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Modern Trends in Human Leukemia IX

New Results in Clinical and Biological Research Including Pediatric Oncology

Organized on behalf of the Deutsche Gesellschaft für Hämatologie und Onkologie, Wilsede, June 17–21, 1990

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"Clinical Aspects"

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Wilsede Joint Meeting
Hamburg
16. 6. 1990
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Ladies and Gentlemen,
Dear Friends

In 1988, after the last Wilsede meeting, Elena Frolova and her friends had the marvelous idea of organizing a Volga Wilsede meeting. Today, I am very pleased that this idea became true.

Every day, we dream of freedom and peace. A small part of this dream has today come into our hands.

Everybody here has done his best to come together for good science and in friendship.

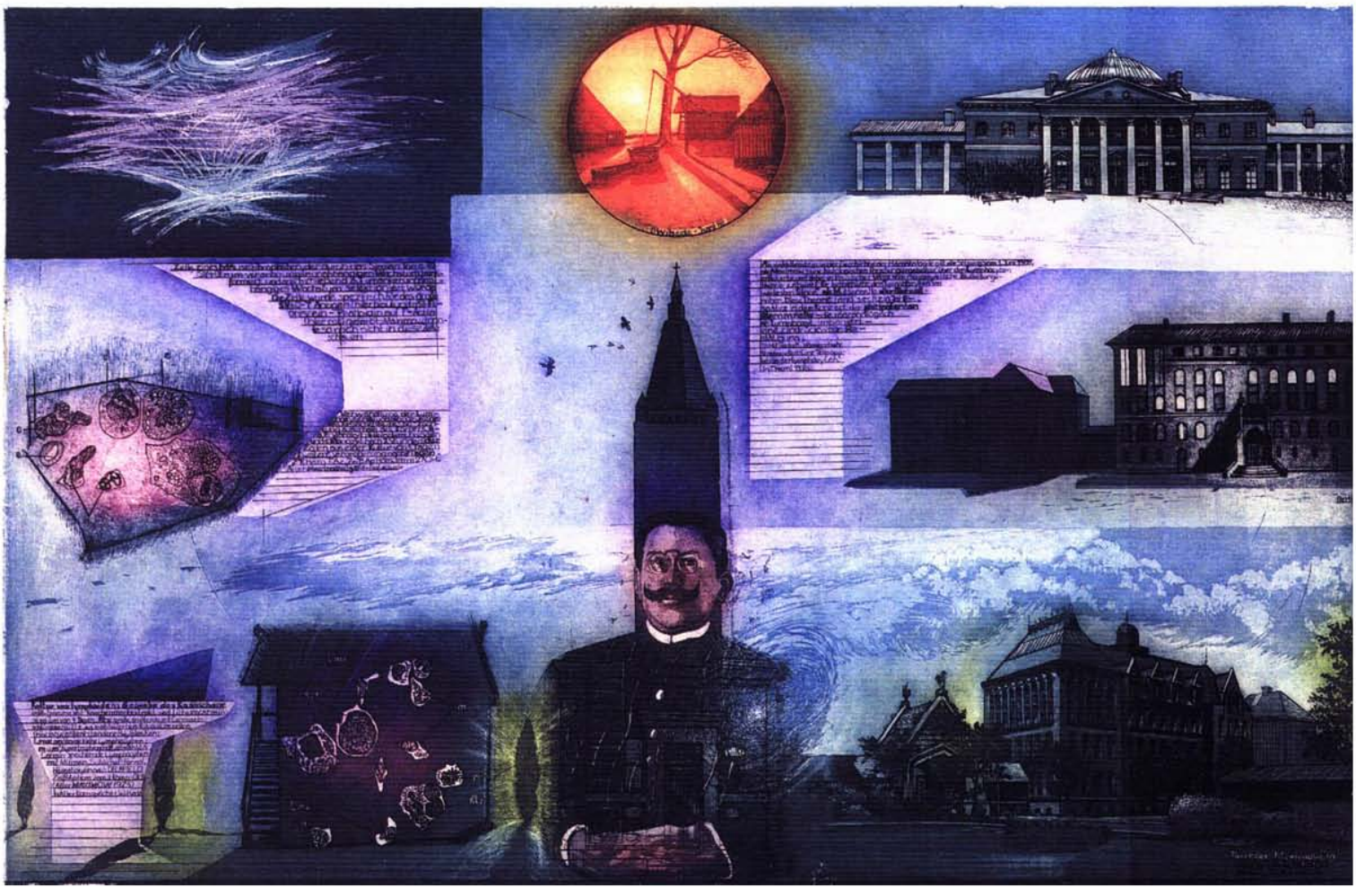
I am sure that this Volga Wilsede meeting and the Neva Wilsede Meeting will become part of the international tradition of good science in a peaceful human atmosphere.

For us this door is open: let us go through.

Moscow, 13 June 1990 *Rolf Neth*

Den völligen Gegensatz zum Antlitz bildet das Wort Maske.

Pavel Florenski 1882–1937 in:
„Die Ikonostase"



Dr. Rolf Neth asked me to say a few words about my last work, which I called "Alexander Maximov's world". I could think that many of you had to learn or to study his experiments and results, when they were students. Well, I am not competent enough to give a valuation of his work or meaning in histology. But the few things I read about his work and life gave me a great deal of respect and admiration. Specially how he drew and painted the results of his experiments in such a filligran and precise way.

Amongst scientists, often the work of a colleague is not always considered with great admiration rather more with a feeling of respect or competition. So, when I put up some kind of a monument for Alexander Maximov, it is the painters admiration for a scientist, who probably drew much better than I ever would do in histology.

Now to my work: There is the real and spiritual world of Alexander Maximov. The polarisation of the idea is seen on the right side which shows the three stations in his life.

First: St. Petersburg, where he worked at the Military Medical Academy for some years.

Second: In Berlin, the Charité Hospital, where he worked like his famous colleagues Arinkin and Botkin before.

Third: And last station of his life was the University of Chicago where he had to emigrate with heavy heart after october revolution.

On the left side you see two blood pictures which have been drawn by Alexander Maximov. The upper picture which looks like a Super Nova is a cell of a mouse fibroplast which Alexander Maximov in his time could not see like this, as the electronic microscope was'nt invented then. This is the contemporary level of this work together with the circled Wilsede motif over the tower.

If you look closer and I could help everybody with my magnifying glass, you discover that Maximov's two blood pictures are drawn in the silhouette of the Emhof (up side down) and the Granary.

The Center shows the portrait of Alexander Maximov about the time as a young man in St. Petersburg and it shows close to his heart the entrance to his laboratory in the Military Academy.

To end now, I sum up the key idea may be called 5 seconds of inspiration and five weeks of rather hard work. Alexander Maximov as a tower surrounded by the real buildings St. Petersburg, Berlin and Chicago and on the other hand surrounded by his spiritual buildings, the blood pictures in the silhouette of Wilsedes granary and the Emhof.

So I thank you for accompanying my explanations of Alexander Maximov from St. Petersburg to us here in Wilsede.

Michel Weidemann

Maximov's world
Etching 40 × 60 by Michel Weidemann

Scientific and private discussions on the Volga and around Uglich

Scientific and private discussions in and around "The Emhof"

Photographs: R. Völz (Hamburg)

Contents

Clinical Aspects

Cell Biology

Molecular Genetics

Virology

Immunology

Early Life and Evolution

Dr. Mildred Scheel Memorial Lecture

Hamburg, June 18, 1988

In Memoriam Dr. Mildred Scheel

Klaus Munk

The Wilsede Meeting is also supported by the Wilsede Fellowship Programme of the Dr. Mildred Scheel Stiftung, which is part of the Deutsche Krebshilfe. Since that Foundation was established by Dr. Mildred Scheel, it is appropriate that we should reflect and comment on the great contribution which she made to cancer prevention, treatment and research.

Who was Mildred Scheel? What were her ideas and what did she achieve with her Foundation?

Mildred Scheel was born in Cologne in 1932, daughter of a physician and radiologist. She studied medicine and specialized in radiology. Later, she married Mr. Walter Scheel before he was appointed Minister for Foreign Affairs. When Mr. Scheel subsequently became President of the Federal Republic of Germany, she became the "First Lady" of this country. No doubt this helped her to fulfil her noble ambition to contribute to the fight against cancer. As a consequence of this, she founded the Deutsche Krebshilfe in 1974. From that time on, all her efforts were directed towards encouraging people to contribute money for this crucial purpose. She developed many significant ideas for organizing cancer prevention, early diagnosis and treatment that was applicable on a large scale. She initiated the establishment of the first five cancer centers in this country. Once they were functioning successfully, she was able to convince the Government to assume full responsibility for maintaining them. She then prepared to launch new undertakings. It became apparent to people that she had unique qualities that enabled her to initiate new ideas for fighting cancer, and this added significantly to her personal success. She also supported in particular the treatment of childhood cancer in many hospitals, and initiated the psychosocial after-care of patients and their families. In addition, she aided individuals who were economically affected by having cancer.

The Dr. Mildred Scheel Stiftung was established to promote and support cancer research. It supports a great number

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

of research projects in many institutes and provides a fellowship programme for scientists to work and study at institutions abroad. Included in that programme is the Wilsede Fellowship Programme. The Dr. Mildred Scheel Stiftung is now an important body in the Federal Republic of Germany for the granting of fellowships. Many of Mildred Scheel's initiatives were not broadly accepted at first, but through her continued energy they are now accepted as common practices in the oncological field in this country.

When she had a particular goal in sight, no obstacles could prevent her from reaching it. Yet, for all her tenacity, Mildred Scheel was a warm, loving and sensitive person who had special understanding for cancer patients, together with a human touch. She was always very hard-working and enthusiastic, and stimulating for all of us. None of those who, like myself, had worked with her in the Foundation for over 10 years can remember her ever missing a meeting of the board or the scientific councils of the Deutsche Krebshilfe or the Dr. Mildred Scheel Stiftung, until the last few weeks of her life. During those meetings she listened carefully to the experts, although sometimes she came to her own conclusions when she was convinced that a particular step forward had to be made. She never lost her enthusiasm for helping others, even when she realized what would be the consequence of her own illness. She always seemed to be positive in her attitude and could always stimulate others with her spirit and her personality. She could have done so much more in the future and she is sadly missed by all of us. We all will always remember her with great devotion.

The Mildred Scheel Memorial Lectures are our tribute. The second lecture will be held by Axel Ullrich, a classic molecular biologist, who has made important contributions to the understanding of cancer in the field of molecular biology.

Growth Factor Receptors: Role in Normal Mitogenic Signalling and Oncogenesis

A. Ullrich [1] and J. Schlessinger [2]

Growth factors, differentiation factors, and polypeptide hormones are crucial components of the regulatory system that coordinates development of multicellular organisms. Many of these factors mediate their pleiotropic actions by binding to and activating cell surface receptors with an intrinsic protein tyrosine kinase (PTK) activity. Figure 1 presents a schematic representation of the known growth factor receptors that bear PTK activity. Growth factor receptors with PTK activity, or receptor tyrosine kinases (RTKs), have a similar molecular topology. All possess a large, glycosylated, extracellular, ligand-binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain which contains a PTK catalytic domain (Hanks et al., 1988; Yarden and Ullrich 1988, Schlessinger 1988; Williams 1989).

Primary sequence homology and distinct structural characteristics of different RTKs allow the classification of these receptors into subclasses (Fig. 1). The structural features characteristic of the four subclasses include two cysteine-rich repeat sequences in the extracellular domain of monomeric subclass I receptors, disulfide-linked heterotetrameric $\alpha_2\beta_2$ structures with similar cysteine-rich sequences in subclass II RTKs, and five or three immunoglobulin-like repeats in the extracellular domains of subclass III and IV RTKs, respectively. The tyrosine kinase domain of the latter is interrupted by hydrophilic insertion sequences of varying length. The availability of RTK cDNA clones has made it possible to initiate detailed structure-function analyses of the mechanisms of action of RTK family members. Numerous mutants of insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), colony-stimulating factor 1 (CSF-1), and other receptors have been characterized in regard to their biological and biochemical properties. This has led to the establishment of a receptor domain function map and model for RTK-mediated signal generation (Fig. 2).

Ligand binding to the extracellular domain of the receptor results in conformational change and subsequent oligomerization [Schlessinger 1988]. Receptor oligomerization is a universal phenomenon among growth factor receptors. It has been detected in living cells, in isolated membranes, and in preparations of solubilized and purified receptors [Schlessinger 1986; Yarden and Schlessinger 1985, 1987 a, b; Cochet et al., 1988]. It may be induced by either monomeric ligands, such as EGF, which cause receptor oligomerization by inducing conformational changes [Greenfield et al. 1989] resulting in receptor-receptor interactions [Lax et al. 1990] or by bivalent ligands, such as PDGF and CSF-1, which mediate dimerization of neighboring receptors [Seifert et al. 1989; Heldin et al. 1989; Hammacher et al. 1989]. Oligomerized growth factor receptors possess elevated PTK activity [Yarden and

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Haematology and Blood Transfusion Vol. 35
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R. Neth et al. (Eds.)

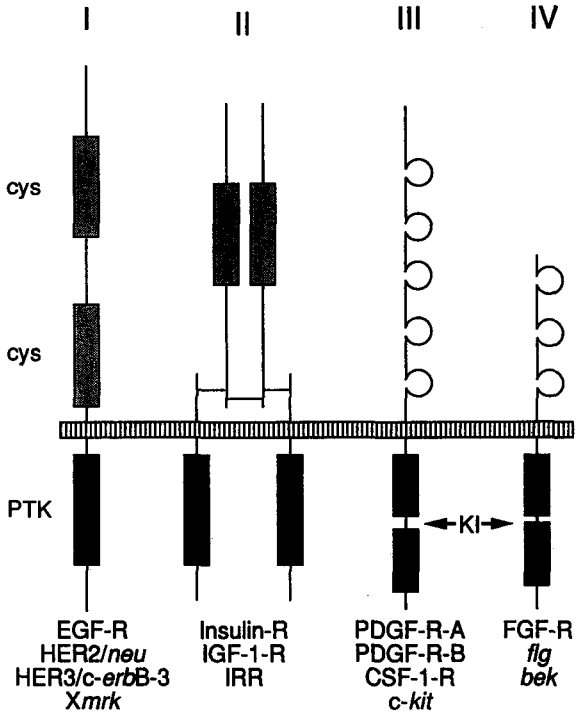

Fig. 1. Schematic representation of receptor tyrosine kinase subclasses. For details, see Ullrich and Schlessinger (1990)

Schlessinger 1987a, b; Böni-Schnetzler and Pilch 1987], which leads to phosphorylation of tyrosine residues of the receptor polypeptide chain and of cellular substrates.

Receptor phosphorylation releases an internal constraint by stabilizing a conformation that is competent to interact with and phosphorylate cellular substrates [Honegger et al. 1988a, b]. The recent observation that phosphorylation of EGF and insulin receptors can occur by intermolecular cross-phosphorylation both in vitro and in living cells [Honegger et al. 1989, 1990; Ballotti et al. 1989; Lammers et al. 1990] further supports the importance of receptor oligomerization in the process of receptor activation.

The chain of events that is initiated by tyrosine phosphorylation of cellular substrates is still poorly understood. Several RTK substates of potential biological importance have recently been identified (Figure 3). Both PDGF and EGF can induce tyrosine phosphorylation of phospholipase Cγ(PLC-γ) in vitro and in living cells [Margolis et al. 1989; Meisenhelder et al. 1989; Wahl et al. 1989]. In addition, PLC-γ was observed to associate with the activated receptor kinases in a ligand- and kinase-dependent manner [Margolis et al. 1989, 1990a; Kumjian et al. 1989]. However, growth factor-induced inositol triphosphate (IP_3) generation appears not to be the

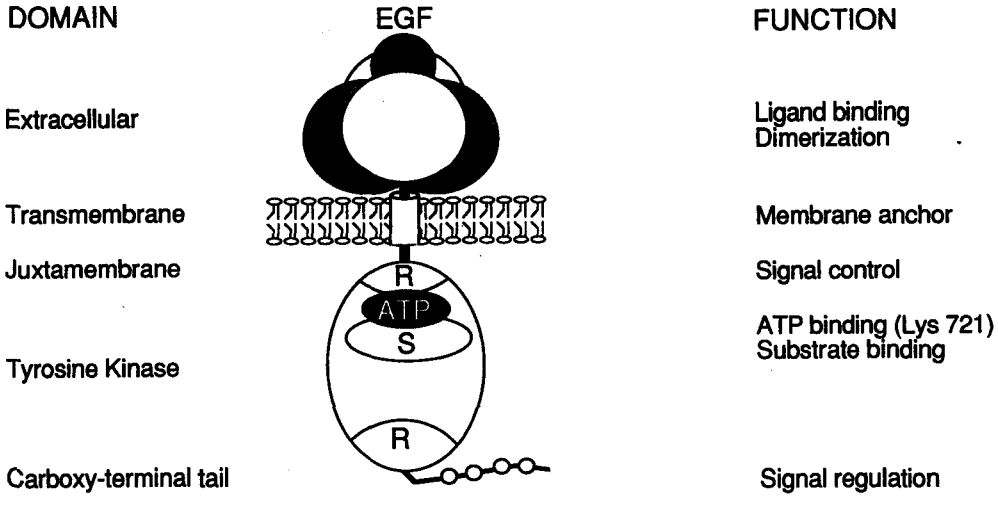

Fig. 2. Proposed structure-function topology of the EGF receptor. Subdomains II and IV (*stippled*) represent the cysteine-rich regions of the extracellular domain. Most of the structural determinants that define EGF binding affinity are proposed to be located in the cleft formed by subdomains I and III. The symbols *S* and *R* within the PTK domain represent proposed interaction sites for substrates and regulatory factors [Ullrich and Schlessinger, 1990]

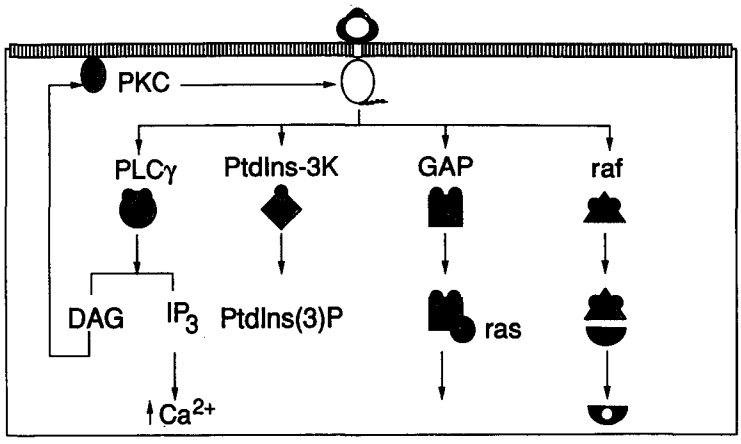

Fig. 3. Receptor-mediated multiple signalling pathways. Direct phosphorylation (*black dots* on symbols) of substrates, PLC-γ, PtdIns-3K, GAP, and *raf* leads to secondary events, including enzymatic activation and metabolite formation (DAG, IP_3, PtdIns(3)P), activation of enzymatic functions by association, and Thr/Ser phosphorylation (*white dot* on symbol) of substrates [Ullrich and Schlessinger, 1990]*PtdIns-3K:* phosphatidylinositol 3-kinase; *GAP:* GTPase-activating protein; *PtdIns(3)P:* phosphatidylinositol 3-phosphate

sole mechanism leading to the initiation of DNA synthesis [Downing et al. 1989], which is compatible with the notion that the phosphatidylinositol (PI) signalling pathway does not play an essential role in the mitogenic response [Lopez-Rivas et al. 1987; L'Allemain et al. 1989; Margolis et al. 1990b].

Other RTK substrates that have recently been identified include PI kinase and the *ras* binding protein GAP [Kaplan et al. 1987; Varticovski et al. 1989; Molloy et al. 1989] (Fig. 3). Similarly, it has been suggested that the c-*raf* protooncogene product becomes phosphorylated in response to PDGF receptor activation [Morrison et al. 1989]. Intriguingly, all proteins identified thus far as RTK targets are either components of second messenger pathways, protooncogene products, or factors that regulate the activity of protooncogene products.

The importance of allosteric regulation of receptor activation and signal transduction is further emphasized by the fact that a large variety of structural alterations found in RTK-derived oncogene products lead to constitutive kinase activation and, consequently, subversion of molecular control mechanisms and alteration of receptor signals. Thus, transforming RTK derivatives serve as valuable model systems not only for studying the mechanisms of oncogenesis but also for the analysis of normal structure-function relationships for these signal-transmitter molecules. Constitutive activation of RTK signalling functions can be achieved in a number of ways. For example, in the cases of v-*erb*-B and v-*kit*, deletion of the extracellular binding domain eliminates the negative control that this structure normally exerts on the cytoplasmic domain. Even point mutations within the extracellular domain can lead to intracellular activation, as in

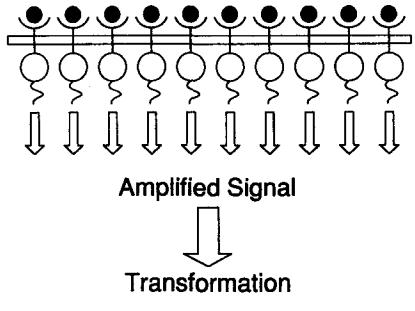

Fig. 4. Transformation by receptor amplification. Schematic representation of proposed transformation model by autocrine stimulation of overexpressed receptor tyrosine kinases. Ligand (*black dots*) is activating receptors in the plasma membrane of a tumor cell, resulting in an amplified transforming signal

the case of v-*fms* mutations at residues 301 and 374 [Woolford et al. 1988; Roussel et al. 1988] (Fig. 4). These mutations appear to induce and stabilize a conformational change equivalent to that triggered by ligand binding and possibly dimerization. Another dramatic effect of a single point mutation is exemplified by the Val/Glu conversion in the *neu* transmembrane domain [Bargmann et al. 1986], which suggests that this part of the putative receptor is involved in an overall conformational alteration that occurs upon interaction with the yet unidentified ligand. In this case, the transmembrane mutation results in constitutive receptor oligomerization [Weiner et al. 1989]. Another type of structural alteration has been identified in the EGF receptor/*erb*-B system and involves mutations in the PTK core region [Massoglia et al. 1990]. Despite the presence of an intact extracellular domain, these mutations render the EGF receptor competent for mitogenic and transforming signalling without autophosphorylation. RTK-derived oncogenes possess other structural lesions such as cytoplasmic point mutations, deletions, and C-terminal truncations which appear to enhance and modulate the transforming signal [Khazaie et al. 1988; Woolford et al. 1988].

For human cancer, activating RTK mutations appear to be of minor importance. The most common cellular lesion found in human cancers involves autocrine activation in conjunction with receptor overexpression (Fig. 4). Many tumors and tumor cell lines have been found to coexpress growth factors and their receptors, including TGF-α, PDGF-A, PDGF-B, acidic fibroblast growth

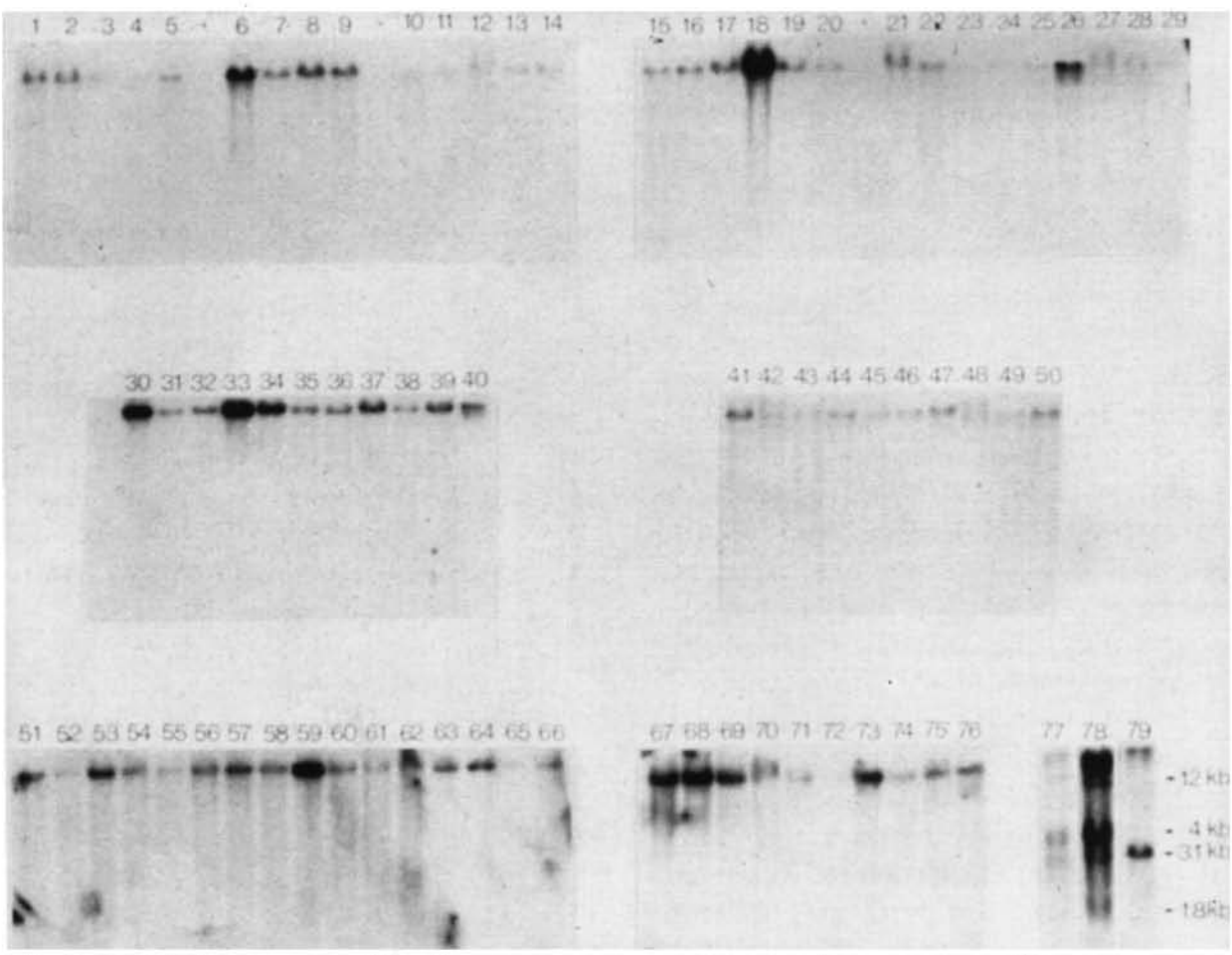

Fig. 5. HER2/*neu* gene amplification in mammary carcinoma. Southern blot hybridization analysis of chromosomal DNA from primary mammary carcinoma tumors [Slamon et al. 1987]

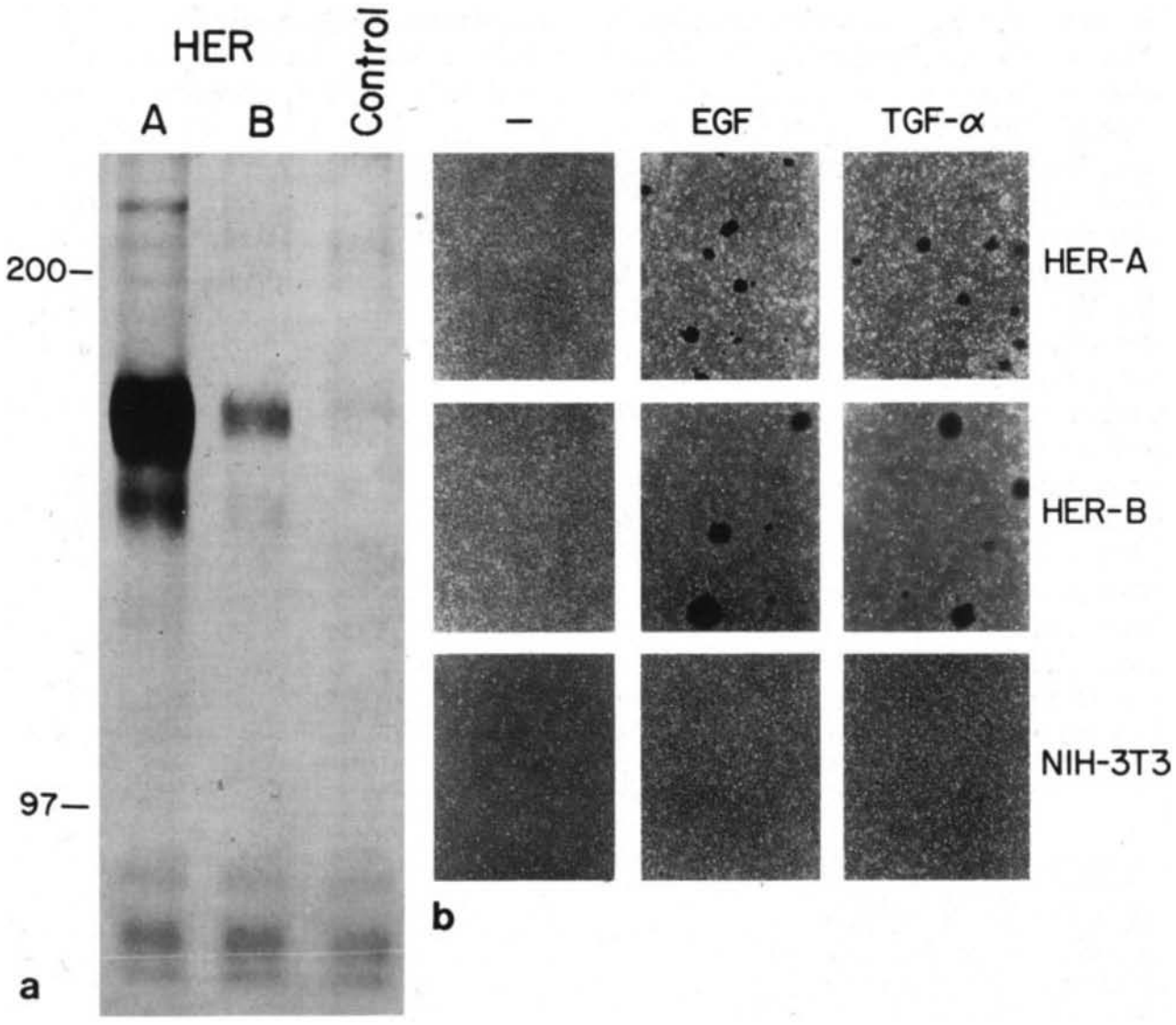

Fig. 6a,b. Cell transformation by EGF receptor overexpression. NIH-3T3 cell lines HER-A and HER-B overexpressing the human EGF receptor (**a**) were stimulating with EGF or TGF-α and tested for their ability to grow in soft agar (**b**)

factor (FGF), basic FGF, and their specific RTKs. Thus, autocrine receptor activation represents yet another scenario of subversion of normal growth control. For mammary and ovarian carcinoma, extensive studies have demonstrated a direct correlation between the extent of overexpression of $p185^{HER\,2/neu}$ and a patient's prognosis, a result which strongly suggests a critical role for this EGF receptor-like RTK in tumor progression and perhaps even tumor initiation [Slamon et al. 1989] (Fig. 5). This possibility is further supported by efficient induction of mammary carcinoma in mice by an activated *neu* gene product [Muller et al. 1988] and transformation of NIH-3T3 cells by overexpression of unaltered $p185^{HER\,2/neu}$ [Hudziak et al. 1987]. Analogous experiments with the EGF receptor indicated that autocrine stimulation of the overexpressed receptor was essential to achieve a transforming effect [Di Fiore et al. 1987; Velu et al. 1987; Riedel et al. 1988] (Fig. 6).

