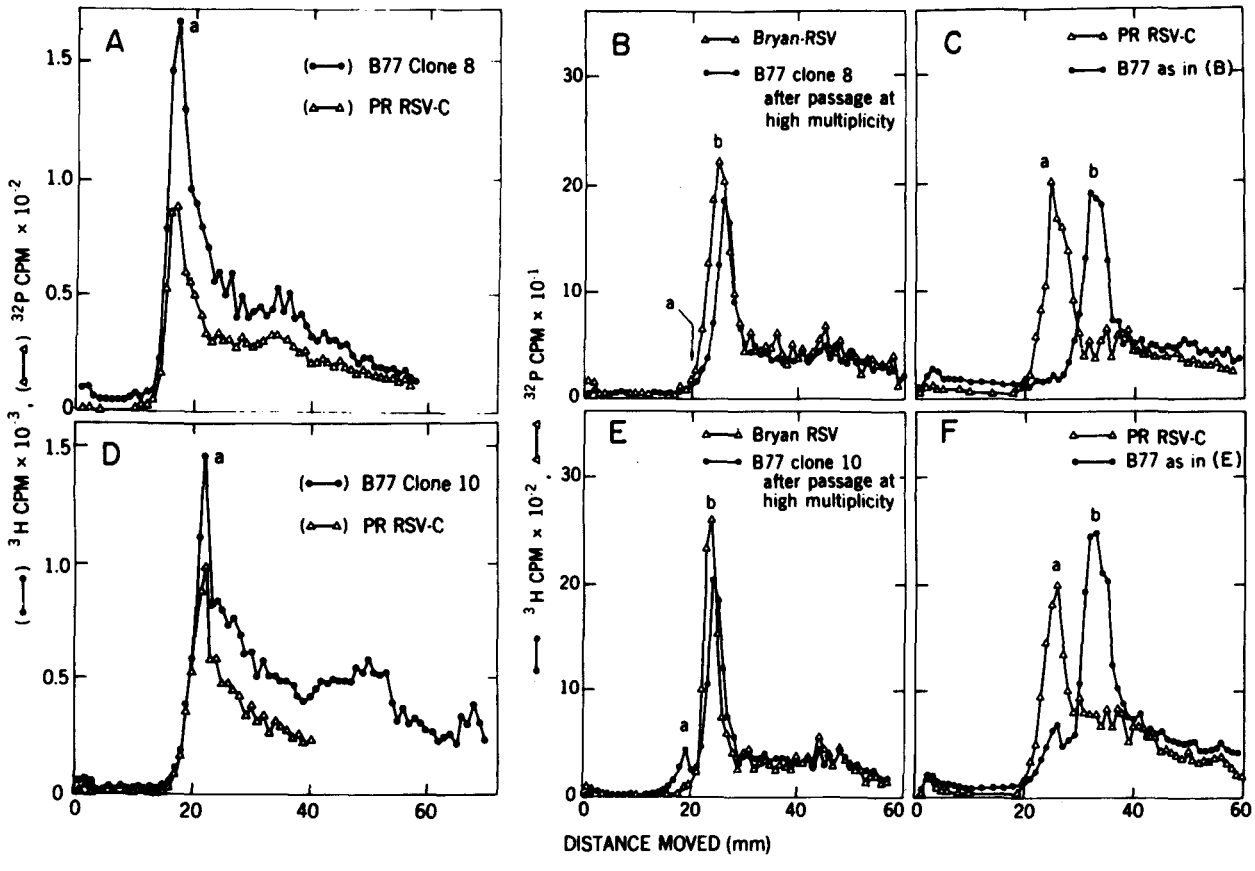


Fig. 6: The 60—70S RNAs of 2 clones of B77 RSV-C before (A, D) and after (B, C and E, F) five passages of the virus at high multiplicity of infection in chick embryo fibroblasts, after heat-dissociation and electrophoresis in polyacrylamide. (A, D) show the RNA of clone 10 and Clone 8 of B77 RSV-C with a class a RNA standard of cloned PR RSV-C as described for Fig. 1. (B, C and E, F) show the RNA of the 2 clones of B77 RSV-C after passage at high multiplicity with a standard of Bryan RSV(RAV) containing only class b RNA (B, E) and of cloned PR RSV-C RNA containing only class b RNA (B, E) and of cloned P RSV-C RNA containing only class a RNA (C, F). The data are from Duesberg, Vogt, Maisel, Lai und Canaani (26).

assembled into noninfectious virus particles (8, 28). Some of the murine sarcoma viruses show a similar degree of defectiveness (S+L-particles, [29, 30]). However in others, for example in Kirsten MSV the defects are more extensive so that no synthesis of viral structural proteins can be detected in the transformed cells (9).

Early analyses in our laboratory of the RNAs of Bryan RSV and MC 29, two defective sarcoma viruses, indicated absence of a subunit (5,6). One excuse to explain the absence of a subunit in these viruses was the following consideration. Since the defective sarcoma viruses studied were associated with a large excess of helper leukosis virus, electrophoretic detection of a subunit could have been obscured by the large preponderance of b subunit from leukosis virus. Alternatively the RNA of a defective sarcoma virus could have been smaller than a, due to deletions of structural and/or replicative functions of the RNA. Such a defective class a subunit may be only detectable if it were electrophoretically significantly different from class b subunit of associated helper virus or if cloned defective sarcoma virus, free of helper virus were available for analysis.

Scheele and Hanafusa have reported that the RNA subunits of RSV(-), presumably the cloned defective sarcoma virus of the Bryan RSV, have the same size as the b subunit of RAV-2, a typical leukosis virus (28). This is compatible with our earlier reports on the RNAs of Bryan RSV (5) and MC 29 (6). Because of the striking biological differences between these focus forming, replication-defective viruses and avian leukosis viruses, we hypothesize that the class b RNA found in Bryan RSV or in MC 29 differs from class b RNA found in leukosis viruses in two ways: (i) The RNA of defective sarcoma virus should contain sequences typical of its transforming ability and not present in leukosis viruses and (ii) it should lack sequences required for replication, which are present in leukosis viruses (compare model in Fig. 8).

Because of its defectiveness Kirsten MSV is of necessity associated with a helper murine leukemia virus (MLV). We have recently shown that when propagated in rat kidney cells Ki MSV is favored about 10–100 fold over its helper Ki MLV (31). Thus the RNA of the mixture of MSV and MLV released from such cells should be predominantly MSV RNA. Given a source of predominantly Kirsten MSV RNA, it was possible to compare Kirsten MSV RNA to that of pure Kirsten MLV RNA by electrophoresis in polyacrylamide gels. Kirsten MSV RNA was found to be smaller than that of its leukosis helper virus (31) (Fig. 7). However, the relationship between the RNAs of Kirsten MSV and Kirsten MLV is not clear at present. Available evidence suggests that the two RNAs share little perhaps no homologous sequences (31). Therefore we cannot say whether the RNA of defective Ki MSV resulted from deletions of an unknown nondefective murine sarcoma virus, which must be largely unrelated to Kirsten MLV, or whether the RNA of Kirsten MSV is perhaps cellular oncogenic information transducible by MLV.

DISCUSSION

Nondefective sarcoma viruses contain the complete information for virus reproduction and for cell transformation. The RNA which corresponds to this complete genetic endowment is size class a. From these sarcoma viruses, transformation-defectives can

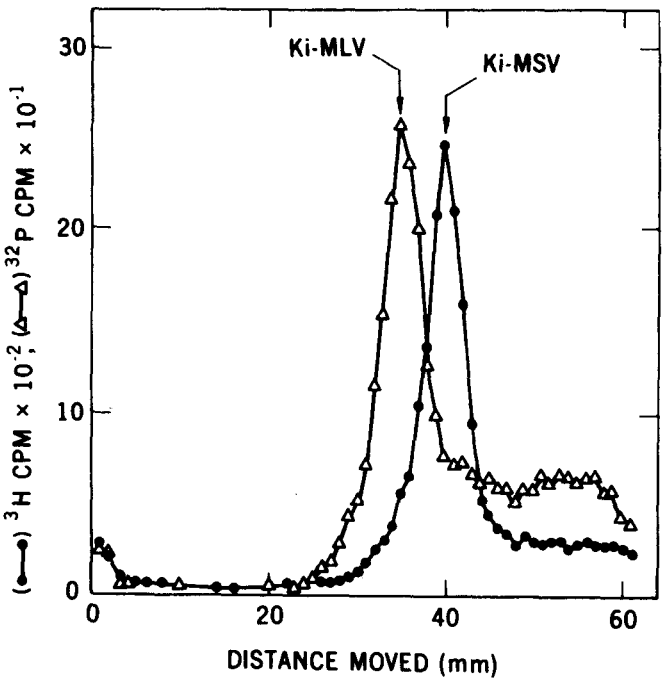


Fig. 7: The 50–70S RNA of Kirsten murine leukemia virus (Ki MLV) and Kirsten murine sarcoma virus (Ki MSV) after heat-dissociation and electrophoresis in polyacrylamide. The data are from Maisel, Klement, Lai and Duesberg (31).

be derived which resemble leukosis viruses in tissue culture: they replicate efficiently but do not induce transformation in fibroblasts. These transformation-defectives as well as leukosis viruses proper contain 30–40S RNA subunits of a smaller size, class b. Since transformation-defective derivatives share many properties with the parental sarcoma viruses, class a and b RNA must have genetic information in common. Chemical analyses directly indicate that class b RNA probably contains only sequences which are also represented in class a RNA. However, class a RNA contains some sequences which are not present in class b. Therefore we proposed their relation may be a = b + x, where x represents genetic information necessary for focus formation and b contains all information required for the synthesis of progeny virus particles. Some replication-defective viruses such as Bryan RSV and MC 29 lack class a RNA despite an ability to form foci in tissue culture. In an electrophoretic comparison the RNA of these replication-defectives was indistinguishable from the RNA of transformation-defective viruses and leukosis viruses. Nevertheless, it appears unlikely that 30–40S RNA of size class b obtained from transformation-defective and leukosis viruses contains the same basic information as class b RNA of replication-defective viruses. Both may be derived from class a but, while transformation-defectives have a deletion of focus forming genes resulting in 30–40S RNA of class b the replication-defectives may have a similar size deletion in replicative genes, resulting in defective 30–40S RNA of class a (Fig. 8). This relationship could be expressed by, defective a = a – y wherein a defective a contains all of the genetic information necessary for focus formation and y represents replicative genes deleted from a. More extensive replicative defects correspond to a smaller RNA, as might be indicated by the observations with Kirsten sarcoma virus.

Besides the known defective viruses which contain lesions in either focus forming ability or in replication, other defectives may occur which lack transforming as well as replicative information. These would be replication-defective leukosis, or replication and transformation-defective sarcoma viruses (Fig. 8). Such viruses would be difficult to detect directly but could perhaps be demonstrated by marker rescue or be detected as radioactive defective virions. It has been postulated that defective viruses represent deletions of nondefective sarcoma viruses (4, 5, 6, 10) or are actually incomplete viral intermediates evolving from cellular genes (10), as proposed by Temin's protovirus hypothesis (32). We conclude that the various size classes of tumor virus RNA can be formally derived from class a RNA of nondefective sarcoma viruses by various deletions in replicative or transforming genes (Fig. 8).

We can only speculate as to how defective tumor virus RNAs arise. It could happen during transcription from RNA to DNA or DNA back to RNA; it could also be due to posttranscriptional cleavage or to an event similar to recombination between multiple DNA proviruses in the cell. The phenomenon of defective viral RNAs is not limited to RNA tumor viruses. The first historic example is the von Magnus phenomenon of influenza virus (23, 27). It describes the origin of defective virus after passage at high multiplicity. We have found in 1968, that von Magnus virus contains RNAs smaller than that of standard virus (33). Vescicular stomatitis virus was shown to form defective interfering particles with RNAs shorter than those of complete virions (25) and other RNA and DNA viruses were shown to produce defective and interfering particles containing an incomplete complement of nucleic acid (24, 5). Even soluble

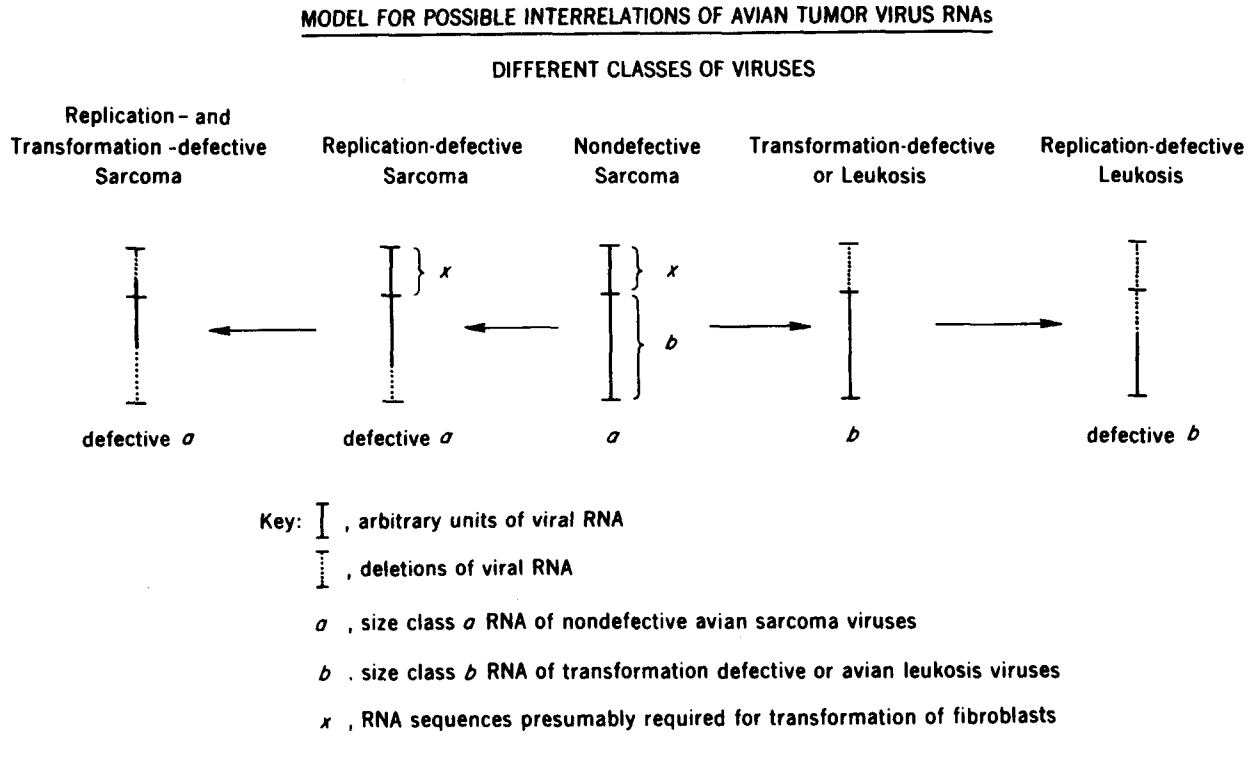


Fig. 8: Models for possible relationships between the RNAs of different classes of RNA tumor viruses.

Qß-phage replicase, given Qß RNA as template, synthesizes under conditions of high selective pressure, little "monster" RNAs which are only 17 % as large as the complete Qß RNA template (34, 5).

The origin of all these defective viruses or RNA molecules has one principal in common: They arise at high multiplicity of infection presumably in highly competitive conditions. These conditions may be more readily established by RNA tumor viruses than by cytocidal viruses, because most RNA tumor viruses do not kill the cell and can virtually saturate the cell with particles competing for replication. So perhaps defective virus particles are not so bad after all, by competing and interfering with their nondefective counterparts, they may function as natural contraceptives for viruses.

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LIFE CYCLE OF RNA ONCOGENIC VIRUSES

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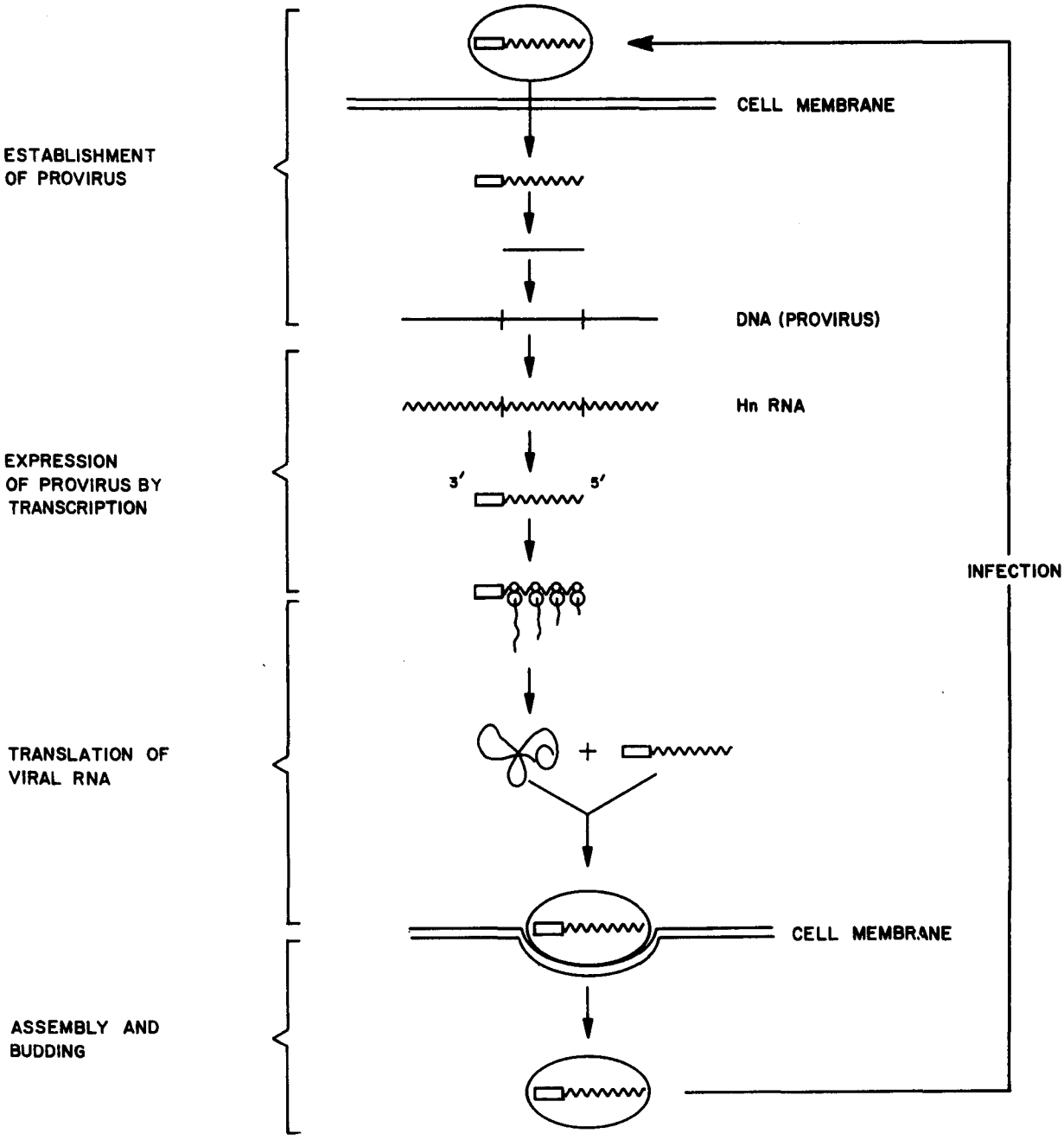
In view of the increasing evidence suggesting that human cancer cells contain molecular footprints related to murine and primate type-C RNA tumor viruses (1–7), it is appropriate in this symposium to discuss a simple scheme for the life cycle of RNA tumor viruses. It is hoped that this scheme will be useful in searching for virus related proteins, nucleic acids and particulates in human leukemic cells, and in providing a working hypothesis for further studies on the mechanism of viral replication. The scheme is illustrated in the attached figure.

Virus particles

The RNA viruses contain a single stranded RNA with a molecular weight of 10^7 daltons which normally sediments at 70S and some other smaller RNA molecules which sediment at 35S, 28S, 18S and 4–5S. (For a review see ref. No. 8). The RNA contain a sequence of poly (A) with a size of about 200 nucleotides (9–11) which is probably located at the 3′ end of the RNA chain (12, 13). In avian RNA viruses, the 70S RNA can be dissociated to subunits of 30 to 40S (14, 15, 16). However, in mammalian RNA tumor viruses, the subunit structure of the 70S RNA is not well characterized (17, 18). The RNA is located in the core of the viruses (19–21). Some other important molecules such as reverse transcriptases (22, 23) and group specific (gs) antigens (24) are also located in the viral core. Generally, the cores are enveloped in a lipoprotein membrane (25). The average density of whole virus particles is about 1.16 gm/cm^3 measured in a sucrose density gradient while that of the cores is about 1.25 gm/cm^3 (26, 27). (For a recent review see ref. 28.)

DIAGRAMMATICAL REPRESENTATION OF LIVE CYCLE OF RNA TUMOR VIRUSES



SYMBOLS :

- : POLY (A)
- : VIRAL RNA WITH POLY (A)
- : MONOSOMAL RIBOSOME
- : POLYPEPTIDE
- : DNA
- Hn : HETEROGENOUS RNA

Possible States of Provirus Gene Expression in Balb/C 3T3 Cells

Classification of Cells	State of Expression	Cell State[1]	Induction of Infectious Viruses	Detection of Virus-Like		Examples
				Reverse Transcriptase	Nucleic Acid	
Uninfected	Unexpressed	N_T	Yes	Not Tested	Not Tested	Uninfected 3T3 CLS,
Non-Producer	Partial	T	Yes	Yes	Yes	Balb/K 3T3[3] Balb/M 3T3[4]
Producer						
A) Defective Particles	Partial	T	Yes	Yes	Yes	S^+L^-[5]
B) Infectious Particles Transforming	Full	T	N.A.[2]	Yes	Yes	MuSV (MuLV) Infection
Non-Transforming	Full	NT	N.A.	Yes	Yes	MuLV Infection

[1] N.T. - Not Transformed; T – Transformed

[2] N.A. - Not Applicable

[3] Balb/C K3T3 – Non-Producer of Balb/3T3 Infected with Kirsten Murine Sarcoma Viruses.

[4] Balb/C M3T3 – Non-Producer of Balb/3T3 Infected with Moloney Murine Sarcoma Viruses.

[5] S^+L^- - Any Cells Containing Sarcoma Virus Genetic Information, but not Leukemia Virus Genetic Information. In Case of Murine S^+L^- Cells, they are Producing Defective Particles.

Establishment of Provirus

Upon infection, the envelopes of the viruses which are presumably responsible for attachment and penetration of virus to cells are decoated. The location of the decoating is debatable (29, 30) and its mechanism is not known. The genetic information residue in the exposed RNA is then transcribed into DNA form. This transcriptional step is mainly catalyzed by RNA directed DNA polymerase (conventionally called reverse transcriptase or RNA dependent DNA polymerase) (31–34; for recent review see reference No. 35). Some other enzymes such as ribonuclease H (viral and/or cellular) (34–39), exonuclease and DNA ligase (41) might also participate in this transition. The subcellular location of this transition is still unclear (29, 30, 42). Two lines of information suggest that this "reverse" transcriptional step is essential for viral transformation and/or viral infection. These are: (a) A mutant of Rous sarcoma viruses (RSV) designated as RSV (0) which is noninfectious (43), also lacks RNA directed DNA polymerases (43); (b) A treatment of virus particles with various derivatives of rifamycin SV, inhibitors of reverse transcriptase, resulted in a loss of viral infectivity in culture (45) and in animals (46). The size of DNA product inside the infected cells is not known, although the size of DNA product in most *in vitro* systems has been found to be 4–5S (47–49). The poly (A) sequence of RNA probably is not copied because it was reported that in an *in vitro* endogenous reaction system, the DNA product did not contain a sequence of poly (T) (50). The DNA product *in vivo* is termed the *provirus*. It is not known whether the provirus exists in the infected cell in a free form as an episome, in an integrated part of some episome or in an integrated part of the host chromosomal structure. In avian systems, some preliminary experiments suggested that proviruses are in integrated forms (51). Nothing is known about the mechanism of integration.

State of the provirus

The genetic information of the provirus may stay unexpressed, partially expressed or fully expressed. Examples of these are shown in Table 1. In uninfected murine fibroblasts there is no sign of infection or transformation. However, intact viruses (termed "endogenous virus") can be produced from some of these cells on treatment with a halogenated deoxyuridine (52, 53). For example, two types of infectous viruses (Balb virus –1 and Balb virus –2) are induced from Balb/3T3 cells by IdU (54). It appears than that the genetic information of the provirus in these cells is not expressed. Alternatively, some expression of the proviral information may occur, and not be detectable due to lack of known characteristics. Normally murine sarcoma viruses are always associated with murine leukemia virus but murine leukemia viruses can be obtained without association of sarcoma viruses. Therefore, the outcome of viral infection by the murine sarcoma leukemia complex depends on the composition of sarcoma viruses and leukemia viruses in the inoculants. If cells are infected with a sarcoma-leukemia virus complex which is dominated by leukemia viruses almost all of the infected cells are transformed and are able to produce both sarcoma and leukemia infectious particles (55). However, if cells are infected with sarcoma-leukemia virus complex, dominated by sarcoma viruses, again most of the infected cells are transformed and are producing infectious particles but some of the transformed cells are

either nonproducers (56, 57) or S^+L^- (58, 59). S^+L^- is defined as any cells containing sarcoma virus genetic information but not that of leukemia virus. In case of murine S^+L^- cells, they are producing defective particles. These virally transformed nonproducer and S^+L^- cells, can be considered examples of cells exhibiting partial expression of the proviral genome. In infected but not virus producing cells, although no release of virus particles has yet been detected (56, 57), nucleic acid sequences (RNA) homologous to nucleic acid sequences (DNA products) of infected virus and virus-like reverse transcriptase have been observed. The role of virus-like RNA is understandable, however, the significance of reverse transcriptase in nonproducing cells deserves some thought. The possibility that the presence of reverse transcriptase is due to "leakiness" in gene regulation with no apparent function of the enzyme cannot be ruled out. Alternatively, reverse transcriptase might be involved in gene amplification of proviral genomes. Whatever the role of reverse transcriptase is, the presence of virus-like reverse transcriptase in a nonproducing cell provides us with a "footprint" for the presence of viral information, in the cells in question. This concept of possible presence of viral information in nonproducers is, in fact, substantiated by the fact that the expression of genetic information required for virus production can be induced either by helper virus or by chemical agents such as 5-iodo-2-deoxyuridine (IdU) or 5-bromo-2-deoxyuridine (BrdU) (52, 53, 60). Human leukemic cells can be considered as a type of nonproducers because they are transformed cells which do not produce virus particles, at least not in most circumstances as far as we can tell, but they contain nucleic acids homologous to those of the primate and murine RNA tumor viruses (3, 4, 5) and their reverse transcriptases (1, 2) share common antigenitity with primate and murine RNA tumor viruses (6, 7). Obviously, only the induction of infectious virus particles from human leukemic cells is the proof of infection. The other example of partial expression of the provirus is the S^+L^- cells (58). These cells are producing virus particles but the particles are defective in their content of both viral nucleic acid (61) and of viral reverse transcriptase (62). Just like the nonproducers, S^+L^- cells also contain (a) nucleic acid sequences homologous to viral nucleic acid sequences (61) and (b) virus-like reverse transcriptase (63). Furthermore, infectious particles (both of sarcoma and leukemia nature) can be induced by helper virus (58, 64) or by the chemical agents (IdU or BrdU) from S^+L^- cells (58). Apparently, both nonproducer and S^+L^- cells contain sufficient information for viral replication, but the degree of expression is regulated. Finally in case of virus producing cells after infection, they can be divided into transformed and nontransformed cells. In general, the sarcoma-leukemia virus complexes cause transformation both in animals and in culture, while the leukemia viruses cause transformation in animals but not in cultured fibroblasts.

Expression of Provirus

The RNA of RNA tumor viruses is similar to cellular m-RNA in at least two aspects. Both contain a sequence of poly (A) (9–11) which is probably at the 3′ end (13, 14; 65–67) and both can be used as for cell free protein synthesis (68, 69). With this information in mind, we previously proposed that the process of expression of proviral genome upon induction is probably similar or identical to processing of cellular

m-RNA (66, 70). Heterogenous large RNA molecules would be synthesized first, reactions catalyzed by host RNA polymerases of host origin (since no novel RNA polymerases in the virus infected cells has yet been found [Sethi and Gallo, unpublished data]). When synthesized, the heterogenous RNA molecules are much larger than that of m-RNA (71), but they are cleared and degraded to a smaller size accompanied by an addition of a sequence of poly (A). This final RNA is the mature viral mRNA, similar to cellular m-RNA (65, 67); the poly (A) sequence of viral RNA is probably added to 3′ end sequentially rather than segmentally. It takes about 10 minutes to complete the process from synthesis of heterogenous RNA to the addition of poly (A) sequence for cellular m-RNA (67, 71) and poly (A) synthesis can be preferentially inhibited by cordycepin (3′-deoxyadenosine) (66). The necessity of poly (A) synthesis in viral replication was suggested by the fact that virus production induced by IdU from BALB/K3T3 cells can be inhibited by a relatively low concentration of cordycepin (70, 72). The enzymes required for poly (A) synthesis and for degradation of heterogenous RNA are not known.

Little is known about synthesis of virus specific proteins *in vivo*. Recently, it has been shown that *in vitro* the viral RNA can be used as templates in a cell free protein synthesis system (69, 73). In all of these reports, the results are rather preliminary either due to lack of species specificity (only works well with an *E. coli* cell free system), or due to the fact that the protein products are not specific. However, these *in vitro* findings suggest that the viral RNA, after transportation, can serve as templates for the synthesis of virus specific proteins by host translational machinery. Recently, virus-specific mRNA and nascent polypeptides was demonstrated in the polyribosomes of transformed cells replicating murine sarcoma-leukemia viruses (74). This finding further supports the proposal of using translational machinery for viral replication. Involvement of a unique species of tRNA, quantitative change in pre-existing tRNA or appearance of specific modifying enzymes (such as tRNA-methyl-transferases) are possible regulations at this step. Recently, we observed that dexamethasone and many other glucogenic corticosteroids were able to stimulate virus production 3–25 fold from nonproducer (Balb/K3T3 cells) induced by IdU (72). Similar stimulation of virus production was observed in virus producing cells (75). Studies on the effective time course for the hormone effect indicated that these steroids acted after poly (A) synthesis, since a low concentration of cordycepin could nullify the stimulatory activity of the hormone (72, 75).

After virus specific proteins are made, the packaging of virus particles is then feasible. Again, little is known about assembly of virus particles. It is possible that the same viral RNA which were used as a m-RNA in translation were then packed and released as virus particles (74). The final step in the release of virus particles is generally characterized by budding. The budding can occur either intracytoplasmically and/or extracellularly (76). With avian viruses, soon after budding, the size of RNA is about 35S. Somehow the 35S RNA molecules are converted to 70S RNA at the relative high culturing temperature (37–40 °C) (77). The relationship of infectivity before and after conversion of RNA to 70S is not clear. It is not known whether there is such a conversion in the mamalian system.

Conclusion

The above scheme for the life cycle of type-C RNA tumor viruses is a simplified, and perhaps biased summary of observations regarding their replication mixed with some speculation for the sake of completion. The portions derived from speculations may be useful as working hypotheses for studying the mechanism of viral replication in animal systems, and for evaluating results of studies looking for molecular "footprints" of these viruses human cancer cells. The analogy of human leukemis cells and murine nonproducer cells is of particular interest. In fact, it has been shown that the virus related nucleic acids in human leukemic cells have a higher degree of homology with sarcoma viruses than with those of leukemia viruses (both from mouse and monkey) (5). Since the murine nonproducers also contain the sarcoma genome, this observation may be of particular relevance for understanding putative viral oncogenesis of human leukemia.

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RNA TUMOR VIRUSES AND HUMAN LEUKEMIAS

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RNA Tumor Viruses and Human Leukemias

Considerable information is available concerning the etiological role of RNA tumor viruses in leukemias, lymphomas, sarcomas, and breast cancer in a variety of animals, e. g., chicken (1), mice (2), cats (3), etc. In animals, causative proof has been achieved by inoculation experiments that satisfy Koch's postulates. In man, such experiments obviously cannot be undertaken, and other more indirect evidence has to be assembled to clarify the etiology of human malignancies with its implications for prophylaxis and therapy.

The data reported here on the presence of viral-like particles in human leukemias are based on the technique of molecular hybridization of nucleic acids (4—10) and of the simultaneous detection of high molecular weight RNA associated with a reverse transcriptase at physical densities characteristic of oncogenic RNA viruses (11—17).

The general biochemical and physical properties of the RNA tumor viruses include a characteristic density of 1.16 g/cm^3. Inside an outer shell they contain nucleoids with a density of 1.23 g/cm^3. As genetic material they possess a high molecular weight, with 60 to 70S-sedimenting RNA, which can dissociate into 35S subunits. The RNA is associated with an RNA-dependent DNA polymerase (reverse transcriptase) (18, 19). With this enzyme, a radioactively labeled DNA can be synthesized that is complementary to the tumor virus RNA (5).

Several considerations (5, 7) led us to the assumption that the RNAs of different mammalian leukemia viruses might be related to each other with regard to their base sequence. To test this assumption, radioactive DNA , which had been synthesized on RNA of a leukemia virus of a mouse (RLV), was hybridized to RNA isolated from leukocytes of a leukemic rat (7). After a positive outcome of this comparison involving two closely related animal hosts, a similar experiment was undertaken by hybridizing RLV-^{3}H-DNA to RNA isolated from the cytoplasm of human leukemic cells (7). In Fig. 1 the density profiles are shown of hybridizations with RNAs from four human leukemias. The ^{3}H-DNA mainly bands in the DNA density region of the gradient (ρ = 1.45 g/cm^3), but some of the radioactive DNA can be seen banding in the RNA density region of the gradient (ρ = 1.65 g/cm^3) complexed to the denser RNA. Homologies to mouse leukemia virus RNA were found, whether

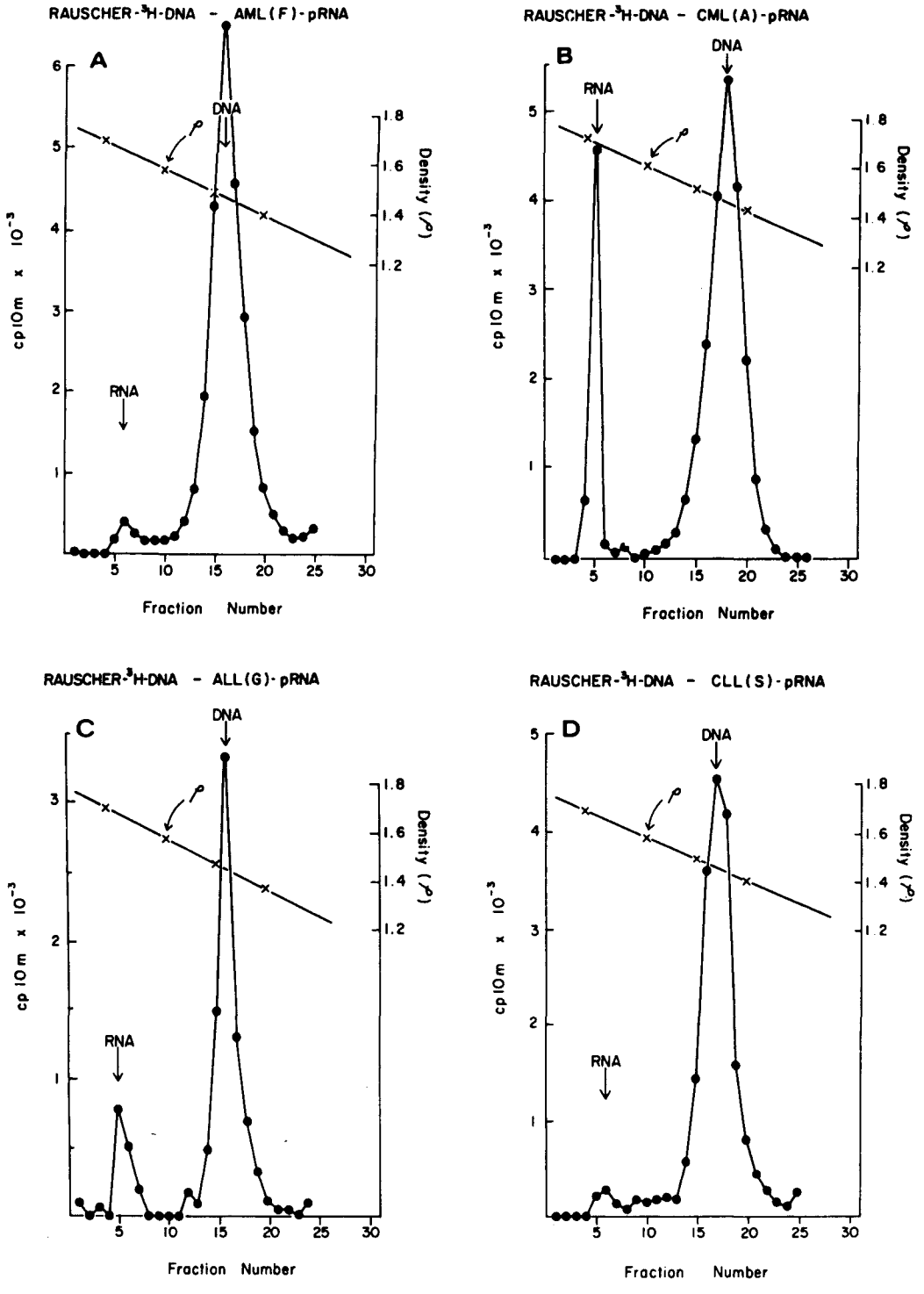

Fig. 1: Cs_2SO_4 density profiles of hybridization reactions between ^{3}H-RLV-DNA and cytoplasmic RNA isolated from human leukemic white blood cells. ^{3}H-RLV-DNA and cytoplasmic RNA were prepared as described (7). About 350 µg RNA was hybridized to 2000 cpm of purified, heat denatured ^{3}H-RLV-DNA (10,000 cpm/pmol) for 18 hr at 37 °C in the presence of 0.4 M NaCl and 50 % formamide (total vol 60 µl). After incubation, the reaction mixture was added to 10 ml half-saturated Cs_2SO_4 (ρ = 1.52 g/cm^3) in 5 mM EDTA and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15 °C. Fractions of 0.4 ml were collected from below and assayed for TCA-precipitable activity. The cytoplasmic RNA was derived from A) AML (F), B) CML (A), C) ALL (G), D) CLL (S).

the human leukemic RNA was derived from lymphoblastic or myelogenous, acute, or chronic leukemias. Positive outcomes were observed in 28 out of 31 leukemic patients, i. e., in more than 90 % the RNA isolated from leukemic leukocytes showed homology to the RNA of a mouse leukemia virus. In contrast, more than 50 normal control tissues, including normal leukocytes, did not show this kind of response.

The position of the RNA-DNA-hybrids in the Cs_2SO_4 density gradients indicates that the human leukemia-specific RNA determines the density of the hybrids and therefore is considerably larger than the 5–8S large radioactive DNA. It was logical to assume that this large RNA is of the 60–70S type and possibly associated with a reverse transcriptase in a complete viral particle.

The resolution of these and related questions was made feasible by the apllication of a technique for the simultaneous detection of a high molecular weight RNA associated with a reverse transcriptase (11, 12). The basis for the simultaneous detection of 60–70S RNA and reverse transcriptase stemmed from the observations (20–22) that the initial DNA product is found complexed to its 70S-RNA template. If early DNA product is found on sedimentation analysis to travel as if it were a 70S molecule, and if supplementary evidence is provided that its apparent size is due to its being complexed to a 70S-RNA molecule, evidence is provided for the presence of reverse transcriptase that uses a 70S-RNA template.

Figure 2 shows representative results of experiments along these lines with leukocytes of leukemic patients (13, 16). After an endogenous reverse transcriptase reaction and deproteinization of the nucleic acids a portion of the ^{3}H-DNA sediments in discrete 70S and 35S peaks in glycerol-sedimentation-gradients. After RNase digestion, the entire radioactive DNA is found in the low molecular weight region of the gradient, as exemplified in Fig. 2B. This proves that the ^{3}H-DNA was complexed to a high molecular weight RNA. In Fig. 2, experiments with cell material from patients with ALL (A) and CML (B) are shown. In total, more than 50 leukemic cell samples gave the specific DNA-synthesis resulting in ^{3}H-DNA complexed to a 70S RNA whereas only one out of 27 normal white blood cell samples yielded evidence for this type of activity. In this case, the available white blood cells did not permit further characterization by hybridization and thus an unambiguous statement as to the nature of this positive reaction cannot be made.

After extensive alkali digestion to destroy all RNA present, the ^{3}H-DNA was recovered and annealed to viral-enriched RNA of the same leukemic cells that had been used for the specific DNA synthesis. From Fig. 3A, it is clear that about 20 % of the ^{3}H-DNA is shifted from the DNA region to the hybrid and RNA regions of the gradient due to formation of RNA-DNA hybrid structures. When the same human ^{3}H-DNA is annealed with an equivalent amount of AMV-70S-RNA, no evidence of hybrid formation is seen (3B). This back hybridization completes the operational definition of a reverse transcriptase and shows that human leukemias contain 70S RNA-associated reverse transcriptase. These data are in agreement with the finding by other groups of reverse transcriptase in human leukemic cells (23, 24).

By density fractionation of the leukemic cytoplasm, it could be shown that this 70S-RNA-DNA polymerase complex has a density of 1.16 g/cm^3 (Fig. 4), which is

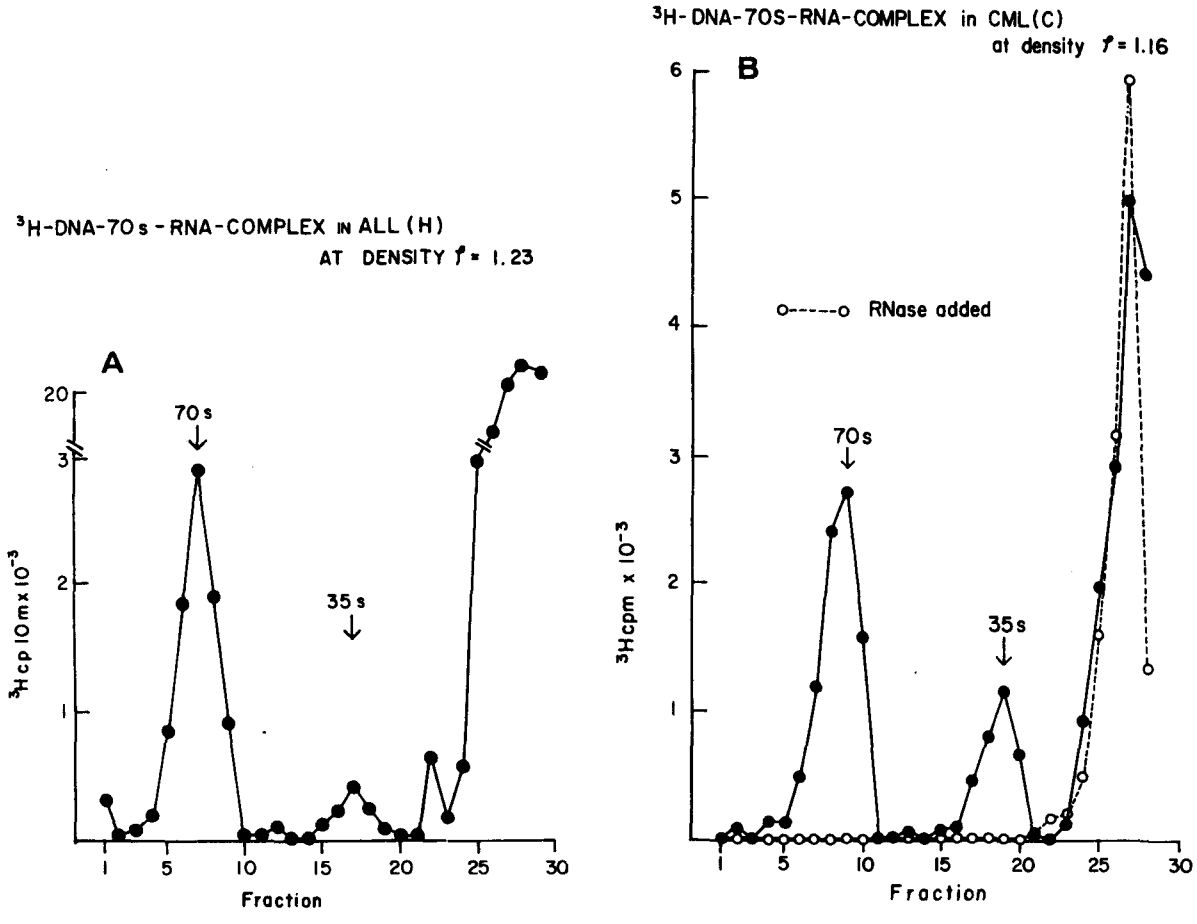

Fig. 2: Simultaneous detection of 60—70S RNA and reverse transcriptase in human leukemic white blood cells. 0.5—2 g of white blood cells were disrupted in TNE buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA) with a Dounce homogenizer. Nuclei were removed by centrifugation of the cell homogenate at 4000 X g for 10 min at 0 °C, and the supernatant was centrifuged at 10,000 X g for 10 min at 0 °C. The resulting postmitochondrial supernatant fluid was then layered on a 20-ml column of 20 % glycerol in TNE and spun at 100,000 X g for 1 hr at 1 °C in a Spinco SW-27 rotor. The resulting pellet was resuspended in TNE buffer (2 ml per 1 g of cells). Insoluble debris was removed from this suspension by centrifugation at 4000 X g for 10 min. For further purification and density determination, the suspension was then layered on several preformed 20—60 % sucrose TNE gradients and spun for 3 hr at 100,000 X g and 4 °C. Seven to 10 fractions were collected from each gradient. Each fraction was diluted in TNM buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$), pelleted (100,000 X g, 1 hr, 4 °C, Spinco SW-27 rotor), resuspended in 0.3 ml of 0.1 M NaCl – 0.01 M Tris-HCl, pH 8.3, and assayed for endogenous reverse transcriptase activity: After preincubation with 0.1 % Nonidet P-40 at 0 °C for 15 min. 3 µmol $MgCl_2$, 0.4 µmol each of dATP, dGTP, dCTP, 1 mCi ^{3}H-TTP (NEN, 50.1 mCi/mmole) and 100 µg/ml actinomycin D to inhibit DNA-instructed DNA synthesis were added to give a final vol of 0.5 ml. The reaction was incubated at 37 °C for 15 min. After adjustment to 0.2 M NaCl to stabilize hybrid structures, the reaction was terminated by addition of 0.5 % SDS and subsequent deproteinization by phenol-cresol extraction. The aqueous phase was then layered on a 10—30 % glycerol in TNE gradient and centrifuged at 40,000 rpm for 3 hr at 1 °C (SW-41 rotor, Spinco). Fractions of 0.4 ml were collected from below and assayed for TCA-precipitable radioactivity. 18 and 28S r-RNA and 70S AMV-RNA served as markers. The sedimentation profiles of two representative simultaneous detection tests with the appropriate density fractions are shown. A) ALL (H) at density ρ = 1.23, B) CML (Con) at density ρ = 1.16 g/cm^3. RNase sensitivity was tested by incubating the reaction mixture with 80 µg/ml ribonuclease A (Worthington) for 15 min at 37 °C prior to sedimentation analysis.

characteristic of RNA tumor viruses (16). Further, the DNA synthesized by the leukemic cell reverse transcriptase on its own endogenous template is related in sequence to RLV RNA (13). Note that this last result is complementary to and completes the logic of our earlier experiments in which the DNA synthesized on RLV RNA was used as a probe to find the virus-related information in leukemic cells.

The experiments reported here demonstrate the presence in human leukemic cells of four parameters characteristic of RNA tumor viruses: 1) a high molecular weight RNA of the 60–70S type, 2) a reverse transcriptase, 3) a base sequence of the RNA, which is related to that of a mouse leukemia virus, and 4) a physical density for the 70S RNA-DNA-polymerase complex characteristic of RNA tumor viruses. The findings are individually suggestive of a viral agent, but do not prove a viral etiology of human leukemia. The data, however, do encourage further efforts along these lines.

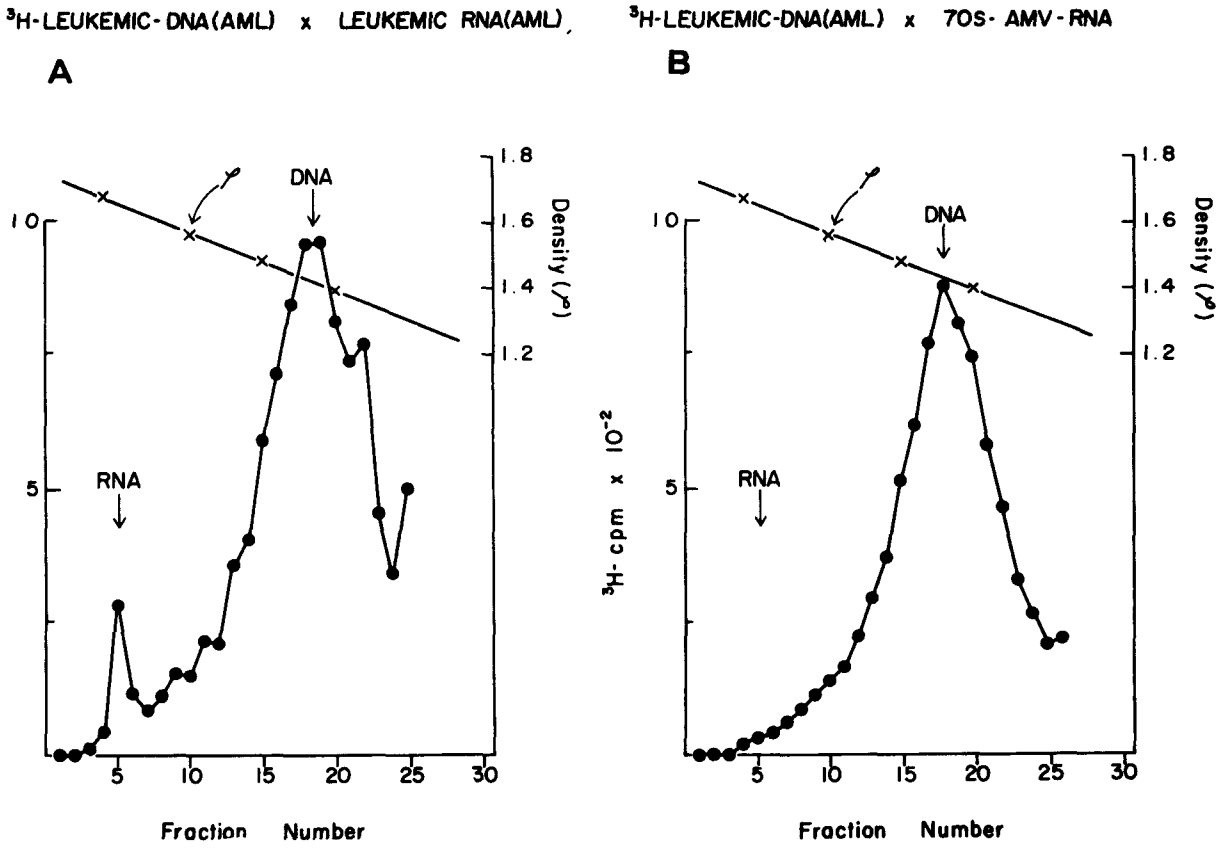

Fig. 3: Cs_2SO_4 equilibrium density gradient centrifugation of annealing reactions of human leukemic (AML [T]) ^{3}H-DNA to (A) 85 μg human leukemic (AML [T]) RNA enriched in viral sequences by prior density fractionation of cytoplasmic material and subsequent RNA extraction from the 1.10–1.26 g/cm^3 density region, and (B) 0.5 μg highly purified 70S AMV-RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legend to Fig. 2. The high molecular weight RNA ^{3}H-DNA complex obtained after glycerol sedimentation centrifugation was digested with 0.4 M NaOH for 18 hr at 37 °C to remove all RNA present. The ^{3}H-DNA product was then annealed to the respective RNAs in a vol of 60 μl in the presence of 50 % formamide and 0.4 M NaCl. After annealing for 18 hr at 37 °C, the reaction mixture was subjected to Cs_2SO_4 gradient centrifugation as described under Fig. 1.

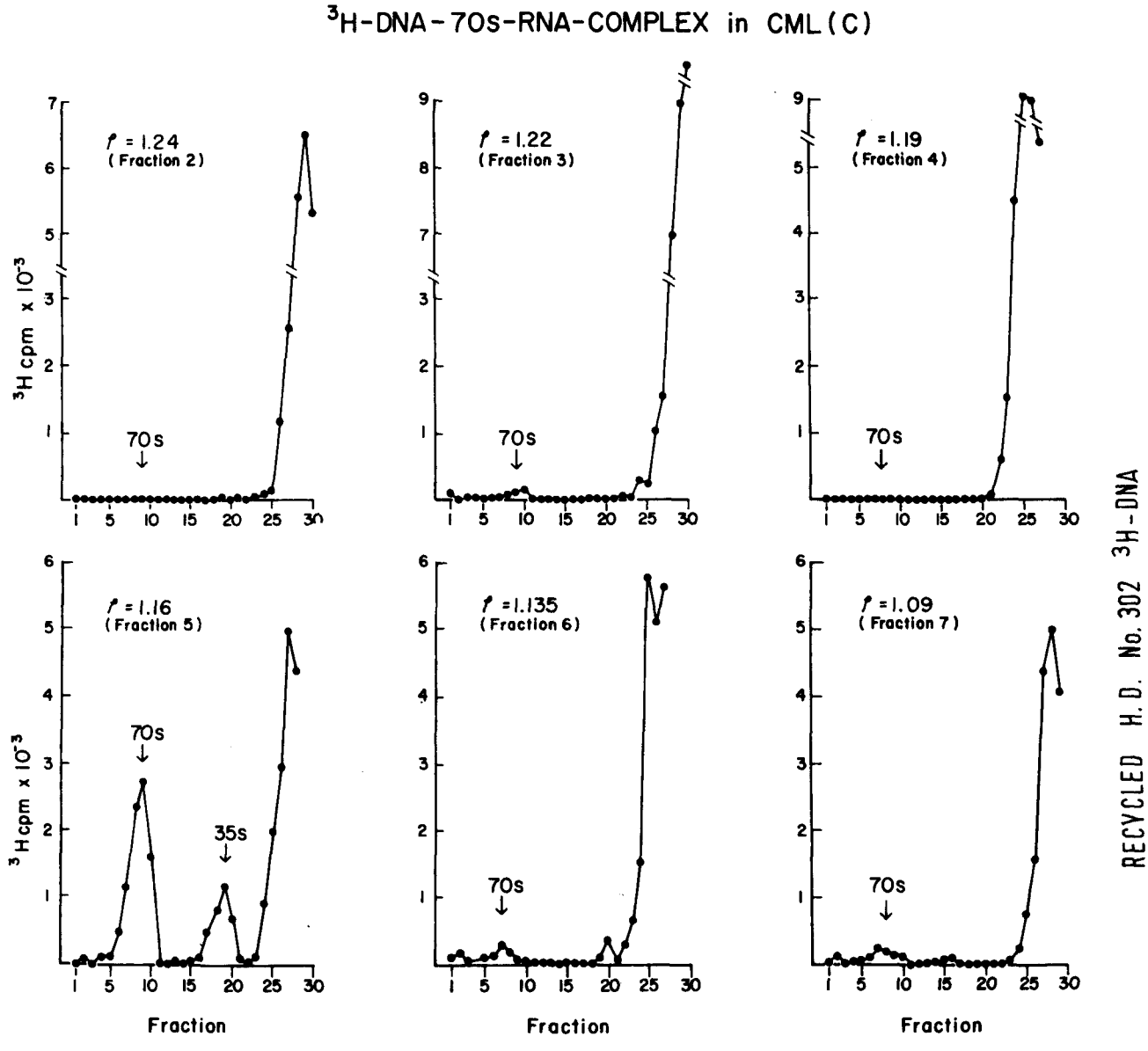

Fig. 4: Sedimentation profiles of a complete set of simultaneous detection tests after sucrose density fractionation of leukemic cytoplasm. 2 g of white blood cells from a patient with CML (Con) were prepared and examined as described in Fig. 2.

Acknowledgments

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RNA TUMOR VIRUSES AND HUMAN LYMPHOMAS

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We have used molecular hybridization to detect virus-specific RNA in tumors (1), and have found that corresponding neoplasias of murine and human origin exhibit remarkable similarities. Thus, human breast carcinomas contained (2) RNA possessing sequence homology to that of mouse mammary tumor virus (MMTV). This type of RNA was unique to the malignant adeno- and medullary-carcinomas, being undetectable in normal breast tissue and in such benign pathologies as fibrocystic disease and fibroadenoma. In keeping with the known unrelatedness of the murine leukemogenic and mammary tumor viruses, we found that breast cancer RNA did not hybridize to DNA complementary to the RNA of Rauscher leukemia virus (RLV). Finally, and more compelling, was the demonstration that human leukemic cells (3) and human lymphomas (4), including Burkitt's disease (5), contain RNA showing homology to that of Rauscher leukemia virus and not to that of mouse mammary tumor virus.

An Analysis of the Significance of Viral-related RNA in Human Neoplasia

The existence of viral-related RNA in human neoplasias does not of course establish a viral etiology for this disease. One must now perform experiments designed to answer the following questions: (1) How large is the RNA being detected? (2) How much homology does it in fact have to the RLV RNA? and (3) Is the viral-related RNA associated with a reverse transcriptase (RNA-instructed DNA polymerase) and is it located in structures characteristic of incomplete or complete virus particles? The requisite techniques resolving these issues were developed (6—9) and were applied to extracts of human tumors and normal tissues in a search for evidence of oncogenic RNA viruses.

The enrichment of possible virus particles is accomplished by disruption of the cells in the presence of EDTA to destroy the ribosomal structures. After removal of nuclei and mitochondria, the cellular supernatant is centrifuged at 98,000 X g through 20 % glycerol in TNE (0.01 M Tris-HCl, pH 8.3, 0.15 M NaCl, 0.01 M EDTA). The cytoplasmic pellet is then treated with NP-40 to disrupt possible virus particles and used in a standard endogenous reverse transcriptase reaction to generate [^{3}H]DNA after 15 min of synthesis at 37°. The reaction product was freed of protein by treatment with SDS and phenol and subjected to sedimentation analysis in a 10 to 30 % glycerol gradient with suitable markers to determine the apparent

size distribution of the DNA synthesized. In a reaction mediated by a B- or C-type virus, [^{3}H]DNA will sediment in a 70S region of the gradient representing the 70S RNA : [^{3}H]DNA early reaction product.

Such features as sensitivity to ribonuclease and the requirement for all four deoxyribonucleoside triphosphates can also be used to demonstrate that the appearance of the 70S-RNA : [^{3}H]DNA complex is in fact the result of a reverse transcriptase reaction. However, due to the possibility of nontemplated end addition reactions, the most definitive proof finally demands that the [^{3}H]DNA synthesized is hybridizable to an RNA derived from a known oncogenic virus.

The successful use of the simultaneous detection technique to detect RNA viruses in mouse and human milk (7, 8) led to the demonstration of complexes of 70S RNA and reverse transcriptase in peripheral white blood cells of 95 % of leukemic patients (10) and in 79 % of human breast cancers examined (11). In both of these malignancies, high molecular weight RNA has been found to be encapsulated with reverse transcriptase in a particle possessing the density characteristic of the RNA tumor viruses.

The Simultaneous Detection of 70S RNA and Reverse Transcriptase in Human Lymphomas

Figure 1A shows a representative outcome of a 70S RNA : DNA complex synthesized by the pellet fraction from an involved spleen of a patient with Hodgkin's disease. In certain samples additional peaks have been observed at positions of 52S and 35S. As shown in Fig. 1B, peaks are detected at both the 70S and 52S positions. It is further demonstrated that these complexes are due to an RNA-dependent reaction, as prior treatment with RNase eliminates both of the peaks. When equivalent quantities of either a normal spleen or an uninvolved spleen from a patient with Hodgkin's disease are analyzed by the same technique, no incorporation of [^{3}H]TTP is detected in a rapidly sedimenting structure (Figs. 1C and 1D).

Simultaneous detection assays were performed on 36 human lymphomas, which include 28 Hodgkin's disease specimens, 6 lymphosarcomas, and 2 reticulum cell sarcomas (12). The control series consisted of 14 uninvolved spleens and 5 cases of hypersplenism. The number of cpm in the 70S region of the glycerol gradient as determined by external size markers was taken as a measure of the presence and extent of the reaction. The average cpm in the 70S region for the control series was 14, whereas in contrast, the malignant tissues yielded an average of 302. In view of the low value of the controls, we have arbitrarily assigned any reaction yielding more than 30 cpm in the 70S region as being positive. With these criteria, all of the control samples were negative and 80.6 % of the malignant tissues were positive. Fifteen of the positive tumors were tested for ribonuclease sensitivity of the 70S-DNA complex, and in each of them the complex was degraded. It should be noted that 3 cases of hypersplenism yielded positive responses, which were sensitive to ribonuclease. The significance of this and its possible relation to premalignant conditions requires further investigation.

The demonstration of 70S RNA : DNA complex that is sensitive to ribonuclease already argues for the presence of an RNA-dependent reaction. However, due to the

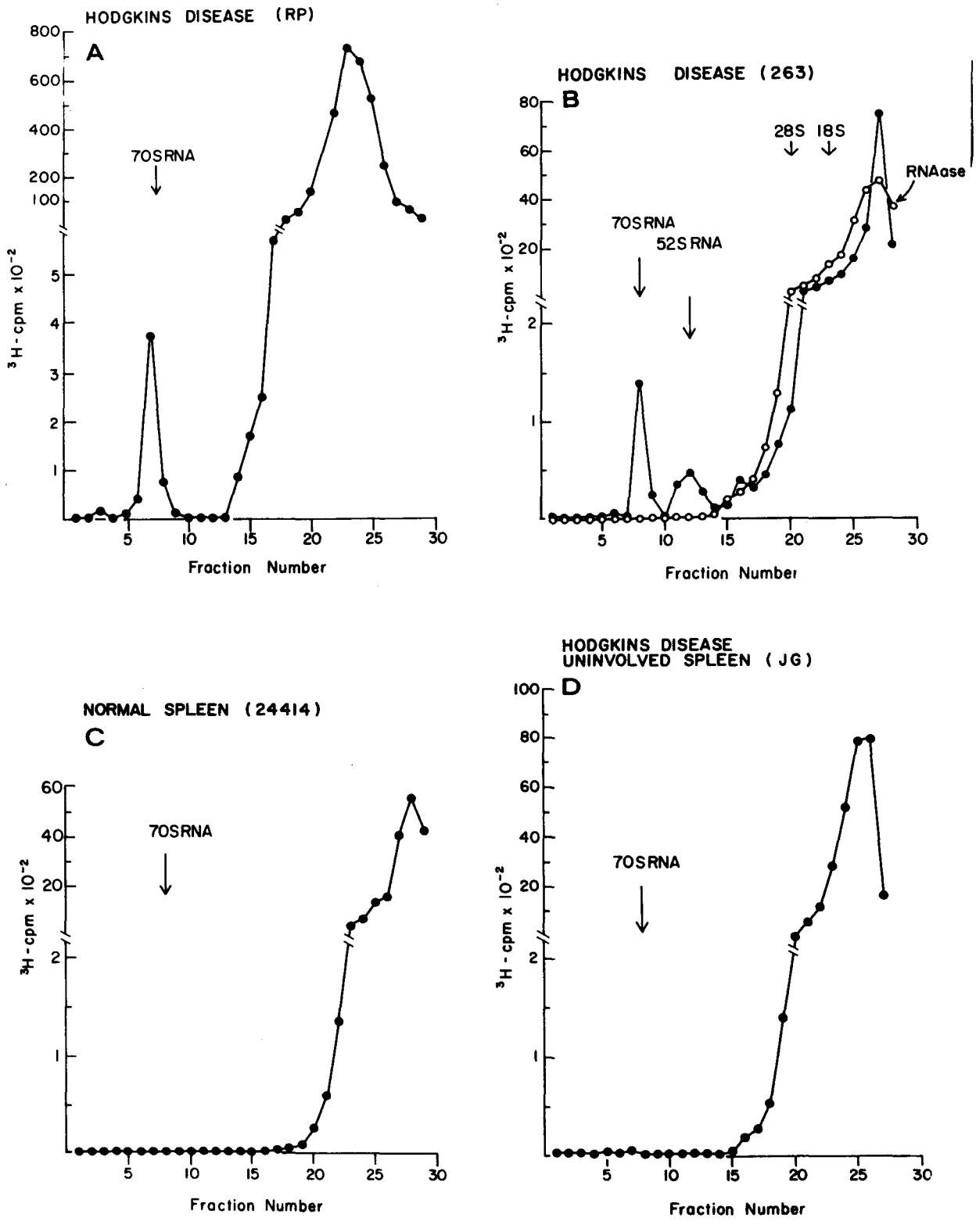

Fig. 1: Detection of 70S RNA-[^{3}H]DNA in human lymphoma tissue: A), and B), Hodgkin's disease; C), normal spleen; D), uninvolved spleen from a Hodgkin's disease patient. Five g of tissue were finely minced and disrupted with a Silverson homogenizer at 4° in TNE buffer (0.01 Tris-HCl, pH 8.3, 0.15 M NaCl, 0.01 M EDTA). This suspension was centrifuged at 4000 X g for 10 min at 2°. The resulting supernatant fluid was then layered on a 13-ml column of 20 % glycerol in TNE and spun at 98,000 X g for one hr at 4° in an SW-27 rotor (Spinco). The resulting pellet was resuspended in 0.5 ml 0.01 M Tris-HCl, pH 8.3, brought to 0.1 % Nonidet P-40 (Shell Chemical

Co.) and incubated at 0° for 15 min. DNA was synthesized in a reverse transcriptase reaction mixture (final volume 1 ml) containing: 50 μmol of Tris-HCl, pH 8.3, 20 μmol NaCl, 6 μmol $MgCl_2$, 100 μmol each of dATP, dGTP, dCTP, and 50 μmol-[^{3}H]dTTP (50 curries/mmole). 50 μg/ml actinomycin D was added to inhibit DNA-instructed DNA synthesis. After incubation at 37° for 15 min, the reaction was adjusted to 0.2 M NaCl and 1 % SDS. An equal volume of a phenol-cresol (7 : 1) mixture containing 8 hydroxyquinoline (0.2 g per 100 ml of mixture) was added and the final mixture was shaken for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10—30 % in TNE) and centrifuged at 40,000 rpm for 180 min at 2°. Fractions were collected from below and assayed for TCA-precipitable radioactivity. In Fig. 1B, one aliquot of the product was run directly on the glycerol gradient, while the other aliquot was incubated in the presence of RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37° prior to sedimentation analysis.

possibility of nontemplated end addition reactions, a more definitive proof demands that the [^{3}H]DNA synthesized is hybridizable to the relevant "oncogenic" RNA template. One approach is to hybridize the [^{3}H]DNA with 70S RNA prepared from Rauscher leukemia virus (RLV). A positive outcome would be expected from the earlier demonstration that human lymphoma pRNA hybridizes to synthetic DNA complementary to RLV-RNA (4, 5). If the hybridization to RLV-RNA is specific, the lymphoma [^{3}H]DNA should not hybridize to the 70S RNA of the avian myeloblastosis virus (AMV).

Figure 2A shows a Cs_2SO_4 equilibrium gradient profile of an annealing reaction between [^{3}H]DNA from Hodgkin's disease spleen (#211) and the RLV-70S RNA. It is clear that approximately 10 % of the [^{3}H]DNA has shifted to the RNA region of the gradient. Upon annealing an equivalent amount of AMV-70S RNA to the same [^{3}H]DNA, no significant shift to the RNA or hybrid region is observed (Fig. 2B). Note that this last result is complementary to and completes the logic of our earlier experiments in which the DNA synthesized on RLV-RNA was used as a probe to detect the virusrelated information in lymphoma cells.

The Simultaneous Detection of 70S RNA and Reverse Transcriptase in Burkitt's Tumors

Figures 3A, B, and C show representative 70S RNA-DNA complexes synthesized by the pellet fractions of biopsy specimens of Burkitt's tumors. In certain samples, additional peaks have been observed at positions of 35S. It is further demonstrated, as exemplified in Fig. 3C, that these complexes contain a 70S RNA molecule since prior treatment with RNase eliminates the peak. When equivalent quantities of peripheral white cells of a patient with infectious mononucleosis were analyzed by the same technique, no incorporation of [^{3}H]TTP into a rapidly sedimenting struktur was detected (Fig. 3D).

Of the 15 Burkitt's tumors examined, 13 or 87 % gave unambiquous evidence for the presence of 70S RNA : RNA-instructed DNA polymerase complexes (13). The Burkitt's tumors as a group yielded an average of 304 cpm in the 70S region compared with an average of 11 cpm for non-Burkitt control material. Six non-Burkitt samples were examined by the simultaneous detection test. These were chosen as controls for examination since they are particularly relevant to the question of the relation of the Epstein-Barr virus to the RNA particles being detec-

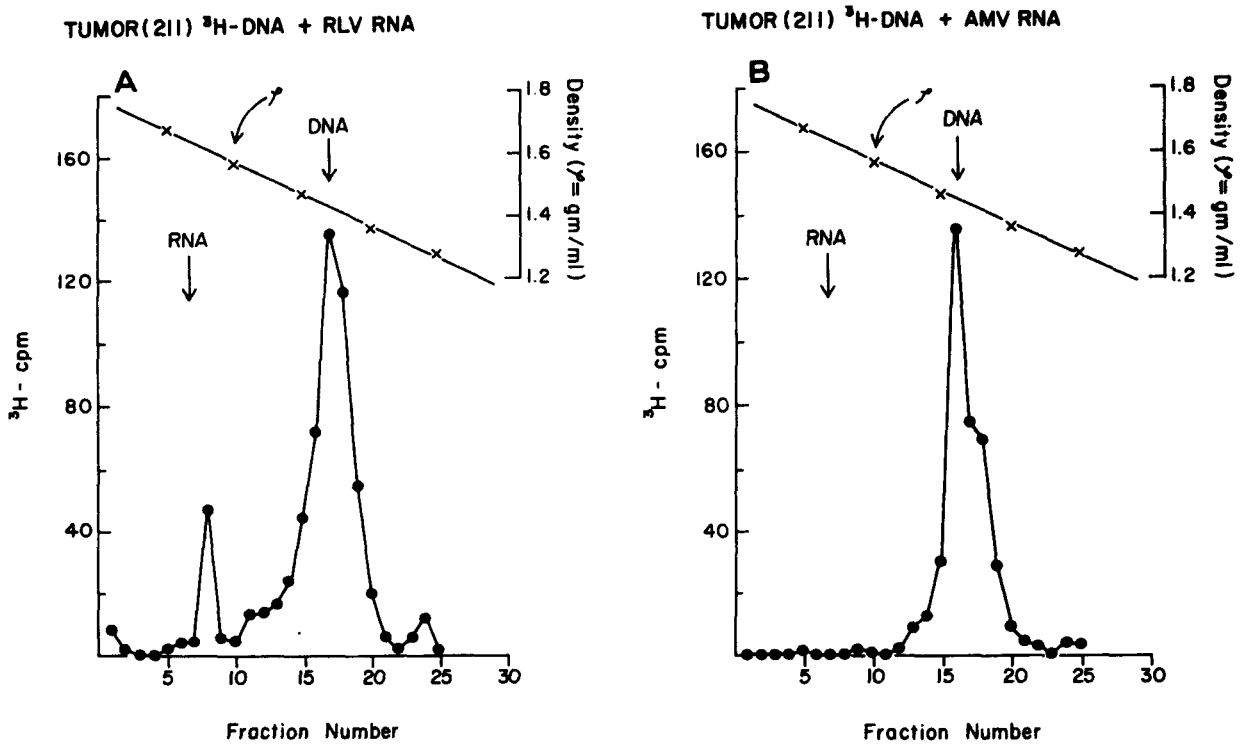

Fig. 2: Cs_2SO_4 density profiles of annealing reactions of human lymphoma #228 [^{3}H]DNA to A), RLV-70S RNA and B), AMV-70S RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legend to Fig. 1, except that the glycerol gradient sedimentation step was omitted. The aqueous phase was instead subjected to Sephadex G-50 column chromatography and the [^{3}H]DNA isolated and precipitated with two volumes of ethanol. The precipitate was digested in 0.4 M NaOH for 24 hr at 43° and neutralized. The [^{3}H]DNA product was then annealed to 1 μg of RLV-RNA and 1 μg AMV-RNA. The hybridization reaction (50 μl) was performed in the presence of 50 % formamide and 0.4 M NaCl. After annealing for 24 hr at 37°, the reaction mixture was subjected to Cs_2SO_4 density analysis.

ted in the Burkitt's tumors. One control is derived from the peripheral white blood cells of a patient with infectious mononucleosis, a self-limiting non-neoplastic condition in which prospective studies (14, 15) have strongly implicated the Epstein-Barr virus. Another control is a cell line derived from the lymphocytes of an infectious mononucleosis patient. This line and four other lymphoblastoid lines used are known to contain the Epstein-Barr genome. All the controls were negative for the 70S RNA-directed DNA polymerase. If a more extensive study confirms this pattern in infectious mononucleosis, evidence would be provided identifying the RNA particles as unique components of neoplastic tissues. In any case, it is clear that the existence of EBV information is not mandatorily linked to the detectable presence of the RNA particles found in the Burkitt's tumors.