On the basis of these findings, strategies involving antireceptor antibodies were designed for the treatment of mammary and ovarian carcinoma. Monoclonal antibodies, such as the anti-HER2/*neu* antibody 4D5, are able to interfere with autocrine activation of the receptor, which results in inhibition of tumor cell growth in tissue culture and nude mouse models (Ullrich et al., unpublished).

In principle, every receptor with PTK activity has oncogenic potential. One can anticipate that many more types of activating mutations, as well as specific instances of RTK overexpression, will be detected in animal and human tumors. The molecular identification and characterization of these mutants will not only provide important insights into fundamental mechanisms underlying receptor activation and normal growth control, but may also enhance our understanding of oncogenesis and open new avenues for diagnosis and therapy.

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Yuri Ovchinnikov Memorial Lecture

Moscow, June 13, 1990

In memoriam Prof. Yuri Ovchinnikov

V. T. Ivanov

Who was Yuri Ovchinnikov? What were his ideas and what did he achieve during his dynamic although brief carrier? Why do we remember him at this Wilsede meeting? I do not think that these questions really ought to be answered, since Yuri Ovchinnikov belonged to the elite of international scientific community, being extremely well known not only for the results of his own research but also as an outstanding leader of chemical and biological sciences in the Soviet Union and a champion of international scientific collaboration.

A separate chapter of this book describes the path along which the main achievements of Ovchinnikov as a biochemist were reached. Here, I would like to offer the reader a glipse of Ovchinnikov as a human being. He was born in 1934 in Moscow; in 1952 he entered the Chemical Department of Moscow University and in 1957, after graduation, he became a professional researcher. Many facets of his unique personality showed up already in these early years. He had a phenomenal memory and was a leading actor at the University studio.

Yuri loved sports. He was a University champion in free-style wrestling, and was also a keen swimmer and cross-country skier. These skills he maintained for many years to come. It seems that the famous Robert Woodward has little

Fig. 1. Moments of relaxation (1970): Yuri Ovchinnikov (*left*) and Nobel prize winner Prof. R. Woodward (USA)

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Fig. 2. Yuri Ovchinnikov lecturing (1972)

Fig. 3. Deep in thought (1975)

chance of surviving in the billiard game with Yuri shown in Fig. 1.

Being a loving husband and father and generally a very warm personality, Ovchinnikov had a talent for collecting teams of young enthusiastic people and helping them in their research.

A clear vision, high motivation, the burden of responsibility – these are some

Fig. 4. At Yaroslavl airport (1982)

of the characteristic Yuri's qualities and feelings which are well reflected in Figs. 2, 3. A favorite among many of those who knew Yuri well is Fig. 4, where we see his firm stand, the boyish mischief, the invincible optimism and the belief that "we shall overcome", whatever the obstacles.

Indeed, he overcame much, but one thing proved too hard even for him: disease, the disease which could have been defeated if we knew more about the hemopoietic process. Maybe this is one more reason for dedicating the Volga Wilsede meeting to the memory of Yuri Ovchinnikov.

Prof. Yuri Ovchinnikov

V. T. Ivanov and V. A. Nesmeyanov[1]

Yuri A. Ovchinnikov started his carrier within the precincts of Moscow University at the Chemical Department under Professor Yu. A. Arbuzov. The project of his masters' degree (1957) provided material for the first publication on a new technique for the synthesis of pyrrolidine and thiophan derivatives. By that time, the gifted student had already shown a disposition toward synthetic organic chemistry. It was at this period that his belief took shape that the chemistry of living organisms was by far the most attractive area for an organic chemist to enter. Therefore, having begun his postgraduate course at the Chemical Department, Y. A. Ovchinnikov readily accepted an invitation to participate in the project on the complete synthesis of an important group of antibiotics, the tetracyclines. While working toward his doctorate, Yuri Ovchinnikov met M. M. Shemyakin, the leader of the project. The joint work led to a long-lasting collaboration between the two scientists, whose contribution to the foundation and advancement of physicochemical biology in the USSR was outstanding.

After finished his postgraduate course, Ovchinnikov joined the Institute for Chemistry of Natural Products of the USSR Academy of Sciences, set up not long ago. Here, Professor Shemyakin proposed that he go into peptide chemistry.

The subject under study depsipeptide antibiotics, atypical peptides containing hydroxy and amino acid residues. The problems of synthesis of the optically active N-methylated amino acids, reversible protection of the hydroxyl function of hydroxy acids, and cyclization of linear depsipeptides were rapidly solved and compounds with the structures proposed in the 1940s by Swiss researchers for antibiotics enniatins A and B were prepared. However, the samples obtained were devoid of antimicrobial activity and their physicochemical properties differed much from those of the naturally reliably confirmed, it remained to conclude that the formulae proposed for enniatins A and B were incorrect. Several alternative structures differing in ring size were suggested and accordingly synthesized. Two of them were indistinguishable from the natural enniatins A and B, which meant a solution to structural problems.

Later (1964–1970), Ovchinnikov and his colleagues performed a series of elegant syntheses of some other naturally occurring depsipeptides (sporidesmolides I-IV, angolide, serratamolide, esperin, beauvericin). he was awarded a D.Sc. in 1966 for the synthesis of natural depsipeptides and their analogs.

In 1967, Shemyakin, Ovchinnikov, and their team formulated the original (so-called topochemical) principle of transformation of biologically active peptides: novel molecules can be designed by such deep structural modifications as reversal of the acylation direction and the configuration of asymmetric centers, replacement of ester bonds by amide bonds and vica versa, cyclization of linear molecules,

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

etc. The conditions favorable for retaining the original stereoelectronic parameters and, consequently, biological properties of the molecule were found. Ideas from this pioneer research were taken up by many laboratories and served to create novel highly active peptides (hormones, antibiotics, neuropeptides, enzyme substrates, and inhibitors).

The experience accumulated during this synthetic work served as a basis for the next and the culiminative step in studying the depsipeptide antibiotics. Bearing in mind the recently discovered ability of valinomycin and enniatins to induce permeability of lipid membranes to alkali metals ions, Y. A. Ovchinnikov and his colleagues undertook a study of the physicochemical basis of the phenomenon. It appeared that valinomycin binds potassium ions in solution, yielding stable complexes, and shows a unique K/Na-selectivity of complex formation unsurpassed in nature. Enniatins bind virtually all alkali and alkali-earth cations, though with a lower selectivity. These complexes are the ion-transporting species, and selectivity of ion binding is the origin of the selectivity of transmembrane ion transport. Further, the three-dimensional structures of the free antibiotics and their complexes were established. It was shown for the first time that such sophisticated structures can be resolved not only by X-ray analysis but also in solution by spectral methods. The bound ion appeared to reside always in the center of the depsipeptide molecular cavity and be kept in place by ion-dipole interactions with the carbonyl oxygens. The size of the valinomycin cavity is limited by a bracelet-like system of six intramolecular hydrogen bonds that accounts for its inability to adapt to smaller sized ions such as sodium or lithium. Enniatin structures are more flexible, which enables adjustment of the cavity to the size of the bound ion. The molecular periphery of both valinomycin and enniatin complexes is fully hydrophobic, which allows them to migrate freely across lipid zones of the membrane. Several laboratories outside the USSR were about to get similar results, but "the train had already gone."

Step-by-step protein compounds, the major working bodies of any living system, began to occupy the prominent place in Ovchinnikov's research activity. The 1970s witnessed a series of studies on the primary structures of porcine aspartate aminotransferase, and toxins from the venoms of cobra, bee, scorpion, etc. As a result, more than 20 structures were added to international data banks and atlases of protein structures. Inspired by these advances, Ovchinnikov and his group tackled the deciphering of the primary structure of *E. coli* DNA-dependent RNA polymerase, a key transcription enzyme investigated in many laboratories. Ovchinnikov had a very strong team, but even for them the problem seemed extremely difficult, since RNA polymerase is built of several subunits, among them two very large β- and β'-subunits (each over 1300 amino acid residues). Indeed, after rapid sequencing of the α-subunit (over 300 amino acids) it became clear that analysis of the β- and β'-subunits exclusively by conventional methods of protein chemistry could take many years. A decision was made to utilize the methods of genetic engineering and to analyze the sequences of genes coding for the subunits. In those days, such an approach was new for this country, and elsewhere it was at the early stages of development.

Genes for large subunits of DNA-dependent RNA polymerase form the so-called operon rpoBC and contain about 10000 base pairs. They were isolated, inserted into plasmids, and sequenced. Structures of peptides of large subunits were detected in parallel and independently. That was of use: when the structural analysis of genes was completed and the structures of corresponding proteins were derived according to the genetic code, they appeared to coincide with the peptide structures and, consequently, were determined correctly. Soon after that, other laboratories reported the gene

fragments but not the complete gene. It is worthwhile noting that the structures of these fragments contained errors. Only the combined use of the methods of protein and nucleotide chemistry provided reliable results.

The structural analysis of RNA polymerase served as a basis for a thorough investigation of the mechanism of action of the enzyme, for numerous genetic and biochemical studies.

That was in the late 1970s. More and more laboratories outside the USSR were successfully applying genetic engineering methods to microbiological synthesis of practically important proteins. Yuri Ovchinnikov was the first in the USSR to assess the prospects. He united enthusiasts and headed the work on improving the methods of chemical synthesis and directed mutagenesis of DNA to create microorganisms producing alien peptides and proteins. As a result, strains producing an opioid neuropeptide, leucine-enkephalin (1979), the antiviral and antitumour human protein interferon-α_2 (1981), and the precursor of human insulin, proinsulin (1983), were obtained.

Despite these advances of Yuri Ovchinnikov in genetic engineering and biotechnology, the bioorganic chemistry of peptides and proteins was always his major interest and devotion.

In the mid-1970s, he, N. Abdulaev, and a group of colleagues focused their interest on the molecular mechanisms of photoreception. By that time, a series of substantial discoveries had been made that paved the way for solving the problem of how light energy is transformed into the electric energy of the nerve impulse by rhodopsin, a well-known light-sensitive protein from the animal retina.

Soon after wards, there appeared data on the membrane protein – bacteriorhodopsin – found in microorganisms living in salt lakes. The protein was given that name because of its similarity to the visual rhodopsin (the presence of the bound retinal, light-sensitivity, etc.). Though bacteriorhodopsin functioned as a light-dependent proton pump, from the viewpoint of the primary photochemical properties it was very similar to rhodopsin. At the same time, bacteriorhodopsin is more readily available in large amounts and has a simpler structure than the visual rhodopsin, the main effort was initially directed to that protein. It was also considered that bacteriorhodopsin was (and still is) an ideal model for structure-functional analysis of membrane proteins. Simaltaneously with Prof. G. Khorana of the USA, the Nobel prize winner, Ovchinnikov succeeded in determining the amino acid sequence of bacteriorhodopsin, is was the first time that the chemical structure of the membrane protein had been deciphered (1987). Ovchinnikov and his team were then pioneers in solving the structure of rhodopsin from bovine retina (1981).

Research into the topography of polypeptide chains of these proteins in native membranes and elucidation of the structure of their active sites and disposition of functionally important groups were the next steps in this project. Using a variety of approaches including chemical modification, enzymatic treatment, and immunochemical methods, Yuri Ovchinnikov and his colleagues demonstrated that the two rhodopsins are arranged in the membrane in a similar way – as seven extended protein segments spanning the membrane's width and connected with each other on the two sides of the membrane by short peptide links.

In the mid-1980s, Y. Ovchinnikov and V. Lipkin focused their attention on the studies of other proteins involved in transmission and amplification of the visual cascade – transducin and cyclic GMD phosphodiesterase. In 1985, the primary structures of the γ- and α-subunits of transducin from bovine retinal rods were sequenced. Interestingly, the γ-subunit is characterized by the two adjoining cysteine residues also connected by a disulfide bridge. The residues are apparently involved in the formation of

the transducin-photoactivated rhodopsin complex.

An exciting page in the scientific biography of Yuri Ovchinnikov was his last project, devoted to studies of the system of active ion transport, i.e., Na,K-transporting adenosine triphosphatase and related proteins. In the late 1970s, Ovchinnikov initiated research into the structure of Na,K-ATPase. At the beginning, oligomeric organization of the functionally active complex in the native membrane was unraveled and the asymmetric arrangement of the subunits described. Further progress depended upon determination of the amino acid sequence of the subunits. Around 1985–1986, Ovchinnikov's team completed studies of the nucleotide sequences of genes for subunits and amino acid sequences of their polypeptide chains, which led to the complete primary structure of Na,K-ATPase from pig kidney outer medulla. Some research centers outside the USSR were also working intensively in these areas. The teams of S. Numa (Japan) and A. Schwartz (USA) simultaneously reported amino acid sequences of similar enzymes from other sources.

However, the approach chosen by Ovchinnikov extended far beyond the primary structure determination. Complemented by spectroscopic and molecular modelling studies, it resulted in the first detailed model of the Na,K-ATPase spatial structure. Here, the α-subunit (1016 amino acid residues) forms seven transmembrane segments and the major portion of its hydrophilic region accommodating the catalytic site is located inside the cell. The β-subunit (302 residues) spans the membrane once and the main part of its polypeptide chain forms an extracellular glycosylated domain.

As for the Na,K-ATPase active site, Ovchinnikov and his team employing affinity modification by ATP analog succeeded in identifying an unknown component of the catalytic site, thus experimentally confirming its dynamic changes during enzyme functioning.

Yuri Ovchinnikov, together with Eugene Sverdlov and their groups of researchers, obtained novel data on the regions of the human genome encoding the systems of active ion transport that seem to be of general biological significance. A family of at least five genes was defined in the human genome coding for several isoforms of the Na,K-ATPase catalytic subunit as well as other structurally similar ion-transporting ATPases.

The discovery of the multigene family gave rise to new concepts on regulation of the active ion transport through changes in the activity of the appropriate genes. This was supported by experiments on the expression level of various genes for Na,K-ATPase in healthy and pathological human tissues. Thus, ideas on the mechanisms of genetic regulation of ion-transporting enzymes received a solid foundation.

Lately, the problems of immunology and hematology attracted the attention of Yuri Ovchinnikov, who believed that chemistry and biology should do more to help solving medical problems in the USSR. Intense investigations of naturally occurring regulators of immunity and hemopoiesis have been started at the Shemyakin Institute. Some presentations at this symposium deal with these problems.

Above, we have outlined the scientific interests of Yuri Ovchinnikov, who was also in the driving seat in leading the chemical and biological scientific communities of his country. Ovchinnikov could not imagine how the science could evolve without intensive international cooperation. He excellently presented the advances of the Institute, and promoted scientific contacts, giving impetus to a series of bilateral symposia such as USSR–FRG, USSR–USA, France–USSR, Sweden–USSR, and Italy–USSR in various fields of physicochemical biology, many of which have now became a tradition. The remarkable symposia on Frontiers in Bioorganic Chemistry and Molecular Biology in Tashkent (1980) and Moscow-Alma-Ata (1984)

were also organized and presided over by him.

Of Yuri Ovchinnikov occupies a prominent place in the world's scientific heritage. We can only guess at what his further endeavors would have been, if he were still alive. It is our hope that this numerous works will inspire many generations of bioorganic chemists to come, providing the key to solving a diversity of problems and demonstrating again and again the beauty and the attractive power of the world of science.

Yuri Ovchinnikov Lecture

Human Leukemia Genes: Search for the Villains

J. D. Rowley

This Ovchinnikov Lecture provides an occasion to review our progress in a central area of cancer research, namely the genetic changes that occur within the cancer cell that are critically involved in the transformation of a normal to a malignant cell. To concentrate on genes to the exclusion of cell biology would be too narrow and shortsighted a perspective. Nonetheless, I am convinced that until we have isolated the genes that are centrally involved in at least some of the malignant processes in different cell types we will be unable to answer the fundamental questions about malignant transformation. More importantly, we will be unable to answer the questions with precision. I will limit my consideration to those changes that have been detected by analyzing the karyotypic pattern of human cancer cells using chromosome banding, and in particular to those found in leukemia.

We are living in a golden age of the biomedical sciences. Increasingly sophisticated instruments and creative scientific strategies allow remarkably precise understanding of some aspects of cancer biology. It is clear that during the course of the last three decades, the scientific community's assessment of the role of chromosome changes in the complex process of malignant transformation has changed from considering them to be merely trivial epiphenomena to recognizing their fundamental involvement at least for some tumors. This change in attitude has occurred for at least two reasons. First, the demonstration of specific recurring chromosome rearrangements, including translocations and deletions, that were often uniquely associated with a particular type of leukemia, lymphoma, or sarcoma provided clear evidence that these rearrangements *were* critically involved in malignant transformation [1–3]. About 70 recurring translocations as well as many nonrandom deletions and other structural abnormalities are listed in the chapter on structural chromosome changes in neoplasia included in *Human Gene Mapping 10* [4]. The evidence for the presence of recurring chromosome abnormalities in a wide variety of human neoplasms was the result of 30 years of painstaking chromosome analysis by my cytogenetic colleagues around the world.

Second, and I believe an even more powerful force acting within the general scientific community to reassess the role of karyotypic alterations, was the identification of the genes involved in some of the chromosome rearrangements and the discovery that some of these genes were the human counterparts of the viral oncogenes [5]. In a sense, each group of investigators gave the other scientific validity. The fact that oncogenes were directly involved in chromosome translocations demonstrated that *both* translocations *and* oncogenes were critically involved in human cancer.

The genetic changes that occur in different types of malignant cells are quite varied, and clearly several different changes occur in the same cell as it is altered from a normal to a fully malignant cell. Cytogenetic analysis has been the key to defining at least two major categories of rearrangements, namely recurring translocations and consistent de-

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

letions. One of the first translocations, identified in 1972, was the 9; 22 translocation in chronic myeloid leukemia [6]. There are now at least 70 recurring translocations that have been detected in human malignant cells. The identification of consistent chromosome deletions has been equally important because it has provided the absolutely essential information regarding the chromosome locations of the genes that are involved in cancer. I submit that the retinoblastoma gene would not have been cloned, or at least not yet, if cytogeneticists had not identified deletions of the long arm of chromosome 13, and specifically of band 13q14, in patients with constitutional chromosome abnormalities who had a high incidence of retinoblastoma [7]. This is not to detract from the careful and exciting work of many scientists is actually cloning the gene, but at least they knew where to look [8]. This triumph has now been joined by the recent cloning of the *DCC* (deleted in colorectal carcinomas) gene on chromosome 18; the fact that a gene important in the transformation of colorectal cells was located on chromosome 18 was the result of cytogenetic analysis of colon cancer cells that revealed that loss of chromosome 18 was a recurring abnormality [9–10].

I must acknowledge that it has been a source of great disapointment to me that we have progressed so slowly in cloning most of the genes located at the breakpoints in the recurring translocations or inversions in human leukemia. This emphasizes the fact that knowing the location of the breakpoint is very helpful in selecting the genes to use as probes for these rearrangements. However, a chromosome band contains at least five million base pairs and the likelihood that the DNA probe that you "pull off the shelf" is at the breakpoint and can detect a rearrangement on Southern blot analysis is vanishingly small. The lymphoid leukemias and lymphomas are the major exceptions to this slow progress, because the immunoglobulin genes in B cell tumors and the T cell receptor genes in T cell tumors have provided the essential DNA probes to clone several dozen translocations [11–13].

Fortunately, the rapid progress being made in mapping the human genome, coupled with major advances in working effectively with large pieces of DNA, has already made important contributions to the successful mapping of some of the recurring translocations in the acute leukemias and sarcomas. The use of cosmids or yeast artificial chromosomes (YAC) as probes to screen much larger segments of DNA for rearrangements provides a new strategy for the analysis of these chromosome abnormalities. We have used these probes in in situ hybridization with biotin labeling of the DNA and detection with a fluorescein isothiocyanate-(FITC)-tagged avidin–antiavidin conjugate. The focus of our research has been the analysis of chromosome translocations involving band 11q23. This band is of great interest because it is affected in a large number of different recurring rearrangements. The translocations may occur in either acute lymphoblastic or acute myeloid leukemias, especially of the monoblastic or myelomonocytic subtype. Finally, about two-thirds of chromosome abnormalities in leukemia cells of children under 1 year of age involve 11q23, regardless of the morphological classification of the leukemia.

We have used a series of cosmid probes as well as a yeast clone containing two YACs to map the 11q23 breakpoint in four different translocations, namely t(4;11), t(6;11), t(9;11), and t(11;19) [14]. The cosmid probes were isolated by Evans et al. [15] and they were mapped to the region 11q22 to 11q25 by Lichter et al. [16]. The yeast clone with the YACs was identified using polymerase chain reaction primers specific for the *CD3G* gene. We showed that this yeast clone contains two YACs of 320 and 275 kb that differ only because of a 45 kb deletion in one of the YACs; the deletion is in the end opposite the *CD3G*/*CD3G* complex. With the use of cosmid probes we

obtained essentially the same results in all four translocations. The cosmid probes 3.16, 23.20, 1.16, 4.13, ZB6, and *CD3D* all remained on chromosome 11. The cosmid probes XH5, XB1, ZC9, *PBGD*, 9.4, ZA7, *THY1*, 8.5, *SRPR*, XB2, *ETS1* 23.2, and 5.8 all were translocated to the other chromosome. Seven cosmid probes (XH5, XB1, ZC9, *PBGD*, 9.4, ZA7, and *THY1*) were deleted in one t(9:11) patient, presumably simultaneously with the translocation.

The *CD3G* YACs localized only to chromosome 11 in normal cells. However, in addition to labeling the normal chromosome 11, the YACs were split in cells with the four translocations; thus one portion remained on chromosome 11 and the other was translocated to the other chromosome. There was no labeling of any other chromosomes in these cells. Thus the breakpoint in these translocations, which occur in both lymphoblastic and myeloid leukemia, is within the same 320 kb region of human DNA. We have no evidence, at present, of whether the break involves the same segment in the different translocations. Thus the use of YAC clones provides a new strategy for screening large pieces of DNA and for focusing intensive molecular analysis only on the segment that is shown cytogenetically to be of interest. YACs will also be of great benefit in defining the genetic boundaries of chromosome deletions. These probes also provide powerful tools for detecting these same rearrangements in interphase cells.

Using more conventional techniques, a colleague of mine, Dr. Timothy McKeithan, has cloned the translocation breakpoint found in some patients with B cell chronic lymphatic leukemia [17]. The translocation involves the immunoglobulin heavy chain locus (*IGH*) located at chromosome band 14q32 and a previously unknown gene that we have called *BCL3* on chromosome 19 (band 19q13). They cloned the translocation breakpoint from two of our patients as well as from several others from material provided by other laboratories. There was evidence for rearrangement adjacent to one of the *IGH* constant regions in each case. In four of the cases, this rearranged band has been cloned; all showed a rearrangement with sequences from chromosome 19. Three of the breakpoints on chromosome 19 were within 170 bp of each other; the fourth lay 19 kb centromeric. Overall, a region of about 35 kb surrounding these breakpoints has been cloned and mapped.

A cluster of CG-containing restriction sites was found close to the cluster of breakpoints on chromosome 19. These "CpG islands" are usually associated with the 5′ ends of genes. The presence of a CpG island adjacent to the cluster of t(14;19) breakpoints was confirmed by sequencing. Probes from this region were used in Northern blot experiments, which detected a 2.1–2.3 kb transcript in many hematopoietic cell lines. S1 protection experiments confirmed this result and showed that transcription occurred in a direction away from the breakpoint toward the telomere.

The *BCL3* cDNA was cloned and sequenced [18]. A basic protein of 446 amino acids and a molecular weight of 46741 is predicted, which shows a remarkable structure. The N-terminus is highly enriched in proline (25%), and the C-terminus in proline (23%) and serine (28%). Almost the entire remainder of the protein (about half) is made up of seven tandem repeats of 33–37 amino acids. Comparison with proteins in the available data bases showed significant homology to the *Drosophila* Notch protein. The homology is in the region of the repeats. Notch has six repeats with clear similarity to the repeat in BCL3. These repeats have been found in three additional proteins – namely, lin-12 of *Caenorhabditis elegans* (six repeats), cdc10 in *Schizosaccharomyces pombe* (two) and SW16 in *Saccharomyces cerevisiae* (two) [reviewed in 18]; the repeats are generally referred to in the literature as cdc10 repeats. The role of this motif is not known.

Total RNA from two patients with chronic lymphocytic leukemia (CLL) and the t(14;19) – one with a break on chromosome 19 close to *BCL3* and one with a break more than 25 kb away – was hybridized on Northern blots to determine the level of *BCL3* expression in cells containing the t(14;19). The samples were compared with total RNA from the peripheral blood of two other patients with CLL, as well as with three cell lines derived from the prolymphocytic variant of CLL; none of the cell lines contain the t(4;19). In addition, five other hematopoietic cell lines were examined. The level of message in the two CLL samples with a t(14;19) was higher than that found in any other sample examined. By hybridization to blots containing various quantities of RNA, the two t(14;19) samples were found to contain 5–7 times and 10–15 times the level of message present in the CLL cell line with the greatest quantity of message [18]. The message present in the cells with the t(14;19) was identical in size to that present in normal hematopoietic cells, as would be expected from the fact that the translocation breakpoints occur upstream of the transcription start site. The apparent normality of the message suggests that the increased message level results from increased transcription and not from an increased message stability arising from changes in the structure of the transcript itself.

The known functions of the other proteins containing the cdc10 motif may offer a clue to the function of BCL3; unfortunately, however, the divergent structure and function of these proteins makes it difficult to image a common role for the motif. Notch (in *Drosophila*) and lin-12 (in the nematode *Caenorhabditis*) are transmembrane proteins involved in cell lineage determination. On the other hand, the two yeast proteins are not transmembrane proteins and they share functions involved in control of the cell cycle. *cdc10* is one of two genes in *Schizosaccharomyces pombe* known to be required for commitment to the cell cycle; this control point, in G1, is known as "start". Much more is known about the function of the other required gene, *cdc2*, encoding a protein kinase which is highly conserved among eukaryotes and is required both for start and for mitosis. Little is known about the function of the cdc10 protein. Recently, a specific antibody to cdc10 was shown to detect a protein of similar size in mammalian cells, suggesting that, like cdc2, the protein may be conserved throughout the eukaryotes.

SW16 is one of several genes known to be required for transcription of the *HO* gene, which encodes the endonuclease which initiates mating type switching in *Saccharomyces cerevisiae*. *HO* is activated immediately after commitment to start, and a particular repeated sequence in the 5′ flanking region of the gene has been shown to be responsible for cell cycle control of its transcription. *SW16* and *SW14* (whose sequence has not yet been reported) are the only genes known to be specific for this control element. These two genes appear to be at least partially interchangeable since neither single mutation is lethal, but double mutations are nonviable. While the function of *SW16* strongly suggests that it is a nuclear *trans*-activating protein, it has not been directly shown to interact with DNA or even to be a nuclear protein.

If the cdc10 motif is involved in protein-protein interactions, there may be little commonality in function between the two yeast proteins and the two invertebrate proteins. Nevertheless, there are a few plausible models in which the proteins could have related functions. For example, BCL3, cdc10, and SW16 may be peripheral membrane proteins which interact with the cytoplasmic domains of transmembrane proteins and are involved in signal transduction. According to this interpretation, the ancestor to the *lin12* and *Notch* genes could have resulted from the fusion of two genes in evolution – one encoding a transmembrane protein, and the other, a cdc10-related protein.

The increased levels of *BCL3* message following mitogenic stimulation and the homology of the gene to cell cycle control genes suggest that abnormally large quantities of the protein present in CLLs with the t(14;19) may lead to an increased proliferative rate in these cells. This superficially seems inconsistent with the very low mitotic rate of CLL cells. Perhaps this mitotic rate, while low, is nevertheless greater than that of normal CD5+ B lymphocytes. Alternatively, a subpopulation of CLL cells, perhaps those present in pseudofollicular growth centers in lymph nodes, may show an abnormally high rate of proliferation.

One of the major reasons to concentrate on cloning the genes involved in rearrangements is that the consistent chromosome changes pinpoint the location of the genes whose functions are critical in the growth potential of that cell type. The chromosome changes that we concentrate on are present in all of the malignant cells; thus they are not random events affecting one or a few cells in the involved tissue. Moreover, they are clonal in origin and are derived from a single cell in which the intial chromosome change occurred. These changes are somatic mutations in individuals who otherwise virtually always have a normal kyrotype in their uninvolved cells. These observations provide the evidence that cancer is a *genetic disease*. This notion seems self-evident today, but it was not generally accepted several decades ago when many of us began working in cytogenetics. Clearly, I am using "genetic" in a special way, referring to changes in genes within the affected cell, not in the more usual sense of a constitutional genetic disease such as hemophilia or color blindness.

I will conclude with some comments regarding the longer-term potential impact of discovering new genes via chromosome rearrangements. Once these genes are identified, many previously unknown, *BCR* or *BCL3* for example, they become the focus of very active investigation. Scientists try to find answers regarding the function of these genes in normal cells; how are they altered by the chromosome rearrangements, and how does this relate to malignant transformation? The questions are endless. The answers will provide insights into cell biology that have very profound implications. Within the next decade or two, we should be able to define the major genetic abnormalities in many types of cancer and to identify the specific changes in the tumor cells of many patients. For most leukemias, lymphomas, and sarcomas, unique chromosome changes are often associated with a particular subtype of these neoplasms.

Cloning of the genes involved in these chromosome changes will provide specific DNA markers that will have diagnostic importance. For some solid tumors, on the other hand, current evidence suggests that deletions of the same chromosome region may occur in different types of tumors, such as the deletions of 13q in retinoblastoma, osteosarcoma (not secondary to radiation for retinoblastoma), breast cancer, and lung cancer. The deletion of the same region does not necessarily imply that the same gene is involved or that the change within the gene is identical, witness the fact that two different translocations in band 22q11 involve different genes, namely, the lambda light chain gene in the 8;22 translocation in Burkitt's lymphoma and the *BCR* gene in chronic myelocytic leukemia (CML); furthermore, the breakpoints within *BCR* in Ph^1-positive leukemia are also somewhat variable.

The multistep process of malignant transformation is complex. In the leukemias and lymphomas, we often see specific chromosome translocations combined with loss or gain of particular chromosome segments. Some combination of alterations in dominantly acting proto-oncogenes and in recessively acting tumor-suppressor genes certainly act synergistically to enhance the malignant phenotype.

In the future, the precise definition of the genetic changes in the malignant cells

of a patient will be used to select the most appropriate treatment for cells with these genetic defects. This treatment will be less toxic for the normal cells in the patient. Moreover, this genetic profile may allow monitoring of the patient's course and early detection of relapse. These same genetic markers may be used to detect the involvement of other tissues such as bone marrow, spleen, or lymph nodes. These changes in treatment strategies will clearly benefit the patient. Of more general scientific importance, however, will be the identification of dozens of genes, many hitherto unknown, that can be used to study the complex process of the regulation of cell growth and differentiation. This development may be the most significant result of our success in understanding the genetic changes that occur in cancer cells.

I would like to conclude with a more personal note based on my continuing amazement at the interrelatedness of the biomedical sciences. This should be no surprise to me, but it is. Many investigators have found that a successful system for carrying out some function in primitive organisms has evolved and then this system is used repeatedly with varying modifications as the organisms become more complex. As a cytogeneticist, I had to learn something about the cell cycle and DNA replication, about chromosome structure, and about various agents that can alter both of these. More recently, I have had to become an amateur tumor virologist at least with regard to the action of viral oncogenes and their cellular counterparts, the proto-oncogenes. With the cloning of translocations, especially some of the recent ones, a knowledgeable cancer cytogeneticist must understand cell cycle control genes in yeast (*cdc10* and *SWI4* and *6*) and developmentally regulated genes in *Drosophila* (*Notch* for example) and nematodes (*lin-10*, *glp-1*). I have already described in some detail, the cloning of the *BCL3* gene by McKeithan et al. [17, 18]. If I am to understand the possible roles this gene plays in B cell transformation, I must understand how its homology to portions of the *cdc10* and *Notch* genes might provide clues as to its functions in both normal and malignant cells. As more genes involved in translocations and deletions are defined, many of us in cancer research will continue to have to "go back to school" to be able to incorporate the knowledge provided by molecular geneticists and cell biologists into our concepts of carcinogenesis. The golden age in biology and in medicine are nourishing one another as never before in history.

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Yuri Ovchinnikov Lecture

Human Retroviruses: Linkage to Leukemia and AIDS

R. C. Gallo [1] and L. S. Nerurkar [1]

Introduction and Background

This review will discuss interactions of retroviruses with the cells of the hematopoietic system. Such interactions have been studied in the past as tools to insert genes in the cells to study their regulation or to study cellular and molecular basis of transformation in vitro. The emphasis of this review will be on viruses which cause diseases, particularly in man.

A decade ago there was no general acceptance of the concept that genes were critical to leukemias and lymphomas or to disorders of hematopoietic cells. In a somewhat analogous way there was also a general feeling that viruses did not cause human cancers, and that retroviruses, in particular, did not exist in human beings. We now know that viruses, either directly or indirectly, either as a cofactor or as a direct cause, play a role in more than 40% of human cancers. We have also learned that human retroviruses do exist and in multiple types.

During the 1970s, there was also a feeling in the United States that serious or fatal, epidemic or pandemic diseases were things of the past. Infectious diseases that would become global epidemics were no longer a problem for the so-called "industrialized nations." Such diseases were really a problem for the less-privileged nations. We had preventive and curative measures like vaccines and antibiotics in addition to better sanitary conditions and public health measures. In retrospect, we should have remembered that the last great pandemic that affected the United States, Europe and the world was only about 70 years ago. It was the great influenza epidemic of 1918–1920. And if one reviews the history of microbiology, there were often periods where epidemics disappeared and mysteriously reappeared after more than 60 or 70 years, or even for 100 or 200 years. Perhaps we were overconfident in thinking that epidemics belonged to the past: an epidemic or pandemic of the acquired immunodeficiency syndrome (AIDS) as now been with us for a decade. There was also a feeling that pandemic diseases were not possible unless the causative agents or the microbes were casually transmissible. We now know that we have a pandemic of AIDS and the agent is not casually transmissible, but transmissible only by close contact and with the exchange of body fluids.

The failure to appreciate the coming of these events was probably because of the failure to remember some of the lessons of past medical history; that often there are major changes in diseases following some major changes in the society. The major changes in the post-World War II era were: a great increase in air travel; the use of blood and blood products, often going from one nation to another; the insane habit of intravenous drug abuse; and the increase in sexual contacts. All these things made it possible to transmit something that was remote or rare so that it become relatively common and global. Comparing the epidemics of the past, the AIDS epidemic is not particularly novel,

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Haematology and Blood Transfusion Vol. 35
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nor is the response to the epidemic by the public as is often portrayed by the media. The only novel feature of this epidemic is the nature of the microbes that are causing the epidemic. The novel properties of these microbes are: they are newly discovered (but are not new); they are microbes that are often difficult to find because they do not replicate much or do not infect many target cells; they are only produced by the infected cells, primarily during the proliferating phase of the cell's life cycle. In fact, this is true for all human retroviruses.

For the same reasons that they are difficult to find, the viruses are difficult to transmit. Almost always, they have a very long latency period. This is an important characteristic and has allowed their transmission to become global as they are present in the host from the time of infection until death (lifelong infection) and during that period can be transmitted to others. This is a major difference between retroviruses and other viruses, which we tend to think are transmitted while a person is sick or in the early phase of incubation, which can be a few days, a few weeks, or at the most a few months. The very long latency period of retroviruses means that it may be several years or several decades from the time of infection before the first manifestation of disease will be noted. They often cause serious diseases, e.g., central nervous system disease, malignancies, and immune deficiency. These agents have thus become increasingly important because of the serious and often fatal consequence of their infection.

Discovery of Human Retroviruses

There are four human retroviruses well characterized by now [1–3]. Human T-lymphotropic (leukemia) virus type I (HTLV-I) was found by Gallo and coworkers in the late 1970s and first reported in 1980. Its relative, HTLV-II, was also found in our laboratory a year or two later. The human immunodeficiency virus type 1 (HIV-1) or AIDS virus causing the epidemic we now face was found in 1983 by Barre-Sinoussi et al. at the Institute Pasteur [4] and established by our laboratory as the cause of AIDS in early 1984 with many isolations of the virus and the development of the blood test. The related virus from West Africa, called HIV-2, is neither as pathogenic as HIV-1 nor is it spreading like HIV-1. It appears to be almost limited to West Africa.

The technology developed in the 1970s, particularly, the sensitive assays for reverse transcriptase (RT), was crucial for the discovery of human retroviruses. The discovery of RT, by Temin [5] and independently by Baltimore [6], was quickly extended by the finding of a similar enzyme in human leukemic cells from unusual cases by Gallo and his colleagues during the 1970s [7]. Enzymes from at least four or five patients which had the properties of the viral enzyme were partially purified. More important was the development of synthetic template primers, e.g., synthetic polymers (oligo-dT-poly-A and oligo-dG-poly-C) that made the assays for these enzymes specific and sensitive. This improved the detection of retroviruses several-fold compared to the electron microscopic method used for decades. Also, the assay using RT is much simpler and cheaper and can be done continuously while the culture is ongoing. Electron microscopy does not offer that possibility. Retroviruses, including those affecting humans, complete their replication cycle much more efficiently during the proliferating phase of that cell's life cycle. RT assays performed continually on the cells in culture can reveal short-term viral replication which otherwise may be missed by electron microscopic techniques.

The second important technology was the ability to grow human T cells, particularly with interleukin-2 (IL-2), discovered by Morgan, Ruscetti and Gallo in 1976 [8]. Developments in the field of immunology such as monoclonal antibodies have allowed defining subsets of

lymphocytes by surface markers or by other assays to understand different functions of T-cell subtypes. A third factor contributing to the discovery of human retroviruses is the fact that they spread globally in the 1960s and the 1970s, and became much more common. We believe that this may have increased the chances of detecting and isolating them considerably. And the last point which is worth mentioning was the perseverance in looking for them, even though most scientists did not think they existed.

Classification of Human Retroviruses

Human retroviruses belong to two entirely different subclasses which differ in their morphology, some aspects of their genomic organization, and some aspects of their biology. The HTLVs belong to the more classic type of animal retroviruses known in most species as type C or oncorna retroviruses, whereas the HIVs belong to the category known as lenti-retroviruses. "Lenti" is not an accurate term, as it means slow. HIV does not replicate slowly compared to HTLVs. HTLVs are much more slowly replicating viruses, and thus the class names can be misnomers. Until the discovery of HIVs, lenti-retroviruses were only known to occur in ungulates, the hoofed animals like horses, sheep, cows, and goats [9]. One has to be careful in not drawing too much of an analogy between HIV and these ungulate lenti-retroviruses. However, there are some common characteristics, e.g., they infect cells of the macrophage lineage and morphologically their core structures appear similar. But there are major differences in other aspects. For example, some of the ungulate lenti-retroviruses can be transmitted casually. The visna virus of sheep is thought to be transmitted by fomites in crowded sheep that are herded together in a closed environment. None of the ungulate lenti-retroviruses target $CD4^+$ T lymphocytes and they are not known to be associated with the increased frequency of the development of a malignancy. More recently, we have other animal models, particularly the simian models, in which lenti-retroviruses have been isolated that are closer to the humans [10].

Morphology. The size and shape of HTLVs and HIVs are roughly the same. However, the core structure of the leukemia viruses is much different from that of the AIDS virus. The latter is much more condensed and cylindrical in shape compared to that of the leukemia viruses (see Fig. 1).

Biological Properties. The HTLVs and HIVs show many parallels in their biological characteristics. Both viruses infect $CD4^+$ T lymphocytes but they vary in the consequence of infection. The overall effect depends on the extent of virus replication and on the functions of some of the genes the virus carries. HIV-1 infection kills the $CD4^+$ T cells. HIV-2 essentially behaves in a similar manner. On the other hand, HTLV-I and HTLV-II, like most animal retroviruses, have no lytic activity on their target cells, but can alter the function of that cell. Some infected T cells become immortalized in vitro and may contribute in the same manner to the development of leukemia in vivo. Both classes of viruses remain latent in the patient or in the cell for their lifetime. Another feature both HIVs and HTLVs have in common is the tight control of the DNA-integrated provirus. Following the infection of a $CD4^+$ T cell by these viruses there is integration of their DNA forms into the host chromosomes, but the DNA forms do not induce expression of RNA or proteins. So an infected cell will have no viral RNA or viral protein immediately after infection. This means that the immune system cannot find the infected cells. This is one way these viruses escape the immune system. Other mechanisms which allow these viruses to escape are, for example, by infecting the brain and by undergoing considerable genomic variation from isolate to isolate (particularly in the case of HIV).

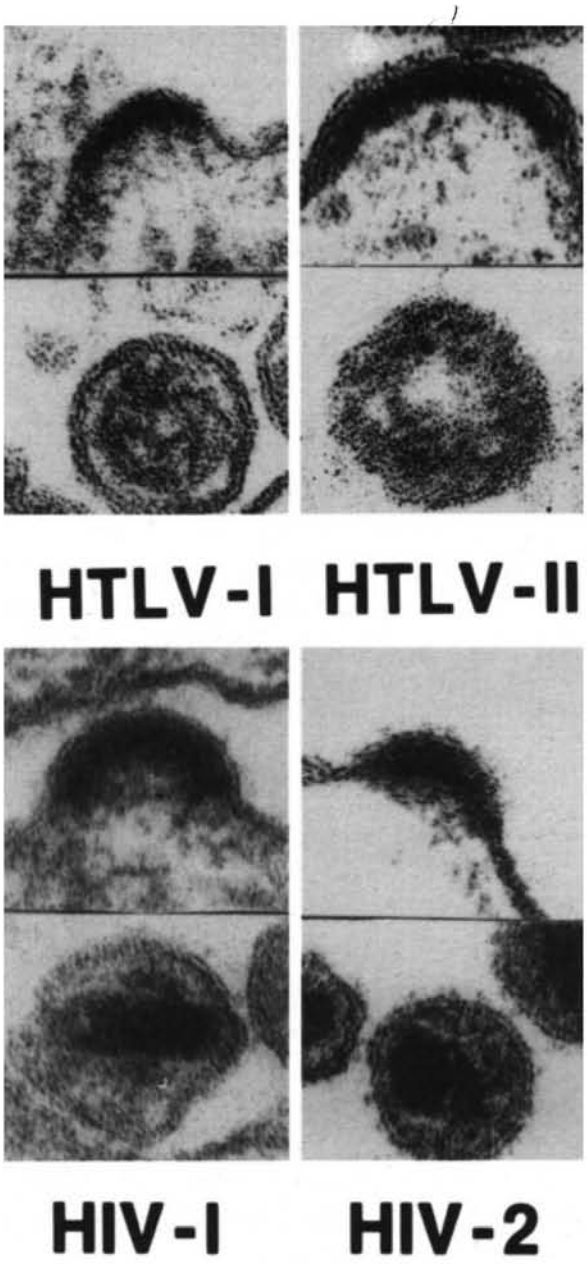

Fig. 1. Morphological structures of HTLV-I, HTLV-II, HIV-1, and HIV-2. The *top panels* represent the budding from the cell membranes, the *bottom panels* show the cross section of the mature virons

In addition, HIV destroys the cells of the immune system which are crucial in the immune surveillance itself, thereby escaping the immune attack.

When the T cells are immune-stimulated, perhaps by another infection, the viral genes become active along with a variety of other cellular genes and viral proteins are expressed on the cell surface. This allows the immune system to see the infected cell. Such immune clearance may be too late. The virus released from such cells infects other cells. In this manner, the HIV-infected host who has other chronic infections is more likely to spread this virus.

Modes of Transmission. The HIVs and the HTLVs have common modes of transmission. They are transmitted by blood or sex and from mother to child. For HTLV-I, males chiefly get infected from their mothers, and women chiefly get infected from their male sexual contacts. The mode of transmission from mother to child is *in utero*, transplacentaly as well as by milk or in the actual birth process. Blood transfusions and the use of blood products are, of course, also modes of transmission of both viruses, the major target cell being the $CD4^+$ T cell [11].

Analogous Animal Retroviruses. The closest relative of the human leukemia viruses, called simian T-cell leukemia/lymphoma viruses, are found in African monkeys [12, 13]. They are not found at all in New World monkeys, i.e., in monkeys from the North American continent and those from Asia [14]. But the viruses in African monkeys are closer to the human viruses than to those from Asian monkeys. Similarly, the closest relative of HIVs are also found in African monkeys. No relatives of HIV have been found in New World or Asian monkeys. Because of the fact that the closest relatives of all human retroviruses are present in African primates, the ancestral origin of these viruses is almost certainly African. That does not mean that the recent epidemic of AIDS came from Africa. As far as one can tell, the epidemic of AIDS began almost simultaneously in parts of Central Africa, some of the Caribbean islands, particularly Haiti, and the United States, and perhaps in Europe.

Geographical Distribution of HTLV-I

HTLV-I transmission is extremely tightly controlled and if one did not have a

handle on the virus (virus isolation or virus detection using probes), the diseases it causes, e.g., leukemia or neurological diseases, could be mistakenly thought to be genetically inherited. HTLV-I is endemic in Subsaharan Africa. It is not present in all parts of Subsaharan Africa, but seems to be restricted to certain tribes or geographical areas and is not casually transmitted. HTLV-I is also endemic in the Caribbean basin, including the northern part of South America, Central America, most of the Caribbean islands, and parts of the southeastern United States. Some Caribbean islands do not have any HTLV-I. It depends on where in Africa the island inhabitants have their origin. If the ancestral tribe is positive, then the descendants in some Caribbean islands are positive. Similarly, if the ancestral tribe is negative, then the descendants in another Caribbean island are negative for the most part. HTLV-I is also endemic in the southern islands of Japan in Shikoku, Kyushu, Okinawa, and other neighboring islands. Seroepidemiologic studies have suggested that clusters of HTLV-I, or a virus like it, are observed in some villages in Spain and in southeastern Italy in a region called Apulia. Manzari of Rome and Varnier of Genoa believe that the virus in Apulia in southeast Italy is endemic. It is not a coastal introduction from outside in recent times; rather, it is found in the people living in the interior hills. Recent molecular analysis studies of some of the isolates from that region indicate that it is not the classic HTLV-I, but may be another retrovirus related to HTLV-I. There have been clusters of HTLV-I-related leukemia reported in Amsterdam and London in migrating populations from the Caribbean. The rate of developing leukemia after HTLV-I infection is identical in populations which have migrated and in the nonmigrating population, indicating that no other environmental factor is needed for the cause of leukemia, at least as far as the epidemiology can determine [11]. For a great part of the world, we have very little data. For example, we do not know very much about infection by HTLV-I in the Soviet Union.

Nature of the Diseases Caused by HTLV-I

Leukemia. The picture of the first patient from whom a retrovirus was isolated is given in Fig. 2. This patient was a young black male and came from the southeastern part of the United States. He had no interesting past history, either medical, familial, or occupational. He developed a severe acute T-cell malignancy of the $CD4^+$ T lymphocytes. The skin manifestation in this disease is due to infiltrates of leukemic cells in the skin, which is a common feature in this disease [15, 16]. Frequently, there is high blood calcium, which can lead to death of the individual. Liberation of some lymphokines is suggested as a possible molecular mechanism for high blood calcium [17]. There is also an increased incidence of opportunistic infections and slight immune impairment can be observed in infected people. However, when a disease begins to develop, the course is very rapid. It resembles the chronic myelogenous leukemia going into blast crisis. Death usually follows in less than 6 months.

These manifestations of the disease are common, occurring in about 70%–80% of people who have leukemia with HTLV-I. Another 20%–30% show a more chronic course, and the diagnosis is of chronic lymphocytic leukemia of a $CD4^+$ T-cell type, mixed cell lymphoma, or histiocytic lymphoma of a $CD4^+$ T-cell type. So, in any $CD4^+$ T-cell malignancy, one has to consider the possibility of HTLV-I, and particularly if the disease is as aggressive as described above.

Neurologic Disease. It is now known that HTLV-I also causes a serious and fatal progressive neurologic disease which is similar to multiple sclerosis but can be distinguished from it. There is some confusion in this area because some labo-

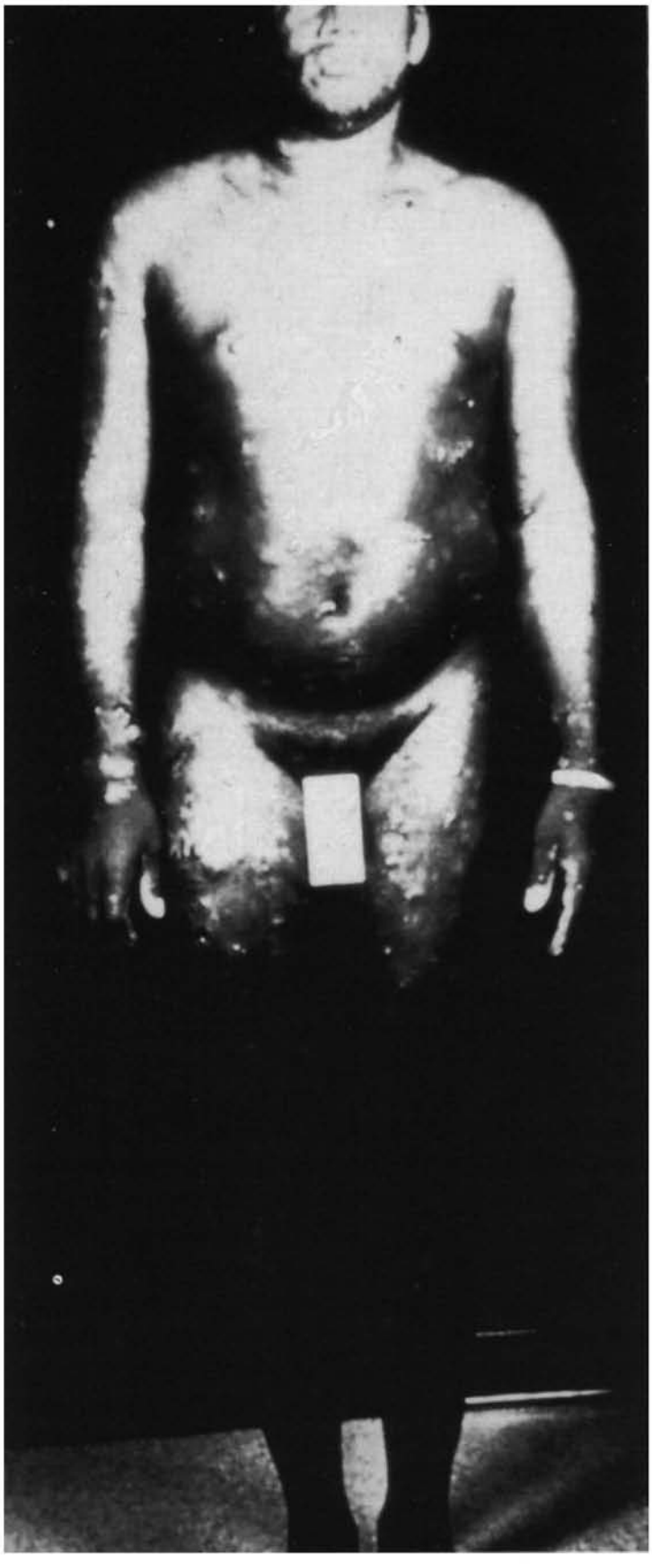

Fig. 2. The first patient with T-cell leukemia caused by HTLV-I

ratories have reported HTLV-I or a closely related virus as being involved in multiple sclerosis itself. The data are not consistent from laboratory to laboratory. More evidence is required to implicate HTLV-I or a relative of HTLV-I in playing a definitive role in multiple sclerosis. However, the neurologic disease that has been called tropical spastic paraparesis or Jamaican neuropathy, and sometimes misdiagnosed as multiple sclerosis or a variant of multiple sclerosis, is certainly linked to HTLV-I [18], although the disease mechanism is not understood. The HTLV-I-associated disease differs from multiple sclerosis in that it does not have exacerbations and remissions like multiple sclerosis: it is progressive. It is characterized by incontinence of the bladder, impotency in males, loss of bowel function and spasticity of the lower extremities. The disease can occur rapidly after infection with HTLV-I. It appears to depend on the dose of the virus.

There is a recent report of a Frenchman who received a transfusion with HTLV-I-positive blood, developed the neurologic disease in 5 weeks, and transmitted the virus to his wife during that period. This implies that all of the blood supply should be tested for HTLV-I as well as for HIV [19]. However, the neurologic disease could take many years to develop and there is some indication that genetic factors are important. There are some reports from Japan showing an HLA class 2 association and that certain patterns have an increased frequency of developing the neurologic disease. A known fact is that the virus is not found in the central nervous system tissues, e.g., brain cells or cells of the spinal cord, but only in the cerebrospinal fluid.

The other known facts are that people who develop the neurologic disease have a very high titer of antibody, much higher than the healthy carriers or the leukemic patients. Even more interesting are the recent results of Jacobson, McFarlin, and their coworkers, who describe high levels of cytotoxic T lymphocytes reactive against *tax* and *env* gene products [20]. This has led to the speculation that the immune response to the virus produces an autoimmune disease. Recent reports indicate that HTLV-I is also involved or linked epidemiologically to other diseases listed in Table 1.

Table 1. Diseases caused by or associated with human retroviruses

1. Adult T-cell leukemia (ATL)
2. Occasional other T4 leukemias/ lymphomas
3. Tropical spastic paraparesis (TSP) or HTLV-associated myeloneuropathy (HAM)
4. Mild immune impairment
5. Polymyositis
6. Rheumatoid arthritis-like disease (?)
7. Retinitis (?)
8. B-cell lymphocytic leukemia (B-CLL), indirect (?)
9. AIDS progression, possible role as cofactors
10. Guillain-Barre syndrome
11. Chronic lung disease
12. M-proteinamia
13. Chronic renal failure

Genome of HTLVs. Like any retrovirus, HTLV-I has long terminal repeat sequences at each end. These sets of nucleotides are involved in regulation of the viral gene expression and form sites of covalent attachment to cellular sequences on each side of the integrated provirus.

Like any animal retrovirus, it has the three genes for structural proteins, the *gag* gene for the viral core proteins, the *pol* gene for the enzymes, including RT, and the *env* gene for the envelope (Fig. 3). These give the retrovirus the ability to reproduce itself.

When the molecular analyses of HTLV-I and HTLV-II were completed, it became evident that they have new genes present at the 3′ end of the genome. Originally, one of the genes was called *tat*, but with the revised terminology, this gene is now called *tax*. The *tax* makes a 40000-dalton protein localized in the

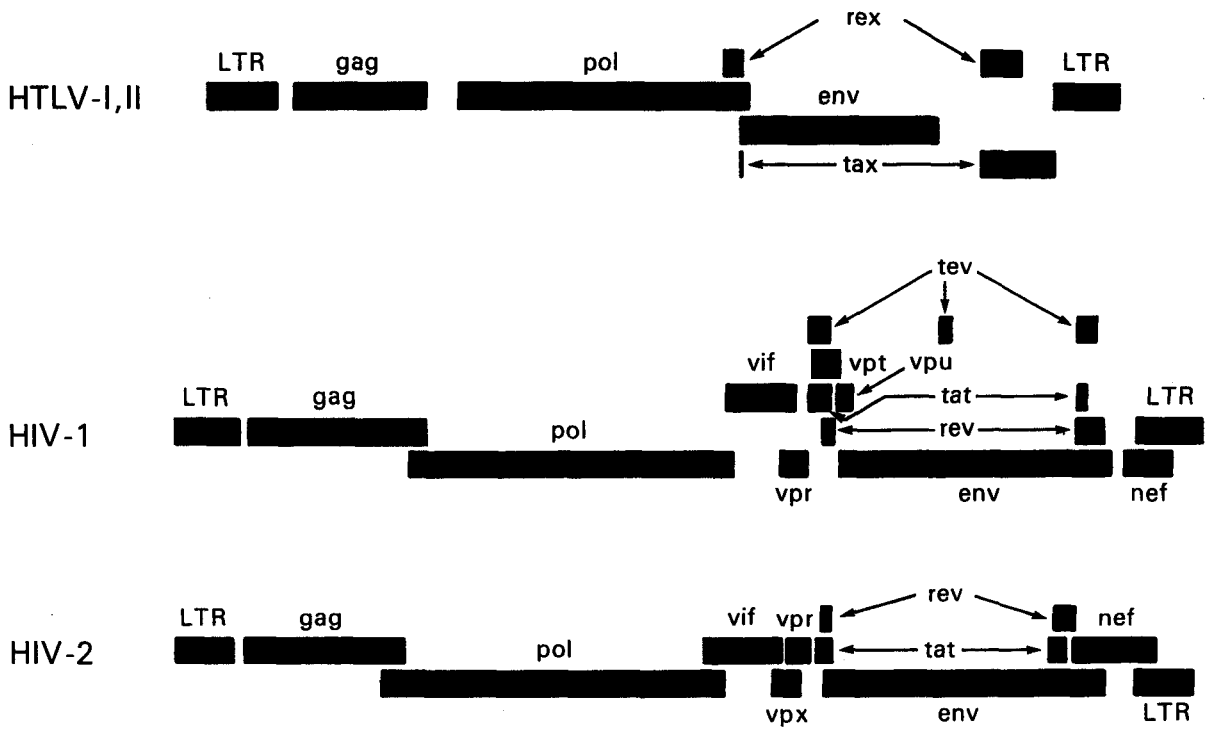

Fig. 3. Genomic structures of human retroviruses

nucleus or the infected cells. The *rex* is the second gene in the 3′ region of HTLV-I and HTLV-II. These genes are coded from two segments of the genome and are products of doubly spliced messenger RNAs. This phenomenon (double or even triple splicing) was new in human retrovirology. It was soon realized that the protein products of these genes are absolutely essential for the replication of HTLV-I and HTLV-II. They are also essential for the biological activity of these viruses.

The products of the *gag*, *pol*, and *env* genes are formed from unspliced or singly spliced messenger RNA molecules. This is similar to what was known among animal retroviruses.

Replication Cycle of HTLVs. The replication cycle of HTLVs can be divided into two parts (Fig. 4). The first part, like any animal retrovirus, involves a phase of attachment to the cell membrane. The receptors for HTLV-I or HTLV-II are unknown, as for most of the animal retroviruses. However, the chromosomal site of the HTLV-I receptor has been determined [21]. Following the attachment, fusion of the viral envelope with the cell membrane occurs, followed by emptying of the viral core into the cytoplasm of the cell. The viral RNA is transcribed in the cytoplasm, with formation of the double-stranded linear DNA, which then enters the nucleus and integrates into the chromosomal DNA.

With most animal retroviruses, after provirus integration into a permissive target cell, virus replication and its expression start immediately. There are sufficient cellular factors, and sufficient viral and cellular machinery allowing quick transcription of the DNA provirus, to reform viral RNA in the nucleus. The viral RNA then traverses to the cytoplasm and assembles at the cell membrane with viral proteins that have been formed by translation of unspliced or singly spliced messenger RNAs in the cytoplasm. The viral proteins are processed, particularly by cleavage through viral and cellular proteases (the former for viral core proteins, the latter for viral envelope proteins). After assembly, budding and release of the newly formed virions completes the replication cycle of the viruses.