The data thus far described indicate that Burkitt's tumors contain particles that encapsulate reverse transcriptase and a 70S RNA related in sequence to that of the Rauscher leukemia virus. It was of interest to see whether the particles possessed the density characteristic of an RNA tumor virus. To this end, a pellet fraction was prepared from a Burkitt's tumor and subjected to equilibrium centrifugation in a linear gradient of 15 to 55 % sucrose. The gradient was then divided into 10 equal fractions that were diluted to 15 % sucrose and again spun at 100,000 X g for 1 hr.

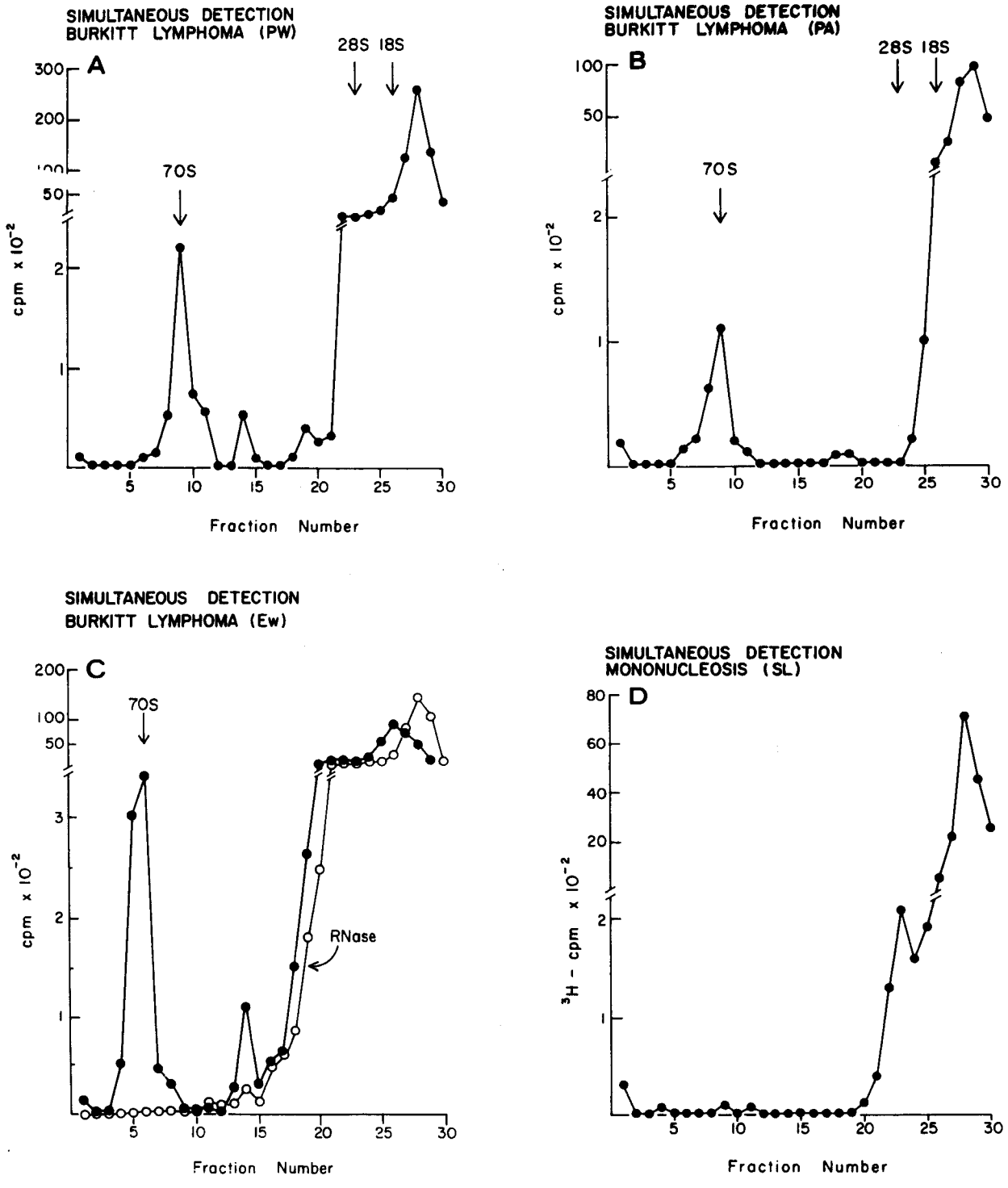

Fig. 3: Detection of 70S RNA-[^{3}H]DNA: in Burkitt's lymphoma tissue, (A), (B), (C); and (D), peripheral white blood cells from a patient with acute mononucleosis. The procedure employed is as described in the legend to Fig. 1. In Fig. 3C, one aliquot of the product was run directly on the glycerol gradient, while the other aliquot was incubated in the presence of RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37° prior to sedimentation analysis.

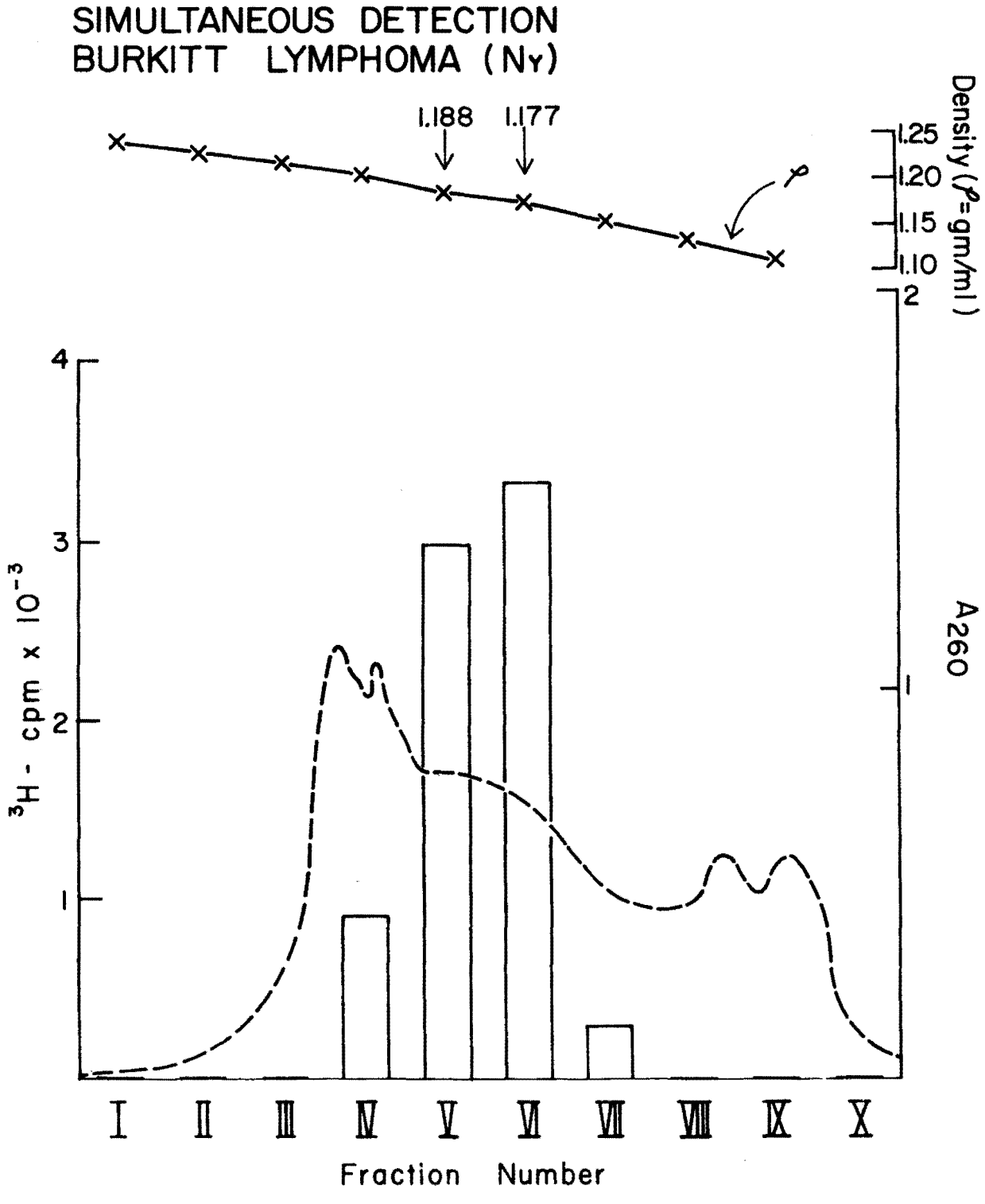

Fig. 4: Sucrose gradient localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of Burkitt's tumors. A pellet fraction was prepared from Burkitt's tumor (Ny) as in the legend to Fig. 1. The pellet was resuspended in TNE and layered on a linear gradient of 15–55 % sucrose in TNE and spun in a SW-27 (Spinco) rotor at 4° for 210 min. The gradient was dripped from below through a recording Gilford spectrophotometer at A_{260}, and ten equal fractions were collected. Each fraction was diluted with TNE to a sucrose concentration of less than 15 % and then spun at 100,000 X g. The pellet obtained from each of the ten fractions was then subjected to the simultaneous detection assay as in the legend to Fig. 1, and the amount of 70S RNA : [^{3}H]DNA synthesized from the ten different density regions was determined by glycerol velocity centrifugation.

Simultaneous detection tests were then carried out on the pellet from each fraction to determine the distribution in the density gradient of 70S RNA-instructed DNA synthesizing activity. It can be seen from Fig. 4 that the particles possessing 70S RNA-instructed DNA polymerase localize within a density of 1.16 to 1.19, the density range characteristic of the oncogenic RNA viruses. Three Burkitt's tumors and one African histiocytic lymphoma were analyzed in a similar manner and all of them gave the same results.

Particle-related Sequences in Lymphoma Nuclear DNA (16)

As in our previous studies with human leukemia (10) and breast cancer (11), the experiments described here were performed to elucidate the possible etiologic significance of our earlier (4) detection in human lymphomas, including Burkitt's tumors (5) of RNA uniquely homologous to that of the Rauscher leukemia virus. The data obtained here show that at least a portion of the RNA we were finding exists in the form of a 70S RNA associated with an RNA-instructed DNA polymerase in a particle having a density between 1.16 and 1.19 g/ml. The particles thus identified in the cells from both of these malignancies have four of the biochemical and physical features diagnostic of the RNA tumor viruses. Ultimately, final proof of the contributory or causative nature of these particles requires the demonstration that they are infectious and transforming agents. At this time, however, a logical approach in attempting to further define their involvement would be to demonstrate that the particle-related sequences are unique to the DNA of malignant cells.

These human particles can be used to generate radiocative DNA probes, which in turn could be used to probe for viral-specific information in human nuclear DNA. The requisite methodology has been developed and applied in a study of eight leukemic patients (17). In each case, the DNA of leukemic cells contained particle-related sequences undetectable in the leukocyte DNA of normal individuals.

As outlined in the studies of human leukemia, the methods used in this investigation are as follows: 1) Isolate the particles encapsulating 70S RNA and reverse transcriptase from human lymphoma specimens; 2) Use the particle fraction to endogenously synthesize [^{3}H]DNA in the presence of a high concentration of actinomycin D to inhibit host and viral DNA-directed DNA synthesis; 3) Purify the [^{3}H]DNA by Sephadex chromatography and hydroxyapatite; 4) Remove the [^{3}H]DNA sequences shared with normal DNA by exhaustive hybridization in the presence of vast excess of normal DNA followed by hydroxyapatite chromatography to separate paired from unpaired [^{3}H]DNA. 5) Test the unpaired residue for specific hybridizability to lymphoma DNA.

[^{3}H]DNA probes were synthesized from five Burkitt's lymphoma and three Hodgkin's disease specimens. In all instances, the lymphoma [^{3}H]DNA probe hybridized 35 to 40 % to normal spleen nuclear DNA. The observation that the [^{3}H]DNA synthesized by the lymphoma particles hybridized to normal DNA was not surprising in view of previous experience with murine, avian and human leukemic systems (18–25).

The sequences in [^{3}H]DNA common to normal nucelar DNA were then removed by exhaustive annealing with normal spleen DNA in vast excess. In carrying out this step,

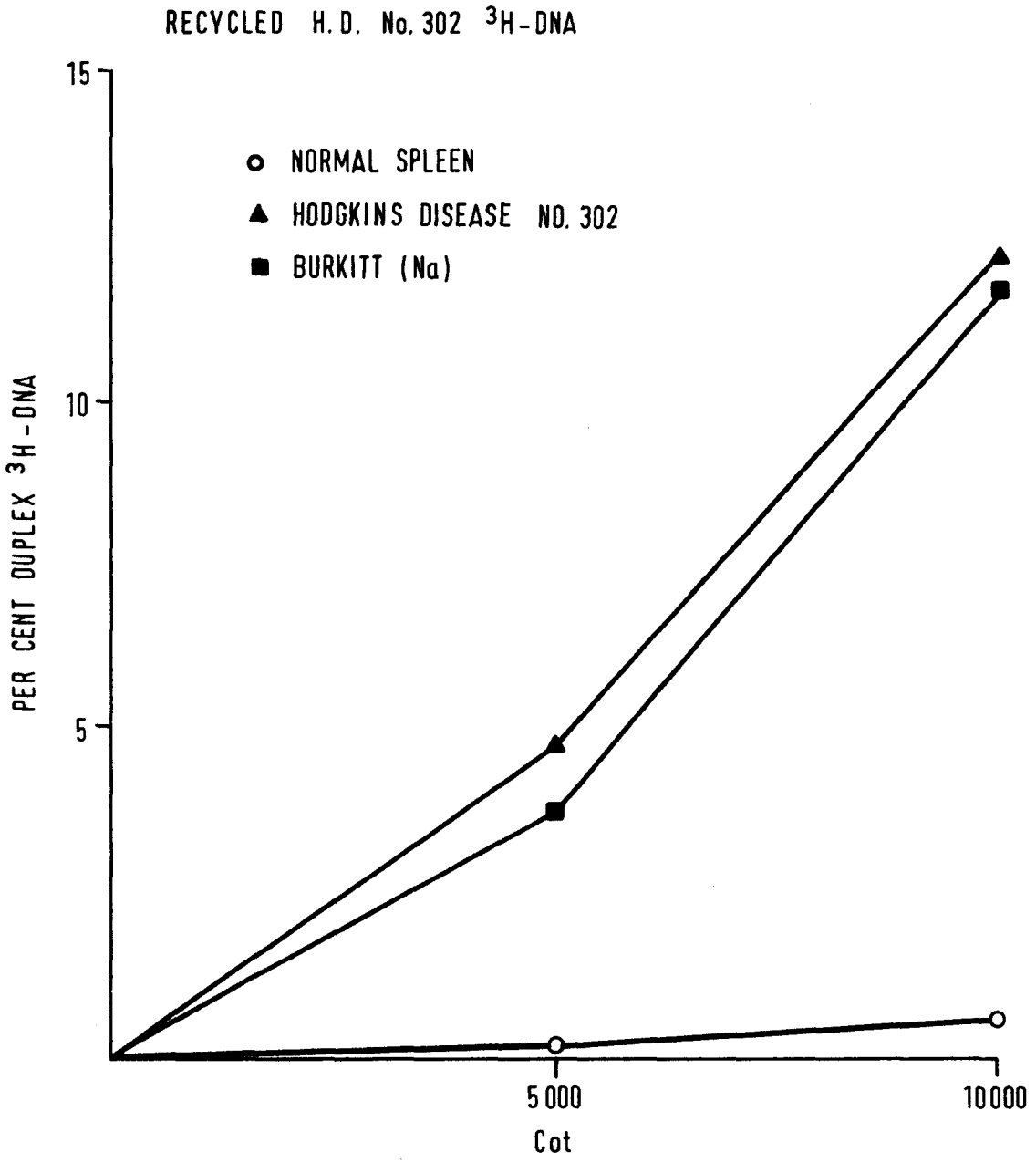

Fig. 5: Hybridization of recycled Hodgkin's disease #302 [^{3}H]DNA to nuclear DNA isolated from normal spleen (O- - - - O), Hodgkin's disease #302 (▲- - -▲), and Burkitt's lymphoma Na (■ - - ■). Tritiated DNA probe synthesis, nuclear DNA preparation, and annealing conditions are detailed elsewhere (16). Hybrid formation was analyzed by hydroxyapatite chromatography at a phosphate buffer elution concentration of 0.15 M. Five fractions of 4 ml were collected at each of four temperatures (60°, 80°, 88°, 95°) and the [^{3}H]DNA counts in each fraction were assayed by scintillation counting in 10 ml Aquasol (NEN). The method identifies unpaired strands that elute at 60° and poorly paired duplexes that disassociate at 80°. *Only the duplexes disassociating and eluting at 88° to 95° are counted here as hybridized.*

annealing reactions were set up to contain 60 A_{260} units of normal cellular DNA, 0.1 pmol of [^{3}H]DNA (1000 cpm) and allowed to anneal to a C_0t of greater than 10,000. After this reaction, the recovered unpaired strands should no longer contain sequences complementary to those found in normal DNA. Exclusive hybridizability of such recycled [^{3}H]DNA to lymphoma DNA would then establish that the genome of lymphoma cells contains specific sequences not present in normal DNA.

Figure 5 shows the outcome of hybridizing such recycled Hodgkin's disease [^{3}H]DNA to normal spleen nuclear DNA, Hodgkin's disease nuclear DNA, and to Burkitt's lymphoma nuclear DNA. The input counts for each C_0t point is 1500 to 2000 cpm. Only those stable duplexes disassociating and eluting above 88° are counted here as hybridized. Taking into account normal background on our hydroxyapatite columns, it is evident that no stable complexes are formed with normal DNA. On the other hand, over 10 % of the input recycled Hodgkin's disease [^{3}H]DNA forms well-paired duplexes with both Hodgkin's and Burkitt's disease nuclear DNA. In all eight instances, the lymphoma [^{3}H]DNA hybridized to nuclear DNA from the same pathological type of lymphoma from which the probe was synthesized. In several, but not all cases, Burkitt's [^{3}H]DNA hybridized to Hodgkin's disease nuclear DNA indicating that there is some, but not complete homology between their viral-specific sequences. A similar situation was obtained in hybridizing Hodgkin's disease [^{3}H]DNA with Burkitt's lymphoma nuclear DNA. It is pertinent to point out that the lymphoma [^{3}H]DNA did not show significant levels of hybridizability with nuclear DNA from either carcinoma of the breast or colon. This indicates that the lymphoma particle information is specific for lymphomas but not for all types of malignancy. Furthermore, our particle-specific information cannot be related to the Epstein-Barr virus due to the fact that the [^{3}H]DNA does not hybridize to the nuclear DNA of NC37 cells or of infectious mononucleosis cells.

As in the experiments in human leukemia (16), the results described here for human lymphomas strongly argue against the applicability of the virogene theory to these diseases. The experiments designed here involve nuclear DNA and consequently all genes, expressed or silent. It is clear that particle-specific information, undetectable in normal nuclear DNA, is present in each lymphoma nuclear DNA examined. This implies that the addition of particle-related information was required for the conversion of the normal to the malignant cell. The virogene hypothesis on the other hand demands that the lymphoma-specific information be present in the genomes of both normal and malignant cells. The relationship of these findings to the epidemiologic evidence of time-space clustering in both Hodgkin's and Burkitt's disease remains to be determined.

Acknowledgments

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BIOCHEMICAL EVIDENCE FOR THE PRESENCE OF RNA TUMOR VIRUSES IN HUMAN LEUKEMIC PLASMA

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Recent biochemical techniques have enabled the detection of murine RNA tumor virus-related particle in human malignancies. Molecular hybridization has shown that human breast tumors contain RNA homologous to murine mammary tumor virus (1, 2). Similar analysis of human leukemias (3), sarcomas (4), and lymphomas (5) showed the presence of RNA related to the Rauscher leukemia virus.

Finding the appropriate oncornavirus-related RNA in the corresponding human neoplasia stimulated the search for RNA tumor viruses in human malignancies. The presence of 70S RNA and RNA-instructed DNA polymerase in the RNA tumor viruses provided a sensitive technique for the detection of such viruses (6). The possibility of a concomitant test for 70S and reverse transcriptase was suggested by the nature of early reaction intermediates (7–9). The initial DNA product was found to be hydrogen-bonded to the 70S RNA template and the structure could be detected due to the co-sedimentation of newly synthesized small [^{3}H]-DNA product with the 70S RNA. The procedure revealed the presence of 70S RNA and RNA-instructed DNA polymerase in human milk particles (11). Extension of the simultaneous detection assay to tumor tissue (12) showed the presence of particles containing 70S RNA and RNA-instructed DNA polymerase in human leukemia (13, 14), human breast tumors (15), Hodgkin's disease (16), and Burkitt's lymphoma (17). The particles were also shown to possess the density (1.17 g/ml) characteristic of the RNA tumor viruses (14–17). The presence of virus particles in plasma of Rauscher leukemia virus-infected mice (18) and in chickens infected with avian myeloblastosis virus (19) suggested a search for RNA tumor viruses or their components in the plasma of human leukemic patients. Electron microscopy has revealed electron-dense particles in leukemic patients' plasma (20, 21), and, further, work of Kiessling *et al.* (22) has shown the presence of a DNA polymerase. Gallo and his colleagues have partially purified RNA-directed DNA polymerase from leukemic cells (23) and have recently confirmed (24) the sequence relatedness of animal tumor viruses to the RNA of human leukemic particels (3, 13, 14).

Further work has shown the presence of 70S RNA and RNA-instructed DNA polymerase in the plasma of 74 % of the patients examined (25).

Examination of leukemic plasma for 70S RNA-instructed DNA polymerase.

The plasma is enriched for virus-like particles by pelleting at 100,000 X g and the pellet is washed with EDTA-containing buffers and centrifuged at 150,000 X g for 1 hr to yield a pellet (p-100). The p-100 is solubilized with 0.1 % NP-40 and a standard DNA polymerase reaction is then performed. The DNA synthesis is monitored by the incorporation [^{3}H]TTP into acid-insoluble products. Figure 1 shows the kinetics of DNA synthesis from the p-100 pellet of a patient with chronic lymphatic leukemia. For simultaneous detection of 70S RNA and RNA-directed DNA polymerase, the standard DNA polymerase reaction is deproteinized after 20 min of incubation at 37°, and the nucleic acids are subjected to sedimentation analysis in glycerol gradients with external size markers.

Figure 2 shows the results of simultaneous detection assays on the plasma of two patients with acute lymphatic (ALL-Bl) and acute myelogenous (AML-Kl) leukemia. The DNA synthesized is seen travelling with a sedimentation coefficient of 70S. The 70S-DNA complex observed with p-100 pellet from a leukemic patient's plasma is due to a complex involving a 70S RNA, because a prior treatment with RNase eliminates the [^{3}H]DNA from the 70S region of the gradient (Fig. 3).

Table 1 summarizes our findings with plasma specimens from 19 leukemic patients all in the active phase of their disease, and 13 normal blood bank donors. Included, where available, are the peripheral leukocyte counts (WBC) at the time of sampling. P-100 pellets (Fig. 2) were prepared from the plasma specimens and assayed for their ability to synthesize 70S RNA : DNA complex. The cpm in the 70S region of the glycerol gradient were taken as a measure of the presence and extent of the reaction. The reaction was termed positive if the cpm in the 70S region of the gradient exceeded 30. On this basis, 14 out of 19 leukemic plasmas examined were positive. The leukemic specimens produced an average of 184 cpm in the 70S region, with a few producing several hundreds of cpm (Table 1). Unfortunately, in many cases the amount of [^{3}H]DNA found in the 70S region was not sufficient to permit a concomitant assay for ribonuclease sensitivity. However, 4 of the 19 plasmas could be checked and in all cases the reaction was sensitive to ribonuclease, as exempliefied by Fig. 2. Similar analyses of 13 normal blood bank donors produced an average of 8 cpm in the 70S region (Table 1). One plasma produced 100 cpm, but on examination of a parallel aliquot, the cpm in the 70S region were insensitive to ribonuclease and the significance of this reaction remains obscure. There is no evidence in Table 1 of a relation between the level of leukocytosis and the activity found by the simultaneous detection test. However, the numbers available are too small to permit any definitive statements.

Characterization of the [^{3}H]DNA product. Synthesis of the ribonuclease-sensitive 70S RNA : [^{3}H]DNA complex in the p-100 pellet of leukemic plasma suggests the presence of an RNA-instructed DNA polymerase. However, definitive proof requires a demonstration that the [^{3}H]DNA synthesized is in fact complementary to the 70S RNA template. To this end, high molecular weight RNA : [^{3}H]DNA complex was pooled, alcohol-precipitated, and extensively treated with alkali to digest the RNA. [^{3}H]DNA was then used to show its specificity to its presumed template. RNA was extracted from the p-100 pellet obtained from leuke-

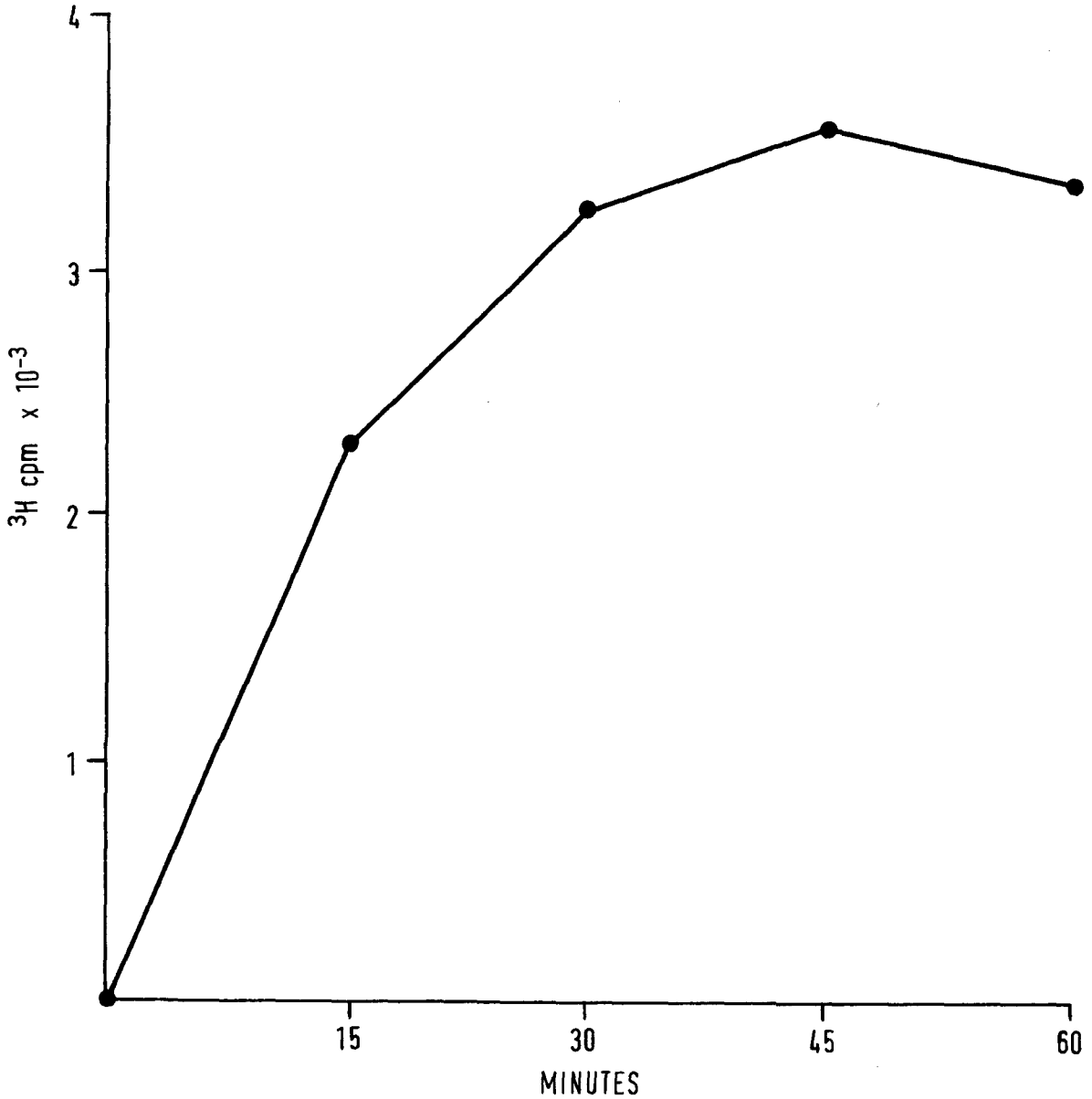

Fig. 1: Kinetics of an endogenous reaction in a pellet fraction isolated from the plasma of a chronic lymphatic leukemia (CML) patient. Plasma was centrifuged at 10,000 X g for 10 min at 3°. The clear zone between the lipids and the precipitate was processed as described (25). A 125 µl reaction containing 6.25 µmol of Tris-HCl (pH 8.3), 1 µmol $MgCl_2$-1.25 µmol NaCl-0.2 µmol each of dGTP, dCTP, dATP, and 0.2 mCi of [^{3}H]TTP (50.1 Ci/mmol) was performed. At different times, 20-µl aliquots were withdrawn and assayed for acid-precipitable radioactivity.

mic and normal blood bank donors' plasma. These RNAs were then annealed to the [^{3}H]DNA product. After 24 hr of annealing, the reaction mixtures were analyzed on cesium sulphate density equilibrium gradients. Figures 4A and B show that 92 % of the DNA hybridizes specifically to the leukemic RNA and not to the RNA isolated from normal blood bank donors.

Table 1. Simultaneous Detection of 70S RNA and Reverse Transcriptase in Leukemic Plasma Pellets

Leukemias	Volume Ml	WBC	Simultaneous Detection 70S-cpm	Reaction
Acute lymphatic				
Bl	60	900,000	1780	+
Du	62	27,500	148	+
Acute Myelogenous				
#196	6	–	32	+
Ra	30	26,800	56	+
Si	40	50,000	193	+
Ba	45	7,800	135	+
Gu	75	40,000	171	+
El	45	77,000	50	+
McL	100	60,000	285	+
Kl	115	16,500	110	+
Chronic Lymphatic				
Mil	75	150,000	61	+
Sc	120	9,300	0	–
Chronic Myelogenous				
Mi	28	80,000	0	–
To	100	–	80	+
Co	10	62,000	0	–
Te	1.5	–	100	+
Mo	37	114,000	300	+
Acute Myelomonocytic				
Be	72	52,000	0	–
Acute Leukemia (non-sp.)				
#379	9	–	0	–
Non-leukemic				
Le (Polycythemia)	50	60,000	0	–
Fi (Polycythemia)	90	9,500	20	–
Za (High WBC Counts)	90	82,000	15	–

Summary of simultaneous detection assay for 70S RNA and reverse transcriptase in plasma pellets. The sum of cpm in the 70S position monitored by external size marker is recorded (Methods) and is designated as positive if the cpm exceeds 30, which is three times the background count. All of the leukemic patients examined were in the active phases of their disease. Peripheral leukocyte counts (WBC) at the time of blood sampling are indicated where available.

Table 1. Continued

Leukemias	Volume Ml	WBC	Simultaneous Detection Reaction 70S-cpm	
Normal Pooled Plasma	75	–	0	–
Normal Plasma A	75	–	0	–
" " B	75	–	0	–
" " C	75	–	0	–
" " D	75	–	4	–
" " E	75	–	0	–
" " F	75	–	0	–
" " G	75	–	100	? *
" " H	75	–	14	–
" " I	75	–	2	–

* = Ribnuclease-insensitive as determined on another aliquot of the same sample.

We have previously shown [3] that the DNA product of virus-like particles in human leukemia shows homology to the RNA from Rauscher leukemia virus. It was therefore of obvoius interest to test the homology to the RNA from Rauscher leukemia virus of endogenous DNA product synthesized in the presence of actinomycin D by the p-100 pellet of leukemic plasma.

Figures 4C-D show the outcomes of the annealing reaction of the [^{3}H]DNA product from a leukemic plasma pellet to 70S RNA isolated from the Rauscher leukemia virus and to that from avian myeloblastosis virus. It is clear from Fig. 4C that 20 % of the [^{3}H]DNA product anneals specifically to the 70S RNA of the RLV, whereas no hybridization occurs with the 70S RNA of AMV (Fig. 4D). This result is in complete accord with the earlier studies on particles from leukemic cells (3, 13, 14, 24, 25).

The earlier finding in human leukemia cells (3, 13, 14, 24, 25) of particles possessing four of the biochemical and physical properties of murine RNA tumor viruses suggested an analogy of the disease in humans and animals. The presence of RNA tumor viruses in the plasma of animals with neoplastic growth stimulated a search for virus-like particles or their components in the plasma from human leukemic patients.

On examining plasmas from 19 leukemic patients and 13 normal blood bank donors, 74 % of the leukemic patients showed evidence for the presence of particulate complexes containing 70S RNA and reverse transcriptase. No such complexes were identified in the plasma from normal blood bank donors.

Particularly interesting was the observation that the [^{3}H]DNA synthesized by the RNA-instructed DNA polymerase on its own endogenous RNA template hybridizes specifically to RNA from leukemic plasma (Fig. 4A) and to RLV RNA (Fig. 4C), in agreement with previous studies on particles found in the white blood cells of

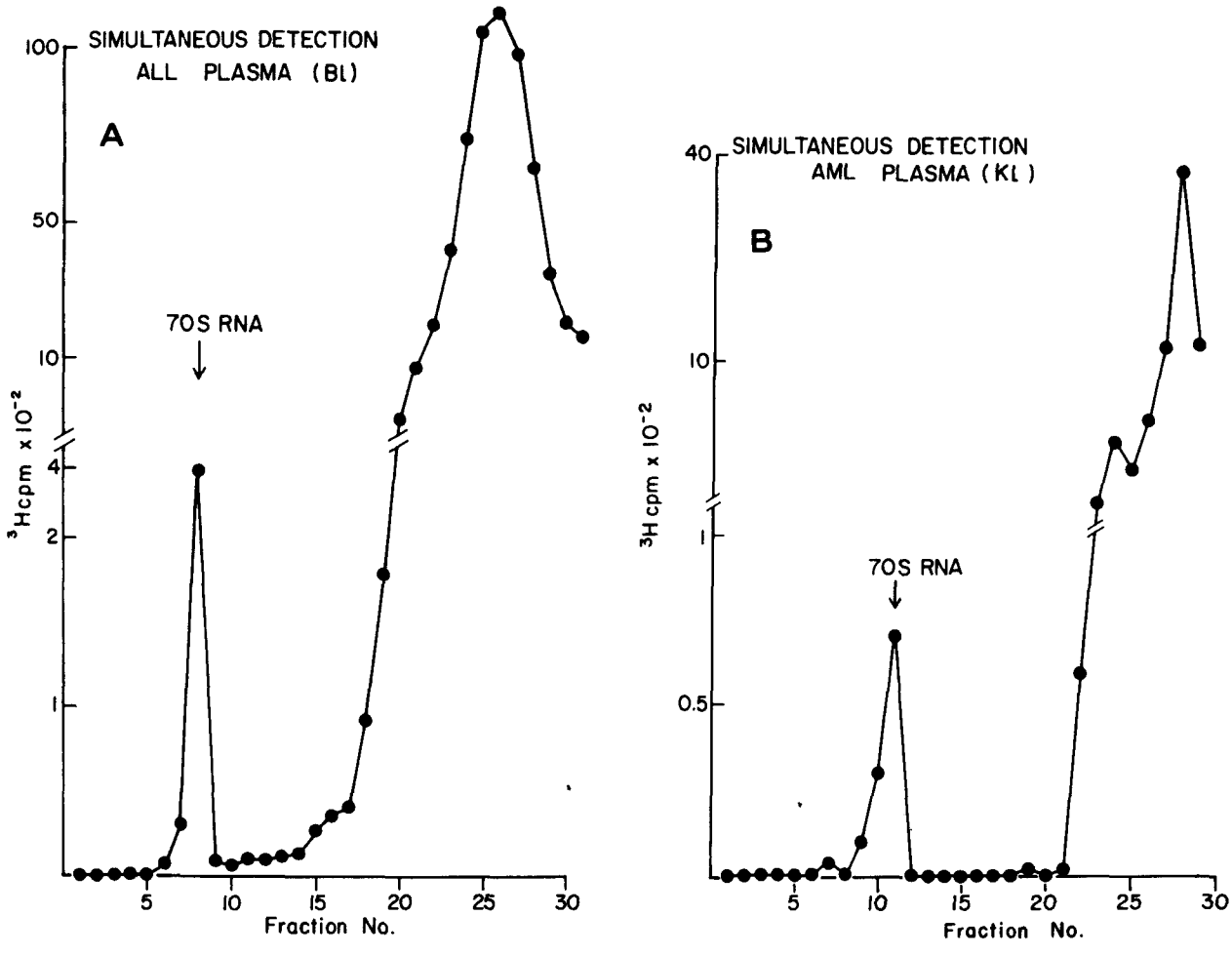

Fig. 2: Simultaneous detection of 70S RNA : [^{3}H]DNA complex in pellets of human leukemic plasma. A) acute lymphoblastic leukemia (ALL) and B) acute myelogenous leukemia (AML). Preparation of the pellet fraction from human plasma and the reagents used in a standard endogenous RNA-instructed DNA polymerase assay are described in Fig. 1. After a 20-min incubation at 37°, the reaction was terminated by the addition of NaCl and sodium dodecyl sulphate to final concentrations of 0.4 M and 1 %, respectively. An equal volume of a phenol-cresol (7 : 1) mixture containing 8-hydroxyquinoline (0.1 g/100 ml of mixture) was added, and the final mixture was shaken at 25° for 5 min and centrifuged at 5000 X g for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10—30 % in TNE) and centrifuged at 50,000 rpm for 90 min at 4° (Spinco SW-50.1 rotor). [^{3}H]-labeled 70S RNA from avian myeloblastosis virus was used as an external marker. Fractions were collected from below and were assayed for acid-precipitable radioactivity.

leukemic patients (3, 13, 24). The lack of hybridizability to AMV RNA (Fig. 4D) and RNA extracted from normal blood bank donors (Fig. 4B) eliminates the possibility that complexing is due to the poly(A) stretches found in RNA tumor viruses (26—28).

Finding the 70S RNA-reverse transcriptase complexes in the leukemic plasmas is of course not unexpected in view of our previous studies of human leukemias (3, 13, 24). The nucleic acid homologies shown in Fig. 4 parallel those found for leukemic cell particles and suggest that the plasma complexes are similar to or are derived from the particles detected in the leukemic cells. Unfortunately, the amounts found in the plasmas were not sufficient for a definitive characterization with the techniques currently available.

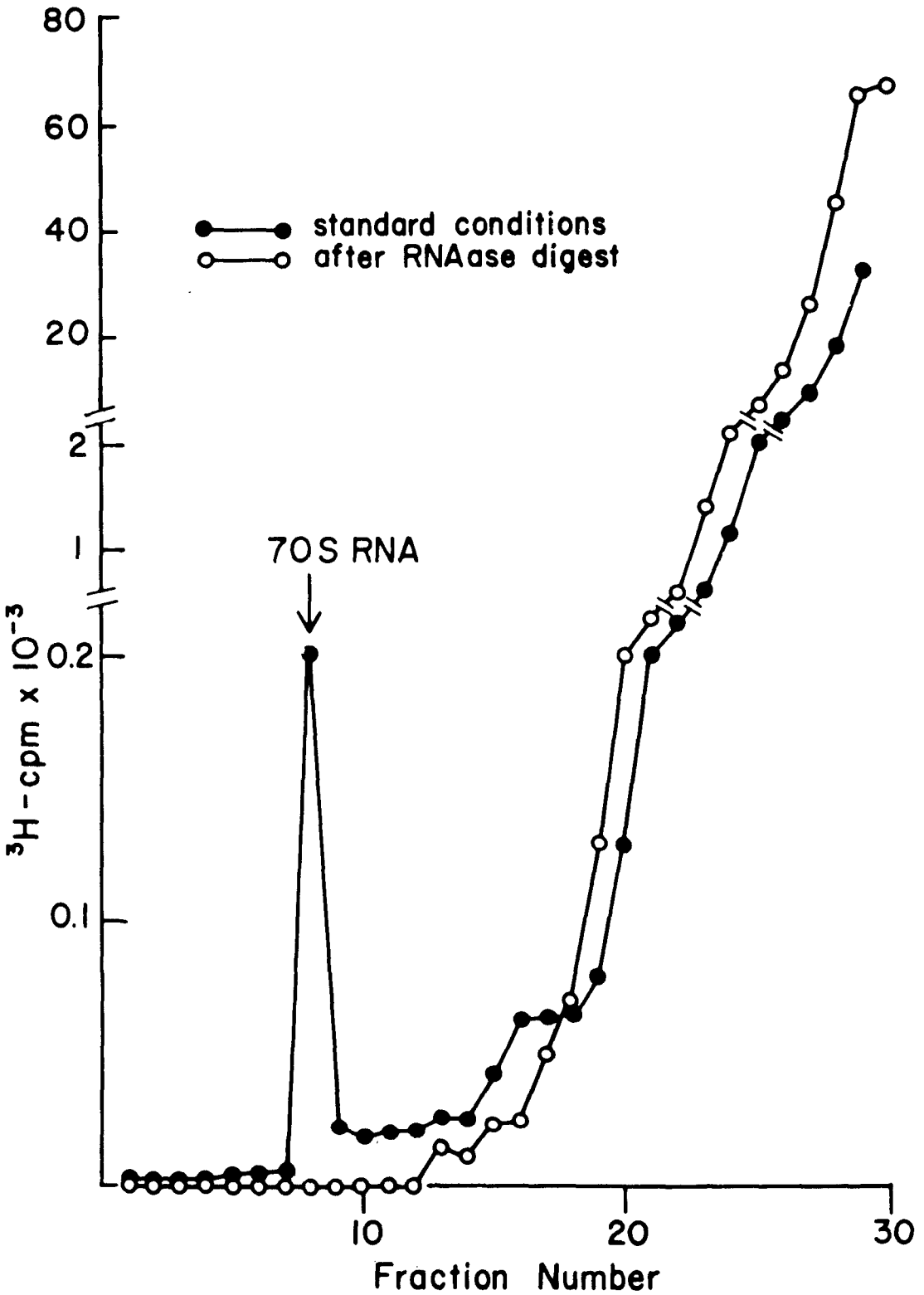

Fig. 3: Effect of ribonuclease on the 70S RNA : [^{3}H]DNA complex. Plasma pellet of a chronic myelogenous leukemic patient was processed and assayed as described. After 20 min of incubation at 37°, the nucleic acid was extracted and divided into two equal parts. Onehalf was left untreated (●——●), whereas the other half was treated with RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37° (0——0). The two parts were then layered on separate gradients and analyzed for 70S RNA : [^{3}H]DNA complex by the procedure described (25).

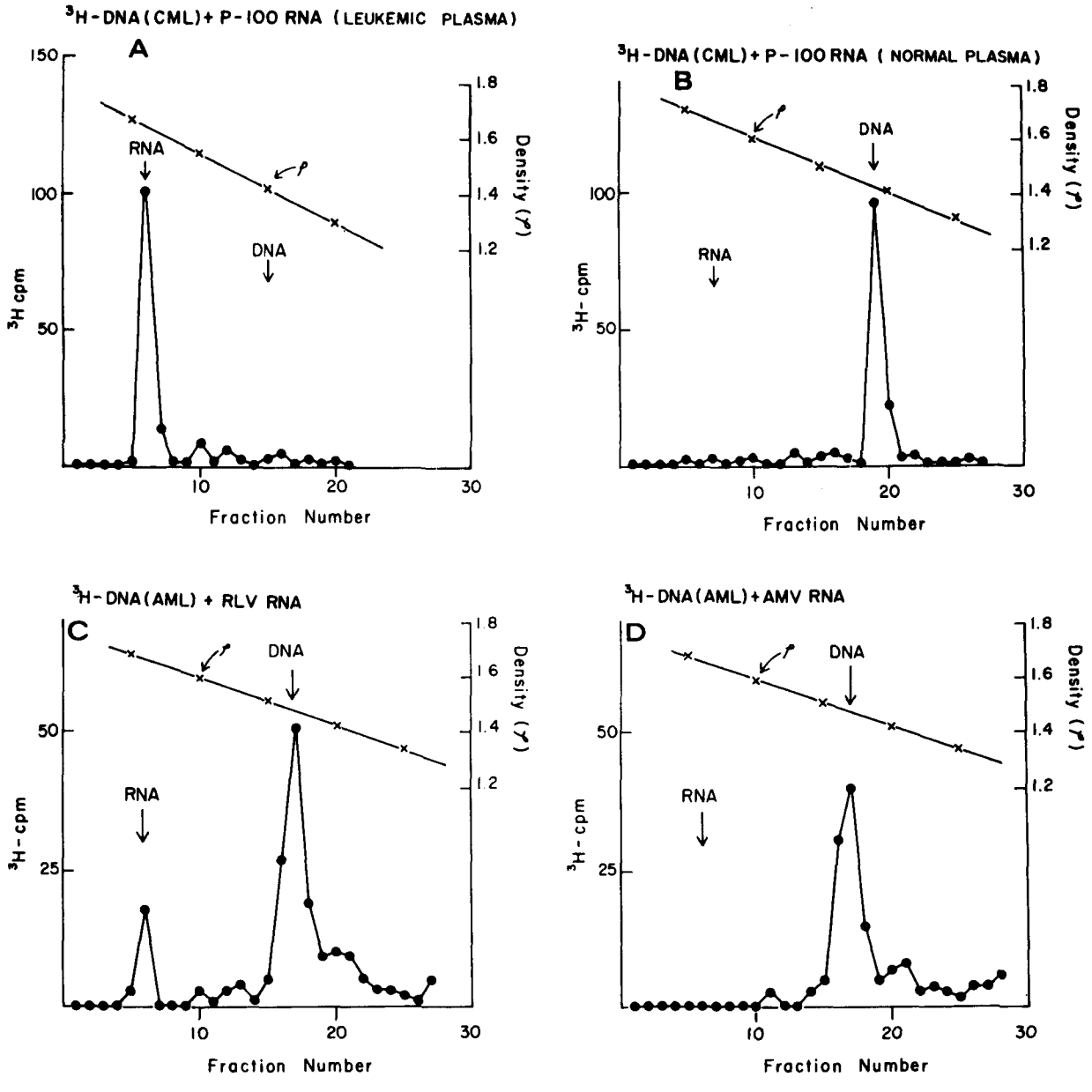

Fig. 4: Cesium sulphate equilibrium density gradient analysis of annealing reactions of leukemic plasma pellet [^{3}H]DNA to: A) RNA from leukemic plasma, B) RNA from normal plasma, C) RLV-70S RNA, and D) AMV-70S RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legends to Figs. 1 and 2. The 70S RNA: [^{3}H]DNA complex obtained following velocity centrifugation was digested with 0.4 M NaOH for 8 hr at 37° to destroy all RNA present. The [^{3}H]DNA product from a plasma pellet of a CML patient (Te) was then annealed to 12 μg of plasma pellets RNA (A, B). The [^{3}H]DNA product from an AML patient (Si) was annealed to 1 μg of 70S RNA from Rauscher leukemia virus (C) and avian myeloblastosis virus (D). After annealing for 18 hr, the reaction mixture was analyzed by cesium sulphate isopycnic gradient centrifugation (25).

The presence of these complexes in the leukemic plasmas raises a number of interesting questions and possibilities with respect to their relation to the disease and its state. It is conceivable that the levels of these complexes could be used as infor-

mative parameters in both diagnosis and treatment. The simultaneous detection test (6) and molecular hybridizations used to identify these complexes are highly informative since they focus attention on the relevant genetic information. However, it must be emphasized that these techniques are too sophisticated and laborious for the routine use demanded by extensive clinical studies. An obvious pathway for exploiting the potential usefulness of these RNA-enzyme complexes is to develop a radioimmune assay (29) for their protein components. This approach, if successful, would possess the simplicity, sensitivity, and speed required for realistic clinical applications. Attempts along these lines are in progress.

Summary. Complexes containing 70S RNA and RNA-directed DNA polymerase were detected in 74 % of the human leukemic plasmas, whereas none was observed in normal plasmas. The DNA product synthesized by these complexes hybridized to the RNA of Rauscher leukemia virus and to RNA obtained from leukemic cells and did not hybridize to the RNA of normal leukocytes or to the RNA of the unrelated avian meyeloblastosis virus.

ACKNOWLEDGEMENTS

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RELATEDNESS OF RNA AND REVERSE TRANSCRIPTASE FROM HUMAN ACUTE MYELOGENOUS LEUKEMIA CELLS AND FROM RNA TUMOR VIRUSES

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Abstract

Complexes of RNA tumor virus-like RNA (35S or 70S RNA) and DNA product, synthesized endogenously from deoxynucleotide triphosphates by a particulate cytoplasmic fraction, were detected in leukemic cells from seven of eight cases of human acute myelogenous leukemia. In one case, the DNA reaction product was shown to have extensive sequence homology to RNA from a primate C-type virus. In two other cases, a RNA-directed DNA polymerase with biochemical and immunological relatedness to reverse transcriptase from primate C-type viruses was isolated from the endogenous, DNA-synthesizing cytoplasmic fraction. Thus, human leukemic cells contain virus-related RNA and RNA-directed DNA polymerase (reverse transcriptase), which are associated in a fashion similar to analogous molecules in RNA tumor viruses. These findings suggest that C-type viruses are etiologically related to leukemia in man, as in lower species, and that immunobiochemical products from primate C-type viruses may provide sensitive probes for further exploring the role of viral information in human leukemia. They also substantiate our earlier reports on reverse transcriptase in human leukemic cells.

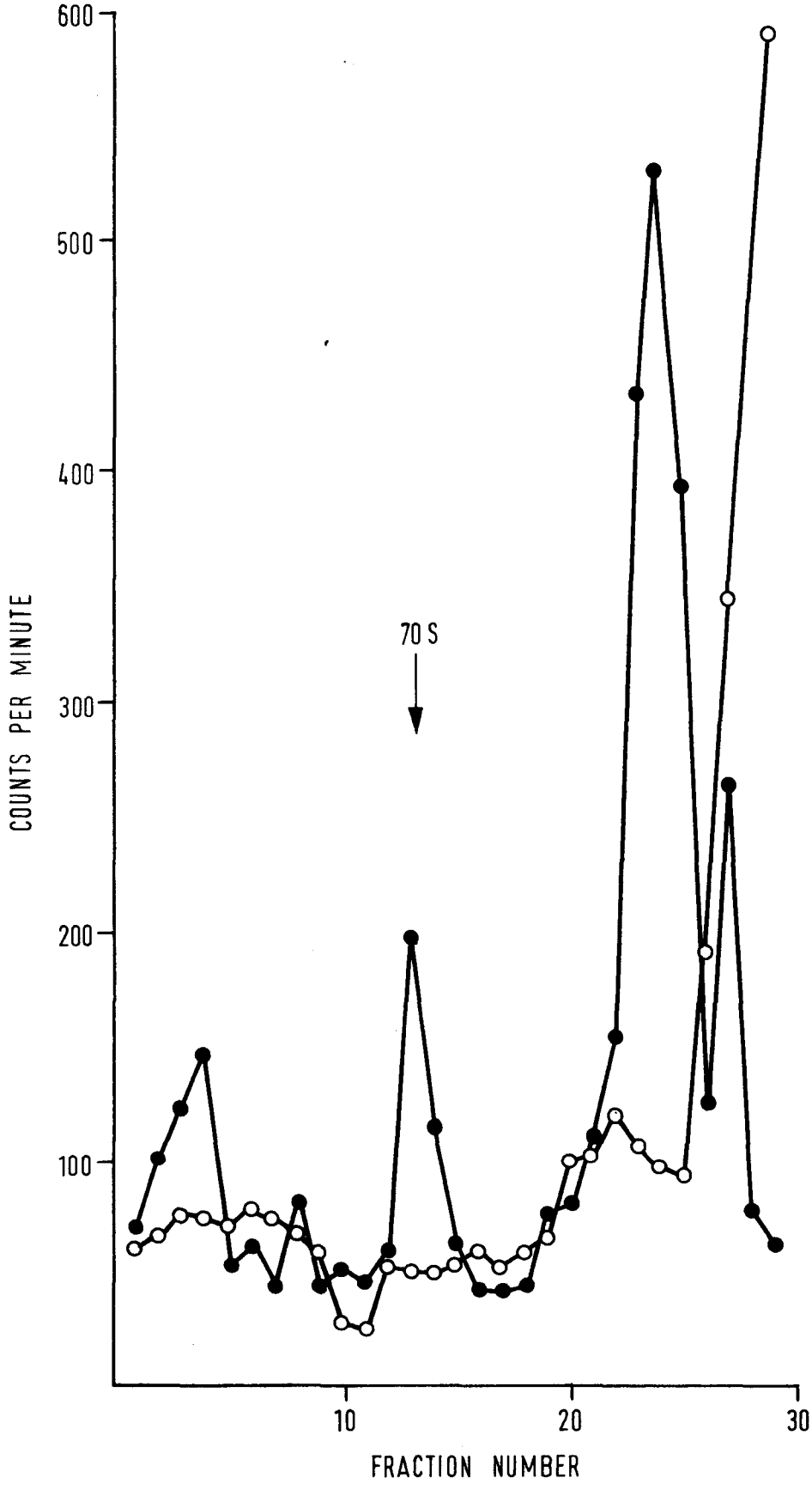
Introduction

In the past two years evidence has accumulated that human leukemic cells contain molecules with marked similarity to analogous components from RNA tumor viruses (1–7). One experimental approach, followed by Gallo and his associates, has resulted in the identification and purification from both acute lymphoclastic and acute myeloblastic leukemia cells of RNA-directed DNA polymerases, which use synthetic template-primers and natural RNA-primers in a similar fashion to purified reverse transcriptase from RNA tumor viruses (1, 2, 7). Recent evidence further indicates that leukemic RNA-directed DNA polymerase is inhibited by antisera to reverse transcriptase from some mammalian C-type viruses (ref. 3 and *vide infra*). A second experimental approach, advanced by Spiegelman and his colleagues, has accomplished the identification in acute and chronic myelogenous and lymphatic leukemia cells of cytoplasmic RNA's, which has small but detectable sequence homology with RNA

from a mouse type-C leukemia virus (Rauscher) but not with RNA from avian leukemia virus or from mouse mammary tumor virus (5, 6). Recently, our laboratory has found more extensive homology of RNA from human acute leukemic cells to primate C-type sarcoma virus RNA (ref. 4 and *vide infra*). Both experimental approaches indicate that the RNA tumor virus-like polymerase and RNA are complexed, as determined by the endogenous synthesis of DNA on the RNA template (2–5), and that the polymerase-RNA complex is present in a cytoplasmic particle which has the density (1.14–1.17 g/ml) characteristic of RNA tumor viruses (3–5). The latter suggests that some virus-like form exists in leukemic cells, however, no discrete morphological entity or infectious particulate activity has yet been identified. Also, both experimental approaches have failed to find RNA tumor virus-like molecules in normal leukocytes, although this could be due to inadequate sensitivity of the techniques or to the unavailability of completely appropriate control cells, i. e. normal myeloblastic or lymphoblastic stem cells.

In the present report, we summarize some of our recent findings supporting the presence of RNA tumor virus-like RNA and RNA-directed DNA polymerase in human acute myelogenous leukemic cells. These data are related to results obtained by velocity glycerol gradient analyses of endogenous DNA reaction products from a particulate cytoplasmic fraction of leukemic cells from eight cases of acute myelogenous leukemia. The preparation of the cytoplasmic pellet fraction was modified from a previously reported method (2). The velocity gradient assay for the "simultaneous detection" of RNA template and associated DNA polymerase is a slight modification of the previously described method (8).

Fig. 1: Velocity glycerol gradient analysis of endogenous ^{3}H-DNA product synthesized by the cytoplasmic "pellet" fraction of acute myelogenous leukemia cells (P. C.). The cells (0.75 gm), suspended in 5 volumes of buffer A (0.05 M Tris, 5pH 7.5, 5 mM $MgCl_2$, 0.02 M dithiothreitol and 0.5 mM EDTA) containing 0.1 m sucrose, were homogenized to approximately 75 % cytoplasmic rupture in a tightfitting Dounce homogenizer. The nuclei and mitochondria were successively removed by centrifugation for 15 min. at 1000 xg and 12,000 xg, respectively. The supernatant was layered over a 10 ml column of buffer A containing 25 % (w/w) sucrose in an SW27 rotor and centrifuged at 98,000 xg for 1 hour. The supernatant was removed and the "cytoplasmic pellet fraction", was evenly suspended in 0.5 ml buffer B (0.05 M Tris, pH 7.5, 5 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol and 0.5 mM EDTA). Triton X-100 was added to a final concentration of 0.1 %, and the suspension was incubated at 4 °C for 15 min. In a final volume of 1.25 ml containing 0.25 ml of the pre-incubated pellet, 50 μg/ml actinomycin D, 0.05 M Tris, ph 8.3, 60 mM KCl, 15 mM KF, 10 mM $MgCl_2$, 5 mM ATP, 320 μM dGTP and approximately 30 μM each ^{3}H-TTP (Amersham, 54 Ci/mMole), ^{3}H-dATP (Schwarz, 12.5 Ci/mMole) and ^{3}H-dCTP (Schwarz; 23 Ci/mMole), DNA synthesis was performed at 27 °C for 30 min. The reaction was terminated and the DNA product purified as described by Reitz, et al. (9). The purified DNA product was applied to a 10–30 % gradient of glycerol in TNE buffer (0.01 M Tris, pH 7.5, 0.1 NaCl and 1 mM EDTA) and centrifuged in a SW41 rotor at 40,000 RPM for 4 hr. Samples were collected and processed as previously described (9). An external marker of ^{3}H-70S RNA from avian myeloblastosis virus was included in the centrifuge run. ●——●, untreated DNA product; ○——○, DNA product treated with RNase A (Worthington), 20 μg/ml, at 37 °C for 30 min.


600
500
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COUNTS PER MINUTE
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Fig. 1

Methods and Results

Myelogenous leukemic cells used in these studies were obtained by plasmaphoresis from patients with the clinical diagnoses of acute myelogenous leukemia (AML), acute myelomonocytic leukemia (AMML) and chronic myelogenous leukemia in the acute, blastic phase of the disease (CML). The cytoplasmic pellet fraction of the leukemic cells was prepared by first removing the nuclei and mitochondria from homogenized cells by differential centrifugation and by then further centrifuging the post-mitochondrial supernatant fraction at 98,000 x g for 1 hour. The material thus "pelleted" was used for the endogenous synthesis of DNA from four added deoxynucleotide triphosphates (see the legend to Figure 1 for details) and for further purification procedures described in the text.

Velocity Glycerol Gradient Analyses of Endogenous DNA Product

As illustrated in Figure 1 and summarized in Table 1, ^{3}H-DNA product associated with a rapidly-sedimenting, ribonuclease-sensitive complex was detected in 4 of 8 cases in which fresh or fresh-frozen (−70° C in 10 % dimethylsulfoxide) myelogenous leukemia cells were studied. The rapidly sedimenting ^{3}H-DNA-RNA complex was not observed if one deoxynucleotide triphospate was omitted from the endogenous DNA synthesizing reaction mixture (Table 1; Figure 2A, 3B) indicating that template-independent, end-addition reactions were not being detected (5). In all cases, the ^{3}H-DNA-RNA complex sedimented at the location of ^{3}H-70S and ^{3}H-35S

Table 1. Velocity gradient analyses of ^{3}H-DNA products from the 98,000 xg cytoplasmic pellet fraction of human leukemic blood cells*

		Rapidly-Sedimenting (RS) DNA				
Patient	Diagnosis	Estimated Size	cpm RS DNA	Per cent Total cpm	RNase Sensitivity	−1 dXTP (% of cpm)
P.C.	AML‡	70S	495	7.8	+	N.D.+
N.N.	AML		N.D.			
G.F.	AML	35S	1830	50.4	+	7.6
J.C.	AMML§		N.D.			
C.P.	AMML	35S	110	10.7	+	N.D.
J.Wh.	CML¶		N.D.			
J.Wi.	CML		N.D.			
A.W.	CML	35S	95	2.1	+	N.D.

* Procedures used for the preparation of the cytoplasmic pellet fraction, for the synthesis and purification of ^{3}H-DNA product, and for velocity glycerol gradient analysis are described in Figure 1.

\+ N. D., not detected.

‡ AML, acute myelogenous leukemia

§ AMML, acute myelomonocytic leukemia

¶ CML, chronic myelogenic leukemia in blast crisis.

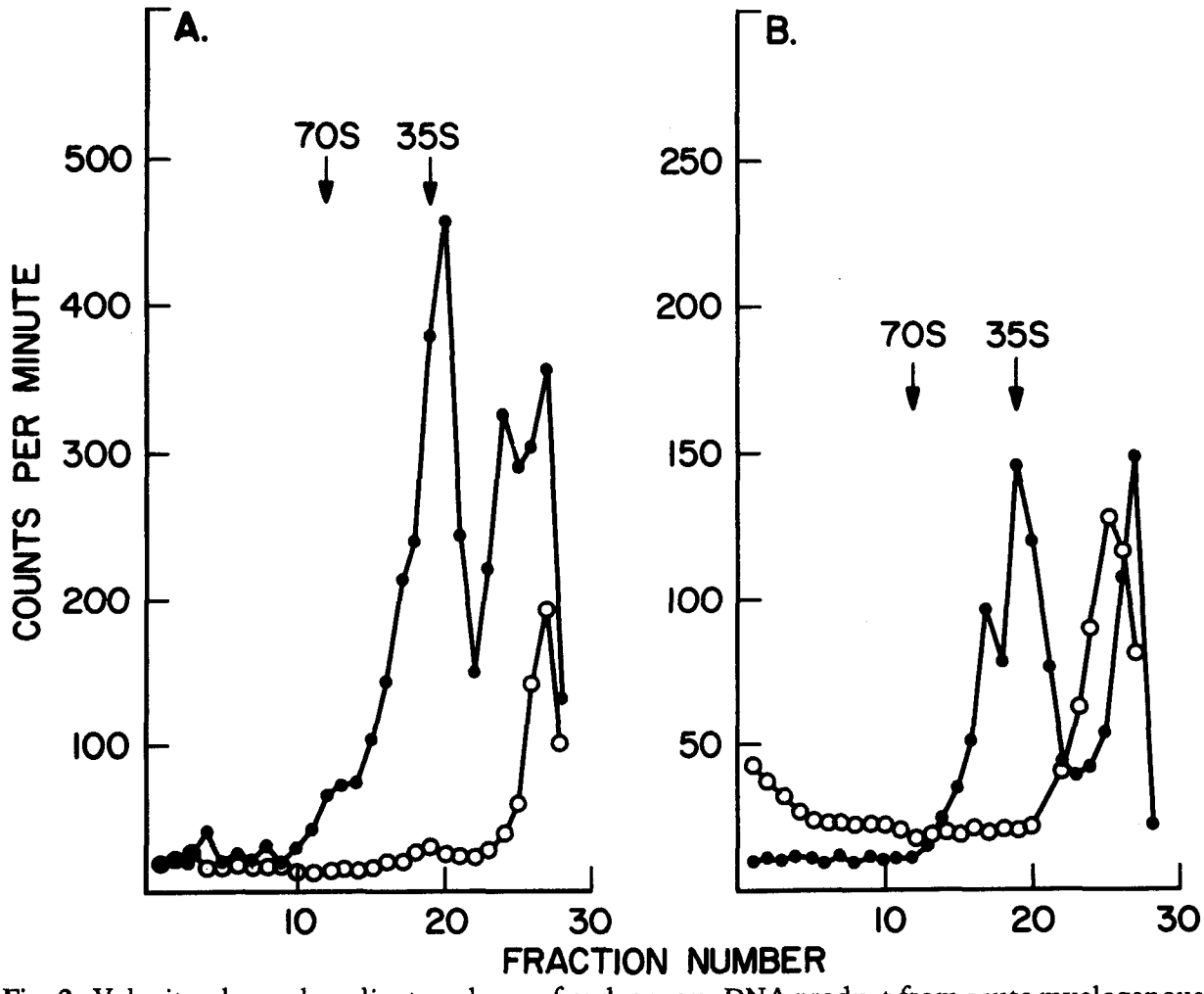

Fig. 2: Velocity glycerol gradient analyses of endogenous DNA product from acute myelogenous leukemia cells (G. F.). Procedures are described in Fig. 1. (A) Endogenous DNA product from the 98,000 xg "crude" cytoplasmic pellet fraction: ●——●, all 4 trinucleotides present in the reaction mixture; ○——○, dGTP omitted from the reaction mixture. (B) Endogenous DNA product synthesized by the 1.15 to 1.17 density fraction after sucrose equilibrium density gradient centrifugation (21): ●——●, untreated; ○——○ after RNase treatment.

Table 2. Velocity gradient analyses of ^{3}H-DNA products from leukemic blood cells after short-term culture*

Patient	Diagnosis	Rapidly Sedimenting (RS) DNA			Results From Fresh Cells
		Estimated Size	cpm RS DNA	Per Cent Total cpm	
N.N.	AML	35, 70S	98, 83	12.4, 10.6	N.D
G.F.	AML	70S	180	10.5	35S
J.C.	AMML	35S	795	15.3	N.D.
C.P.	AMML	35S	135	2.6	35S
J.Wh.	CML	35S	101	5.2	N.D.

* Leukemic blood cells were placed in stationary culture at 1 x 10^7 cells/ml, using RPMI 1640 containing 20 % fetal calf serum, at 37 °C for 6 to 8 days. All methods and designations are given in Table 1.

RNA from purified RNA tumor viruses. Occasionally, as in Figure 1, some RNase-sensitive ^{3}H-DNA complex was noted at the bottom of the glycerol gradient and in the 12 to 28S region of the gradient. The former probably represents molecular aggregates; the latter may represent DNA associated with partially degraded RNA template, although other explanations are possible.

In several instances, 0.5 to 1.0 gm of leukemic cells were placed in short-term tissue culture (6 to 8 days) before preparing endogenous DNA product for glycerol gradient analysis. After culture, DNA product from three leukemic cytoplasmic pellet preparations which had previously failed to show any rapidly-sedimenting DNA, was demonstrated to sediment in part as ribonuclease-sensitive 35S and 70S complexes (Table 2). Similar results were obtained in the presence of 5′ – iodo-deoxy-uridine (20 μg/ml for the first 48 hours in culture), a compound capable of "inducing" leukemia virus from non-producing mouse tissue culture cells (10).

Overall, in 7 of 8 cases of leukemia involving myeloblastic cells endogenous DNA product was detected as a complex with RNA characteristic in size of intact (70S) or

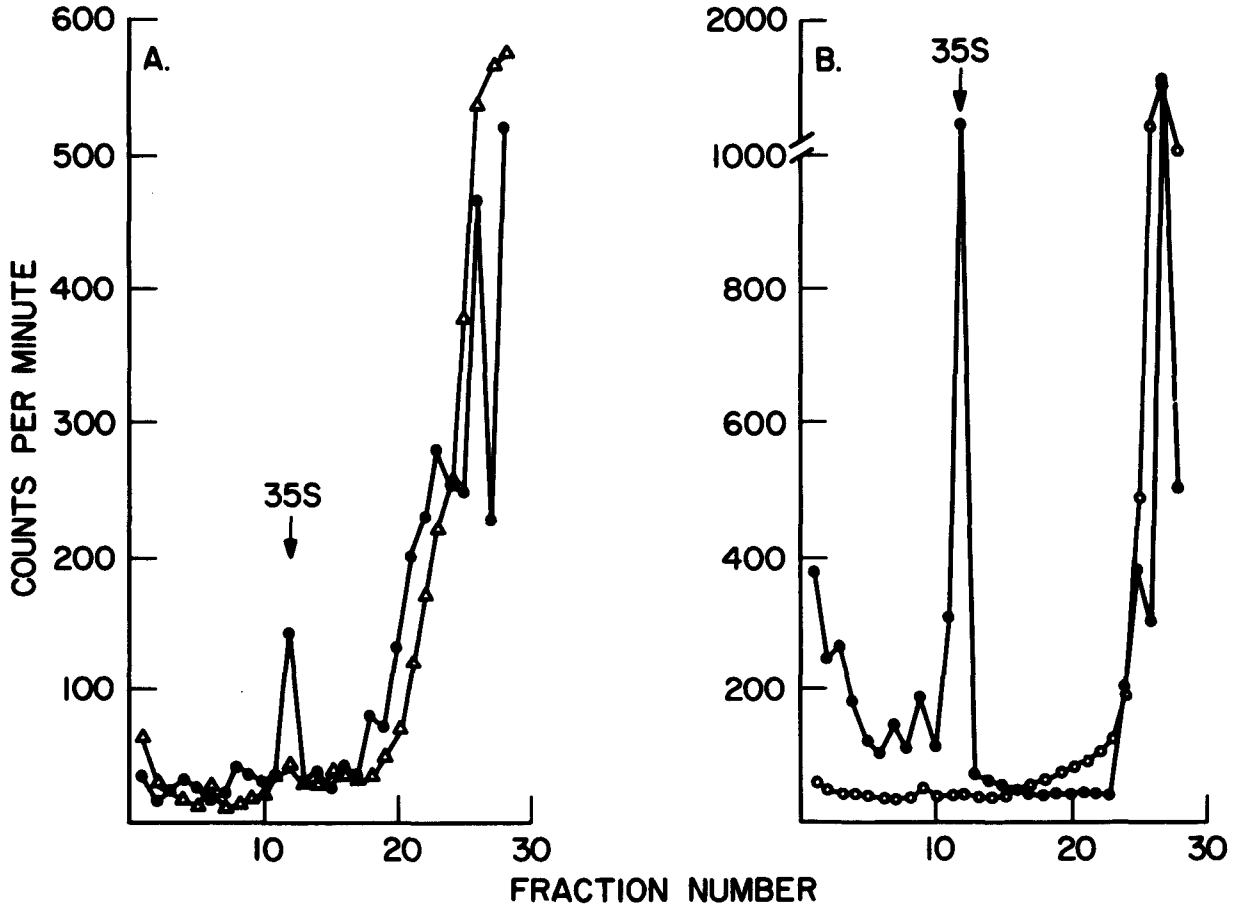

Fig. 3: Velocity glycerol gradient analyses of endogenous DNA product from acute myelomonocytic leukemia cells (C. P.). Procedures are described in Fig. 1. (A) Endogenous DNA product from the 98,000 xg "crude" cytoplasmic pellet fraction: ●——●, untreated; △——△, after ribonuclease treatment. (B) Endogenous DNA product synthesized by the 1.13 to 1.15 density fractions after equilibrium sucrose gradient centrifugation (21): ●——●, all 4 trinucleotides present; O——O, dGTP omitted from the reaction mixture.

subunit (35S) RNA from RNA tumor viruses. Similar analyses of three sets of normal, peripheral blood buffy coat leukocytes and three sets of phytohemagglutinin-stimulated lymphocytes showed no evidence of rapidly sedimenting DNA-RNA complexes. These data confirm the report by Baxt, et al. (5), that nearly all human acute

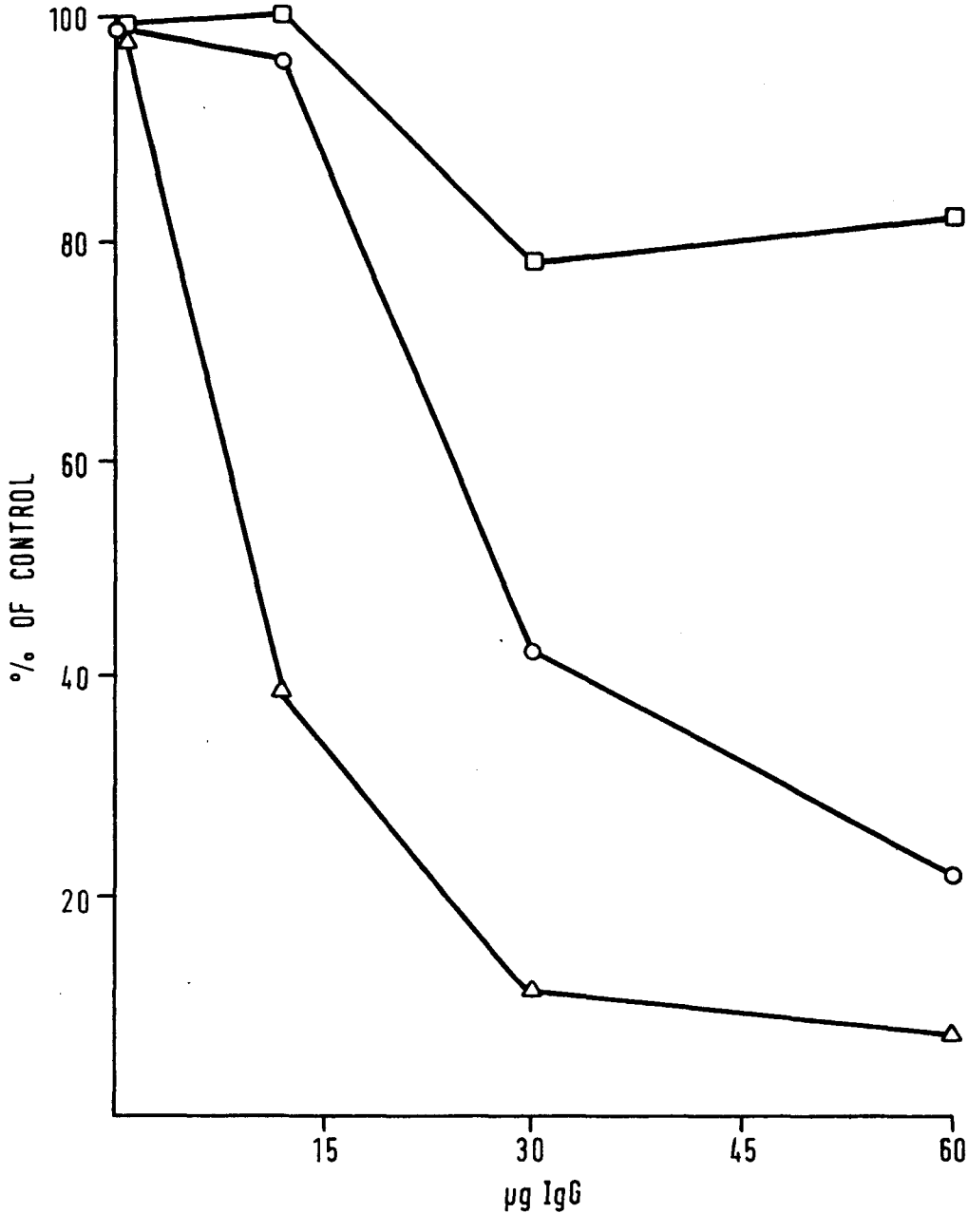

Fig. 4: The effect of variable concentrations of IgG from antisera to reverse transcriptase from primate and mouse C-type viruses on the leukemic cytoplasmic pellet enzyme. The preparation of the post-sepharose enzyme used in this experiment is outlined in the text, and detailed procedures for the preparation of antisera and for the performance of enzyme reactions are presented elsewhere (3, 21). □——□, murine leukemia virus (Rauscher) antipolymerase IgG; ○——○, gibbon ape lymphosarcoma virus antipolymerase IgG; △——△, simian sarcoma virus antipolymerase IgG.

myelogenous leukemia cells contain RNA which is the same size as RNA from RNA tumor viruses, and the template function of this RNA with its associated DNA polymerase, verifies (by a different approach) the earlier reports from our laboratory of reverse transcriptase in human leukemic cells (1–3).