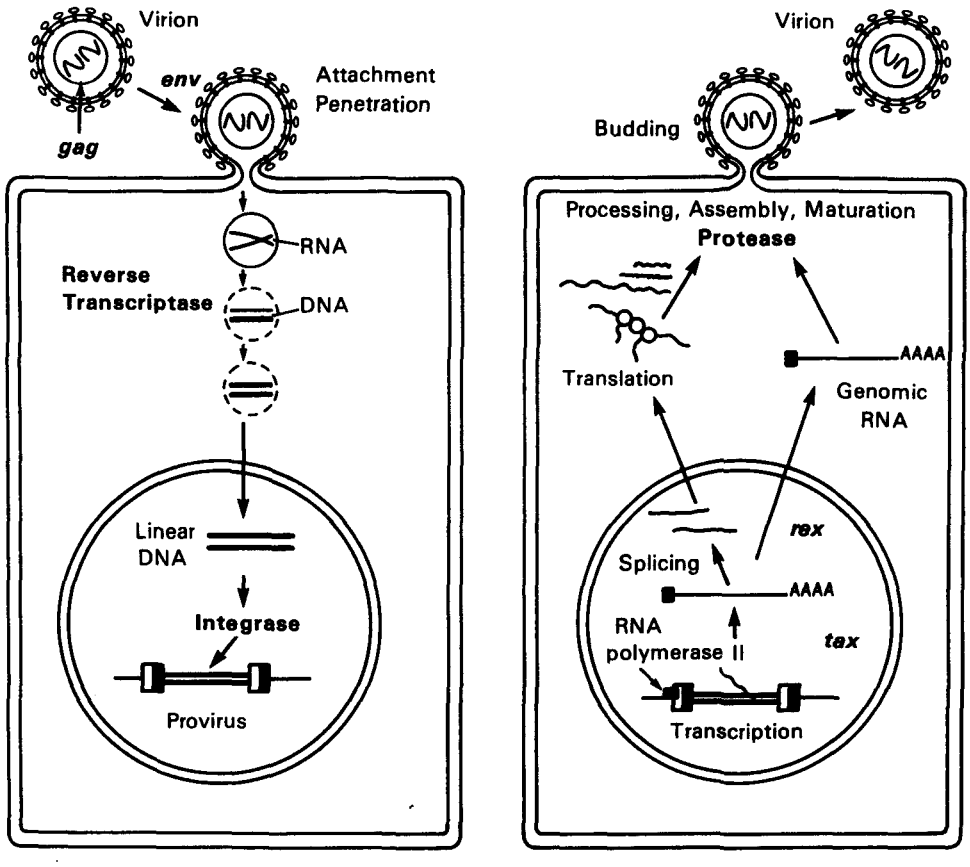

Fig. 4. Life cycle of HTLV-I and HTLV-II

HTLV-I and HTLV-II have introduced a new complexity into our understanding of the replication cycle, and that complexity relates to the events which take place in the nucleus. In order to have successful transcription of the DNA provirus to viral RNA, first there is the expression of an early gene product. This phenomenon, although known in some DNA viruses, was newly discovered in retroviruses. The first genes to be expressed are *tax* and *rex* (Table 2). What turns on the expression of *tax* and *rex* is unknown, but the *tax* gene product (TAX) is essential for the early transcriptional events to make the viral RNA. The function of the *rex* gene product (REX) is not only newly observed in retrovirology, but it has introduced some new mechanisms into all of molecular biology. The REX protein is involved in removal or transport of the messenger RNAs for the viral structural proteins, i.e., the messenger RNAs that are unspliced or singly spliced. In other words, in the absence of REX, the only messenger RNAs that are made are the messenger RNAs that are doubly spliced, i.e., the messenger RNAs for *rex* and *tax*. But once the REX protein is made, the formation of the unspliced RNAs or the singly spliced RNAs for the viral structural proteins is favored. This is an interesting mechanism because once the REX protein is made, it down-regulates its own expression. It also down-regulates *tax* and allows the formation of the viral proteins so that there is a sudden release of virus during this narrow window in which these human retroviruses have to complete their cycle. This mechanism is evident even in HIV but not in the lenti-retroviruses of animals. This may suggest a convergent evolution of mechanisms for infection of human T cells by two entirely different classes of human retroviruses.

Mechanism of Leukemogenesis. TAX protein plays an important role in leukemogenesis. It acts in *trans* and is involved in the mechanism of transcription of viral RNA. TAX protein also activates cellular

Table 2. Accessory genes of human retroviruses

Immunogenicity		Size	Cellular localization	Function	Replication competence of (−) mutants
vif	+	p23	Cytoplasm/inner membrane	Infectivity	±
tat	+	p14	Nucleus/nucleolus	Transcriptional and post-transcriptional activation	−
rev	+	p19	Nucleus/nucleolus	Expression of structural proteins, modulation of transcription	−
nef	+ +	p27	Cytoplasm	Negative regulator	+ +
vpr	+	p18	Nucleus	Rapid viral growth (?)	+ +
vpu (HIV-1)	+	p15	Cytoplasm/membrane	Assembly and release (?)	+ +
vpx (HIV-2)	+	p15	Cytoplasm	?	+ +

genes indirectly. It complexes to some cellular proteins and transcriptional factors that are involved in the turning on of genes important for T-cell proliferation such as those for IL-2 and IL-2 receptor (IL-2R) [22, 23]. It is somewhat ironic that the protein (IL-2) used to grow T cells to isolate the virus is the very protein that the virus uses or turns on in its first stages of leukemia. At least this is the way we think about it today. The TAX protein is also involved in turning on other cellular genes, e.g., the c-*fos* proto-oncogene. The development of adult T-cell leukemia (ATL) by HTLV-I is summarized in Fig. 5. Perhaps about one-third of T cells may be infected by HTLV-I, but only a small fraction expresses the

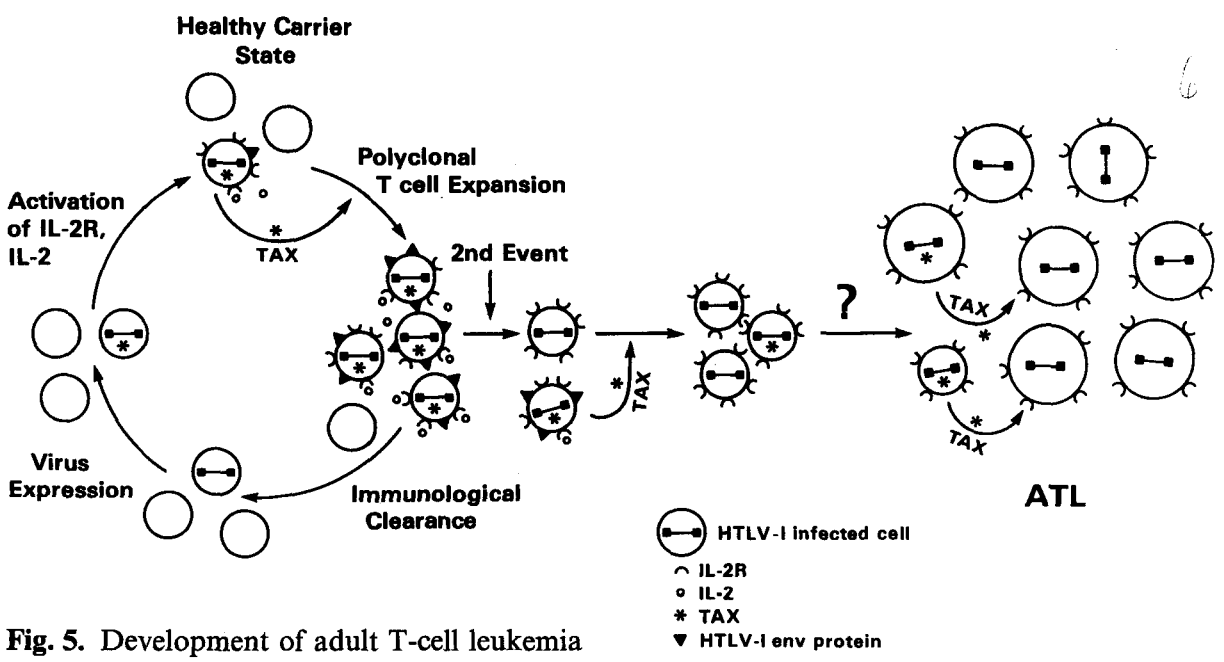

Fig. 5. Development of adult T-cell leukemia

virus. The immune system cannot see the cells which do not express the virus and cannot attack them.

At some stage, the *tax* gene is turned on. What exactly leads to the turning on of *tax* is unknown, but once it occurs genes for other viral proteins can be turned on. The *tax* gene also turns on the IL-2 and IL-2R genes. The IL-2R has a complex structure and is made of different polypeptides. The high-affinity polypeptide of IL-2R that binds best to IL-2 is activated by *tax*.

This may lead to autocrine and paracrine phenomena allowing polyclonal T-cell expansion. At this stage it is not a malignancy but it can be documented in many people infected by HTLV-I. The immune system attacks and clears the proliferating cells expressing viral proteins. The cycles of appearance and clearance of virus-expressing cells occur for years and maybe for decades. The virus continues to increase the expansion of proliferating T cells. As estimated recently in Japan, 3%–5% of the infected individuals will be able to develop monoclonal expansion of a T cell within their lifetime, most likely mediated by another as yet unknown genetic event. This event could be an accident, a mutation, or a rearrangement, but appears to be a chance event ultimately leading to true leukemia. A third genetic event which leads to the blast crisis may be necessary, analogous to chronic myelogenous leukemia. There is no complete agreement on specific chromosomal changes to account for the second or the third event. There are some that are common, but not consistent.

HIV-1 and AIDS

The idea that AIDS might be caused by a $CD4^+$ T-cell lymphotropic retrovirus came from discussions between R. Gallo and M. Essex and his colleagues in Boston who had worked on feline leukemia virus. Discovered in the 1960s by W. Jarrett et al. [24] in Scotland, it was shown by W. Jarrett, O. Jarrett, and others [25] that feline leukemia virus can be transmitted horizontally and cause immune deficiency as well as leukemia. Essex, in his epidemiologic studies in the early 1980s, highlighted the greater importance of this feline virus in immune suppression than in causing leukemia, whereas Gallo suspected from the experiences with HTLVs a possible involvement of a retrovirus in AIDS. These experiences were: studies of HTLV-I epidemiology showed that the AIDS virus was, like HTLV-I, endemic in Central Africa; the causative agent, like the HTLVs, targeted $CD4^+$ T cells; the modes of transmission by sex, blood, and the maternal/fetal route were similar; AIDS was associated with immunosuppression and the HLTVs can be immune suppressive (although modestly); HTLV-II had just been discovered, providing impetus to the idea of there being more human retroviruses. All these things led to thoughts that a new human retrovirus existed perhaps derived from a mutation or a recombinant change in an HTLV-I emerging from Africa, moving to Haiti and then to the United States. This was the notion that led people, ourselves and scientists in Paris, to look for a new retrovirus. However, ironically, we soon learned that, though AIDS is caused by a retrovirus, the virus is not a variant of HTLV-I or a recombinant with HTLV-I, but is due to a different category of human retrovirus(es) that simply has (have) many properties in common, although with a much different genomic organization as well as classification.

There are several components of the overall pathogenesis of AIDS, the major one being the immune deficiency with opportunistic infections. Because of the lifestyle of the individuals there is an increased incidence of infection with real pathogens which include mycobacteria, herpesviruses, HTLVs, and hepatitis and papilloma viruses. In addition, there is infection of the brain in 40%–50% of infected people. Subsequent to infection of the brain, there is a thinking disorder and some acute psychosis. The develop-

ment of two types of tumors is very common (Kaposi's sarcoma and B-cell lymphoma) and must be thought of as involving mechanisms distinct from the other manifestation of AIDS.

Immunodeficiency. The essence of the AIDS problem is immune suppression and immune deficiency. Part of the envelope of HIV, the gp120 molecule, interacts with the CD4 molecule. This interaction has been described as being much tighter and with much greater affinity than many antigen-antibody interactions.

The CD4 molecule is expressed on the surface of cells that are important for the immune system, including T helper lymphocytes, peripheral blood monocytes [26, 27], and cells of the macrophage lineage such as microglial cells of the brain [28], Langerhans cells of skin [29], which are widely distributed in the body, and the follicular dendritic cells of the germinal center of the lymph nodes; this allows the AIDS virus, immediately upon infection, to alter the most pivotal cells of the immune system. An idea to use the soluble CD4 in therapy of people who are infected has already been launched and animal systems are being investigated for that purpose. To use CD4 as a molecular decoy to bind virus before it finds CD4 on the cell surface seems to be the most rational approach to the therapy of this disease. Unexpectedly, however, CD4 is rapidly excreted by humans, and so the results have been extremely disappointing. Much research in the United States and Europe is focused on modification of CD4; for example, Genentech's use of immunoglobulin attached to the CD4 molecule seems to prolong the half-life of CD4, diminishing its rate of excretion.

Genomes of HIVs. The genomes of HIV-1 and HIV-2 are significantly more complex than that of HTLV-I or HTLV-II (Fig. 3). In addition to the three genes that all retroviruses have, *gag*, *pol*, and *env*, HIVs have two regulatory genes called *tat* and *rev* which are analogous to the *tax* and *rex* genes of HTLV-I and HTLV-II and are absolutely essential to the replication of HIVs and probably critical to the biological ability of HIVs to cause AIDS or other manifestations. Many other genes are discovered in the genomes of the AIDS viruses: *vif*, which is essential for cell-free infection by these viruses; *vpr*, which was recently found in our laboratory to be essential for infection of primary human macrophages, but not T cells; *vpu*, whose function is not yet well known; *nef*, which is controversial as to whether it does nothing to virus replication or slightly down-regulates it; and at least two more genes discovered in the last year or so, particularly by Haseltine and his colleagues, whose functions are not yet well understood (Table 2).

The early steps of the life cycle of HIVs are the same as those of animal retroviruses (Fig. 6) and involve attachment and penetration of the virus into the target cell. It is known that the CD4 molecule is the receptor or at least part of the receptor for HIV. Once HIV penetrates the $CD4^+$ T cell, RNA to DNA transcription and DNA integration into the cell follow. Actually, it is not established whether HIV provirus integrates into macrophage. Like HTLV-I and HTLV-II, following integration, there is a silent or latent period even for HIVs. After T-cell activation due to any stimuli, expression of the DNA provirus takes place to form viral RNA and viral proteins. The products of the *tat* and *rev* genes have the same kind of functional corollary as those for *tax* and *rex* of HTLVs. They act as regulatory switches in the replication cycle. In the long silent period after infection nothing happens to the cell if virus is not expressed. But if that cell is immune-stimulated the replication cycle is completed. The virus comes out in a burst, and the cell dies. So, contrary to the notions of some, that retroviruses can not be cytopathic and cytolytic, HIV is certainly cytopathic. Actually, the earlier work of Howard Temin with avian retroviruses also showed the cytopathic and even cytolytic nature of some of those viruses.

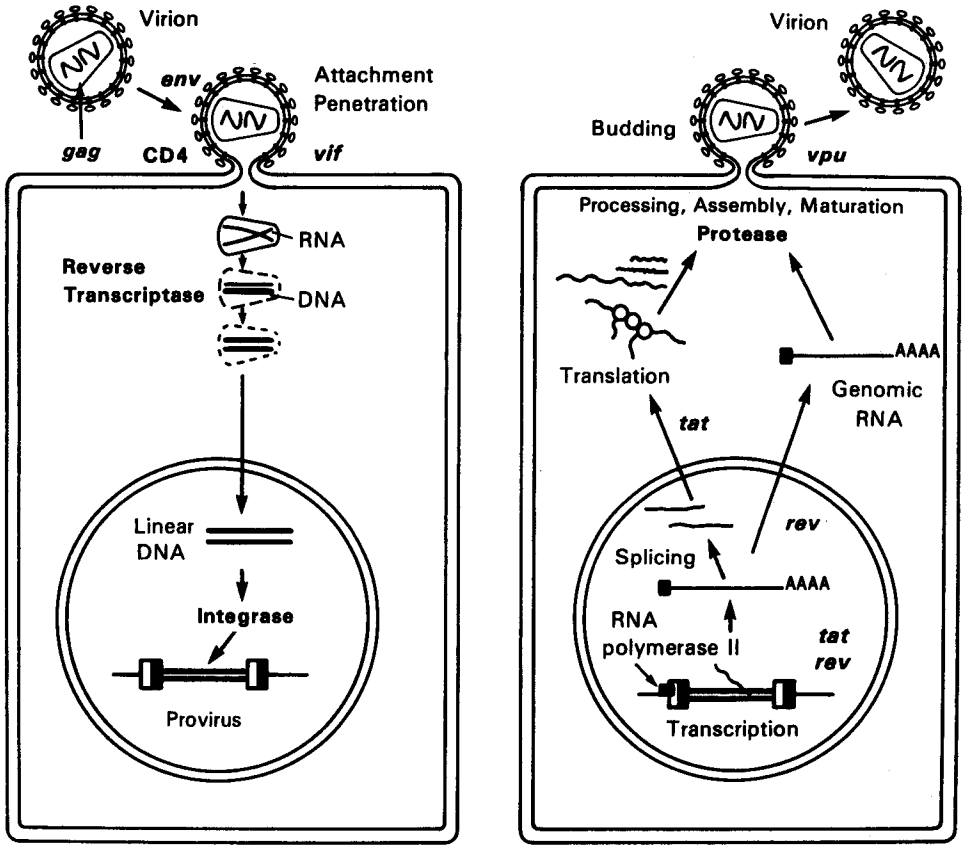

Fig. 6. Life cycle of HIV-1

Mechanisms of HIV Pathogenesis

Role of HIV and HIV Proteins. The question is often asked "Why do the $CD4^+$ T cells become depleted in AIDS?" HIV may be involved in direct killing of infected T cells. HIV also has the capacity to form multinucleated giant cells. When the virus is forming, the envelope protein gp120 is on the cell surface. If there are uninfected cells nearby expressing the CD4 molecule, there will be binding and fusion of the two cells; and this can give rise to fusion of literally several cells together. Such cells have aberrant function and die prematurely. Based on the laboratory observations, one can speculate on other ways which could account for the $CD4^+$ T cells depletion. Extrapolation of these to the in vivo situations may still be remote. It is important to mention that the gp120 falls off the virus easily. In vitro studies show that the gp120 can interfere with T-cell activation. It can also lead to the down-regulation of IL-2 expression in uninfected T cells when this protein binds to CD4 molecules. There are still other indirect mechanisms that could permit the depletion of T cells (see Table 3).

Escape from the Immune System. The infection of the macrophages by HIV shows a very unique feature, namely, infectious whole virus in vesicles inside the cytoplasm of the cell [26]. This happens in only a small fraction of all macrophages.

The important question, however, is: "What if the immune system attacks this cell and destroys it?" Would there be a release of more infectious virus? In laboratory studies the answer is yes. If we take an infected macrophage and we break it manually or by attack from cytotoxic T cells, more infectious virions are indeed released. Therefore, immune therapy that kills infected macrophage must also consider the need for a direct antiviral attack, for example, azidothymidine or neutraliz-

Table 3. Mechanisms of $CD4^+$ T-cell depletion in AIDS

1. Direct killing by HIV following immune stimulation and virus expression
2. Cell death following syncytia formation
3. Decreased IL-2 production
4. Cell-mediated cytotoxicity against uninfected cells mediated by free gp120 complexed to CD4 and antibodies against this complex
5. Some viral protein products inhibit T-cell proliferation
6. Another virus, HHV-6, upon replication is T4 cell-lytic; HHV-6 is common in HIV-infected people and may replicate more in them
7. Defective antigen presentation leads to lower T4 cell proliferation
8. Inappropriate release of certain cytokines, e.g., tumor necrosis factor-α, can decrease T-cell proliferation
9. The gp120-specific class II-restricted cytotoxic lymphocytes can lyse activated ($1a^+$), autologous, uninfected T4 lymphocytes. The CD4 receptor-mediated uptake of gp120 is a critical event for this lytic process. This mechanism could allow destruction of a large number of activated lymphocytes responding to many pathogens

ing antibodies. Neutralizing antibodies against the HIV-1 work by complexing to a certain region of gp120 and blocking entry into the cell.

Virus Variation. Another important question is "Why does the HIV-infected person continue to spread the virus?" We know that HIV varies from person to person. We discovered in 1984 the heterogeneity of HIV for the first time [30]. We found that no two viruses were the same, and the variation was up to 4%–15% in the genomes of different HIV variants. The variation was predominantly in the envelope region. We also showed later that within any one virus isolate there are minor variants. That is to say, if you isolate the virus from one person with AIDS, although most of the viral particles will be very closely related, there is still some variation. And this, in time, has been shown to have biologic significance [31]. For instance, in one virus strain there are many virions with minor differences. At time zero, one variant may predominate and the neutralizing antibody could neutralize almost all of this virus variant. Some time later, another minor variant, perhaps with as little as one amino acid change in the envelop, may emerge, and this may not be neutralized by the original antibody. We have been able to study this in a laboratory worker who, while mass-producing the virus in another laboratory, was infected by accident. One can follow such a person in time. This seems to be an important way by which this virus continues to escape the immune system. Variant-specific antibody develops and can neutralize the virus; new minor variants then emerge, but are not neutralized. One would expect the variation to occur in the region of the neutralizing epitope which exists in the hypervariable region of the envelope. This is true not only with neutralizing antibody, but with cellular immunity as well. In addition to variation in this region, we have seen mutation in completely distant regions, e.g., in the transmembrane region of the envelope protein gp41. Such a mutation also affects the interaction of antibody with this site. More likely the mutations at a distance bring about conformational changes [32, 33].

AIDS and Cofactors. In a small study of homosexuals in Trinidad, Bartholomew et al. studied dual infections with HIV and HTLVs and concluded that HTLV-I may be a cofactor in AIDS [34]. There are additional reports now that agree with this, from Japan in hemophiliacs and from New Jersey with drug addicts [35]. HTLV-I is not needed to get AIDS at all, but the rate of progression may be accelerated in the presence of HTLV-I. Several mechanisms are possible. HTLV-I can lead itself to minor T-cell impairment. The TAX protein of HTLV-I can also activate HIV if the cells are infected

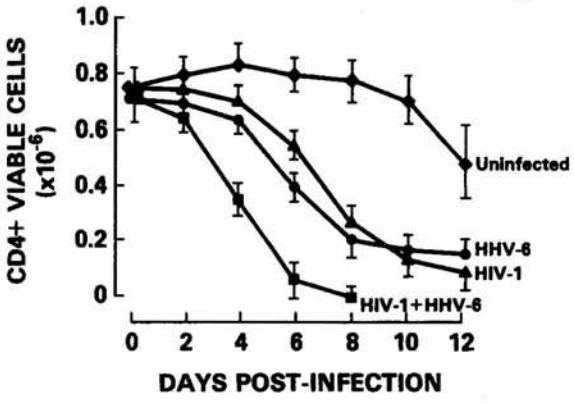

Fig. 7. Killing of $CD4^+$ T cells by HHV-6

with both viruses. In addition, HIV-1-infected T cells can be activated by the simple interactions of HTLV-I with the cell membrane. We have also shown that HTLV-I and HIV can form mixed virus particles, which gives HIV the ability to affect cell types it normally could not affect [36].

The new herpesvirus, human herpesvirus type 6 (HHV-6), which we discovered and isolated in 1986 from B cells [37], actually principally infects $CD4^+$ T cells. This herpesvirus can also kill T cells (Fig. 7) [38]. In the United States, 70%–80% of all people infected with this virus are seropositive. Therefore, in most people, it obviously causes no problem. It is the cause of roseola in babies, which is not a very serious disease. Adults who have antiviral antibodies and cellular immunity can control the replication of the virus. It is possible that in AIDS with immune impairment, there is increased replication of this virus. If so, one must consider damage to the immune system by direct killing of T cells by this virus. In addition, this herpesvirus can activate the HIV genome. It has a gene which makes a protein that can *trans*-activate the expression of HIV [39], analogously to the HTLV-I TAX protein.

Finally, we recently showed that this human herpesvirus is, as far as we know, the only biological agent existing naturally that turns on the CD4 gene at the transcriptional level, and to our knowledge this is the first time it is known that one virus can turn on the receptor of another [40]. In $CD8^+$ T cells and in some epithelial cells, infection by HHV-6 turns on CD4 so that they can become targets for HIV infection. All these aspects of

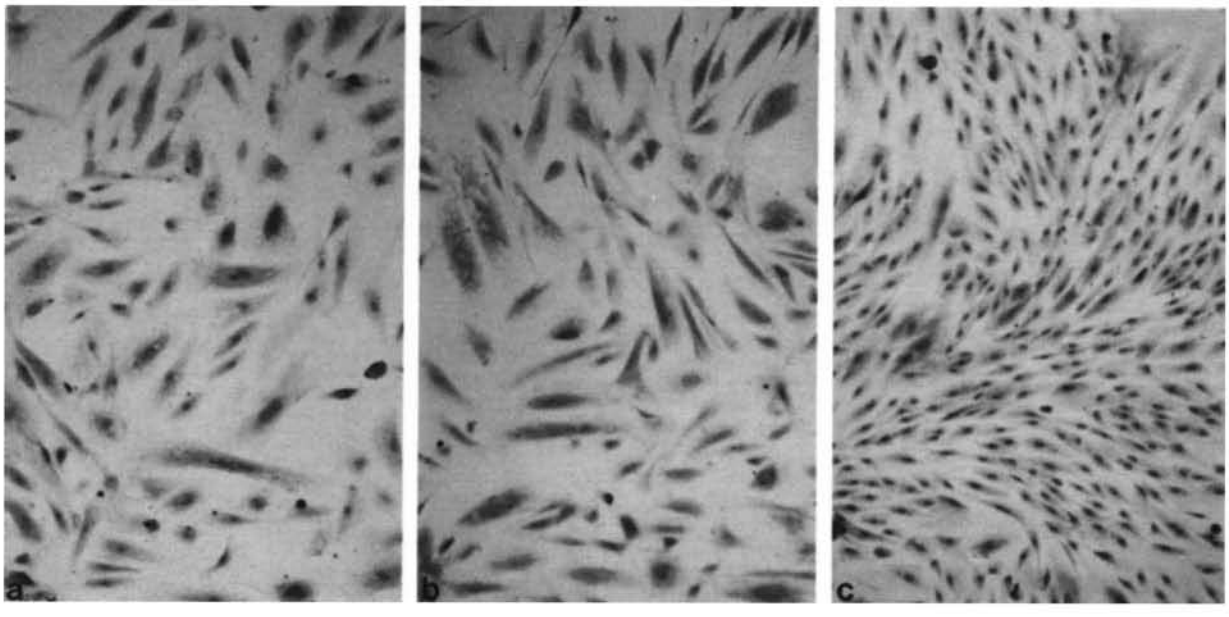

Fig. 8a–c. Spindle cells of Kaposi's sarcoma. **a** Culture in standard medium (RPMI 1640 plus FCS 15%). **b** Standard medium plus endothelial cell growth factor (ECGS) 30 µg/ml and heparin 45 µg/ml. **c** Standard medium plus HTLV-II CM (20% v/v). (From [42], with permission; Copyright 1988 by the American Association for the Advancement of Science)

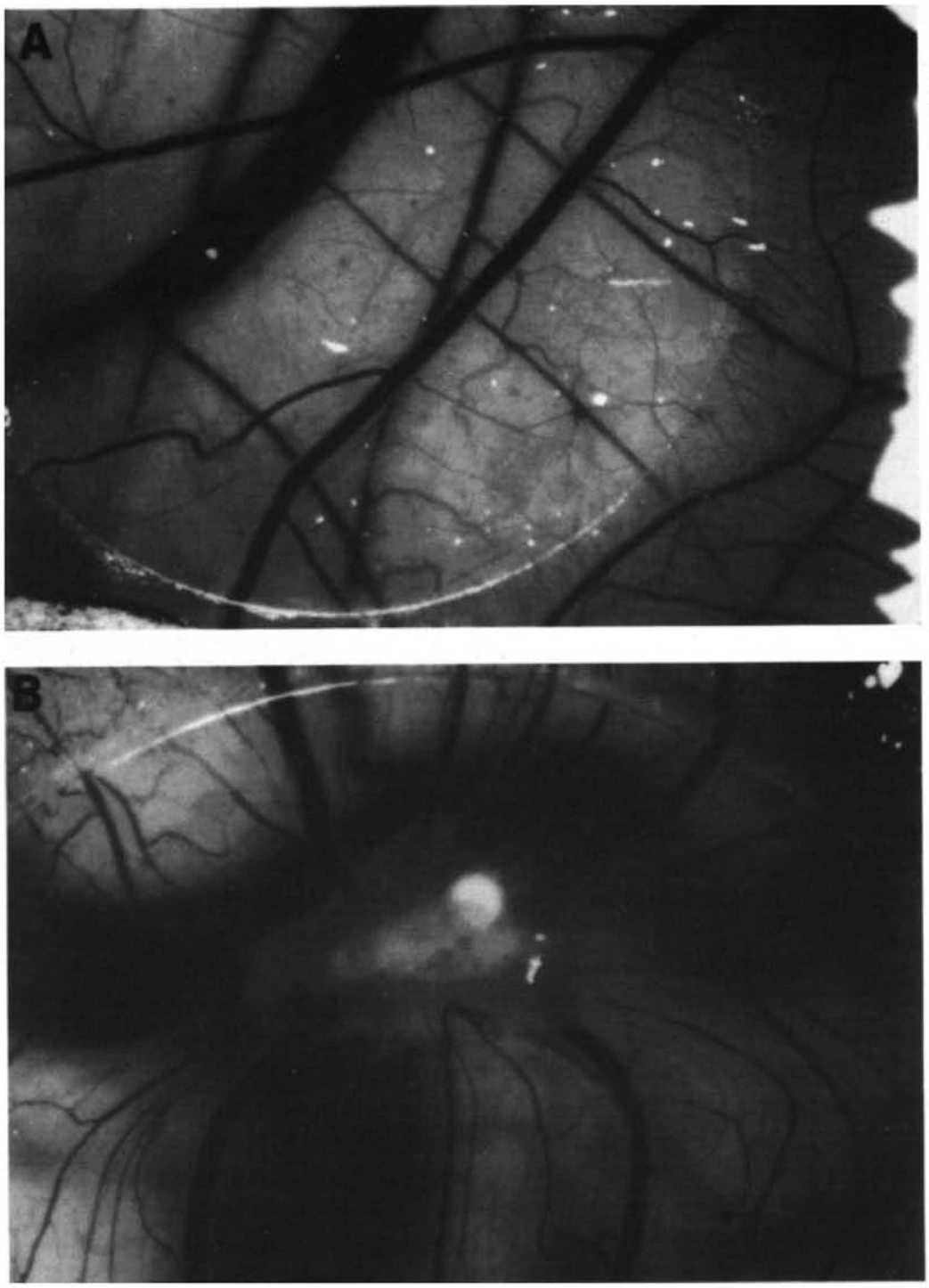

Fig. 9 A, B. Angiogenesis in chick chorioallantoic membrane. **A** Fixed (0.00125% glutaraldehyde) cells gave a negative result. **B** Metabolically active cells gave a strongly positive result. (From [42], with permission; Copyright 1988 by the American Association for the Advancement of Science)

HHV-6 lead us to propose that this virus may contribute to the impairment of the immune system in people already immune suppressed by HIV.