Molecular Hybridization of Endogenous DNA Product

Endogenous DNA product, synthesized from the cytoplasmic pellet fraction of patient G. F. (AML) in the presence of actinomycin D (50 μg/ml), was shown to be complexed approximately 50 % to 35S RNA by velocity glycerol gradient analysis (Figure 2A; Table 1). After further purification of this pellet fraction by equilibrium sucrose density gradient centrifugation, the particulate fraction synthesizing DNA product complexed with 35S RNA was localized at a density of 1.15 to 1.17 g/ml (Figure 2B), the density characteristic of RNA tumor viruses. DNA product synthesized from this sucrose density fraction was annealed for 8 days to denatured viral RNA immobilized on 7 mm phoshocellulose discs (11). As shown in Table 3, over half of the input DNA hybridized to 70S RNA from simian sarcoma virus (SiSV; derived from a woolly monkey fibrosarcoma; 12). Approximately, 20 % and 5 % of the input DNA was bound, respectively, to murine sarcoma virus (Kirsten) and murine leukemia virus (AKR) RNA. Further information related to DNA product from patient G. F. and hybridization data with similar results from a patient with chronic myelogenous leukemia in blast crisis (J. Wh.) are presented in detail elsewhere (4). The pattern and magnitude of these hybridization results leave little doubt that RNA from the cytoplasmic pellet fraction of human myelogenous leukemia cells have nucleotide sequences in common with genomic RNA from RNA tumor viruses.

Identification of an RNA Tumor Virus-Related RNA-Directed DNA Polymerase in the Endogenous, DNA Synthesizing Cytoplasmic Pellet Fraction

Biochemical Properties

Endogenous DNA product from the cytoplasmic pellet fraction of patient C. P. (AMML), was complexed to 35S RNA, and the proportion of 35S-sedimenting DNA synthesized was considerably enriched after sucrose density-gradient banding of the pellet fraction (Figure 3). This enrichment phenomenon has been observed in several cases and is probably due to purification of the RNA and reverse transcriptase containing particulate fraction from contaminating degradative enzymes. Similarly, the endogenous, ribonuclease-sensitive DNA polymerase activity from the pellet fraction has also been augmented by sucrose gradient banding, and in several cases, marked purification of this activity at a density of 1.14 to 1.18 has occured after repetitive sucrose gradient bandings (4), suggesting the presense of discrete virus-like particles in human leukemic cells.

In the present case (C. P.), DNA polymerase activity which slightly preferred the synthetic template-primer $(A)_n \cdot dT_{12-18}$ to $(dA)_n \cdot dT_{12-18}$ (1.45 to 1) was observed in the 1.13 to 1.15 g/ml sucrose density fractions. The DNA polymerase activity favoring RNA template $(A)_n$ was further purified, not by convential techniques for

the purification of polymerase enzymes, but by sepharose 4B chromatography, a procedure for analyzing high molecular weight molecules or complexes (exclusion = 20 x 10^6 daltons). After this procedure, the endogenous, ribonuclease-sensitive DNA polymerase activity and most of the DNA polymerase activity preferring $(A)_n \cdot dT_{12-18}$ (now by 8 : 1 over $(dA)_n \cdot dT_{12-18}$) was localized in the exclusion volume of the sepharose column, indicating the presence of a large complex containing 35S RNA and RNA-directed DNA polymerase.

When reaction conditions were optimized by adjusting the MnC1 and KC1 concentrations, respectively, to 1 mM and 100 mM, the preference of the post-sepharose enzyme for $(A)_n \cdot dT_{12-18}$ over $(dA)_n \cdot dT_{12-18}$ increased to greater than 40 : 1 (Table 3). This enzyme, subsequently termed the "leukemic pellet enzyme," had an absolute requirement for divalent cation with Mn++ markedly preferred to Mg++. $(dA)_n \cdot dT_{12-18}$ was poorly utilized under all reaction conditions tested. This clearly differentiates the leukemia pellet enzyme from DNA polymerases I and II from normal leukocytes, which strongly prefer $(dA)_n \cdot dT_{12-18}$ over $(A)_n \cdot dT_{12-18}$ (13). This criterion does not differentiate between the leukemic enzyme and the "R-DNA polymerase," which has been demonstrated in several types of mammalian cells by Weissbach and his associates (14). However, as further shown in Table 3, the leukemic pellet enzyme utilized $(C)_n \cdot dG_{12-18}$ approximately 30 %, and in repeat studies up

Table 3. Hybridization of the (^{3}H)-DNA Product of the Endogenous RNA-Directed DNA Polymerase from Human Leukemic (GF) Cytoplasmic Pellet Fraction to RNA Isolated from RNA Tumor Viruses*

RNA+	cpm‡	cpm (−AMV)	% Hybridization
SiSV (NRK)	550	450	53
MuSV (Kristen)	259	159	19
MuLV (AKR)	145	45	5
AvLV (AMV)	100	–	–
Input	840	–	–

* ^{3}H-DNA product was synthesized from the cytoplasmic pellet fraction, banding at a density of 1.16 g/ml, after equilibrium sucrose density gradient centrifugation (4). This DNA product was purified and annealed for eight days in 0.1 ml of 50 % formamide- 3XSSC (final concentrations) to 0.2 μg of immobilized 70S viral RNA (4, 11).

+ The tumor viruses used as a source of RNA are abbreviated as follows: SiSV (NRK) = Simian sarcoma virus grown in normal rat kidney cells (RK); (Kirsten) = a sarcoma-leukemia virus complex grown in NRK cells which originated by repeated infection of rats with a Gross-type mouse leukemia virus; MuLV (AKR) = a Gross-type mouse leukemia virus grown in mouse fibroblast cells and originating spontaneously from AKR mice; AvLS (AMV) = avian leukosis virus, strain avian myeloblastosis.

‡ Data are presented as crude filter-bound radioactivity in counts per minute (cpm), as the same data corrected for radioactivity trapped by a heterologous RNA (cpm minus AMV), or as the corrected data expressed as percent of the input DNA recovered in a hybrid structure (% hybridization).

Table 4. Template-Primer Specificity of the Leukemic Cytoplasmic RNA-Directed DNA Polymerase After Sepharose 4B Purification*

Additions	^{3}H-Deoxyribonucleotide Triphosphate	pmole Incorporated+ X 10^{-1}
$(A)_n.dT_{12-18}$	TTP	7.56
$(dA)_n.dT_{12-18}$	TTP	0.17
$(C)_n.dG_{12-18}$	dGTP	2.13
dT_{12-18}	TTP	0.35
	dGTP	0.01
dG_{12-18}	TTP	0.08
	dGTP	0.06

* Methods for the preparation of the post-sepharose enzyme are indicated in the text and described in detail in Ref. 21.

\+ The pmoles of TMP or dGMP incorporated in 30 minutes at 37 °C in a 0.05 ml reaction mixture containing 50 mM Tris, pH 8.3, 1 mM $MnCl_2$, 3 mM dithiothreitol, 6 μM ^{3}H-TTP (54 Ci/mMole) or ^{3}H-dGTP (10 Ci/mMole), 0.01 ml of the post-sepharose enzyme ("activated" by pre-incubation for 10 minutes at 37 °C in 0.1 % Triton X-100), and 3860 pMoles (expressed as pmoles of mononucleotide) of oligomer (dG_{12-18} or dT_{12-18}) with or without an equimolar amount of complementary polymer ($[C]_n$, $[A]_n$, or $[dA]_n$).

to 50 %, as well as $(A)_n \cdot dT_{12-18}$. This level of activity with $(C)_n \cdot dG_{12-18}$ has previously been observed only with true viral reverse transcriptase (3, 14, 15). Recently, Fry and Weissbach reported the use of $(C)_n \cdot dG_{12-18}$, approximately 5 % as well as $(A)_n \cdot dT_{12-18}$, by R-DNA polymerase from mouse L-cells (15); this result must be interpreted with reservation since mouse L-cells are infected with RNA "tumor" virus (16). We find that extensive utilization of this template-primer is a specific indication of virus polymerase, in agreement with Baltimore and his colleagues (3, 17, 18). Finally, since no significant activity was demonstrated with the primers dT_{12-18} and dG_{12-18} (Table 3), this enzyme is not terminal deoxynucleotidyl transferase, which was recently reported to be present in leukemic cells from one patient with acute lymphocytic leukemia (19). Moreover, as shown in Table 4, there is no activity with primer alone (i. e., without templates), the assay used for detection of terminal transferase (19).

Immunological Properties

In order to further investigate the relatedness of the leukemic reverse transcriptase to viral reverse transcriptase, we tested for possible inhibition of the enzyme by purified IgG from antisera prepared against reverse transcriptases from various RNA tumor viruses. As shown in Figure 4, the leukemic enzyme was markedly inhibited by antipolymerase IgG to the two known primate C-type viruses, simian sarcoma virus (12) and gibbon ape lymphosarcoma virus (20). A slight degree of inhibition was noted with antipolymerase IgG against reverse transcriptase from mouse leukemia

virus (Rauscher) (Figure 4). In other studies, antisera to reverse transcriptase from avian sarcoma virus and the non-C-type Mason-Pfizer monkey virus did not inhibit the leukemic enzyme. These results, which are reported in greater detail elsewhere (21), are very similar in pattern to those recently reported for RNA-directed DNA polymerase from another case of acute myelogenous leukemia (3). We have further demonstrated that these leukemic enzymes are not inhibited by antisera to DNA polymerase I from phytohemagglutinin-stimulated lymphocytes (23).

These immunological results confirm the biochemical data by demonstrating that the human leukemic reverse transcriptase is related to virus reverse transcriptase but not to normal cellular DNA polymerase I. It seems probable that the leukemic enzyme is, also, not related to cellular DNA polymerase II (13), since antisera to reverse transcriptase from RNA tumor viruses do not inhibit either of the major cellular DNA polymerases (22). The immunologic results extend the biochemical data by demonstrating that the leukemic enzyme has close homology to reverse transcriptase of C-type viruses from primates but not from lower species. These results are similar to those reported from a study of the immunologic relationships of primate oncornaviruses and agree with the specificity of the nucleic acid hybridization results previously mentioned (4).

Discussion

The data presented here indicate with near certainty that human acute myelogenous leukemic cells contain RNA and RNA-directed DNA polymerase related in function and structure to genomic RNA and to reverse transcriptase from RNA tumor viruses, particularly from primate C-type viruses. These findings make "evolutionary sense" in that both the RNA and RNA-directed DNA polymerase are related in general biochemical function to analogous molecules in distantly-related species but by exact, primary structural criteria (nucleic acid hybridization and immunologic cross-reactivity) have extensive homology specifically to RNA and reverse transcriptase from closely-related, primate species. Although these data do not establish the cause of human myelogenous leukemia, they do provide powerful support for the concept that C-type viruses have an etiologic role in leukemia in man, as in other species. Also, these data do not indicate whether the leukemic viral information comes from endogenous genetic information or from exogenous sources. The finding of similar nucleic acid hybridization patterns of leukemic DNA product (4) and of similar immunologic reactivity patterns of "reverse transcriptase" from different patients with myelogenous leukemia suggests that a common viral element may be involved, although more discriminating studies with specific molecular probes are required to resolve this issue.

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GLUCOCORTICOSTEROIDS AND RNA TUMOR VIRUSES

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Abstract

Glucocorticosteroids at physiological concentrations stimulated type-C virus production 2 to 50-fold from mouse fibroblasts induced by 5-iodo-2′-deoxyuridine. They also stimulated virus production from chronically infected mouse myeloid leukemia cell line. Some potential practial applications of these findings are discussed.

In 1971, D. R. Lowy, et al., reported that murine leukemia virus can be induced from previously non-viral producing mouse cells using 5-iodo-2′-deoxyuridine (IUdR) (1). This phenomenon of virus induction from previously non-producing cells is now well established in several systems. The accumulated data from the induction experiments provide strong evidence that the genetic information for virus production is present in every cell of an inducible clone. We have previously suggested that the expression of the provirus genome (to form viral RNA) probably utilizes normal cell mechanisms for transcription and translation of mRNA. Compatible with this proposal are the following recent observations: (A) RNA tumor viruses contain a sequence of poly (A) like mRNA (2); (B) Cordycepin (3′-deoxyadenosine) an inhibitor of poly (A) synthesis (3) which preferentially affects the maturation of mRNA, inhibits virus induction by IdU (4); and (C) cells infected with leukemia virus do not have new RNA polymerase (5).

The glucocorticoids are a group of agents which have been shown to affect host transcriptional and translational mechanisms. It was of interest to establish the role of these and other hormones in virus induction by IdU from "non-producer", and their effect on virus producing cells.

The cells used in this study were BALB/C-3T3 fibroblasts and the same clone transformed by Kirsten murine sarcoma virus (K-MSV) (designated BALB/K-3T3) (both "non-producers"). A mouse myeloid leukemia cell line was chosen as a "producer" (designated.M_1, courtesy of Dr. Y. Ichikawa, Kyoto, Japan). Induced cells were treated with IdU for 24 hours and in most of the experiments the hormones were present throughout the whole course of the experiment.

The Effect of Dexamethasone on Virus Production Induced by IdU

The production of K-MSV (after IdU) was measured by assaying reverse transcriptase in particulate fractions released into the media as previously described (4). The effect of dexamethasone on induction is shown in Figure 1. Basically the kinetics of virus induction are the same in the control group (IdU alone) and in those treated with hormone and IdU. Maximum augmentation in this particular experiment is in the second day after induction where relative virus production was stimulated about 50-fold and at the peak (day 6) about 25-fold. The concentration of the hormone was 10^{-6} M. As shown in Table 1, the optimal concentrations for stimulation are between 10^{-5} and 10^{-6} M.

At this point, it was necessary to show that the increase in the reverse transcriptase activity truly reflects an increase in virus production. One approach was to measure the preference of the enzyme for $(dT)_{12-18}$. (rA)n over $(dT)_{12-18}$. (dA)n as template-primer, a characteristic useful in distinguishing viral from normal cellular polymerase (6, 7, 8, 9). The enzyme derived from the IdU treated cultures showed a substantial preference for $(dT)_{12-18}$. (rA)n as template-primer over $(dT)_{12-18}$. (dA). This fact together with the fact that the enzyme activity is found in the medium and after high speed centrifugation in the particulate pellet and not found in the control, implies that we are almost certainly dealing with enzyme from viral origin.

A second and more definitive approach was to measure viral biological activity by determining the number of focus forming units (f. f. u.). The results of such an experiment are summarized in Table 2. Both the polymerase activity and the number of f. f. u. increased (5 and 50-fold, respectively). The later assay is a much more sensitive one as it measures single units and provides direct proof that dexamethasone did indeed stimulate virus production.

Table 1. Stimulation of the IdU Induced Virus Production From BALB/K-3T3 Cells By Various Concentrations of Dexamethasone and Prednisolone

Hormone Concentration (M)	Relative Virus Production* pmole/ml	
	Dexamethasone	Prednisolone
0	3.2	3.2
10^{-8}	7.7	N.T.
10^{-7}	22.7	7.6
10^{-6}	56.0	40.4
10^{-5}	29.0	47.8
10^{-4}	N.T.	32.3

* Virus production was measured by RNA directed DNA polymerase activity present in the virus particles. The data were obtained from the peak activity of each culture as shown in Figure 1 (the second day, and third day after induction for prednisolone and dexamethasone, respectively). The data are expressed in picomoles per milliliter of unconcentrated medium. The value of the uninduced control (0.05 pmole/ml) was subtracted from all values. 40 μg/ml of IdU was used in each experiment.

N. T. = not tested

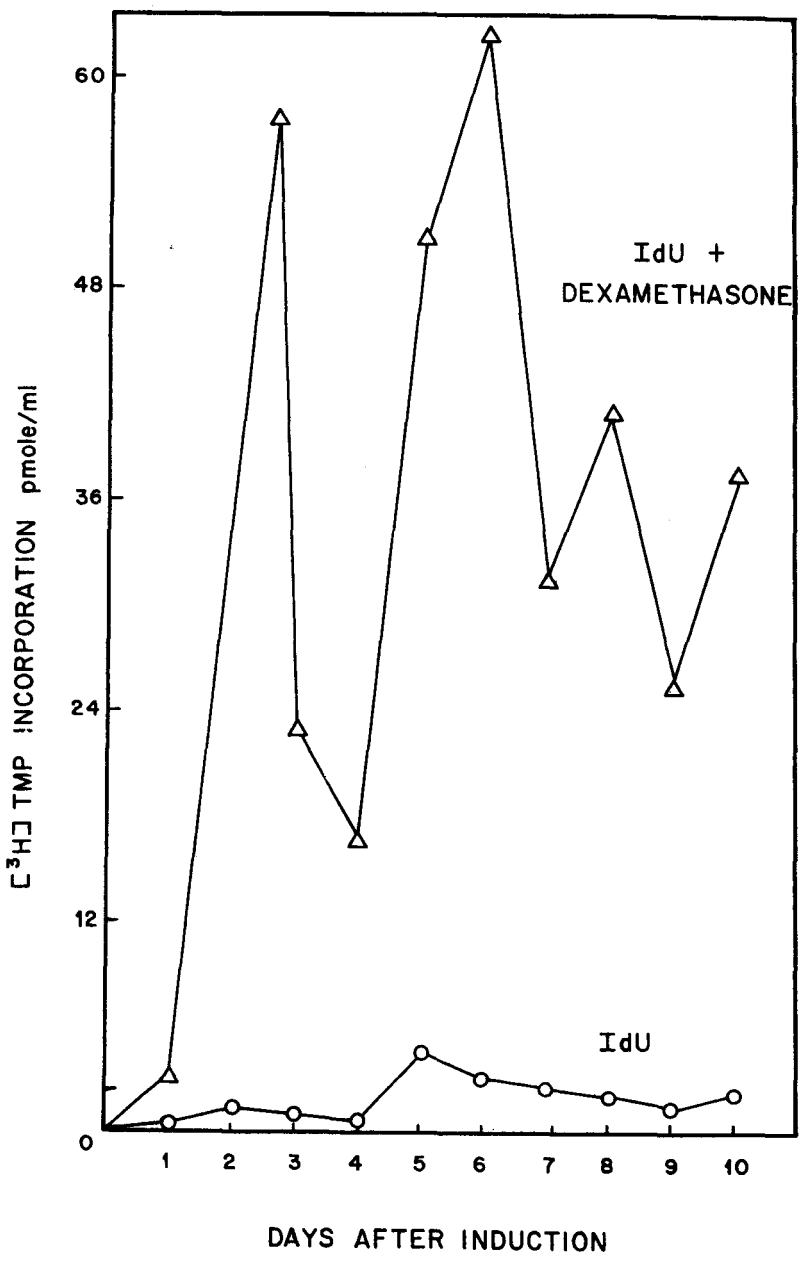

Fig. 1. Kinetics of induction of virus production by IdU and its stimulation by dexamethasone in BALB/K-3T3 cells. 10^6 cells were plated in 100 mm petri dishes. Dexamethasone, when present, was added immediately after plating the cells, and thereafter, daily throughout the course of induction. IdU was added one day after plating the cells for 24 hours. Days after induction started with the addition of IdU, the harvested medium was concentrated 30 fold by ultracentrifugation at 82,000 g for 60 minutes at 4°C. The pelleted particles were resuspended in buffer containing 10 mM Tris HCl, pH 7.9, 20 mM KCl, 1 mM EDTA, 1 mM Dithiothreitol (DDT), and 50 % glycerol. The polymerase activity was assayed in a 50 μl reaction mixture containing 40 mM Tris HCl, pH 7.9, 60 mM KCl, 1 mM DDT, 1 mM Mn Acetate, 0.2 mM EDTA, 20 mM NaF, 0.1 % Triton X-100, 10 μg/ml $(dT)_{12-18}$. (rA)n (Collaborative Research), 5.4 μM H^3-TTP (Schwarz-Mann Biochemicals, specific activity 13,000 c.p.m./pmole) and 20 μl of virus solution. (dT)n was synthesized at 30°C for 45 minutes and the precipitable radioactivity was then determined. Since the yield of viral enzyme after centrifugation was estimated to be about 20 %, the enzyme activity was corrected accordingly.
Symbols: o---o, Control; Δ---Δ, IdU + Dexamethasone

Table 2. Relationship Between RNA Directed DNA Polymerase Activity and Viral Transforming Activity*

Dexamethasone	RNA Directed DNA Polymerase Activity** (pmole/ml)	Viral Transforming Activity*** (f. f. u./ml)
None	10.7	0.4×10^3
10^{-6} M	49.1	20×10^3
Fold Stimulation By Hormone	4.6	50

* The cultured cells (BALB/K-3T3 cells) in the absence and in the presence of hormone were induced with IdU and the media were collected daily and assayed for RNA-directed DNA polymerase and focus forming ability in normal rat kidney cells (NRK). The values presented in the Table were obtained from results 3 days after IdU, the time of maximum virus induction. The polymerase assays were the same as those described in the legend to Figure 1.

** The focus forming assay was assayed as follows: 0.2 ml of virus containing medium was added to DEAE-Dextran pretreated normal rat kidney cells (NRK) in 60 mm plates. After one hour absorption, all plates were washed with 2 ml phosphate-buffered saline and fed with 3 ml DMEM supplemented with 5 % fetal calf serum. Media were changed on days 2 and 4, and the foci were stained with giemsa and counted on day 6.

*** Specific Viral Activity is defined as the amount of RNA-directed DNA polymerase activity (pmole/45 min./ml) per f. f. u.

\# Fold stimulation is defined as follows:

$$\frac{\text{Activity in the presence of hormone}}{\text{Activity in the absence of hormone}}$$

Virus Induction and Cell Growth

Cultures treated with IdU grow slower and reach a lower cell density saturation (4). We, therefore, measured the effect of dexamethasone on cell growth to exclude the possibility that the hormone effect was only to enhance cell growth and as a consequence, more virus production. Cell number was determined daily during the course of induction. The results of this experiment are shown in Figure 2. The upper panel shows the profile of the accumulated cell number during the induction course and the lower panel shows an estimate of virus production by polymerase assay. The hormone had no effect on cell number in the IdU treated or untreated cultures indicating that cell number is not a determining factor for the hormone effect (cells treated with hormone alone did not release any detectable virus).

Optimal Time for Stimulation

It has been reported that IdU incorporation into DNA is essential for virus induction by this agent (1), and only 1 % of the cells (in one study) in the IdU treated culture are actually induced to release virus. Since the hormones used in this study were present throughout the whole course of induction, it is possible that the action of the hormone is a preinduction event, i. e., the steroids might increase the number of cells susceptible to virus induction. We have two pieces of evidence that indicate that

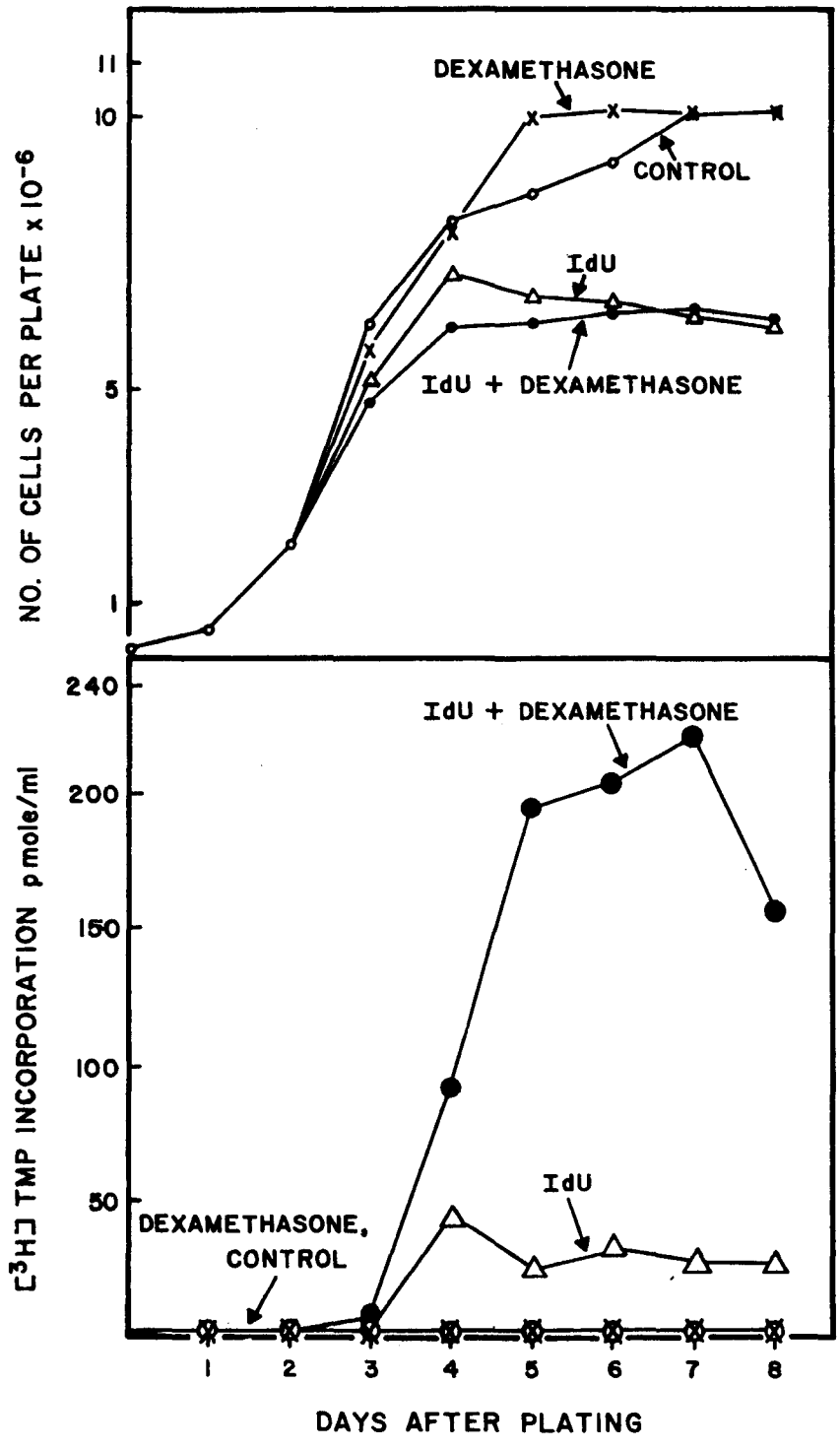

Fig. 2: Effect of dexamethasone on cell number and virus production induced by IdU from K-3T3 cells.
Upper Panel: The accumulated cell number during the course of induction of viruses by IdU in cells.
Symbols: o---o, Control; △---△; ●---●, IdU + Dexamethasone; x---x, Dexamethasone alone.
Lower Panel: The rate of induction of virus production assayed by RNA directed DNA polymerase activity. The enzyme assays were performed as described in the legend to Figure 1.

this is not the case, The first, which we won't go into detail about, is the absence of a significant increase in the number of infectious centers (10). The second derives from results obtained from a time course experiment. In these experiments, dexamethasone was added at various time intervals during the course of virus production. The results are shown in Table 3.

Maximum stimulation of virus production occurs when dexamethasone is present between 24 to 48 hours after IdU. Since IdU was present only in the first 24 hours, stimulation by dexamethasone must occur in the absence of IdU. The results show also that pretreatment of the cells with dexamethasone and its presence at periods other than that between 24 to 48 hours are not very effective in promoting virus induction.

Dexamethasone Increases the Detectability of Induced Viruses

The optimal IdU concentration for viral induction is between 40 and 200 μg/ml, and concentrations below 10 μg/ml apparently are not very effective. Table 4 summarizes the results of an experiment in which we used as low as 4 μg/ml of IdU. As indicated above, very little or no virus induction can be detected at this concentration of IdU. However, when dexamethasone was added virus was readily detected. Further, when 10 μg/ml of IdU and hormone are utilized, virus induction was greater than with 40 or 200 μg/ml of IdU alone.

The Effect of Dexamethasone on Chronically Infected Cells

In order to determine the effect of the hormone on chronically infected cells which are low producers, a mouse myeloid leukemia cell line (M_1) was treated with dexamethasone at a concentration of 10^{-6} M. Table 5 summarizes the results of these experiments. It was shown that after 6 days in culture, the treated cells produced five

Table 3. Dexamethasone Enhances Type C Virus Production After Removal of IdU

Presence of Dexamethasone*	Relative Virus Production#
(hours)	(pmole/ml)
none	119.2
−24 to 0	161.5
0 to 24	446.2
24 to 48	692.3
48 to 72	126.9

* The time of addition of IdU is taken as 0 time.

The procedure for virus induction from BALB/K-3T3 cells is the same as that described in the legend of Fig. 1 except that dexamethasone was added at the time indicated. IdU was present only in the first 24 hours of induction.

Table 4. Stimulation of IdU Virus Production from BALB/K-3T3 Cells by Dexamethasone

IdU μg/ml	Relative Virus Production* pmole/ml	
	– Dexamethasone	+ Dexamethasone
4	1.8	3.6
10	3.1	23
20	5.5	47
40	10.9	60
200	12.5	75

* Relative virus production is expressed as pmole/ml of RNA directed DNA polymerase activity.

Table 5. Stimulation of Virus Production from Mouse Myeloid Leukemia Cell Line (M_1) by Dexamethasone

Days in Culture	Relative Virus Production pmole/ml/10^6 Cells*	
	– Dexamethasone	+ Dexamethasone (10^{-6}M)
1	33	39
2	26	86
4	29	96
6	46	230

* Relative virus production is expressed as pmole/ml of RNA directed DNA polymerase activity per 10^6 cells.

times more virus than the non-treated cells. It is worthwhile to mention here that the hormones do not augment virus production from cells that already produce large amounts of virus.

Effect of Other Steroid Hormones on Virus Induction

All the natural and synthetic glucogenic hormones we tested augmented the induction of virus as seen in Table 6. The magnitude of stimulation with the BALB/K-3T3 cells range from 2-fold (cortisone) to 25-fold (dexamethasone) and with the BALB/3T3 cells from 1.8-fold (cortisone) to 7.5-fold (dexamethasone). Apparently this stimulatory activity is specific for the glucocorticosteroids.

Insulin was shown to enhance a post-transcriptional synthesis of TAT in rat hepatoma cells (11). To test whether insulin could also control virus production stimulated by dexamethasone the effect of insulin alone and together with dexamethasone was measured. As shown in Table 6, insulin does not enhance IdU induced virus production or the stimulation by dexamethasone. On the contrary, a small degree of inhibition was observed in both cases.

Table 6. Effect of Various Steroid Hormones and Insulin in Induction of Type C Virus by IdU from BALB/K-3T3 and BALB/3T3 Cells

Hormones*	Relative Virus Production** pmole/ml	
	.BALB/K-3T3	BALB/3T3
None	3.7	4.4
Dexamethasone	92.5	33.5
Prednisolone	81.0	18.5
Fludrocortisone	76.5	17.5
Hydrocortisone	61.0	14.8
Corticosterone	41.2	10.4
Cortisone	7.2	7.6
Androstenedione	5.9	2.5
Testosterone	4.4	2.5
Progesterone	3.4	4.7
B-estradiol	2.4	1.2
Insulin	1.8	–
Dexamethasone + Insulin	50.0	–

* The concentration of all steriod hormones is 10^{-6}M. The concentration of insulin is 5 to 10 μg/ml.

** Relative virus production is expressed as pmole/ml of RNA direct DNA polymerase activity. For details of these data, see the footnote to Table 1.

Discussion

This phenomenon of stimulation of RNA tumor virus production by steroid which was shown to be unique to a glucocorticosteroids (12) might have several applications among which include: 1) increase in the level of detectability of virus production which might be useful in attempting to induce viruses from human tumor cells. Advancement of this application may be important in the understanding of the etiology of human oncogenesis and as a diagnostic tool; 2) greater production of type-C virus from mammalian virus producing cell lines; and 3) in the light of the recent biochemical evidence for footprints of viral information in human leukemic cells such as virus-like reverse transcriptase (13, 14, 15), antigenic cross reactivity between this enzyme and primate viral enzyme (16), and the relatedness of nucleic acid sequences between human leukemic cells and murine type-C viruses (14) and particularly to primate (ape and monkey) type-C sarcoma viruses (17) it may be appropriate to consider our results with corticosteroid prior to their use in diseases where their value is in question such as myeloblastic leukemia (18).

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REVERSE TRANSCRIPTASE OF AN ONCORNAVIRUS-LIKE PARTICLE DERIVED FROM HUMAN HeLa CELLS

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Abstract

Oncornavirus-like particles have been observed and isolated from HeLa cells that contain a DNA polymerase activity with properties similar to RNA tumor virus reverse transcriptase. The virions have a density of 1.16–1.17 g/cm^3 and contain nucleic acid species that sediment as RNA (1.68 g/ml) in a Cs_2SO_4 equilibrium density gradient. Antigenically the HeLa virus DNA polymerase is related to the Mason-Pfizer monkey virus DNA polymerase.

The presence of virus-like particles, morphologically similar to those of the known RNA tumor viruses, has been recently observed in HeLa cells (1). Further electron microscopical analysis revealed maturation of an intracellular A-type particle at the cell membrane releasing a particle with properties of both B- and C-type virus particles (2). Comparative studies suggested these virus particles to be morphologically similar to the Mason-Pfizer monkey virus (MP-MV).

From these results, it was of considerable interest to examine the biochemical and serological properties of these particles which is currently under investigation. It is the purpose of this communication to report some physical and biochemical properties of the HeLa virus and a particle-associated DNA polymerase activity.

Materials and methods

Unlabelled deoxyribonucleoside triphosphates, bovine pancreatic ribonuclease and oligodeoxythymidylate (10) – polyriboadenylate [oligo (dT) – poly (rA)] were products of Boehringer-Mannheim. Tritiated thymidine 5' – triphosphate (48 Ci/mmole) was purchased from New England Nuclear. Cesium sulfate was obtained from Merck and Nonidet-P40 was kindly supplied by Deutsche Shell Chemie Gesellschaft mbH. *Viruses.* HeLa virus was produced in culture from a line of HeLa cells originally obtained from Dr. W. A. K. Schmidt, Düsseldorf. Cells had been cultivated for three years in Homburg/Saar and one year in Berlin in Eagle's medium supplemented with 4–10 % heat-inactivated calf serum. Cultures were continually examined for contamination by *Mycoplasma salivarium* and were treated with anti-PPLO (Tylocine, Grand Island Biological Company).

Culture supernatants were collected every 24 hr and subjected to low speed centrifugations of 3000 x g for 15 min and 16,000 x g for 20 min before the material was pelleted by high speed centrifugation (90,000 x g, 60 min, 4 °C). The

pellets were then resuspended in TNE, pH 7.4 (0.01 M Tris, 0.1 M NaCl and 0.003 M EDTA), centrifuged 5 min at 2,000 rpm and the virus-containing supernatant was layered over a linear 20–70 % (w/v) sucrose density gradient for equilibrium density centrifugation (15 hr, 4 °C, 23,000 rpm in a Spinco SW 25.1 rotor).

Mason-Pfizer monkey virus (MP-MV) was a gift of H. Daams, Amsterdam and avian myeloblastosis virus (AMV) was obtained by methods previously described (3) from the blood of chicks in the terminal stage of myeloblastic leukemia.

$^{32}PO_4$-labelled virus was prepared by first washing HeLa cell cultures with Eagle-Dulbecco medium (Flow Laboratories) lacking phosphates (10 ml/dish). The cultures were then incubated for 20 hr at 37 °C in phosphate-free medium containing 100 μCi/ml of ^{32}P-orthophosphate (carrier free, Radiochemical Centre, Amersham, England) and 10 % dialyzed calf serum. $^{32}PO_4$-labelled nucleic acid was extracted by the method of Kacian *et al.* (4) from virus banding in the region of density 1.15–1.18 g/cm^3 of a sucrose equilibrium density gradient.

For DNA polymerase studies, a pool of the region of density 1.15–1.18 g/cm^3 was made, diluted to 30 ml with TNE buffer (0.01 M Tris-HCL, pH 7.4, 0.1 M NaCl, 0.003 M EDTA) and centrifuged 60 min at 4° and 23,000 rpm in a Spinco SW 25.1 rotor. The concentrated pellet was resuspended in 0.5 ml of TNE buffer and stored in ice. Unless otherwise indicated this was the source of virus DNA polymerase.

Before use, the material was disrupted 15 min at 4 °C with Nonidet –P40 detergent (0.05 % for endogenous and 0.2 % for exogenous-templated reactions) and then standard reaction mixtures (100 μl) for DNA polymerase were prepared essentially as described previously. The specific activity of ^{3}H-TTP was 200–400 cpm/pmole for the synthetic RNA-DNA hybrid and DNA-templated reactions and 3,500 cpm/pmole for the endogenous DNA polymerase reactions.

Assays for neutralization of HeLA DNA polymerase activity by anti-MP-MV DNA polymerase IgG were carried out as described previously (5). Purified IgG fractions from normal rabbit serum (obtained prior to immunization) and rabbit anti-Mason-Pfizer monkey virus DNA polymerase serum were kindly supplied by Dr. A. YANIV, Institute of Cancer Research, Columbia University, New York, N. Y.

RESULTS

Density of the virions and detection of DNA polymerase activity.

Culture fluids of the HeLa cells were collected daily and the virus was concentrated and purified by sucrose equilibrium density centrifugation as described in Methods. Individual gradient fractions were then tested for DNA polymerase activity using the synthetic RNA-DNA hybrid, poly (rA)-oligo (dT). Figure 1 illustrates the presence of DNA polymerizing activity at a density of 1.17 g/cm^3, characteristic of RNA tumor viruses. Examination of the gradient fractions by electron microscopy revealed the presence of virus-like structures only in the region of density 1.17 g/cm^3, corresponding to the DNA polymerase activity.

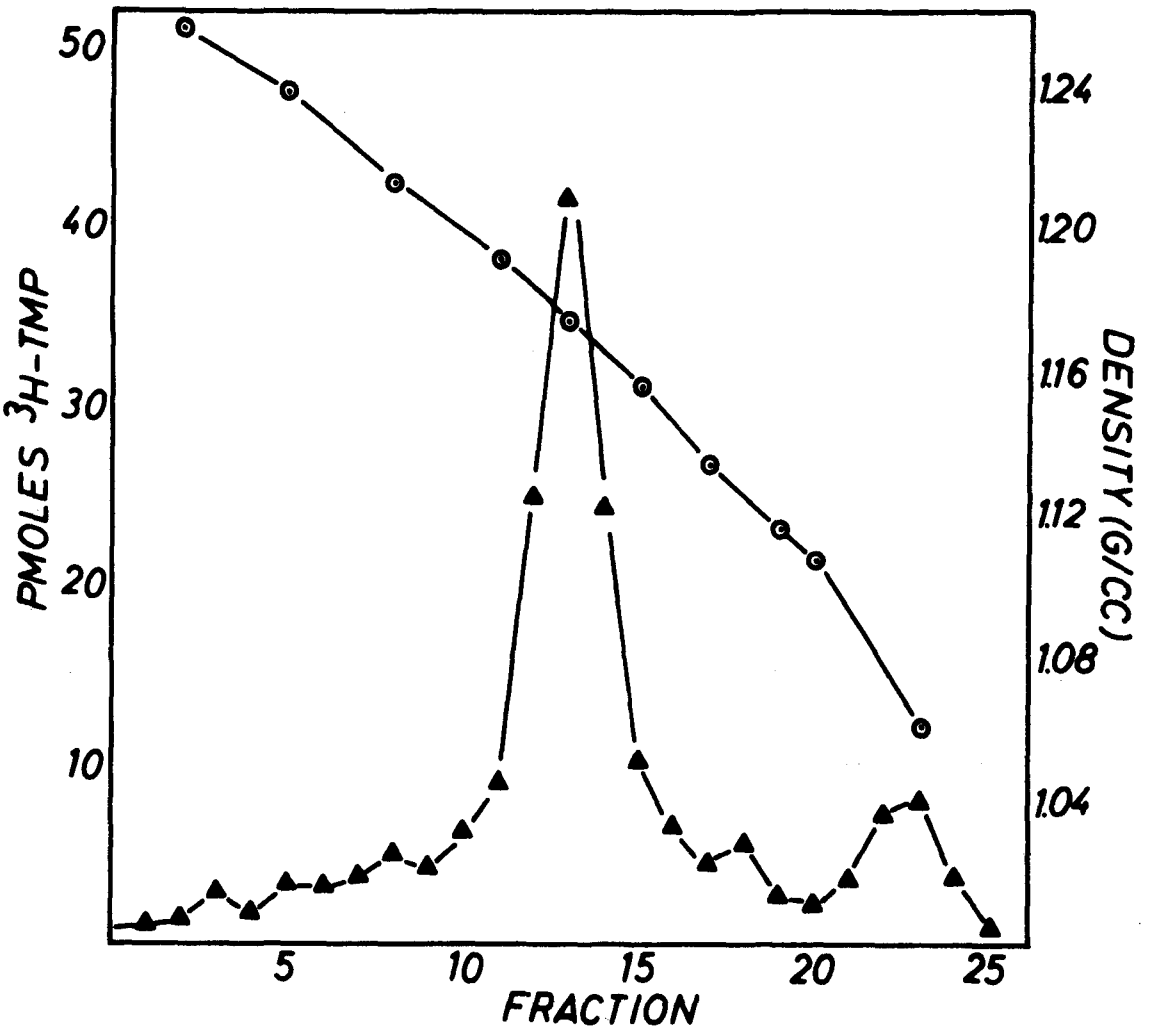

Fig. 1: Sucrose equilibrium density gradient centrifugation of HeLa virus and detection of DNA polymerase activity. HeLa virus was concentrated and subjected to sucrose gradient centrifugation as described in Methods. A 10 μl aliquot of gradient fractions was added to standard DNA polymerase reaction mixtures containing synthetic $(dT)_{10}$-poly (rA) as the primertemplate and ^{3}H-TTP as the labelled substrate.

Conditions	Activity (pmoles ^{3}H-TMP
Complete	0.31
Minus Mg^{++}	0.001
Minus Mg^{++}, plus Mn^{++}	0.005
Minus dATP	0.006
Minus dATP, dCTP	0.003
Minus Nonidet-P40	0.02

Table 1. Requirements of HeLa virus DNA polymerase.
Standard DNA polymerase assays were performed with omissions and additions as indicated. ^{3}H-TTP was the labelled substrate (specific activity, 3,500 cpm/pmole) and 10 ul of density gradient purified virus suspension was used as the source of endogenous DNA polymerase activity.

Properties of DNA polymerase activity.

Response of the DNA polymerase to its resident nucleic acid (endogenous reaction), activated DNA and poly (rA)-oligo(dT) is illustrated in Figure 2. The kinetics of incorporation of ^{3}H-TMP into acidprecipitable product was linear in all cases for more than 90 min. As illustrated in Figure 3, the rate of the endogenous reaction was proportional to the amount of virus suspension added to the reaction mixture. Also pretreatment of the detergent-disrupted virus with pancreatic ribonuclease (50 μg/ml) completely inhibits incorporation of radioactive substrate into DNA product (Fig. 3), suggesting RNA as the instructive agent in this reaction.

Chemical requirements of the endogenous reaction are shown in Table 1. A nonionic detergent (Nonidet-P40) pretreatment is necessary for expression of polymerizing activity as is the presence of all four deoxyriboside triphosphates. Magnesium ions are required and manganese ions do not substitute as the divalent action.

Further evidence that the endogenous reaction is instructed by RNA is shown in Figure 4. A simultaneous detection assay (6) designed to indicate the presence of RNA-instructed DNA polymerase and high molecular weight (60–70S) viral RNA template was performed. Our results indicate DNA product sedimenting with species with estimated sedimentation coefficients of 20–30S. Analysis of the nucleic acid content of the HeLa virus could explain these results.

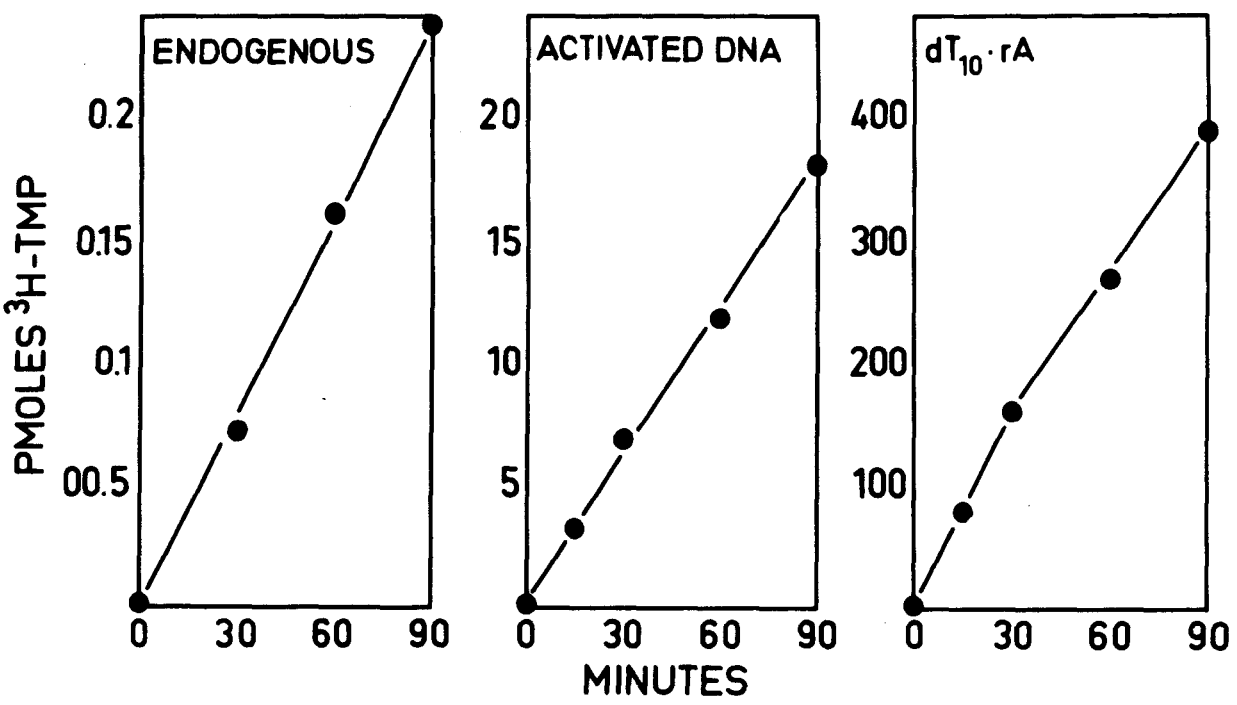

Fig. 2: Response of HeLa virus DNA polymerase to various nucleic acid templates. Standard DNA polymerase reaction mixtures (0.4 ml) were prepared and 100 μl aliquots were removed at the indicated time points. Activated calf thymus DNA, prepared by the method of Aposhian and Kornberg (12), was added at a concentration of 15 μg/ml, while $(dT)_{10}$-poly (rA) was added at a concentration of 5 μg/ml.

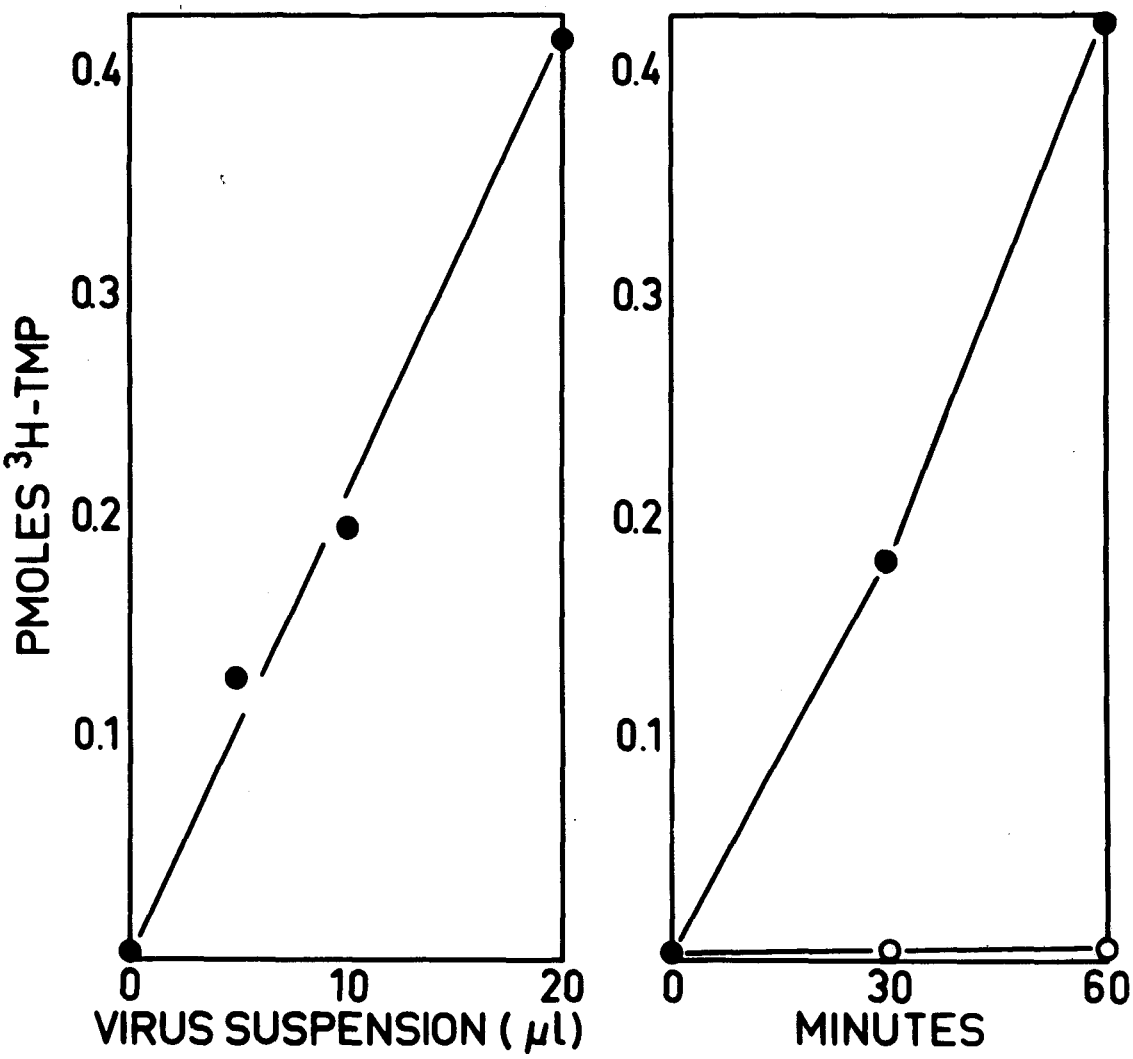

Fig. 3: Response of HeLa virus DNA polymerase activity to increasing virus concentration and to ribonuclease treatment. Standard endogenous DNA polymerase reaction mixtures were prepared with increasing amounts of virus suspension added (left figure). For sensitivity to ribonuclease (right figure), two reaction mixtures (0.3 ml) were prepared. To one, pancreatic ribonuclease (50 µg/ml final concentration) was added, while to the other an equal volume of 0.01 M Tris, pH 8.2 was added. Both mixtures containing 30 µl of virus suspension were then disrupted 15 min at room temperature with 0.05 % Nonidet-P40. After addition of Mg^{++} and the deoxriboside triphosphates, the mixtures were incubated at 37 °C, removing 100 µl aliquots at 0,30 and 60 min. The radioactivity was analyzed as previously described in Methods. The control is designated by the closed circles and the open circles represent the reaction containing ribonuclease.

Characterization of the viral nucleic acid.

Following labelling of HeLa cell cultures with $^{32}PO_4$ and purification of the virus through the sucrose density gradient step, nucleic acid was extracted from the density region of 1.16–1.17 g/cm^3 and further analyzed by velocity gradient centrifugation. Figure 5A shows a profile containing $^{32}PO_4$-labelled nucleic acid with sedimentation coefficients of 30S, 18S and 4–8S. Analysis by Cs_2SO_4 equilibrium

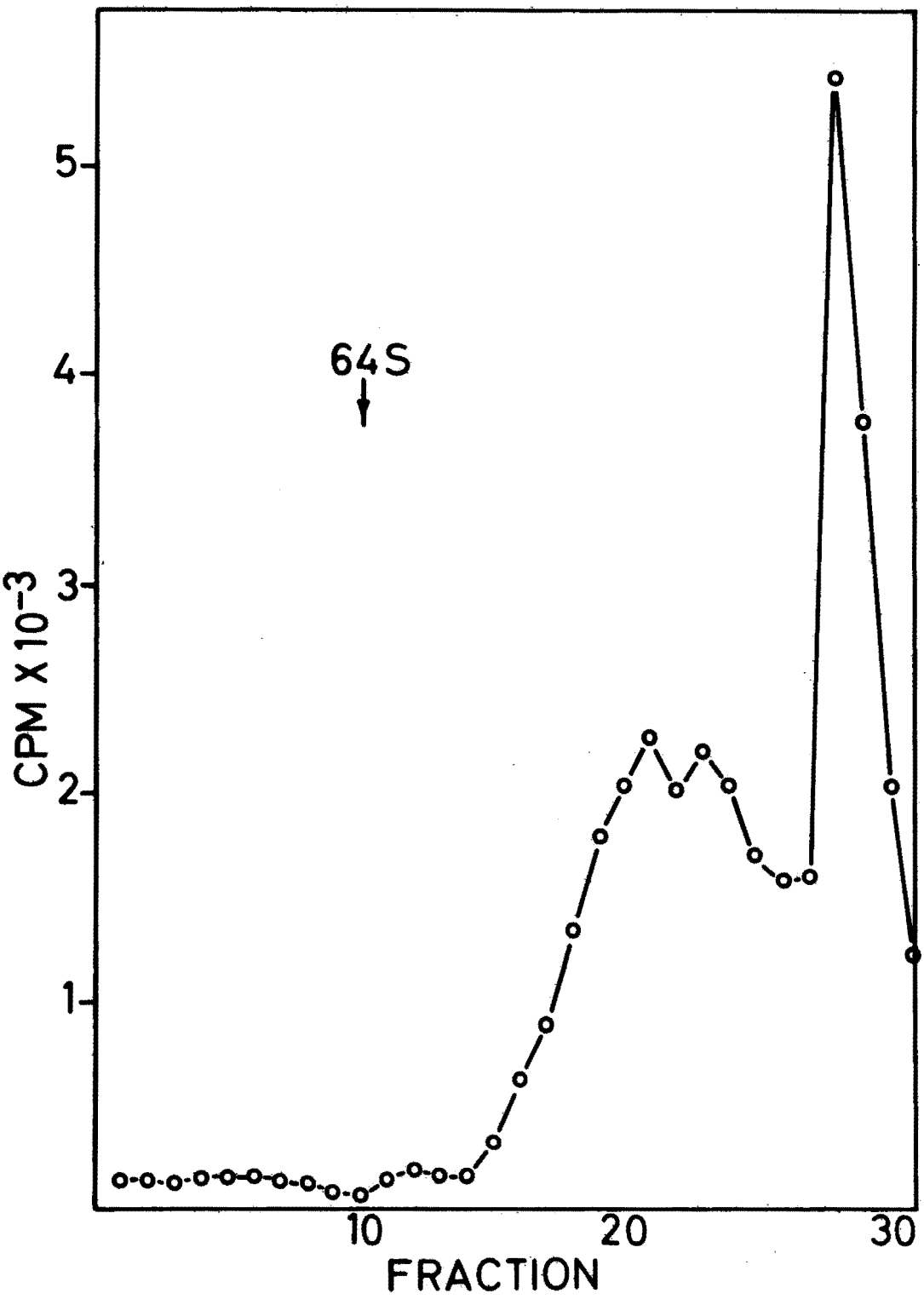

Fig. 4: Simultaneous detection of RNA-directed DNA polymerase activity and viral RNA. Following disruption of 0.1 ml of the peak activity fraction from Fig. 1 for 30 min in ice with 0.2 % Nonidet-P40 and 10 mM dithiothreitol, a standard endogenous DNA polymerase assay mixture (0.5 ml) was prepared containing ^{3}H-TTP as the labeled substrate with a specific acti-

vity of 1.9 x 10^4 cpm/pmole. After 30 min at 37°, the reaction was terminated with the addition of sodium dodecyl sulfate to a final concentration of 0.5 %, and an equal volume of phenol (pH 7) containing 8-hydroxyquinoline (0.1 g per 100 ml) was added. After shaking 3 min at room temperature, the mixture was centrifuged 5 min at 3,000 rpm and 20 °C. The aqueous phase was layered over a preformed linear gradient of glycerol (10—30 %) and centrifuged 90 min at 50,000 rpm. and 4 °C in a Spinco SW 50.1 rotor. Gradient fractions were collected and processed as described in Fig. 5A. AMV RNA used as an external marker being centrifuged under identical conditions.

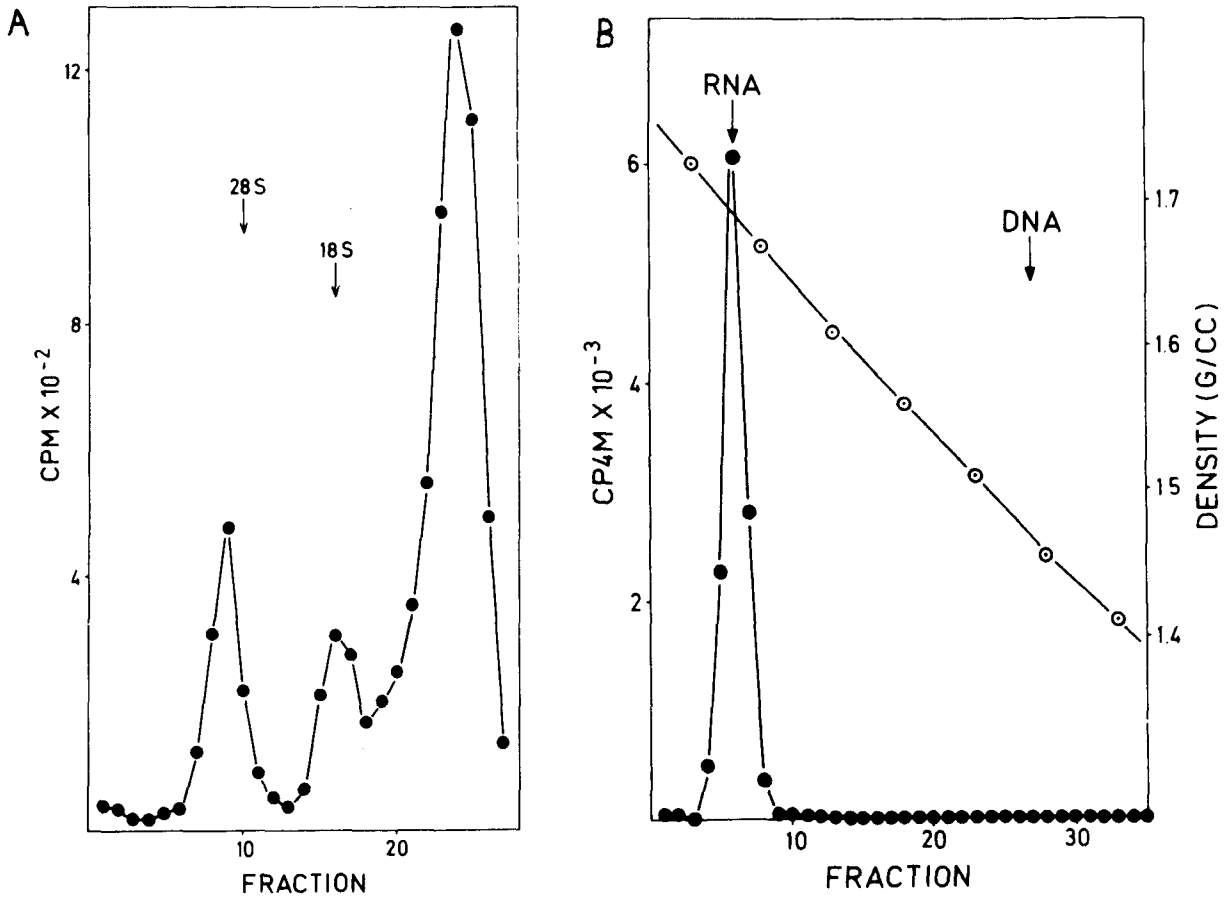

Fig. 5: (A) Sedimentation analysis of nucleic acid from HeLa virus. $^{32}PO_4$-labelled nucleic acid was extracted from the 1.15—1.18 g/cm^3 region of a sucrose equilibrium density gradient as described in Methods. The nucleic acid solution was layered over a 5 ml preformed 10—30 % glycerol gradient and centrifuged 3.5 h at 50,000 rpm and 4 °C in a Spinco SW 50.1 rotor. 12 drop fractions were collected from the bottom of the tube, 20 µl aliquots were precipitated with trichloroacetic acid and the radioactivity determined as previously described (KACIAN et al., 1971). ^{3}H-uridine-labeled 28S and 18S ribosoral RNAs from chick embryo fibroblasts were included as markers. (B) Cs_2SO_4 equilibrium density gradient centrifugation of ^{32}P-HeLa 30S nucleic acid. An aliquot containing 4,000 cpm was added to 2 ml of TNE buffer, pH 7.4, mixed with an equal volume of saturated Cs_2SO_4, and centrifuged at 31,000 rpm in a Spinco SW 39 rotor for 60 h at 20 °C. Fractions were collected and processed for acid-precipitable radioactivity as described above (Fig. 5A). (^{3}H)-uridine-labeled 28S ribosomal RNA and 30 µg of calf thymus DNA were added as markers.

density gradient centrifugation demonstrated that each size specie had the characteristic properties of RNA. Figure 5B illustrated the density properties of the 30S specie.

Antigenic properties of HeLa virus DNA polymerase.

A technique useful for comparative studies of primate RNA tumor viruses involves serological characterization of the viral DNA polymerases and has been described (7). Because of the morphological similarities of HeLa virus with MP-MV, it was of interest to compare the antigenic properties of the MP-MV DNA polymerase and the HeLa virus DNA polymerase.

Using isolated IgG fraction from antisera produced against partially purified MP-MV DNA polymerase, our data demonstrates that MP-MV DNA polymerase and HeLa virus DNA polymerase are both inhibited to an equivalent extent by the anti-MP-MV DNA polymerase IgG. AMV DNA polymerase activity is not affected by this same IgG fraction (Fig. 6).

Discussion

Some physical and biochemical properties of a RNA tumor virus-like particle from HeLa cell culture supernatants have been described. Equilibrium density centrifugation in sucrose of this concentrated material revealed a DNA polymerase activity (Fig. 1) and the presence of RNA tumor virus-like particles at 1.17 g/cm^3, characteristic of all known RNA tumor viruses.

Analysis of the endogenous DNA polymerase activity demonstrated a requirement for Mg^{++} as the divalent action and the four deoxyriboside triphosphates as substrates (Table 1). Sensitivity of this reaction to pancreatic ribonuclease suggested RNA as the instructive agent for synthesis of DNA (Fig. 3).

Investigation of the endogenous DNA polymerase reaction by the simultaneous detection assay (Fig. 4) supports the presence of RNA-instructed DNA polymerase, although no 60–70S RNA specie was observed. Rather, DNA product sedimented with molecular weight species similar to those found to be present as RNA in the virions (Fig. 5). The significance of these species is not understood. However, they may represent sub-unit RNA structures known to be products of 60–70S tumor virus RNA after heat or dimethylsulfoxide treatment (8, 9).

Identity of this DNA polymerase with the R-DNA polymerase from HeLa cells (10) is unlikely, in that their primer-template, ion and temperature requirements are quite different.

In a further attempt to relate biochemical properties of this virus and its DNA polymerase activity with the RNA tumor viruses and particularly with the morphologically similar MP-MV that contains a reverse transcriptase activity (11), antigenic properties of the DNA polymerase activity of the two viruses were compared. The results indicate that both DNA polymerase activities were inhibited to an equivalent extent by anti-MP-MV DNA polymerase IgG (Fig. 6) suggesting a serological relationship between the two enzymes. AMV DNA polymerase was unaffected by the anti-MP-MV DNA polymerase IgG.

Therefore, we believe we are observing a virus containing a DNA polymerase activity characteristic of the RNA tumor viruses, particularly the MP-MV. However, to establish that we are, indeed, dealing with a functionally enzymatic viral reverse transcriptase will require back-hybridization of RNA-instructed DNA product to its homologous RNA template.

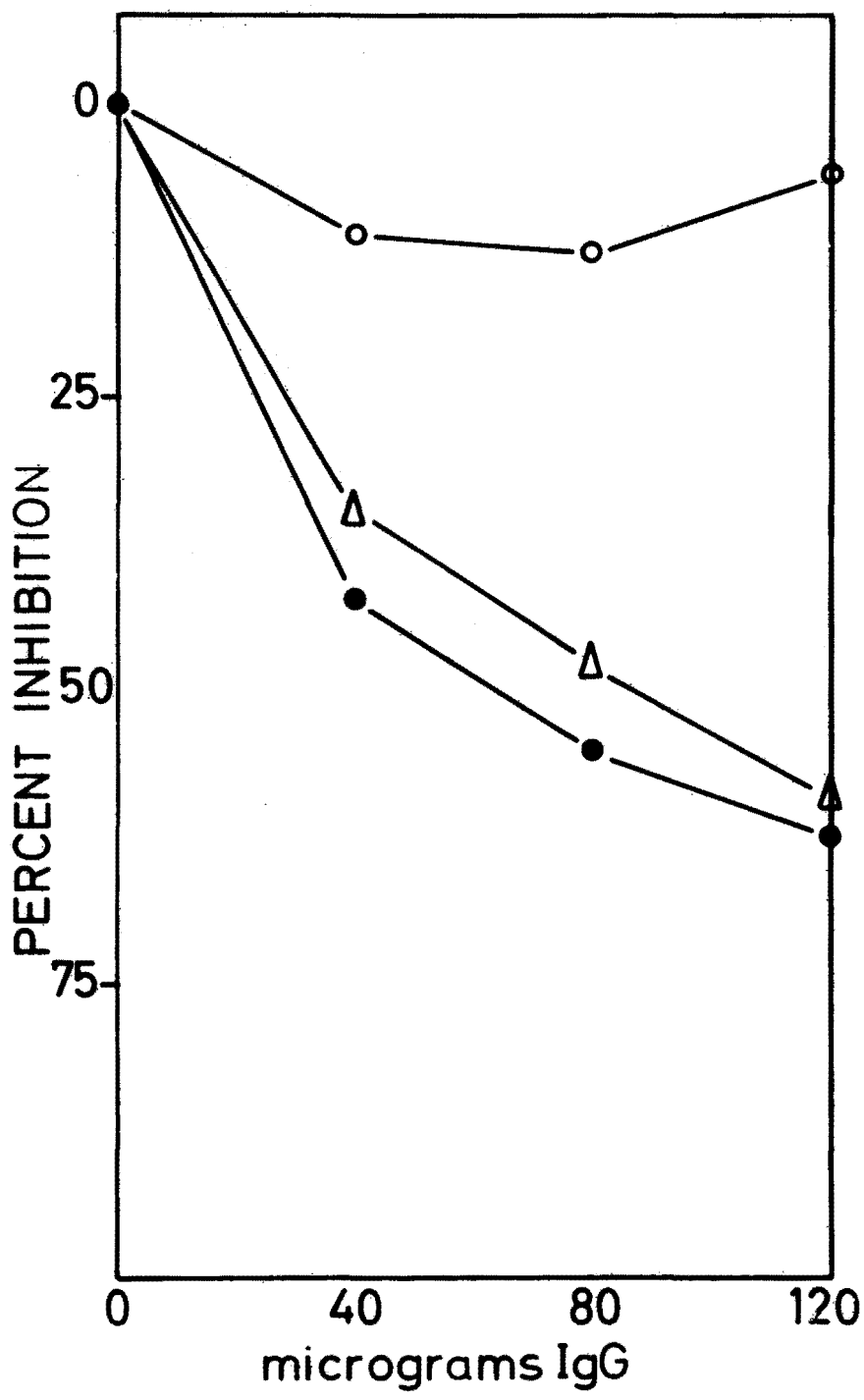

Fig. 6: Neutralization of HeLa DNA polymerase activity by a purified IgG fraction from anti-MP-MV DNA polymerase serum. Neutralization assays were carried out essentially as previously described (WATSON et al., 1972). Equivalent amounts (amount of virus suspension necessary to incorporate 1 pmole of ^{3}H-TMP/min with oligo(dT)-poly(rA) as primer-template) of detergentdisrupted HeLa virus, MP-MV and AMV were incubated with increasing amounts of anti-MP-MV DNA polymerase IgG and purified normal rabbit IgG as control. The results are expressed as percent inhibition by anti-Mp-MV DNA polymerase IgG compared to the same amount of control normal rabbit IgG. Neutralization of DNA polymerase from HeLa virus (closed circles), MP-MV (open triangles), AMV (open circles) is demonstrated.

As HeLa cells were derived from a human cervical carcinoma, the origin of this virus and the relationship of its genetic information to primates and their viruses are of particular interest. These studies are presently in progress.

The authors wish to thank Dr. H. GELDERBLOM for electron microscopic analysis of HeLa virus materials. K. F. WATSON was the recipient of a research training fellowship from the International Agency for Research on Cancer.

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INHIBITION OF DNA POLYMERASES ONCORNAVIRUSES BY MODIFIED NUCLEIC ACIDS.