AIDS-Related Kaposi's Sarcoma. Since there has been a great increase in the incidence of Kaposi's sarcoma in HIV-infected people, more so in male homosexuals, one can speculate that HIV infection plays some role. It is not known whether any new or unknown viruses play a role in AIDS-related Kaposi's sarcoma. We started to explore the possibility of other virus(es) but did not find any. In the process, we developed a system for studying Kaposi's sarcoma [41, 42].

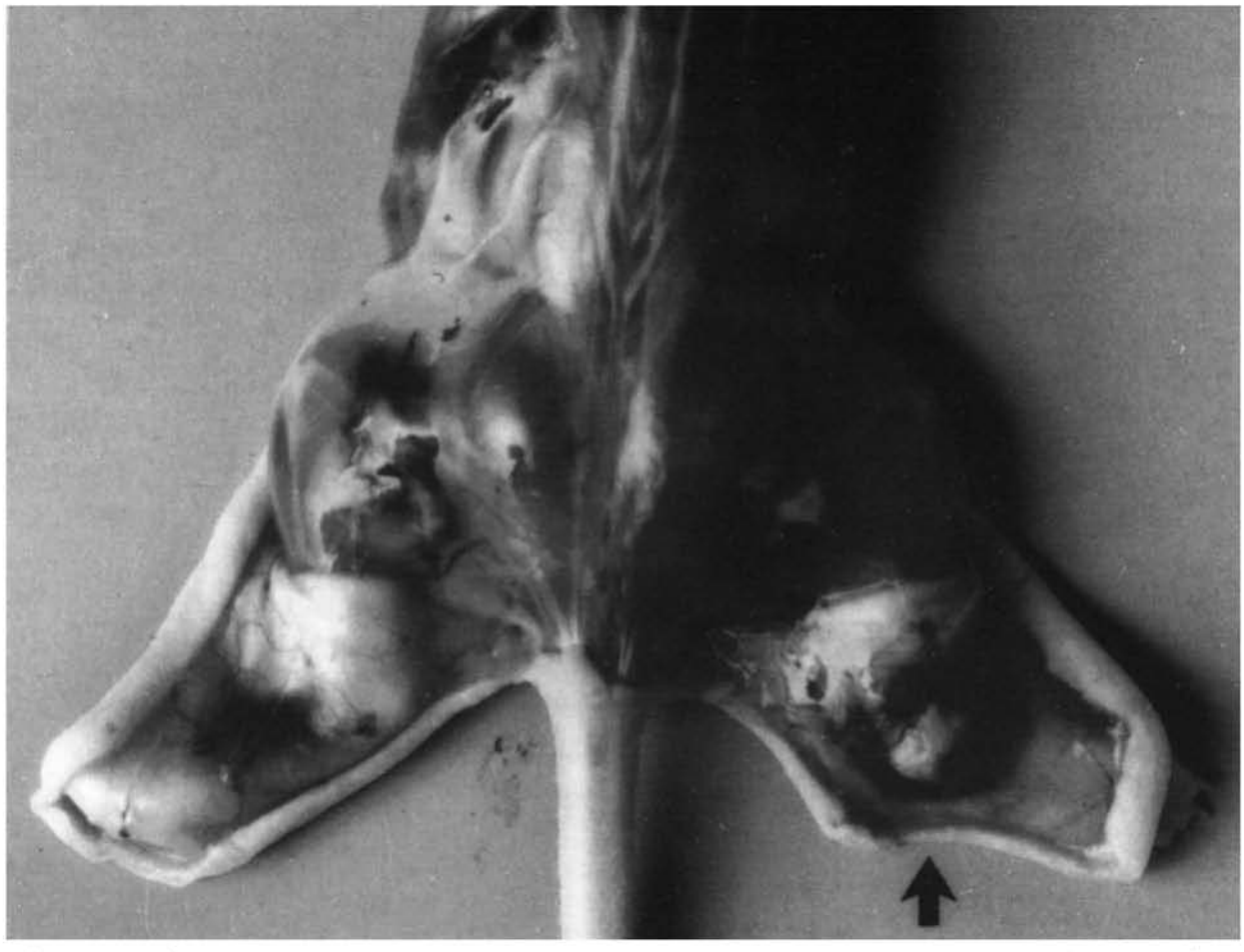

Fig. 10. Lesion in nude mouse induced by Kaposi's sarcoma spindle cells (4×10^6 metabolically active cells injected subcutaneously). *Arrow* indicates positive result. The *left side* was injected with fixed cells

The important thing that came out of our studies over the last few years is that we have a system in the laboratory for studying Kaposi's sarcoma. We can grow the spindle cells which are believed to be the tumor cells of Kaposi's sarcoma. Figure 8 shows the spindle cells derived from a person with Kaposi's sarcoma which were grown for several months in culture. We have several such cell cultures now. These spindle cells have been analyzed in collaboration with Judah Folkman and his associates from Harvard University [43]. They have the properties of primitive smooth muscle cells of vascular origin, as well as some properties of endothelial cells. We think then that the precursor cell of Kaposi's sarcoma is a mesenchymal, primitive precursor of cells of the blood vessel walls. Although we could not find any virus, particularly HIV-1, in these cells, it was found that they release a number of cytokines that have powerful angiogenic activity, which is a key feature of Kaposi's sarcoma. Figure 9 shows angiogenic activity released by the spindle cells grown in the culture tested in the normal chick chorioallantoic membrane [41]. One can take either the intact spindle cells or the concentrate of factors released from them and apply it to the membrane. Distinct angiogenic activity is observed in both instances.

More interestingly, these spindle cells, when put into a nude mouse, cause a tumor similar to human Kaposi's sarcoma to develop (Fig. 10). The lesion develops near the site of inoculation of the spindle cells within 10 days. When the spindle cells regress, the lesion dies out. We examined the lesion histologically. It appears like early Kaposi's sarcoma with blood vessel proliferation, fibroblasts,

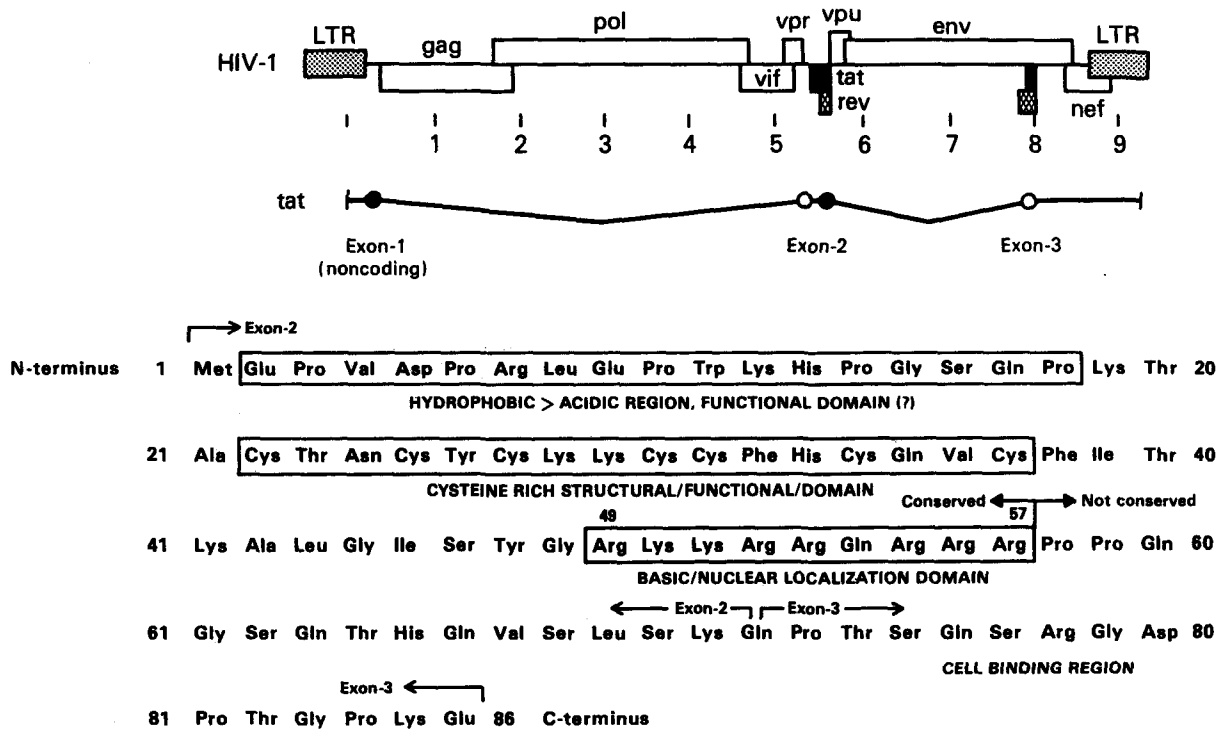

Fig. 11. Different structural and functional domains of the TAT molecule

infiltration with leukocytes, and spindle cells. The conclusion is that the spindle cells secrete factors that are responsible for the early lesion of Kaposi's sarcoma [42]. We have evaluated the cytokines it makes. It appears that IL-1 and basic fibroblast growth factor are the most important ones. These molecules can have angiogenic activity and promote growth of fibroblasts and endothelial cells directly or indirectly. In addition to these, other factors such as granulocyte-macrophage colony-stimulating factor, tumor growth factor-β, IL-6, and low levels of acidic fibroblast growth factor and platelet-derived growth factor are also detected [44].

The manner in which we succeeded in growing the spindle cells is interesting in itself. We grew the spindle cells by using lymphokine(s) made by chronically activated $CD4^+$ T cells. The major active lymphokine for this effect is currently being purified in our laboratory and is the most potent growth factor for AIDS Kaposi's sarcoma spindle cells. In addition, we have found that the TAT protein is released in very small amounts (nanograms) by HIV-1-infected T cells and acts as a growth factor for the spindle cells [45].

The TAT molecule has different regions which are responsible for different activities (Fig. 11). We think one region is particularly important for the growth-promoting activity on the spindle cells [45]. This small protein of 10000 daltons is very complex. It has a region that is important for the *trans*-activation activity of the virus and a basic domain important for the nuclear localization.

Human Retroviruses and Tumorigenesis

The direct effects of a retrovirus like HTLV-I where the virus infects its target cell, can immortalize that cell, and makes it abnormal have been discussed earlier. We find the viral sequences in every cell in the sample place, indicating their clonal derivation from the original transformed cell. HTLV-I can thus be called a directly acting tumor virus. We refer to HIV as having indirect effects that can lead to the increased possibility of tumor develop-

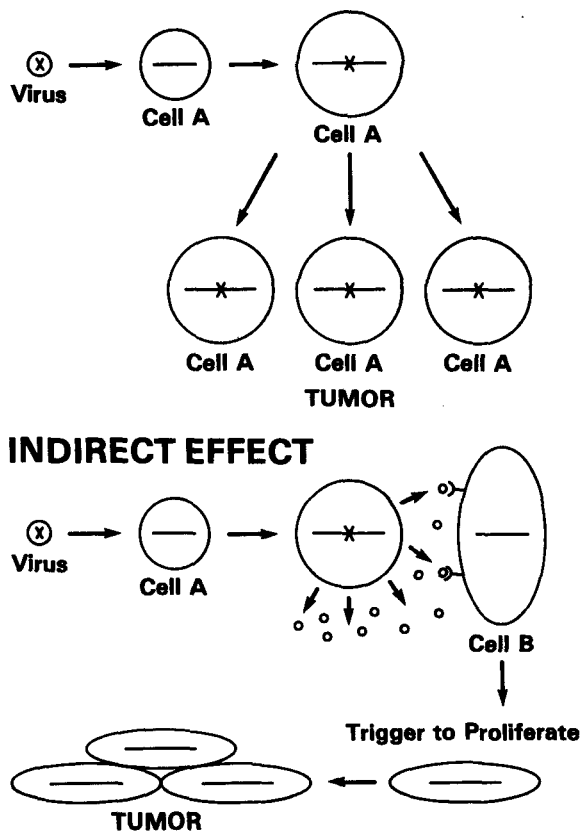

Fig. 12. Direct and indirect mechanisms of tumor induction by human retroviruses

ment. HIV probably increases the possibility of Kaposi's sarcoma developing in at least two ways:

1) it infects T cells and releases TAT protein;
2) its proteins activate immune cells (T cells and B cells) which release lymphokines.

Some of these lymphokines can have an effect on the primitive mesenchymal cell that has lineage to smooth muscle and endothelium and which is the precursor of the spindle cell of Kaposi's sarcoma. This cell in turn releases a series of cytokines that act to form a complex mixed tumor that we call Kaposi's sarcoma. In summary, human retroviruses can induce tumors, directly or indirectly, in addition to their suppressive effects on the immune system and abnormal effects on the nervous system (Fig. 12).

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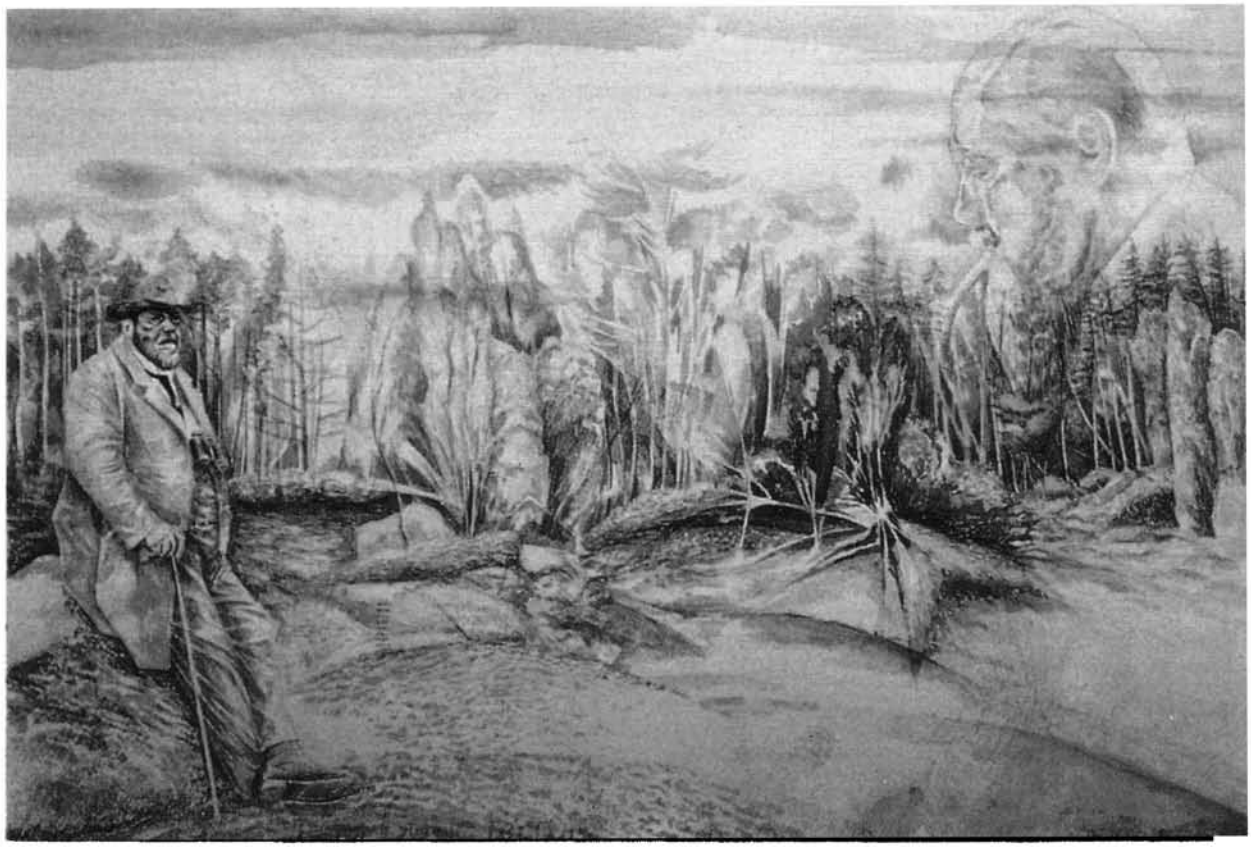
Pastor Bode Lecture

Wilsede, June 20, 1990

N. Avrion Mitchinson:
Escape from the Red Queen

„Heidepastor“ Wilhelm Bode



Michel Weidmann (Aquarell)

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Wilhelm Bode was born on October 20, 1860, in Lüneburg, in Germany, as the third of ten children. His father was a teacher at the seminary in Lüneburg. There is a legend that the father, on the night of the birth of each of his sons, took the child and, placing hin in the wide pocket of his coat, diaper and all, walked up the nearby Kalkberg hill. There, he held up the child facing the town spread out peacefully below, and said, "Behold, my son! This is your home country! Remain true to it, hold it dear to your heart, and protect it!"

Wilhelm, the third son, inherited his father's inclination towards nature and his enthusiasm for it, and indeed for everything unspoiled and free, more strongly than any of the other children. Wilhem often accompanied his father on the long excursions he habitually took in the school holidays. And he showed from the first a particular love of nature lore and science.

The youth grew up in a tightly knit family. The father's favorite saying was, "The rich man is not the one with many possessions, but the one with few needs." In 1880, upon fishing school, the 19-year-old Wilhelm started theological studies, first in Göttingen, then in Strasbourg. Even as a student, his interests ranged wide: zoology, botany, history, and German romantic literature fascinated him in addition to his main subject of study. For several weeks during one holiday, he even joined a wandering circus.

After completing his theological studies, he was assigned to the parish in Egestorf, six miles to the east of Wilsede Hill. This was to become the scene of his life's work. His first serom, on August 15, 1886, was delivered on the theme *We together*. "You are my parishioners," he said, "and I am your pastor; and if two people are going to live together, and take up housekeeping together, it is a good thing for each to have a clear notion of the rights and duties that each has towards the other." "Do not demand that I demonstrate all the social graces," he went on, "or that I be worldly wise, or a flashy speaker, or anything else other than a servant of the true Teaching! Take care what is said from this, your pulpit, and watch jealously that it not be profaned! The parish that requires nothing of its pastor is asleep; but the one that requires much is alive... The first premise for beneficial cooperation is an unreserved and mutual give and take between us. We shouldn't say; 'Here are the parishioners, and there is the pastor,' but rather 'We together!' This is may task."

Pastor Bode tried to make lessons in the schools more interesting, and checked to see that the teachers really began lessons on time. He himself organized programsfor official school celebrations; he arranged for the children to be provided with their school books, with the exception of Bible and psalm book, at the cost of the school district. He advocated putting an atlas and a book on science and languages in the hands of each pupil; and he considered sports, gymnastics, to be one of the most important subjects. It was Pastor Bode, with his farmers, who in 1888 founded the first savings and loan bank in the Lüneburg Heath. Later, a cooperative for the insurance of farm animals, the *Kuhkasse* or "cow found," was added. The cooperative purchase of feed and fertilizer was organized, followed by a water cooperative, which used wind power to provide the village with running water.

This was the one side of his work: practical, active help so that the people entrusted to him could improve their standard of living.

Pastor Bode's other side seems marked by a sort of natural piety: his sermons breathe the air of freedom and nature. His passion for the heath is not an ideology; rather, it was a part of his pastoral teaching to win each of his farmers to an appreciation of the land that he farmed. On a walking trip with his father, the young Bode had passed from Egestorf via Aue and Radenbach to Wilsede, through the untouched natural beauty of the open heath with its juniper bushes, with the lustrous dark green of

the bordering pine woods. At one point, his father said, "My son, if a man could preserve this landscape for future generations, he would have accomplished a great work, a good work."

Many years later, when Bode found that a considerable parcel of land, the Totengrund, was to be sold and used for construction, he tried to prevent the sale. After many fruitless attempts, he found a valuable ally in Professor Thomsen from Münster: this man was prepared to supply funds for the purchase of the Totengrund, thus saving it from the development that threatened it. After very difficult negotiations, Bode succeeded in purchasing the Totengrund in 1906 for the sum of 6000 marks. This piece of land was to become the seed from which the Lüneburg Heath Nature Park was to grow.

Bode carried out his next project in cooperation with a Herr Dageförde, a teacher from Tangendorf. This teacher had assembled an extensive anthropological collection which filled the schoolhouse to overflowing. On the initiative of Pastor Bode, a piece of land was purchased in Wilsede. Thus, Bode became one of the founders of the Wilsede Heath Museum Society. Dageförde acquired (quite cheaply, as it was going to be torn down) a fine old farmhouse in Hanstedt dating from 1750. This house was disassembled, and then rebuilt on the lot in Wilsede. It opened on August 15, 1907, as "The Old House", or in the North German idiom, "*dat ole Huus*." Tourism increased. The Society, only recently formed, enthusiastically erected an inn, the "Inn at the Heath Museum." Pastor Bode wrote the advertising pamphlets himself, and argued: "No paved road! No nickelodeon!"

As Pastor Bode learned that a dance hall was to be built on Wilsede Hill, he managed to delay the sale of the land. District Counsellor Ecker from Winsen/Luhe, a member of the Nature Park Society, sent the author of *Kosmos*, Dr. Curt Floericke, to look over the situation. Impressed, Floericke wrote a decisive report. Ecker, as representative in the Prussian Legislature, succeeded in arranging for public funds to be made available: the Nature Park Society was then able to purchase this parcel as well.

When Pastor Bode died on June 10, 1927, he was mourned by large numbers of people. It was his request that his ashes be scattered to the winds from the top of the Wilsede hill. This wish was granted him.

Acknowledgment. I thank Griffin Andersen for the English translation.

Hanne-Lore Neth

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Pastor Bode Lecture

Escape from the Red Queen

N. A. Mitchison [1]

Immunological diseases follow a characteristically fluctuating course of relapse and remission, such as is illustrated in Fig. 1. This is true most obviously of diseases such as rheumatoid arthritis which have a major autoimmune component, but it holds equally well for chronic infectious diseases such as leprosy in which hypersensitivity plays an important part. It is likely, but not definitely established, that the fluctuations reflect an imperfect balance between opposing forces within the immune system, and that these in turn reflect the activity of opposing control genes. Among such control genes, those of the major histocompatibility complex (MHC) are likely to be the most important.

Studies on the MHC and disease have tended to focus on detrimental genes, that is, those which are positively associated with the disease in question, predispose for it, and presumably act as causal factors. Some of the autoimmune diseases are tightly associated with particular HLA genes, such as is ankylosing spondylitis (and certain forms of reactive arthritis) with HLA-B27. For others the tightness of the association has become apparent only as seemingly unrelated predisposing genes have been discovered to share sequences in common. Thus an epitope shared between HLA-DR1 and HLA-Dw4 explains well why both of these genes predispose for rheumatoid arthritis [1]. The existence of a tight association suggests that the disease process may be driven by presentation of a self-peptide by the HLA molecule. Not only does this provide an attractively simple picture of how the disease develops, but it also points the way forward to new modes of treatment. From the tight associations spring the present flurry of excitement concerning HLA-blocking peptides and monoclonal antibodies.

In comparison, beneficial HLA genes have suffered neglect. This seems a pity, if only because it makes sense to try to understand what makes a patient get better. The negative associations between HLA and disease seem on the whole to be weaker than the positive ones, although this has not been categorically established. Another reason for neglect is that it is less obvious how an MHC gene could inhibit an immune response, in the way that these beneficial genes seem to do.

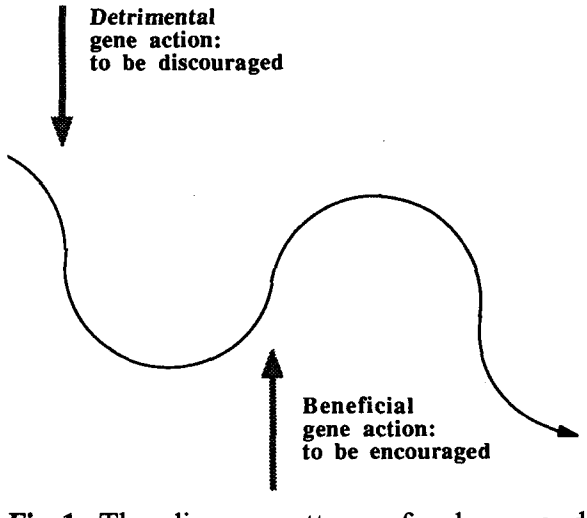

Fig. 1. The disease pattern of relapse and remission, characteristic of immunological diseases, suggests that opposing activities operate within the immune system

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

One has to think seriously about suppressive activity and suppressor cells, and those are subjects that immunologists have learned to be cautious about. Nevertheless they are the subject of this paper.

Inhibitory MHC Genes

Examples of inhibition of immune responses by MHC genes are not hard to find. Our recent survey lists some 20 in mouse and man [2]. What that listing did not include are the significant but only moderately impressive negative associations between HLA and autoimmune disease that have often been recorded, usually as by-products of surveys aimed principally at verifying positive associations. Figure 2 gives an example, showing the apparently beneficial effects of three HLA-DR genes on rheumatoid arthritis in a recent study [3].

If I had to choose just one example of a disease study, it would be the joint work carried out by groups from Leyden and Caracas on HLA and lepromatous leprosy [4]. Not only does this contain beautiful data, but also it provides an amusing sidelight on paternity and family life in Venezuela. Since then leprosy has become an arena for testing ideas about genetic control of suscepbility to chronic infectious disease, and more recent presentations of the topic are available [5]. Rather than go over the whole list of inhibitory genes in this and other areas again in detail, it seems more useful on the present occasion to offer the following generalizations.

1. As evidence of an immunoinhibitory effect, a negative association between HLA and an autoimmune disease (or other immunological disease, such as allergy) is equivalent to a positive association with an infectious disease.
2. Evidence of such associations often springs initially from population surveys. Such data need eventually to be supported by the stronger evidence that multiple-case family studies can provide. In the mouse, studies on panels of recombinant inbred mice provide the equivalent of family studies in man. It should be noted, however, that families with multiple cases tend to have a high susceptibility background, and that this will diminish the impact of protective genes (I am

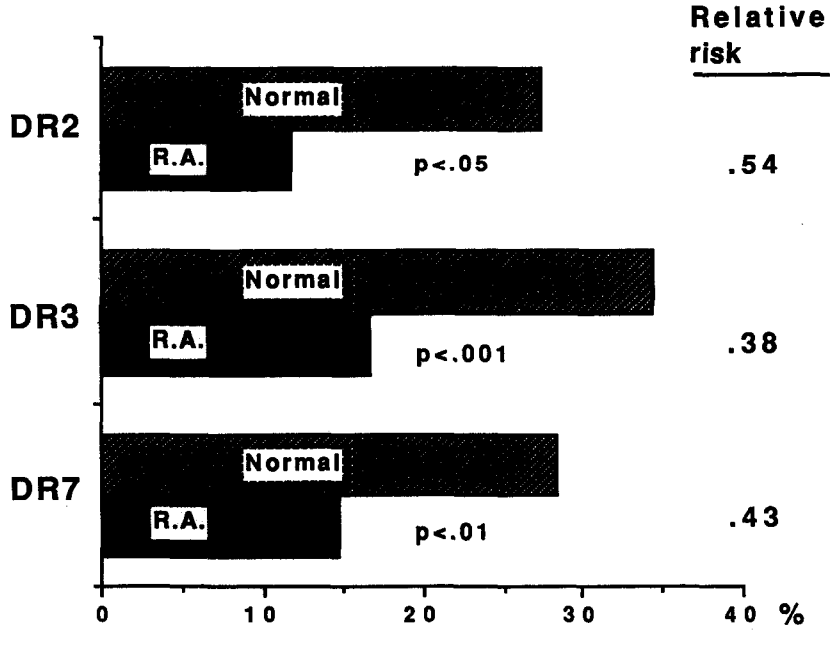

Fig. 2. Negative associations between class II HLA genes and rheumatoid arthritis, detected in a UK survey

grateful to H. O. McDevitt for pointing this out to me).

3. As mentioned above, many immunological diseases have strong positive HLA associations. This will tend to produce negative associations for other HLA genes, and the more frequent such a gene is in the study population, the more likely is this negative association to reach significance.
4. Although a negative MHC association may be taken as prima facie evidence of immunoinhibition in an immunological disease (and likewise a positive one in an infectious disease), detailed immunological study would be needed to substantiate the claim. This might involve exploring the possibility of relieving the inhibition by in vitro (or in the mouse by in vivo) treatment with anti-MHC monoclonal antibodies, or other procedures.
5. The clear-cut inhibitory MHC genes have all so far turned out to belong to class II. This is surprising, in view of the fact that T cells able to mediate an inhibitory effect often have the CD8 phenotype. One can think of explanations, for instance, along the lines of the phenomenon known in inbred mice where, when an active H-2K allele (in a cytotoxic response) is replaced by an inactive one, the previously inactive H-2D allele become active (and vice versa). But the absence of class I genes still seems odd, and perhaps further research will change the picture.
6. No MHC gene has been found to mediate inhibition exclusively. All the genes which inhibit a response act positively in others (this statement requires some qualification as regards HLA-DQ in man, where most of the evidence for a positive effect comes from in vitro studies with cloned T cells of "helper" phenotype).
7. Nevertheless a certain bias in the location of inhibitory activity within the MHC is evident, both in mouse and man. This is respectively towards H-2E and HLA-DQ. These genes are not, of course, homologous in evolution, but they do share certain features in common. Both seem to be secondary class II genes, with relatively few T cells restricted by them, with relatively low expression, and with relatively low polymorphism (none of these features are definitively documented, unfortunately). Function seems to have flipped from one gene to another during the evolutionary divergence of the two species.