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The DNA polymerase activity in virions of the RNA tumor viruses can be studied in two different ways: 1) The endogenic reaction: the disrupted virions are incubated with substrates in the absence of any added template, and synthesis of RNase

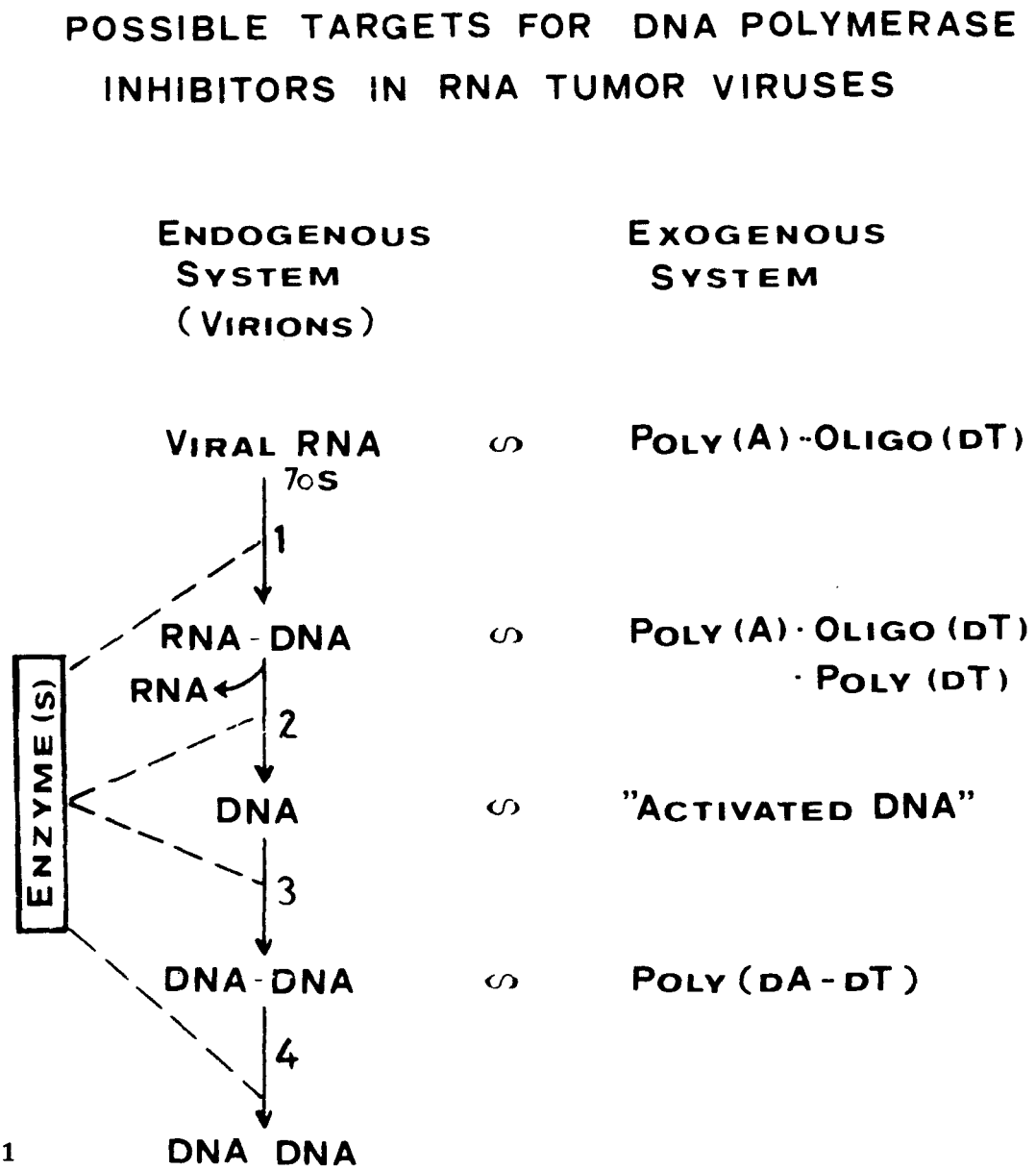

Fig. 1

sensitive DNA synthesis is measured, which indicates the transcription of endogenous viral RNA (60–70s); 2) The template-dependent reaction: this reaction can be studied by purifying the solubilized enzyme, or by using disrupted virions. The reaction can be carried out in the presence of a viral RNA, activated DNA, or synthetic polynucleotides of various composition (see Fig. 1).

A large number of compounds have been reported to inhibit the DNA polymerase activity of oncornaviruses in the absence (endogenic reaction) and in the presence of a variety of templates (see Table 1). On the basis of template specificity of the reactions which are most sensitive to these inhibitors, possible targets have been postulated in Table 1.

Most of the inhibitors reported so far act by forming complexes with the template(s), so that their specificity is not strictly for the viral enzyme, and bacterial as well as mammalian enzymes will also be inhibited by such inhibitors; the exception to this generalization is an inhibitor which specifically will bind to 70S viral RNA. An inhibitor of latter type has not been reported so far. Another way to develope specific inhibitors is to search for compounds which will specifically bind to DNA polymerases of oncornaviruses. The present report describes the inhibition of DNA polymerases of oncornaviruses by a novel template analogue, a partially thiolated (5-SH) polycytidylic acid (MPC), which acts by complexing with the enzyme(s), and shows some selectivity in its mode of action on the viral DNA polymerase.

Table 1. Reactions inhibited by various inhibitors of DNA polymerases of oncornaviruses

Inhibitor	Target Reaction (see Fig. 1)	References
Dideoxythymidine-Triphosphate (ddTTP)	1,2 und 4	SMOLER et al., 1971
Actinomycin D	2,3 and 4	GURGO et al., 1971 MÜLLER et al., 1971
Rifampicin derivatives	1-4	GURGO et al., 1971 KOTLER and BECKER, 1971 TING et al., 1972 CHANDRA et al., 1972a
Ara-CTP	1	MÜLLER et al., 1972
Distamycin derivatives	3 and 4	CHANDRA et al., 1972b
Daunomycin derivatives	3 and 4	CHANDRA et al., 1972c
Tilorone-HCl	(1), 4	CHANDRA et al., 1972d
Ethidium bromide	1,2	HIRSCHMAN, 1971 MÜLLER et al., 1971 FRIDLENDER & WEISSBACH, 1971
Anthracyclines	1	APPEL and HASKELL, 1971

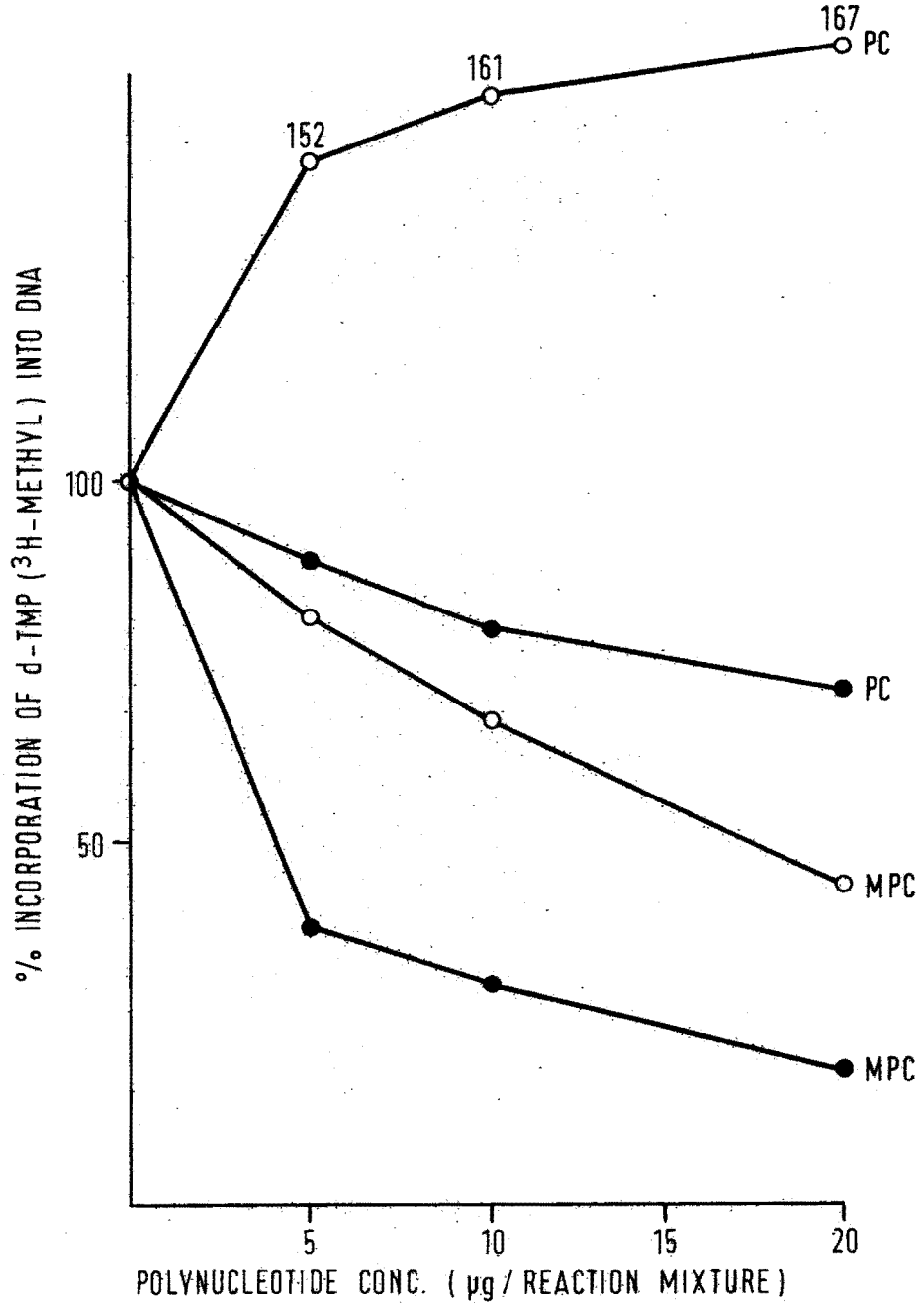

Fig. 2: Effects of thiolated polycytidylic acid (MPC) and unmodified polycytidylic acid (PC) on the DNA polymerase activities from MSV(Moloney) in the presence of poly rA. $(dT)_{14}$ (●——●) and poly (dA-dT) (○——○) as templates.

Fig. 2 shows the effects of 5-mercapto-(8.68 %)-polycytidylic acid (MPC) and unmodified polycytidylic acid (PC), respectively, on the incorporation of ^{3}H-TMP into DNA by the DNA-polymerases of the MSV(Moloney), in the presence of either poly (dA-dT) or poly rA. $(dT)_{14}$ as the template. The results obtained with the

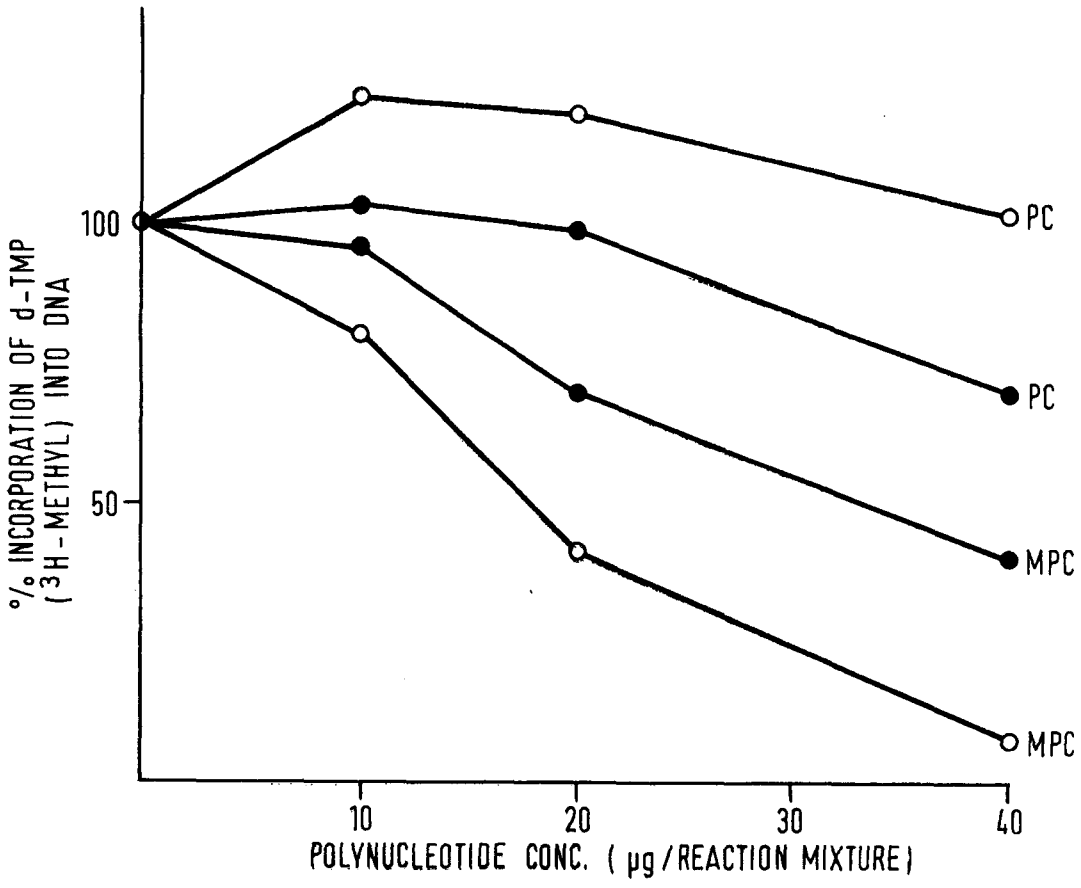

Fig. 3: Effects of thiolated polycytidylic acid (MPC) and unmodified polycytidylic acid (PC) on the DNA polymerase activities from FLV (Friend) in the presence of poly rA. $(dT)_{14}$ (●——●) and poly (dA-dT) (○——○) as templates.

same pair of modified and unmodified polycytidylic acid samples in the FLV DNA-polymerase assay, using the same pair of templates, are graphically represented in Figure 3.

It is clear from these graphs that the modified polynucleotide, MPC, significantly inhibits the DNA polymerases present in both viral extracts; furthermore, the inhibitory activity of MPC is very nearly the same in the two systems when poly (dA-dT) is used as the template (50 % inhibition at 18 µg/0.25 ml of reaction mixture), but in the presence of poly rA. $(dT)_{14}$ as the template, MPC acts as a much more potent inhibitor of ^{3}H-TMP incorporation in the MSV(M) assay system (50 % inhibition at 4 µg/reaction mix.) than in the FLV system (50 % inhibition at 35 µg/reaction mix.). In contrast, the unmodified polynucleotide, PC, stimulates DNA polymerase activity in both viral systems with poly (dA-dT) as the template, and it shows slight inhibitor activity in the presence of the poly rA. $(dT)_{14}$ template.

A time study of the effect of MPC, added at various periods after incubation of the reaction mixture showed that once the formation of the enzyme-template complex has occured, this RNA-directed polymerase is no longer sensitive to MPC.

The interpretation of the mode of MPC action on DNA polymerases of oncornaviruses needs a clarification of many questions, one may ask: 1) Is the inhibition dependent on the degree of thiolation of the polymer? ; 2) Does it inhibit DNA

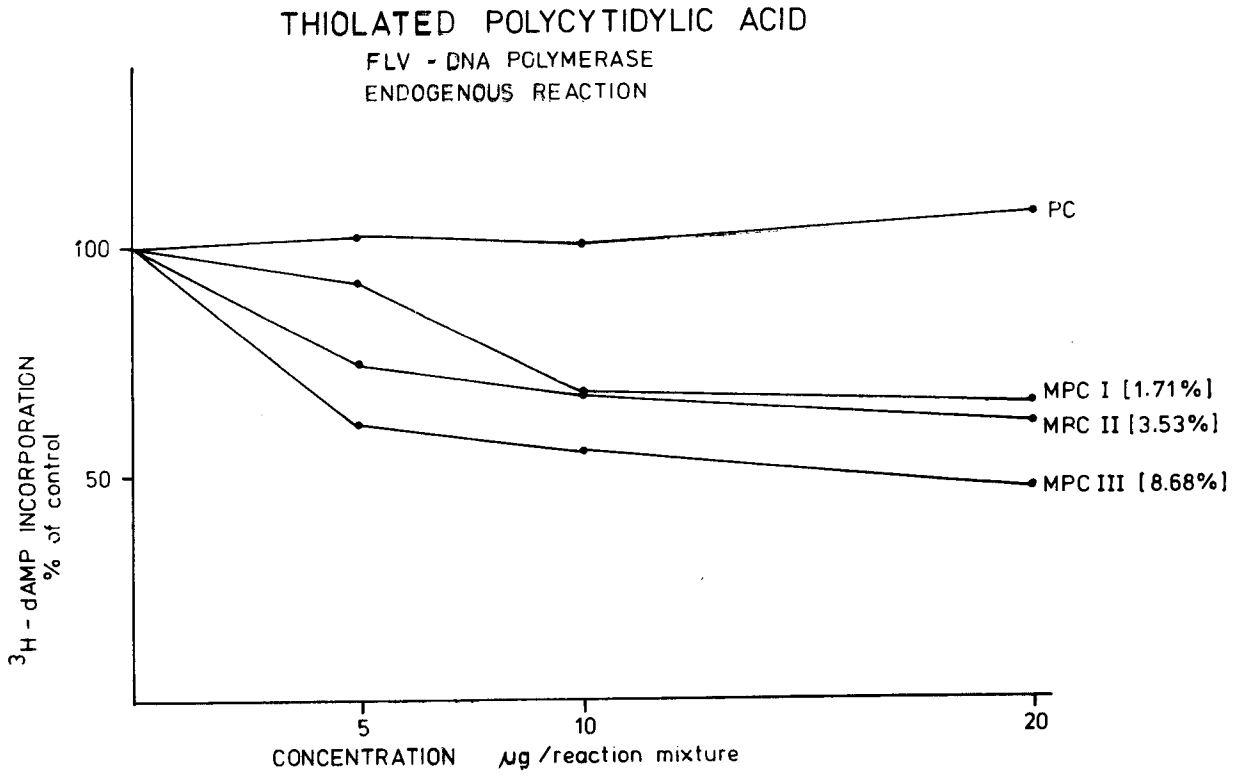

Fig. 4: Inhibition of FLV-DNA-polymerase activity in the absence of any added template by thiolated polycytidylic acids (MPC), containing 1.71 %, 3.53 % and 8.68 % of 5-SH-cytidylate units.

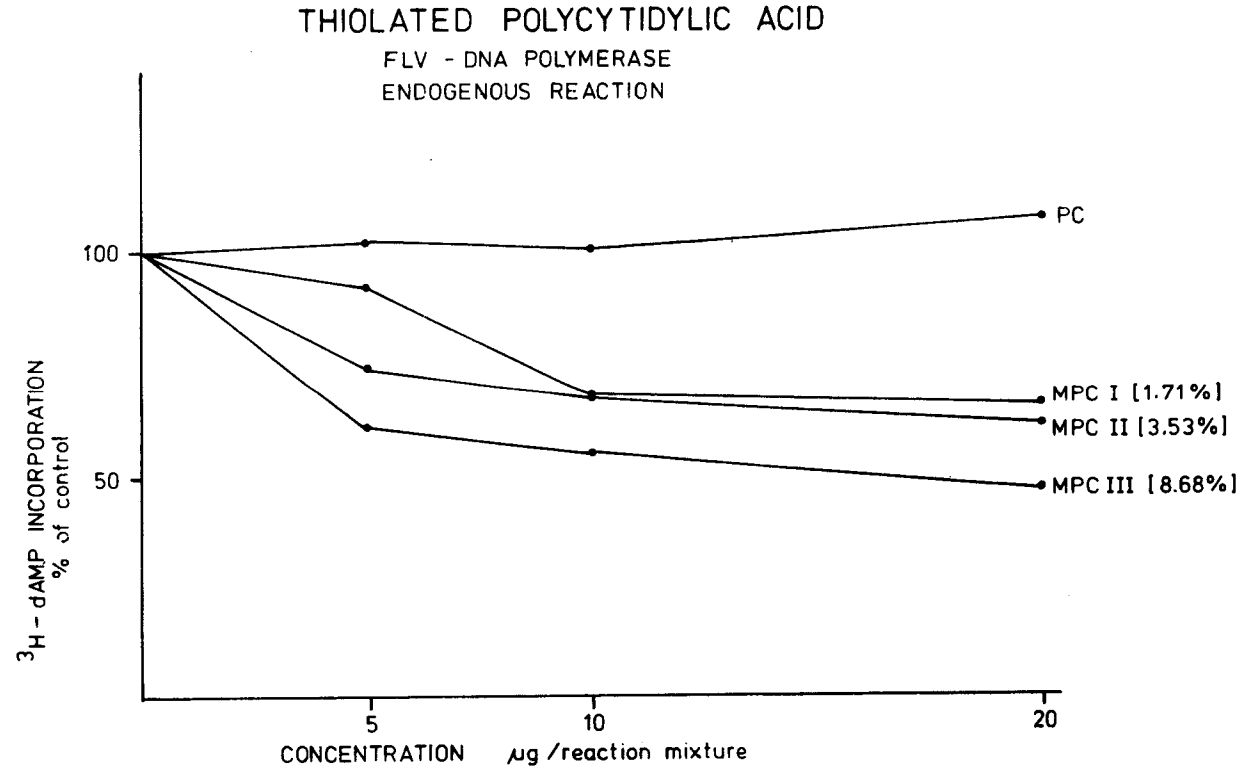

Fig. 5: Inhibition of FLV-DNA-polymerase activity in the presence of poly rA. $(dT)_{14}$ by thiolated polycytidylic acids (MPC), containing 1.71 %, 3.53 % and 8.68 % of 5-SH-cytidylate units.

polymerases from other sources and, 3) How is the affinity of this compound towards enzymes from other types of virus particles?

The study with polycytidylic acid preparations with varying degree of thiolation showed a clear dependence of inhibiting capacity on the degree of thiolation of the polymer. These studies were carried out with MPC containing 1.71 %, 3.53 % and 8.68 % of SH-cytidylate units in the polymer. The degree of thiolation was estimated by neutron activation analysis.

As follows from Fig. 4, the endogenic reaction in FLV is inhibited by all MPC preparations, and the amount of inhibition is dependent on the degree of thiolation of the polymer. Similar results were obtained in the FLV-reaction catalyzed by poly rA. $(dT)_{14}$, a template known to be specific for viral DNA polymerases.

Regarding the selectivity of MPC action we have done some experiments on the DNA-polymerase of *E. coli* K_{12} cells, isolated by the procedure of RICHARDSON (1966).

Table 2. Effect of polycytidylic acid (PC) and mercapto-polycytidylic acid (MPC) on DNA-polymerase of E. coli K_{12} in the presence of denatured DNA

Compound added+	^{3}H-dAMP Incorporation into DNA (cpm/reaction mixt.)	% of control
None	575	100
PC	608	106
MPC++–I	572	99.5
MPC –II	554	96
MPC –III	591	103

+ All compound were added at a concentration of 5 µg/reaction mixt. (0,30 ml);
++ MPC-I, MPC-II and MPC-III represent mercaptocytidylic (poly) acids with 1.71 %, 3.53 % and 8.68 % 5-SH cytidylate units respectively.

As follows from Table 2 the DNA polymerase reaction of bacterial cells is insensitive to MPC; even at higher concentrations no inhibition occured. More experiments are necessary to understand the specificity of MPC in viral systems. These studies are now being substantiated in collaboration with Dr. Robert C. Gallo.

The basic idea to initiate these studies was to find out the role of such modifications on natural viral templates, 60–70 sRNA. We, therefore, have done some model experiments with partially thiolated DNA and various types of RNA, isolated and purified from Ehrlich ascites cells.

In these experiments best results were obtained using modified soluble RNA (sRNA) in the MSV-DNA polymerase reaction. As follows from Table 3 the unmodified sRNA shows a slight inhibition of the endogenic and poly rA. $(dT)_{14}$-catalyzed reactions; however, the modified sRNA inhibits both the reactions very significantly.

Table 3. Effect of soluble RNA (sRNA) from Ehrlich ascites cells on the DNA polymerase of MSV(M)

Compound added	^{3}H-TMP incorporation into DNA (% of control)		
	-Template	+poly rA. $(dT)_{14}$	+poly (dA-dT)
Non-thiolate sRNA (μg/reaction mix.)			
10	93	98	104
20	92.5	86	100
40	92	71	102
Thiolated (1-3 %) sRNA (μg/reaction mix.)			
10	77	69	56
20	75	58	43
40	63	49	40

Conclusions

The present results indicate that modification of polynucleotides and nucleic acids may lead to the development of useful inhibitors of DNA polymerases of oncornaviruses. Model studies with partially thiolated polycytidylic acid (MPC) show that 1) MPC inhibits the DNA-polymerase activity of oncornaviruses by forming a complex with the enzyme, 2) the inhibition can be potentiated by increasing the number of 5-SH-cytidylate units in the polymer, and the inhibition is to some extent selective. The last point needs more experiments which, we hope our future studies will substantiate. The studies regarding the implication of this inhibition on biological activity of these compounds are in progress.

Acknowledgement:

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THE RELEVANCE OF RNA-DIRECTED DNA POLYMERASE ACTIVITY TO HUMAN NEOPLASIA

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I have been asked to write a short article on some biological aspects of RNA-directed DNA polymerase activity that are relevant to the theme of this workshop. Because I was not able to attend the workshop, I shall write a general article without data or specific literature references. At the end of the article, I have listed some of my articles which give detailed references.

Why are RNA tumor viruses and RNA-directed DNA polymerase activity considered relevant to the etiology of human leukemia? In animals, especially in mice and chickens, much of the leukemia is caused by RNA tumor viruses. Furthermore, RNA tumor viruses and other viruses with apparent RNA-directed DNA polymerase activity are widely distributed in some normal animals and have been isolated from many animals, although not yet from man. It appears to be a reasonable working hypothesis that RNA tumor viruses have some relevance to the etiology of human leukemia.

RNA tumor viruses are characterized by a medium-sized, enveloped virion with a characteristic morphology and a density in sucrose of about 1.16 g/cm^3. Infectious RNA tumor virus virions contain a 60–70S RNA complex and a DNA polymerase. The genome of an infectious, strongly transforming RNA tumor virus, such as Rous sarcoma virus, contains genes for virion structural proteins, also sometimes called the virogene, which includes genes for two external glycoproteins, three or more internal proteins, and a DNA polymerase. The internal proteins and the DNA polymerase have group-specific antigenicity. The genes for neoplastic transformation are often called the oncogene and may consist of at least four complementation groups. Thus, the genome of an infectious, strongly transforming RNA tumor virus, whether as virion RNA or as DNA provirus, equals the virogene plus the oncogene. (There are also non-transforming RNA tumor viruses that do not have an oncogene.)

RNA tumor virus replication involves attachment of the infecting virus to receptors of a sensitive cell, entrance into the cell and uncoating, synthesis and integration of the DNA provirus, activation of RNA transcription by normal passage of the infected cell through the replicative cell cycle, synthesis of virus-specific RNA and protein, formation of the virion core, and formation of the virion by budding from the modified cell plasma membrane.

The major descriptive questions about RNA tumor viruses are what genes exist in DNA proviruses in normal and neoplastic cells and what products of these genes are

formed in normal and neoplastic cells. The major genetic questions are what is the origin of these genes and what determines which products are formed.

The virion RNA-directed DNA polymerase has provided a way to answer some of the descriptive questions. The DNA product of RNA-directed DNA polymerase activity has allowed synthesis of radioactive DNA products, which can be used in nucleic acid hybridization experiments to find DNA and RNA sequences related to RNA tumor virus RNA. (Similar experiments have been done with cellular messenger RNA's.) Viral RNA has also been used in nucleic acid hybridization experiments to find cellular DNA sequences related to RNA tumor virus RNA. Limitations of this approach are the necessity to start with RNA of a known RNA tumor virus and the apparent lack of nucleotide sequence homology between the RNA from different RNA tumor viruses, even among those from different groups growing in the same cells (for example, avian leukosis viruses and reticuloendotheliosis viruses) and among those from antigenically related viruses (for example, mouse and feline leukemia viruses).

A similar requirement for a known RNA tumor virus exists when looking for most of the virion structural proteins. Only known proteins or proteins related to known ones can be studied. However, RNA-directed DNA polymerase activity is characteristic of RNA tumor viruses and thus can be looked for without starting with a known RNA tumor virus.

A limitation in the last approach is that endogenous RNA-directed DNA polymerase activity with the biochemical characteristics of RNA tumor virus RNA-directed DNA polymerase activity has been found in normal, uninfected chicken embryos and in normal, uninfected chicken fibroblasts and amnion cells in culture. This activity is unrelated to known viruses and has no other properties of a virus. It was isolated from apparently uninfected cells, which are not producing virus.

Therefore, without an infectious human RNA tumor virus, it may be hard to answer the descriptive questions about possible proviruses and their expression in normal and neoplastic human cells.

While there are fairly complete answers to some of the descriptive questions about RNA tumor viruses, the genetic questions about RNA tumor viruses are still unanswered in animals where many RNA tumor viruses are known. The current hypotheses regarding the occurrence of oncogenes are that oncogenes exist a) only in neoplastic cells or b) in normal and neoplastic cells. The hypotheses as to the origin of oncogenes are that they arise a) from the provirus of an infectious, strongly transforming RNA tumor virus, b) from the vertically transmitted provirus of a strongly transforming RNA tumor virus, c) from variational processes involving normal cellular elements called protoviruses, or d) from mutation of cell genes.

Much more work will have to be done to clarify these questions in animals before we can hope to shed much light on the etiology of human neoplasia. A further sobering thought is that, although RNA tumor viruses have been known since the early 1900's to cause some chicken leukemia, effective means of prevention or treatment of this leukemia are still unknown.

Further Reading

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On the Origin of Human Acute Myeloblastic Leukemia: Virus-"Hot Spot" Hypothesis

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Cellular Aspects

These remarks are directed only to one specific type of leukemia, acute myeloblastic (granulocytic) leukemia (AML). In this disease, as discussed elsewhere in this workshop, there is a progressive expansion of myeloblasts that appear morphologically somewhat different from normal myeloblasts. The important concept that these cells are *usually* not rapidly dividing but, in fact, may even have generation times much longer than normal bone marrow myeloblasts has led us to speculate (1–3) that the pathophysiology of this disease most likely involves a block in the normal process of maturation and differentiation of these cells to granuloctyes. Normally, myeloblasts make up less than 5 % of the total nucleated cell population of the bone marrow. The size of the pool of these cells appears to be constant. These cells, of course, are capable of DNA synthesis and of mitosis. During differentiation, a process that occurs in the bone marrow for the life time of the organism, it appears that on usual division of these cells, one daughter cell feeds back into this pool while the other begins to mature to a more differentiated end stage cell, the granulocyte. During the maturation process, several cell stages can be morphologically identified. These are listed below: myeloblast ——→ promyelocyte ——→ myelocyte ——→ metamyelocyte ——→ band cell ——→ mature granulocyte

The major morphological changes are: (1) the nucleus becomes progressively more condensed and stains more darkly (so-called pyknosis) while nucleoli disappear; (2) the cytoplasm becomes less basophilic (presumably less RNA) and begins to show the appearance of granules some of which are lysosomes. Functionally, the nuclear morphological changes indicate that the nuclear material is becoming "metabolically inert," i. e., DNA synthesis terminates and the cells are no longer able to divide. The functional cytoplasmic changes are manifold but, in general, I like to think of it as representing a change in the pattern of proteins that are made. The immature cells, for instance, contain high amounts of enzymes involved in the anabolic processes for DNA and RNA synthesis, while the proteins in the more mature cells are primarily concerned not with the "selfish" process of self-reproduction, but in serving the whole organism. There are many other analogous cell systems, e. g., in the formation of the anucleated mature red blood cell from the proliferative erythroblast the entire process appears directed to the purpose of forming a carrier of hemoglobin for the benefit of the organism while quite obviously losing its proliferative potential. The granulocyte

contains, for instance, high concentrations of hydrolytic enzymes (within the lysosomes) which, of course, are essential to the normal function of the granulocyte, phagocytosis and digestion of foreign material, especially micro-organisms. The capacity to synthesize DNA and divide probably terminates at the metamyelocyte stage, and the cell which is released under normal circumstances into the peripheral blood from the bone marrow is the granulocyte. This cell is thought to live approximately eight days. Now, in AML we see an expanding population of myeloblasts, abnormal in appearance, which have variable generation times but most of which apparently divide no more rapidly and often slower than normal myeloblasts. It is obvious why we proposed that to account for these observations, the simplest explanation is that these myeloblasts are blocked in their maturation process so that a pool of immature cells capable of proliferation gradually expands and results in the clinical disease we call acute myeloblastic leukemia. I naturally do not infer that these cells are not abnormal or very "sick" cells as Dr. Torelli likes to call them, I only emphasize that to account for their expansion the simplest explanation is the one described above.

The "Inciting" Agent

In thinking about the origin of the above described cellular changes, we should take into account pertinent information in man and in animal model systems. Some of these are listed here. 1) Some chemicals, e. g., benzene and radiation, can increase the incidence of leukemia in man and animals. 2) Genetic considerations are clearly of great significance both in animal and human leukemias. The most striking example is with identical twins. If one twin develops leukemia the other has a very high risk of subsequently developing leukemia. Genetic factors are also critical to animal leukemias, e. g., very high frequency of leukemia in AKR inbred mice and the almost non-existence of the disease in NIH Swiss mice, yet no greater amount of virogenes was detected in high frequency leukemia strains (AKR) than in intermediate (C-57) or low frequency strains such as the NIH Swiss mice (6). 3) In animals of several species, acute leukemias can be induced by certain type-C RNA tumor viruses. (Although it must be admitted that these are generally lymphocytic leukemias, and we really have no appropriate models for myeloblastic leukemias.) It appears that at least some normal tissues of at least some animals may contain *endogenous* type-C viruses which so far do *not appear to be tumorigenic*. Thus, it appears that type-C viruses may or may not contain oncogenic information. Presumably, this depends on the nature of the genomic RNA, and there is evidence that this RNA can be changed depending on its recent "history." For example, infecting a different cell with virus may lead to aquisition of new sequences from this cell (4). *(It may be best to look upon these viruses as all potentially capable of picking up information that leads to oncogenic potential, if they are not already frankly oncogenic.)*

It appears that type-C viruses are most frequently found in transformed cells but components of virus (in the absence of complete virions) have been noted in many normal tissues, especially embryonic, and as stated above, whole virions can be induced from some previously non-virus producing normal cells. It should also be noted that type-C mammalian *leukemia* viruses, generally do not transform cells *in vitro*; type-C

sarcoma viruses transform *in vitro*, yet leukemia viruses are, of course, leukemogenic *in vivo*. Moreover, Duesberg has emphasized that sarcoma viruses contain extra information apparently not found in leukemia viruses (5), although this evidence is limited to avian systems. 4) Type-C RNA tumor virus components have now, I believe, been unequivocally demonstrated in human acute leukemic cells (see below).

Evidence for Type-C Virus Related Information in Human Acute Leukemic Cells

In 1970, shortly after the discovery of reverse transcriptase in type-C viruses, we reported on the detection of an RNA-dependent DNA polymerase activity in some fresh human acute leukemic cells, an activity we could not detect in normal lymphocytes even after stimulation with PHA (7). Since that report, this enzyme has been purified and extensively characterized, particularly in AML (8—14). We can say that it has the known biochemical properties of virus reverse transcriptase. These biochemical properties include the following: a) it catalyzes an endogenous (i. e., using a *native* RNA template-primer) RNA primed-RNA directed DNA synthesis in a cytoplasmic particle; b) the enzyme purified from this particle will transcribe heteropolymeric portions of viral 70S RNA; c) the enzyme responds to the relatively specific synthetic template-primer dG_{12}.rC and favors dT_{12}.rA over dT_{12}.dA. These are all characteristics like viral reverse transcriptase and different from the major DNA polymerases of normal cells or *other* DNA polymerases from leukemic cells; d) the molecular weight of a recently purified enzyme from a patient with acute myelomonocytic leukemia (AMML) was shown to be approximately 70,000 daltons (see R. Gallagher, et al., elsewhere in this book), this is the known estimate of virus reverse transcriptase; e) the RNA template appears to be 70S (15, 16) or 35S (14, 15) in size, the size of virus RNA and its subunits, respectively. Recently, immunological observations have shown that this enzyme is specifically related to the reverse transcriptase from two known primate type-C RNA tumor viruses, the woolly monkey (simian sarcoma virus) and gibbon ape leukemia virus (17, and R. Gallagher, et al., elsewhere in this book).

Nucleotide sequence relatedness has been demonstrated between the nucleic acids of these particles from human acute leukemic cells and the genomic RNA of murine (10, 15, 16) and especially primate (simian sarcoma) virus (10). In these recent studies, we showed that a small amount of homology exists between leukemia virus RNA and the DNA product of the human leukemic reverse transcriptase. However, to our surprise, more than 50 % homology was found with the RNA of some (primate and murine) *sarcoma* type-C viruses (10).

Finally, we have noted that these viral related components are present in an intracytoplasmic particle which bands with the density typical of type-C virus (1.14 to 1.17 g/ml) (8, 10, 11, 14, 16). We interpret these results as indicative that at least some human acute leukemic cells contain defective (i. e., doesn't infect, replicate, etc., no budding form membrane and release is seen) type-C virus which is sarcoma related (Mammalian sarcoma viruses are not released in the absence of helper type-C "leukemia" virus.) A recent observation from Spiegelman's lab indicates that leukemic cells DNA contains sequences different (or extra) than found in normal cell DNA. Some of the DNA sequences made from leukemic cytoplasmic RNA by the

human leukemic cells reverse transcriptase apparently are not found in normal cell DNA but are detected in the DNA of leukemic cells (20). These results were interpreted as evidence against the oncogene theory of Huebner and Todaro (21). The evidence I have discussed here for type-C virus related components in human leukemic cells has been recently reviewed in more detail elsewhere (18, 19).

Virus "Hot Spot" Hypothesis

I believe the following model may be useful in thinking about leukemogenisis in man. In any case, it is in keeping with what we know and accounts for some apparent discrepencies on the obervations of factors which induce leukemia.

(1) There are "virogenes" in normal cells. These "viral" genes play an important role in growth and perhaps early differentiation of primitive embryonic cells. We know that type-C viruses may bud from cell membranes. Presumably, a genetic component of these viruses may affect host cell membranes. Normally, these gene products are repressed with the progression of undifferentiated primitve embryonic type cells to more mature cells. These genes are all included in type-C leukemia viruses and most are included also in type-C sarcoma viruses. In addition, other genomes repressed in the more primitive cells become induced during maturation. The active "virogenes", characteristic of undifferentiated cells, are functional in maintaining the growth of these cells, and are critical to transformation.

(2) Operationally, myeloblasts and similar cells of the normal bone marrow may be considered as "embryonic" cells (similar metabolic state) while the granulocyte (myeloblast derived) may be regarded as a "mature adult" cell.

(3) Physically adjacent or functionally related to the virogenes, resides a set of nucleotide sequences ("hot spot") which are unusually sensitive to events which alter nucleotide sequence (by mutation or by recombination). Among other things, these regions are sensitive to influence of some chemicals, radiation and hereditary factors. These changes in sequences may also influence chromosomal structure. These genes (virogenes and "hot spot") are not limited to one chromosome. The chromosomal changes frequently observed in leukemias (and many other neoplasias) are probably secondary to these events and need not in themselves be causally related to leukemia. Regulator genes may control the expression of both virogene and "hot spot". The regulator genes need not be in close proximity to the virogene or "hot spot." In this respect, Rowe and his associates have evidence of genes controlling viral gene expression which apparently are present in different chromosomes. The *normal* function of the "hot spot" may involve genetic diversity, useful to the embryonic state, and in antibody producing cells perhaps also to generation of antibody diversity. Some component directly bears on membrane function and or structure.

(4) The "hot spot" then is present and is susceptible to modification by recombination and/or by mutagenesis in *all* individuals. In some individuals, there is "hypermutability" of the "hot spot" (again by either mutation or recombination). These people then may already contain aberrent sequences and by heredity or by congenital factors may be passed on from one generation to another. Direct modification of the "hot spot" (by radiation, chemical carcinogenes, or by integration of new information as from virus infection) or indirect, e. g., by mutation in the

regulator gene which controls the "hot spot" and virogene may account for expression of these sequences at a time when they should be "repressed," leading to a return to, or maintenance of, the "embryonic" state. Some individuals are predisposed due to a hypermutable "hot spot," depending on genetic factors (e. g., hereditary ataxia telagniectasia, Bloom's syndrome, Down's syndrome [mongoloids]-all hereditary or congenital disorders with a high incidence of leukemia may already contain aberrant sequences within the "hot spot" or are highly susceptible to develop changes). The congenital problems such as mongoloids occur because of *in utero* changes. Thus, "normal" cells *could* contain oncogenic information (as the oncogene proposal demands) but not necessarily, and by this model *usually* they would not, but they would *always* contain the set of "viral" genes. Thus, the presence and expression of the virogene is not sufficient for leukemia, and nucleotide sequence change is a pre-requisite. In hereditary disorders, the event which modified the "hot spot" (directly or indirectly) occured generations ago.

(5) The type-C leukemia RNA tumor viruses may contain only these virogenes, but by virtue of the propensity of the provirus DNA (synthesized from the genomic RNA via reverse transcriptase after infection) to integrate in the region of the "hot spot" (which is subject to change not only by mutagenesis but also by recombination) it may induce transformation by modifying the "hot spot." The type-C sarcoma viruses contain most of the nucleotide sequences as the leukemia viruses, but they also contain the modified information from the "hot spot."

When virogene activity is expressed completely, virus particles may form. Normally, even in embryonic type cells complete expression does not occur. Partial expression can lead to defective (non-infectious) non-tumorigenic particles, e. g., viral-like particles such as the so-called A particles, or infectious particles such as some of the endogenous type-C viruses referred to earlier, or virus specific products not assembled into particulate forms. The *normal* information from the "hot spot" relates to normal membrane function and expressed with the viral genes can affect virus replication (budding from cell membrane, etc.).

(6) When the "viral gene" expression occurs these RNA transcripts have a propensity to physically and functionally associate with RNA transcripts from the "hot spot". When the "hot spot" is aberrant and expressed with virogene, sarcoma viruses may be produced as well as endogenous type-C viruses which may or may not contain oncogenic information. Mechanisms which induce expression of the "hot spot" may co-induce components of the virogene. With infection from without by type-C viruses, the new virogenes will have a propensity to integrate around this "hot spot" region. The "hot spot" will also have high content of reiterated nucleotide sequences. With expression of an *aberrant* "hot spot" only partial expression of the virogene usually occurs. This component is essential to formation of mature budding virions, hence sarcoma genome products are defective, unable to be released as mature virions to infect additional cells, leaving the organism "protected" to a transformation of only one cell. When complete virogene expression occurs, sarcoma virions are formed and released, potentially transforming several clones or even spreading the disease from organism to organism (exogenous infection). The type-C endogenous virus may also be formed after transformation *without sarcoma virus.* Presumably in this instance, there are other mechanisms which inhibit the

the formation of the mature sarcoma virus. These different possibilities then are:

1) Transformation (expression of aberrant "hot spot")→→→→no virus but virus components.
2) Transformation (expression of aberrant "hot spot")→→→→endogenous virus.
3) Transformation (expression of aberrant "hot spot")→→→→infectious sarcoma virus.

(7) For neoplastic transformation of any one cell then, whole virus is not needed nor is virus infection from without. Transformation will be accompanied, however, by the expression of at least some virogenes when they normally should be repressed-since induction of the expression of the "hot spot" is accompanied usually, if not always, by induction of some of the virogenes. (Virogenes and "hot spot" may be under the co-ordinate control of one regulatory gene.) Thus, leukemia occurs with at least some virogene expression (the result of the transformation rather then the cause), which may occur with release of virions which are either infectious, defective, or tumorigenic. If the latter, these particles may transform other cells and on occasion, infect another person so that in this instance the disease is directly caused by exogenous infection.

(8) Some type-C viruses may be without immediate oncogenic potential. In the past few years, some endogenous type-C viruses have been induced from some normal cells (22–24). These induced apparently endogenous virions in most instances do not appear to be oncogenic for their species of origin. However, they do contain reverse transcriptase and hence the ability to synthesize a DNA provirus some or all of which may integrate into the "hot spot" region. They, therefore may evolve into oncogenic particles. They may acquire nucleotide sequences (by repeated RNA→DNA DNA→RNA reactions) which allows them to eventually induce expression of an aberrant "hot spot" or make a normal "hot spot" aberrant. It is proposed that in most cases type-C leukemia viruses which are, in fact, leukemogenic *in vivo* act by affinity of this information to integrate around the "hot spot" sequences and induce expression of these sequences (see number 6). The critical factor to leukemogenesis then is the control of the expression of this "hot spot". The information when abnormal may be because of sequence change or addition of new sequences.

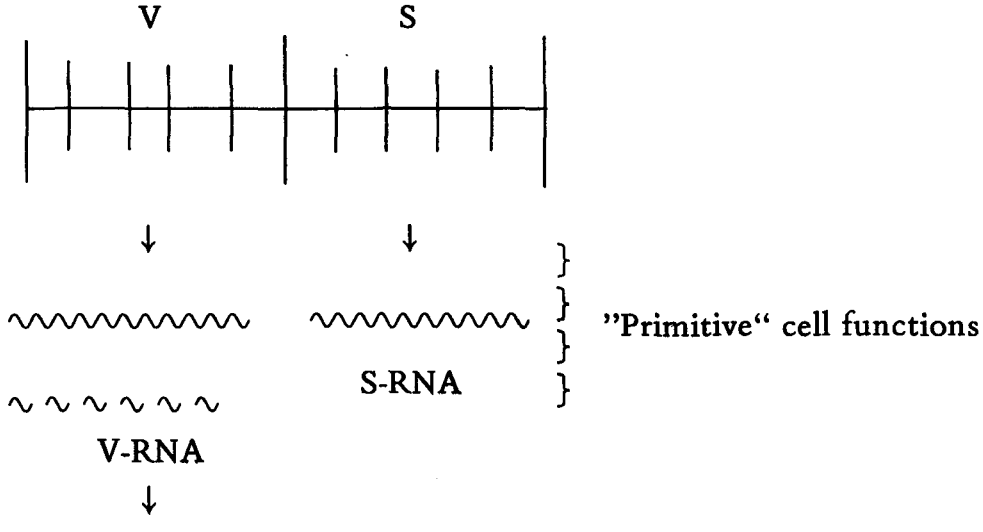
(10) One function of the "hot spot" (as well as one component of the virogenes) as described above has, at least in part, an affect on cell membrane function and/or structure. When altered, cell membranes are modified so that they do not respond properly to the usual regulator proteins. For instance, a greater concentration of the "maturation" proteins (colony stimulating activity) may be needed for normal maturation. Maturation arrest ensues which is potentially reversible *in vitro*, but should not have long term therapeutic benefits *in vivo* because of the need for its continued presence in relatively high concentrations. In this respect, another disease should be mentioned. Paroxysmal nocturnal hemoglobinuria, an apparent "in born error" is a membrane alteration and terminates in leukemia in a greater than predicted frequency.

A schematic illustration of the model is shown below where V = virogene and S = hot spot.

This model is testable in some of its components, and it predicts the following:

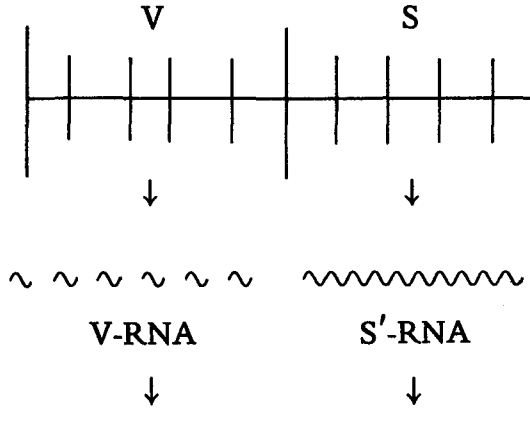
(1) Leukemic cells will contain some nucleic acid sequences different from normal cells of most, but not all, people, and these special nucleotide sequences in leukemic cells may be found in some "normal" cells of certain hereditary disorders, or congential disorders, e. g., possibly in mongoloids.

1) Normal Cell (immature)



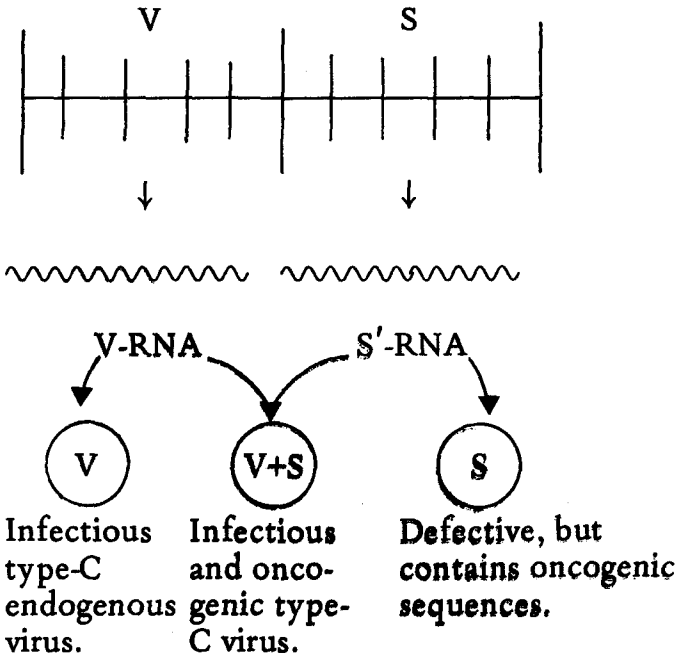
Some degradation of V-RNA (virus particles usually not found)

2) Normal Cell (mature)

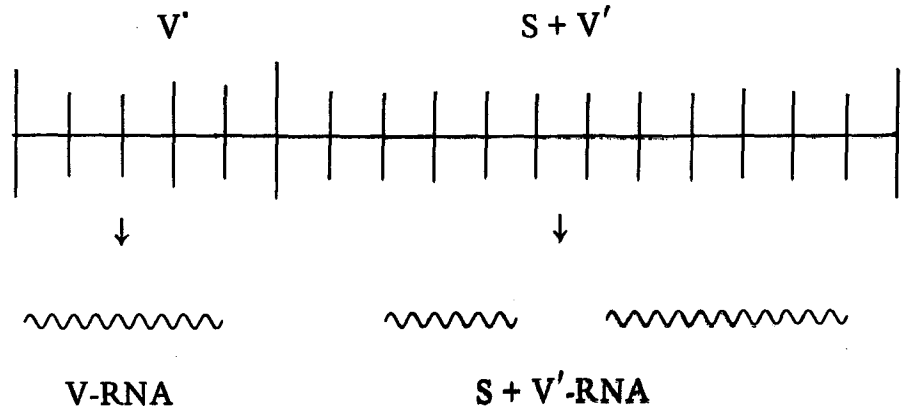


Most is degraded or it is not even expressed.

3) Transformed Cell (by mutation)



4) Transformed Cell (by recombination-addition of sequences, e. g., by infection with virus)



(Consequences are as for 3.)

(2) Information within sarcoma viruses is the oncogenic information not only for sarcomas but also leukemias.

(3) Normal cells always contain some genes in common with type-C viruses. These genes are normally expressed in embryonic type cells to some degree and always in transformed cells, but not necessarily completely. When completely expressed, viruses are formed. The viruses then are the *result* of the transformation in this case.

(4) Infection from without by oncogenic type-C viruses may be a direct cause of

leukemia in some instances. It is possible, that it is always caused by exogenous viral infection. The model does not bear on the question – wether this is common or not.

(5) Type-C leukemia viruses activate or create "sarcoma" sequences.

(6) More type-C virus information is expressed in transformed cells than in normal adult tissues, and perhaps some virogene expression occurs in some normal mature cells.

(7) The sequences in the "hot spot" or in a regulator gene controlling the "hot spot" and virogenes of *some* hereditary disorders with high incidences of leukemia will be *different* (nucleotide sequence mutational change or addition by recombination of new sequences) than normal cell DNA.

(8) Finally, I propose that unlike some traditional concepts of viral transformation *in vitro*, a more mature cell is not induced to de-differentiate, but instead the target cell for a leukemia inducing agent will be the pluripotential stem cell or the progenitor blast cell (myeloblast) which will be blocked in its maturation.

The virus "hot spot" proposal differs from the oncogene theory of Huebner and Todaro (21) in the following ways: a) It is aimed chiefly at a specific neoplasia – leukemia in man in contrast to all neoplasia; b) It attempts to explain the phenotype changes in the cells and not just the "genotypic" origin of neoplasia; c) Most importantly, in the consideration of the genotypic origin of neoplasia, it does not demand oncogenes in all somatic cells of all vertebrates, as the oncogene theory proposes. The latter states that regulator (switch on- switch off mechanisms) are sufficient to cause neoplastic transformation in any somatic cell. In contrast, this proposal states that this certainly may occur *but* nucleotide sequence change or addition in DNA is a pre-requisite; although this change could be "in born" it is not present in most cells of most people. The virus "hot spot" proposal differs from Temin's protovirus hypothesis (25) as follows: a) Again, its aim is somewhat different. The protovirus hypothesis is directed primarily at explaining the origin, evolution, and replication of RNA viruses that may become oncogenic; b) The protovirus hypothesis also does not attempt to deal with the phenotypic changes in leukemia; c) The requirement for DNA nucleotide sequence change in the virus "hot spot" proposal is similar to the protovirus theory. However, the protovirus theory puts the emphasis on somatic mutation and reverse transcriptase and its RNA template as pivotal to this change, i. e., the emphasis for mutation is in the RNA template for the reverse transcriptase, reverse transcriptase itself, or the DNA product made by reverse transcriptase. The virus "hot-spot" proposal states that the nucleotide sequence change critical to leukemogenesis may be by mutation or and perhaps more commonly, by addition of information through recombination. In addition, the change may be brought about by modification of nuclear DNA sequences not involving reverse transcriptase reactions.

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POLY(A)-CONTAINING MOLECULES IN HETEROGENEOUS NUCLEAR RNA: DIFFERENCES BETWEEN NORMAL LYMPHOCYTES AND ACUTE LEUKEMIA BLAST CELLS

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Rapidly labeled nuclear RNA from mammalian cells can be divided into two major classes. The first class is formed by the 45S ribosomal precursor RNA and its products of cleavage 41S, 36S and 32S RNA (1, 2). Experiments with HeLa cells have shown that these molecules are synthesized and processed in the nucleolus (3). The base composition of these molecules is similar to that of ribosomes, and they are further characterized by the presence of methylated bases and sugar moieties (4) and by the absence of polyadenylic acid sequences (5).

The second type of nuclear RNA consists of molecules ranging in size up to 120S. The base composition of these molecules resembles that of DNA (6), and they are further characterized by the absence of methylated bases and sugar moieties and by the presence of polyadenylic acid sequences (7). These molecules are generally considered to be completely heterodisperse, so that this class of RNA is called heterogeneous nuclear RNA. However, fractionation of these molecules by gel electrophoresis as performed in our laboratory, has shown that, at least in human blood cells, the pattern is more complex than previously considered (8, 9).

As shown in fig. 1, by analyzing nuclear RNA from human small lymphocytes several discrete fractions were resolved behind the 28S ribosomal RNA component. Some of them may be identified with the already mentioned short-lived products of cleavage of the 45S ribosomal precursor RNA, but several of these discrete fractions run more slowly than this latter molecules. Evidence has been presented (9) that they are not technical artifacts and that they can also be observed in PHA-stimulated lymphocytes, provided that the period of incubation with the labeled precursor was not longer than 15 minutes (Fig. 2 and 3).

One of the most recently demonstrated characteristics of heterogeneous nuclear RNA is the presence, in this RNA, of polyadenylic acid sequences covalently linked. In a group of experiments carried out in our laboratory we have assayed the proportion of poly(A)-containing molecules in electrophoretically separated fractions of nuclear RNA obtained from unstimulated and PHA-stimulated normal human lymphocytes. The assay was carried out by measuring: 1) the amount of radioactivity bound to nitrocellulose filters after filtration of radioactive RNA dissolved in buffer with high salt concentration; 2) the amount of radioactivity bound to glass fiber filters containing poly(U).

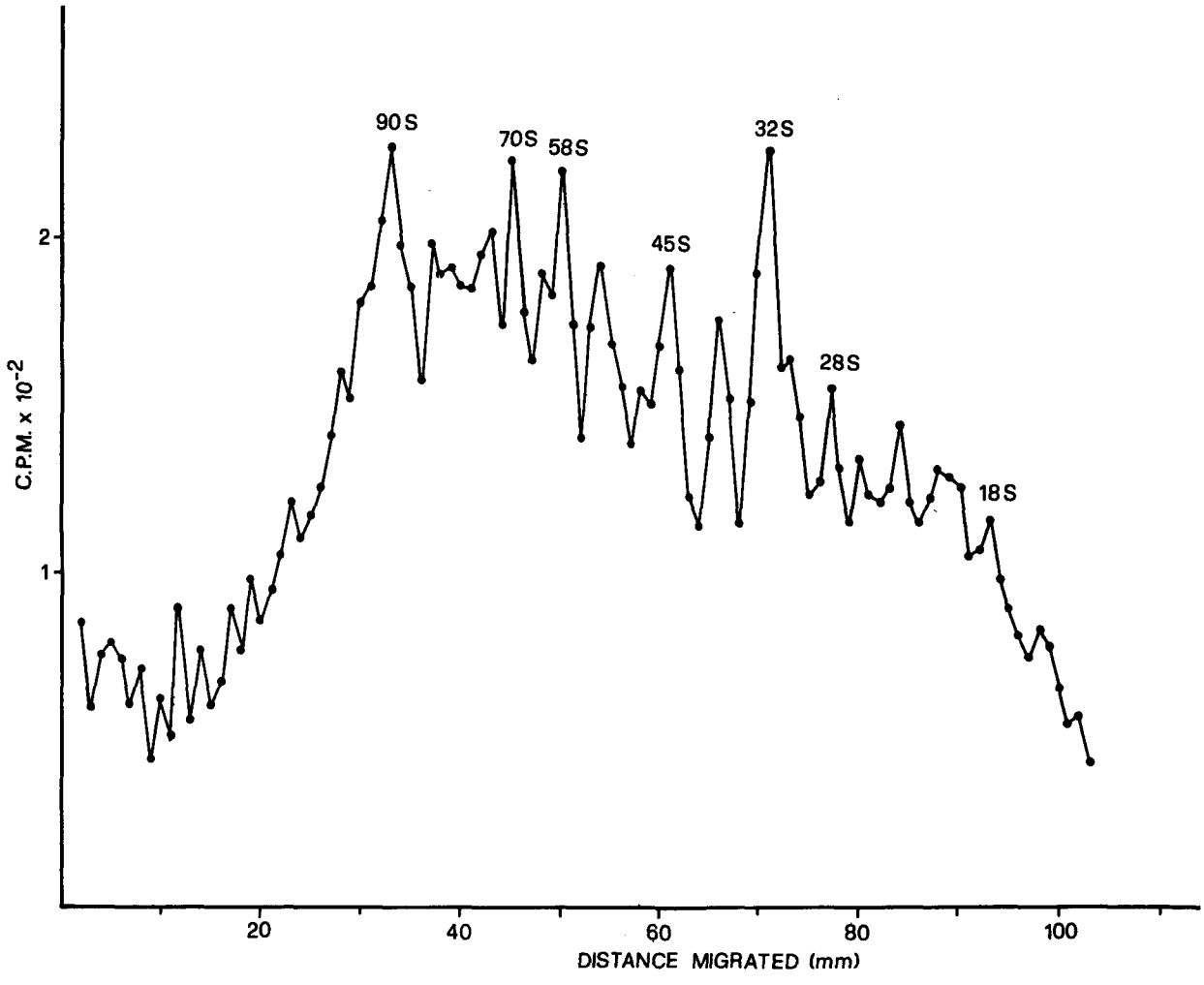

Fig. 1: Electrophoretic profile of newly synthesized nuclear lymphocyte RNA. The cells were incubated with ^{3}H-uridine (10 μc/ml) for 6 hours. Nuclei were separated and extracted together with an amount of unlabeled KB cells sufficient to give enough 28S and 18S RNA as markers. Electrophoresis was für 180 minutes at 3 mA/gel. After the electrophoretic run the gel was scanned at 260 mμ and sliced in 1 mm slices. Nominal S values were assigned on the basis of the linear relationship between the logaritm of the molecular weight of a given RNA component and its relative electrophoretic mobility. The S values were therefore calculated from the known S values of the two RNA components of the ribosomal subunits (18S and 28S).

To evaluate the binding of labeled RNA to nitrocellulose filters, 2 ml of buffer (0.01 M Tris, pH 7.4, 0.5 M KCl, 1 mM $MgCl_2$) containing the RNA recovered from the gel were diluted to 10 ml with the same buffer and then filtered at room temperature through Millipore filters (HA 0.45 μm, Millipore Filter Corp., Bedford, Mass.) previously soaked in the same salt solution. The filters were then washed twice with 10 ml of this salt solution, dried and counted in toluene scintillation mixture. Input values were obtained by drying 0.5 ml aliquots of the RNA solution on Millipore filters previously soaked in high salt buffer, and counting. To evaluate the binding of labeled RNA to glass fiber filters containing poly(U), RNA in gel slices was solubilized in 3 ml of binding buffer (0.01 M Tris. KCl (pH 7.5), 0.12 M NaCl). 2 ml of this solution were brought to 10 ml with the same buffer, then filtered at 2 ml per min through glass fiber filters (Whatman, GF/C 2.5 cm) on which poly(U) had been previously immobilized by the technique of Sheldon et al. (10).

The filters were washed with 20 ml of binding buffer at 25 °C, followed by 20 ml of ice cold 5 % TCA and 10 ml of 95° ethanol, then dried and counted. Table I shows that an average 20 % of the labelled molecules in the size range between 49S and 120S bind to nitrocellulose filters, whereas a reduced amount of filter-bound radioactivity was found in fractions corresponding to S values lower than 48. This may be explained by the observation that ribosomal RNA and its precursors lack poly(A) segments (5). Little differences were found between RNA from unstimulated and PHA stimulated lymphocytes. The proportion of poly(A)-rich molecules was a littel lower in the former cells, at least in the fractions corresponding to S values from 16 to 60. The average proportion of labeled molecules bound to glass fiber filters containing poly(U) was about 11 %, as shown in table II. The poly(A)-rich molecules of total nuclear RNA were also analyzed by gel electrophoresis. The results show that whereas total nuclear RNA was resolved in several discrete peaks, the poly(A)-rich molecules of the same RNA were heterogeneously distributed through the gel (Fig. 4).

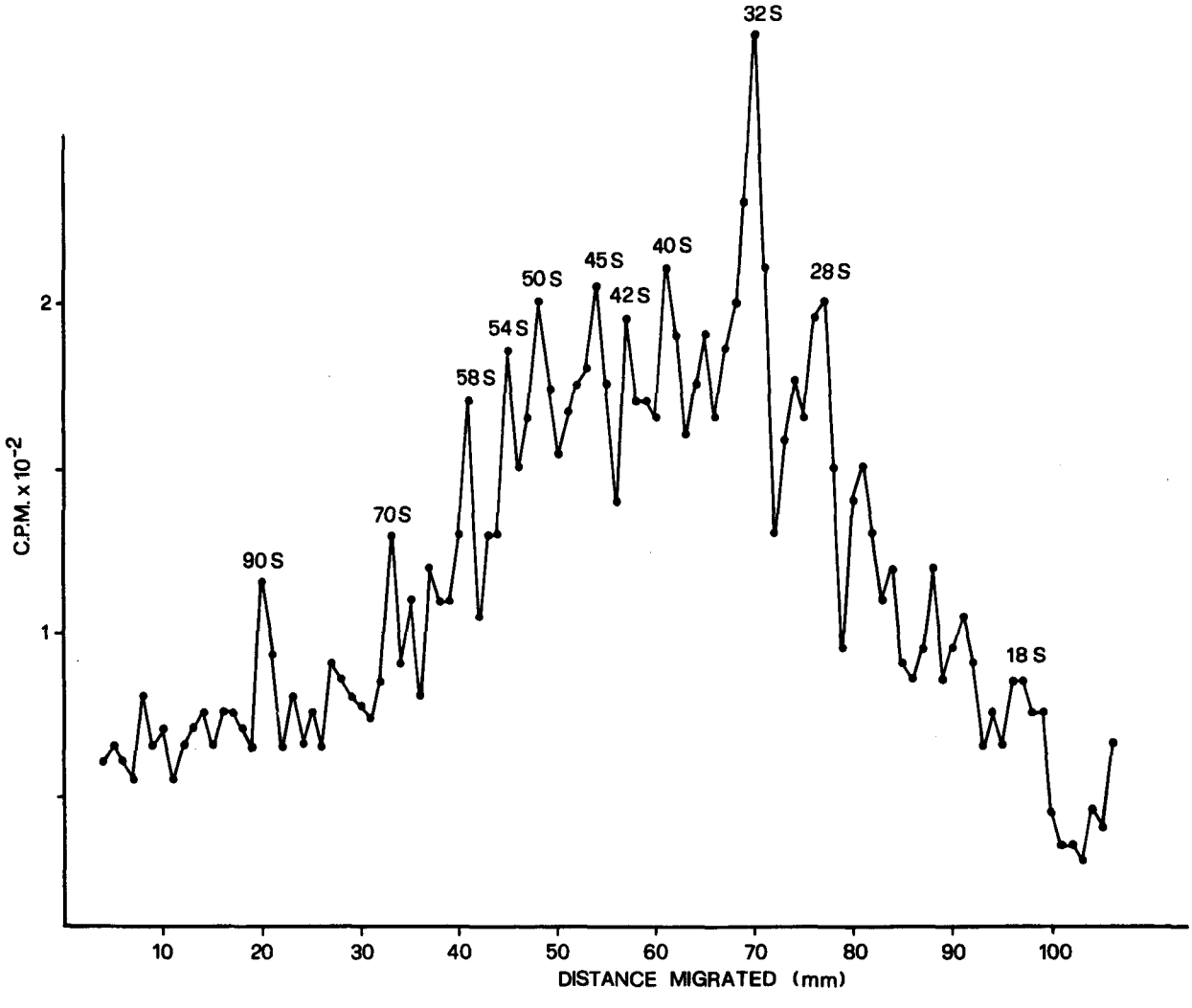

Fig. 2: Electrophoresis of labeled whole cell RNA extracted from PHA-stimulated lymphocytes incubated for 12 minutes with ^{3}H-uridine (20 µc/ml). Run for 200 minutes at 3 mA/gel.

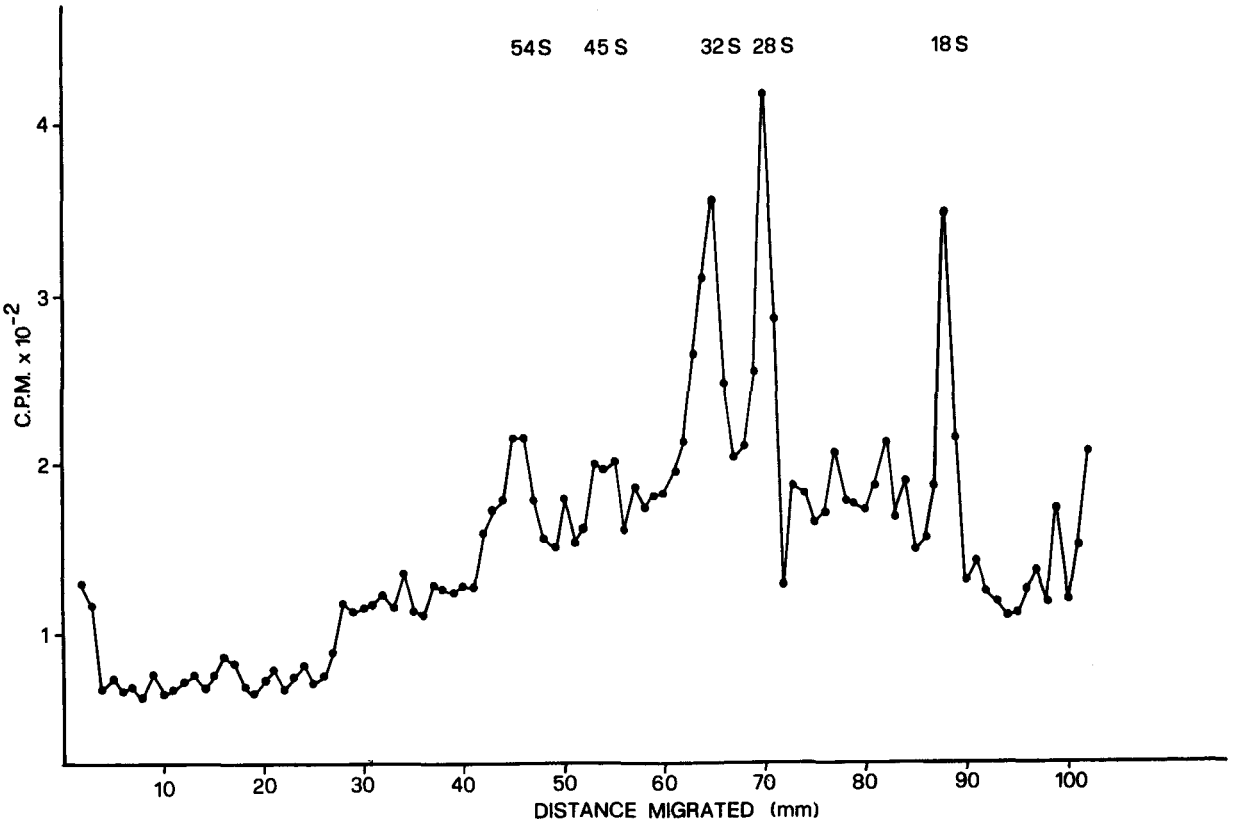

Fig. 3: Labeling pattern obtained by electrophoresis (200 minutes, 3 mA/gel) of whole cell RNA extracted from PHA stimulated lymphocytes incubated with ^{3}H-uridine (10 μc/ml) for 45 minutes.

Table I Binding of labelled nuclear RNA from unstimulated and PHA-stimulated normal lymphocytes to nitrocellulose filters

RNA fraction size range (S values)	Percent of input radioactivity bound to filters	
	unstimulated lymphocytes	PHA-stimulated lymphocytes
16 – 20	9	10
21 – 25	7	11
26 – 30	8	10
31 – 37	7	10
38 – 42	9	13
43 – 48	11	16
49 – 53	15	21
54 – 59	15	20
60 – 65	22	23
66 – 73	21	23
74 – 82	22	22
83 – 89	20	21
90 – 98	23	21
99 – 120	23	22

Several different techniques were employed in the past three years in our laboratory to detect possible differences between heterogeneous nuclear RNA of normal lymphocytes and that of acute leukemia blast cells. No difference between normal and leukemic RNA was observed by gel electrophoresis of whole cell RNA and nuclear RNA (8). The same discrete fractions resolved in RNA of normal small lymphocytes were found in RNA of acute leukemia blast cells.

More revealing findings were obtained in experiments carried out to compare nuclear RNA from normal PHA-stimulated lymphocytes and acute leukemia blast cells by competition hybridization techniques. The results of a first group of experiments have been already reported (11). Further studies were performed in our laboratory to extend our earlier findings. RNA extracted from isolated nuclei of blast cells from ALL patients and of PHA-stimulated lymphocytes was tested in competition hybridization assays. The results have confirmed that unlabeled nuclear RNA from normal lymphocytes is unable to inhibit hybridization of nuclear leukemic RNA with leukemic cell DNA. These results add further evidence to our previous data suggesting that leukemic blast cells are transcribing RNA sequences which are not transcribed in normal lymphocytes (11).

To obtain a better characterization of the heterogeneous nuclear RNA of leukemic cells we have assayed the proportion of poly(A)-containing RNA molecules in different electrophoretic fractions of nuclear RNA obtained from leukemic blast cells. Six cases of acute leukemia have been so far examined. In most fractions, the

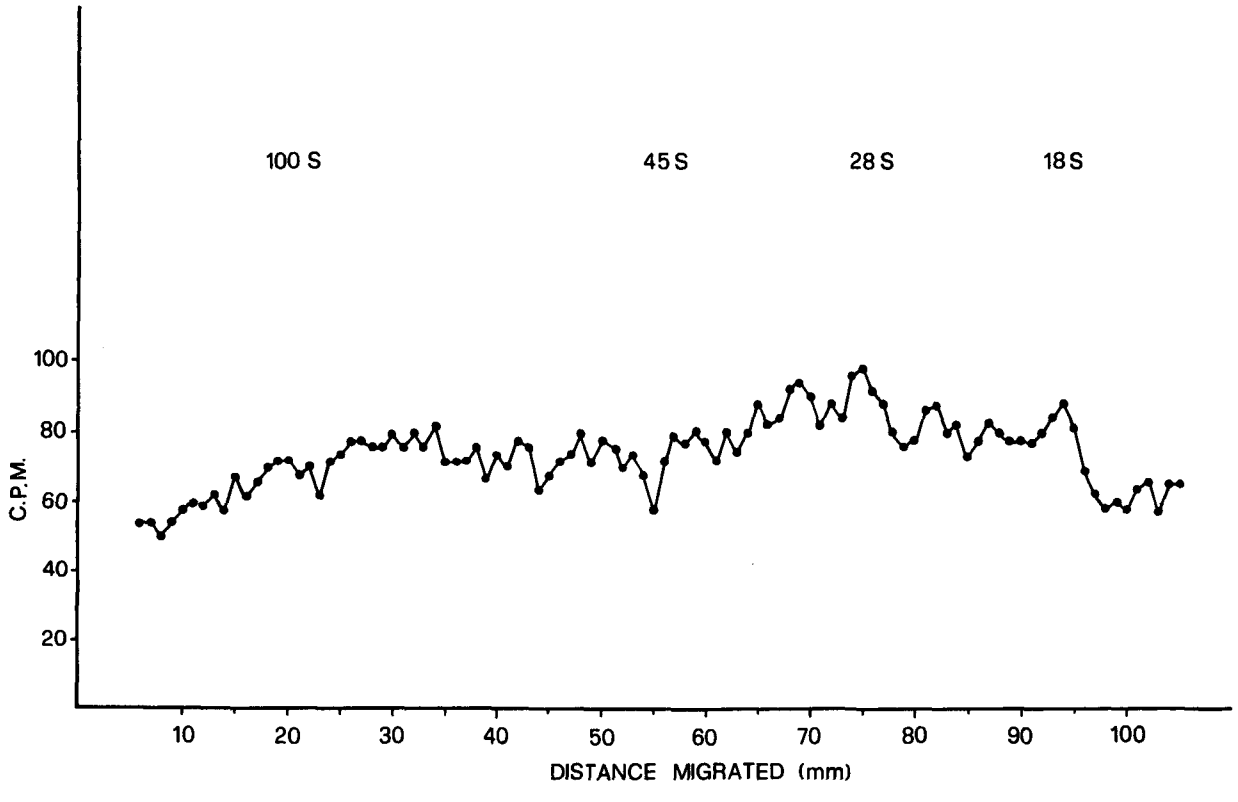

Fig. 4: Electrophoretic profile of the poly(A)-containing fraction of the nuclear RNA extracted from small unstimulated lymphocytes. The RNA was filtered through a Millipore filter, then 2 small discs, 6 mm in diameter, were cut from the filter and put on top of a gel column. The discs were then covered with electrophoresis buffer containing unlabeled KB cell RNA as a carrier. Run was prolonged for 4 hours at 3 mA/gel.

Table II Binding of labelled nuclear RNA from unstimulated and PHA-stimulated normal lymphocytes to Poly (U)-containing glass-fiber filters

RNA fraction	Percent of input radioactivity bound to filters	
	unstimulated lymphocytes	PHA-stimulated lymphocytes
16 – 20	5	6
21 – 25	4	5
26 – 30	4	5
31 – 37	5	6
38 – 42	5	7
49 – 53	8	10
54 – 59	10	13
60 – 65	11	11
66 – 73	11	11
74 – 82	11	10
83 – 89	12	12
90 – 98	11	12
99 – 120	12	10

Table III Binding of labelled nuclear RNA from acute leukemia blast cells to nitrocellulose and Poly (U)-containing glass fiber filters

RNA fraction	Percent of input radioactivity bound to filters	
	Millipore filters	glass fiber filters
16 – 20	17	14
21 – 25	19	25
26 – 30	20	12
31 – 37	25	11
38 – 42	26	11
43 – 48	24	28
49 – 53	36	36
54 – 59	34	39
60 – 65	26	29
66 – 73	31	18
74 – 82	28	12
83 – 89	26	11
90 – 98	20	12
99 – 120	22	10

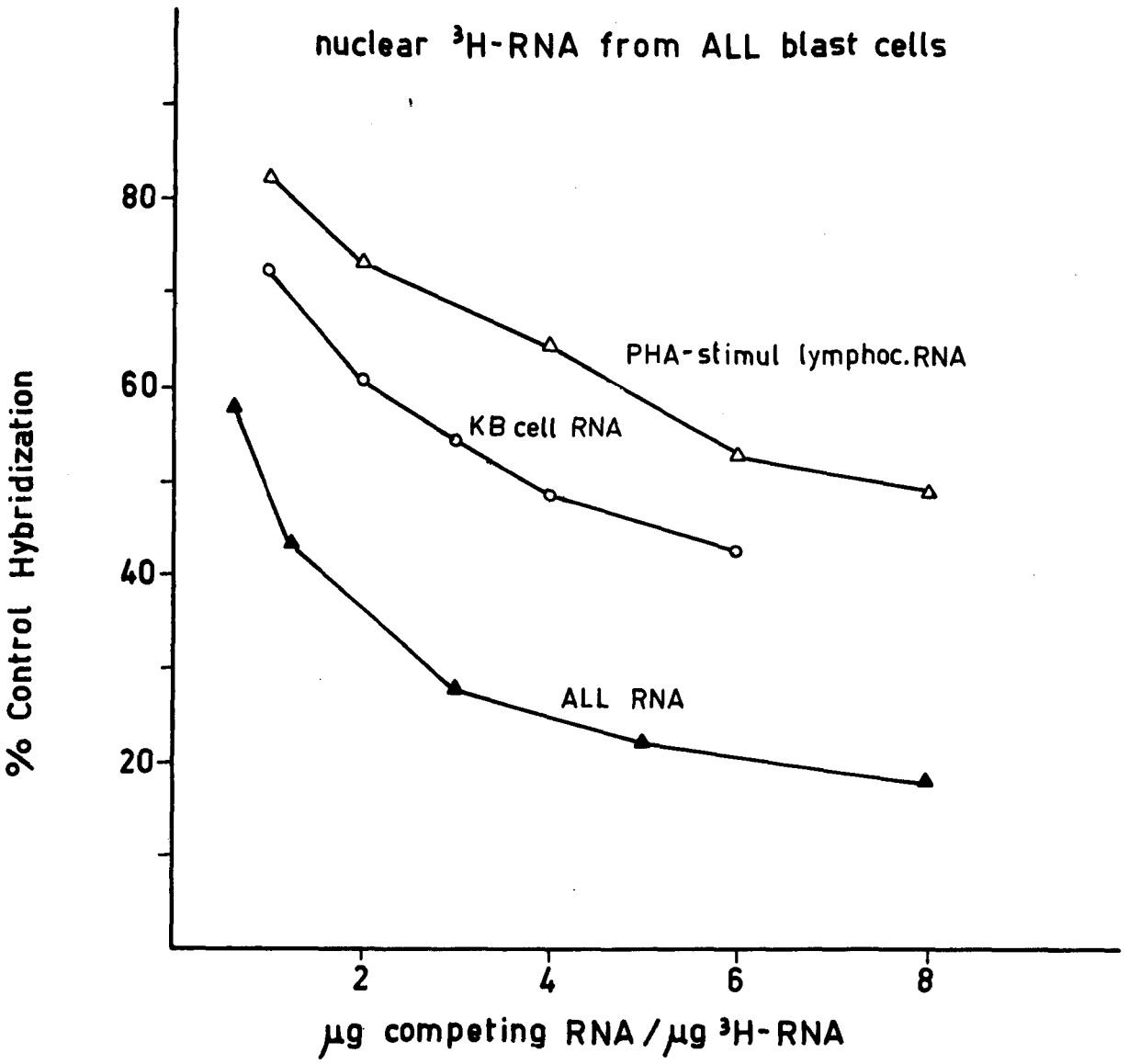

Fig. 5: The competition of unlabeled nuclear RNA from ALL, KB cells and PHA stimulated lymphocytes in hybridization reaction of labeled nuclear RNA from ALL cells with ALL DNA. Filters loaded with DNA were incubated with unlabeled RNA for 18 hr at 67 °C, drained, soaked twice for 30 min in 20 ml of 2 x SSC at 65 °C and then returned to vials for further incubation with labeled RNA.

proportion of poly(A)-containing molecules was definitely larger than that of the corresponding fractions of normal lymphocytes (Fig. 6). The largest proportion was found in all cases in the fractions formed by molecules in the size between 49 and 59S. In many fractions, corresponding to S values lower than 60, the proportion of poly(A)-containing molecules was twice as much as that of the corresponding normal fractions (table III). Furthermore, in normal RNA the proportion of poly(A)-containing molecules was strictly similar in all fractions corresponding to S values greater than 49, whereas leukemic RNA was characterized by a marked variability in the occurence of poly(A)-containing molecules in the different fractions.

It is difficult at present to offer any explanation of the increase in proportion of poly(A)-containing molecules in heterogeneous nuclear RNA of acute leukemia

cells. It is well known that the polyadenylic acid sequences have been considered a necessary "ticket" for those nuclear RNA sequences which become cytoplasmic messenger sequences (7). However, the ability to process heterogeneous nuclear RNA in poorly differentiated, slowly growing cells such as the leukemic cells is presumably lesser than normal. Furthermore, the results of our competition hybridization experiments suggest that in heterogeneous RNA of leukemic cells are transcribed "new" sequences which are not transcribed in normal lymphocytes. We must therefore seek an explanation for two facts which may be related, the transcription of new sequences and the increase in the proportion of poly(A)-containing molecules. Although no definite evidence has been so far obtained, we feel justified to postulate that at least part of the new sequences carry poly(A) segments.

Support to this hypothesis comes from the result of recent experiments carried out in our laboratory. The poly(A)-containing molecules in nuclear RNA of leuke-

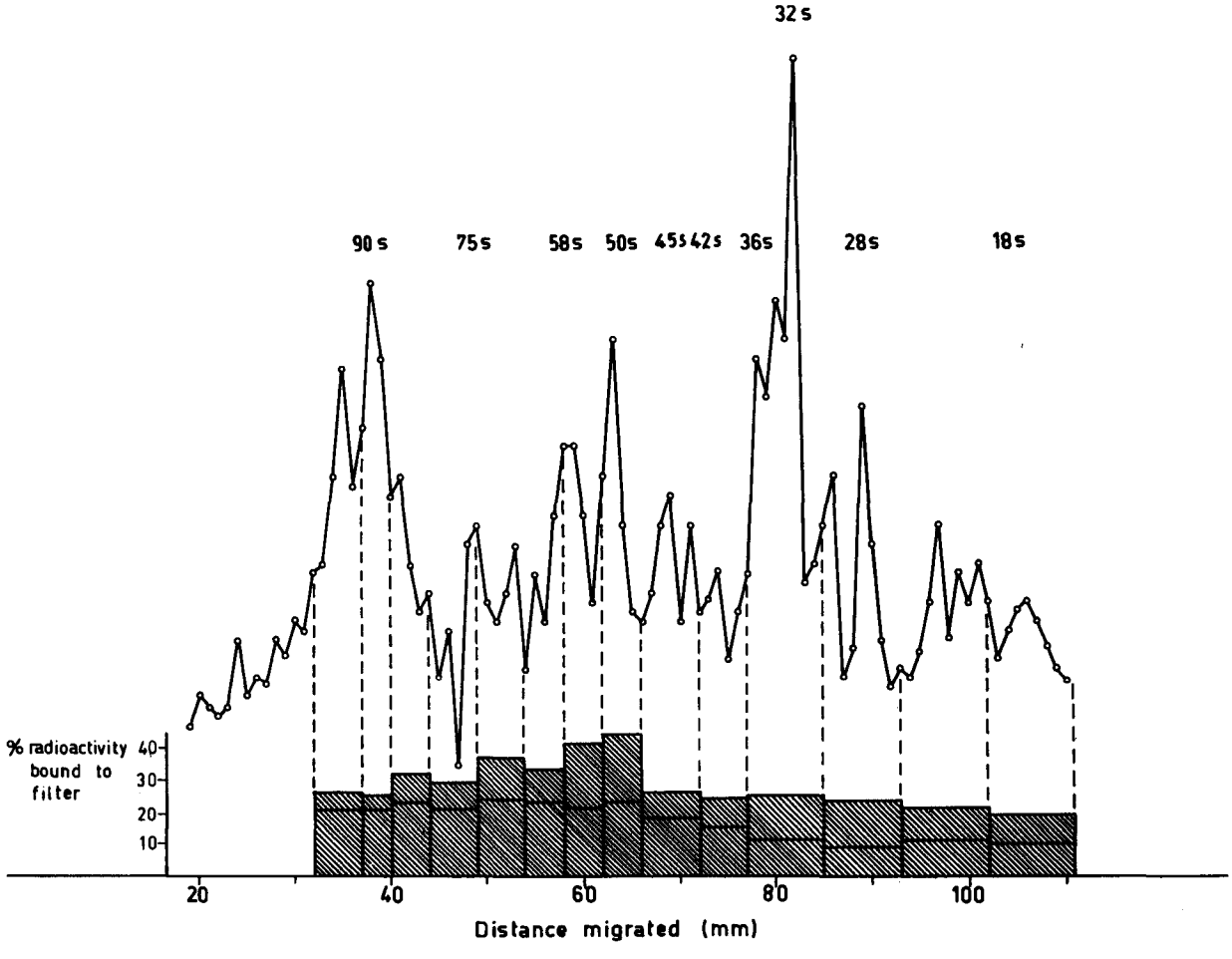

Fig. 6: Electrophoretic profiles of labeled nuclear RNA from a case of AML. Two samples of the same RNA were run on parallel columns. After scanning at 260 μm one of the columns was sliced in 1 mm slices, which were then counted. The second column was divided in fractions comprising molecules in a size range of about 5 S. The dotted lines indicate the limits of those fractions which were finely sliced to recover the RNA to be assayed for the poly(A)-containing molecules.

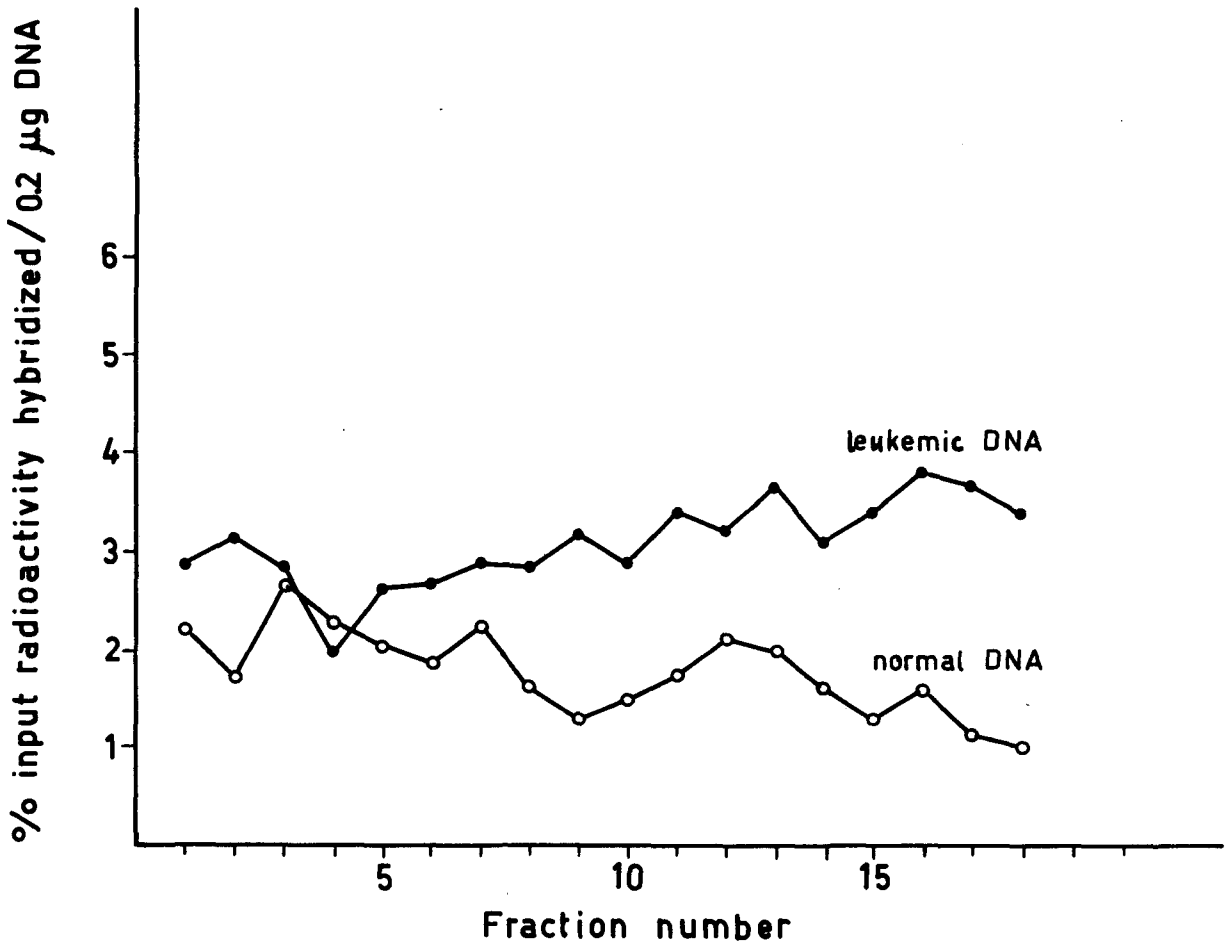

Fig. 7: Hybridization of poly(A)-containing nuclear RNA molecules from ALL blast cells to normal and leukemic DNA. The molecules were fractionated in 18 fractions by gel electrophoresis, using unlabeled KB cell ribosomal RNA as carrier.

mic cells were separated from those lacking poly(A) by hybridization with polyuridilic acid followed by chromatography on hydroxylapatite, according to the technique of Greenberg and Perry (12). The poly(A)-containing molecules were precipitated with ethanol after addition of electrophoretically purified 28S and 18S RNA from KB cells. The redissolved RNA was then fractionated by agarose acrylamide gel electophoresis and the gel column was cut in 0.3 mm slices. Groups of slices corresponding to 18 fractions were extracted with 1 ml of 2 x SSC. DNA was prepared from normal human lymphocytes and from leukemic blast cells by the method of Marmur (13). The DNA was denaturated by boiling and loaded on Millipore filters. Disks 4 mm in diameter containing 0.2 µg of DNA were punched out of each filter and placed in 1 ml vials, together with a blank filter to monitor non specific binding of RNA. Labeled RNA from each gel fraction, in 0.5 ml of 2 x SSC was placed in the vials which were sealed and incubated at 65 °C for 40 hours. The filters were then removed, washed with 2 x SSC, digested with RNAse, washed again and counted. The results of one of our experiments are shown in

fig. 7. It is apparent that the ability of poly(A)-rich nucelar RNA from leukemic cells to hybridize to leukemic DNA was always greater than the ability to hybridize to normal DNA. This result, which has been so far obtained in two cases, suggests that poly(A)-rich RNA from nuclei of leukemic blast cells contains a fairly large amount of sequences which are complementary to DNA sequences present in leukemic DNA but absent from normal DNA. These RNA sequences may then explain the inability of nuclear RNA from normal cells to inhibit the hybridization of leukemic nuclear RNA with DNA.

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DNA POLYMERASES IN LYMPHOID CELLS

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Introduction

Three major DNA polymerases have been identified in soluble extracts from eukaryotic cells (1). One is a high molecular weight enzyme (about 150,000 daltons), found predominantly in cytoplasmic fractions, which we refer to as DNA polymerase C (1–4). The second is a lower molecular weight form (about 40,000 daltons), which is recovered primarily from nuclei (but also present in cytoplasm), which we refer to as DNA polymerase N (1–4). A third activity has also been seen, which can copy the adenylate-containing strand of the synthetic homopolymers poly(A) · oligo(dT) or poly(dA) · poly(dT), in the presence of Mn^{++} (1, 5, 6). We call this enzyme DNA polymerase A.

We have developed a method which allows us to rapidly separate, quantitate, and identify these activities in crude cell or tissue extracts. In the course of screening a variety of cells and tissues both for these normal DNA polymerases and for the unique reverse transcriptase of RNA tumor viruses (7), we have discovered that certain lymphoid cells have unique DNA metabolizing enzymes.

Normal Mouse Bone Marrow Fibroblasts

Figure 1 shows the three major polymerase recovered from a monolayer culture of mouse fibroblasts called JLS-V9 cells, which are not producing detectable C-type particles (8). Cells are prepared for these assays as follows: cell disruption is accomplished by repeated freeze-thawing. The crude homogenate is treated with non-ionic detergent to solubilize membrane-bound enzymes. High speed centrifugation yields a particle-free supernatant containing the solubilized enzymes, which is analyzed directly by chromatography on a phosphocellulose column. A salt gradient is passed through the column, and fractions are assayed for DNA synthetic activity.

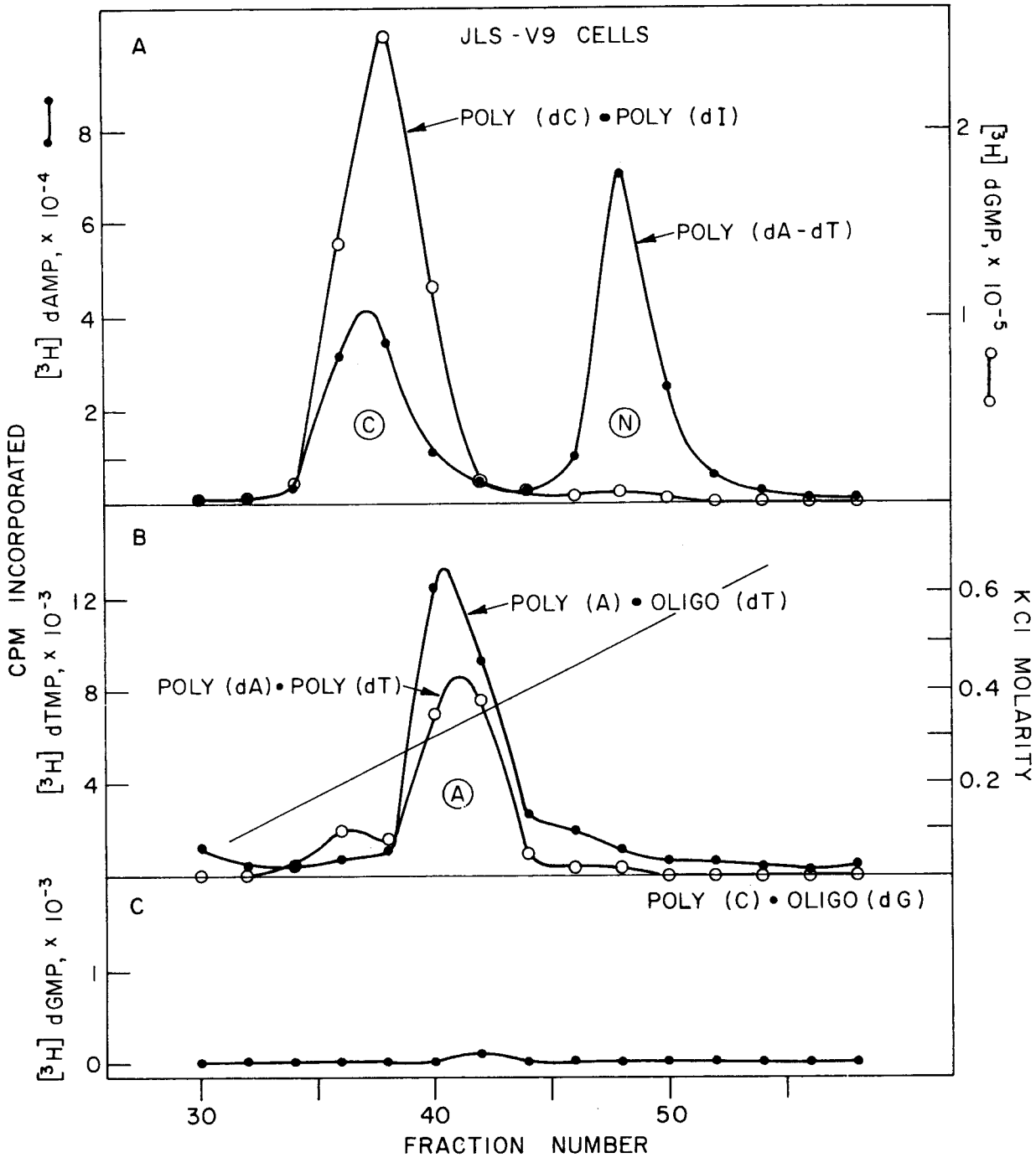

Fig. 1: DNA polymerases of uninfected murine fibroblasts, (JLS-V9 cells).
The individual panels represent assays of the fractions from a single phosphocellulose column using various templates, primers, and substrates. Assay conditions were as defined in reference 1.

Figure 1A shows two activities able to copy the alternating co-polymer, poly(dA-dT). These activities correspond to DNA polymerases C and N. Polymerase C is also evident when dGMP incorporation stimulated by poly(dC) • poly(dI) is measured. Figure 1B shows the activity of DNA polymerase A. It elutes as a separate peak, identifiable by its ability to synthesize poly(dT) when stimulated by poly(A) • oligo(dT) or poly(dA) • poly(dT). Figure 1C shows that there is no enzyme in this tumor virus-free mouse cell line which can copy poly(C) • oligo(dG). This will become a critical negative finding as discussed below.

Polymerases in Virally Infected Cells

A culture of JLS-V9 cells was infected with Moloney murine leukemia virus (MLV), and then became known as JLS-V11 cells (8). Figure 2 shows some of the DNA polymerase activities present in these infected cells. Figure 2A demonstrates polymerases C and N, as defined by activity with poly(dA-dT). Figure 2B shows that a major new activity is demonstrable in these cells, which we call DNA polymerase V. This enzyme polymerizes dGTP onto an oligo(dG) primer, directed by a poly(C) template. No such activity is present in the uninfected JLS-V9 cells (Fig. 1C). Polymerase V (termed V because of its restriction to the virus-infected cells) could be completely separated from polymerase C by re-chromatography on DEAE-Sephadex. Viral polymerase isolated directly from Moloney leukemia virions behaves exactly as does polymerase V on phospho-cellulose and DEAE-Sephadex. In fact, much of polymerase V probably comes from recently-made virions on the cell surface.

Specificity of Poly(C) • Oligo(dG)

We have screened a variety of viruses, cells, and tissues for an enzyme activity able to synthesize poly(dG) in response to poly(C) • oligo(dG). Such activity is demonstrable in virions of all RNA tumor viruses we have tested (7). It is also present in all cells known to be infected by, and producing oncogenic RNA viruses. Cells from 12

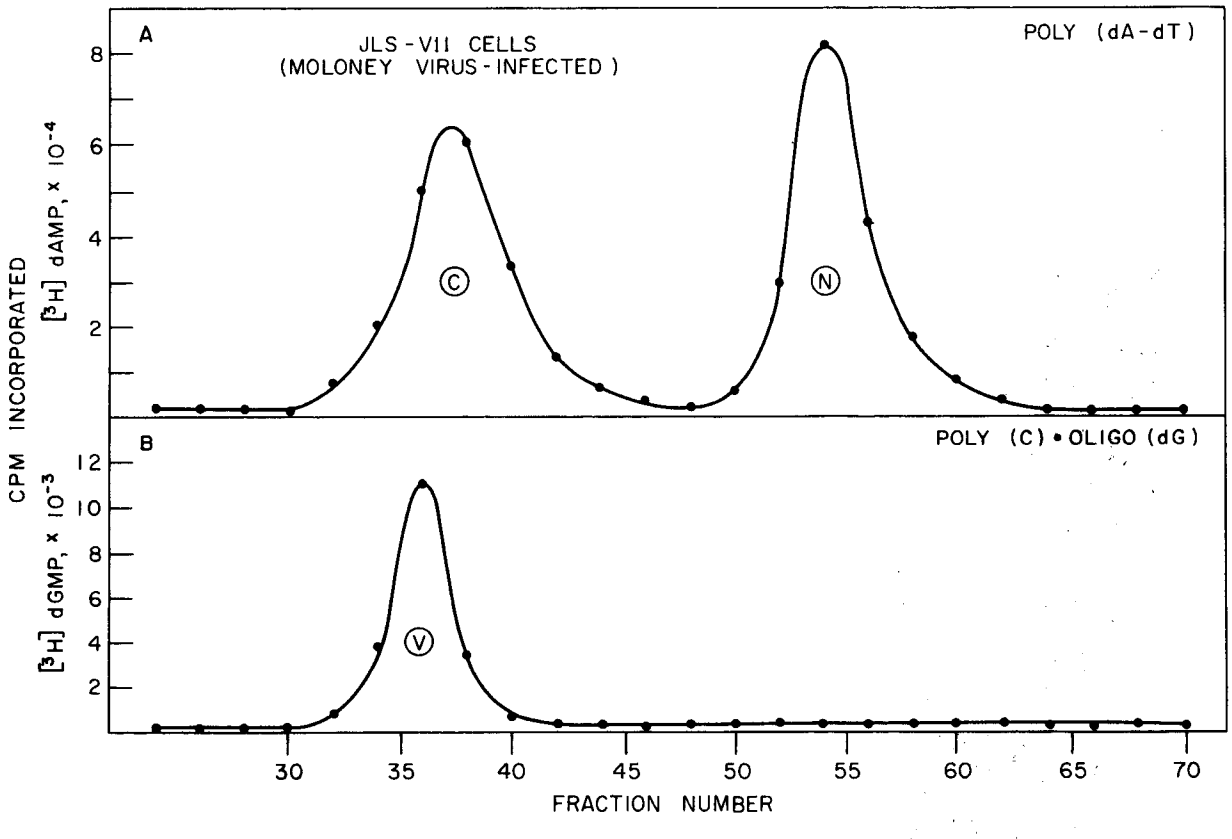

Fig. 2: DNA polymerases of murine fibroblasts productively infected with Moloney murine leukemia virus (JLS-V11 cells).

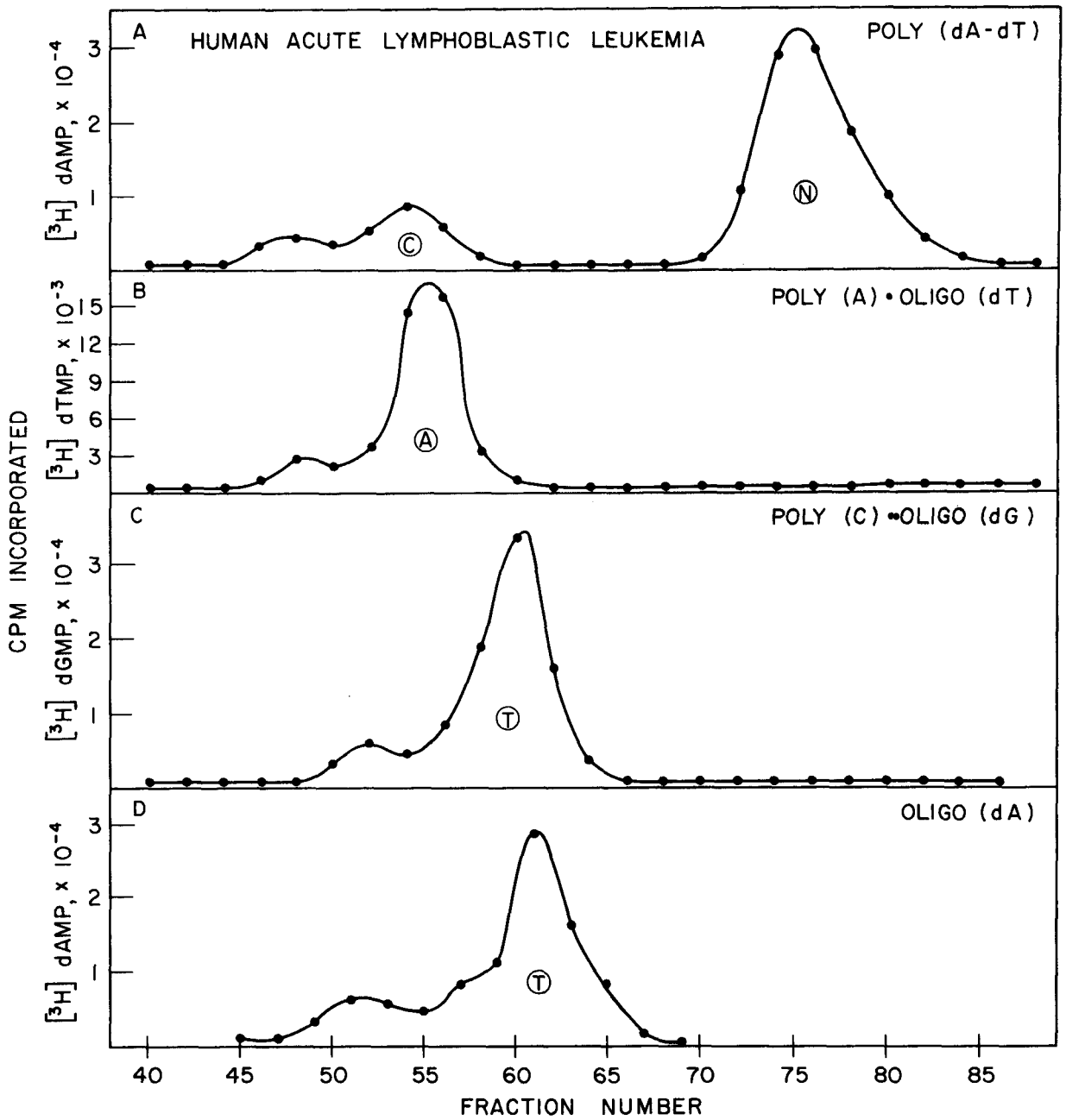

Fig. 3: DNA polymerases of circulating leukemic cells from a case of childhood acute lymphoblastic leukemia.

Reaction conditions are contained in reference 1. Taken from reference 1 by permission of the publishers.

cases of human leukemia (lymphoblastic, myeloblastic, lymphosarcoma, and chronic lymphatic), when assayed in this manner were all negative for polymerase V activity while they all contained polymerases C, N, and A.

Polymerases in Childhood Acute Lymphoblastic Leukemia

In the circulating leukemic cells from three out of five cases of childhood acute lymphoblastic leukemia we have noted a unique DNA polymerase in addition to the polymerases C, N and A which are evident in Figures 3A and 3B. This new enzyme

we have labeled DNA polymerase T (Figs. 3C and 3D). It scored on our initial screen for polymerase V in these cells, using poly(C) · oligo(dG). (Fig. 3C). However, as shown on Table 1 and in Figure 3D, it is completely template-independent. It can polymerize a deoxynucleoside triphosphate when supplied only an oligomer as initiator. In this respect it behaved similarly to calf-thymus terminal transferase, previously described by Bollum and co-workers (9, 10), an enzyme later shown to be unique to thymus tissue by Chang (11). Her study showed that in several animal species, only thymus had activity while the following tissues contained no such end-addition enzyme: bone marrow, spleen, lymph nodes, circulating lymphocytes, liver, lung, and bursa of Fabricius (from chickens).

Comparison with Human Thymus

We compared the lymphoblastic leukemia cell polymerases with the polymerases recovered from normal human infant thymus processed in the same manner. Figure 4 shows this data. The three normal enzymes are present (Figs. 4A and 4B), plus a fourth (Fig. 4C) activity which elutes from the phosphocellulose column at the same salt concentration and has the same primer requirement as does the terminal transferase from the leukemic cells.

Comparison with Other Human Cells

A variety of other human cells have been assayed using these same procedures. Only in ALL and normal thymus has an oligomer-stimulated end-addition enzyme (terminal transferase) been noted. Negative cells include three cases of lymphosarcoma cell leukemia, two cases of chronic lymphotic leukemia, two cases of acute myelo-blastic leukemia, normal spleen, normal circulating lymphocytes, infectious mononucleosis cells in culture, lymphosarcoma cells in culture, a line of lymphocytes derived from a patient with Burkitt's lymphoma, and HeLa cells.

DNA Polymerase in Chick Bursa

The occurrence of a DNA synthetic enzyme unique to the thymus, the organ which gives rise to T-lymphocytes, led us to ask whether the organ which processes

Table 1. Dependence of dGMP incorporation on poly(C) and oligo(dG)

Additions	Avian myeloblastosis virus DNA polymerase	Moloney leukemia virus DNA polymerase V	All polymerase T	Terminal transferase
	(pmol of dGMP incorporated)			
Poly(C) oligo(dG)	140	5.5	4.0	137
Poly(C) alone	< 0.1	< 0.4	0.4	–
Oligo(dG) alone	< 0.1	< 0.4	4.8	112

Adapted from reference 1. Data on avian myeloblastosis virus taken from reference 14.

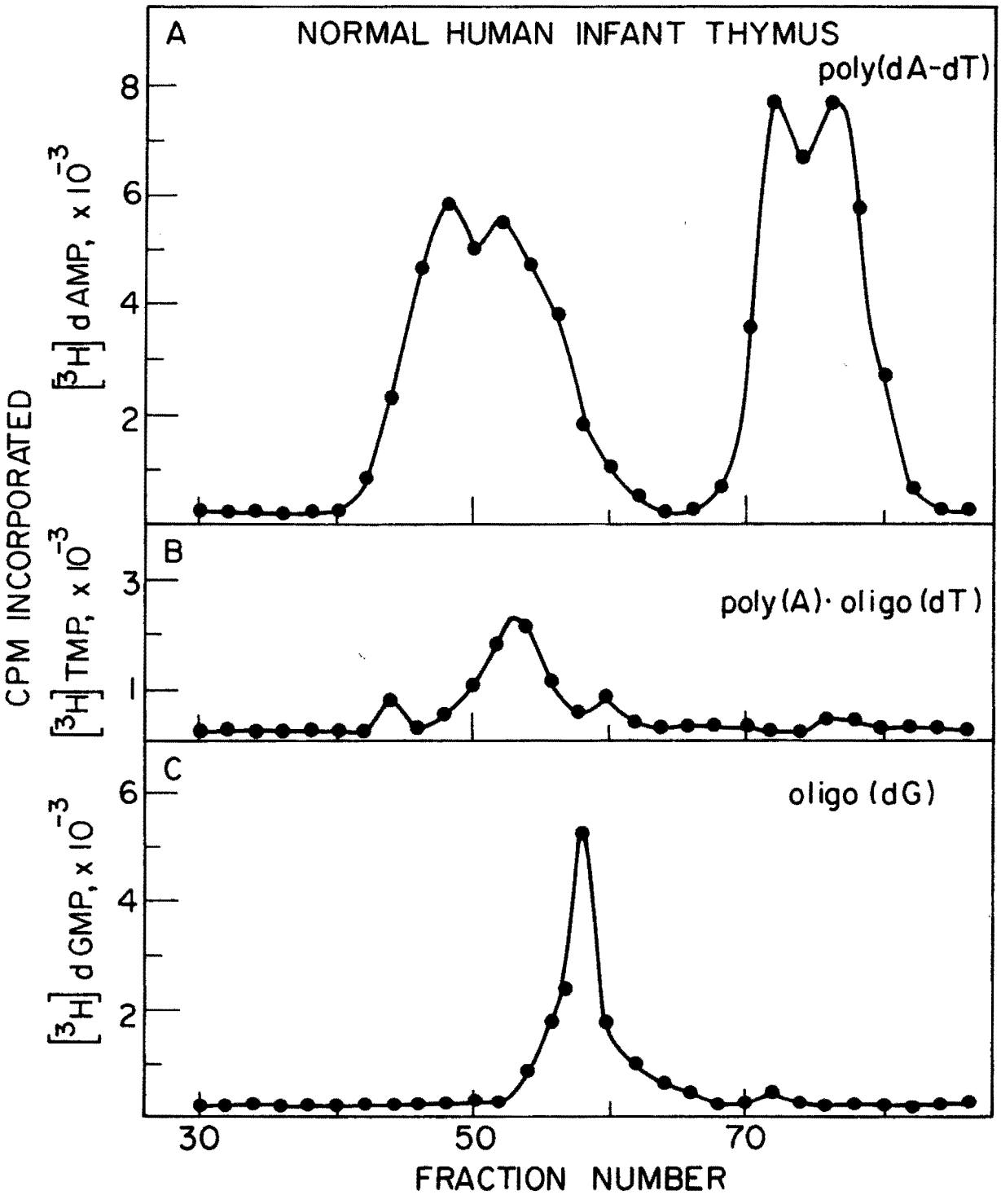

Fig. 4: DNA polymerases of normal human infant thymus.
Taken from reference 1 by permission of the publishers.

B-lymphocytes might similarly have a unique DNA polymerase. In chickens such an organ exists, and is called the bursa of Fabricius. Figure 5 shows the DNA synthetic activities present in 3–4 week old chick bursa tissue processed as described for other tissues here. A variety of activities are present. Figure 5A shows two major peaks with poly(dA-dT), which presumably are polymerase C and N from bursal cells. A third, new activity stimulated by poly(dA-dT) is also noted eluting at 0.6 M KCl. Figure 5B

shows that when activated DNA and a single deoxynucleoside triphosphate (here dTTP) are supplied to the various column fractions, a very significant peak of incorporation is noted eluting at about 0.6 M KCl. At lower salt concentrations a broad band of similar activity is seen. When a second deoxynucleoside triphosphate (here dCTP) is added, the enzyme eluting at 0.6 M KCl is dramatically inhibited; the broad low salt peak is inhibited to a lesser degree. Figure 4C shows a small peak of activity eluting at 0.25 M KCl when the bursal fractions are assayed for thymic-type terminal transferase. Under these conditions, i. e., oligo$(dA)_{14}$-stimulated incorporation of dGTP, the chick thymus shows an extremely active enzyme eluting from

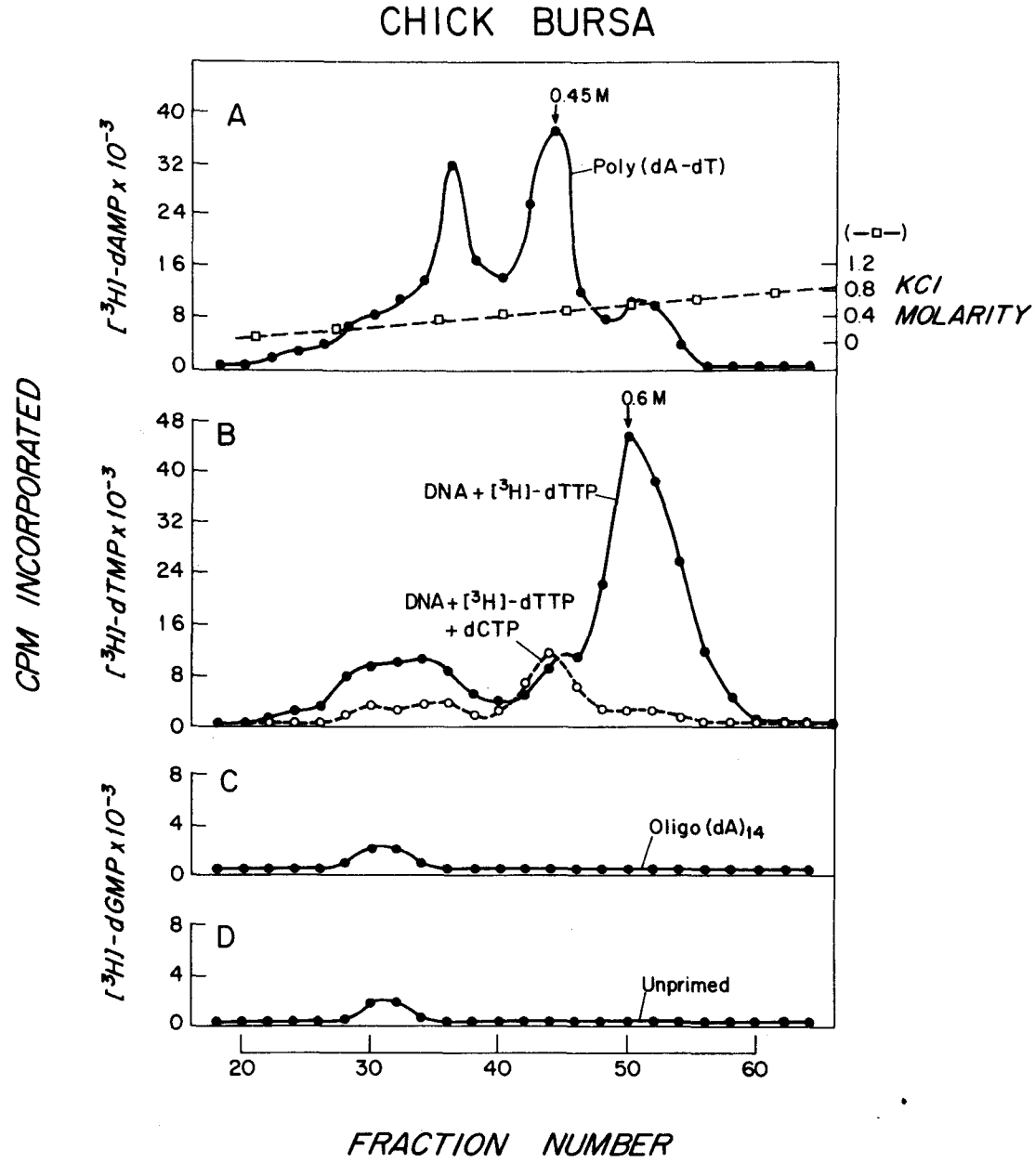

Fig. 5: DNA polymerases of chick bursa.

For panel B, 5 μg of DNase I treated calf thymus DNA, 200 picomoles of ^{3}H-dTTP (8000 cpm/pmole), 2000 picomoles of dCTP, and 0.6 mM $MnCl_2$, were used under reaction conditions noted in reference 1.

Table 2. DNA polymerases of chick tissues

Tissue	Terminal Transferase	Enzymes 0.6M „Bursal Enzyme“	Low Salt „Bursal Enzyme“	Replicative DNA Polymerases
Thymus	+++	–	±	+
Bursa	–	+++	++	+
Spleen (young)	–	++	+	+
Spleen (adult)	–	+	±	+
Liver	–	–	±	+
Muscle	–	–	±	+

Tissues were processed as described in text. Minus(–) signs indicate little or no ability to function under the assay conditions as defined in the text and reference 1. Plus(+) signs indicate increasing levels of activity. "Replicative DNA polymerases" refers to DNA polymerases C and N.

phosphocellulose at 0.4 M salt. Although this activity in the bursa has not yet been studied in any detail, the fact that we see essentially as much activity in the absence of oligo$(dA)_{14}$ (Fig. 4D), suggests that it is not related to terminal transferase. Further study of this activity is contemplated.

We have screened a number of tissues from 3–4 week old chicks for these activities, as shown in Table 2. Thymus contains the oligomer-stimulated terminal transferase. It lacks the activity seen in the bursa which elutes at 0.6 M KCl. All chick tissues contain the low salt broad peak, maximally active with nicked DNA, and a single deoxynucleoside triphosphate. Beside the bursa of Fabricius, only the spleen, an organ thought to be populated in part by lymphocytes from the bursa (12, 13), contains the enzyme that elutes at 0.6 M KCl. Our tentative conclusion is that this particular enzyme is unique to developing bursal (B-) lymphocytes.

Conclusions

We have developed a method which allows us to separate the three major soluble DNA polymerases present in eukaryotic cells by a single step purification from a crude cell extract. Using this methodology we can also detect viral reverse transcriptase as an additional fourth activity in cells which are producing virions.

In studying a variety of cells and tissues, we have noted that lymphocytes from different sources contain unique DNA polymerases. Certain cases of human acute lymphoblastic leukemia have circulating leukemic cells which have terminal transferase, an enzyme previously known to be present only in thymus. Cells (presumably lymphocytes) from the bursal system of chicks contain a unique, and hitherto undescribed enzyme, which is maximally active with nicked DNA and a single triphosphate.

The role of these unique enzymes in B-lymphocyte and T-lymphocyte precursor cells remains to be defined.

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DNA-DEPENDENT DNA AND RNA POLYMERASES AND tRNA-METHYL-TRANSFERASES IN HUMAN LEUKEMIA AND DIFFERENTIATING FRIEND VIRUS LEUKEMIA CELLS*

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DNA-dependent DNA and RNA polymerases play a central role in the process of gene replication, gene expression, gene amplification, and also gene regulation. At present, RNA-dependent DNA polymerase (reverse transcriptase) has attracted particular interest in connection with the problem of a viral etiology of human leukemia. Considering the importance of DNA-dependent RNA polymerases for transcription and replication, these enzymes also must be important for the process of malignant transformation. Evidence for direct participation of RNA synthesis in DNA replication has been reported for phages, bacteria, and, recently, for eucaryotic cells (1). RNA polymerases have been highly purified from bacteria. It has been shown, that the complete or so-called holoenzyme could be separated into several subunits with defined functional properties (2). Holoenzyme consists of the sigma factor and the core enzyme with its subunits alpha 1, alpha 2, beta, beta′, and a number of smaller factors called psi, kappa, rho. Sigma catalyses the specific initiation of RNA synthesis on the promoter site of DNA. Core enzyme is able to synthesize RNA but lacks the ability for specific initiation. Beta subunits are responsible for the binding to DNA, kappa for the arrest, and rho for the termination of transcription (3). ZILLIG and coworkers demonstrated that, after infection with bacteriophage T_4, E. coli RNA polymerase is altered in two consecutive steps. The purified enzyme from phage-infected cells does not contain sigma, and exhibits structural changes in all subunits of core enzyme (4, 5, 6).

Polymerases from eucaryotic cells are extremely unstable enzymes complicating purification and analysis. Thus very little is known about properties and function of mammalian RNA polymerases up to now. DNA-dependent RNA polymerases are differentiated according to their sensitiveness to the mushroom toxin alpha-amanitin and their preference of native or denatured DNA templates (Table 1). The first group of polymerases, termed A, is not inhibited by alpha-amanitin and prefers native DNA templates, while the second group of RNA polymerases, called B, is completely inhibited by low doses of alpha-amanitin, and prefers denatured DNA templates. In

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Enzyme	A			B			
Characteristic	A I	A II	A III	B I	B II	C	"mit"
Subcellular localization	nucleolus	nucleolus	nucleoplasm	nucleoplasm	nucleoplasm	cytoplasm	mitochondria
Size	16 S						
Molecular weight	500 000			500 000	500 000	500 000	
Function	rRNA synth.			mRNA synth.			mitRNA synth.
Template preference ratio nat./denat. DNA	20	16	1	0.5		0.74	0.57
Divalent cations ration Mn/Mg	1.1	0.7	3	5		3.5	0.55
Optimum ionic strength							
$(NH_4)_2SO_4$	40 mM	55 mM	40–200 mM	100 mM			10 mM
KCl	200 mM	200 mM					
Optimum pH	8.0	8.0		8.7			
Elution from DEAE cellulose							
$(NH_4)_2SO_4$	0.17 M		0.4 M	0.2 M	0.35 M		0.25 M
Ionic strength	0.2			0.3		0.22	
Elution from phosphocellulose							
NH_4CL	0.45 M			0.4 M		0.35 M	
KCl	0.50 M	0.65 M					
+ 0.1 µg/ml α-amanitin	100 %			0 %		75 %	
+ stimulating factor "B"	115 %			270 %		109 %	

Table 1: Reported properties of DNA dependent RNA polymerases from mammalian cells.

addition, RNA polymerase is found in a deoxyribonucleoprotein complex, demanding high ionic strength for optimum transcription. Besides these polymerase activities, located in the nucleus, a cytoplasmic enzyme with intermediate sensitivity to alpha-amanitin (7) and a mitochondrial RNA polymerase (8) have been described. RNA polymerase A synthesizes rRNA und tRNA precursors, RNA polymerase B catalyzes the transcription of mRNA precursors (heterogenous nuclear RNA). Polymerase A has been fractionated into two large subunits. Three large subunits have been described for the B enzymes. In addition, each enzyme molecule possesses several subunits of lower molecular weight (9). In contrast to the E. coli enzyme, no defined functional properties can be associated with the subunits of these eucaryotic enzymes, so far.

In the first stage of our investigations, RNA polymerases A and B were characterized in nuclear fractions from isolated lymphocytes, granulocytes, or total leukocytes. Cells were isolated from 10–40 ml of venous blood by accelerated sedimentation

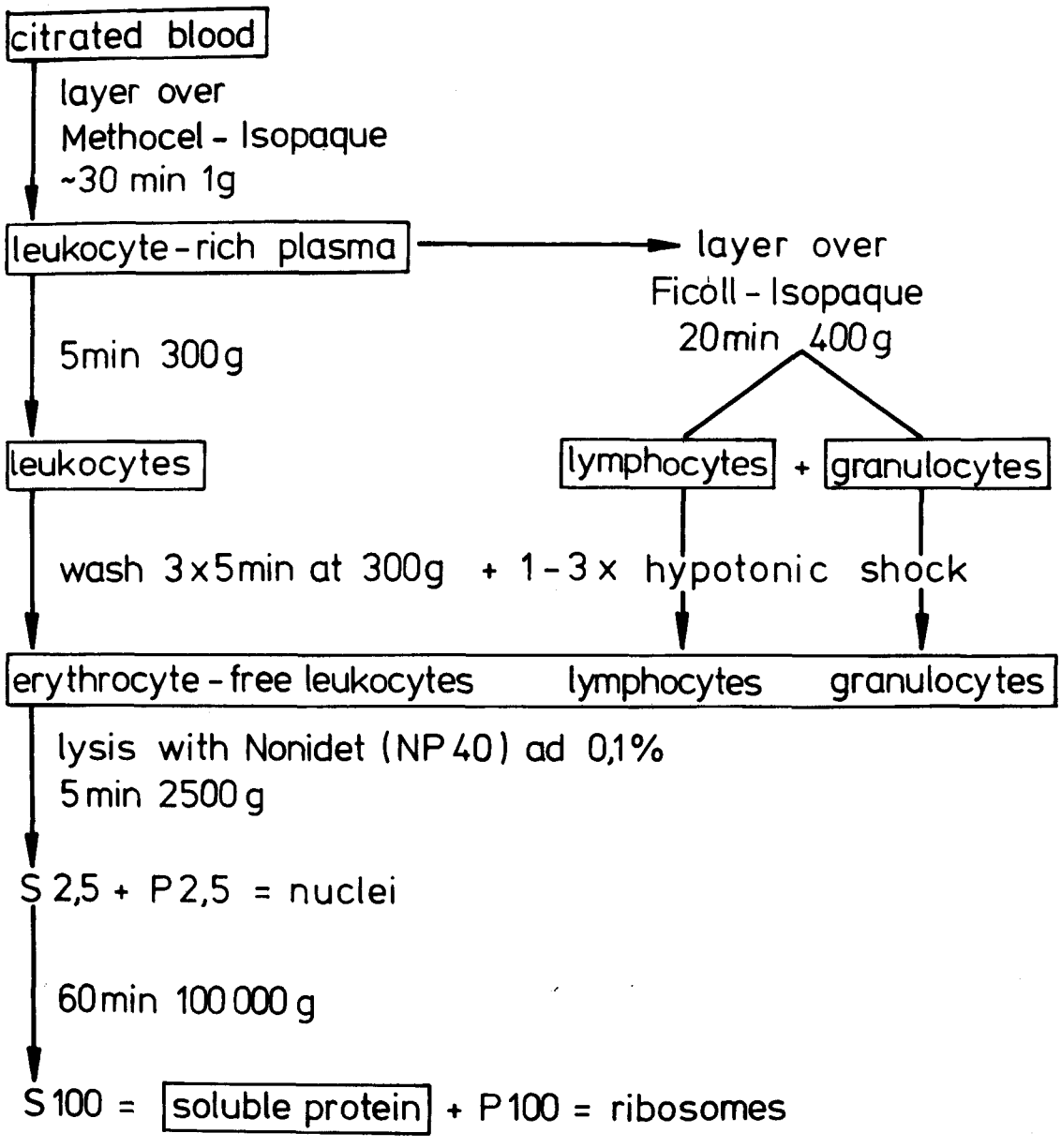

Fig. 1: Isolation of leukocytes, separation into lymphocytes and granulocytes, and preparation of nuclei and 100 000 g supernatant.

The method is a modification of the procedure of Böyum*). 10 ml citrated blood (3.8 % Na-citrate 1 : 5) are layered on top of 5 ml of a Methocel-Isopaque mixture (16 parts Methocel, Fluka, 2 % in aqueous solution mixed with 10 parts Isopaque = Ronpacon 75 %, Cilag, sterilized 30 min at 120° C). The leukocyte-rich plasma was diluted to 15 ml by isotonic buffer (IB: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM K-EDTA, 90 mM NaCl) and layered over 7 ml of a Ficoll-Isopaque mixture (24 parts Ficoll, Pharmacia Uppsala, 9 % in aqueous solution, 10 parts Isopaque = Ronpacon 75 %, sterilized 30 min at 120 °C). After 20 min centrifugation at 400 xg the lymphocytes banded at the interface, the granulocytes together with a few contaminating erythrocytes precipitated to the bottom of the tube. Cells were washed with IB (3 x 5 min 300 g) and erythrocytes lysed by 1—3 hypotonic shocks (by addition of 2 ml bidistilled water and after 20 sec 2 ml 1.8 % NaCl). Cells were resuspended in IB to a concentration of 50 000/µl and lysed by addition of 0.1 % Nonidet (NP 40, Shell). Nuclei were immediately spun off (5 min 2500 g). The supernatant was centrifuged 60 min at 100 000 g.

*)Böyum, A.: Separation of leukocytes from blood and bone marrow; Scand. J. Clin. Lab. Invest. *21*, Supp. 97 (1968).

(Fig. 1). The resulting leukocyte rich plasma was separated into lymphocytes and granulocytes on a Ficoll-Ronpacon gradient. Leukocytes were disrupted by sonication or by addition of 0.1 % NP-40, and nuclei spun off at 2500 x g (5 min). The resulting pellet, consisting of nuclear debris, was resuspended, homogenized, and used for investigation of RNA polymerase activity (fraction II P. For details of methods cf. 10, 11). Polymerase activities were differentiated by appropiate incubation conditions: RNA polymerase A was tested in the presence of 10 mM $MgCl_2$, 50 mM ammonium sulphate (AmS), native DNA templates and 0.02 µg alpha-amanitin, RNA polymerase B in the presence of 2.5 mM $MnCl_2$, 100 mM AmS, denatured DNA templates, chromatin-bound RNA polymerase in the presence of 2.5 mM $MnCl_2$, 400 mM AmS, being neglegibly stimulated by addition of DNA templates.

The nuclear fraction, obtained as described, contains a high amount of endogenous DNA, nevertheless RNA polymerases A and B are highly dependent on exogenous template DNA (11). This could be caused by a very high degree of repression of endogenous chromatin DNA. No significant qualitative differences have been found between RNA polymerases A, B, and the chromatin bound enzyme from normal and leukemic leukocytes, regarding the efficiency of different DNA templates (Tab. 2), the dependency on divalent cations and ionic strength (Tab. 3), and the inhibition by

RNPASE	A	A	B	B
DIAGNOSIS	NL	CLL	NL	CLL
CONDITIONS	%	%	%	%
complete	100	100	100	100
- DNA	15	56	13	6
- denat., + nat. DNA	100	100	23	15
- nat., + denat. DNA	56	45	100	100
TQ	0.56	0.45	4.2	5.8

TQ = quotient of transcription of denatured versus native DNA template

Table 2: Influence of DNA template on DNA-dependent RNA polymerases in isolated nuclei from human peripheral blood leukocytes.

NL = normal leukocytes; CLL = chronic lymphatic leukemia; RNA polymerase activity was measured by the incorporation of ^{3}H-UMP into acid precipitable polynucleotides in the presence of the following components: ^{3}H-UTP (spec. act. 10 Ci/mmole) 1 µM; ATP, CTP, and GTP 1 mM; 20 µg DNA (native for RNA polymerase A, denatured for RNA polymerase B); 200 µg protein of fraction II P; Tris-HCl pH 8.5 200 mM; 2-mercaptoethanol 8 mM; glycerol 9 % (v/v); and 0.3 mM K-EDTA in a final volume of 100 µl (divalent cations and ammonium sulphate concentrations as described in the text).

RNA Polymerase	A	A	B	B
Diagnosis	NL	CLL	NL	CLL
Conditions:				
complete	100 %	100 %	100 %	100 %
- $MgCl_2$, + $MnCl_2$	206 %	198 %	100 %	100 %
- $MnCl_2$, + $MgCl_2$	100 %	100 %	26 %	25 %
- $(NH_4)_2SO_4$	75 %	73 %	32 %	27 %
ratio				
$MnCl_2$/ $MgCl_2$	2.1	2.0	3.8	3.9
+ $(NH_4)_2SO_4$/ - $(NH_4)_2SO_4$	1.3	1.4	3.1	3.8
+ 0.2 µg/ml α-amanitin	100 %	100 %	16 %	14 %

The complete system contains 10 mM $MgCl_2$ - 60 mM $(NH_4)_2SO_4$ - 0.02 µg α-amanitin for RNPase A and 2.5 mM $MnCl_2$ - 100 mM $(NH_4)_2SO_4$ for RNPase B in a final volume of 100 µl (30 min at 37°).

Table 3: Properties of DNA-dependent RNA polymerases in isolated nuclei from human peripheral blood leukocytes. For incubation conditions cf. legend to Tabl. 2.

alpha-amanitin and cytostatic substances like distamycin A, actinomycin D, and daunomycin. The specific activities of the DNA-directed nucleic acid polymerases are, however, significantly elevated in CLL, CML, and Hodgkin's disease stage IV, as compared to normal controls (Tab. 4). During remission, near normal values have been observed in chronic myelocytic leukemia. No elevations of the specific activities were found in reactive granulocytosis or lymphocytosis.

This phenomenon cannot be explained by a different degree of depression of endogenous DNA template. It must be assumed, that there are more polymerase molecules present in leukemic cells, or that the RNA polymerases of leukemic cells are more active than normal enzymes.

There appears to be a correlation between nucleic acid polymerase activities and the response to a cytostatic regimen. In a patient with a blastic crisis in CML, 3 mg of vincristin were applied initially. Polymerase activities fell to nearly normal values, even before peripheral leukocyte counts dropped in response to therapy. In another

patient with AML who did not respond to the COAP regimen, nucleic acid polymerase activities kept on rising, the patient became resistent to cytostatics, and died a few weeks later.

In order to verify the results obtained in the nuclear system and to further investigate normal and pathological RNA polymerase molecules, we have started to purify these enzymes. Polymerases are solubilized from isolated leukocyte nuclei by sonication and a buffer of high ionic strength (0.20 M AmS). RNA polymerase A is eluted from DEAE cellulose at 0.13 M AmS, RNA polymerase B at 0.35 M AmS. RNPase B can be further fractionated into polymerases B I and B II by re-chromatography on DEAE cellulose. B I is eluted at 0.2 M, B II at 0.35 M AmS. Using this method, RNA polymerase A has been purified 16 fold, with a specific activity of 40.3 U/mg protein, RNPase B 200 fold with a specific activity of 200 U/mg protein (1 unit = 1 pmole of labelled nucleotide incorporated/30 min 37°, Tab. 5). Parameters of the purified enzymes have been found identical to those determined in the nuclear system (Tab. 6).

A second series of experiments has been carried out in order to investigate the possibility of deranged control mechanisms in leukemic cells on the translational level. tRNA, the smallest known RNA species, exhibits a most complex structure. Numerous modifications of its bases are known which determine structure and function.

Diagnosis	RNPASE A	RNPASE B	cbRNPASE
normal leukocytes	100 % n = 9	100 % n = 9	100 % n = 8
chronic lymphatic leukemia	265 % n = 9	174 % n = 6	505 % n = 5
chronic myelocytic leukemia (untreated)	450 % n = 4	774 % n = 4	663 % n = 4
chronic myelocytic leukemia (remission)	245 % n = 4	333 % n = 4	350 % n = 4
HODGKIN `s disease (stage IV a)	215 % n = 9	288 % n = 9	220 % n = 8
FRIEND murine virus leukemia	611 % n = 4	62 % n = 4	220 % n = 4

Table 4: Specific activities of DNA-dependent RNA polymerases in nuclei from normal leukocytes and from leukocytes in malignant hematological disease. Specific activity = pmoles ^{3}H-UMP incorporated/mg protein. 45 min at 37 °C.

Table 5: Purification of DNA-dependent RNA polymerase from Friend virus murine leukemia cells.

Fraction	Total Protein	RNPase A				RNPase B			
		Total Units	Specific Activity	Yield	Purification	Total Units	Specific Activity	Yield	Purification
	mg		units / mg protein	%			units / mg protein	%	
I Homogenate	120	310	2.59	100	1	104	0.87	100	1
II S 30	48.6	245	5.04	79.0	1.95	121	2.50	116	2.87
III AmS precipitate	21.2	181	8.56	58.4	3.31	71	3.37	68.2	3.87
IV DEAE cellulose									
0.13 M eluate (A)	8.2	263	40.3	84.8	16.1				
0.35 M eluate (B)	3.1					406	204	390	236
V DEAE cellulose									
0.2 M eluate (B I)	0.24					16.4	68.4	15.8	78.6
0.35 M eluate (B II)	0.60					34.3	58.3	32.9	67.0

AmS = ammonium sulphate
1 unit = 1 pmole of labelled UMP incorporated in 30 min at 37°
Specific activity = most active fractions of column eluate.

Table 6: Properties of purified DNA-dependent RNA polymerases from human leukocytes and from Friend virus murine leukemia cells. For incubation conditions cf. legend to Tabl. 2.

RNA polymerase	A	A	B	B	B I	B II
Diagnosis	FR	FR	CML	FR	FR	FR
Conditions:	%	%	%	%	%	%
complete	100	100	100	100	100	100
- $MgCl_2$, + $MnCl_2$	121	364	100	100	100	100
- $MnCl_2$, + $MgCl_2$	100	100		18.6	75.6	54.9
ratio Mn^{2+} / Mg^{2+}	1.21	3.64		5.38	1.32	1.82
+ 0.2 ug / ml α-amanitin	100	100	2.0	3.9	2.0	2.0

The complete system contained 10 mM $MgCl_2$ - 60 mM $(NH_4)_2SO_4$ - 100 µg/ml native calf thymus DNA for RNA polymerase A and 2.5 mM $MnCl_2$ - 100 mM $(NH_4)_2SO_4$ - 100 µg/ml denatured calf thymus DNA for RNA polymerase B (30 min 37°).

Name	Diagnosis	Leuko / mm^3	Therapy	pmoles CH_3- bound / 20µg Protein		
				Lympho	Granulo	Leuko
N.N.	normal (n = 4)	6600 - 9200	-	-	-	<0,5 - 1,4
N.N.	normal (n = 13)	5000 - 7100	-	<0,5 - 3,2	< 0,5	-
N.N.	virus infection (n = 4)	7550 - 16250	antibiotics	1,4 - 2,0	< 0,5	-
F.M., 63	CML	93500	-	< 0,5	< 0,5	-
G.S., 59	"	85000	MYL	1,4	< 0,5	-
G.D., 40	"	24800	MYL	-	-	< 0,5
K.S., 46	" blast crisis	68200	MYL,URB,VCR	-	-	21,7
I.N., 49	" "	41000	MYL	-	-	6,6
I.B., 18	" "	17900	ARA-C	-	-	5,5
"	" "	2150	" + SH-G	< 0,5	< 0,5	-
P.P., 30	AML	13750	-	< 0,5	< 0,5	-
E.H., 42	"	3800	-	1,6	< 0,5	-
A.Z., 14	"	17500	-	-	-	< 0,5
"	"	5400	SH-G,URB	< 0,5	< 0,5	-
A.B., 40	"	5300	VAMP	-	-	< 0,5
A.B., 11	"	102500	SH-G,MTX,URB	-	-	1,3
"	"	53800	" " "	-	-	3,5
"	"	214000	+ ADR	-	-	< 0,5
G.V., 8	"	45500	-	-	-	6,2
"	"	57500	VCR,SH-G,URB	-	-	4,8
"	"	152500	" " "	-	-	5,2
A.K., 68	CLL	10900	URB, ENX	-	-	0,6
W.S. 65	"	46100	URB	2,8	< 0,5	-
H.M. 69	"	95500	URB	< 0,5	< 0,5	-
A.R., 7	ALL	85000	ENX	-	-	6,0
A.A., 9	"	BM	6MP, MTX	-	-	12,3
F.S., 40	"	4600	ENX	6,9	< 0,5	-
"	"	2700	"	4,2	< 0,5	-
M.G., 32	AL remission	13200	URB	1,7	< 0,5	-
N.G., 21	" "	14400	URB	< 0,5	< 0,5	-
H.P., 14	" (aleukemic)	6050	URB	-	-	1,3
J.K., 5	"	286000	URB,ADR,ARA-C	-	-	8,9

differentiation

Table 7: Specific activities of tRNA methylases in normal and pathological leukocytes.

The reaction mixture contained, in a final volume of 100 µl : 5 µmoles of Tris-HCl, pH 7.5, 1 µmole of $MgCl_2$, 0.5 µmole of dithiothreitol, 1nmoles of (^{14}C)-S-adenosyl-L-methionine (55 Ci/mole, Amersham), 2nmoles of E. coli tRNA, 5 to 50 µg of protein in the enzyme fraction. After 90 min incubation at 37 °C the tRNA was precipitated with 0,5 % cetyltrimethylammonium bromide and a second time with 2 volumes of ethanol. The (^{14}C)-methyltransfer was measured by counting aliquots of the redissolved tRNA in a Packard Tricarb liquid scintillation counter at an efficiency of 74 %. Enzyme concentration curves were determined in each case and the values of the specific activities (expressed as pmoles of CH_3-groups bound by 20 µg of protein in 90 min) were taken from the initial linear range for these curves. For the cytological and cytochemical differentiation of leukemias see Beckmann, H. and R. Neth, this symposion. A = acute, C = chronic, L = lymphatic leukemia, M = myelocytic, AL = acute undifferentiated leukemia, BM = bone marrow;

ADR = Adriamycin, ARA-C = Cytosin-Arabinosid, ENX = Endoxan, 6MP = 6-Mercapto-Purin, MTX = Methotrexat, SH-G = Thioguanin, URB = Urbason, VCR = Vincristin.

Methylation of bases is the most frequent of these modifications. Transfer ribonucleic acid methyltransferases (tRNA methylases) are strictly species specific enzymes. They transfer methyl groups from S-adenosyl-L-methionin (SAM) into definite positions of specific bases of unmethylated tRNA precursor molecules. Results regarding the biological function of tRNA methylation, indicate changes in the chargeability with amino acids, binding to ribosomes, and codon recognition of the tRNA species. Elevated activities of tRNA methylases have been found in embryonic tissues, in proliferating tissue cultures, and in malignant growth. Thus tRNA methylases seem to possess important regulatory properties during growth, differentiation and malignant transformation of eucaryotic cells (12, 13). GALLO and coworkers reported elevated tRNA methylase activities not only in leukemic but also in proliferating normal human blood cells (14). High tRNA methylase levels, thus

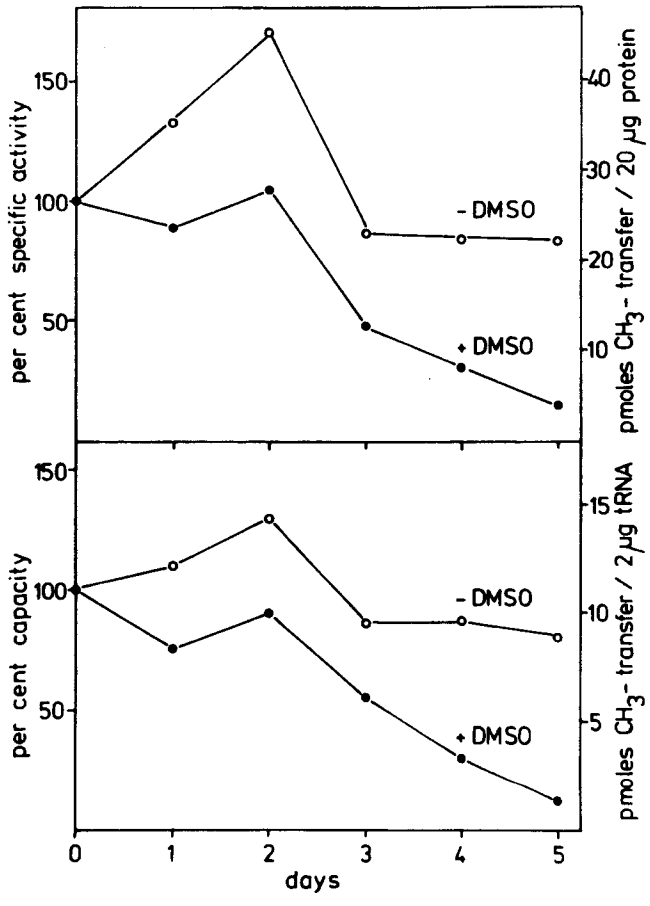

Fig. 2: Specific activity and capacity of tRNA methylases in untreated and DMSO stimulated cultures of Friend virus mouse leukemia cells.