One of the important issues of the day in this area is the claim that the presence of asp-57 in HLA-DQ protects against insulin-dependent diabetes. The most recent publication on this subject amounts to a vigorous rejection of this claim on the basis of a segregation study in multiple-case families [6], but the qualification about this type of study, mentioned in point 2 above, means that judgement should be suspended. I doubt if we have yet heard the last word on this matter.

Finally let me mention the recent study that raised my interest in the present issue [7]. It showed that substitution of H-2A^b for H-2A^k strongly inhibits the response of mice to F liver antigen and does so more weakly for their response to Thyl antigen. This provides a promising system for further study of mechanism.

Mechanisms of Inhibition

A comprehensive conceptual framework within which to consider mechanisms of inhibition is much needed, and the main purpose of this paper is to present one in the outline form shown in Fig. 3. In doing so, I gladly acknowledge the benefit derived from discussions with C. S. David and H. O. McDevitt, and from the trenchant commentary of Nepom [6].

This classification begins with a distinction between intracellular and intercellular mechanisms, so that only in the former does the inhibitory MHC product operate within the same cell as the positive MHC (immune response, Ir) product. Intercellular mechanisms corre-

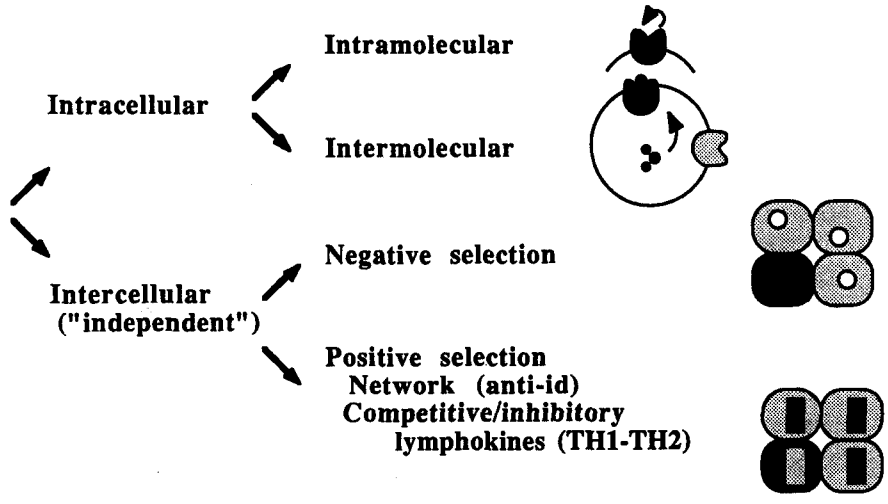

Fig. 3. A classification of immunoinhibitory mechanisms mediated by class II major histocompatibility complex molecules. The inhibitory molecule or repertoire is *shaded darker*

spond roughly to what Nepom designates as "independent" inhibitory activity, a category whose existence he questions.

Intracellular mechanisms further subdivide into intra- and intermolecular ones. Intramolecular inhibition, for instance, might involve an amino acid substitution in the $\alpha 1$ helix inhibiting the activity of an Ir gene previously defined by substitution in the $\alpha 2$ helix. This is a makeshift, as eventually the combination would be designated simply as a new neutral allele. On the other hand, the category of intracellular, intermolecular inhibitions has several examples, mostly involving class I genes in the mouse. These I have discussed some time ago [8]. The phenomenon of competition in the antiviral cytotoxic response between H-2K and H-2D mentioned above belongs here, as also does the competition between heterozygous alleles at H-2K or H-2D that has been noted in the same type of response. More relevant to our present discussion of class II inhibitory genes, perhaps, is the competitive suppression that my group has studied in the anti-Thyl response [9]. There the presence of an allogeneic MHC molecule on a Thyl antigenic cell (but not on a neighboring cell) can inhibit the response. That this is truly a competitive inhibition is strongly supported by our (unpublished) data demonstrating loss of inhibition when the dose of antigen is increased. We do not known for certain whether the allo-MHC antigen in this experiment is presented by self-class II molecules in the usual way, but if it is then the gene(s) which encode them could be regarded as inhibitory for the anti-Thyl response. In summary, the known instances of intracellular, intermolecular inhibition boil down to antigenic competition.

The intercellular mechanisms subdivide into those that result from negative or positive selection. In negative selection the product of one MHC gene inhibits the activity of another by deleting a part of its repertoire. The ikon for this category in Fig. 2 depicts the T cell repertoire as subdivided into four parts according to their restriction elements (e.g. H-2A^a, H-2A^b, H-2E^a, H-2E^b); the part restricted by the inhibitory gene is shaded darker, and there are holes in the other parts of the repertoire. This phenomenon is now familiar in the context of superantigens, such as H-2E, or the *mls* product. It has not yet been identified (to the best of my knowledge) as a mechanism of inhibition resulting from class II allelic substitution, although that may well be disclosed in the future.

Suppression mediated by positive selection is the most challenging category,

for this is our old friend the suppressor T cell revived. In the formal sense used here, inhibition results from positive selection when the product of one MHC class II gene enables a group of T cells to develop that are able to inhibit the activity of another group of T cells that would otherwise perform a response. The ikon shows the part of the repertoire that is restricted by the inhibitory gene as biased towards inhibition, while the rest of the repertoire is biased away from that activity (note the patches of shading). As mentioned in the figure, current theory is that inhibitory T cells could operate in alternative ways. One would be, by engaging in inhibitory anti-idiotypic recognition, thus involving Jerne's network. An alternative would be the secretion of inhibitory or competitive lymphokines, such as do TH1 and TH2 cells in the mouse [10]. Other possibilities could be cited, such as antigen-specific suppressor factors, but these seem too remote to be included in the discussion.

This is not the place to discuss these last alternatives in detail. Inhibition via the network has a long history and much experimental support (see my reviews [11, 12]) and can be regarded from various points of view. As K. Rajewsky has pointed out to me, it could be no more than a form of mopping up, needed only to prevent inescapable network interactions getting out of control. Or, as I. R. Cohen proposes, self-macromolecules such as heat-shock proteins or myelin basic protein could induce a positive response within the immune system, which would normally be contained by anti-idiotypic suppression, but which would on occasion break out in the form of autoimmune disease. As regards lymphokines, the evidence in the mouse is convincing of mutual inhibition mediated by γ-interferon produced by TH1 cells, and interleukin (IL)-4 and IL-10 produced by TH2 cells. In man the position is less clear; perhaps atopy and its control by therapeutic vaccination may offer the best example of inhibitory T cell activity [13].

Now that we have this classification, are we yet in a position to assign any of the known immunoinhibitory effects to their correct slot within it? For the effects which matter in human disease, of the type shown in Fig. 2, the answer is, not yet. For mouse models some assignments can be made to the category of positive selection and others to that of negative selection. V-gene usage provides an important clue to the operation of negative selection, and enhancement of the response by anti-class II antibody does likewise for positive selection. But it must be emphasized that assignments made on these bases are only provisional, because it is always hard to exclude the possibility that some additional mechanism is operating.

This discussion has focussed on ways of carving up CD4 class II-restricted T cells, the main regulatory compartment of the immune system and arguably the single most numerous and most important group of lymphocytes. They can be subdivided according to restriction element, involvement in the network, lymphokine secretion profile, and positive versus negative effect (and also according to markers such as CD45R which discriminate between naive and memory cells). Some of these are lineage markers while others are not, and one needs to use ones wits when mixing the two [14]. It seems to me that the alignment of these various characteristics is one of the most important items on the agenda of cellular immunology.

Why Are Immunoinhibitory Genes so Frequent?

It is hard to believe that autoimmune disease occurs with sufficient frequency, or in a young enough age group, to have had much evolutionary impact. As we have argued elsewhere [15], the driving force is more likely to have been the hypersensitivity induced by chronic infection. Most or all of the major tropical diseases are associated with hypersensi-

tivity, and nowhere is this more conspicuous than in leprosy. In that disease immunopathological mechanisms are most threatening in borderline cases. It is as though individuals at either end of the spectrum are protected: at the tuberculoid pole (and in the much larger number of individuals who are infected but never show clinical symptoms) the immune system functions in its usual protective mode, while at the lepromatous pole its protective functions are inhibited and the parasite becomes free to multiply. Immunoinhibitory genes may thus occur in human populations in the developed world largely as a result of past selection for inhibition of infection-associated hypersensitivity.

The association noted above between immunoinhibitory activity of MHC genes, low expression, and low polymorphism now begins to make sense. The predominant activity of MHC genes is positive where they function as immune response genes. Such genes are driven to become intensely polymorphic, as a result of what the evolutionary biologists have come to call "the Red Queen strategy." By this is meant that any one species lives within an environment provided by other species, and as one evolves so must the others. The final result is a great deal of evolutionary change but little real progress, just as in Lewis Carrol's *Through the Looking Glass* where Alice and the Red Queen hold hands and run, without getting anywhere. Nowhere does this apply with greater force than in the coevolution of immune response genes in the host and the antigen genes in parasites to which they are opposed. This ceases to apply to MHC genes in respect of their inhibitory function. In that case the interests of the host and the parasite coincide; the Red Queen, so to speak, comes out of play. We can therefore expect immunoinhibition to associate with diminished polymorphism. The association with diminished expression may occur because, on balance, such genes prove less valuable in an evolutionary sense; they may even be on their way to total elimination from the MHC gene pool. It is tempting to suppose that reduced class II expression may provide a mechanistic signal for suppression within the immune system, thus closing the evolutionary circle.

All this is of course highly speculative. The value of the evolutionary arguments is that they focus attention on particular mechanisms, and also that they identify the need for particular types of immunoepidemiological data.

New Therapies: Combatting or Enhancing Immunoinhibition

The proof of these ideas about inhibitory activity is whether they lead on to new forms of therapy. In this context three lines of current research look particularly promising. The first two concern chronic infectious diseases in which immunoinhibitory activity has long been suspected of preventing recovery, and where a novel form of therapy offers hope of breaking through that barrier. The third concerns the opposite problem, autoimmune disease in which the lack of adequate immunoinhibitory activity may help cause the disease, and where a novel form of therapy might rectify that defect.

This year a group from the Rockefeller University collaborating with local researchers in Addis Ababa published their results on sublesional administration of IL-2 in leprosy [16]. This is the first trial of lymphokine treatment in a chronic infectious disease, and it used the lymphokine at something approaching physiological concentrations (far less than has been used in cancer trials). The results were encouraging, as judged by the histological response determined in skin biopsies, and treatment with other lymphokines is planned. From the point of view expression above, treatment of this sort carries great promise as well as some hazard. If patients are to be shifted along the spectrum towards the tuberculoid pole, it is essential that they be moved out of the intermediate zone of hypersensitiv-

ity and not into it; that will require careful patient selection. While these results do not provide direct support for the TH1-TH2 concept, they are at least compatible with it.

Last year there appeared a full report on the treatment of cutaneous leishmaniasis by immunotherapy in the form of vaccination with bacille Calmette-Guérin (BCG) plus killed leishmania organisms [17], a form of treatment that has also been applied in leprosy. Results as good as those of conventional chemotherapy were obtained. That reports includes a detailed and thoughtful discussion of the possible mode of action; once again, while many other possibilities remain open, an interpretation in terms of competing lymphokines seems attractive.

The third attractive line of research is gene therapy. After long debate and much hesitation, we are about to witness the first gene therapy trials in man, starting probably with cancer and with congenital enzyme deficiencies. A strong case can be made for following these with trials in the hemoglobinopathies. If all goes well, it would seem reasonable to consider such therapy for cases of autoimmunity which have proved refractory to other forms of treatment. The type of gene that one would wish to implant are those shown in Fig. 2, or possibly an asp-57 HLA-DQ if the doubts mentioned above can be resolved. I am well aware of the difficulties: how to protect an implanted allogeneic major transplantation antigen, for example; and, for those genes which operate their inhibitory effect through positive selection, how to obtain expression in thymic epithelium. But with the technologies that are becoming available these obstacles do not seem insuperable. Now many be the time to start a serious research effort towards that goal.

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Frederick Stohlman Jr. Memorial Lecture

Wilsede, June 21, 1978

Moloney, William C.: Memorial Tribute to Dr. Frederick Stohlman

Gallo, Robert C.: Cellular and Virological Studies Directed to the Pathogenesis of the Human Myelogenous Leukemias

Pinkel, Donald: Treatment of Childhood Acute Lymphocytic Leukemia

Wilsede, June 18, 1980

Klein, George: The Relative Role of Viral Transformation and Specific Cytogenetic Changes in the Development of Murine and Human Lymphomas

Kaplan, Henry S.: On the Biology and Immunology of Hodgkin's Disease

Wilsede, June 21, 1982

Greaves, Melvyn, F: Immunobiology of Lymphoid Malignancy

Thomas, E. Donnall: Bone Marrow Transplantation in Leukemia

Wilsede, June 17, 1984

Klein, Jan:
Introduction for N. Avrion Mitchison

Mitchison, N. Avrion: Repertoire purging by medium-concentration self-macromolecules is the major factor determining helper and suppressor repertoire differences

Wilsede, June 18, 1984

Gallo, Robert C.:
Introduction for Peter H. Duesberg

Duesberg, Peter H.: Are Activated Proto-*Onc* Genes Cancer Genes?

Wilsede, June 22, 1986

Vogt, Peter K.:
Introduction for Harald zur Hausen

Harald zur Hausen:
Viruses in Human Tumors

Moore, Malcolm A. S.:
Introduction for Donald Metcalf

Metcalf, Donald: Hemopoietic Growth Factors and Oncogenes in Myeloid Leukemia Development

Wilsede, June 19, 1988

Rowley, Janet D.: Molecular Analysis of Rearrangements in Philadelphia (Phl) Chromosome-Positive Leukemia

Moore, Malcolm A. S.: Interactions Between Hematopoietic Crowth Factors: The Clinical Role of Combination Biotherapy

Wilsede, June 17, 1990

Broder, Samuel: Clinical Research Using 3'-Azido-2',3'-Dideoxythymydine (AZT) and Related Dideoxynucleosides in the Therapy of AIDS

Grosveld, Frank: The Human β-Globin Locus Control Region

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Frederick Stohlman Jr. Memorial Lecture

Clinical Research Using 3'-Azido-2',3'-Dideoxythymidine (AZT) and Related Dideoxynucleosides in the Therapy of AIDS

S. Broder [1]

Introduction

Pathogenic retroviruses play an etiologic role in the acquired immune deficiency syndrome (AIDS) and its related disorders. While no cure for diseases caused by these agents is available, we are now in an era in which therapy against pathogenic retroviruses is a practical reality. Therapies against the etiologic agent of AIDS [1–3] were made possible by the discovery that a retrovirus, now called the human immunodeficiency virus (HIV), caused the disorder [4–6]. This discovery and the ability to grow the virus in large quantities enabled the development of in vitro techniques to find drugs that inhibit the replication of HIV [7, 8]. Substances that acted against HIV in vitro could then be identified for further research, and the orderly development of drugs thus proceeded. The work was in part a outgrowth of early research on animal retroviral systems in a number of laboratories [9–12]. No one person or group can take full credit for these discovieries, and a great debt is owed to many scientists who pioneered this research. More recently, our group and other groups have observed that certain members of a class of compounds called dideoxynucleosides are potent inhibitors in vitro of the replication of HIV in human T cells [8, 13–26]. In all the compounds, the hydroxy (–OH) group in the 3'-position on the sugar ring is replaced by a hydrogen atom (–H) or another group that cannot form phosphodiester linkages.

3'-Azido-2',3'-dideoxythymidine (also called zidovudine, 3'-azido-3'-deoxythymidine, azidothymidine, or AZT), the first of these compounds to be tested clinically, reduced the morbidity and mortality associated with severe HIV infection [27, 28]. Volberding et al. [29] reported that AZT is effective in delaying progression to fulminant AIDS in asymptomatic patients infected with HIV. Other dideoxynucleosides are now in various stages of clinical testing. Other substances that act at various stages of HIV's replicative cycle also have been shown to block replication in vitro, and some are undergoing clinical testing [7, 31–59]. This article reviews certain clinical applications of one antiretroviral agent, AZT, and discusses the status of several related compounds. It also addresses other approaches to antiretroviral treatment. While an ultimate cure for AIDS will require further basic research, the knowledge already at hand might make a major impact against the death and suffering from this disease in the coming decade. During the decade of the nineties, AIDS is expected to increase, and the disease is likely to become a major cause of death in men, women, and children. In some parts of the world, infant and child mortality could be as much as 30% greater than what one would have expected [60].

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Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Potential Mechanisms of Action Against HIV

Although there are substantial diffferences among the dideoxynucleosides, we can make several general comments about their mechanisms of action. Each drug likely inhibits reverse transcriptase but must first be activated to a 5′-triphosphate form by various enzymes of a target cell [61–65]. It is the triphosphate form that is active against HIV [61, 66]. The activation process, called anabolic phosphorylation, involves a series of enzymes (kinases) [61–65, 67]. AZT and other dideoxynucleosides, as triphosphates, exert their antiretroviral activity at the level of reverse transcriptase (viral DNA polymerase) [12, 61, 66, 68, 69]. Reverse transcriptase is essential in the replicative cycle of HIV. A great deal is now known about the overall structure of the polymerase domain of HIV reverse transcriptase, about the active site, and about the secondary structure within the active site (e.g., see [69]. When HIV enters a target cell, this enzyme makes a complementary strand DNA copy of the viral genomic RNA and then catalyzes the production of a second, positive strand DNA copy. The genetic information of HIV is thus encoded in a double-stranded form of DNA. Two mechanisms may contribute to the effect of AZT and other dideoxynucleosides on reverse transcriptase. First, as triphosphates, they compete with the cellular deoxynucleoside-5′-triphosphates that are essential substrates for the formation of proviral DNA by reverse transcriptase with inhibition constants generally in the range of 0.005–0.2 μ*M* [61, 70–73]. These concentrations can be attained in cells exposed to the drugs [61–65]. Second, such 5′-triphosphatases act as chain terminators in the synthesis of proviral DNA. Because of the 3′ modification of these compounds, once viral reverse transcriptase adds them to a growing chain of viral DNA, the DNA is elongated by exactly one residue and then terminated [66]. In contrast to HIV reverse transcriptase, mammalian DNA polymerase alpha is relatively resistant to the effects of these drugs (inhibition constants, 100–230 μ*M*) [12, 61, 63, 70], which is one reason for their selective antiretroviral activity in cells that can phosphorylate them. However, mammalian DNA polymerase gamma, found in mitochondria, and DNA polymerase beta are also sensitive to these compounds (inhibition constants, 0.016–0.4 μ*M* and 2.6–70 μ*M*, respectively), and this may be a basis for drug toxicity [12, 61, 63, 70]. It should always be borne in mind that additional mechanisms of activity and toxicity might be at work. It is possible that AZT works through an as yet unidentified intermediate.

AZT and related dideoxynucleosides have activity against certain retroviruses, including HIV type 2, human T-cell lymphotropic virus type I, animal lentiviruses, and murine retroviruses [9–12, 68, 74–78]. (Some congeners also have in vitro activity in an animal model of hepatitis B virus [79] that, although a DNA virus, replicates through an RNA intermediate using a reverse transcriptase-like DNA polymerase [80].) This conservation of activity suggests that the sensitivity of reverse transcriptase to these agents (as triphosphates) is linked to an essential feature of the viral enzyme. Nevertheless, two groups recently reported that certain isolates of HIV from patients who had taken AZT for 6 months or longer had reduced sensitivity to AZT in vitro [81, 82]. Some of the known mutations would be likely to affect the charge or alpha helix content of the catalytic site, or at least the probable catalytic site, of reverse transcriptase. Larder et al. [83] previously showed that the induction in vitro of certain mutations in reverse transcriptase by site-directed mutagenesis could make the enzyme less sensitive to inhibition of AZT triphosphate and phosphonoformate (but also reduce enzymatic activity). However, HIV isolated from patients receiving long-term AZT therapy retains its in vitro sensitivity to inhibition by

most other dideoxynucleosides (such as 2′,3′-dideoxycytidine) and phosphonoformate [82]. Also, preliminary studies suggest that the sensitivity of the reverse transcriptase obtained from these resistant viral isolates to 3′-azido-2′,3′-dideoxythymidine-5′-triphosphate (AZT-TP) did not change [82].

These preliminary findings suggest that while changes in reverse transcriptase may account for the loss of sensitivity to AZT, additional studies are needed to clarify these issues. Many clinical investigators believe that the emergence of AZT-insensitive isolates is a marker of impending clinical progression, but the clinical importance of the reduced viral sensitivity to AZT is not fully known. The potential problem, however, underscores the urgency for additional experimental therapeutic agents and for regimens that employ multiple agents.

Biochemical Pharmacology of AZT

Unlike most nucleosides that enter cells by specialized transport systems, AZT can enter mammalian cells by passive, nonfacilitated diffusion [84]. Once inside, the drug is phosphorylated to a triphosphate form by a series of kinases that usually phosphorylate thymidine [51]. 3′-Azido-2′,3′-dideoxythymidine-5′-monophosphate (AZT-MP) is first produced by thymidine kinase, and two additional phosphates are then added by the sequential action of thymidylate kinase and nucleotide diphosphate kinase to form 3′-azido-2′,3′-dideoxythymidine-5′-triphosphate (AZT-TP), the active moiety [61, 72, 73]. The addition of a second phosphate group to AZT-MP by thymidylate kinase is probably the rate-limiting step for this process [61]. The reaction occurs much more slowly (relative maximal velocity, 0.3%) than the phosphorylation of thymidine-5′-monophosphate, the usual substrate for this enzyme [61], and human cells exposed to AZT accumulate relatively high levels of AZT-MP, but low levels of AZT-TP [61]. AZT-MP binds efficiently to thymidylate kinase, but comes off slowly, thus tying up the enzyme. In certain T-cell lines exposed to very high concentrations of AZT (50–200 μM), decreased phosphorylation of thymidine and decreased levels of thymidine-5′-triphosphate (the normal DNA building block that competes with AZT-TP for reverse transcriptase) have been reported [61, 85]. This may result from the inhibition of thymidylate kinase by AZT-MP (inhibition constant, 8.6 μM) [61]. However, decreased concentrations of thymidine-5′-triphosphate have not been found in other cell lines or with lower concentrations of AZT [85, 86], and whether thymidine kinase inhibition contributes to the bone marrow toxicity induced by AZT is uncertain. Furman et al. [61] originally reported that the levels of deoxycytidine-5′-triphosphate, another building block of DNA, fell in the presence of AZT, but later they concluded that the finding was due to a technical error [87]. It should be stressed that murine cells handle AZT in a very different way from human cells.

Pharmacokinetics of AZT

In culture, AZT inhibits new HIV infection of lymphocytes at levels of 1–5 μM (even under conditions of high multiplicity of infection) [8]. The initial pharmacokinetic studies demonstrated that AZT absorbed well orally (average oral bioavailability, 63%) and that peak plasma levels of 3–4 μM are attained 30–90 min after an oral dose of 200 mg is ingested [27, 88, 89]. The peak plasma concentration is proportional to the amount administered, over a wide range of doses [89]. The serum half-life is only 1.1 h [27, 89, 90]. For this reason, a dosing every 4 h was chosen for phase II testing in patients with advanced disease and then adopted as the recommended schedule. It should be stressed, however, that there is not yet enough information to determine the optimal dose or dosing schedules for AZT. Less frequent dosing

schedules and total daily doses work, but further research is needed. It is likely that total doses of approximately 600 mg per day using an interval of eight hours between doses would be adequate in most adults. The levels of intracellular AZT-TP (the activity moiety of AZT) have a half-life of about 3 h [61]. Thus, effective anti-HIV activity may theoretically be attained with an oral dosing interval of 8 or 12 h. Alternatively, for the optimal therapeutic benefit, it may be necessary to maintain constant plasma drug levels. Resolving these issues will require careful controlled trials.

Approximately 15%–20% of an administered dose of AZT is excreted unchanged in the urine, and 75% is metabolized by hepatic glucuronidation to form 3′-azido-2′,3′-dideoxy-5′-glucuronylthymidine, an apparently inert metabolite that is also excreted into the urine [90]. The enzyme or enzymes responsible for the glucuronidation of AZT may be inhibited by other compounds that share this pathway, and such compounds may prolong the half-life of the drug [91]. In this regard, probenecid inhibits both hepatic glucuronidation and renal excretion and thus reduces the total body clearance of AZT by 65%. Other drugs that undergo hepatic glucuronidation and may, theoretically, inhibit the metabolism of AZT include nonsteroidal anti-inflammatory agents, narcotic analgesics, and sulfonamide antibiotics. Until the interactions of such drugs with AZT have been carefully investigated, clinicians should be aware that they may affect the metabolism of AZT, and AZT may affect theirs. Finally, the metabolism of AZT could be slower in patients with severe hepatic disease. It should be stressed that different nucleoside analogs will show individual pharmacokinetic profiles [27–30, 32, 63, 89–98].

Because HIV can infect cells in the central nervous system and cause dementia, antiretroviral agents used in the treatment of AIDS should be capable of penetrating the brain. Three to four hours after a dose of AZT has been administered, levels in cerebrospinal fluid are approximately 60% of those in plasma (range, 10%–156%) [27, 38, 89, 99], which indicates that AZT can enter the brain by diffusion from the cerebrospinal fluid or possibly through capillaries in the brain. The clinical improvement that occurs in patients with HIV dementia who are given AZT suggests that the drug reaches the site of viral replication in the central nervous system [28, 99]. However, AZT may not cross the blood-brain barrier in all species [100].

Evidence suggests that cells belonging to the monocyte-macrophage series are the most important target cells of HIV infection in the brain [101, 102]. These nonproliferating cells have lower levels of kinases than lymphocytes [103, 104] and dideoxynucleosides may not be efficiently phosphorylated in them. One study indicated that AZT is poorly phosphorylated in peripheral blood monocytes and macrophages and does not protect these cells against infection by the lymphadenopathy-associated virus strain of HIV in vitro [104]. This study is not correct. Two subsequent studies demonstrated that low concentrations of AZT and other dideoxynucleosides profoundly inhibited the replication of a monocytotropic strain of HIV in monocytes and macrophages [105, 106]. These results are consistent with the observation that dementia induced by HIV may be at least temporarily reversed by AZT [21, 99, 107]. The potent activity of AZT in monocytes and macrophages can perhaps be explained by the observation that such cells have very low levels of thymidine-5′-triphosphate, the normal nucleotide that competes with AZT-TP at the level of reverse transcriptase [105]. Thus, the ratio of AZT-TP to thymidine-5′-triphosphate may actually be higher in monocytes than it is in T cells [105]. Additional studies indicate that granulocyte-monocyte colony-stimulating factor, which stimulates the replication of HIV in monocytes [108, 109], increases the entry of AZT into these cells

and potentiates its activity against HIV [109]. However, it is important to use caution in extrapolating these data to clinical applications.

Clinical Application of AZT

In the initial clinical studies of AZT at the National Cancer Institute and Duke University Medical Center, patients with AIDS or AIDS-related complex had immunologic, virologic, and clinical improvement during 6 weeks of therapy [27, 88]. (Three of the 19 patients from the original phase I study, each of whom had AIDS-related complex or Kaposi's sarcoma when they entered the trial, were alive 3.5 years after the initiation of therapy.) Also, several patients with HIV dementia who were given AZT had substantial improvement in their intellectual function, accompanied in some by a normalization in the pattern of use of cerebral glucose (as assessed by positron emission tomography) [99, 100]. On the basis of these results, Wellcome Research Laboratories began a multicenter, randomized, controlled trial of AZT in February 1986 among 282 patients with AIDS (after their first episode of *Pneumocystis carinii* pneumonia) or severe AIDS-related complex [29, 111]. The trial demonstrated a reduced mortality in the patients receiving AZT. By September 1986, 19 patients taking placebo but only one taking the drug had died [28]. After 36 weeks, 39.3% of those taking placebo had died compared with 6.2% of those taking AZT, and after 52 weeks the cumulative mortality in the patients treated with AZT was still only 10.3% (no comparable figure is available for the placebo group, because most were given AZT after September 1986, thus ending the control arm) [28]. Suppressive prophylaxis for *Pneumocystis* pneumonia was not a formal protocol option in the 7-month randomized trial, although a few patients randomly distributed between the two arms received it. Also, 19 of the 144 originally assigned to AZT received more than 6 weeks of such prophylaxis during the 52 weeks after their entry into the study. Eliminating these patients from the analysis does not affect the basis conclusion of the trial. Subsequent studies have suggested that patients with AIDS who receive AZT in conjunction with prophylactic therapy for *Pneumocystis* pneumonia may have a lower mortality than those who receive AZT alone [112]. However, it is important to stress that the precise role of *Pneumocystis* pneumonia prophylaxis in patients who are receiving antiretroviral therapy has not been defined.

The phase II study also showed that patients receiving AZT had a temporary increase in their $CD4^+$ lymphocyte counts (average, $80/mm^3$), fewer opportunistic infections, and an average weight gain of about 0.5 kg, as compared with those receiving placebo [28]. Furthermore, the results confirmed an observation in the phase I study; some patients with cognitive dysfunction induced by HIV improved when given AZT [99, 107]. Finally, patients who received AZT had a decreased viral load as compared with the placebo group in assessments made by measuring levels of serum HIV p 24 antigen [113]. The ability to isolate HIV from cultured lymphocytes, however, was not affected, although there was a delay in the appearance of HIV in cultures [114]. Although serum HIV p 24 antigen levels are an experimental clinical measure of HIV replication, they may be affected by antibodies to p 24 and other factors. Better methods of assessing viral load are urgently needed. The polymerase chain reaction will likely prove useful in this regard [115]. On the basis of this trial's results, AZT was approved for the treatment of severe HIV infection in most countries. In the United States, it was approved in March 1987 for patients who have had *P. carinii* pneumonia or whose CD4+ cell count is below $200/mm^3$. Evidence from the New York State Department of Health indicates that the survival of patients given a diagnosis of AIDS in 1987 increased substantially

over that of patients whose disease was diagnosed in previous years. It is exceedingly likely that the widespread use of AZT contributed to this trend [116]. The dose used in the phase II study is now known to be higher than is necessary for optimal effects.

In both the phase I and phase II trials, it became apparent that the increase in the number of CD4+ lymphocytes induced by AZT may be transient [27, 29, 117], particularly in patients with fulminant AIDS, whose CD4 count often returns to baseline after 16–20 weeks of therapy [28]. In this trend, the contributions of direct drug toxicity, altered host defense mechanisms, and changes in viral sensitivity to AZT are unclear. It is highly probable that no antiretroviral agent will work to maximal advantage if the host immune response is severely damaged. Some patients have had late increases in levels of HIV p 24 antigen even while receiving a constant dose of AZT [118]. In addition, AZT has a number of toxic effects; the most frequent is suppression of bone marrow cells, and anemia is its most frequent manifestation [27, 28, 111, 117]. An increase in the mean corpuscular volume of erythrocytes often occurs before frank anemia [27, 111], but the dose should not be modified on the basis of this measure. Patients receiving AZT may have megaloblastic changes in bone marrow, maturational arrest of erythrocyte lineage, or hypoplastic (rarely aplastic) changes. Hypoplastic changes can occur without an increase in mean corpuscular volume [119, 120]. Bone marrow toxicity occurs more frequently in patients with established AIDS, and in the phase II study, 45% of the patients who had *P. carinii* pneumonia required transfusions or a reduction in dose during the first 6 months of AZT therapy [111]. In a subsequent open trial, only 21% of the patients with AIDS could complete 6 months of full-dose AZT treatment without a reduction in dose or the interruption of therapy [121]. Marrow toxicity is also more frequent in patients with underlying anemia, low CD4+ cell counts, or low (or low-normal) serum folic acid or vitamin B_{12} levels before therapy begins [27, 67, 111]. Pending further study, vitamin-replacement therapy may be useful in patients with low levels of these vitamins. The platelet count is generally spared until late in the course of AZT therapy. In fact, the drug can actually induce increased platelet counts in patients with thrombocytopenia induced by HIV [122, 123].

Other toxic effects of AZT include nausea, vomiting, myalgias, myositis (particularly in patients who receive the drug for more than a year), headaches, abnormalities of liver function, and bluish nail pigmentation [27, 111, 124–126]. Very high doses can cause anxiety, confusion, and tremulousness [27, 117, 127]. These symptoms occasionally develop in patients receiving the current recommended dosage. Finally, a few patients have had seizure, an encephalopathy similar to Wernicke's or Stevens-Johnson syndrome [128–130]. Thus, although AZT decreases morbidity and mortality among patients with severe HIV infection, its use can be associated with substantial toxicity, particularly in those with advanced disease.

Slightly more than 2 years after AZT was first observed to inhibit the replication of HIV in vitro, it was approved by the Food and Drug Administration for the treatment of AIDS. Because of this extraordinarily rapid development, a number of questions regarding its use remain unanswered. In AIDS, as in perhaps no other condition, the line between approved and experimental therapy is difficult to draw.

Physicians frequently ask whether AZT should be administered early in the course of HIV infection. The drug appears to be relatively well tolerated at this stage [131], and recent results of a randomized trial have indicated that HIV-seropositive patients with less than 500 CD4 cells/mm^3 who were given AZT had less frequent progression to severe AIDS-related complex or AIDS than those given placebo [29]. As we learn

more about the factors that make a progression to AIDS highly likely (e.g., high serum HIV p 24 antigen levels or β_2-microglobulinemia) [132, 133], it may be possible to target AZT therapy to patients who are at high risk. At present, we cannot say that starting AZT early in an asymptomatic phase of HIV infection provides a survival advantage over waiting until more symptomatic disease supervienes.

In considering early intervention with AZT, it is of particular concern that the drug may be carcinogenic or mutagenic [134]. Its long-term effects are unknown. Rodents exposed to high doses of AZT for long periods can develop vaginal neoplasms (principally nonmetastasizing squamous cell carcinomas). Male rodents did not develop tumors. The implications of these sex- and site-specific tumorigenic effects in rodents are not clear at this time, but the results provide a warning against complacency when using this class of drug. It is worth emphasizing again that AIDS itself makes the development of certain cancers more likely, and AZT may be associated with the higher incidence of cancers in patients whose immunosurveillance mechanisms are disturbed, simply because it increases their longevity. This has occurred in certain immunodeficiency disorders of childhood, in which advances in the treatment of infections have allowed patients to survive longer [135]. Lymphomas have developed in a significant subset of the original phase I patients between 1 and 3 years after AZT therapy began.

The use of AZT in children with AIDS is an area that is only now being investigated. The high incidence in certain cities of cord blood samples that are seropositive for HIV (for example, 1 in 80 newborns in New York City are seropositive [116, 136]) indicates that the number of children with AIDS will grow in the near future. AIDS is dramatically altering the landscape of obstetrical and pediatric care in many countries. The manifestations of HIV infection in children can differ from those in adults. Neurologic dysfunction and high-grade bacterial infections are much more evident, for example [137], and the patterns of drug toxicity may differ. Ongoing studies at the National Cancer Institute suggest that administration of AZT by continuous intravenous infusion can reverse certain neurologic symptoms associated with HIV in children with AIDS [138]. In some patients, the intelligence quotients returned to what they had been before the disease developed [138]. However, the problems of bone marrow suppression limit that treatment. In both adults and children who have dementia associated with AIDS, considerable bone marrow suppression may be tolerable if antiretroviral therapy can reverse major neurologic deficits. Another unresolved issue is whether AZT can prevent new HIV infection if it is given at the time of viral exposure. Kittens can be protected against feline leukemia virus (a retrovirus) by the administration of AZT at the time of infection [78]. Also, fetal mice can be protected against retroviral infection by the administration of AZT to their mothers [139]. A short course of AZT at the time of exposure (e.g., after a serious needle-stick or laboratory accident) may therefore be useful. However, because it is mutagenic (and carcinogenic in rodents) and can induce chromosomal abnormalities [134], its use in such a setting cannot be recommended except in an approved protocol.

Finally, even in patients for whom AZT is recommended, there is much to learn. The available evidence suggests but does not prove that patients should continue to receive therapeutic doses for as long as they can tolerate the drug. AZT was determined to be effective because it lowered the risk of opportunistic infections and prolonged life. The late decline in the CD4+ cell counts is thus not an indication to stop therapy. However, it is not yet clear how patients who have hematologic toxicity, evidence of clinical progression, or increased levels of serum HIV p 24 antigen while receiving AZT should be managed. Certain factors that

stimulate bone marrow, such as erythropoietin, granulocyte-stimulating factors, or granulocyte-monocyte colony-stimulating factors [109, 110], may reduce the suppression of bone marrow associated with AZT, and these approaches need further research in an academic center.

Biochemical Pharmacology of Other Antiretroviral Dideoxynucleosides Including 2′,3′-Dideoxyinosine (Didanosine)

As noted, a number of dideoxynucleosides other than AZT have antiretroviral activity in vitro [13–26], and several studies of such agents are now enrolling patients. There are substantial differences in the rate at which human cells phosphorylate these compounds and in their enzymatic pathways. These differences are profoundly important to their antiretroviral activities. 2′,3′-Dideoxycytidine (ddC) is, for example much more potent than 2′,3′-dideoxythymidine (ddT) in most human cells because of differences in its phosphorylation [31, 61–65, 67]. Since their rates of phosphorylation differ between species [68], one cannot draw conclusions about their activities in human cells on the basis of their performance in animal cells. 2′,3′-Dideoxyadenosine (ddA), 2′,3′-dideoxyinosine (ddI), and ddC are three compounds with potent activity against HIV in human T cells and monocytes in vitro under study [13, 14, 105] in clinical trials. One of the most active dideoxynucleosides is ddC, a pyrimidine analog [13, 14]. Unlike many cytidine analogs, it is resistant to deamination by the ubiquitous enzyme cytidine deaminase [62, 63]. It is, therefore, stable in plasma and bioavailable after oral administration. After entering a cell, ddC is phosphorylated by a set of enzymes that usually phosphorylate deoxycytidine [62, 63, 141, 142]. Thus, ddC is activated by a different pathway than AZT. Also, ddC does not affect the levels of its competing dideoxynucleoside triphosphate, deoxycytidine-5′-triphosphate [14]. However, its anabolic phosphorylation and activity may be affected by other nucleosides (e.g. thymidine) [143]. ddC is exceptionally potent and the optimal dose to avoid neuropathy is still under study. Total doses of 2 mg per day or less are being tested in adults.

It is worth emphasizing that this entire class of nucleoside analogs represents a new area of clinical research. These drugs have antiretroviral activity; however, they also have considerable potential for side effects. These drugs should be administered only by physicians who are well versed in their properties.

ddA and its immediate metabolite ddI are purine analogs with in vitro activity against HIV [13] and, unlike AZT, relatively little toxicity against bone marrow precursor cells [144]. Within cells, ddA can be phosphorylated to its active 5′-triphosphate moiety [64]. It is also susceptible to deamination by adenosine deaminase and forms ddI [64]. In human plasma and cell extracts, this conversion occurs almost instantaneously [26, 64]. As mentioned, ddI has potent in vitro activity against HIV [13] because it can be metabolized in human cells to form ddA-5′-triphosphate through a complex series of reactions [65]. Interestingly, ddI uses the enzyme 5′-nucleotidase to undergo the initial phosphorylation it needs for activation and ultimate salvage back to ddATP. Thus, for many purposes the two drugs can be considered identical. Once ddA and ddI are converted to ddA-5′-triphosphate in cells, they remain there for a relatively long time – their intracellular half-life is more than 12 h [145]. Thus, even with their short plasma half-life, they may be clinically effective when administered relatively infrequently (e.g., every 8–24 h). Unlike AZT or ddC, ddA and ddI undergo solvolysis (cleavage) in acid reactions to form a purine base and dideoxyribose [64]. This may lower their capacity for oral absorption, and they must be used with antacids or buffers. High concentrations of the free purine base of ddA, adenine, have been reported

to cause renal damage [146]. The free base of ddI, hypoxanthine, does not have similar toxicity, and it may, therefore, be preferable for oral administration. Preliminary results from a phase I trial and ddI suggest that it is an active antiretroviral drug [9]. Some patients have now received this drug for more than 2 years. The major side effects to data have been a reversible peripheral neuropathy and acute pancreatitis. In some cases, the pancreatitis may be lethal. It appears that a prior history of pancreatic disease is risk factor for this complication. Significant diarrhea and hypokalemia may occur. These side effects seem to be dose related. Patients who have advanced disease or who are debilitated have an increased risk for toxicity. At doses less than 8 mg/kg per day, serious side effects are significantly less common than at higher doses. In an average adult, total doses should not exceed 500 mg per day. It is possible that even lower doses eventually will be found active. The use of alcohol is contraindicated in patients receiving ddI due to the possibility of pancreatitis.

Certain analogs of ddA (e.g., 2′,3′-dideoxy-2′-fluoro-ara-adenosine) are resistant to acid hydrolysis [21] and, therefore, may have better bioavailability than ddA. Also, certain 2-halogen-substituted forms of ddA are resistant to deamination [26], and they may be directly phosphorylated and not follow the ddI pathway. Whether clinical studies with such forms will produce compounds superior to ddA or ddI is not known; ddI has potent in vitro activity against HIV in its own right [13], and the issue of acid instability can be addressed by simple measures such as the buffering of gastric secretion.

Like AZT, a number of analogs of ddT have been tested for activity against HIV in vitro. Many were inactive, but a few blocked the replication of HIV in human T cells [14, 16, 17, 22, 147]. An unsaturated form of ddT called 2′,3′-didehydro-2′,3′-dideoxythymidine is about as active as AZT on a molar basis [16, 17]. Unlike AZT, it does not affect the activity of thymidylate kinase [148], whether it induces bone marrow toxicity remains undetermined. The dose-limiting toxicity is peripheral neuropathy. Finally, a 3′-substituted uridine analog, 3′-azido-2′,3′-deoxyuridine, which appears to be activated by the same enzymes that phosphorylate AZT, has some anti-HIV activity in vitro [19, 149]. All these dideoxynucleosides have an intact oxacyclopentane (sugar) ring. However, several acyclic compounds (adenallene, cytallene, and a phosphonyl-methylethyl purine derivative) also have activity against HIV in vitro as single agents. Such compounds provide new relation between structure and activity and may be of value in developing a new class of anti-HIV agents.

Clinical Research with Dideoxycytidine

Although AZT can prolong the lives of patients with AIDS, there are some limitations to its use. The hematologic toxicity of AZT is not inextricably linked to its antiviral effect, and we can expect that other agents will be worth exploring or have different patterns of toxicity. In vitro testing and studies of animal toxicology can provide clues as to which drugs are likely to have favorable therapeutic results. Ultimately, however, the issues can be resolved only by testing in patients, and an effort is now under way to test several of these agents in patients with AIDS or related conditions. The first to be studied clinically (after AZT) was ddC, which has potent activity against HIV in vitro at concentrations of 0.01–0.5 μM, depending on the viral dose used in the assay system [13, 69, 70]. It is well absorbed when given orally, and peak levels of 0.1–0.2 μM can be attained after the oral administration of 0.03 mg/kg body weight [30]. Like AZT, ddC has a half-life of slightly more than 1 h. It differs from AZT in that it is excreted by the kidneys [30, 150]. Finally, ddC penetrates at least partially the cerebrospinal fluid [30, 150].

Both the initial study of ddC [30] and a subsequent trial [151] found evidence of clinical activity against HIV. Nearly all the patients who received daily doses of between 0.06 and 0.54 mg/kg had decreased levels of serum HIV p24 antigen [30, 151], and most had small increases in the number of CD4+ cells by week 2 [30]. Furthermore, some had an increase in antigen-induced T-cell proliferation in vitro [30]. The decrease in levels of p24 antigen persisted in some patients for at least several weeks after the drug was withdrawn. In others, however, the immunologic and virologic values moved toward baseline after several weeks despite the continued administration of ddC [30]. One purpose of these studies was to define the dose-limiting toxic effects of ddC. In a number of patients, particularly those receiving higher doses, maculovesicular cutaneous eruptions, aphthous oral ulcerations, fever, and malaise developed after 1–4 weeks of therapy [30, 151, 152]. These symptoms usually resolved in 1–2 weeks even with continued therapy. However, after several months of continuous therapy with daily doses of 0.06 mg/kg or more, most patients had a painful sensory motor peripheral neuropathy (involving mainly the feet) that became the dose-limiting toxic effect [30, 151]. This neuropathy appeared earlier, was more severe, and lasted longer when the highest doses were tested; some patients receiving the highest doses still had persistent, moderate sensory loss and pain a year after the drug was discontinued [151, 153]. Neurotoxicity resolved much more quickly, however, in patients receiving lower doses [30, 151, 153]. One metabolic product of ddC in human cells is dideoxycytidine diphosphate choline [62], which could conceivably contribute to the neuropathy. Alternatively, the neuropathy may result from an inhibitory effect of ddC-5′-triphosphate on mitochondrial DNA polymerase gamma (inhibition constant, 0.16 μM) [63, 154]. Thus, a search for ddC congeners which would not affect mitochondrial DNA synthesis is under way. Scientists at Hoffmann-La Roche have begun studying a fluorinated version of ddC which may spare mitochondrial DNA polymerase.

A continuation of the study of Merigan et al. [151] and a separate study organized by M. Gottlieb and W. Soo (personal communication) have shown that many patients can tolerate lower doses of ddC (0.03 mg/kg per day) for 6 months or more; mild, readily reversible neuropathy developed in a minority of patients. This dose of ddC was associated with a decline in HIV p24 antigen levels and an increase in the number of CD4+ lymphocytes in most patients. Since the toxicity of ddC is strikingly different from that of AZT, combining the two agents may reduce overall toxicity. To test this approach, a group of patients with AIDS or AIDS-related complex followed a regimen alternating AZT (200 mg every 4 h) and ddC (0.09 or 0.18 mg/kg per day) therapy in 7-day periods [30, 155]. It was hoped that neuropathy would not occur or would occur later with the intermittent administration of ddC. Preliminary results suggest that the toxicity of both agents can be significantly reduced. Some patients have now tolerated the regimen for more than 36 months [155] (unpublished data). Overall, the patients had an average increase of more than 70 CD4+ cells/mm^3 at week 22, sustained decreases in serum p24 antigen levels, and a mean weight gain of 5 kg (not caused by fluid retention) [30, 155]. It is interesting to note that on low-dose or intermittent dosing regimens, once patients pass the 6-month mark without neuropathy, they may have a significant probability of avoiding serious neuropathy on continued administration of ddC. Next to AZT, ddC has been given to patients longer than any other dideoxynucleoside. It is probable that ddC will find its best use as part of a combination regimen with AZT.

Other Anti-HIV Agents in Preclinical and Clinical Development

This article has focused on the use of dideoxynucleosides as antiviral agents, in part because they are bioavailable after oral administration and because data from several studies support their virustatic activity in vitro. This is, however, by no means the only approach being investigated for the treatment of AIDS. The genome and replicative cycle of HIV are very complex, and several stages of replication may, therefore, be potential targets for antiretroviral therapy [31, 67, 156, 157]. Already, a number of agents that may act at various stages have been defined. Although an extensive review of these other approaches is beyond the scope of this article, a few points are worth stressing. Certain agents under study appear to act by inhibiting the initial binding of HIV to its CD4 glycoprotein receptor on target cells [36–38, 41, 42, 52–56, 158–160]. Using molecular biologic techniques, several groups recently reported truncated soluble forms of CD4 that lack the transmembrane and cytoplasmic domains [52–56]. At concentrations of 2–20 μg/ml, these forms inhibited the binding of HIV to T cells, the formation of syncytia, and the infection of T cells [52–56]. A potential advantage of this approach is that soluble CD4 is likely to inhibit, to some degree, all forms of HIV that use CD4 as the cell receptor. Also, agents that act at the cell surface may block cell death induced by syncytia, which can occur even when the target cell is not infected by HIV [38, 161, 162]. Phase I trials of recombinant CD4 are now under way. Second-generation versions of CD4 (such as CD4-immunoglobulin hybrid proteins) retain their activity against HIV in vitro, but may gain other desirable properties, such as a longer circulating half-life [163]. A phase I trial of such CD4-immunoglobulin hybrids is now under way at the National Cancer Institute and at other academic centers. Also, forms of recombinant CD4 linked to *Pseudomonas* endotoxin or ricin selectivity kill cells expressing HIV envelope proteins in vitro [164, 165]. In patients, such agents might selectively kill cells that can replicate HIV without being killed by the virus (e.g., macrophages). Unfortunately, CD4 does not necessarily bind to the gp120 of primary isolates (as opposed to laboratory isolates) with high affinity. This may mean that very high doses of CD4 need to be used.

Recently, low-molecular-weight dextran sulfate (7000–8000) was found to inhibit the infectivity of T cells by HIV [36–38]. This polyanionic polysaccharide also appears to inhibit the initial binding step [38]. A phase I/II trial of orally administered dextran sulfate suggested that it had little toxicity but also little effect on the number of CD4+ cells or serum p24 antigen levels [166]. However, dextran sulfate has since been found to be very poorly absorbed when given orally, and studies of intravenous dextran sulfate are needed in order to assess this agent. Other molecules in this class are worth studying by parenteral administration.

Recent advances in our understanding of the biochemistry of HIV replication have made the testing of new approaches to therapy possible. For example, antisense phosphorothioate oligodeoxynucleotides, which can bind to specific segments of the HIV genome, have sequence-specific inhibitory effects that may result from the arrest of translation after its hybridization to messenger RNA [48]. Interestingly, such compounds may also inhibit the replication of HIV in a manner that is not sequence specific [47]. Alteration of the sugar moiety of viral glycoproteins (e.g., by inhibitors of trimming glucosidases) reduces the infectivity of the resulting viruses [57, 58]. In addition to dideoxynucleosides, other agents may act at the level of reverse transcriptase. In particular, phosphonoformate, a pyrophosphate analog with activity against herpesvirus, has activity against HIV in vitro. Several pilot trials suggest that this drug can reduce serum HIV p24 antigen levels in patients with

HIV infection [167, 168]. However, no reliable oral formulation is available, and this remains one drawback of this drug.

There is a growing interest in developing drugs that inhibit the protease of HIV. During the next few years, it is likely that several protease inhibitors will enter clinical trials.

Several agents that act at different stages of viral replication (e.g., interferon-α) have synergy with AZT in vitro [34, 36, 40] and this could theoretically result in better treatment in patients. Interferon-α may be particularly interesting in this regard, because it also has a direct antitumor effect against cutaneous Kaposi's sarcoma [169–172]. In a similar vein, the antiherpes drug acyclovir, which has little activity against HIV alone, can potentiate the anti-HIV activity of AZT in vitro [14, 31]. In a pilot clinical trial, patients with AIDS or AIDS-related complex tolerated these drugs together for 10–30 weeks [32]. A theoretical advantage of the regimen is that suppressing the replication of herpesvirus may secondarily reduce the replication of HIV since a product of herpesvirus, ICPO (infected-cell protein), can increase the initiation of HIV transcription [173, 174]. The possible suppression of human herpesvirus 6 (human B-cell lymphotropic virus), which can infect lymphoid cells [175], may also be relevant. (In a related fashion, adenovirus enzyme-immunoassay product can also amplify HIV transcription [173]. Certain dideoxynucleosides can inhibit the replication of adenovirus [176] and thus may conceivably reduce the replication of HIV.) Acyclovir has been reported to be at least additive with AZT in inhibiting the replication of Epstein-Barr virus, and it could theoretically benefit patients infected with both that virus and HIV [177–179]. Whether AZT and acyclovir together offer a therapeutic advantage over AZT alone is not yet clear. Only properly controlled clinical trials can answer this point.

Not all combinations of anti-HIV drugs have synergistic or even additive effects. For example, the nucleoside analog ribavirin inhibits the phosphorylation of AZT in vitro and blocks its activity against HIV [50]. Ribavirin, however, increases the phosphorylation of purine analogs such as ddA through complex mechanisms involving its ability to inhibit inosine monophosphate dehydrogenase [180]. Ribavirin can be given orally and may in theory potentiate the anti-HIV effects of ddA or ddI. Unfortunately, one cannot predict from first principles whether this kind of potentiation would be good or bad. Once again, only carefully controlled clinical trials can resolve this issue. These in vitro observations should alert clinicians to the possibility of unexpected interactions among agents, and they are an argument against ad hoc experimentation with antiretroviral therapies outside approved clinical trials.

Conclusion

In this article a number of new therapeutic agents and strategies have been discussed. We now have at hand a number of approaches that can inhibit the replication of HIV in vitro. These approaches, as well as a number of additional developments which are in the offing, can be expected to induce clinical improvement and prolong life even in patients with advanced AIDS. The progress against the mortality caused by AIDS is noteworthy in its own right, but there have been a number of advances that have improved the quality of life. For example, the incidence of dementias ascribable to AIDS has been noted to have decreased after the introduction of AZT [181].

HIV infection is probably a lifelong process. It now appears highly likely that a complete latency phase does not exist. Rather, many, if not all patients, have circulating infectious HIV particles present in their plasma even when the disease is clinically quiescent. Thus, it is perhaps unrealistic to expect a single drug to provide therapy for all patients. The experiences with cancer therapy, as well

as the experiences with other serious infections, suggest that a combination of drugs will produce superior clinical outcome and less toxicity than any single therapy used alone [182]. Combination therapy may also delay or prevent the emergence of viral resistance. Just as in the treatment of certain leukemias or advanced bacterial diseases, optimal therapy against HIV may require at least three different phases: induction, consolidation, and maintenance. It is worth noting that the drugs and biological agents, as well as the relevant doses of such drugs and agents, may vary in each phase. At present, the only formally approved antiretroviral agents are AZT and ddI. AZT has been proven to reduce morbidity and mortality above and beyond any effect of aerosolized pentamidine in severe cases of AIDS [183]. Nevertheless, several virustatic drugs in the same general family are being tested in patients, and it seems highly probable that AZT is not the only agent which eventually will prove effective against HIV. As with a number of other therapies used in life-threatening disorders, AZT may have a relatively low therapeutic index in some patients. Therefore, it is very important that clinicians pay close attention to its clinical pharmacology and to the specific patient responses that occur following initial therapy. As new experimental agents are tested and become more widely available, it is important that careful adherence to the principles of clinical trials be a major priority if we are to succeed in the mission of developing better therapeutic options. As simpler assays to measure plasma drug levels become available [184, 185], their results conceivably may provide useful data in the optimal management of HIV infections. A number of studies are now under way to test whether various agents should be administered to patients with early HIV infections and to explore other therapeutic regimens. In the coming decade, it seems highly probable that major advances will occur against the death and suffering caused by HIV, but this progress can be ensured only if the principles of scientific drug development and controlled trials are maintained.

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Frederick Stohlman Jr. Memorial Lecture

The Human β-Globin Locus Control Region*

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Introduction

The human β-globin gene cluster spans a region of 70 kilobases (kb) containing five developmentally regulated genes in the order 5′-ε,$\gamma_G\gamma_A$,δ,β-3′ (Fig. 1). The haematopoietic tissue in the early stages of human development is the embryonic yolk sac and the ε-globin gene is expressed. This is switched to the γ-globin genes in the foetal liver and the δ- and β-globin genes in adult bone marrow (Fig. 2; for review, see [9]). High levels of these genes are expressed in circulating red blood cells (RBC), giving rise to 90% of the total soluble protein. RBC are derived from a pluripotent stem cell which can differentiate along alternate pathways to erythrocytes, platelets, granulocytes, macrophages and lymphocytes. During the transition to erythroblasts which have lost the capacity to proliferate, the β-globin genes become transcriptionally activated achieving messenger RNA (mRNA) levels of more than 25000 copies per cell.

A large number of structural defects have been documented in the β-globin gene locus (for review, see [9, 44]). These defects are responsible for a heterogeneous group of genetic diseases collectively known as the β-thalassaemias, which are classified into β, δβ, γδβ, etc. thalassaemia subgroups according to the type of gene affected. In a related condition, hereditary persistence of foetal haemoglobin (HPFH), γ-globin gene expression and hence HbF (fetal haemoglobin) production persist into adult life. These clinically important diseases provide natural models for the study of the regulation of globin gene regulation during development. Most interesting in terms of transcription are the promoter mutations and deletions. The δβ-thalassaemias and a number of the HPFHs are associated with an elevated expression of the γ-genes in adult life as a result of deletions of varying size. Analysis of these deletions has suggested that they act over considerable distances, to influence differential gene expression within the human β-globin domain.

The Locus Control Region

The existance of a region that activates the entire β-globin gene cluster first became apparent from the study of a heterozygous γβ-thalassaemia (Fig. 1) [31]. This patient contained one deletion allele which lacked 100 kb, eliminating the entire upstream region but not the β-globin gene [57], which was shown to be completely normal [31, 64]. The other allele was expressed in the patient and it was therefore not a lack of *trans*-acting factors which silenced the mutant chromosome but an important control region had be missing. A set of developmentally stable, hypersensitive sites, 5′ HS1, 2, 3 and 4, were shown to be present upstream in the deleted region, and these

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

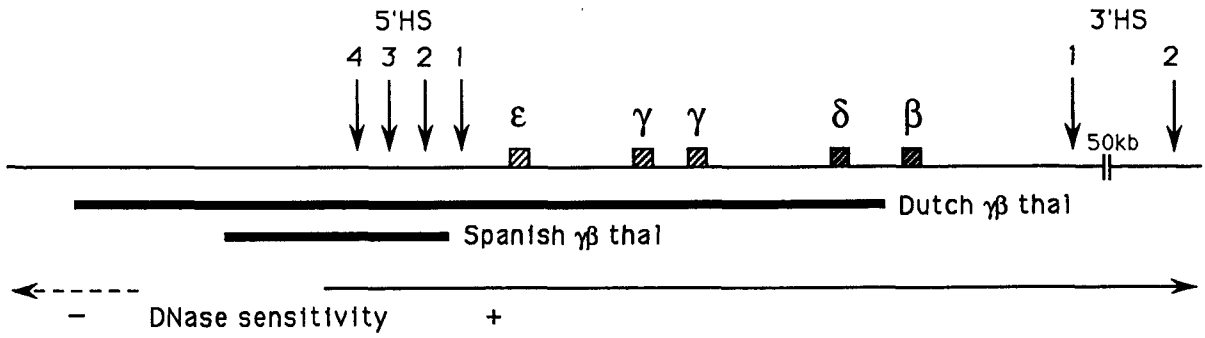

Fig. 1. Schematic representation of the β-globin locus. *Boxes* indicate the different genes which are all transcribed from left to right. The *vertical arrows* indicate DNase hypersensitive sites. The *four arrows* mark the LCR containing 5'HS1, 2, 3 and 4 upstream of the ε-gene. The *two arrows* downstream of the β-gene are 3'HS1 and 2. The *black bars* represent two in vivo deletions which eliminate the function of the LCR. The *horizontal arrows* indicate low (−) and high (+) sensitivity of the chromatin to DNaseI digestion

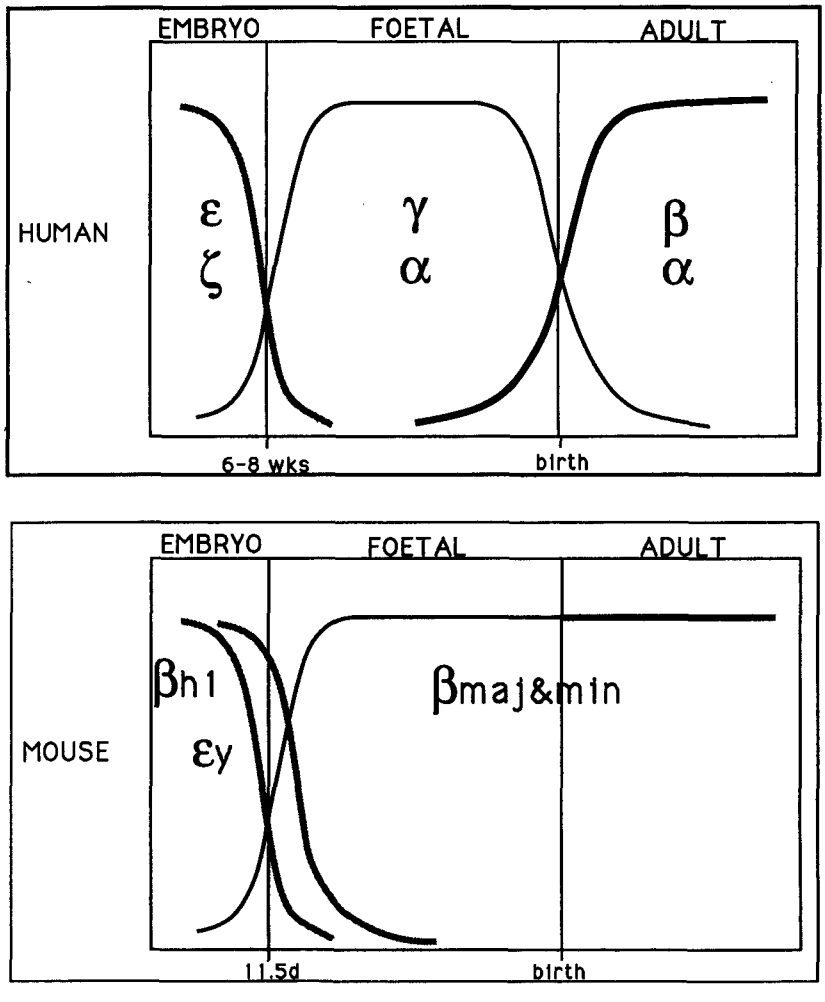

Fig. 2. Schematic representation of the developmental expression patterns of globin genes in human and mouse. The curves refer to the β-like genes only

were potential candidates for such a locus control region (LCR; Fig. 1) [17, 27, 60]. Linkage of this region to a cloned β-globin gene resulted in erythroid-specific high-level expression of the gene in transgenic mice and in tissue culture cells. This expression is dependent on the copy number of the transgene and independent of the integration site in the host genome [3, 27], a phenomenon which had not previously been observed in transgene expression. This posed two questions: How is independence of the site of integration achieved? And how is the LCR involved in globin gene switching?