Cells (FSD-1/clone F 4) were grown in Eagle's medium with Earle's balanced salt solution, supplemented with twice the usual amount of amino acids and vitamins, five times the usual amount of glutamine and 15 % foetal calf serum. Cultures were started with 200 cells/µl. On day 0 DMSO was added to a final concentration of 1.5 % and the culture flasks were closed. Controls without DMSO were treated in the same way. Cells were harvested by centrifugation, disrupted by sonication and tRNA methylases were measured as described in the legend to table 7 except that additional capacity experiments were performed with limiting amounts (0.04 nmoles) of tRNA. Values are expressed in per cent activity of that at day 0 (= 100 %).

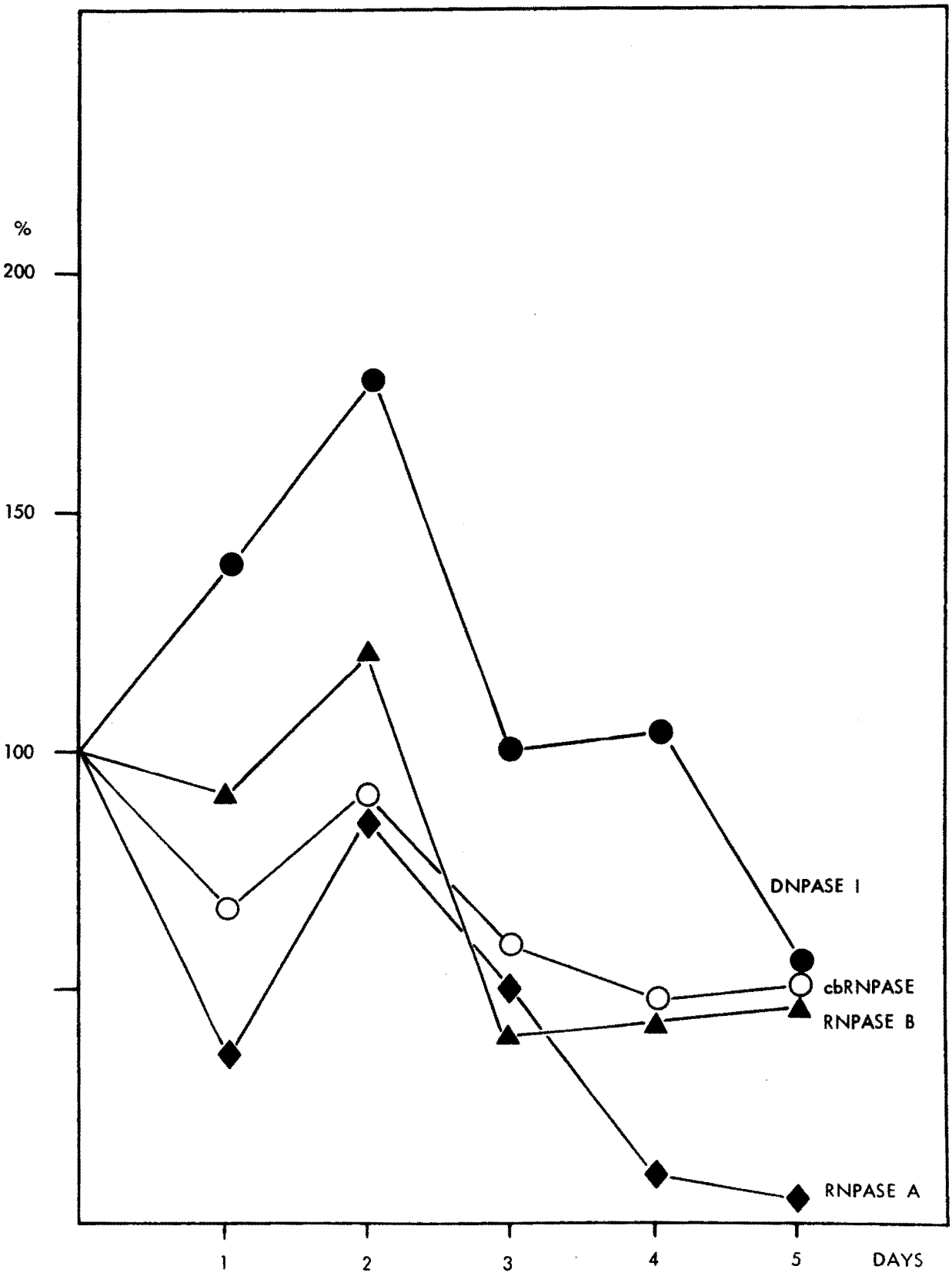

Fig. 3: DMSO-stimulated Friend virus murine leukemia cells: polymerase activities in percent of control cultures. For culture conditions cf. legend to Fig. 2.

appear to be an indicator of proliferation in general rather than of malignancy per se. Tab. 7 shows the specific activities of tRNA methylases in normal and pathological white blood cells (for details of methods cf. 10). Normal, unfractionated leukocytes exhibit very low methylase activities, sometimes even below the sensitivity of the assay. Normal lymphocytes, however, always show measurable activities. In virus induced lymphocytosis, there were no increases in lymphocyte tRNA methylase activities as compared to normal controls. We never observed demonstrable methylase activities in isolated granulocytes in all patients investigated so far. In CML, tRNA methylases have been found elevated in blastic crisis, in AML in the most undifferentiated forms only. tRNA methylase activities are elevated in some cases of CLL, and always in ALL. Of the four cases of acute undifferentiated leukemia (AL), investigated as yet, two cases in remission and one newly detected aleukemic case exhibited low activities. A fourth case, examined in the final stage of the disease, was strongly positive. No correlation has been observed between the number of white blood cells in the peripheral blood and tRNA methylase activities. Regarding high tRNA methylase activities as a sign of rapid proliferation, i. e. immaturity, a decrease of methylase activities should be expected, when immature leukemic cells further differentiate, spontaneously or after exogenous stimulation. A suitable in vitro model for investigation of this hypothesis are the DMSO-stimulated Friend virus leukemia cells. These cells start to synthesize hemoglobin three days after addition of 1.5 % DMSO (15). Hemoglobin production reaches its maximum after five days. Fig. 2 shows that there is a decrease of the specific activities as well as of the capacities of tRNA methylases in DMSO-stimulated cells after day three, when the exponential growth phase is terminated. At the same time, control cultures exhibit persisting levels of enzyme activities. Simultaneous determination of DNA-dependent DNA and RNA polymerase activities in these cells (Fig. 3) demonstrates an immediate increase of DNA polymerase I (for details of methods cf. 16) after addition of DMSO, reaching a maximum on the second day. RNA polymerase B reaches its maximum activity also on the second day with about 120 % of the activity of the control cultures. The enzyme responsible for rRNA and tRNA synthesis, RNA polymerase A, never exeeds the level of the control cultures. From day three to five, while increasing numbers of hemoglobin positive cells are appearing, DNA polymerase I, RNA polymerase B, and the chromatin bound RNA polymerase activities approximate 50 % of control values, while RNA polymerase A drops to nearly zero, as well as the tRNA methylase activities.

In conclusion, elevated tRNA methylase activities, determined in native leukocytes, do not indicate the malignancy of hematological disease in general, but appear to be found when differentiation of leukemic cell lines is blocked at an early, undifferentiated stage.

The results about DNA dependent RNA polymerases in human leukocytes suggest a correlation of enzyme activities and the clinical course of the disease and the response to therapy. They have been found elevated in leukocytes in all malignant hematological diseases investigated so far, decreasing during successful cytostatic therapy and in remission.

We hope, that further analysis of biochemical parameters in the course of human leukemias will contribute new parameters for diagnosis, therapy, and prognosis in addition to the classical morphological and cytochemical criteria.

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OPENING REMARKS TO THE TRANSLATION SECTION

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Looking over the lecture program of this session shows two things. Many of the problems of in vitro protein synthesis have been addressed but in not one of these cases has tumor virus RNA been used. This fact reveals that although there is a general awarness of the significance of in vitro protein synthesis for studying gene expression of tumor viruses, the experimental approach to this field is still being sought.

Tumor viruses do not stop the protein synthesis of the infected cell. Thus, there are only two ways to study their genetic information and its expression. First, one can look at the effects of using a conditionally lethal (temperature sensitive) mutant of the virus. Second, one can attempt to find proteins encoded for on the virion RNA by in vitro translation.

The first question is, can the virion RNA act as a messenger or does a messenger RNA, complementary to the nucleotide sequence of the virion RNA, have to be synthesized in the cell. We have been able to show (1) that at least some of the structural proteins of AMV are encoded on the virion RNA. Similar results for various other tumor viruses have been reported (2, 3). On the grounds of theoretical considerations (1) the conclusion seems justified that in tumor viruses the whole genetic information is present in the form of messenger RNA.

Surprisingly, the cited results have been found in a heterologous system, namely in cell-free extracts of E. coli. To date it has not been possible to develope a well functioning in vitro system out of the actual host cells of the tumor viruses. It is conceivable that in the heterologous system it will become possible to answer the next important question: are there encoded on the RNA non-structural proteins which are perhaps involved in the transformational process? Or in other words, does the synthesis of virus-specific proteins lead to the expression of the so-called oncogenes?

A further set of questions which need to be mentioned in closing can only be answered in a homologous but not a heterologous system. It would be important to know if special (perhaps specifically inhibitable) initiation or elongation factors are involved in the translation of viral RNA, or if specific tRNA species are required, etc.

Some of the following contributions, although not concerned with tumor viruses, show how such studies can, in priciple, be performed. Others show how an in vitro system can be made using material isolated from various sources and what it is able to do. If, through these contributions and their discussion, we can come only a step closer toward creating a homologous system, then we can be quite satisfied.

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THE TRANSLATION OF VIRAL RNAs IN FROG OOCYTES

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It is desirable to be able to translate viral RNA in frog oocytes for two reasons. First, viruses provide RNA in an exceptionally pure form, and as such constitute an unusually favourable source of mRNA for studying the control of protein synthesis in development. Second, viral functions can be studied independently of the limitations imposed by the normal infection cycle in their host cells. With the exception of viral coat proteins, very little is known about the proteins coded for by viral genes, because it is hard to be sure which of the new proteins synthesized in virus-infected cells are the products of virus-coded genes as opposed to virus-activated host cell genes. This difficulty can be overcome if the virus RNA can be translated in a cell-free protein-synthesizing system, but it is not yet clear that this can be achieved satisfactorily with any of the oncornaviruses.

The first viral function to be investigated by microinjection into frog eggs was the replication of polyoma DNA. Laskey and Gurdon (1) made use of radioactive and density labels to provide evidence for the replication of purified polyoma DNA injected into unfertilised eggs.

The successful translation of viral messenger RNA in injected frog cells was first achieved by Laskey, Gurdon and Crawford (2). For these experiments, purified virion RNA of a picornavirus, Encephalomyocarditis, was injected into oocytes of *Xenopus laevis* according to the methods described by Gurdon, Lane, Woodland and Marbaix (3) and shown by Lane, Marbaix and Gurdon (4) to translate mammalian haemoglobin mRNA correctly. The method of analysis which has proved exceptionally useful, both in economy of time and in the precision of results obtainable, is to label injected oocytes in ^{35}S-methionine, analyse labelled proteins by SDS-poly acrylamide gel electrophoresis, and then quantitate the results by autoradiography. If oocytes are incubated in the saline solution, which we have found very satisfactory (5) in the presence of ^{35}S-methionine at 250 μCi/ml, each oocyte will incorporate up to 10^6 dpm of radioactivity into proteins within a few hours. Oocytes have a small pool of methionine, by comparison with most other amino acids, and perhaps for this reason most of the label in the medium is incorporated into proteins within 18 hours, assuming that oocytes are incubated in 5 μl of medium per oocyte at 250 μCi/ml.

The results of analysing oocytes injected with EMC virion RNA have been reported by Laskey, Gurdon and Crawford (2). As judged by size, all of the virus-coded proteins which could be detected in EMC-infected ascites cells could be found in oocytes after

injection of EMC viral RNA. None were found in control saline-injected oocytes. Three of the newly synthesized proteins were removed from gels, digested with trypsin, and the methionine-labelled peptides analyzed by thin layer chromatography and electrophoresis. In this way they were shown to be indistinguishable from known virus-coded proteins. We conclude that the oocyte probably translates the entire EMC genome.

These experiments are of interest from two points of view. First, they demonstrate that the RNA of a virus which normally infects the respiratory system of mice can be translated successfully, and probably completely, in frog oocytes. This at least raises the possibility that other mammalian virus messages may be translated in frog oocytes. Second, the virion RNA of many picornaviruses is a polycistronic message. It is initially translated into a large polypeptide, which is subsequently cleaved in a series of steps down to about 10 stable proteins. Rather surprisingly this cleavage process appears to proceed completely normally in frog oocytes. We do not know whether this is because oocytes and perhaps all vertebrate cells contain the necessary cleavage enzymes, or because part of the initially translated virus polypeptide folds up into an enzyme which cleaves itself. The first possibility seems more likely because oocytes appear to carry out a number of secondary modifications to proteins which they never normally contain (review by Lane and Knowland (6), and by Gurdon (7)). In this case it is again likely that frog oocytes may prove of general value for translating heterologous messenger RNAs.

At the time of writing no fully documented reports of the translation of RNA from other viruses in frog oocytes have been published. We are aware of attempts to translate Avian myeloblastosis virus in injected oocytes by several laboratories. In collaboration with Salden and Bloemendal we have injected the virion RNA of Rauscher murine leukaemia virus, in 65s form, or as 37s RNA with the low molecular weight RNA obtained from the 65s RNA by heat denaturation. In none of these cases has a positive result been obtained, but it would be premature to claim that the injected RNAs are not translated. The virion RNAs of various bacteriophage viruses, such as f2 and R17 have been injected into oocytes. The results (3) and Knowland (unpublished) have failed to reveal the synthesis of any of the three expected proteins, though in each case a general stimulation of protein synthesis, by 4–5 times, was observed. The apparent inability of bacterial messages to be translated in frog cells is not surprising in view of the difference between pro- and eu-karyote ribosomes. We do not yet know whether the lack of success, so far, in translating oncornavirus RNAs is due to a deficiency of the oocyte, such as the need for special translational "factors" or a deficiency in the RNA such as a low proportion of biologically active molecules, or the need to assume or unfold a special secondary structure.

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HAEMOGLOBIN MESSENGER RNA

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Abstract

This is a short review on the isolation and translation in heterologous systems of a 9S RNA which carries the information of haemoglobin.

Rabbit haemoglobin (Hb) messenger RNA (mRNA) was the first eukaryotic genetic message to be isolated, quite a long time ago (1). This was mainly due to the fact that mammalian reticulocytes offer the favourable opportunity of being highly specialized cells whose protein synthesis is almost totally concerned with haemoglobin. Haemoglobin mRNA should amount to about 90 % of total polysomal mRNA. Furthermore, α and β globin chains have such lengths that their mRNAs must sediment at approximately 9S. Isolation of the latter by sucrose gradient centrifugation was therefore quite easy (Hb mRNA must sediment between the 4S region and the 18S ribosomal RNA region if one analyzes total reticulocyte polysomal RNA).

These criteria and others allowed us to isolate from rabbit reticulocyte polyribosomes a 9S RNA fraction presenting several properties the globin mRNA must possess (1) (2). The putative mRNA could also be detached as a specific 14S ribonucleoprotein when polyribosomes were dissociated by EDTA or CMC treatment (3). This 14S mRNP has been extensively studied in our laboratory (4) and its obtention now constitutes the first step in the preparation of 9S Hb mRNA.

But the definitive proof that the 9S RNA fraction was or contained Hb message could only be obtained if this RNA would direct Hb synthesis when added to an heterologous protein synthesizing system. A preliminary indication in this direction was obtained by SCHAPIRA and coworkers (5) but the first clearcut result was presented by LOCKARD and LINGREL in 1969 (6). They added mouse polysomal 9S RNA to a rabbit reticulocyte cell-free system and identified the mouse globin β chain in the synthesized products. Somewhat later, GURDON and coworkers showed that 9S rabbit reticulocyte RNA could be extensively translated into α and β globin chains when injected into frog oocytes (7). Translation could proceed for several days. The synthesized products were characterized by gel filtration, ion exchange chromatography (7) and finger printing (8). JONES and LINGREL also showed that the 9S RNA fraction isolated from mouse reticulocyte polyribosomes,

acted as the message for mouse α and β globin chains (9). The 9S mouse reticulocyte RNA has also been translated in the Krebs ascites cell-free system (10) and the 9S rabbit mRNA has been translated in the rat liver system (11). SCHERRER and coworkers reached quite similar conclusions using the duck reticulocyte system (12).

As a conclusion, it is clearly established that the 9S RNA fraction isolated from reticulocyte polyribosomes contains the messages for globin chains. This, of course, does not exclude the possibility that other RNAs be present in the 9S fraction.

A fractionation procedure using adsorption to cellulose (13) was applied to 9S reticulocyte mRNA, purified by sucrose gradient centrifugation. So far, we detected a clearcut heterogeneity in the preparation: 70 % to 80 % of the 9S RNA were adsorbed onto the cellulose in high ionic strength conditions whilst 20 % to 30 % were excluded. The excluded fraction was much less active than the retained one in directing globin synthesis in oocytes. Work is now in progress to further analyze these subfractions and determine their nature and function.

On the other hand, we focused our attention on nucleated erythroïd cells from spleens of anemic rabbits. This material constitutes a useful tool for the study of the selection mechanisms of the nuclear genetic information and of the transfer of this information from the nucleus to the cytoplasm. Avian reticulocytes have already been used for this purpose by another research group (14). In our laboratory we chose the spleen of anaemic rabbits as a source of nucleated erythroïd cells: this should allow us to compare the molecular characteristics of globin mRNA in young nucleated and old, anucleated erythroid cells.

So far, we have been able to prepare active globin 9S mRNA from the spleen of anaemic rabbits (15) and are now comparing its properties to those of the 9S mRNA from reticulocytes of the same animals.

Acknowledgements:

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CONTROL OF GLOBIN SYNTHESIS DURING DMSO-INDUCED DIFFERENTIATION OF MOUSE ERYTHROLEUKEMIC CELLS IN CULTURE

by

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A small fraction of Friend or Stansly Virus (SFFV) transformed erythroleukemic cells of spleen, liver or peripheral blood, if maintained under tissue culture conditions (1–5), continuously differentiate into erythroblasts. Addition of aprotonic solvents, such as DMSO (2) or dimethoxyethane (Ostertag et al. unpublished results), to these cells in culture, stimulates their differentiation along the erythrocytic line much further. We have shown that under favorable conditions the cells are able to synthesize up to 50 % hemoglobin. Usually a 20–30 % globin synthesis is found. Adult α and β globin chains are synthesized (4, 6).

Induced hemoglobin synthesis is a good marker to study the processes involved in cellular differentiation. The differentiation, as evidenced by a 40–100 fold increase in globin synthesis, can be due to a) increased transcription of the mRNA for proteins characteristic for the differentiated state of the cell; b) processes occurring after transcription and leading to translatable globin mRNA (transport and processing of globin mRNA); c) increased translation.

From our experiments published elsewhere (6) it seemed likely that there is an increase in available globin mRNA during induction as evidenced by the appearance of at least two new RNA species in the 8–9 and 11–13S regions. This conclusion was confirmed by other authors using the reverse transcription product of mouse

reticulocyte globin mRNA as a complement for titrating globin mRNA in other strains of mouse erythroleukemic cells (7, 8). However, a positive identification of translatable globin mRNA in these cells has been lacking.

I. Globin mRNA during stimulation with DMSO

In all experiments we used the erythroleukemic cell clone FSD1/F4 since it has very little or no detectable spontaneous globin synthesis in the unstimulated state. In the stimulated state we usually obtain 15–25 % globin synthesis after 5 days of exposure to DMSO (6).

Cells were grown as described (Fig. 1) and 15 min. before harvesting the cells, cycloheximide was added to prevent run-off of monosomes (9). The cells were collected and exposed to 0.5 % NP40. The NP40 cytoplasmic suspension was layered on 12–32 % sucrose gradients. The polysomes were separated from the monosomes and the post-ribosomal supernatant (Fig. 1). The indicated fractions were treated with EDTA, SDS at a high pH and low cation concentration (6, 10) and extracted with a mixture of phenol, cresol, chloroform, isoamyl alcohol (6, 7, 10). The RNA of each fraction was separated on 8–50 % sucrose gradients. Fig. 2 shows the separation of the polysomal RNA of stimulated leukemic cells and Fig. 3 of the poly-

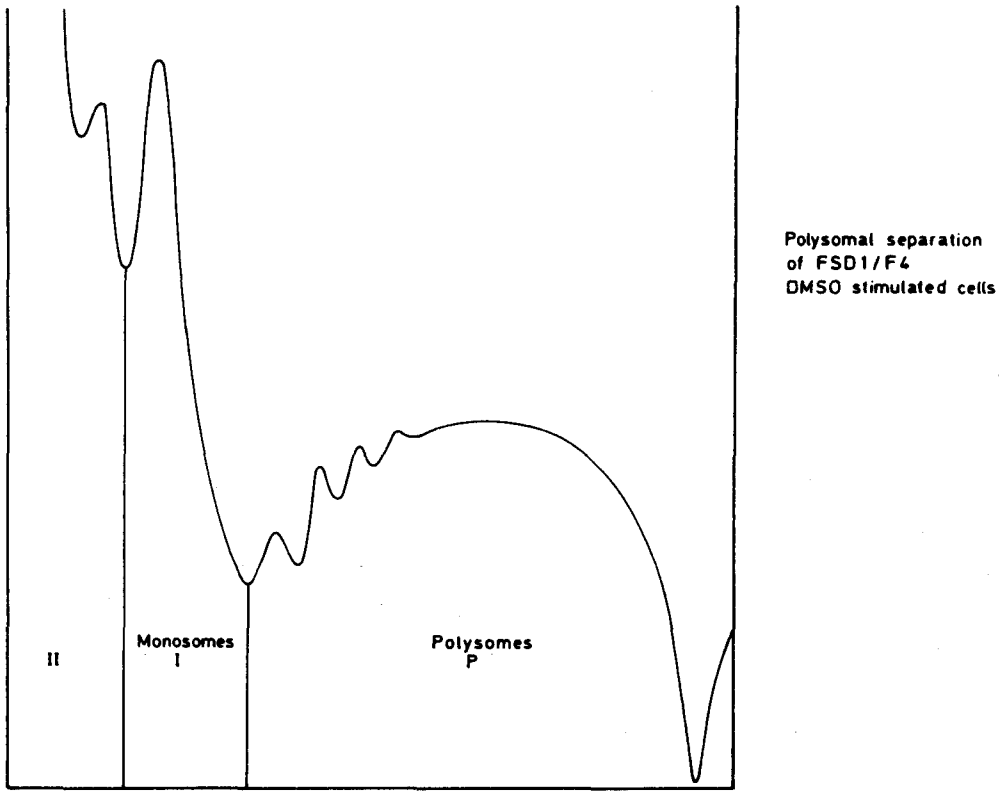

Fig. 1: Polysome profile of stimulated cells. Cells were grown in modified Eagle's medium (6). The stimulated cells were kept in 1.5 % DMSO containing medium for 5 days. Unstimulated and stimulated cells were then exposed to cycloheximide 100 µg/ml for 15 min. at 37 °C. The cytoplasmic NP40 soluble supernatant (6) was centrifuged in a linear 12–32 % sucrose gradient containing 0.05 M Tris-HCl, ph 7.4, 0.025 M KCl, 0.005 M $MgCl_2$ in a Beckman SW 27 rotor at 100,000 g for 2 hours. The fractions indicated as P, I and II were collected and the RNA extracted as in Fig. 2.

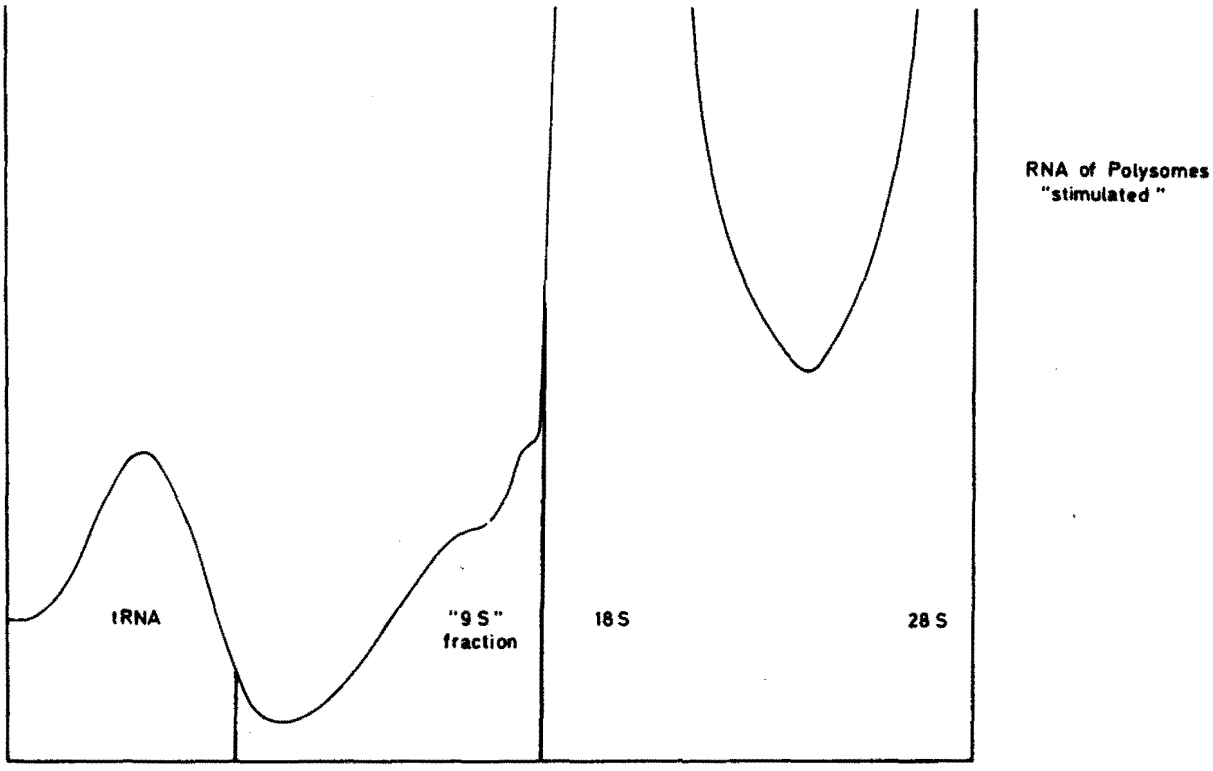

Fig. 2: Polysomal RNA (fractions P of Fig. 1) of stimulated cells separated on a sucrose gradient. The fractions as indicated in Fig. 1 were deproteinized (6, 7, 10). Amoniosalicylate was not used. RNA was applied to 8–50 % sucrose gradients in 0.01 M Tris-HCl, pH 7.4, 0.015 M KCl (SW40, 190,000 g, 16 hours, 4 °C). The 8–16S fraction was used for injection into Xenopus oocytes.

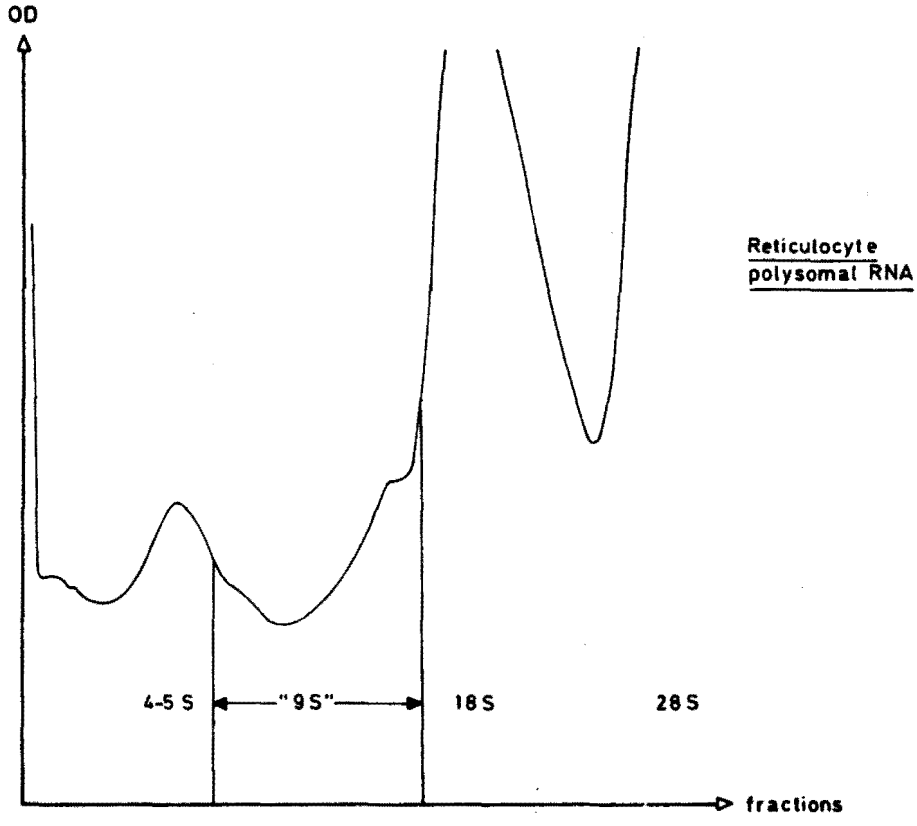

Fig. 3: Polysomal RNA of mouse reticulocytes separated on a sucrose gradient as described in

somal RNA of mouse reticulocytes as a control. It can be seen that the polysomal RNA extracted from reticulocytes, stimulated erythroleukemic cells and nonstimulated cells has a similar distribution profile in the sucrose gradient. However, much more material in the 12–16S region is present in the stimulated post-ribosomal fraction as compared to the same fraction extracted from nonstimulated cells (Fig.4, 5). The recovery of the RNA during the isolation procedure was monitored at each step to allow an estimation of globin mRNA activity per total RNA of an average leukemic cell.

The RNA indicated as „9S" RNA was collected and injected into frog oocytes (10–13). The proteins synthesized by the Xenopus oocytes were applied to carboxymethyl cellulose columns (6) using as carrier proteins either adult mouse globin or alternatively ^{14}C labelled β and α chains. The latter were isolated from DMSO stimulated erythroleukemic cells. Table 1 shows that most of the globin mRNA activity in stimulated cells is in the polysomal fraction (70–80 %), whereas the other two fractions contain much less globin mRNA activity. In all corresponding fractions of the unstimulated cells we found virtually no globin mRNA activity.

Fig. 6 shows the relative increase of synthesis of globin chains in the oocytes after injection of equivalent amounts of polysomal RNA of stimulated and unstimulated cells. The increase in translatable globin mRNA activity confirms our previous conclusions that DMSO induces an increase in translatable globin mRNA in step with the increase in globin synthesis. This increase does not start before the

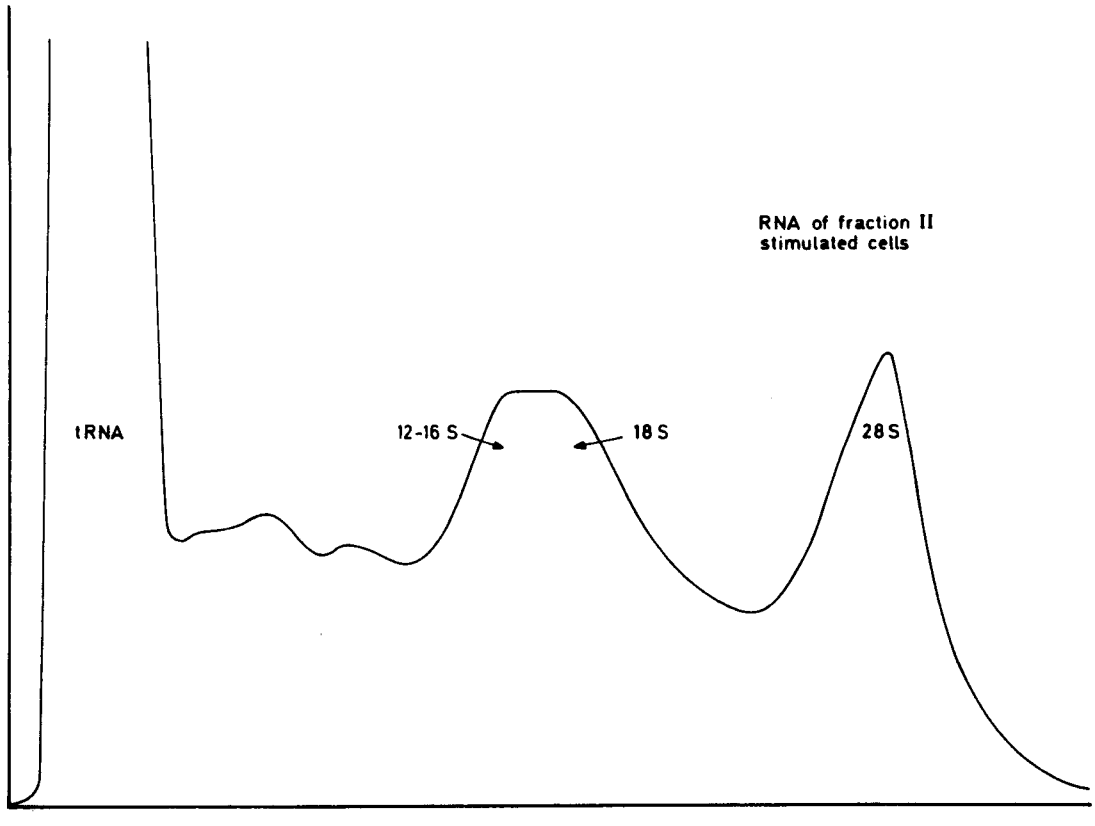

Fig. 4: Stimulated cells. RNA of fraction II of Fig. 1 (post-ribosomal fraction) separated on a sucrose gradient as described in Fig. 2. The fraction in the 8–16S region was again pooled and injected into oocytes.

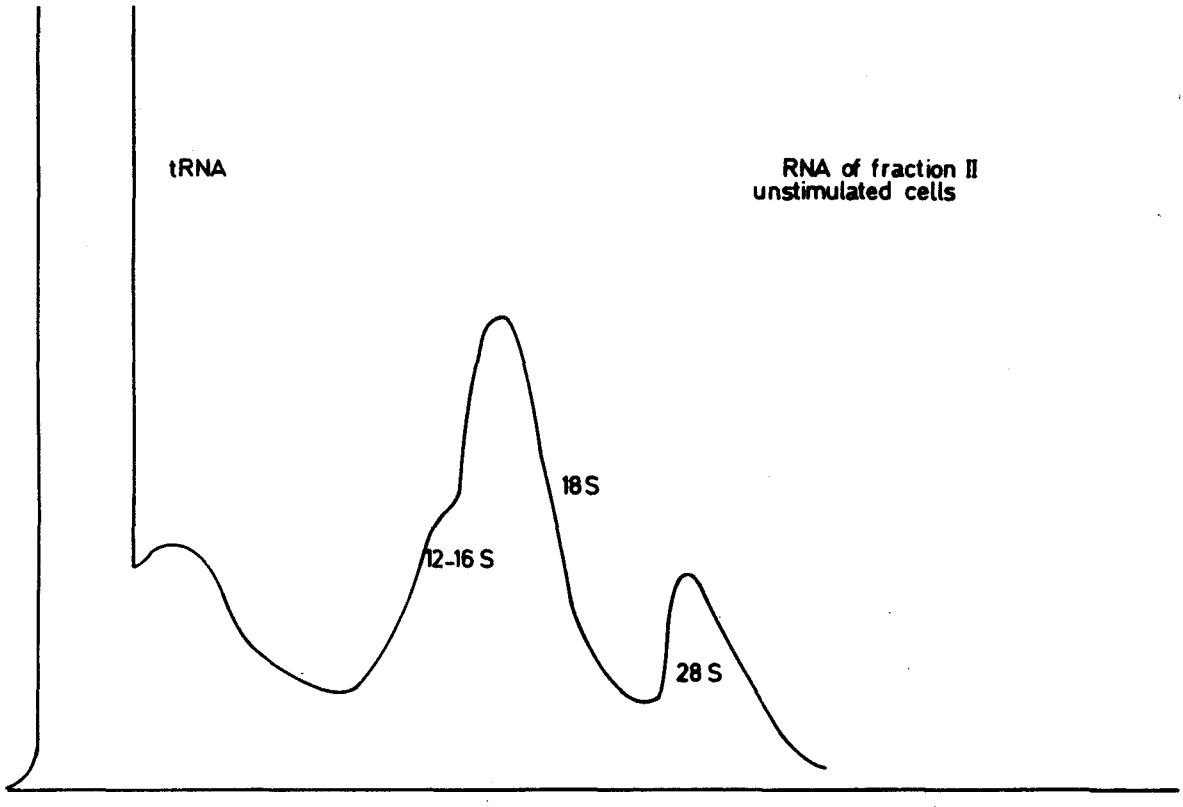

Fig. 5: Unstimulated cells. RNA of fraction II (post-ribosomal fraction) separated on sucrose gradients as in Fig. 2.

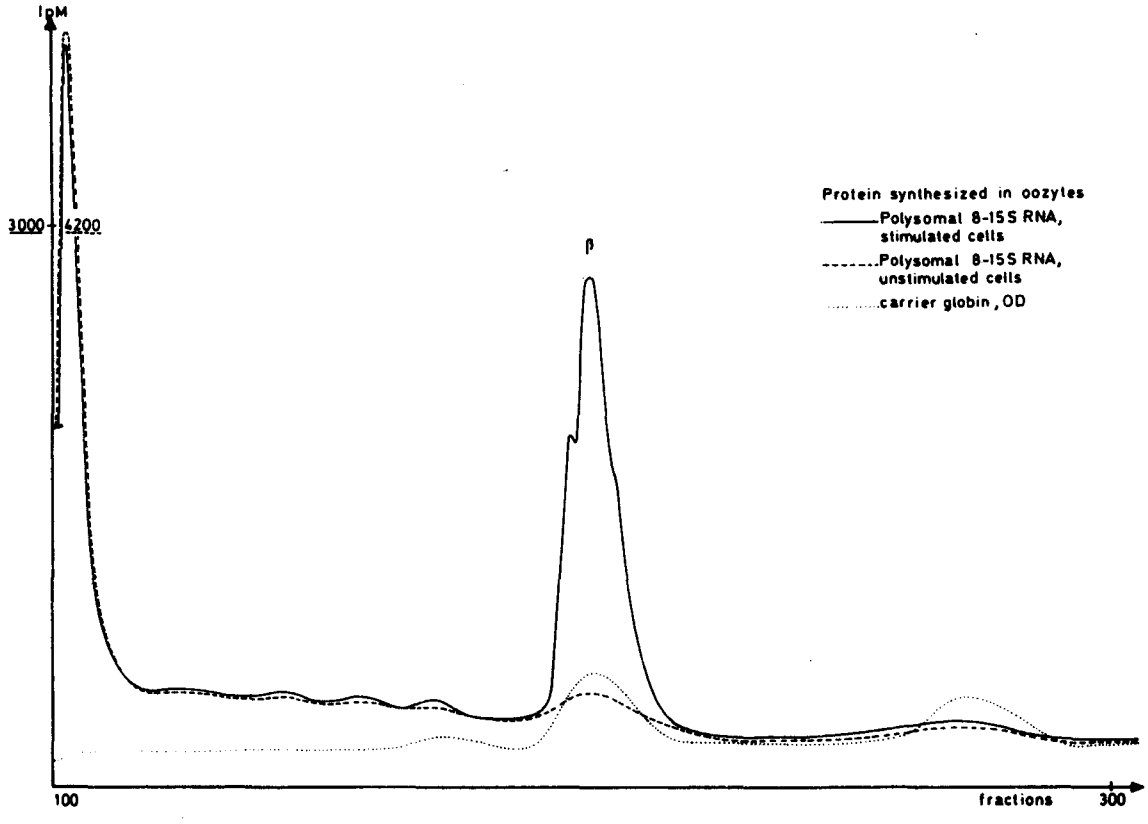

Fig. 6: Relative amounts of globin synthesized in frog oocytes in response to 8—16S RNA extracted from stimulated and unstimulated cells. The experimental details were described previously (10).

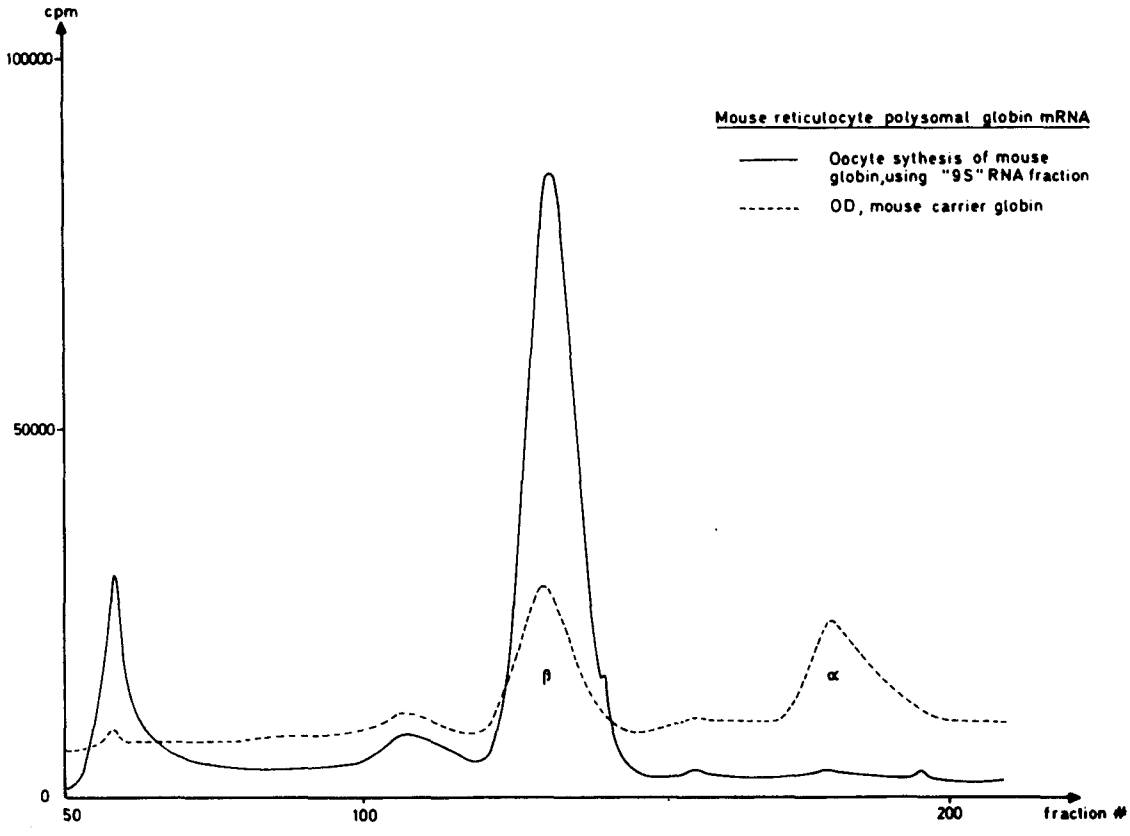

Fig. 7: Globin synthesized in frog oocytes in response to "9S" RNA from mouse reticulocytes.

second day of stimulation with DMSO. It is tempting to conclude that DMSO induces a change in the transcriptional pattern of the cell, although on the base of our evidence, post-transcriptional control mechanisms cannot be ruled out.

Stimulated erythroleukemic cells synthesizing 15–20 % globin contain about 80 % of the amount of globin messenger found in mouse reticulocytes. Leder et al. (14) indicate that only about 1/3 as much globin mRNA can be found in a similar stimulated erythroleukemic cell line. If mouse globin mRNA of reticulocytes and erythroleukemic cells is injected into oocytes we recover very little α globin chains (Fig. 6, 7). The specific messenger activity per RNA fraction per oocyte of mouse 8–12S RNA fractions in our experiments is higher than that of the rabbit reticulocyte RNA described by MOAR et al. (12). It is therefore probable that very few α chains are synthesized, and not that the chains are synthesized, degraded and lost. Another possibility is that the α chain messenger is larger than the messenger for β chain synthesis and lost during isolation on sucrose gradients. However, if we analyze the different size classes of mRNA separated on sucrose gradients or acrylamide gels, we find the highest enrichment of α globin mRNA in the lowest molecular weight region (10). This result argues against the interpretation of a loss of large size mRNA.

A much larger amount of 8–16S material is present in the postribosomal fraction of stimulated erythroleukemic cells as compared to the same fraction of unstimulated cells (Fig. 4, 5). This RNA has very little globin mRNA activity as compared to the polysomal RNA of the 8–16S region. Probably during DMSO stimulation some

ribosomal RNA is degraded, which appears in the post-ribosomal fraction, mainly as 12–16S material. This agrees with observations of ribosomal RNA breakdown during normal reticulocyte maturation (15).

II. Induction of differentiation

In previous experiments we have shown (Kluge et al. unpublished data) that DMSO induces differentiation in erythroleukemic cells grown in serum-free medium. It therefore seems likely that DMSO acts directly on the target cell.

The intention of the experiments which are described below was to show that DMSO induces cellular changes, especially of the transport of small molecules into the erythroleukemic cell. This occurs before the increase of globin mRNA is observed. Table 2 shows that the cell number increases 6.4 fold after stimulation. The unstimulated control cells increase 25 fold. We compared that to the total amount of cytoplasmic RNA recovered in the stimulated cells. There is only a 30 % increase

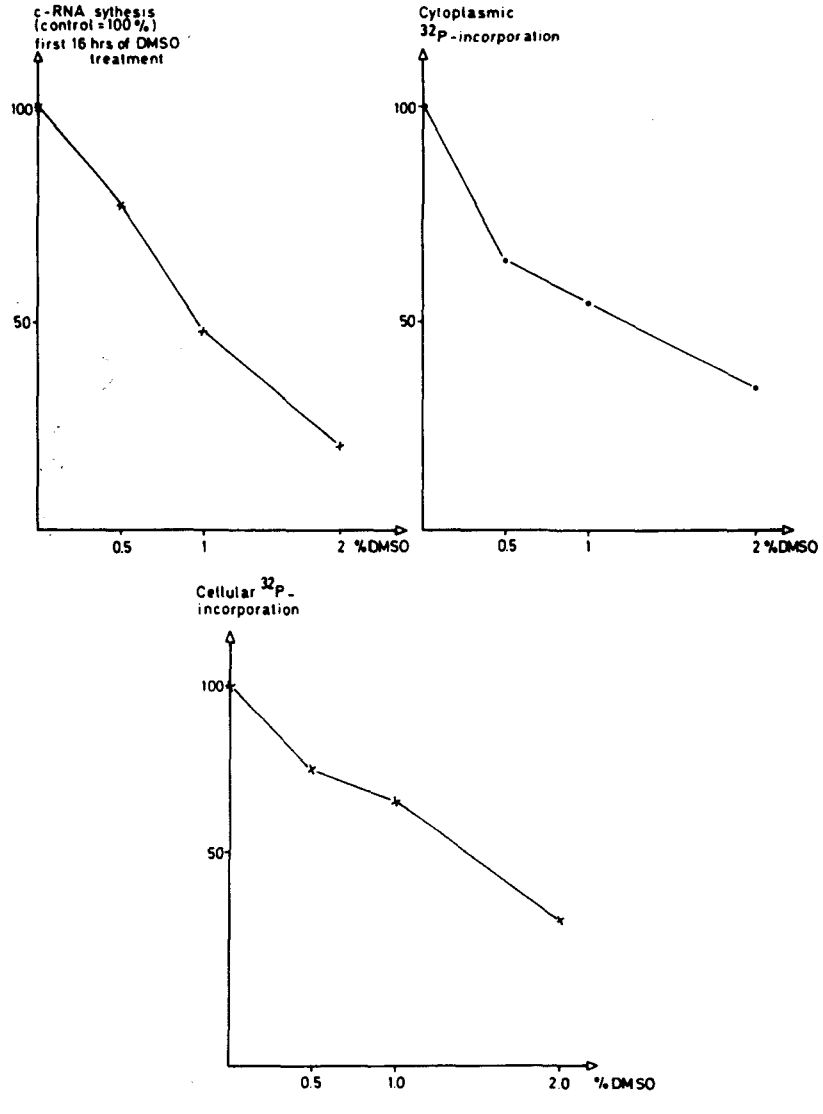

Fig. 8: Decrease of synthesis of cytoplasmic RNA (cRNA), decreased incorporation of $^{32}PO_4$ in cytoplsm and cells the first 16 hours of treatment with DMSO as indicated.

of total RNA, although cells do multiply by a factor of 6.4 (Table 2). This results in an average decrease of RNA content/cell in the stimulated state (Table 2), although globin mRNA activity increases at least 25 fold uring the same time.

The first observed change in cells after adding DMSO before onset of globin synthesis is, however, an immediate decrease of synthesis of cytoplasmic RNA (Fig. 8). Even more interesting, a decrease of ^{32}P incorporation into the cell is found. By adding cAMP to 3T3 cells KRAM et al. (16) observed a similar effect for leucine, deoxyglucose and uridine transport. The similarity of the action of DMSO on inducing differentiation to cAMP found in other systems is further emphasized by the effect of DMSO on decreasing the cell division rate. We have also checked cAMP on the induction of differentiation in erythroleukemic cells. cAMP induces differentiation, however, to a much lower degree as DMSO. A similar degree of stimulation is obtained with the steroid hormone etiocholanolone (Fig. 9).

Summary DMSO induces erythroid differentiation in Friend virus (SFFV) transformed spleen cells in culture. This differentiation results in the appearance of globin synthesis (20 % of the total protein synthesis) and in a correlated increase of globin mRNA. Two days after adding DMSO the first increase of globin mRNA and globin synthesis is observed. In the first 16 hours after addition of DMSO the transport of small molecules (phosphate) into the cells is reduced. Synthesis of cytoplasmic RNA is decreased. Cell division rate is much less reduced after stimulation. The amount of RNA per cell is decreased to 20 % of that of the nonstimulated cells. The effect of DMSO on differentiation shows similarities to hormone and cAMP action.

Acknowledgements This work was supported by a grant from the Deutsche Forschungsgemeinschaft. We would like to thank Angelika Rohmann for her excellent assistance.

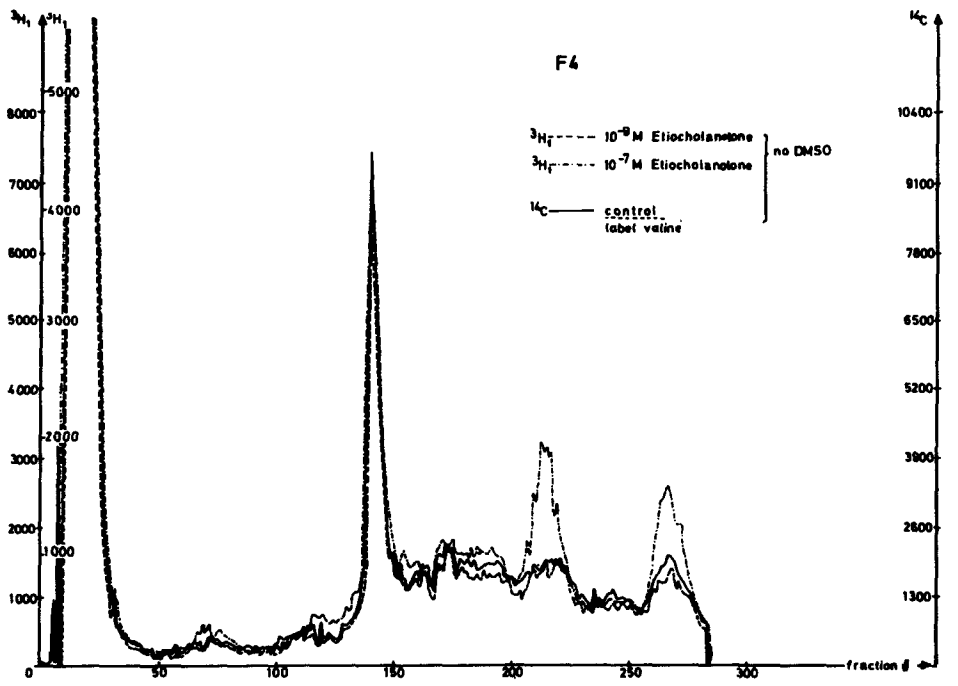

Fig. 9: Stimulation of SFFV-transformed erythroleukemic cells by etiocholanolone. CMC-column chromatography (10).

		cellular globin synthesis in %	relative increase during stimulation	relative globin m-RNA activity corr. for OD	relative increase of globin m-RNA (oocyte test)
unstimulated	polysomal fraction	—	—	‹225	1
	monosomal fraction	—	—	‹1100	1
	postribosomal fraction	—	—	‹150	1
	total	‹0.5	1	‹1500	1
stimulated	polysomal fraction	—	—	25600 (74%)	›100
	monosomal fraction	—	—	5300 (16%)	›5
	postribosomal fraction	—	—	3800 (11%)	›25
	total	20%	›40	34700	› 23
	mouse reticulocyte polysomes	98%	—	29000	—

Table 1: Distribution of globin mRNA activity in different RNA fractions of stimulated, unstimulated cells and mouse reticulocytes.

	unstimulated cells	DMSO stimulated cells
increase in cell number (4 days)	25	6.4
cytoplasmic RNA content per cell in 10^{-11}g	2.0	0.4
total cytoplasmic RNA of all cells in OD units day 0	9	
total cytoplasmic RNA of all cells day 4	225	11.5

Table 2: Cell division and RNA content/cell during stimulation of erythroleukemic cells.

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TRANSLATION OF MAMMALIAN mRNAs IN CELL-FREE EXTRACTS OF ASCITES CELLS AND WHEAT GERM

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Translation constitutes an important and reliable means, though not the only means, for the identification and assay of mRNAs. The value of well-characterized systems capable of translating a variety of heterologous mRNAs has become increasingly apparent over the last 3–4 years. Currently systems of several sorts are available, ranging from highly fractionated cell-free extracts to the unrefined reticulocyte lysate and intact *Xenopus* oocytes. In the present article, we review some properties and applications of systems of an intermediate level of complexity. These systems, preincubated S-30 extracts (post-mitochondrial supernatants) of eucaryotic cells, offer the advantage of a low endogenous background together with the convenience of relatively simple preparation and use. In this regard they resemble the analogous extracts of bacterial cells which have played a major role in investigations of the metabolism of procaryotic mRNAs. These principles are illustrated by reference to S-30 extracts of Krebs II ascites cells and of wheat germ.

Ascites cell system

The Krebs II tumour originated as a spontaneous mouse carcinoma and was passed into the ascitic form in which state it can be conveniently propagated. One mouse can yield some 5 ml packed cells (about 10^9 cells) from an inoculum of $1-2 \times 10^7$ cells given one week previously. It is this fecundity, rather than any unique properties of these cells, which has led to the widespread reliance on this system. Comparable extracts have been prepared from a variety of other cell types, such as rat and mouse liver, Landschutz ascites cells, and several lines of cultured cells (1, 2). These extracts exhibit similar properties to those of the Krebs ascites system.

The key step in the preparation of the ascites system is preincubation of the S-30 under conditions of protein synthesis. This exhausts the endogenous protein synthesizing capacity of the system without destroying its ability to read added mRNAs (3). As a result the response to the addition of exogenous mRNA can be

measured simply in terms of the incorporation of labelled amino acids into acid-insoluble material, and product analysis is simplified. Other steps in the preparative procedure, such as the removal of inhibitory substances by gel filtration, are also important but the distinguishing feature of systems of this type is the means by which a low endogenous background is obtained.

When presented with purified mRNAs such as rabbit globin mRNA, calf lens crystallin mRNA or encephalomyocarditis (EMC) virus RNA, incorporation is stimulated by up to 20-fold. The characteristic products of these messengers are clearly visible over the faint endogenous background (4). Several other cellular and viral mRNAs such as those for histones (5), reovirus proteins (2) and tryptophan oxygenase (6) have also been translated in this system, and at present there is no indication that there are mRNAs which the system is incapable of translating. Whether this truly represents the omnipotence of ascites cells or is an artifact of the conditions of preparation or use of the system is debatable. Messenger purity does not seem to be a major problem in that a preparation containing as little as 0.1 % of the mRNA for tryptophan oxygenase produced detectable amounts of product. Within limits, rRNA contamination seems not to interfere either (3, 7).

As normally prepared, the system appears to be partially deficient in initiation factors (4). When supplemented with a crude initiation factor preparation from reticulocytes there is a several-fold increase in the efficiency of translation of added globin or crystallin mRNAs. EMC RNA is affected less or not at all. The endogenous background is also enhanced suggesting that preincubation does not result in the destruction or permanent inactivation of the endogenous messengers. For many purposes it may be advantageous to augment the system with reticulocyte factors. Some authors have also reported that the system exhibits a stimulation of or dependence on added tRNA (7): most users do not observe this phenomenon, however, and its origin is unclear.

It would be unrealistic not to mention that the ascites system does have some limitations. The period of active chain initiation on an added mRNA is rather short, only about 10 min (3). On the other hand, amino acid incorporation proceeds for a considerable length of time, up to 90 min using EMC RNA as the message. This is a result of the slow rate of chain elongation, which proceeds at about 25 residues/min under typical incubation conditions (M. B. M. and M. Osborn, in preparation). It is not known whether this rate is constant along a message, or whether the ribosomes travel faster along most of the mRNA but become stalled at certain critical points. The latter might account for the phenomenon termed "premature termination" whereby translation of some mRNAs, notably EMC RNA and possibly immunoglobulin light chain mRNA, also tends to cease at certain points (8, 9). In view of the extraordinary length of this mRNA (about 2.6×10^6 daltons) it might be that it is cleaved before it can be fully read. The S-30 contains RNases capable of digesting both single- and double-stranded RNA (10).

Despite these apparent flaws, the ascites system has played an invaluable role in mRNA identification and in studies of the mechanism of protein synthesis in mammals. It will surely continue to render service in these and other directions in the future.

Wheat system

Cell-free protein synthesizing systems from wheat, which can be prepared from either the embryo or the germ, have recently acquired a wholly new significance. Embryos can be isolated from commercial grain by simple physical separation procedures capable of yielding large quantities of material. Alternatively, wheat germ may be obtained commercially. In either case, the material is disrupted by grinding with sand in a suitable aqueous medium, filtered, centrifuged and preincubated under conditions of protein synthesis. This results in a system with a very low endogenous background and an ability to efficiently translate both plant and animal mRNAs. Tobacco mosaic virus (TMV) RNA stimulates incorporation by over 100-fold, and mammalian globin mRNA elicits a 30-fold response (11).

As was the case with the ascites system, the wheat embryo cell-free system was originally developed to translate viral RNA (12). The mRNAs from two plant viruses, satellite tobacco necrosis virus (STNV) and brome mosaic virus (BMV), behave straightforwardly and give rise to virus coat proteins which can be easily identified (13, 14). TMV RNA, the polycistronic mRNA which served as template in the initial studies of the system, presents a more complex case. In the wheat germ system it directs the synthesis of a large number of polypeptides ranging from 10,000–140,000 daltons, but little or no coat protein is detectable on SDS-polyacrylamide gels. Nevertheless we have demonstrated that a portion (at least) of the viral coat protein is translated faithfully (15). For this purpose we exploited a virus mutant which differs from wild-type TMV in possessing a methionine residue in its coat protein. RNA from this mutant directs the synthesis of a methionine-containing tryptic peptide which is absent from digests of wild-type RNA-directed products. This novel peptide has been very thoroughly characterized and shown to correspond to the mutant coat peptide by electrophoretic and partial sequence analysis. The detailed mechanism of TMV RNA translation is far from clear, but there is reason to believe that further cell-free studies may help to elucidate the processes occurring *in vivo*.

The scope of the wheat system has been dramatically widened with the demonstration that it can read various mammalian mRNAs, including those for rabbit globin (both α and β chains) (11) and immunoglobulin heavy and light chains (16). Preliminary evidence indicates that these are not isolated cases, and that the system is capable of faithfully translating several other mammalian cellular and viral mRNAs. The efficiency of the system is high, especially when it is prepared according to the modified procedures (11), and is comparable to the ascites system in this regard.

The advantages of the wheat system are similar to those of the ascites system: large quantities of extract can be easily prepared; the low endogenous background of the preincubated S-30 facilitates messenger testing and product analysis; the system is efficient; and it is capable of synthesizing large polypeptides (up to 1,400 residues in the TMV RNA-directed product). There is no doubt that this system will assume a growing importance in the areas of mRNA assay and identification.

Conclusion.

Both the wheat and the ascites preincubated S-30s can translate a variety of mammalian mRNAs. Both systems are easily prepared from readily available starting materials, perhaps slightly more conveniently from wheat. If it is an advantage to use a system as distantly related as possible, to minimize the chance that the extract contains interfering components, the wheat system might have some advantage for mammalian mRNAs. On the other hand it is conceivable that the wheat system might lack some essential components required for certain mammalian mRNAs, though there is no sign of this at present.

Proteins which are synthesized in the form of longer precursor polypeptides requiring proteolytic action to convert them to the mature form seem to prevent special problems in cell-free systems. The proteolytic enzymes responsible for such cleavages appear to be deficient in cell-free extracts. Thus TMV and EMC coat proteins of the correct sizes are not formed in significant quantities in wheat or ascites S-30s primed by the appropriate viral RNA, though tryptic digests show that these sequences are translated. Crude extracts of poliovirus-infected HeLa cells also fail to cleave the precursor polypeptides which they synthesize (17), but the *Xenopus* oocyte system has at least some ability to carry out cleavages of this sort (18). That this defect of the cell-free systems may be remediable is suggested by recent successes with the wheat system. Incubation of the TMV RNA-directed polypeptides with a tobacco leaf extract results in the accumulation of coat protein which can be incorporated into intact virus particles in a reconstitution experiment (B. E. R., unpublished data).

At the present the wheat system seems to be a very satisfactory substitute for the ascites system: it remains to be seen whether it suffers limitations similar to those of the latter, and the comparative virtues of the two systems remain to be assessed.

Acknowledgements

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STEROID INDUCTION OF THE mRNA FOR HEPATIC TRYPTOPHAN OXYGENASE

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Abstract

Poly (A) containing RNA from rat liver, purified by cellulose chromatography directs the synthesis of subunits of tryptophan oxygenase in a heterologous cell-free protein synthesis system derived from Krebs ascites cells supplemented with reticulocyte initiation factors. The newly synthesized enzyme protein was identified by precipitation with monospecific antibodies prepared against the homogenous enzyme and subsequent sodium dodecylsulfate-polyacrylamide electrophoresis of the immunoprecipitate. The increased *in vivo* rate of synthesis of hepatic tryptophan oxygenase after glucocorticoid treatment is paralleled by an enhanced tissue level of the mRNA for tryptophan oxygenase. The intracellular accumulation of the mRNA is dependent on the dose administered and increases with time during the action of the hormone.

The mechanism of action of steroid hormones has been a very suitable model system for the study of gene regulation in higher organisms, since it has become increasingly apparent that steroid hormones elicit their physiological action by specifically modulating gene expression. The problem of control of genetic expression can be stated in the following way: Since all cells of a multicellular organism contain the same genetic information, the qualitative and quantitative differences in the set of structural and catalytic proteins at various developmental and physiological states must reflect differential expression of the constant set of genetic information. The fundamental action of steroid hormones is to induce specific macromolecular synthesis and we have used the induction of tryptophan oxygenase to obtain insight into the processes controlling gene expression.

The increase in tryptophan oxygenase activity after glucocorticoid treatment was the first demonstration of an inducible enzyme in a mammalian system (1). It was subsequently shown that this increased activity of tryptophan oxygenase activity is due to an elevated level of enzyme protein present (2), which is the result of an increased rate of synthesis of the protein (3).

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Two major modes of action can be visualized to explain the enhanced rate of synthesis of the tryptophan oxygenase protein. The hormone may enhance translation by a more efficient readout of the mRNA for tryptophan oxygenase, the concentration of which however, remains unchanged. Alternatively, the hormone may control the mRNA content itself. Distinction between these alternatives obviously requires the isolation of the mRNA in question, its faithful translation in a heterologous cell-free protein synthesis system and the assay of its intracellular concentration in such a system after hormonal induction of the enzyme.

The successful translation of the mRNA for rabbit globin and for chicken ovalbumin (4), encouraged us to attempt the cell-free synthesis of tryptophan oxygenase (5), even though tryptophan oxygenase represents only a minute fraction of total hepatic protein synthesis.

Results

Fig. 1 (left) shows the effect of rat liver poly (A) containing RNA, purified on cellulose, on protein synthesis in the 30,000 x g supernatant from Krebs ascites cells (S-30 preparation). 6 μg oft this mRNA fraction stimulated the incorporation of L-[^{3}H]leucine into total proteins five-fold. We have used S-30 preparations with a high basal incorporation rate, since they yielded increased synthesis of specific

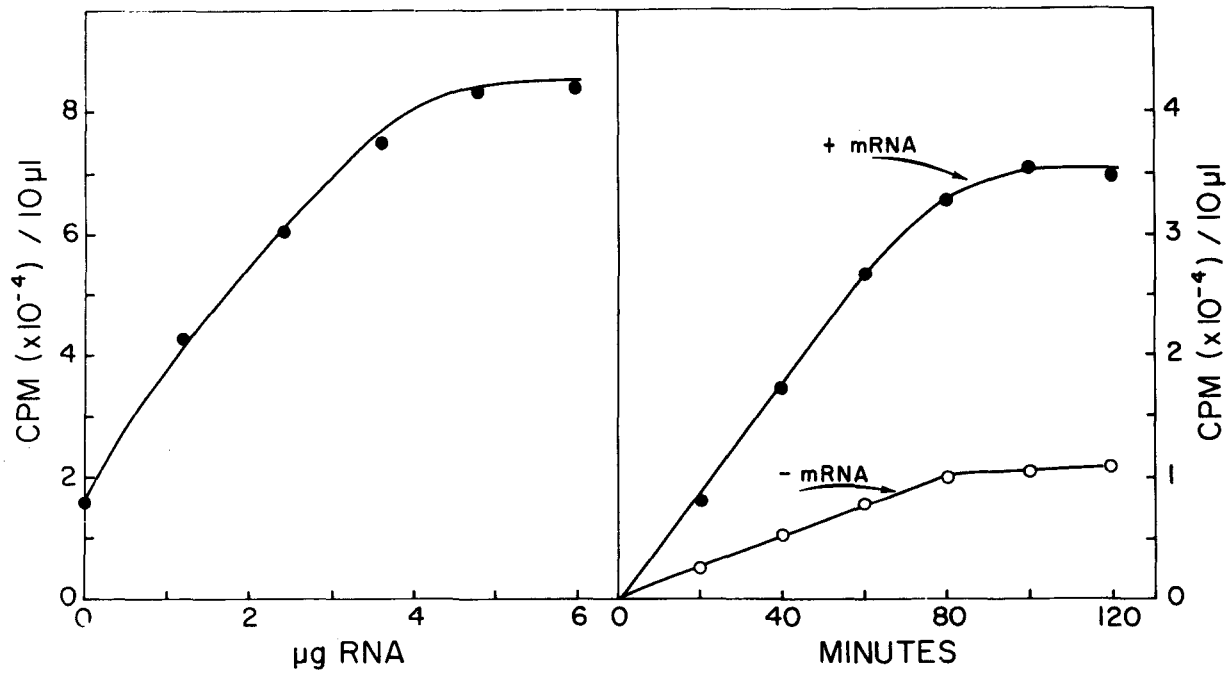

Fig. 1: Effect of rat liver poly (A) containing RNA, on protein synthesis in a Krebs ascites cell-free system.
Left panel: Following incubation for 60 min of 50 μl reaction mixtures with the indicated amounts of cellulose-purified rat liver RNA, the incorporation of L-[^{3}H]leucine was determined (5).
Right panel: Time course of protein synthesis in the absence (○ ——— ○) and the presence (● ——— ●) of 20 μg/ml of cellulose-purified RNA. Different S-30 preparations were used in these two experiments.

products, even though the same mRNA preparation stimulated the protein synthesis up to 15 fold in a S-30 preparation with a much lower background reaction. Protein synthesis in the endogenous as well as in the stimulated reaction is linear for at least one hour (Fig. 1, right).

The newly synthesized tryptophan oxygenase was identified by specific immunoprecipitation and subsequent SDS polyacrylamide electrophoresis of the immunoprecipitate. Since we estimated that only 0.01—0.1 % of the total hepatic protein synthesis could be tryptophan oxygenase, we took great care to obtain monospecific antibodies. Thus, enzyme, purified to homogeneity (6), was used for the preparation of the antibodies. The antibodies obtained yielded a single precipitation line with the purified enzyme as well as rat liver cytosol in an Ouchterlony diffusion analysis. When the purified enzyme is incorporated into the agar the precipitation line with the cytosol is eliminated.

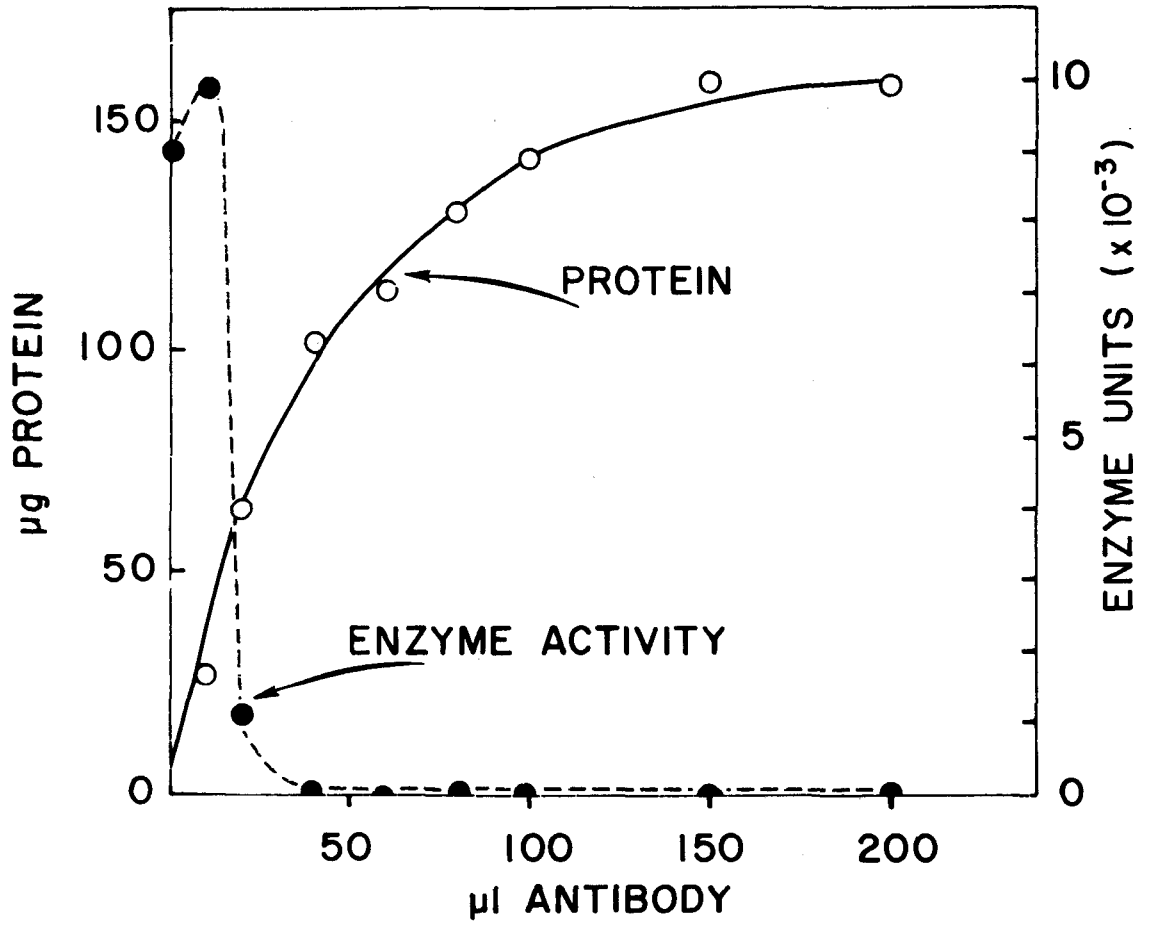

Fig. 2: Determinations of the equivalence point for immunoprecipitation of tryptophan oxygenase by anti-tryptophan oxygenase.
8 µg of tryptophan oxygenase were incubated with increasing amounts of the antibody. The protein content of the washed immunoprecipitates was determined; tryptophan oxygenase activity was measured in the supernatants after removal of the immunocomplexes.

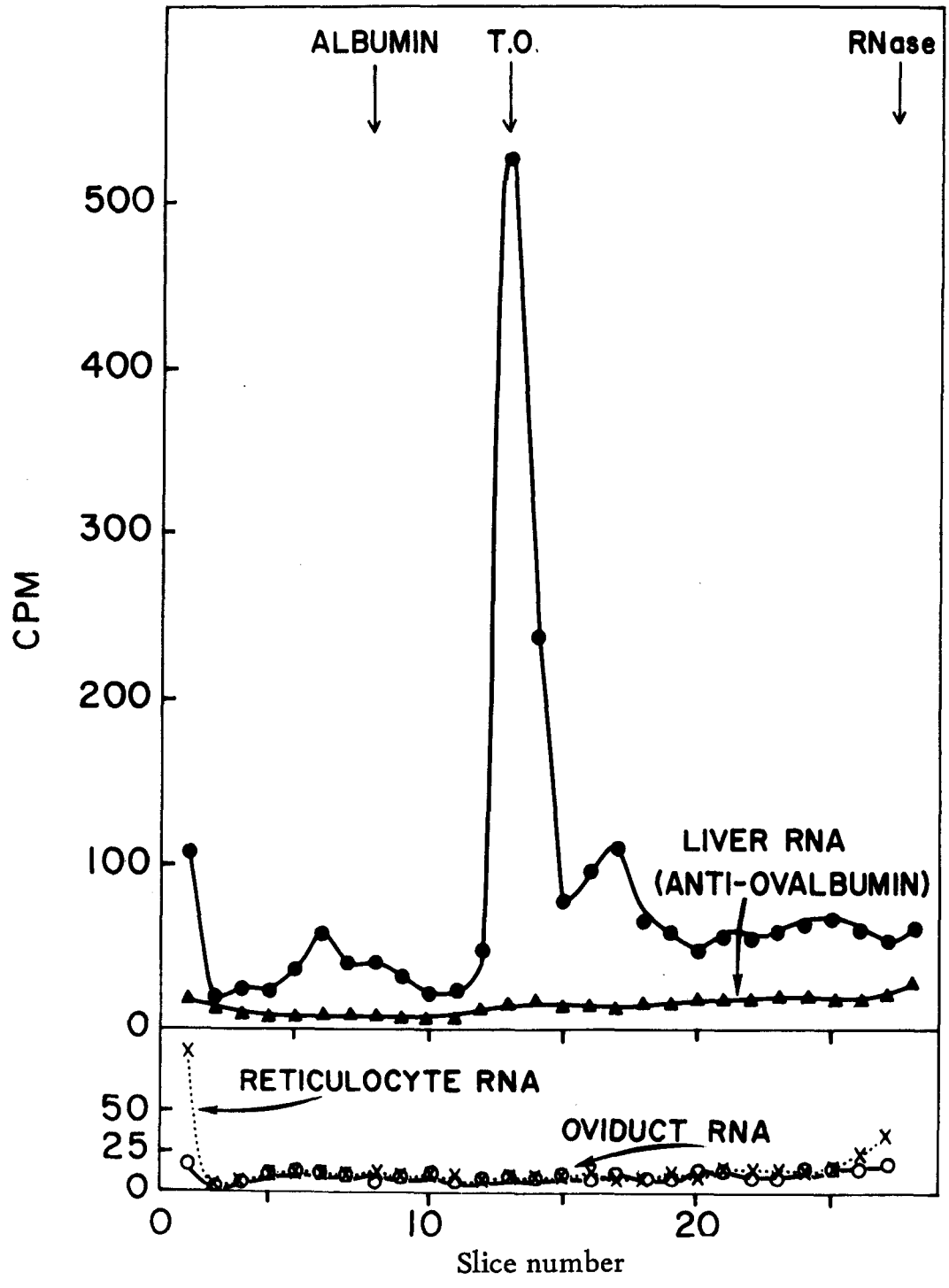

Fig. 3: Sodium dodecyl sulfate-polyacrylamide electrophoresis of *in vitro* synthesized tryptophan oxygenase.
Upper panel: Proteins, synthesized in response to 120 μg/ml of rat liver Poly (A) – containing RNA from animals that had received hydrocortisone were immunoprecipitated with carrier tryptophan oxygenase and anti-tryptophan oxygenase (● ——— ●) and with ovalbumin and anti-ovalbumin (▲ ——— ▲). The washed immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis.
Lower panel: Proteins directed by 14 μg of cellulose purified RNA from rabbit reticulocyte polysomes (X - - - - - - - X) and by 50 μg of cellulose purified RNA from chicken (○ ——— ○) oviducts were treated with carrier tryptophan oxygenase and anti-tryptophan oxygenase.

In Figure 2, the determination of the equivalence point of the homogenous tryptophan oxygenase and anti-tryptophan oxygenase is shown. The antibody inactivates the enzyme and leads to its precipitation at the equivalence point.

To increase the specificity of our identification procedure, the immunologically isolated products were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis. Rat liver tryptophan oxygenase is a tetrameric enzyme ($\alpha_2\beta_2$), with two pair of subunits of identical molecular weight of 43,000 (6, 7); we therefore expected the same electrophoretic behavior of the newly synthesized enzyme components.

Figure 3 shows the results of an experiment which we believe represents the *in vitro* synthesis of tryptophan oxygenase. The major product which is synthesized in response to rat liver mRNA and immunoprecipitated with the specific antiserum comigrates with tryptophan oxygenase subunits upon sodium dodecyl sulfate–acrylamide gels. Two types of control experiments were performed to ensure the specificity of detection for *in vitro* synthesized tryptophan oxygenase.

a. The proteins synthesized in response to rabbit reticulocyte polysomal RNA and chicken oviduct RNA, processed with carrier tryptophan oxygenase and anti-tryptophan oxygenase, did not yield radioactivity above background b.
b. When products directed by rat liver RNA were treated with chicken ovalbumin and anti-ovalbumin (rather than with tryptophan oxyganese and anti-tryptophan oxygenase), the newly synthesized tryptophan oxygenase was not precipitated.

The rate of synthesis of tryptophan oxygenase is proportional to the amount of mRNA added and [^{3}H]leucine incoporation plateaus at the same concentration as the incorporation into total protein (unpublished results), suggesting non-preferential translation of the various liver mRNAs contained in our preparation. Table I compares the inctracellular concentrations of the mRNA for tryptophan oxygenase in livers from control and hormone treated animals. Equal amounts of the mRNA fractions from these livers, processed in identical fashion gave rise to the same incorporation of L-[^{3}H]leucine into total protein; the mRNA from the hormone treated animal leads to a three times higher incorporation of [^{3}H]leucine into tryptophan oxygenase components, suggesting an enrichment in the mRNA content for tryptophan oxygenase after hydrocortisone administration.

Figure 4 shows the accumulation of the mRNA activity for tryptophan oxygenase after hydrocortisone acetate administration for 2 and 4 hours. The observed increase in the mRNA activity, suggesting increase in mRNA levels is as expected from the Actinomycin D inhibition of the induction of tryptophan oxygenase (8).

A correlation exists between the increase in tryptophan oxygenase activity with the tissue level of the mRNA for tryptophan oxygenase after administration of various concentrations of hydrocortisone for 4 hours (data not shown), which again indicates that the increased rate of synthesis of the enzyme is a consequence of the accumulation of its mRNA.

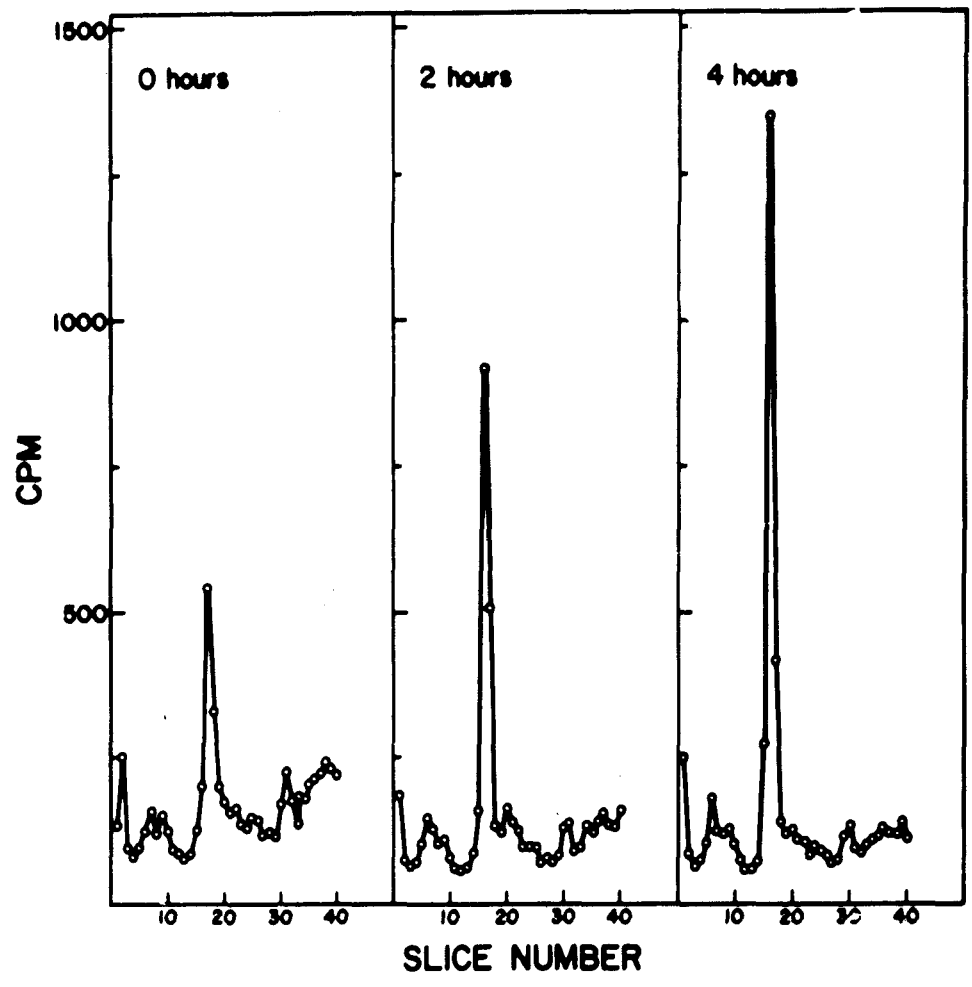

Fig. 4: Accumulation of the mRNA for tryptophan oxygenase after hydrocortisone acetate. Rat liver RNA, extracted from animals that had received no hydrocortisone, and hydrocortisone for 2 and 4 hours respectively were processed as described (5). The released newly synthesized proteins directed by equal amounts of these RNA fractions were analyzed for tryptophan oxygenase (5).

Effect of Hydrocortisone Acetate on the Intracellular Concentration of the mRNA for Tryptophan Oxygenase

	Amount assayed (μg/ml)	cpm incorporated into total proteins	cpm incorporated into released proteins	cpm in tryptophan oxygenase*
Control	120	4.4×10^6	1.25×10^6	290
Hydrocortisone Acetate (3 HRS.)	120	4.2×10^6	1.18×10^6	940

* Represented by the area of tryptophan oxygenase subunits on SDS-polyacrylamide gels.

Conclusion

The increased hepatic concentration of the mRNA for tryptophan oxygenase and its parallelism with the rate of synthesis of the enzyme protein *in vivo* argues in favor of the hypothesis that the accumulation of the specific mRNA activity accounts for the induction of the enzyme. It is at present unknown as to whether this accumulation results from an enhanced rate of transcription of the tryptophan oxygenase gene or from stabilization of pre-existing mRNA, or by activation of the mRNA. It seems that the answer to this question will require the purification of the mRNA for tryptophan oxygenase.

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THE RABBIT RETICULOCYTE LYSATE AS A SYSTEM FOR STUDYING mRNA

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Introduction

In this article we describe the use of the rabbit reticulocyte lysate as an assay system for added mRNA. Some of the techniques we describe are familiar, but we have recently realised that the system can be used in rather subtile ways to provide additional information about the RNA under study. Although our own studies with viral RNA are in their early stages, we believe that the approaches we suggest here will be generally useful to a wide range of workers in all fields of virology. We are taking the opportunity of describing our methodology in considerable detail in the hope that it may be practically useful.

Preparation of reticulocyte lysates

We use rabbits weighing 2 – 3 kg and make them anaemic by injecting them subcutaneously with 2.5 ml of 1.25 % w/v acetylphenylhydrazine on four successive days. We bleed them by cardiac puncture on the ninth day of the schedule. The long gap between the last injection of phenylhydrazine and the collection of the blood allows the damage caused by the phenylhydrazine to be repaired so that although we obtain a lower proportion of reticulocytes the haematocrit is almost 100 % higher, and the reticulocytes are much more consistently active in protein synthesis.

The blood is collected with heparin as anticoagulant, and the reticulocytes collected by centrifugation in a refrigerated centrifuge. They are washed three times with ice-cold saline (0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$) and lysed with 1.5 volumes of ice-cold water per volume of packed cells. The debris are removed by centrifugation at 30,000 g for 15 min at 4°. The supernatant from this spin is stored as 1–2 ml aliquots under liquid nitrogen. Such a preparation seems to be stable indefinitely if stored like this.

Incubation conditions for protein synthesis

The lysate is thawed carefully by rolling in the palm of the hand, and is made 20 μM in haemin before it has completely melted. It is very important not to let the lysate warm up before adding the haemin, and it is also vital to make the haemin solution up correctly – the source of the haemin is not critical. It is made up at a final concentration of 1 mM in approximately 90 % ethylene glycol at pH 8–8.5 by taking a weighed amount of haemin and dissolving it in a small volume (0.2 ml) of

0.5 N KOH together with enough Tris-Cl pH 8.0 to give a final concentration of 50 mM. About 3/4 of the final volume of ethylene glycol is added, and the pH adjusted to around 8 with 1N HCl by rapid swirling or stirring on a pH meter. It is important to avoid local excess of acidity which precipitates the haemin, and to keep the final pH above 7.5. The final volume is adjusted with ethylene glycol, and the concentration checked spectroscopically by adding a sample to 10 mM KCN and reading the absorbance at 540 nm; 1 mM cyanmethaem has an absorbance of 11.1 at this wavelength.

To get protein synthesis by the lysate it is necessary to do three things: adjust the K^+ and Mg^{++} concentrations, add extra amino acids (while the pools of e. g. arginine and alanine are very large, others like leucine, phenylalanine and methionine are present at only a few μmoles/1. Failure to add amino acids causes a serious impairment of the rate and extent of protein synthesis by the lysate). Lastly, a source of ATP and GTP is necessary. For years we added ATP, GTP and creatine phosphate, but in fact creatine phosphate alone is sufficient and much more convenient, since its solutions are neutral, and it does not chelate Mg^{++}. The extent of protein synthesis is linearly dependent on added creatine phosphate up to about 6 mM, and we now add 10 mM to be safe.

So a 'standard incubation mixture' contains the following components: 0,8 ml of lysate, 0.05 ml of salts solution (2 M KCl, 10 mM $MgCl_2$), 0.05 ml of 0.2 M creatine phosphate, 0.05 ml of labelled amino acid solution, and 0.05 ml of an amino acid mixture with the following composition: Ala 3 mM; Arg 0.5 mM; Asn 0.5 mM; Asp 2.0 mM; Cys 0.5 mM; Gln 0.5 mM; Glu 2.0 mM; Gly 2.0 mM; His 2.0 mM; Ile 0.5 mM; Leu 3.0 mM; Lys 2.0 mM; Met 0.5 mM; Phe 1.5 mM; Pro 1.0 mM; Ser 2.0 mM; Thr 1.5 mM; Try 0.5 mM; Tyr 0.5 mM; Val 3.0 mM. One or more of these will be omitted according to the label being used. This mixture is neutralised with KOH to pH 7.5 and is made 10 mM in dithiothreitol to keep Cys and Met in good shape. It is stored, as is the creatine phosphate solution at −20°. Besides these carefully measured components, creatine kinase is added as 'a few crystals' – about 0.1 mg/ml final concentration.

When additions are to be made, one can either add 5–10 % of the final volume and trust that this minor dilution of the standard mixture will have only a slight effect, or alternatively use less lysate and make up the volume with the solution of inhibitor or mRNA. Either way works perfectly well.

Analysis of synthetic products

It is usually wise to monitor the time-course of incorporation of label into TCA insoluble material, since this is often an indication of the nature of problems when they arise (as they often do, particularly with unknown mRNA). Samples of 2–10 μl are removed with a Hamilton microsyringe into 0.2–1.0 ml of distilled water, and the syringe washed up and down 3 or 4 times in the water. This allows one to take successive samples from an array of tubes quickly and accurately with hardly any cross-contamination between samples; the dilution with water stops incorporation completely. When sampling is over, 0.5 ml of 1N NaOH containing roughly 1 mg/ml unlabelled amino acid is added to each sample, and after 15 min at 37° 1 ml of 25 % TCA is added. The samples are filtered into Whatman GF/C

filters, washed with 8 % TCA and glued to cardboard discs for counting in a gas-flow counter. If it is necessary to count the samples in a scintillation counter, the colour of the haemoglobin has to be removed, either by organic solvents like acidified acetone, or by bleaching with H_2O_2. The latter is more convenient, and can be achieved by adding 0.1 ml of 30 % H_2O_2 to the samples before adding the NaOH. After 5 minutes or so, while there is some evolution of O_2, the samples go colourless.

Analysis of samples by SDS-polyacrylamide gel electrophoresis is straightforward if the system of Laemmli is used (1). The interesting thing about these gels is that they allow excellent resolution of proteins with higher molecular weight than globin despite the outrageous overloading with globin (2, 3).

Sucrose gradient analysis

In order to obtain sharp patterns of polysomes and ribosomal subunits from the lysate it is vital to dilute the sample first. Our typical system is to make 50 µl incubations and to stop the reaction by adding 200 µl of ice-cold SMISH buffer (25 mM KCl, 10 mM NaCl, 10 mM Tris-Cl, 1 mM $MgCl_2$, 0.25 mM DTT, pH 7.5). The whole samples is then layered over a 5 ml 15–30 % w/v sucrose gradient in SMISH buffer and spun at 50,000 rpm in the SW 50.1 rotor for periods ranging from 30 min for resolution of polysomes to 2.5 hours for looking at subunits. The temperature is normally 2°. After the run, usually with the brake off (our experience is that faulty or excessive braking is the commonest cause of bad results), the gradients are pumped through a recording spectrophotometer into a fraction collector. The fractions are usually counted by precipitation with 1 % cetyltrimethylammonium bromide (CTAB) in the presence of 0.25 M Na acetate pH 5.1 (the low pH minimises discharge of tRNA) and about 500 µg of carrier RNA. A solution of 2 % CTAB in 10 mM NaAc buffer is added first, followed by an equal volume of 0.5 M NaAc buffer containing the carrier RNA. A precipitate forms at once, and is allowed to clot slightly before filtering and washing with water. This procedure has the advantage of selectively precipitating all RNA species (hence aminoacyl-tRNA and peptidyl-tRNA) while solubilising most proteins, including globin. This gets round the serious problem of colour quenching at the top of the gradients.

Shift assays

The rationale of these assays is described below; this section simply gives some critical experimental details. A lysate is incubated under the standard conditions except that a solution of 2 mM sparsomycin is substituted for the labelled amino acid. The first incubation is usually for 5 min at 30°. The tube is then chilled on ice, and 2 µl of ^{35}S-met-tRNA$_f$ (10,000–100,000 cpm) added, followed by 1–5 µl of mRNA dissolved in water, or an equivalent volume of water for the control. The tubes are incubated for a further 2 min at 30° and are then analysed on sucrose gradients as described above (typically a 2 hr centrifugation). This assay can be modified by using 50 µg/ml diphtheria toxin and 0.15 mM NAD with 0.1 mM puromycin instead of the sparsomycin. The latter system gives a higher yield of

active 40S subunits and hence of initiation complexes, and also allows met-X dipeptides which sparsomycin tends to prohibit (because it inhibits peptide bond formation; diphtheria toxin + NAD is a translocation inhibitor). Another modification, which does away with the need for purified met-tRNA$_f$ is described below.

Translation of added viral RNA

The classic mRNA for bacterial cell-free systems is the genome of the small RNA phages. These studies have allowed many insights into viral physiology to be gained. We presume that similar studies with the genomes of eukaryotic RNA phages will also be helpful in understanding their biology. But we note that eukaryotic cell-free systems translate bacterial mRNA poorly if at all, and feel strongly that studies in which bacterial cell-free systems are used to translate eukaryotic mRNA are irrelevant if not misleading from the true goal.

Most published studies of the translation of viral RNA in eukaryotic systems concern the translation of EMC RNA in a cell-free system from krebs II ascites cells. They have not been terribly illuminating in terms of understanding either protein synthesis or viral physiology; the same can be said for most successful efforts at translating added mRNA in heterologous systems. One succeeds chiefly in adding support to the concept of mRNA, a task once vitally important but rather hackneyed by now. There are several interesting points still to be clarified, however. First, the lysate system is strongly inhibited by added EMC RNA, whereas the ascites system is stimulated (4, 5). The inhibition is relieved somewhat by adding S100 from ascites cells to the lysate, and such an addition also allows some translation of the RNA. This phenomenon is not at all understood; apparently a protein and an RNA are responsible for the effect (4). The inhibition of the lysate by added RNA is common; sometimes it is due to trivial effects such as contamination of the preparation with phenol or heparin, which can be removed by washing with 3M Na Acetate pH 6 or 2M LiCl (6). In other cases it may be that the viral RNA competes effectively for ribosomes with the endogenous globin mRNA, but that factors necessary for the completion of translation are missing – this may be the case with EMC RNA. Another common cause of inhibition is the presence of double stranded RNA. The lysate is sensitive to as little as 0.01 ng/ml dsRNA (7), so that amounts of contamination with viral RF, RE or RI too low to be detected by conventional means can cause trouble. A good example of this was provided by McDowell *et al.*(8) who obtained good synthesis of viral proteins specified by *in vitro* synthesised reovirus RNA, but who also found that synthesis in the lysate stopped abruptly after only 10 min when they added the RNA. Inhibition by added RNA is not necessarily a disaster, therefore. In principle it is possible to abolish this inhibition by treating the RNA with RNase III from E. coli (9), but this is not a commercially available enzyme and is reputedly difficult to purify. It may be that if an RNA inhibits the lysate with the characteristic kinetics of dsRNA (a brief uninhibited phase followed by an abrupt cessation of synthesis) this is the best possible evidence that the RNA does contain true dsRNA. We have tested Sendai RNA in the lysate, and found that it behaved as if it were mostly dsRNA. Of course it would be naive ever to expect the lysate to be stimulated by added mRNA unless a protein

with an unusual content of the amino acid used as label were being synthesised. Most of the ribosomes are already active in protein synthesis, and the most one can hope for is therefore to wean them off globin mRNA and into the added message.

We have recently done some experiments with TMV RNA as a model for this kind of work, and have been impressed with a number of somewhat disturbing features. It is easy to prepare both TMV and extract the RNA from the virions with phenol and SDS, but although the RNA obtained was in our hands about 75 % pure 27S, it inhibited synthesis overall rather strongly by inhibiting initiation of protein synthesis. However, as more TMV RNA is added, viral products predominate over globin. We have not detected the synthesis of coat protein and the products are a collection of proteins with a modal MW of about 70,000; unless that is one uses doses of RNA below 100 μg/ml, in which case there is only one product, a band at 140,000 daltons. At concentrations above 100 μg/ml there is no detectable synthesis of this band. At first we thought that this might be due to preferential use of the smaller RNA fragments present in the preparations of RNA over the intact RNA, but this was shown to be unlikely by adding pure 27S RNA, and finding that it behaved exactly the same way. We do not yet know whether we are seeing false initiation, premature termination or some kind of specific proteolysis. A further finding was that the purified TMV RNA did not inhibit endogenous synthesis at all, even at inputs of 200 μg/ml, at which level the crude material inhibited by 90 %. We cannot account for these findings, but if they have any generality it means that one should probably try to use added mRNA at more physiological input levels, and also interpret the data obtained by this kind of experiment very cautiously.

The shift assay for mRNA

Since a protein cannot be made without initiating its synthesis, one can use the relative numbers of proteins made as a measure of the relative effectiveness of two mRNA species at competing for a limited supply of ribosomes. However, because proteins can be modified – at the extreme destroyed – after their synthesis, and because factors other than the initiation of their synthesis may affect their production it would be very nice to have an assay for initiation which did not rely on protein synthesis. We have recently stumbled on such an assay in the course of studies of the mechanism of initiation of protein synthesis in the lysate. We found that native 40S subunits can be labelled with ^{35}S met-tRNA$_f$ even though protein synthesis is completely inhibited by a wide variety of inhibitors which do not affect the initiation of synthesis – e. g. sparsomycin, cycloheximide and diphtheria toxin. Such 40S/met-tRNA$_f$ complexes are natural intermediates in the initiation of protein synthesis, and the next step in the pathway is the binding of mRNA, followed by attachment of the 60S subunit to give an 80S initiation complex. Thus if one adds labelled met-tRNA$_f$ to a standard incubation which has been preincubated with an inhibitor of synthesis and analyses the reaction on a sucrose gradient, one finds label only at the top of the gradient and on the 40S subunits. When mRNA is added, however, there is a shift of counts from the 40S subunits to the 80S region, and the degree of shift is proportional to the amount of mRNA added. This provides an extremely sensitive assay for mRNA – we can easily detect 0.2 μg of globin mRNA (1 pmole) contained in 1–5 μl of solution. Besides this

sensitivity, the assay has the advantage that one can immediately tell whether the RNA in question has only one or more than one binding sites for ribosomes, since a length of RNA which has two binding sites will cause a shift of label from 40S to the dimer region of the gradient. We have some suspicions about TMV in this respect, but it is quite certain that we can detect such goings-on because random AUG can cause polysome formation when assayed at the appropriate input level. Besides watching the shift and measuring its extent, one can also take the shifted counts and analyse them to see what the met-X dipeptides are. Here again one may pick up multiple initiation sites by finding more than one new dipeptide (though one has to be sure that tripeptides are not being formed). This suggests the possibility of doing direct competition experiments between different mRNA species. Such experiments can be done very easily if the second amino acid specified by each of two mRNAs is known, since one can add labelled tRNA corresponding to these amino acids instead of met-tRNA$_f$. This procedure is complicated by the need to correct for the different pool sizes of each species of tRNA, and yet another way of doing competition experiments is to use labelled mRNA. We have shown that one can use the shift assay to detect binding of labelled sea-urchin histone mRNA and globin mRNA, but so far we have not pursued these studies to their natural conclusion. Neither have we tried the other obvious experiment of using this assay as a means of purifying mRNA from mixtures with neutral RNA.

Finally, it is worth pointing out that one can do shifts without the need for making met-tRNA$_f$, which is the limiting step in these experiments. The problem is to inhibit globin synthesis but still get charging of the tRNA pools with added ^{35}S methionine; the solution is to incubate the lysate with 0.1 mM puromycin before adding the label, and later adding either sparsomycin or diphtheria toxin and NAD followed by mRNA. The background of counts on ribosomes is higher than in the classic shift (10), but is perfectly acceptable, as can be seen in figure 1. We have not yet analysed the dipeptides formed in this reaction.

Conclusion

The reticulocyte lysate has two great advantages over other existing systems which can be used for studying protein synthesis by added mRNA. It is easier to use and prepare than the ascites cell-free system, the Xenopus oocyte system, or the system of Schreier & Staehelin (11). It is much more active than any of these systems, and although its endogenous activity is high, at least this activity is well characterised so that the possibility of confusion of endogenous synthesis with the new synthesis is in practice not much of a problem unless the new products are exceedingly diverse. Its low nuclease content is a further advantage, though we would be wrong in claiming that no nuclease activity exists.

People often accuse the reticulocyte of being an atypical cell and therefore a poor model system for the more interesting aspects of control of protein synthesis. We believe this to be an envious and empty criticism; it may be true that the lysate lacks the ability to translate EMC RNA efficiently, and that intact reticulocytes fail to respond to amino acid starvation in the way that most cells do, but these failures actually make it a better system for studying these effects, since it constitutes an

assay system for the factors involved. In no case, not even the case of the requirement for added haemin to prolong linear protein synthesis, is there an example of the reticulocyte doing something that other cells do not also do, except yield highly active cell-free systems.

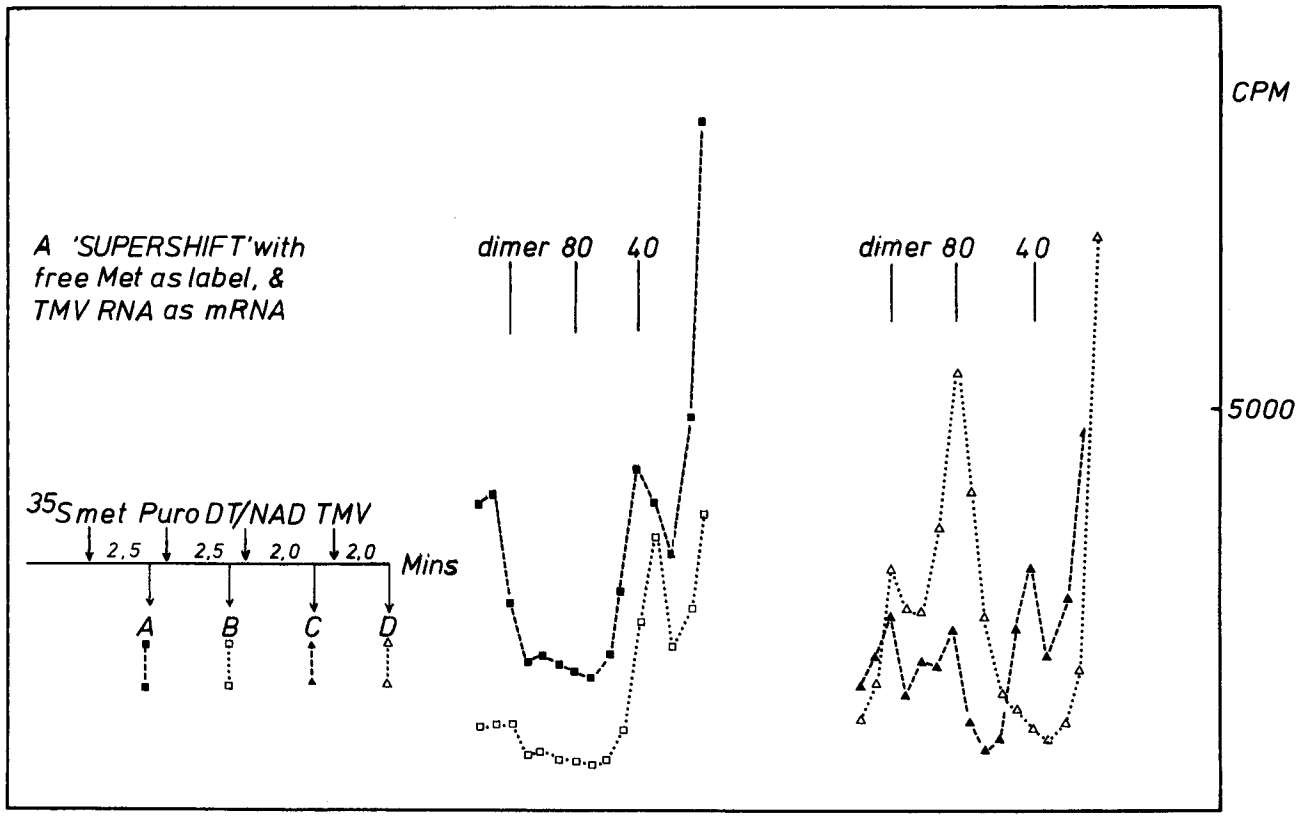

Fig. 1:
A standard incubation mixture was incubated at 30 ° with ^{35}S methionine. After 2mins incubation a sample was removed and diluted with SMISH buffer as described in the methods section. At $2\frac{1}{2}$ min, the mixture was made 1 x 10^{-4}M in puromycin, and another sample of the mixture removed for gradient analysis at $4\frac{1}{2}$min. At 5 min, the mixture was made 50 μg/ml in diphtheria toxin and 0.15 mM in NAD, and a samples removed for analysis at 7 min. Finally, 5 μg of TMV RNA was added to the remaining 50 μl of mixture, and the last sample taken 2 min after this addition. The four samples were then analysed by sucrose gradient centrifugation for 1.5hr on a 10 % – 30 % gradient in SMISH buffer at 45,000 rpm and 2 ° in the spinco 50.1 rotor. The gradients were fractionated and the radioactivity determined by CTAB precipitation.

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STUDIES ON THE BINDING OF ADP-RIBOSYLATED HUMAN TRANSLOCATION FACTOR TO RIBOSOMES

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Translocation factor TF II is one of the two soluble proteins required for the polypeptide chain elongation on the mammalian ribosome. It is involved in the translocation of peptidyl-tRNA from the so-called acceptor site to the donor site of the ribosome (1). TF II promotes the binding of GTP to the ribosome (2–5); furthermore, it itself binds to the ribosome in the presence of GTP, forming thereby a ternary complex (2–6). GTP bound to the ribosome is cleaved into GDP and Pi, and the energy released is utilized for the translocation step (1).

For sometime, we have been studying the interactions of TF II with the ribosome in a purified cell-free system developed from the human lymphatic tissue. This report will describe a recent approach using a modified and radioactively labelled form of the factor to gain insight into the mechanisms of TF II-ribosome interactions.

TF II represents the unique protein in the mammalian cell, known to be attacked by diphtheria toxin (7, 8). The inactivation of the factor by the toxin results in inhibition of protein synthesis. Diphtheria toxin requires the presence of NAD^+ for its effect (7, 8). The reactions resulting in the inactivation of TF II by diphtheria toxin and NAD^+ can be summarized as follows:
Diphtheria toxin + NAD^+ + TF II $\leftrightharpoons$ (Diphtheria toxin • NAD^+ • TF II) $\leftrightharpoons$ Diphtheria toxin + ADP-ribosyl-TF II + nicotinamide + H^+ (9). ADP-ribosyl-TF II represents the translocation factor carrying one residue of covalently bound adenosine diphosphoribose (10). It is inactive in polypeptide synthesis, but still capable of binding to the ribosome (11). Using NAD^+, radioactively labelled in its adenosine, ribose or phosphate moiety, it is possible to label the factor radioactively (9. 10). This makes direct studies on the binding of the factor to ribosomes possible.

The binding of ADP-ribosyl-TF II to the ribosome is demonstrated in Table 1. As can be seen, the binding of radioactivity to ribosomes was strictly dependent upon the presence of both diphtheria toxin and TF II; it was more than three-fold stimulated by the addition of GTP as well as GDP.

The binding of ADP-ribosyl-TF II seemed to be of specific nature as the unmodified factor competed both in the presence or in the absence of GTP apparently for the same binding site(s) on the ribosome. As can be seen in Table 2, increasing amounts of the unmodified factor inhibited the binding of ADP-ribosyl-TF II to the ribosome. As TF II fraction used in this experiment represented a partially purified protein fraction, protein fractions (12) of different TF II-content were added as source of unmodified factor. The total protein amount added was kept constant throughout the

Table 1: Binding of ^{3}H ADP-ribosyl-TF II to ribosomes, as assayed by the recovery of the bound radioactivity on pelleted ribosomes

System	Ribosome-bound ^{3}H ADP-ribosyl-TF II (pmoles / 2.7 A_{260} units)
Complete	8.7
-Diphtheria toxin	0,1
-TF II	0.1
-Diphtheria toxin, -TF II	0.1
-GTP	2.8
-GTP, +GDP	9.7

Assay conditions were as described (11).

Table 2. Effect of the addition of TF II – protein fractions of different purity on the binding of ADP-ribosyl-TF II to the ribosome

Unmodified TF II protein fraction added	pmoles ^{14}C ADP-ribosyl-TF II bound/48.6 pmoles ribosomes		pmoles ^{3}H Phe incorp./ 96 pmoles ribosomes 1 ml, 15 min, 37 °C	
	+ GTP	– GTP	+ GTP	– GTP
None	4.0	2.6	6.0	3.0
117 µg fract. II proteins (= 5.6 pmoles TF II)	4.9	3.2	10.0	2.0
114 µg fract. IV proteins (= 18.2 pmoles TF II)	3.9	2.5	22.0	3.0
114 µg fract. V proteins (= 44.0 pmoles TF II)	2.0	0.9	43,0	6.5
117 µg fract. VI proteins (= 128.0 pmoles TF II)	1.1	0.8	36.0	4.0

74 pmoles ^{14}C ADP-ribosyl-TF II (in 58 µg fraction VI proteins) were incubated in the presence or absence of 1 mM GTP with 3.8 A_{260} units (= 72 pmoles) ribosomes and with or without indicated TF II-containing protein fractions. The reaction mixtures contained the salt concentrations as described (11). TF II-content of the protein fractions added had been determined prior to the experiment by ADP-ribosylation. After the incubation, the ribosomes were isolated by centrifugation and ribosome-bound radioactivity determined as described (11). 0.5 A_{260} units out of resuspended ribosomes were assayed under standard conditions of poly Phe synthesis (11) for ribosome-bound TF II activity. Specific activity of ^{14}C NAD^{+}, used for ADP-ribosylation of TF II was 136 Ci/mole; specific activity of ^{3}H Phe was 1150 Ci/mole. Counting efficiencies for ^{3}H and for ^{14}C were 20 percent and 48 percent, respectively.

assay. The addition of TF II fraction VI proteins containing a nearly two-fold excess of the unmodified factor caused approximately 73 percent inhibition of GTP-dependent binding of ADP-ribosyl-TF II. In the absence of GTP, the inhibition of the binding of ADP-ribosyl-TF II by the unmodified factor amounted to 69 percent. The inhibition of the binding of ADP-ribosyl-TF II by unmodified TF II was accompanied

by an increase of ribosome-bound TF II activity, assayed in poly Phe synthesis. In experiments not shown, the unmodified factor compared to ADP-ribosyl-TF II displayed a significantly higher affinity for its ribosomal binding site. Its binding to the ribosome in the presence of GTP, assayed in poly Phe synthesis, was not inhibited even by the addition of a great excess of ADP-ribosyl-TF II.

Recent studies from several laboratories suggest the existence of overlapping or identical binding site(s) for both AA-tRNA and the translocation factor (13–18). ADP-ribosyl-TF II and Phe-tRNA also displayed a competition for apparently the same or overlapping binding site(s) (Fig. 1). Increasing amounts of Phe-tRNA, prebound non-enzymatically to the acceptor site, significantly reduced the extent of binding of ADP-ribosyl-TF II, again both in the presence or in the absence of GDP which was used instead of GTP in order to avoid any translocation of bound Phe-tRNA. This result appears to suggest that both ADP-ribosyl-TF II and Phe-tRNA bind to a great extent, if not to the same site, at least to the same ribosomes. This

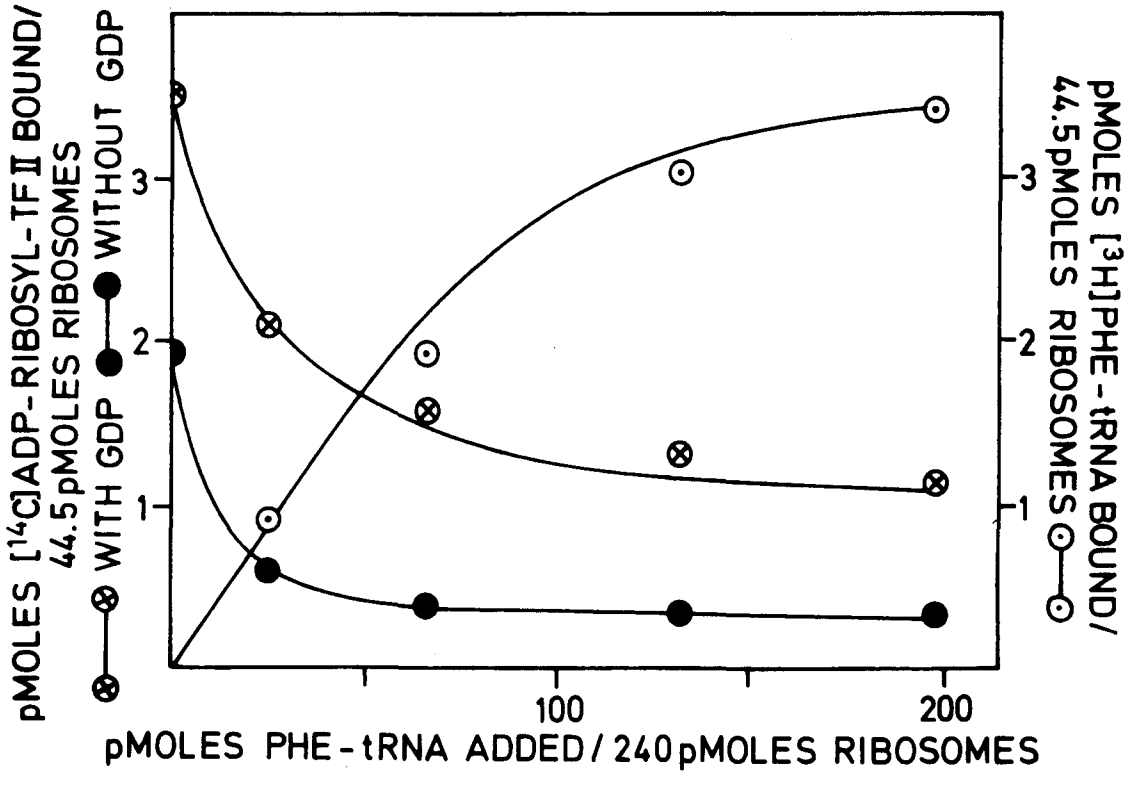

Fig. 1: Effect of Phe-tRNA, bound in the presence of poly U non-enzymatically to the ribosome, on the subsequent ADP-ribosyl-TF II binding. 12.4 A_{260} units (= 240 pmoles) ribosomes were incubated in the presence of 150 μg poly U and, with or without, indicated amounts of $tRNA_{E.coli}$, 2.1 percent charged with ^{3}H Phe, specific activity 1150 Ci/mole; ionic conditions (15 mM $MgCl_2$, 60 mM KCl, 30 mM Tris HCl pH 7.4) of non-enzymatic binding. Ribosomal complexes were isolated by centrifugation and resuspended in the same medium. 4 A_{260} units (= 74 pmoles) ribosomes were incubated under the same ionic conditions in the presence of 83 pmoles of ^{14}C ADP-ribosyl-TF II and, with or without, 1 mM GDP. Thereafter, ribosomes were centrifuged again through a cushion containing the same salt concentrations plus 0.5 M sucrose. Ribosome-bound ^{3}H (counting efficiency 20 percent) and ^{14}C (counting efficiency 48 percent) radioactivities were counted in separate channels of a Packard (Tricarb) liquid scintillation spectrometer as described (11).

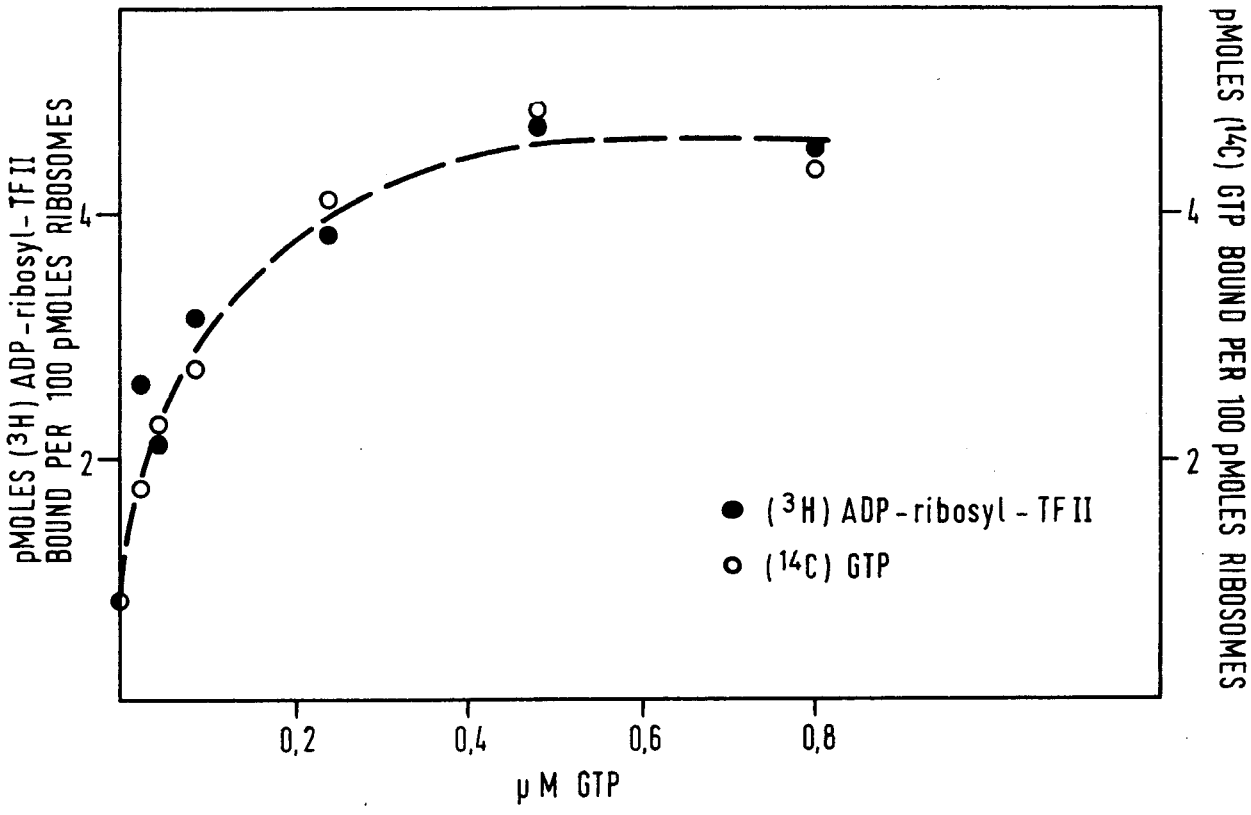

Fig. 2: Effect of increasing concentrations of GTP upon the binding of ADP-ribosyl-TF II to the ribosome. 82 pmoles ^{3}H ADP-ribosyl-TF II were incubated with 6.1 A_{260}units ribosomes and indicated concentrations of ^{14}C GTP, specific activity 36 Ci/mole. The reaction mixtures contained ionic concentrations as described (11). After centrifugation ribosome-bound ^{3}H and ^{14}C radioactivities were determined in separate channels of a Packard (Tricarb) liquid scintillation spectrometer, as described (11).

ribosomal population capable of binding both of the mentioned components of polypeptide synthesis might represent that of „active" ribosomes. Inhibition of the binding of ADP-ribosyl-TF II observed, however, can be explained in terms of some allosteric effects of Phe-tRNA bound as well as by the overlapping or the identity of the binding sites of both of these components.

As demonstrated in Table 3, the modified factor bound primarily to the larger ribosomal subunit. Only slight binding appeared to occur to 40 S particles. Nevertheless, the addition of 40 S to 60 S particles significantly increased the extent of ribosome-bound ADP-ribosyl-TF II, presumably by stabilizing the complex between the modified factor and the larger ribosomal subunit. Similiar results have also been previously obtained with the unmodified factor (20).

Fig. 2 shows the binding of ADP-ribosyl-TF II to the ribosome as a function of GTP. The double labelling used in this experiment (^{3}H ADP-ribosyl-TF II versus ^{14}C GTP) makes the determination of the stochiometry of ribosome-bound factor to GTP possible; approximately 1 mole of the modified factor was bound to ribosomes per 1.4 mole of GTP after subtraction of blanks. In this experiment, 0.4 μM GTP appeared to be nearly sufficient for the maximum stimulation of ADP-ribosyl-TF II-binding to ribosomes.

Table 3. Binding of ADP-ribosyl-TF II to ribosomal subunits

Particles	Ribosome-bound ADP-ribosyl-TF II	
	pmoles/4 A_{260} units	pmoles/100 pmoles ribosomes
40 S	2.9	1.1
60 S	8.1	7.5
40 S + 60 S	14.2	18.5

108 μg TF II fraction VI proteins containing 94 pmoles ^{3}H ADP-ribosyl-TF II were incubated for 5 min at 37 °C in the presence of 1 mM GTP with 5 A_{260} units 40 S or 5 A_{260} units 60 S, or 1.5 A_{260} units 40 S plus 3.5 A_{260} units 60 S particles. The subunits used were less than 3 % cross-contaminated as assayed in poly Phe synthesis (19). Ribosomebound radioactivity was determined as described (11). Counting efficiency for ^{3}H was 20 %. The specific activity of ^{3}H NAD^+ used was 591 Ci/mole. Calculation of molar amounts of ribosomal subunits from A_{260} was based on assumptions described (19).

The results described suggest that use of radioactively labelled ADP-ribosyl-TF II can provide a useful assay system for characterization of the ribosomal binding site of the translocation factor. ADP-ribosyl-TF II has been recently cross-linked by means of bifunctional reagents N,N'-(1,2-phenylene)dimaleimide and 1,5-difluoro-2,4-dinitro-benzene to ribosomal proteins (21). This represents a new possibility for structural work on TF II-binding site on the ribosome.

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PREPARATION AND FUNCTIONAL INTERACTION OF AN AMINOACYL-tRNA SYNTHETASE COMPLEX AND A PEPTIDE INITIATION FACTOR

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Abstract

Five aminoacyl-tRNA synthetases exist in rabbit reticulocytes as an enzyme complex of about 5×10^5 molecular weight. The complex binds to ribosomes. In this state it appears to promote the synthesis of Met-$tRNA_f^{Met}$ bound to ribosomes with a presumptive initiation factor and ApUpG. The molar ratio of the synthetase complex and $tRNA_f^{Met}$ is near unit in reticulocytes. Disruption or removal of the complex from ribosomes may be a primary factor in the relatively low synthetic efficiency for protein synthesis of most fractionated cell-free systems.

Introduction

The primary lesion in neoplasis may involve a block in cell differentiation. This might be at either the level of transcription or translation or at both of these levels. Fractionated cell free systems of high synthetic capacity are needed to search for hypothetical inhibitor or promotor factors. However, fractionated systems for translation of mRNA from eukaryotic organisms have had the invariable characteristic of relatively low synthetic activity compared with the intact cells or unfractionated cell lysates from which they were derived. This is a limiting factor in the detection and assay of regulatory elements that have a quantitative effect in normal or neoplastic cells. A component that would cause a two-fold change in the rate of synthesis of a protein within the intact cell might well have a dramatic effect on its physiology, but probably would not be detected in most fractionated systems. This difficulty may be reflected in the *in vitro* assay of mRNA. Results from a number of laboratories have lead to the widely accepted concept that there is little or no discrimination in the translation of different mRNA's at the level of peptide initiation (1, 2, 3). However, recent work from several laboratories (4, 5) strongly supports earlier indication of factors with specificity for different species or classes of mRNA (6). Also, such systems may be particularly important in studying certain types of cellular modifications such as hypothetical suppressors of nonsense mutations in eukaryotic cells.

We have been interested in the regulatory role tRNA and the aminoacyl-tRNA synthetases may play in intact reticulocytes as well as the upper limit they may establish for the rate of *in vitro* protein synthesis. Previously we demonstrated that some but not all of the aminoacyl-tRNA synthetases are associated to a high degree with ribosomes during their preparation and appear to have been bound to the ribosomes, probably to the 60S ribosomal sununit, in the intact cell (7). Here we describe an enzyme complex that appears to contain five aminoacyl-tRNA synthetases including the enzyme for $tRNA_f^{Met}$. The complex binds to isolated 60S ribosomal subunits. The Met-tRNA synthetase of the complex appears to function in synthesis of Met-tRNA while both deacylated $tRNA_f^{Met}$ and the complex are bound to the ribosomes.

Material and Methods

A detailed description of the preparation of rabbit reticulocytes, reticulocyte ribosomes, reticulocyte peptide elongation enzymes, tRNA and assay conditions for these factors are given elsewhere (8, 9). Detail of the assay procedure used for aminoacyl-tRNA synthetases has been published (7). Generally this activity was determined in 0.5 ml reaction mixtures containing 100 mM Tris · HCl, pH 7.5, 2×10^{-6} M ^{14}C · amino acid (specific activity 100 mCi/mmole), 2 mM ATP (previously adjusted to pH 6.5 with KOH), 20 mM β-mercapto-ethanol, and 100 μg of rabbit liver tRNA. KCl and $MgCl_2$ were added to give the concentrations for each amino acid previously given (7).

The assay and isolation of the 40S ribosomal subunit Met-$tRNA_f^{Met}$ binding factor has been described (10) as has the preparation of the subunits from reticulocyte ribosomes used in these studies (11). Generally, for assay of the tRNA binding factor each assay reaction contained in a total volume of 0.25 ml: 20 mM Tris · HCl, pH 7.5, 100 mM KCl, 8 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μg of poly U, 60 pmoles Phe-tRNA (about 100 μg of tRNA charged generally with ^{14}C · phenylalanine, 100 Ci/mole), 90 μg of 40S ribosomal subunits and binding factor to give 4 to 6 pmoles total of Phe-tRNA bound. ApUpG and Met-tRNA or other codons and aminoacyl-tRNA were substituted for poly U and Phe-tRNA were indicated.

RESULTS

Isolation and Properties of the Met-tRNA Synthetase Complex

A detailed description of the isolation and characterization of the Met-tRNA synthetase complex will be given elsewhere, however, the steps of the procedure and the yield obtained is presented in Table I. The complex has been isolated from both the high speed supernatant and the salt wash fraction removed from rabbit reticulocyte ribosomes with 0.5 M KCl. We prefer the former for isolation of the complex as indicated in Table I, in that it allows the simultaneous preparation of the peptide elongation factor, EF-I, which is separated from the synthetase complex on Sepharose 4B.

The steps shown in Table I provide a purification of about 2300 fold from the high speed supernatant. This purified material has been used in most of the experiments described below. On the basis of centrifugation data in sucrose or glycerol

gradients or in the analytical ultracentrifuge, as shown in figure 1, we estimate that about two-thirds of the protein in the preparation is associated with the synthetase complex. The activity of this preparation for the formation of aminoacyl-tRNA with various amino acids is shown in Table II. The activities for arginine, isoleucine, leucine, lysine and methionine are relatively high and in an approximately constant ratio throughout the final steps of the isolation procedure. The preparation has no detectable EF-I or EF-II, but contains relatively low amounts of synthetase activity for cysteine and glutamine. The activity for these amino acids is nearly eliminated in material isolated from the protein peak in sucrose gradients of the type shown in figure 1. These enzymes appear to sediment more slowly than the main component

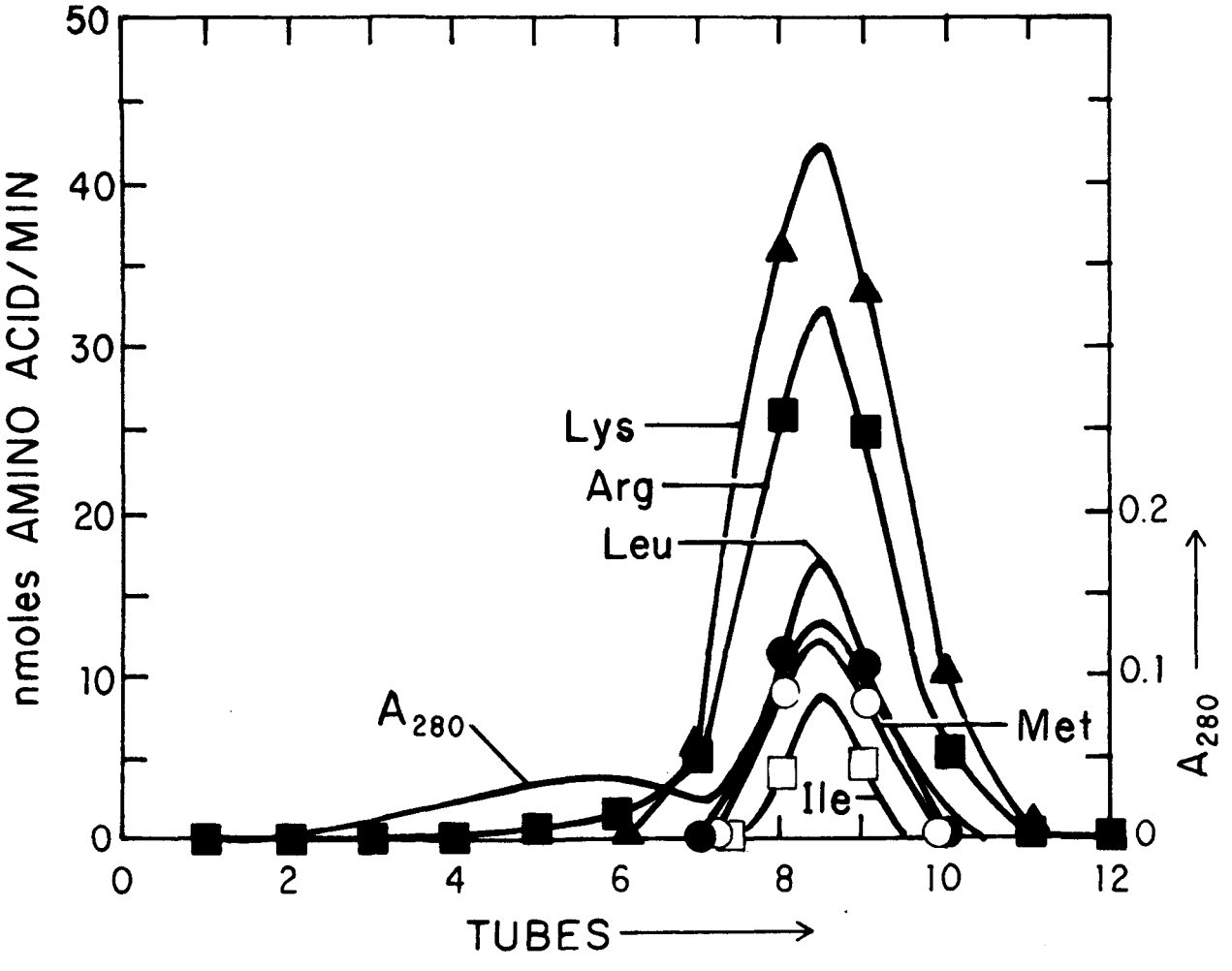

Fig. 1: Distribution on a Sucrose Gradient of Protein and Aminoacyl-tRNA Synthetase Activity of the Enzyme Complex

The Met-tRNA synthetase complex was purified through the fractionation steps shown in Table 1. Then, 0.5 mg of this material was layered on a 10 % to 30 % sucrose gradient containing 100 mM KCl, 20 mM Tris · HCl, pH 7.5, 5 mM β-mercapto-ethanol, and 1 mM dithioerythritol and centrifuged at 40,000 rpm in a SW 41 rotor for 10 hours. Aliquots of 10 µl for arginine, lysine and 50 µl for methionine, leucine and isoleucine from the 1.0 ml fractions of the gradient were used in the standard assay procedure to determine the indicate enzyme activities. The activities of each enzyme were adjusted to a common base to facilitate a direct comparison of the enzyme activities within the complex.

Table 1. Purification of an Aminoacyl-tRNA synthetase complex

Fractionation Step	Total Recovery Protein mg	Enzyme units*	Specific Activity units/mg ptn	Purification
High Speed Supernatant	1.37×10^5	2320	0.017	–
40%–70% $AmSO_4$	5000	1340	0.264	15.7
Sepharose 4B	138	590	4.25	248.0
Hydroxylapatite	10.6	150	14.2	829.0
Phosphocellulose	3.07	124	40.4	2360

*nmoles of Arg-tRNA/minute formed in the standard assay procedure.

Table 2. Aminoacyl-tRNA Synthetase activity in the Met-$tRNA_f$ Complex

Amino Acid	Purified Complex activity*/mg ptn	Purified Complex Total Activity Recovered	Initial Total Activity	% Recovered in Complex	Present in Complex
Arg	32.2	124	2320	5.3	Yes
Cys	1.3	5.0	469	1.1	? (No)
Glu	1.1	4.2	443	0.9	? (No)
Ile	4.5	17.3	1175	6.5	Yes
Leu	12.9	49.7	1344	3.7	Yes
Lys	42.6	164	2547	6.4	Yes
Met	10.5	40.4	603	6.6	Yes
All others	0.5	–	–	0.5	No

*nmoles of aminoacyl-tRNA formed/min in standard assay.
Activity for all other amino acids was less than 0.5 nmoles of aminoacyl-tRNA formed per mg protein in complex and recovery was less than 0.1×10^{-3}.

Table 3. Properties of the Aminoacyl-tRNA Synthetase Complex

1. Molecular weight	about 500,000
2. $S_{w,20}$	14.7
3. SDS Gel Subunits	about 14
4. Met charging for both $tRNA_M^{Met}$ and $tRNA_f^{Met}$	
5. Binds to and charges on the 60S ribosomal subunit	

in sucrose gradients. The significance of their presence in the preparation at this stage of purification is not clear. They may belong to another, slightly smaller complex, however, we conclude that they are not an integral part of what we propose to call the Met-tRNA synthetase complex. The level of aminoacyl-tRNA synthetase activity for all of the other common amino acids is below the limits of the assay system used. Physical characterization of the Met-tRNA synthetase complex has not been completed, however, as summarized in Table 3, it has a sedimentation coefficient of 14.7 S and a molecular weight of about 500,000. As anticipated it gives multiple bonds on SDS gel electrophoresis. We believe we can detect 14 distinct bands, which might be subunits of the various enzymes of the complex, however, this is an approximate value. Nucleic acid including tRNA is below detectable levels at this stage of purification and does not appear to be an integral part of the complex.

A most interesting feature of the Met-tRNA synthetase complex is its propensity toward binding to ribosomes, particularly to the 60S ribosomal subunit, and its apparent ability to carry out its synthetic functions while so bound. The distribution on sucrose gradients of Arg-tRNA and Lys-tRNA synthetase activity in the presence of isolated 40S or 60S ribosomal subunits is shown in figure 2. The complex from the salt wash fraction was mixed with the indicated type of ribosomal subunits then layered on sucrose gradients which were then centrifuged for different times to bring the ribosomal subunits to the indicated position in the gradient. The position of the synthetase complex was determined by assaying appropriate aliquots of fractions taken from the gradient. Similar results have been obtained with binding of purified complex. The five amino-acyl-tRNA synthetases present in the complex give similar patterns, indicating that the complex was maintained as a unit. Nearly all of the synthetase activity was bound to the 60S ribosomal subunits, but very little binding was observed with 40S subunits. Phe-tRNA synthetase present in the salt wash also binds to the 60S subunit but is not present in the purified complex. This reflects tight binding of the complex to the 60S subunit for it to remain associated with the subunit during sucrose gradient centrifugation. Also, it should be noted that the activity of the synthetases were determined by taking aliquots of gradient fractions into reaction mixtures that differed from the gradient only in sucrose concentration and having tRNA, the appropriate amino acid and ATP. In other similar studies we have observed that synthetase activity is maintained when ribosomes are added to reaction mixtures under conditions in which the synthetases will be bound to the ribosomes. These observations appear to indicate that synthetases maintain their synthetic activity when bound to the ribosomes.

Purification and Properties of the tRNA Binding Factor

The relations outlined above carry the implications that the enzymes of Met-tRNA synthetase complex may function in a physiologically significant manner while they are associated with ribosomes within intact reticulocytes. A protein component capable of promoting codon-directed binding of $tRNA_f^{Met}$ to 40S ribosomal subunits, presumably a peptide initiation factor, was used to further test this hypothesis. This protein factor appears to be similar or identical to the factor that has been reported by several laboratories (12, 13, 14, 15) and extensively

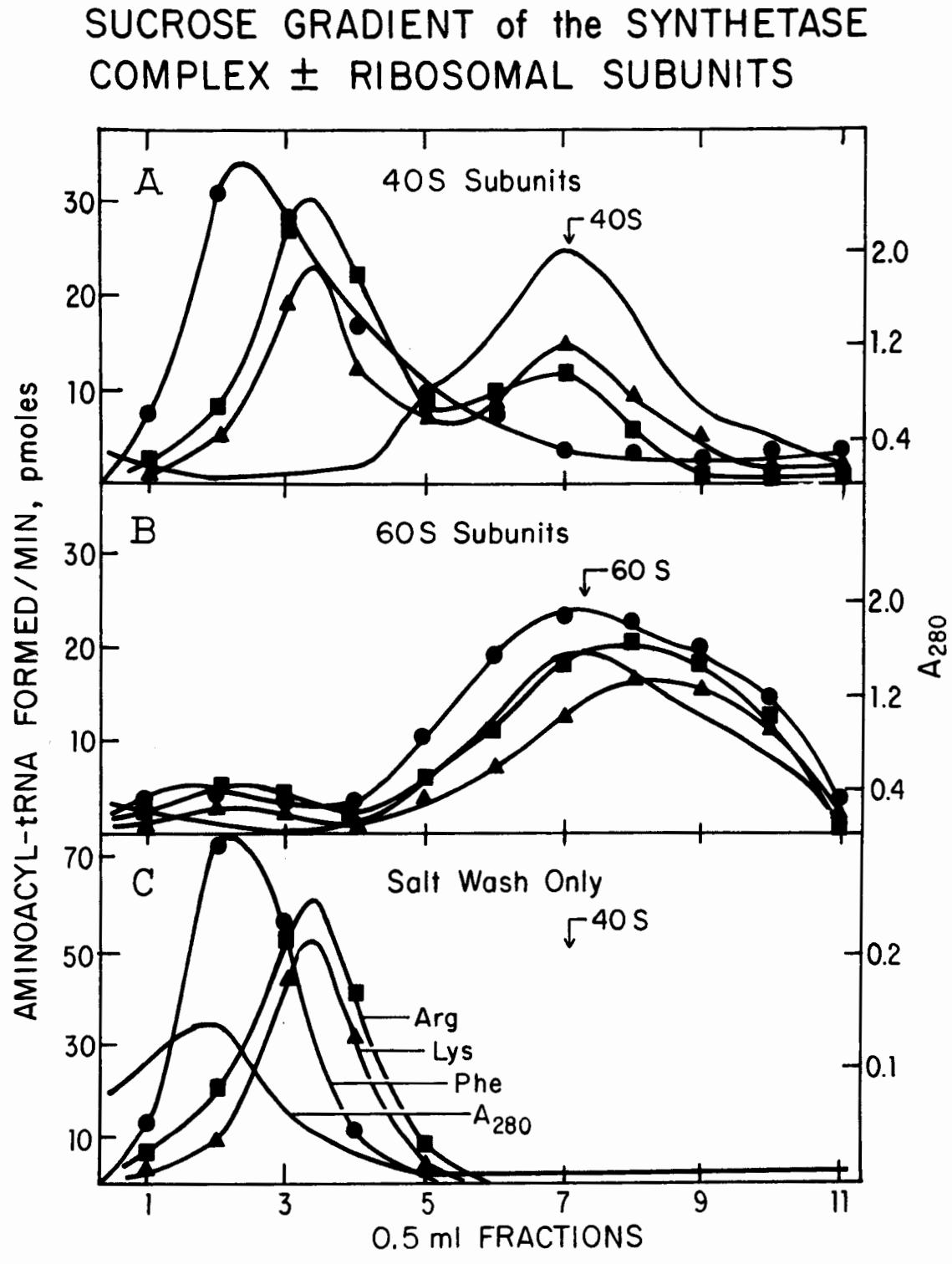

Fig. 2: Distribution of the Met-tRNA Synthetase Complex with Ribosomal Subunits in a Sucrose Gradient

Ribosomal subunits, 0.5 mg, were mixed with 250 μg of salt wash protein in 0.25 ml samples containing 20 mM Tris ·HCl, pH 7.5, 25 mM KCl, 5.0 mM MgCl, and 1.0 mM β-mercaptoethanol. The samples were layered on 5 ml, 10—30 % linear sucrose gradients containing the same salt concentrations as the samples. The gradients were centrifuged 1.5 hr at 60,000 rpm in a SW 65 rotor for gradients containing salt wash alone or 40S subunits. Centrifugation time was reduced to 1.0 hr for the gradient containing the 60S subunit. The distribution of the Met-tRNA synthetase complex is indicated by the activities arginine and lysine. (●) Phe-tRNA; (▲) Lys-tRNA; (■) Arg-tRNA.

purified from brine shrimp by Zasloff and Ochoa (16). The steps and yield of the procedure that we have used to purify the factor from reticulocytes is outlined in Table 4. The procedure provides about a 2500-fold purification of the factor from the supernatant of the high speed centrifugation used to collect ribosomes from the reticulocyte lysate. The product from the procedure appears as primarily a single component on SDS gel electrophoresis and a single component of 3.7 $S_{w,20}$ in the analytical ultracentrifuge. It has a molecular weight of about 86,000 as determined by gel filtration chromatography.

The factor has the capacity to promote binding of $tRNA_f^{Met}$ or, to a less efficient degree, $tRNA^{Phe}$ to 40S ribosomal subunits. This binding is prevented by addition of 60S ribosomal subunits to the reaction mixture. The requirements for binding of Phe-tRNA are presented in Table 5. The binding reaction is strictly codon dependent and inhibited by 60S ribosomal subunits. The factor does not promote ApUpG directed binding of $Met\text{-}tRNA_M$, as shown in Table 6, even though ApUpG directs binding of $Met\text{-}tRNA_M$ at higher Mg^{++} ion concentrations. Met-tRNA bound to 40S ribosomal subunits with the factor has relatively low reactivity for reaction with puromycin even though aminoacylated forms of $Met\text{-}tRNA_f$ react readily under similar conditions. The reaction of $Met\text{-}tRNA_f$ and N-acetylMet-$tRNA_f$ with puromycin are presented in Table 7. For these experiments either Met-tRNA or N-acetylMet-tRNA were bound under standard conditions to 40S ribosomal subunits and then the 60S ribosomal subunits and puromycin were added to the reaction mixtures. $Met\text{-}tRNA_f$ was not appreciably reactive with puromycin and could not be activated with either of the peptide elongation enzymes or ribosomal salt wash fractions that promotes peptide initiation with globin mRNA. In contrast N-acetylMet-tRNA is bound to the ribosomes in relatively large amounts, most of which is reactive with puromycin without the addition of other factors to the reaction mixture. It should be noted that Met-tRNA rather than N-formylMet-tRNA, is thought to function in peptide initiation in eukaryotic organisms.

Table 4. Purification of the Initiator tRNA Binding Factor

Fraction	Total Volume (ml)	Total Protein (mg)	Total Units* ($x10^{-3}$)	Specific Activity (units/mg)	% Yield
High Speed Supernatant	2,000	69,000	278	4	100
40–70 Ammonium Sulfate	120	6,000	120	20	43.2
DEAE Cellulose	80	520	83	160	29,9
Cellulose Phosphate	5.4	6.25	31.2	5,000	11.2
Pulverized glass	3.1	2.06	22.4	10,800	8.1
Hydroxylapatite	2.0	1.54	17.0	11,000	6.1

*pmoles Phe-tRNA bound as determined in the standard assay system.

A detailed description of the fractionation procedure and assay of the factor is presented elsewhere (10).

The tRNA binding factor promotes binding of deacylated $tRNA_f^{Met}$ or $tRNA^{Phe}$ with relatively high efficiency as compared with Met-$tRNA_f$ or Phe-tRNA. Results for Met-tRNA and deacylated $tRNA_f^{Met}$ are shown in figure 3. For these experiments, *E. coli* tRNA estimated to be more than 75 % $tRNA_f^{Met}$ was either charged with ^{35}S • methionine with *E. coli* synthetase or labeled with tritium by exchange procedure as described previously (18, 19). Binding of the uncharged ^{3}H • $tRNA_f^{Met}$ was determined in the standard assay procedure used for Met-$tRNA_f$.

The significance for peptide initiation of this binding of deacylated $tRNA_f^{Met}$ to 40S ribosomal subunits is not clear, however, this property has been used in the experiments described below with the Met-tRNA synthetase complex.

Table 5. Characteristics of the standard assay system

Additions	tRNA Binding pmoles		
	Factor —	Addition +	Factor Activity
Complete*	1.20	4.85	3.65
– poly (U)	0,08	0.04	0
– 40S subunits	0.02	0.01	0
+ 60S subunits	0.55	1.45	0.90
4 mM Mg^{++}	0.20	0.55	0.35
+ GTP	1.20	4.80	3.60
N-acetylPHe-tRNA†	130	4.56	3.26

* The complete system is the standard assay system with 0.36 μg of protein from the hydroxylapatite fraction prepared as described.

† In place of Phe-tRNA.

Table 6. Specificity of $tRNA_f^{Met}$ Binding Factor with ApUpG Codon

tRNA Species	Binding Factor	pmoles bound
Met-$tRNA_f$	—	0.10
	+	2.75
Met-$tRNA_M$	—	0.11
	+	0.24

Binding of Met-tRNA was carried out in the standard assy with system with Met-$tRNA_M$ substituted for Met-$tRNA_f$ where indicated.

Table 7. Puromycin Reactivity of Bound Met-tRNA$_f$ and Ac-Met-tRNA$_f$

Species Bound	Additions	Met-Puromycin pmoles	Formed percent
Met-tRNA$_f$	None	0.23	14
1.65 pmoles	EF-I + GTP	0.20	12
	EF-II + GTP	0.25	15
	EF-I + EF-II + GTP	0.28	17
	Salt Wash + GTP	0.43	26
AcMet-tRNA$_f$	None	2.62	90
2.90 pmoles	Salt Wash + GTP	2.51	87

Binding of Met-tRNA or N-acetyl Met-tRNA was carried out in the standard assay system then 60S ribosomal subunits were added to the reaction mixture to give a 1 : 1 molar ratio with the 40S subunits. Then the other components indicated in the table were added. Quantities added were: salt wash, 80 μg; EF-II, 2 μg; GTP, 0.2 mM; puromycin, 0.5 mM. Then the reaction mixture was incubated 20 minutes at 37° and reactivity with puromycin determined by extraction of the product formed with ethylacetate saturated with methionine by a procedure similar to that described by Leder and Bruztyn (17).

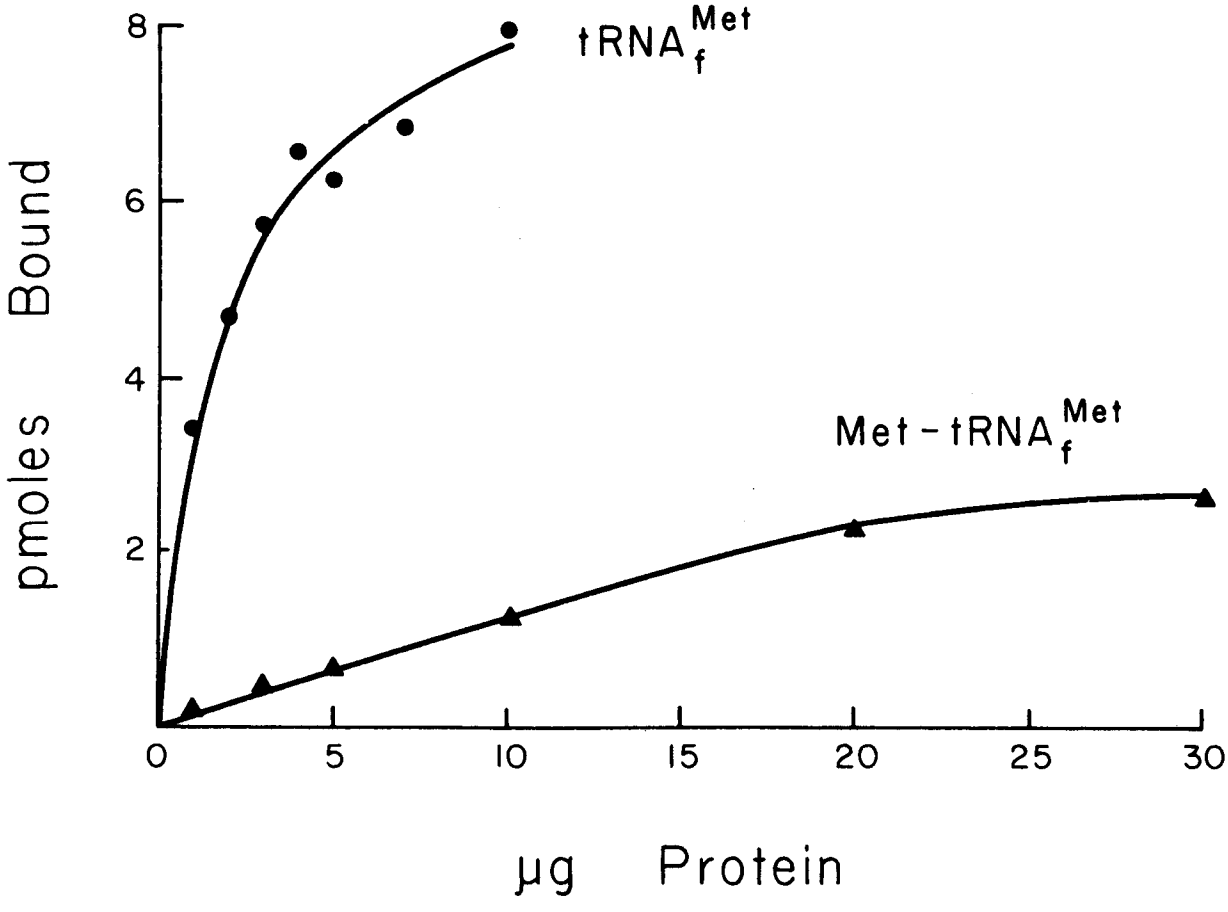

Fig. 3: Binding of tRNA$_f^{Met}$ or Met-tRNA$_f$ with the Binding Factor
Deacylated tRNA$_f^{Met}$ or Met-tRNA$_f$ was bound to 40S ribosomal subunits with ApUpG as described in the text.

Charging of $tRNA_f^{Met}$ Bound to Ribosomes

Earlier studies indicated that $tRNA_f^{Met}$ is accumulated on ribosomes when intact reticulocytes are incubated with NaF (20). NaF blocks initiation of peptides on reticulocyte ribosomes in intact reticulocytes with little or no effect on peptide elongation, thus allowing the breakdown of polysomes by the completion and release of nascent peptides. The block in peptide initiation caused by NaF appears to be beyond the step at which $tRNA_f^{Met}$ presumably as the charged species, is bound to the ribosomes. It is not clear why most of the $tRNA_f^{Met}$ is in the deacylated form. It may be due to the action of a Met-$tRNA_f^{Met}$ hydrolase that has the ability to deacylate this species of tRNA while it is bound to the 40S ribosomal subunit (21). These NaF ribosomes that bear primarily deacylated $tRNA_f^{Met}$ can be easily prepared by procedures similar to those used for regular ribosomes. Like regular ribosomes they have high proportion of the total Met-tRNA synthetase complex present in the cell associated with them at isolation. We have used these ribosomes to test the hypothesis that the $tRNA_f^{Met}$ could be charged while it was bound to the ribosomes.

For these experiments NaF ribosomes that bear both deacylated $tRNA_f^{Met}$ and Met-tRNA synthetase were added to reaction mixtures that contain the concentrations of salts, ATP and ^{35}S • methionine used in the standard assay system. No additional tRNA or enzyme fraction was added to the reaction. After incubation the reaction mixture was diluted about 20-fold by the addition of a solution containing the same salts at the concentrations of those in the reaction mixture, and then the ribosomes were pelleted by centrifugation. The RNA was extracted from the ribosomal pellet with sodium dodecylsulfate and phenol then the tRNA extracted with 1.0 M NaCl as previously described (8). Unfractionated tRNA also containing $tRNA_M^{Met}$ was charged with ^{3}H • methionine and an appropriate aliquots mixed with the ^{35}S • Met-tRNA extracted from the NaF ribosomes. The mixture of ^{35}S and ^{3}H • Met-tRNA's were chromatographed on BD cellulose essentially as described by Kerwar, Spears and Weissbach (22) and the distribution of the two labels followed. The results are presented in figure 4. Nearly all of the ^{35}S • methionine from the NaF ribosomes is in peak I which is Met-$tRNA_f$. Nearly all of the tRNA present on the NaF ribosomes was charged, as indicated by a comparison of the amount of ^{35}S • Met-tRNA formed by first extracting the tRNA and then charging it in a subsequent step.

The experiment described above with NaF ribosomes has served as a model for an apparently comparable experiment carried out with the tRNA binding factor and the Met-tRNA synthetase complex. For these experiments 40S ribosomal subunits were incubated with unfractionated tRNA, tRNA binding factor and ApUpG under conditions of the standard assay. After incubation, the reaction mixture was diluted with a solution containing the same concentration of salts used in the standard reaction mixture, and then the subunits bearing the uncharged tRNA were collected by centrifugation and resuspended in a solution containing 4 mM MgCl, 25 mM KCl, and 10 mM Tris-HCl, pH 7.5. To aliquots of this solution were added 60S ribosomal subunits to give a molar ratio of 60S to 40S subunits of about unity. These ribosomes were added in the amount of 2.0 mg/ml to the standard

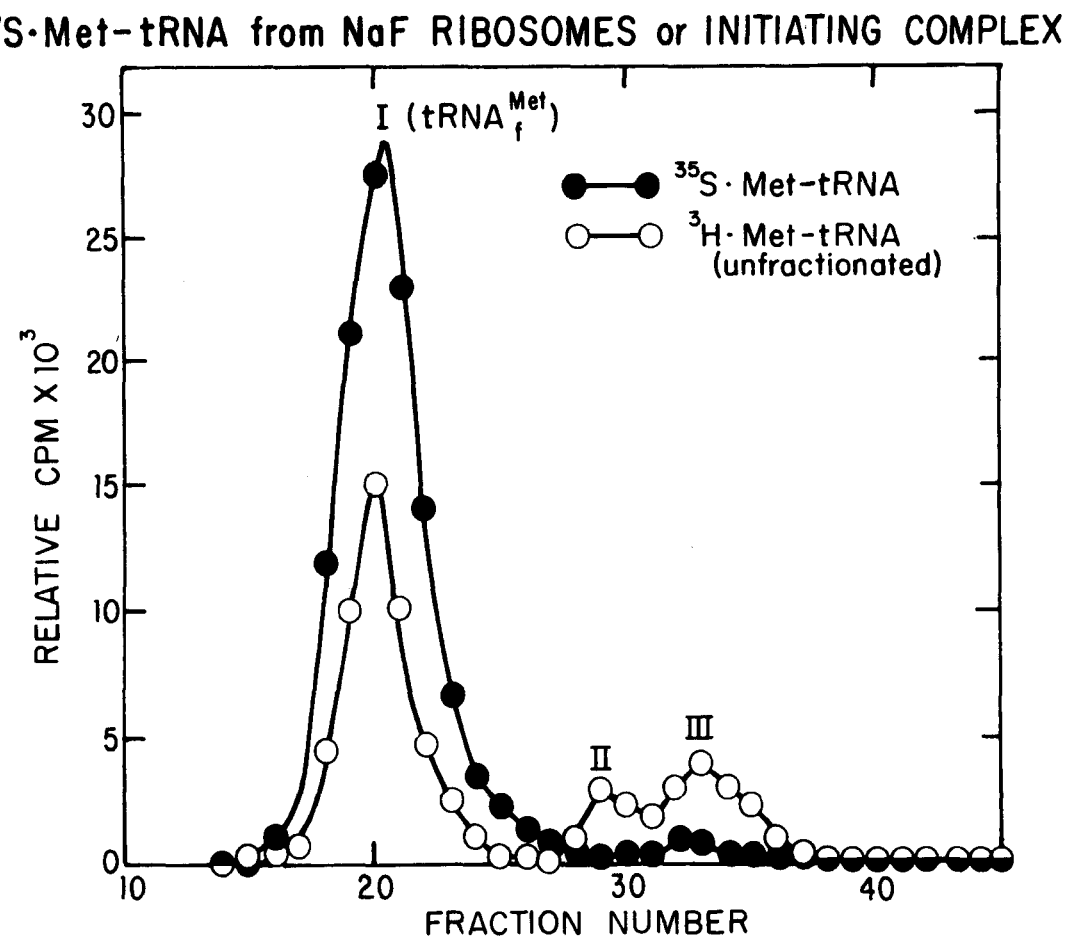

Fig. 4: Distribution of Met-tRNA from NaF Ribosomes or from tRNA Originally Bound to 40S Ribosomal Subunits with the Binding Factor.

Deacylated tRNA bound to NaF ribosomes or to ribosomal subunits with the tRNA binding factor was charged with ^{35}S - methionine, reisolated and chromatographed on BD cellulose with unfractionated ^{3}H - Met-tRNA as described in the text.

reaction mixture modified to contain 2.5 mM KCl. It also contained purified Met-tRNA synthetase complex, ATP and ^{35}S - methionine. After incubation, the tRNA was extracted from the reaction mixture then chromatographed on BD cellulose with unfractionated ^{3}H - Met-tRNA as described above. The distribution of ^{35}S and ^{3}H - methionine were nearly identical to those shown in figure 4. Nearly all of the tRNA bound to the 40S ribosomal subunits was charged during the incubation.

These results with NaF ribosomes and with the tRNA binding factor plus the Met-tRNA synthetase complex appear to indicate that Met-tRNA$_f^{Met}$ can be charged while it is bound with ApUpG and the binding factor to the ribosomes. In addition, the results appear to indicate that with ApUpG the binding factor is able to promote the selective binding of tRNA$_f^{Met}$ with high discrimination against tRNA$_M^{Met}$.

Discussion

We consider the development of a highly active, fractionated cell-free system of primary importance for study of the factors at the molecular level that control and regulate the synthesis of specific proteins. To date this objective has not been accomplished. We believe that the phenomena described here may give some insight into an aspect of the problem that generally is not considered. Classically, it has been assumed that aminoacyl-tRNA is formed free in solution by the respective synthetase enzymes and would be carried to the ribosomes by free diffusion. This may not be the primary mechanism in intact cells.

The molar ratio of tRNA to ribosomes in most cells does not appear to favor a diffusion limited reaction. Maaløe and Kjedlgaard (23) estimated 7–15 molecules of unfractionated tRNA per ribosome in *E. coli*. We find a similar ratio, estimated to be about 10, in rabbit reticulocytes. This is the total tRNA. Minor species may account for 1 % or less of the total. It appears that most tRNA species are present in reticulocytes at less than a 1 : 1 molar ratio. We estimate this ratio to be about 0.3 for Met-$tRNA_f^{Met}$.

The molar ratio of the Met-tRNA synthetase complex to $tRNA^{Met}$ and ribosomes is also an interesting figure. We have attempted to estimate the molar amount of Met-tRNA synthetase complex in intact reticulocytes from the specific activity of the Arg-tRNA synthetase in the purified complex, a molecular weight of 5×10^5 for the complex, and Arg-tRNA synthetase activity in cell lysates in which free arginine and other small molecules had been removed by gel filtration and protein synthesis was blocked with cycloheximide. We estimate 0.2 moles of Met-tRNA synthetase complex per mole of ribosome. Thus, $tRNA_f^{Met}$ and the Met-tRNA synthetase complex are present in similar molar amounts. It should be stressed that value is subject to some error, but we doubt that it is off by more than a factor of 2. Loftfield (24) has pointed out that all measurements of K_D for the dissociation of the enzyme tRNA complex and all K_m's for this reaction fall in a range of 10^{-7} M or less. This appears to indicate that there will be a relatively low proportion of these components that exist as free enzyme and tRNA within the cell. Jacobson has reached a similar conclusion (25). It is conceptually difficult from the physical standpoint to see how the charging reaction could take place on a ribosome in concert with the other reactions of peptide synthesis that occur on the ribosome but this may be indicated.

The data and considerations presented here carry the potential implication that charging of tRNA as well as the reactions of peptide elongation may be carried out on the ribosomes. Disruption of this organized system or changing the concentration or ratio of components in the cell-free system from those in intact cells might account for the characteristic reduction in synthetic capacity.

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INHIBITORS OF MAMMALIAN PROTEIN SYNTHESIS

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The number of antibiotics active on eukaryotic ribosomes has been considerably increased in the last few years. Some of them are active only in eukaryotic ribosomes whereas some others are active on prokaryotic as well as eukaryotic ribosomes (Table 1). There is ample evidence suggesting a similar mechanism for the elongation cycle by prokaryotic and eukaryotic ribosomes. This evidence is mainly based on studies showing that (a) the functions of the eukaryotic ribosomal subunits are similar to those of their prokaryotic counterpart and (b) antibiotics active on prokaryotic and eukaryotic ribosomes act in both cases on the equivalent subunits. Therefore we can tentatively extrapolate and generalize results obtained with some antibiotics concerning their site of action as indicated in Table 2. Similarly we might generalize data concerning the mode of action of these antibiotics. However, most of the data concerning the specific reactions inhibited by antibiotics on eukaryotic ribosomes were obtained using different experimental systems and ribosomes from an enormous variety of biological sources. Consequently we have studied systematically the effect of these antibiotics on similar systems for the reactions of the elongation cycle using in all cases human tonsil ribosomes. For the purpose of comparative studies we have also studied in some cases the effects of some antibiotics on cell-free systems from other types of eukaryotic cells. The results obtained are presented in this contribution.

Materials and methods

Human tonsil ribosomes and elongation factors (EF 1 and EF 2) were prepared as previously described (1, 2). The separation of EF 1 and EF 2 was carried out in Sephadex G-200 chromatography after ammonium sulphate fractionation. EF 1 was further purified by Sepharose 4 B column and hydroxyapatite treatment. EF 2 from the Sephadex G-200 was purified by DEAE cellulose and phosphocellulose chromatography (3).

Yeast ribosomes were obtained as described elsewhere (4). Baker's yeast tRNA (Boehringer) was charged with [^{14}C]Phenylalanine (513 mCi/mmol) (Radiochemical Centre) using a crude synthetase fraction from yeast prepared from the S 100 by means of a Sephadex G-25 column pooling the active fractions.

[14C]Phe-tRNA was separated after phenol treatment and ethanol precipitation and an aliquot was acetylated as described (5). [^{3}H]Phe-tRNA and [^{3}H]Leu-tRNA (prepared from *E. coli* tRNA (Sigma) and either [^{3}H]Phenylalanine (18 Ci/mmol) or [^{3}H]Leucine (52 Ci/mmol)) were acetylated by the same method indicated above. The N-Ac-[^{14}C]Phe-tRNA was further purified by BD-cellulose chromatography.

Table 1. Inhibitors of protein synthesis active on eukaryotic systems

Acting on ribosomal systems of the eukaryotic type	Acting on ribosomal systems of the prokaryotic and the eukaryotic types
* Adrenochrome	Abrin
Anisomycin	Actinobolin
Emetine	Amicetin
Enomycin	Aurintricarboxylic acid
Glutarimide group:	Blasticidin S
Actiphenol	Bottromycin A_2
Cycloheximide	Chartreusin
Strptimidone	Diphtheria toxin
Streptovitacin A	Edeine
Pederine	Fusidic acid
Phenomycin	Gougerotin
Tenuazonic acid	**Griseoviridin
Trichodermin group:	Nucleocidin
Crotocin	Pactamycin
Crotocol	Pyrocatechol violet
Fusarenon X	Poly-dextran-sulphate
Nivalenol	Puromycin
Trichodermin	Ricin
Trichodermol	Sparsomycin
Trichothecin	Tetracycline group:
Verrucarin A	Chlortetracycline
Verrucarol	Deoxycycline
Tylophora alkaloids:	Oxytetracycline
Cryptopleurine	Tetracycline
Tylocrebrine	
Tylophorine	

* Adrenochrome has not been tested in prokaryotic systems.
** Griseoviridin is not active in any of the intact cells of the eukaryotic type which have been tested but it is active on eukaryotic cell-free systems.

CACCA-Ac-[^{3}H]Leu-Ac was prepared from N-Ac-[^{3}H]Leu-tRNA by digestion with T_1 RNAse and separated by paper electrophoresis (6).

Enzymic binding of [^{14}C]Phe-tRNA to the ribosome A site took place at low Mg^{++} concentrations using the EF 1 preparation (7). The complex formed was separated by centrifugation when required, the inhibitors to be tested were added and translocation induced by addition of EF 2 and GTP (Fig. 2). The extent of

translocation was measured by the reaction with puromycin of N-Ac-[^{14}C]Phe-tRNA bound to the P-site. Inhibitors of peptide bond formation were tested in these reactions in controls in which inhibitors were not added prior to translocation (Fig. 2).

Nonenzymic binding of purified N-Ac-[^{14}C]Phe-tRNA was studied at 15 mM Mg^{++} concentration. Under these conditions the N-Ac-[^{14}C]Phe-tRNA binds to the A and P-sites to the same extent. All the substrate bound to the A-site translocated to the P-site in the presence of EF 2 and GTP and reacted with puromycin after translocation.

Peptide bond formation was also studied in the fragment reaction assay using as substrates CACCA-[^{3}H]Leu-Ac-N and puromycin and measuring formation of N-Ac-[^{3}H]Leu-puromycin (4, 7, 8).

Sources of the protein synthesis inhibitors used in this work were as follows: actinobolin and griseoviridin (Parke Davis), amicetin, chartreusin and cycloheximide (Upjohn), adrenochrome and gougerotin (Calbiochem), anisomycin (Pfizer), aurintricarboxylic acid (ATA) (May and Baker), blasticidin S and bottromycin A_2 (Institute of Applied Microbiology, Tokyo, Japan), emetine (Wellcome), fusidic acid and trichodermin (Leo), puromycin (Serva and Nutritional Biochemicals), sparsomycin (National Cancer Institute, Bethesda, USA) and tenuazonic acid (Merck Sharp and Dohme). Edeine A_1 was a gift from Dr. Z. Kurylo-Borowska (Rockefeller University, New York, USA). Pederine was given to us by Prof. M. Pavan (Institute of Entomology, University of Pavia, Italia). Diphtheria toxin was a gift from Dr. E. Bermek (Max-Planck Institute for Experimentel Medicine, Göttingen, Germany).

Methods previously described have been used for the preparation of ribosomes from *Euglena gracilis* (9) and *Phaseolus vulgaris* (10).

[^{3}H]anisomycin (285 mCi/mmol) and [^{3}H]gougerotin (80 mCi/mmol) were obtained by tritium exchange labelling in aqueous solutions and purified as described elsewhere (11). [^{3}H]anisomycin and [^{3}H]gougerotin binding to human tonsil ribosomes was studied following basically the sedimentation method, essentially as described (12) in 50 mM Tris-HCl, pH 7.4, 11 mM $MgCl_2$, 60 mM KCl and 7 mM 2-mercaptoethanol.

Effects of the inhibitors on the steps of the elongation cycle.

Our studies on enzymic binding of [^{14}C]Phe-tRNA and non-enzymic binding of N-Ac-[^{14}C]Phe-tRNA have shown that adrenochrome and pyrocatechol violet are strong inhibitors of substrate binding (Table 1), and confirm previous reports (13, review) showing that ATA, chartreusin, edeine A_1 and tetrycycline are also inhibitors of substrate binding.

In both systems studied we have shown that pederine is a good inhibitor of translocation (Table 2). Diphtheria toxin has been shown previously to act catalytically modifying EF 2 forming ADP-ribosyl-EF 2 which is able to bind to the ribosome (14) but does not induce translocation. Contrary to a number of previous reports (13, review) fusidic acid does not block translocation under the experimental conditions of Table 2. The tylophora alkaloids, cryptopleurine, tylocrebrine and tylophorine are also inhibitors of translocation as already reported for tylocrebrine (15).

Table 2. Inhibitors of protein synthesis acting on eukaryotic ribosomes.
Site of action

40S ribosome subunit	60S ribosome subunit
Aurintricarboxylic acid	Actinobolin
* Edeine A_1	Amecetin
* Pactamycin	Anisomycin
* Poly-dextran-sulphate	Blasticidin S
* Tetracycline group:	Bottromycin A_2
Chlortetracycline	** Fusidic acid
Deoxycycline	Glutarimide group:
Oxytetracycline	Actiphenol
Tetracycline	Cycloheximide
	Streptimidone
	Streptovitacin A
	Gougerotin
	Griseoviridin
	Puromycin
	Sparsomycin
	Tenuazonic acid
	Trichodermin group:
	Crotocin
	Crotocol
	Fusarenon
	Nivalenol
	Trichodermin
	Trichodermol
	Trichothecin
	Verrucarin A
	Verucarol
	Tylophora alkaloids:
	Cryptopleurin
	Tylocrebrine
	Tylophorine

* Edeine A_1, pactamycin, poly-dextran-sulphate and the tetracyclines bind to both the smaller and the larger subunits but the interaction with the smaller subunit appears to be more relevant for the mode of action of these inhibitors.

** Fusidic acid has not been shown to interact directly with the larger ribosome subunit but forms the complex EF 2·GDP·larger ribosome subunit·fusidic acid.

Table 3. Non enzymic binding of Ac-[^{14}C]Phe-tRNA and enzymic binding of [^{14}C]Phe-tRNA to human tonsil ribosomes. Effects of protein synthesis inhibitors

Additions		Ac-[^{14}C]Phe-tRNA binding (% control)	[^{14}C]Phe-tRNA binding (% control)
Adrenochrome	10^{-4}M	25	23
Anisomycin	10^{-4}M	58	51
	10^{-5}M	99	91
ATA	10^{-4}M	1	15
Chartreusin	10^{-3}M	–	37
Edeine A_1	10^{-6}M	26	14
Emetine	10^{-4}M	53	74
Pyrocatechol violet	10^{-4}M	–	13
Tenuazonic acid	10^{-3}M	67	61
	10^{-4}M	96	92
Tetracycline	10^{-3}M	–	28

Assays were carried out under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. Binding of Ac-[^{14}C] Phe-tRNA in the control reactions was 1.6 pmoles whereas [^{14}C]Phe-tRNA binding was 2.4 pmoles.

Table 4. Translocation of Ac-[^{14}C]Phe-tRNA and [^{14}C]Phe-tRNA bound to human tonsil ribosomes. Effects of protein synthesis inhibitors

Additions		Ac-[^{14}C]Phe-tRNA translocation (% control)	[^{14}C]Phe-tRNA translocation (% control)
Cryptopleurine	2.6×10^{-4}M	43	–
Diphtheria toxin	125 μg/ml	36	11
Fusidic acid	2×10^{-4}M	99	97
Pederine	2×10^{-5}M	32	30
Tylocrebrine	2.6×10^{-4}M	43	–
Tylophorine	2.6×10^{-4}M	31	–

Assays were carried out under the experimental conditions indicated above. Figures given in this Table are percentage of control reactions in the absence of inhibitors. Average of Ac-[^{14}C] Phe-tRNA translocated in the controls was 0.8 pmoles (47 % of the total substrate bound) whereas [^{14}C]Phe-tRNA translocated in the control was 0.6 pmoles (21 % of the total substrate bound).

Table 5. Peptide bond formation by human tonsil ribosomes
Effects of protein synthesis inhibitors

Additions		Ac-[^{14}C]Phe-Pur formation (% control)	[^{14}C]Phe-Pur formations (% control)	Fragment reaction (% control)
Actinobolin	10^{-3}M	–	–	29
	10^{-4}M	94	–	75
Anisomycin	10^{-4}M	25	16	5
Blasticidin S	10^{-4}M	–	23	12
Gougerotin	10^{-3}M	–	–	16
	10^{-4}M	97	–	–
Griseoviridin	5×10^{-4}M	–	–	18
Sparsomycin	10^{-4}M	21	19	7
Tenuazonic acid	10^{-3}M	51	–	22
	10^{-4}M	98	50	73
Trichodermin	10^{-5}M	32	–	13

Assays were carried out under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. In the control assays 1.75 pmoles Ac-[^{14}C]Phe-Pur or 0.68 pmoles [^{14}C]Phe-Pur were formed in the puromycin reaction and 1.39 n moles Ac-[^{14}C]Phe-puromycin were formed in the fragment reaction assay.

In the different experimental systems used we have shown that trichodermin and tenuazonic acid block the peptide bond formation step (Table 3). We have confirmed previous evidence that actinobolin, amicetin, anisomycin, blasticidin S, gougerotin and sparsomycin also block the peptidyl transferase centre (4, 8). In most cases the inhibitors preferentially block peptide bond formation in the fragment reaction since it is a more resolved assay. We have also observed that the antibiotic griseoviridin inhibits peptide bond formation in the fragment reaction assay. This finding is interesting since griseoviridin was considered as an antibacterial antibiotic which has neither fungistatic nor antiamebae nor antiprotozoal activity (16). However in cell-free systems we have observed that griseoviridin is indeed an effective inhibitor of poly (U)-directed polyphenylalanine synthesis (results not shown). Results presented in Table 3 show that griseoviridin blocks peptide bond formation by human tonsil ribosomes similarly as in bacterial systems (17), but to a lesser extent.

Differential properties of the peptidyl transferase centre.

For most antibiotics considered in this work there is a similar pattern of inhibition when tested on either human tonsil (7) or yeast (4) ribosomes. However, a number of inhibitors active on the peptidyl transferase centre are more active on human tonsil than on yeast ribosomes. This was indeed observed when the effect of the sesquiterpene antibiotics trichodermin and trichodermol was tested under

identical conditions in the fragment reaction assay catalyzed by either human tonsil or yeast ribosomes (Fig. 1).

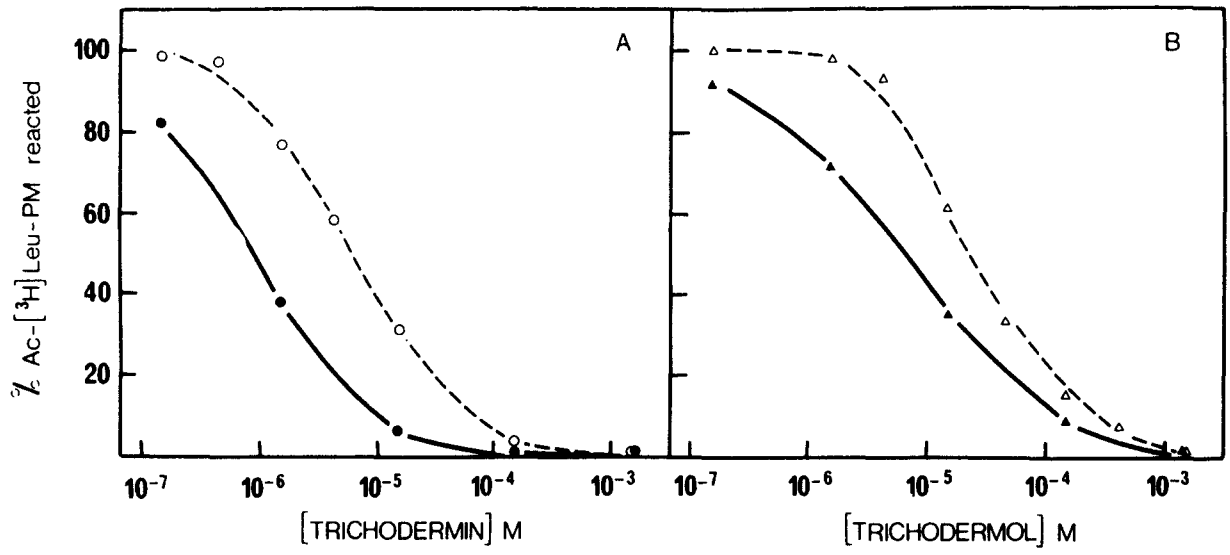

Fig. 1: Effects of sesquiterpene antibiotics on the fragment reaction by human tonsil and yeast ribosomes. *A*: Effects of trichodermin on the fragment reaction by yeast (○- - -○) and human tonsil (●——●) ribosomes. *B*: Effects of trichodermol on the fragment reaction by yeast (△- - -△) and human tonsil (▲——▲) ribosomes. The experimental systems were as indicated under Materials and Methods.

This preferential activity was indeed more striking in the case of tenuazonic acid since this antibiotic showed no inhibitory effect on poly (U)-directed polyphenylalanine synthesis by yeast ribosomes whereas there was a significant inhibition on the human system (Table 6). This differential sensitivity of human tonsil and yeast ribosomes is due only to the larger ribosome subunit and the smaller subunit is irrelevant for this effect. Tenuazonic acid was tested on the puromycin reaction by hybrid ribosomes (Fig. 2) and the results obtained are presented in Table 7. The extent of inhibition by tenuazonic acid on hybrid ribosomes was similar to that which was observed previously in the same system by human tonsil ribosomes (7), whereas the antibiotic does not affect the puromycin reaction when hybrid ribosomes of 60S subunits from yeast and 40S subunits from human tonsils were used.

Furthermore we have also observed that tenuazonic acid does not inhibit peptide bond in the "fragment reaction" assay by yeast ribosomes, has a reduced effect on the reaction catalyzed by *Euglena gracilis* and *Phaseolus vulgaris* ribosomes and is active on pig liver ribosomes (18). Indeed differences between these types of ribosomes were also observed in the peptidyl transferase centre by studying their activity on the fragment reaction after pretreatment with N-ethyl-maleimide (NEM) (Table 8).

Binding of tenuazonic acid to eukaryotic ribosomes

It was not possible to measure binding of tenuazonic acid to ribosomes since the antibiotic is not available radioactively labelled. However, we have prepared [^{3}H]anisomycin and [^{3}H]gougerotin by the tritium exchange labelling procedure

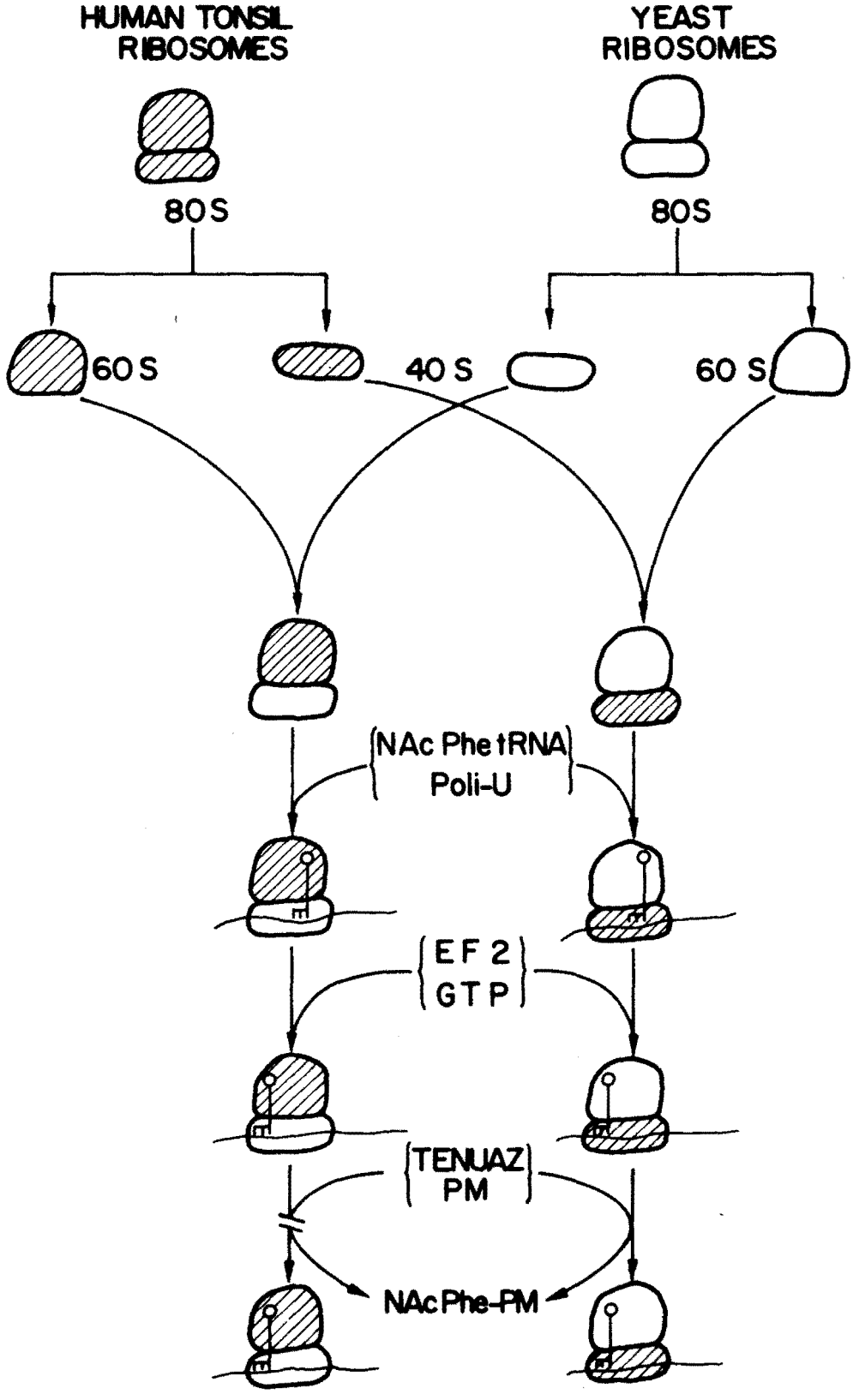

Fig. 2: General scheme followed to study the puromycin reaction by hybrid ribosomes.

(19). We have observed that tenuazonic acid does not affect binding of [^{3}H]gougerotin to either human tonsil or yeast ribosomes. However, tenuazonic acid totally inhibits [^{3}H]anisomycin to human tonsil ribosomes but not to yeast ribosomes (Fig. 3). It certainly appears that tenuazonic acid binds to human tonsil ribosomes to the same set of sites as anisomycin but its interaction with yeast

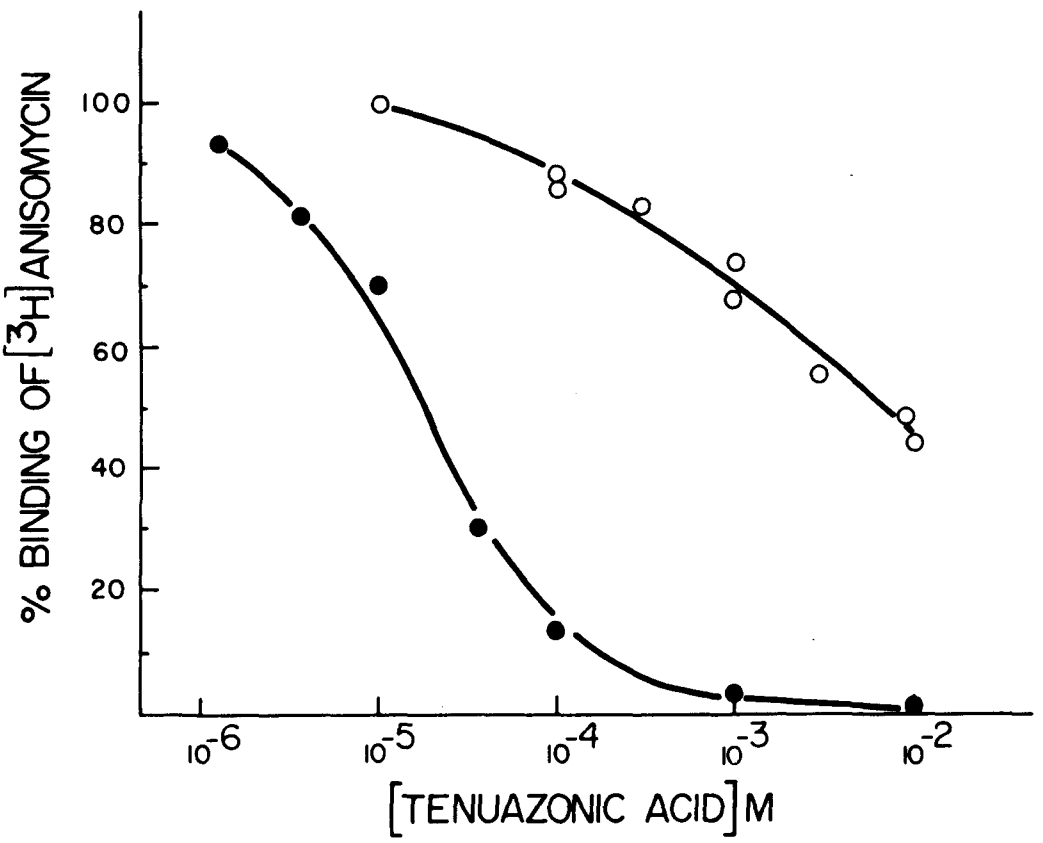

Fig. 3: Effects of tenuazonic acid on [^{3}H]anisomycin binding to ribosomes. (O——O) yeast ribosomes and (●——●) human tonsil ribosomes. Data were taken from an assay following the sedimentation method as described under Materials and Methods. Yeast ribosome concentration was 2.5 x 10^{-6} M. Human tonsil ribosome concentration was 3.5 x 10^{-6} M. [^{3}H]anisomycin concentration was in all cases 10^{-6} M.

ribosomes is negligeable; this explains the lack of effect of tenuazonic acid on yeast ribosomes reported above. Therefore the affinity of tenuazonic acid for the ribosome might be known if tenuazonic acid competes for the binding site of a radioactive antibiotic. We have studied the effects of different concentrations of tenuazonic acid on [^{3}H] anisomycin binding to human tonsil ribosomes and the data obtained were taken to a Klotz plot (Fig. 4). From this plot the value obtained for the dissociation constant for tenuazonic acid is $K_d = 2 \times 10^{-5}$ M following the relationship given to calculate affinity of an unlabelled compound provided that it competes for binding with a radioactive one of known dissociation constant (20).

Table 6. The effect of tenuazonic acid on synthesis of poly-phenylalanine by human tonsil ribosomes

Tenuazonic acid (molarity)	[^{14}C]Phenylalanine incorporated			
	Yeast system		Human tonsils system	
	pmoles	% control	pmoles	% control
None	4.53	100	1.18	100
10^{-5}	4.53	100	1.11	94
10^{-4}	4.58	101	1.04	88
5×10^{-4}	–	–	0.67	57
10^{-3}	4.62	102	0.44	37

Yeast tRNA was charged with [^{14}C]Phenylalanine. Incorporation was studied as described elsewhere using purified EF 1 and EF 2 in the human tonsil system and a crude supernatant fraction in the yeast system.

Table 7. The puromycin reaction by hybrid ribosomes. Effects of tenuazonic acid

Ribosome subunits		Tenuazonic acic (molarity)	Ac-Phe-puromycin formation	
60S	40S		pmoles	% Control
Human tonsils	Yeast	–	1.37	100
Human tonsils	Yeast	10^{-4}	1.37	100
Human tonsils	Yeast	10^{-3}	0.88	64
Human tonsils	Yeast	10^{-2}	0.32	23
Yeast	Human tonsils	–	1.61	100
Yeast	Human tonsils	10^{-4}	1.61	100
Yeast	Human tonsils	10^{-3}	1.66	103
Yeast	Human tonsils	10^{-2}	1.63	101

An experimental system was used similar to that described in Table 5 but using hybrid ribosomes (see Fig. 2).

An identical value of K_d for tenuazonic acid was obtained for two different concentrations of this antibiotic suggesting that this compound involves the same ribosomal set of sites in human tonsil ribosomes as anisomycin but with an affinity 12–13 times smaller.

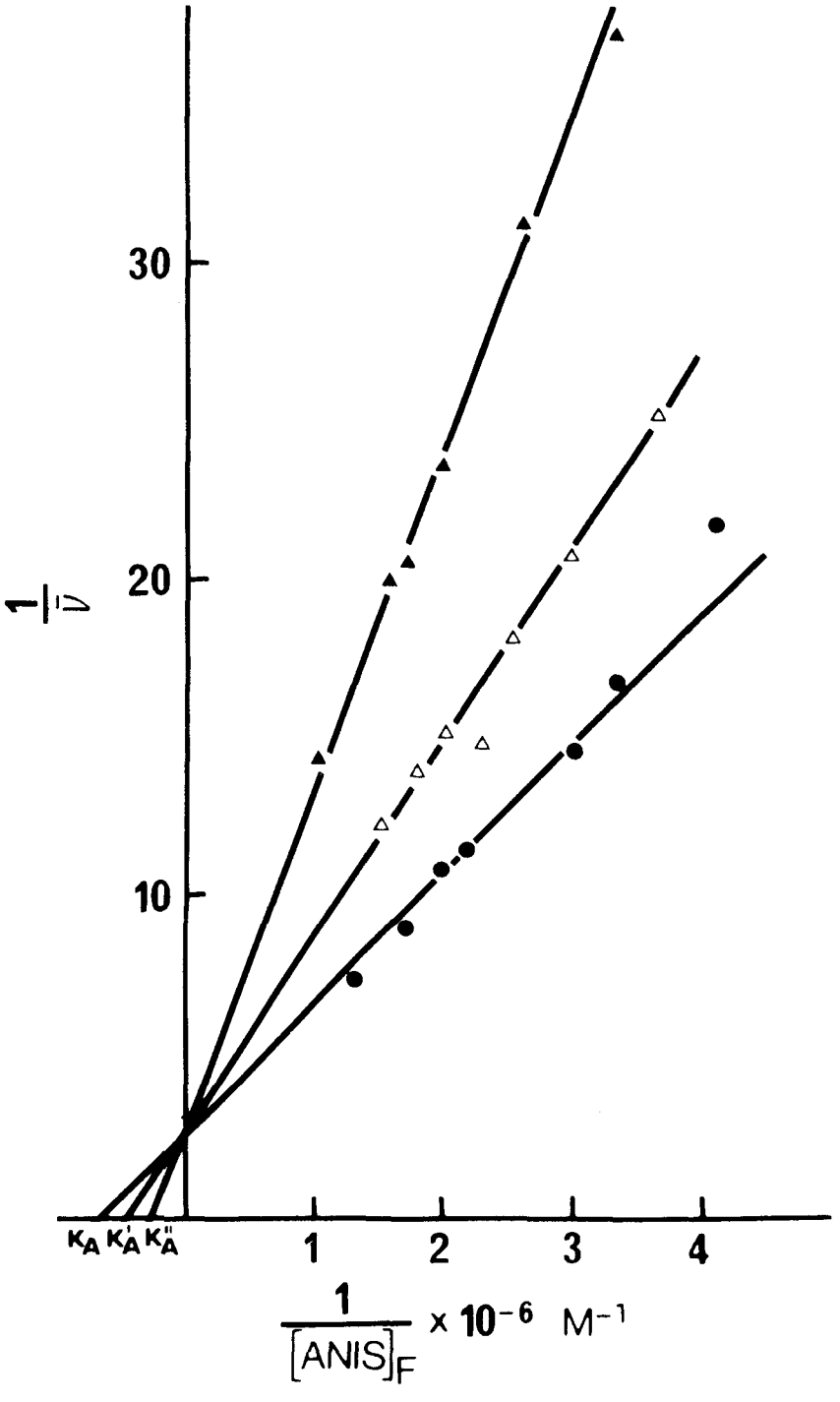

Fig. 4: Calculation of the affinity constant of tenuazonic acid for human tonsil ribosomes. (●——●) Klotz plot for [^{3}H]anisomycin binding in the absence of tenuazonic acid (K_d = 1.7 x 10^{-6} M); (△——△) Klotz plot for [^{3}H]anisomycin binding in the presence of 10^{-5} M tenuazonic acid; (▲——▲) Klotz plot for [^{3}H]anisomycin binding in the presence of 3 x 10^{-5} M tenuazonic acid.

Ribosome concentration was 3 x 10^{-6} M. [^{3}H]anisomycin concentration was ranging from 0.5 to 1.5 x 10^{-6} M. The experiment was carried out at 0° following the sedimentation method quoted under Materials and Methods.

Table 8. Effects of NEM on the peptidyl transferase centre of ribosomes

Source of ribosomes	Additions	Fragment reaction (cpm)	% Control
Human tonsils	None	1710	100
	5 mM NEM	2957	173
Yeast	Non	1276	100
	5 mM NEM	1313	102
Phaseolus vulgaris	None	1950	100
	5 mM NEM	2454	126
Escherichia coli	None	2975	100
	5 mM NEM	3073	103

Fragment reaction assay was carried out as indicated under Material and Method using CACCA-[^{3}H]Leu-Ac (6 nmolar; 6000 cpm/tube) as a donor substrate and puromycin (1 mM) as an acceptor substrate.

Discussion

The results presented in this contribution show that adrenochrome, pyrocatechol violet, ATA, edeine A_1, chartreusin and tetracycline block substrate binding to both the A- and the P-sites of human tonsil ribosomes.

Pederine, diphtheria toxin and the tylophora alkaloids: cryptopleurine, tylophorine and tylocrebrine inhibit substrate translocation from the A- to P-site of the ribosome. Fusidic acid also inhibits translocation but only when either limited amounts of EF 2 or a large excess of free ribosomes are added to the system (21).

Sparsomycin, trichodermin, anisomycin and tenuazonic acid are good inhibitors of peptide bond formation in all the different experimental systems used. Inhibition of peptide bond formation by sparsomycin and anisomycin is common to other eukaryotic systems previously described (4, 8). However, trichodermin is a better inhibitor in human tonsil than in yeast ribosomes (22). The selective action in mammalian ribosomes is more remarkable in the case of tenuazonic acid since this antibiotic is a good inhibitor of peptide bond formation by human tonsil and pig liver ribosomes, has reduced activity on *Phaseolus vulgaris* and *Euglena gracilis* ribosomes and practically has no effect on yeast ribosomes (18). This is indeed the first case in which such a selective activity of an antibiotic in mammalian ribosomes has been reported. Parallel interesting differences were observed in the peptidyl transferase centre of these ribosome preparations since N-ethyl-maleimide strongly enhances the activity of human tonsil ribosomes in the fragment reaction but does not have a similar effect on yeast, *Euglena gracilis, Phaseolus vulgaris* and *Escherichia coli* ribosomes (Table 8).

The antibiotics actinobolin, blasticidin S and gougerotin were found in this work to be very poor inhibitors of peptide bond formation in our model systems for the puromycin reaction but were quite active in blocking peptide bond formation in the fragment reaction since it is a more resolved system for the reaction.

Concerning the site of action of tenuazonic acid on the peptidyl transferase centre it appears that the antibiotic act on the same site(s) as anisomycin and the $K_d = 2 \times 10^{-5}$ M was calculated for its interaction with human tonsil ribosomes whereas a $K_d = 1.7 \times 10^{-6}$ M has been calculated for the anisomycin interaction. This should be expected since anisomycin is 10—20 times more active than tenuazonic acid in the puromycin and fragment reaction by human tonsil ribosomes (7). Tenuazonic acid is hardly active on yeast ribosomes and therefore has no significant inhibitory effect of [^{3}H]anisomycin to yeast ribosomes.

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OPENING DISCUSSION – FREDERICK STOHLMAN, JR., M. D.

The data presented at this meeting seemed to be leading toward the inevitable conclusion that leukemia in man is associated with a reverse transcriptase which may represent the "foot prints" of an RNA tumor virus. From this it may be suggested that human leukemia is in fact of viral origin. The chemical and immunologic properties of transcriptase which have been described in acute myelocytic leukemia, chronic myelocytic leukemia, and the lymphocytic leukemias are similar. The end results, i. e., the leukemia, however differ substantially in their clinical manifestations. One might suggest therefore various subspecies of transcriptase or virus with similar general physical and biochemical properties but each with significant differences that permit the development of varying types of leukemia. Alternatively the determinant as to the type of leukemia may rest within the cell which the virus infects, or perhaps the site of infection within the pluripotent cell.

To date the studies on transcriptase have required fantastic amounts of tissues which have not permitted adequate controls from normal tissues or the sequential evaluation of the leukemia as the patient enters remission and relapse. Perhaps the best control is the phytohemagglutinin stimulated lymphocyte but even this has been criticized by some. Further, the relative insensitivity of the techniques currently available for evaluation of transcriptase have precluded detailed study of the cells in question. The purification of the enzyme promises that specific antibodies may be developed which can be applied with immunofluorescent techniques to evaluation of the single cell. This together with differential separation of bone marrow cells should permit the evaluation of the importance and distribution of transcriptase in stem cells. Development of such technology in my view is critical to gaining meaningful insight into the role of reverse transcriptase and viruses in human leukemia.

In reflecting on the role of reverse transcriptase there are several considerations which come to mind. Leukemia may be viewed as a disease in which there is abnormal information presented to the cell, presumably at the pluripotent stem cell level, which results in abnormal growth patterns. Although there are several different types of myeloid leukemia, e. g. acute myelocytic, progranulocytic, etc., the number is circumscribed. Does this reflect a high specificity of the viral effect at a few sites on the DNA molecule or does it reflect that most of the viral directed effects are lethal and hence a leukemic clone does not develop, there being a random chance that the transcriptase affects the human stem cell in such a way that leukemia eventuates.

I gather from the discussion at this meeting that it is yet to be established whether the viral information is passed vertically and awaits only derepression or whether a more complicated hypothesis must be invoked. It is clear, however, that whatever theories are advanced they must take into account the leukemogenic effects of irradiation, alkylating agents and other leukemogenic drugs such as chloramphenicol. In the case of irradiation there is a suggestion of a dose-effect relationship at high dose levels. Is this due to somatic mutation, derepression of an oncogene or a more complicated method of initiating viral infection?

Clearly information on reverse transcriptase in patients with acute myelocytic leukemia thought to be secondary to irradiation or alkylating agents is of importance. The lag phase between the primary insult and the development of clinical leukemia and the events which transpire at a molecular level need to be explored. Investigation of these relationships, however, is dependent on the development of microtechniques for the evaluation of reverse transcriptase and RNA tumor virus. Without it we are left with gross correlations.

The characteristic of the cell in myeloid leukemia should be considered. These differ in several respects from that of the normal cells. The generation time of the cell in acute myelocytic leukemia may be longer or the same as that of the normal cell; as a result the fractional turnover rate is not increased but, due to the greatly expanded pool size resulting from the failure of differentiation or loss of a "death" function, the total growth of leukemic cells is greater than seen in the normal myeloblast compartment. The leukemic myeloblast or its progenitor may migrate from the marrow into the peripheral blood and proliferate in extramedullary sites, where normal myelopoiesis is not seen. This suggest changes in membrane properties and the interaction between microenvironment and the leukemic cell. Additionally, there is some data to suggest that the leukemic myeloblast may have specific antigens; these are being explored immunologically and may permit improved therapeutic strategies.

A third consideration is that the leukemic cell differentiates partially, i. e. from stem cell to myeloblast and even to progranulocyte but further differentiation usually is not observed. Using the soft agar technique it has been claimed that leukemic clones may differentiate normally under the direction of colony stimulating factor. As I mentioned in my formal presentation, however, the evidence on this point from a morphologic and functional standpoint is inconclusive. Further, it seems to me that to view, acute leukemia solely as a failure of the normal interaction between a granulopoietic regulator and the myeloblast is an over simplifaction. Failure of differentiation and as a result the lack of a "death function" of course leads to an ever increasing number of proliferative cells but the capacity for growth in extramedullary-sites is necessary for the complete evolution of the disease. It is possible of course that if we were able to switch the balance of differentiation from leukemic to normal clones, leukemia could be controlled but in my view this most likely will require manipulation of differentiation of the pluripotent stem cell not the committed myeloid cell or myeloblast.

Discussion following Dr. Stohlman's Talk

Dr. Ostertag: Dr. Stohlman, you are talking about three compartments of the hemopoietic system: the compartment of the pluripotent stem cell, the compartment of committed stem cells and the differentiated compartment of megakaryocytes of myeloid and erythroid elements. You further say that in your view leukemia originates back in the pluripotent stem cell compartment. As supportive evidence you cite the chromosomal change as observed in CML as Philadelphia chromosome, which can be found in cells of all three compartments. Now you do get a *specific chromosomal* change in many patients all suffering from chronic myelocytic leukemia; why can't you get the same kind of chromosomal translocation at the same time in different compartments of the hemopoietic system of the same patient. A viral etiology could easily account for that.

Dr. Stohlman: If we assume for the moment a viral etiology, and I would suggest we are really talking about a virus, then this virus theoretically might affect DNA in a number of ways. The numbers of different types of leukemia are quite restricted, which leads me to think that most effects of viruses are lethal. Whether the immunologic surveillance system identifies and destroys those transformed cells which are recognized as abnormal, perhaps due to membrane changes, or the transformation results in an intrinsically abnormal cell which dies, perhaps after a few divisions, I don't know. The reason a person gets chronic myeloic leukemia, acute myelocytic leukemia or acute lymphocytic leukemia may be either due to the species of virus that transforms the molecule or the same species may hit DNA at random, most of the lesions being lethal, only a few of them being compatible with proliferation; the Philadelphia chromosome is one manifestation and is associated with CML. It would be most improbable for all patients to have three different cell types affected simultaneously.

Dr. Ostertag: I would like to disagree. Let's say a virus is going into the cells affecting the same place in the DNA and breaking the same chromosome in all cells. You would get the same change in all different compartments of the hemopoietic tissue.

Dr. Gallo: That is a perfectly acceptable alternative possibility, and most particularly since a common chromosomal alteration appears limited to CML.

Dr. Stohlman: You also have CML without the Philadelphia chromosome.

Dr. Gallo: Where there are marker chromosomes in AML or ALL you usually do not see them in all three cell lines.

Dr. Stohlman: But you do see the same abnormalities in all three cell lines.

Dr. Hardesty: If leukemia is a stem cell disease, what do you think is the cytological basis of the disease: do the stem cells replicate too rapidly or do their daughters stick around too long? You further mentioned a colony stimulating factor that is involved

in the regulation of myelopoiesis. If one assumes that the normal myeloblast and the leukemic one has a factor, the normal cells should be dominant over the diseased cells. Has somebody tried to make a fusion between leukemic and normal myeloblasts? If the hypothesis is true the normal cells should be able to cure a cancer cell.

Dr. Gallo: To answer your second question first: I don't know if it has been tried yet in vitro. However, many people with abnormal bone marrows were transfused with normal cells. In a few instances apparent transformation of the donor normal cells in the recipient leukemia patient developed. Whether this was due to cell fusion remains doubtful but this would be an argument against your hypothesis (i. e. dominance by a normal cell). To your first question I would say, I am not convinced that leukemias are "stem cell" diseases. If the origin (first cell affected) is always, indeed, the stem cell, then I would say, proliferating stem cells give rise to too many myeloblasts in places they normally should not be. Normal granulocytes go all over the body, they often for instance localize in areas of infection. In short, it might be said that they "metastasize" just like cancer cells, but they know how to terminate, to die. They do not have the potential for continued replication.

Dr. Stohlman: Normal myeloblasts, progranulocytes and myelocytes stay where they are supposed to, namely the bone marrow but abnormal leukemic myeloblasts are seen elsewhere. The abnormal myeloblast grows in extramedullary sites, divides and replicates itself, or in my view it is more likely that the leukemic stem cell is responsible and it and its progeny (the myeloblast etc.) grow in extramedullary sites. The normal fate of a granulocyte is to be released from the bone marrow and to go into the peripheral blood, to go from the peripheral blood to sites of infection. This cell does not have the capacity to divide. The polymorphonuclear granulocyte is an end stage cell just as the red cell, the normal fate of which, after extruding its nucleus, is to enter the peripherial blood, circulate, provide oxygen, and then die in anywhere from 30 days to 10 months depending upon the species. But the myeloblasts and myelocytes do not normally enter the peripheral blood. There is the reason to suggest that this is due to membrane characteristics, namely stickiness and a lack of deformability which prevent early myeloid elements from entering the peripheral blood. Part of maturation is to develop deformability, and lose the "stickiness". In order to release myeloblasts into the peripheral blood there must be an abnormal bone marrow architecture or an abnormal membrane either of which would alter the interaction of the cells with the environment. When you inject myeloblasts into the peripheral blood or stem cells into the peripheral blood, they hone and grow only in the bone marrow or in the spleen.

Dr. Gallo: Let's discuss tuberculosis or other like diseases where immature normal cells including myeloblasts are released into peripheral blood.

Dr. Stohlman: What Dr. Gallo is referring to, is a leukomoid reaction which one occasionally sees in tuberculosis and usually what this means is that immature cells are circulating into the peripheral blood. Myeloblasts are not seen in these situations and moreover the cells in leukomoid reaction don't proliferate.

Dr. Hardesty: What happens when a patient goes into remission? It seems that this is a pertinent question with respect to whether it is a specific block or whether it is something way back.

Dr. Stohlman: I'll give you my thought on that. Normally, you have, what I've been referring to as a pluripotent stem cell and for purposes of conversation it's very easy to consider this a homogenous or uniform compartment. However, it probably is not, one might suggest that a given pluripotent stem cell has a finite limit to the extent of this replication and once exhausted another more immature stem cell is triggered to begin differentiation and you have what is called "clonal" succession. Now, if you treat a leukemic patient with chemotherapeutic agents and induce a remission what may be happening is that a normal stem cell line has differentiated out, remission will last as long as one continues to have normal stem cells differentiate. Relapse comes when either the succeeding stem cell is leukemic or perhaps virus has transformed another normal cell, the presumption being that if you have the capacity for remission then you have normal stem cells present along with leukemic cells.

Dr. Hardesty: You haven't answered the question really. What happens in this respect with Friend virus induced mouse leukemia? In my experience, the spleens are just literally enlarged. The animal is overwhelmed with cells. If there is a remission in this kind of situation do these cells go on and differentiate or do they die?

Dr. Stohlman: In human acute myelocytic leukemia you kill the cells. If you give a patient with acute myelocytic leukemia Daunomycin it wipes out almost all of his cells. You then go through an aplastic phase and then if there is regeneration from a normal pluripotent stem cell, recovery is seen. The Friend virus which produces an erythroleukemia in mice does not have a counterpart in human tissue and hence I am not certain that discussion of this is germane to the present problem.

Dr. Hofschneider: I would like to know the difference between a chronic and an acute leukemia in cellular terms or whatever terms you like. Do you have cells in chronic leukemia that do not grow as fast as in the acute form?

Dr. Stohlman: Chronic myelocytic leukemia is a disease of myeloid cells in the peripheral blood and bone marrow. Immature cells (myelocytes, progranulocytes and myeloblasts) which normally are not present in the peripheral blood are present. It may be associated with anemia, polycythemia, thrombocytosis or thrombocytopenia. Classically there is a decrease in leukocyte alkaline phosphatases. Acute myelocytic leukemia, of the classical variety in young people, is associated with a large increase in the number of myeloblasts in the bone marrow and myeloblasts are present in the peripheral blood without evidence of differentiation such as is seen in the chronic myelocytic leukemia (i. e. in chronic leukemia differentiation is seen). In AML there may be growth of the leukemic cells outside the bone marrow. It could be the lungs, the heart, the brain, the skin and so on. Patients with chronic myelocytic leukemia, at some point develop a blast cell crisis which mimics acute myeloblastic leukemia.

Dr. Hunt: If human leukemias are caused by viruses, do you say the virus infects and is present in the genome of the pluripotent stem cells, and again if that is true, why does it only affect one cell line? What is your explanation for that? Have you ever tried looking in these leukemic patients in any other cell types for the presence of virus particles or viral genomes in the DNA? And what is the answer?

Dr. Gallo: The answer is no, we have not looked at other cell types or tissues, only the affected cells, but it is a very good point. Perhaps I can speculate. I think it's possible that some people are infected with a leukemia virus from without which carries information essential to the induction. This has not been ruled out. However, I don't think infectivity from without is going to be the common mechanism for cancer causation in man. There may be people who disagree with that. By "infectivity from without" I mean active viral infection within in a relatively short period of time relative to the onset of the disease.

Dr. Stohlman: If I may comment on this point. The myeloblast of course predominates in this disease and one can obtain enough cells to examine. But to determine whether there is virus or transcriptase in other cells, erythroid, megakaryocytes etc. will require more refined techniques as there are not enough cells to permit study with current methods.

Dr. Gallo: You're right. We need a better microtechnique. The antibody idea I think is now very feasible. We have available antibodies to primate Type C-virus reverse transcriptase. They cross with the human enzyme, at least some that we've recently isolated. Therefore, we can develop, hopefully a possibility of looking at small quantities of cells by immunochemical methods. This will be a major thrust of our future work in this area.

Discussion following Dr. Gallo's Talk

Dr. Hunt: Dr. Gallo has been reviewing the properties of reverse transcriptase and also informed us about recent experiments to produce antibodies against it. Before we come to the second point, can I ask about one property of this enzyme, its capacity to make double-stranded DNA. What is known about it? Does it in fact make double-stranded DNA?

Dr. Gallo: In most of our experiments the endogenous reaction has been done in the presence of actinomycin D. We, therefore, have not adequately looked at this. However, Peter Duesberg has.

Dr. Duesberg: Yes, they do, but you know we do not know much about this synthesis of DNA other than short pieces of DNA are made perhaps 5 or 6 S in size given a template of 3 or 4 million daltons at least. In the primary reaction it makes complementary DNA, and then the secondary reaction leads to double-stranded DNA also of small size. Whether this is the whole story, that is the complete transcription from RNA that ultimately leads to a complement of double-stranded DNA, as it presumably happens in the cell, is completely open.

Dr. Gallagher: I would like to go back to your (Dr. Gallo) hypothesis that you drew with regard to a hot spot in the DNA because if that is true you might predict a new form of virus and this could get out and infect other cells and so forth. You might be able to test this. Spiegelman's lab could perhaps tell us if there is an increase in the differences between normal and leukemic DNA over a period of time in a leukemic patient, perhaps during the course of CML or something like that. Have they checked it?

Dr. Hehlmann: No, we don't have sequential data in one patient through the course of the disease except one case of ALL, in which we detected viral related RNA during the acute phase of the disease that was not detectable after remission. (Spiegelman and his group have completed these results. There are no leukemic DNA sequences in leucocytes during remission. Moreover, leukemic DNA sequences have been found in the leukocytes of only the leukemic member of identical twins; Proc. Nat Acad. Sci., 70, 269–2632).

Dr. Kufe: At first, according to this hypothesis you said that you think that the malignant state requires the addition of exogenous information and then you went on to evolve the hot spot hypothesis and proposed it was a mutation or addition via recombination of that hot spot. Now is that saying that there was oncogenic potential in that sequence that just had to be altered. Is this just a variation of the oncogene hypothesis only that it requires a base change or something like that?

Dr. Gallo: I believe Dr. Kufe wants to know if the proposed hot spot is either just a sequence that is specially receiving a carcinogenic "boost" thus being just a variant of the oncogene theory, somatic mutation, or added new information? The proposal

demands new information. There was only oncogenic potential by virtue of its unusual susceptability to change. This is clearly distinct from the oncogene hypothesis. However, regarding the nature of the change, I don't think it is useful to attempt to distinguish between the alternatives since as yet the data available, including the important paper that your lab published in this respect, might be explained by amplification, i. e. a difference in some nucleotide sequences between normal and leukemic cells, sequence X after transformation becomes X 50. Your experiment may not differentiate between those two possiblities. Moreover, it is of course, not yet proven that those "extra" sequences are pertinent to leukemogenesis, although I would like to assume with you that they are.

Dr. Kufe: I have to answer that according to the sensitivity of these assays, it would be impossible to have X originally to be amplified. That is, X had to be introduced from the outside because we would have picked up X on the hybridization assay.

Dr. Gallo: Are there viral (type-C RNA tumor vires) genes in some normal cells? Everyone by now must believe that there are some virogenes in at least some normal cells. I would like to know where they came from – or which came first – are these virogenes in fact really cell genes which the virus utilizes? Duesberg should speculate on this.

Dr. Duesberg: That's too much for me. That's like all theories on the origin of life: Where do whales come from, where does God come from, where does a "clean chicken" come from? But I would like to return a question to you, may be somewhat related to that. I think we can at least divide those viruses which cause cancer and those which are sub-virus like things which may be a consequence of cancer. I think that those which are causing cancer may be like the men and the other more or less like the boys. So I think that shouldn't be confused too much. I think these sub-viral particles or endogenous viruses or incomplete endogenous viruses or enzymes might in fact well be a consequence of cancer rather than its cause. But I think there is little doubt that Rous SV or AMV can be the cause of cancer.

Dr. Gallo: I kind of agree with that, at least they cause chicken cancer. I think the information for carcinogenesis may be packaged into only very special type-C RNA tumor viruses. But I wouldn't even make those viruses that you call boys any less important because boys can become men. Moreover, we have now demonstrated that the reverse transcriptase in human leukemic cells and the viral related nucleic acid is related not to endogenous non-oncogenic type-C viruses, but specifically to type-C viruses which in fact *are* oncogenic such as the woolly monkey simian sarcoma virus.

Dr. Duesberg: That is absolutely right. I could have called them girls but I gave you boys. Maybe I could ask one more question. When you talk about leukemia or certain types of myeloblasts that you clinically find are these all genetically or antigenically homogenous? In a given type of luekemia, is there always a unique population of cells? Or could there be heterogeneity, could it be a random thing, just a random messing up of differentiation? Or could it be that it all results from a single cell and

leukemic cells are all identical? I think the identity of leukemic cells would be more compatible with a genetic or viral theory whereas "random" could be regulation or who knows what?

Dr. Stohlman: There are a restricted number on types of leukemia and frequently one sees a monotonous type of cells morphologically. I don't know that anyone has analysed the genetic information from these to say it is identical from cell to cell.

Dr. Duesberg: In these chromosome linked diseases like the Philadelphia chromosome, do you see the change only in the leukemic cells or also in other cells?

Dr. Stohlman: The erythroid (red) cells and the megakaryocytes (platelet precursors) all have the same chromosome.

Dr. Hehlmann: I now refer to Dr. Gallo's very interesting data on antibodies prepared against reverse transcriptases of primate viruses which cross react well with your human leukemic enzyme and offer a new immunologic approach. You have just said you have not prepared an antibody against your human leukemic reverse transcriptase. You had that enzyme fairly purified in the past. What are the difficulties in producing an antibody?

Dr. Gallo: We have been giving it to Bob Nowinski, and he is inoculating rats with the pure enzymes. So far we are losing a lot of enzyme, i. e. no antibody to date. I am becoming very discouraged unless we come in with about a 1 000 grams of leukemic cells so that we can get a lot of this enzyme and are then able to hand him one or two milligrams of enzyme. We tried only twice, we failed, and we didn't relish the idea of losing more of this enzyme.

Dr. Stohlman: I would like to ask Bob one question which shows my immunological incompetence, is it necessary to really clean up and purify this enzyme. Don't some people suggest that you have a better chance of forming good antibodies if things are dirtied up a bit and then you absorb the sera later?

Dr. Gallo: I don't know that I am more competent than you are in immunology but I will answer as best I can. In the first trials when the antibodies to viral reverse transcriptase were prepared, the reverse transcriptase wasn't purified enough and it is true, success was achieved easier in those laboratories which didn't purify as much. It is also apparently true that when you purify more, you reduce antigenic potency. However, if you finally succeed with the purest preparation you are obviously in a much better position. In the long run the results as well as the antigen are cleaner.

Dr. Hofschneider: I have to apologize if I don't ask about reverse transcriptase and such things. I would like to come back to the colony stimulating factor. I have just met, maybe one or two weeks ago, some cell biologists and have asked them for the factor and they told me it is better to forget about it as a specific agent. Apparently, here in the audience are many people who believe in this factor. I would like to have some more information. Is it known what is the chemical nature of the factor, has it

been enriched and to what extent, and has it been applied to animals and what was the effect?

Dr. Stohlman: It's a glycoprotein and various molecular species have been reported from 15.000 to 60.000. It has been given to normal animals. There is a problem in giving it to normal animals in that it is difficult in the experiments done thus far so separate effects of the "release factor", the release of granulocytes from the storage compartment, from true proliferation. The studies to date just don't separate them. I can't answer the question of its physiologic role. When human marrow is cultured with CSF after 12–14 days you get a significant number of eosinophilic colonies, maybe 30, 40, 50 percent, and in the normal human being you certainly don't see this degree of eosinophilic myelopoiesis. So I would raise the question if maybe CSF is a triggering substance, there being other regulators. Most of the evidence suggesting a physiologic role for CSF is inferential. I'm sure it does have one but for various reasons I don't think we have worked it out.

Dr. Torelli: Since I have been under provocation by Dr. Gallo to give my views I would like to say something about the nature of the leukemic cell. I think it is quite evident that we are talking about the etiology of leukemia and we're talking about the virus which is probably being brought into the leukemic cells but we still have to deal with the question, what are the leukemia cells. Because it is quite clear that we are trying to get rid of this question simply by saying: well, these cells do not mature, these cells are blocked in maturation. I think that we should be carefully comparing normal immature cells with leukemic cells. It's quite true that for a long time, in attempting to compare leukemic cells with normal cells, studies were hampered by the fact that many were comparing cells which were proliferating (leukemic) and cells which were not proliferating (normal). I think results at this stage of our studies were useless. We are really faced now with one main question. What is the key difference in the leukemic cell and the appropriate normal cell control. I think that point has to do with the introduction of a viral genome into the cell. This introduction should not bring the cells to limited progression. I think we should look for major changes which are brought into the cell by the introduction of the virogene.

Dr. Gallo: There is one point which I don't think came up at any time in the meeting. I am referring to some cases of bone marrow transplatation. There were two reported cases of recipients that were leukemic who received bone marrow from normal donors and apparently when relapses occured the normal donor cells had transformed in the recipient patient. Now there's a lot of discussion of how valid those observations were; how clear were the results which were based on cytogenetics. If, however, these results are valid, there is obviously a very important lead which directs us to almost only one conclusion, that a transforming agent remains in at least some leukemic patients. We can talk about cell-cell fusion, but I doubt whether this would occur under these conditions *in vivo*. Even *in vitro* under the best conditions, it is difficult. Normal donor cells then apparently can transform in recipient leukemia people, and the most likely interpretation is that the "transforming factor" is still present after destruction of leukemic cells.

Remarks Concerning the Discussion

It was quite impossible to include in this book the whole discussion, which lasted for more than 12 hours, in its entirety. We had to, unfortunately, leave out the greater part, and limit the discussion to the summary reviews of Dr. Fred Stohlman, a clinical hematologist, and Dr. Robert Gallo, a medical molecular biologist. Even from the discussion following Dr. Stohlman's and Dr. Gallo's reviews we were forced to cut a great many interesting critical remarks, and were only able to include 30 to 40 per cent. For the critical selection we thank Drs. R. Hehlmann and T. Hunt.

Because of space limitation many of the authors have included a considerable part of the discussion in their articles and many questions, arising from the different investigational trends, have been summarized in the introduction.

Rolf Neth

Subject Index