Position independence and copy number dependence can theoretically be explained by at least two independent mechanisms; either positive activation by the LCR is always achieved, overriding position effects that could be present, or (and) the region contains a locus border element(s) (LBE) that insulate it from neighbouring regions. Matrix attachment sites (MAR) [22, 30] or "A" elements [4, 53] could be LBEs, and we initially speculated these were part of the LCR in addition to activating sequences [27]. However, preliminary experiments indicate that this is not the case and that such a border may be located further upstream. The latter is based on the fact that the DNaseI sensitivity of chromatin is strongly decreased in the sequences 25–30 kb 5′ to the LCR (Fig. 1) [19, 31, 57]. At least 150 kb of chromatin in the 3′ direction is sensitive under the control of the LCR [19], suggesting that such sequences are not present for a considerable distance 3′ (Fig. 1). The position independence we observe is therefore due to a dominant activation of transcription by the LCR, perhaps by creating very stable interactions between the LCR and the genes. Consequently, positive position effects would only be present in the background and only become apparent in situations where the linked gene is suppressed [12] (see below for discussion). Position effects are not observed at low levels of expression when part of the LCR or mutations in the LCR are used. This indicates that the interaction between the LCR and the promoter is dominant except when the promoter is suppressed [10, 12, 18, 20, 49, 54]. In agreement with the deletion observed in a Hispanic γβ-thalassaemia (Fig. 1) [13], the main activity of the LCR is associated with HS2, 3 and 4 [10, 18, 20, 54, 61], which can each activate a linked transgene, independent of the site of integration. A number of protein binding sites have been mapped to these fragments, in particular, sites for two erythroid-specific factors and several ubiquitously expressed factors. A number of the binding sites are present in all three active sites (Fig. 3) [*43*, *45*, *55*]. One of the shared factors is the erythroid/megakaryocyte-specific factor GATA-1 [38, 48], which has been shown to be essential for erythroid development [42]. Deletion of GATA-1 binding sites prevents erythroid-specific induction of the β-globin gene [11], and the protein has been shown to have transcriptional activation properties [38]. However, the presence of GATA-1 binding sites per se is insufficient to give position-independent expression, e.g. the flanking regions of the human β-globin gene contain at least six GATA-1 binding sites [11, 63], but do not confer integration site-independent expression [32, 34, 58]. However, all three active 5′ HS contain two closely spaced GATA-1 sites in opposite orientations. This arrangement is also observed in the chicken β-globin enhancer, which appears to provide position-independent expression [47]. Possibly an inverted double GATA-1 site is a key component in erythroid-specific, position-independent activation and GATA-1 can interact with itself or one of the other GATA proteins to achieve this [66]. Classical enhancer activity is only associated with 5′HS2 [41, 61], and not with the others. Dissection of the HS2 showed that a number of proteins are bound to the core fragment (Fig. 3) [55]. Attention has been focused on a double consensus sequence for the *jun/fos* family of DNA binding proteins which appeared crucial for HS2 activity [41, 52, 55]. Several pieces of evidence

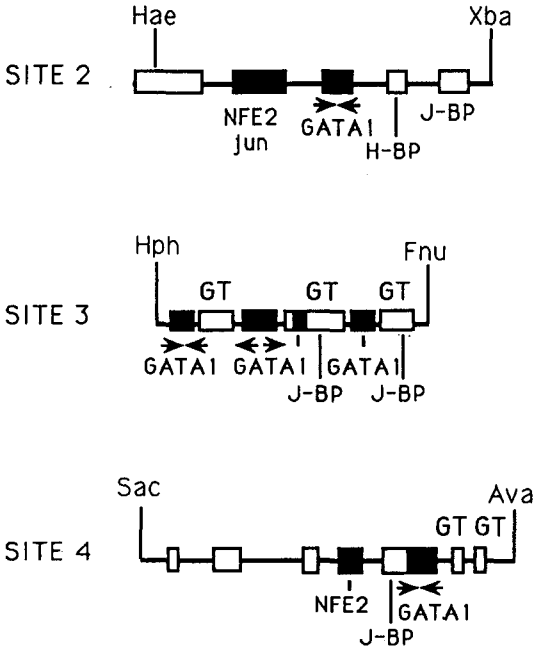

Fig. 3. Summary of factor binding sites to the minimal fragment of 5'HS2, 3 and 4, which provide position-independent expression in transgenic mice. Individual factors are described in the text. *Black boxes* indicate erythroid-specific factors; *open boxes* indicate ubiquitous factors. *GT* indicates a GT-rich motif [43]

show that the functional activator which interacts with the *jun/fos* binding site is NF-E2, originally described as interacting with another erythroid-specific gene, that for porphobilinogen deaminase [39, 40]. Two NF-E2 molecules and at least one other protein binding at two non-equivalent sites are involved [56]. However, the presence of this double NF-E2 sequence alone is insufficient to provide high levels of expression [55] and when the *jun/fos* binding site is removed from the 300 bp core fragment, HS2 retains the ability to activate a linked β-globin gene in a copy number dependent fashion, albeit at low levels (Fig. 4) [56]. We therefore conclude that the 5'HS2 NF-E2 region has strong enhancer activity but that it is not necessarily required to obtain position-independent globin gene activation.

All the other factors which have been shown to interact with LCR sequences, including the factors H-BP and J-BP, are ubiquitous proteins [56]. This suggests that a combination of erythroid-specific and ubiquitous factors may be required to render the β-globin gene independent of its site of integration. The (abundant) ubiquitous factors shared by the three HS of the LCR which have been studied to date are Sp1 and TEF-2 [23, 65], but a simple multimerized combination of a GATA-1 and a Sp1/TEF-2 binding site is not functional (S. Philipsen, unpublished results). We therefore think that other, as yet less well characterised factors may be involved in LCR function.

The LCR and Disease

The discovery, characterization and mapping of the LCR has enabled the pursuit of two novel approaches to the study of globin-related diseases. Firstly it allows high-level expression of disease genes such as the β^s gene which is responsible for sickle cell disease. By linking this gene in combination with human α-globin genes several laboratories have succeeded in producing transgenic mice which show sickle cell disease [26, 50, 59]. High levels of human haemoglobin S can be obtained in mice and the RBC of these mice show a pronounced change in shape when deoxygenated (Fig. 5). We are presently improving this model for two reasons, firstly, to study the effects of sickle cell disease on the progression of infection by different malaria strains, and secondly, to be able to study the progression of sickle cell disease and the treatment thereof by new protocols, in particular the development of gene therapy. The latter has been given new hope by the mapping of the minimal elements that give the full activity of the LCR. The LCR can now be incorporated into retroviral vectors to develop therapy protocols and preliminary experiments (F. Meyer, personal communication) indicate that the LCR will provide high levels of expression in this context in mice.

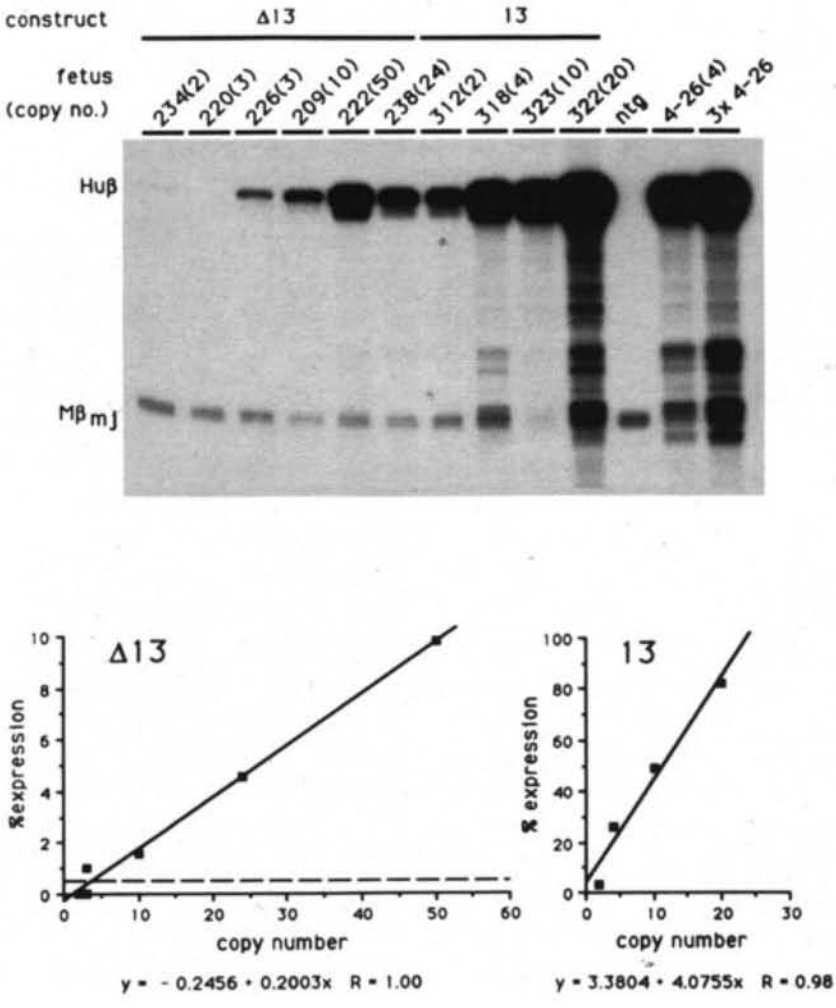

Fig. 4. S1 nuclease analysis of HS2 constructs containing the NF-E2 sites (*13*) or not (*Δ13*) in transgenic mice. Foetal liver RNA (day 13.5) was assayed using a mixed probe S1 nuclease experiment using the 5′ human β-globin probe and the mouse βmaj probe [56]. Specific activities were 10:1 for Hu β:M β. Protected products are indicated on the *left*. The 200 series of transgenic mice contains the Δ13 construct and the 300 series contains the 13 construct. Copy numbers are shown in *parentheses*. *Lower pnael* depicts a quantitation experiment of the S1 protection analysis using the Huβ5′ probe and a mouse α-globin probe as an internal control. The % expression is given as the total Huβ-globin signal divided by the total mouse α-globin signal (adjusted for specific activities). This was plotted against the copy number. The *line* represents the result of a linear regression analysis on the data points. The *R* value, the correlation coefficient, indicates very high correlation with a straight line ($R = 1$). The *dashed line* in the Δ13 graph represents the minimal level that can be measured accurately

Developmental Regulation of the β-Globin Locus

Genetics. The study of globin gene switching has been assisted by the characterization of deletions and point mutations which affect expression of the γ and β-genes. Point mutations in the γ-promoters have been linked to HPFH phenotypes and these can be divided into two groups (Fig. 6).

Those clustered around the distal CAAT box appear to result in the loss of factor binding sites [21, 36], suggesting that this region may contain a binding site for a negative regulator. For example, a 13-bp deletion which removes the distal CAAT box results in a very strong HPFH (60%) [25]. Interestingly, a recently described Japanese HPFH (20%) is associated with a point mutation in the CAAT sequence of the distal CAAT box

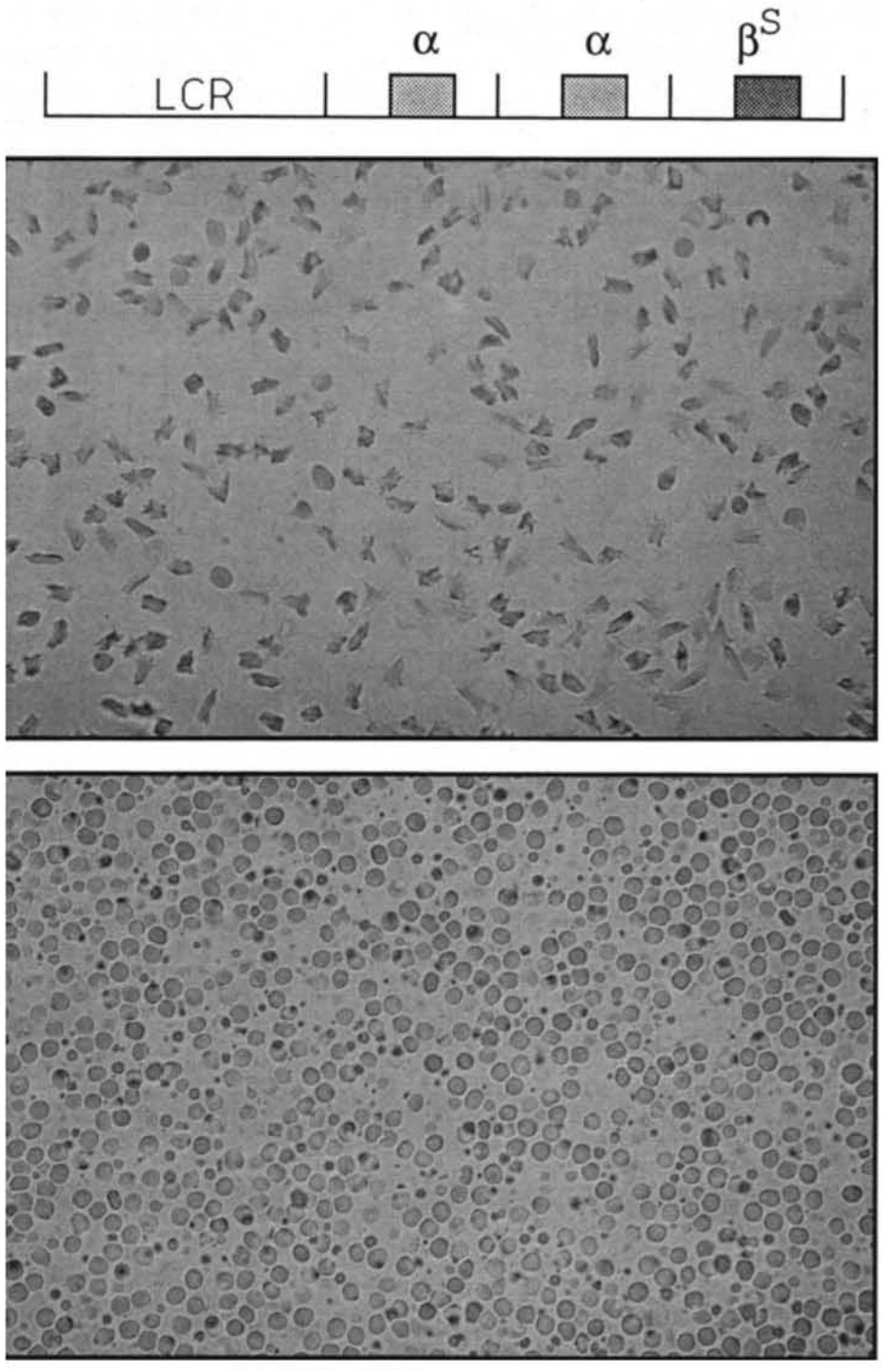

Fig. 5. Sickle cell disease in transgenic mice. The *top line* shows the arrangement of genes and the LCR that was injected into fertilized mouse eggs to obtain transgenic mice. The *top panel* shows sickled cells from one of the transgenic mice [26]; the *bottom panel* shows control nontransgenic red blood cells

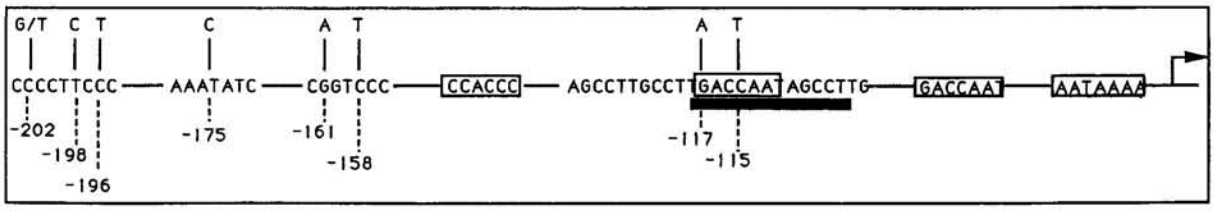

Fig. 6. Summary of mutations occurring in the γ-globin promoter resulting in HPFH phenotypes (see [44])

[21] which reduces affinity for the transcription factor CP1. The −117 mutation associated with Greek HPFH (40%) has been reported to cause reduced binding of the erythroid-specific factor NF-E3 [36]. These findings suggest a model for γ silencing in which factors binding to the distal CAAT box (at −115) compete for interaction with factors bound to upstream promoter sequences preventing the proximal CAAT box (at −87) from forming such interactions. The distal CAAT box is located outside the normal optimal position for CAAT elements, and this is likely to prevent it from functioning as an effective positive promoter element. One would expect this type of silencing mechanism to depend on the topology of the promoter region and it is also likely to be affected by the creation of extra factor binding sites in the upstream sequences. Such sites may partially bypass the competition between the proximal and distal CAAT boxes, resulting in suboptimal transcription. Indeed, a second group of mutations, upstream of −150, result in new or improved binding sites for transcription factors, e.g. Sp1 [16, 28] and GATA-1 [35, 37].

Activation of γ transcription in the nondeletion HPFHs is associated with down regulation of the β-gene. The reduction in β expression (to around 60% in Southern Italian HPFH) is approximately equivalent to the rise in expression of the *cis*-linked γ-globin gene, with only a slight reduction in overall transcriptional output from the locus [24, 62]. This suggests that competition is taking place between the genes and that this is tightly linked to the process of transcription.

However, a drastic reduction or loss of β transcription due to point mutations and deletions in the β-promoter does not significantly increase γ expression (less than 5%; Fig. 7) [44]. Clearly, a γ-gene exerts a negative effect on the β-gene (coupled to transcription) but this effect is not reciprocal. Some β-gene deletions show higher levels of γ expression (Fig. 7) but these deletions are always large (>10 kb). Of these, the Aγδβ-thalassaemias all have deletions which extend into the region of γ transcription. They are uninformative for competition models because enhancers found near the deletion breakpoints may be responsible for the high level, pancellular γ expression observed in the deletion HPFHs [1, 15]. Some increased γ expression is also observed in the δβ- and Dutch β-thalassaemias, but the broad range of values between patients with the same deletion and the heterocellular distribution of γ-protein among the red cells suggest that the increase in γ expression is not solely at the transcriptional level. This is supported by the observation that *non*transcriptional defects (e.g. RNA processing) in heterozygous β-thalassaemias cause elevated levels of γ-chains (up to 5%). Selection of a small proportion of cells expressing γ is a likely mechanism for this increase. Deletion of the δ-gene (which is normally expressed at only 2%−3% of the level of β) also does not seem to be required for the γ-expression observed in the δβ-thalassaemias, since it is intact in Dutch β-thalassaemia, which has a very similar phenotype. Instead, the requirement appears to be a minimum size of deletion (>10 kb). Probably these large deletions perturb the chromatin structure of the locus, resulting in a small increase in γ transcription which is further amplified by the chain imbalance. In conclusion, the genetic data show that strong down-regulation of the β-gene can result from an increase of γ-gene transcription, while there does not seem to be any significant link between transcription of the β-gene and silencing of the γ-genes in adult life.

Transgenic Mice. Attempts to study switching of globin genes have also made use of transgenic mice as a model system. Mice do not possess separate foetal globin genes but instead switch directly from embryonic to adult β-globin expression at 11−13 days of gestation (Fig. 2).

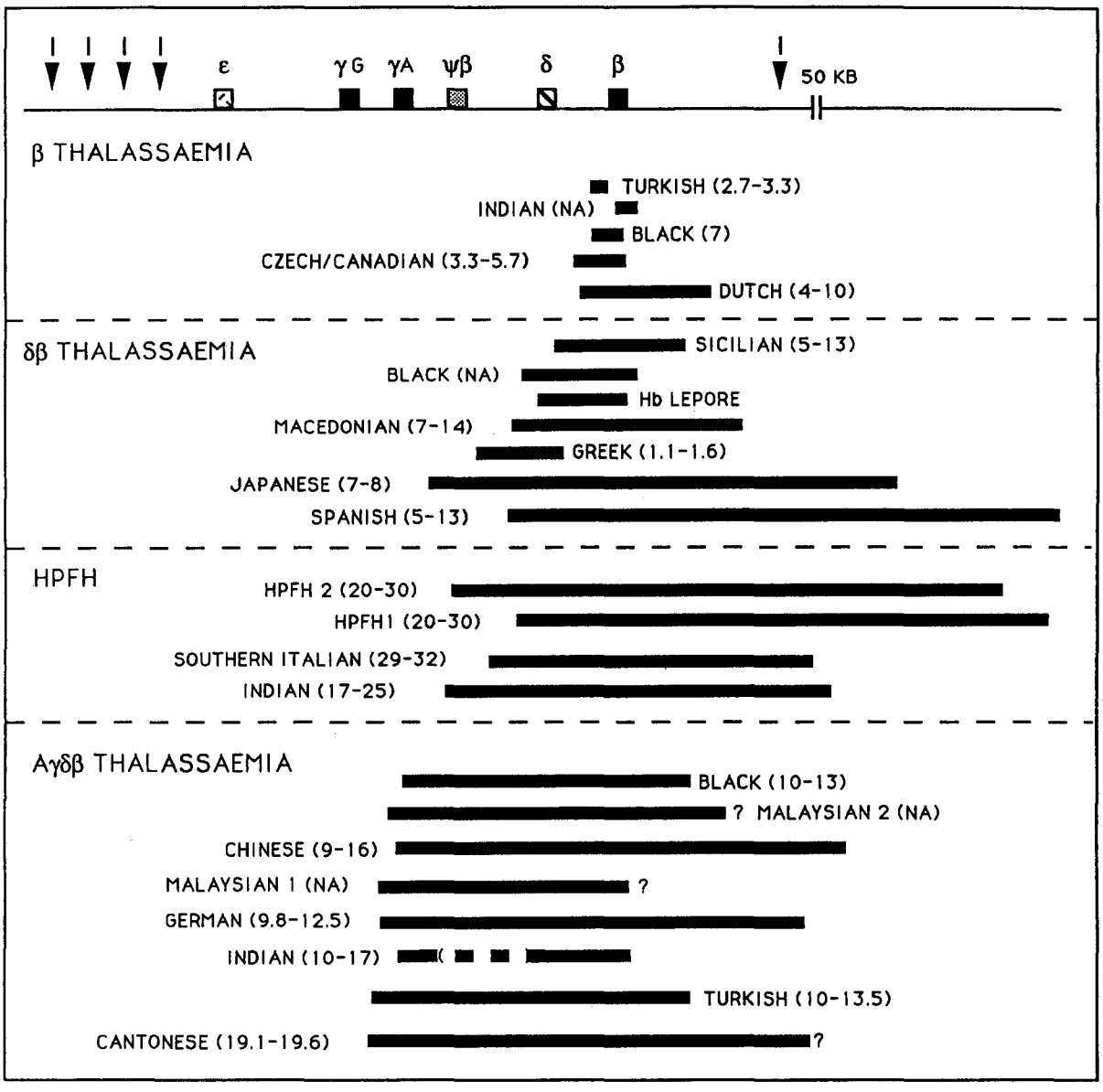

Fig. 7. Schematic representation of the different deletions occurring in the β-globin locus in thalassaemias and HPFHs. *Black bars* indicate the size of the deletion; *numbers in parentheses* indicate the levels of γ-globin expression in heterozygotes

The developmental regulation of the human ε-gene has been analysed in both embryonic stem cells and transgenic mice. In mice the ε-gene is expressed at high levels during the embryonic stage only when linked to the LCR and is completely silenced thereafter [33, 46, 51]. Based on the studies by Cao *et al.* deletion mutants lacking the −200 to −300 promoter region show a small increase in ε expression in adult transgenic mice but the low level indicates that other sequences may also be involved in silencing ε (P. Fraser, unpublished observations).

The human γ-transgene without the LCR is expressed like the mouse embryonic genes [7, 32]. It was initially reported that linkage to the LCR resulted in γ expression at all developmental stages and that the γ-gene was silenced in adult mice only when the β-gene was also present. This appeared to support a competition model where the β-gene is required for silencing of the γ-gene [2, 14]. However, a different result was obtained when the single γ-gene experiments were carried out on animals carrying only one or two copies of the LCR-γ-gene con-

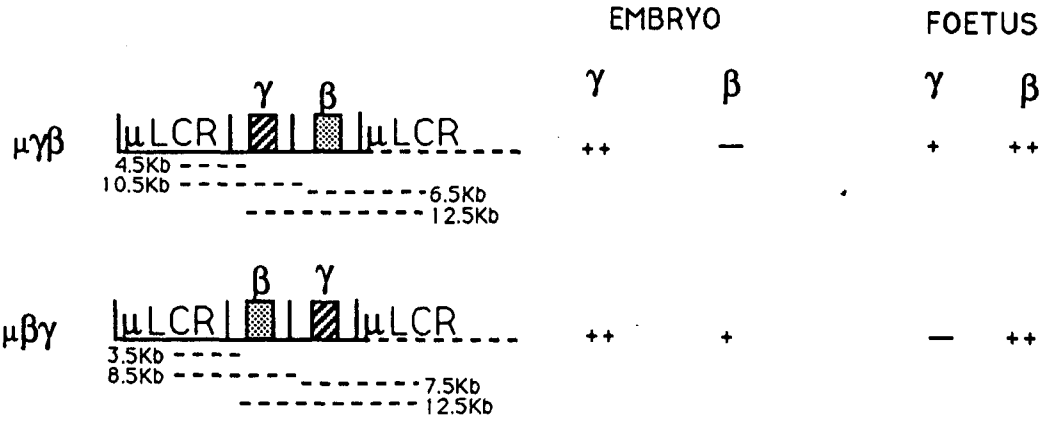

Fig. 8. Microlocus (μLCR) *γβ* and *βγ* constructs [29]. Genes are represented as *shaded boxes*. All genes are in the same trancriptional orientation, 5′ to 3′, with respect to each other and the LCR. The *dotted LCR lines* indicate the situation in multicopy animals to illustrate the distance from a promoter to a 5′ and 3′ LCR. These distances are indicated by *dotted lines* below the constructs. *Plus* and *minus symbols* indicate high, medium, and very low levels of expression

struct. *γ* expression persisted in the early foetal liver, but was silenced at adult stages, independent of the presence of the *β*-gene [12]. Transcription of the LCR-linked *γ*-gene can therefore also be blocked completely by stage-specific negative regulators acting on the sequences immediately flanking the gene, and this removes the basis of the argument that the *β*-gene would be needed for *γ* silencing. The elements responsible for *γ* silencing have not yet been identified but the mutations associated with the nondeletion HPFHs suggest that at least the sequences around the distal CAAT box are likely to be involved (see above). The availability of a transgenic mouse model for *γ*-gene silencing should allow this to be tested and possibly lead to novel approaches for treating thalassaemia and sickle cell anaemia. If *γ*-gene expression were understood at the level of the transcription factors, it might be possible to develop novel therapies that could specifically interfere with the adult suppression of the *γ*-gene and alleviate the clinical problems associated with severe chain imbalance or sickling.

Linkage of the adult *β*-gene to the LCR results in inappropriate expression at the embryonic stage [2, 3, 14, 29, 33], albeit at a low level. Placing a *γ*-gene or a human *α*-globin gene between the *β*-gene and the LCR blocks this expression [2, 14, 29], supporting the idea that competition plays a role in preventing premature *β* expression. However, when the order is reversed and the *β*-gene is placed in the first position, it is expressed at a level similar to that observed for the *β*-gene in the absence of the *γ*- or *α*-gene (Fig. 8) [29]. Silencing of the *β*-gene at the embryonic stage is therefore not caused by reciprocal competition only, but relative distance between the LCR and the genes (i.e. position and polarity) is also important.

Polarity in the locus has long been suggested by the fact that the genes are arranged in the order of their expression during development. The order of the genes is conserved among mammals but there is some divergence in the other vertebrate loci. In chicken, the embryonic *ε*- and *ϱ*-genes are located at opposite ends of the locus, with the adult *β*-genes between them. However, it is important to note that the chicken *β*-globin LCR may have been split as part of an *ε* translocation such that part of it is located between the *β*- and *ε*-genes [8, 47] and that the *ε*-gene contributes only 20% of the total embryonic haemoglobin compared to 80% for *ϱ* [5].

The data reviewed above indicate that developmental regulation of the human

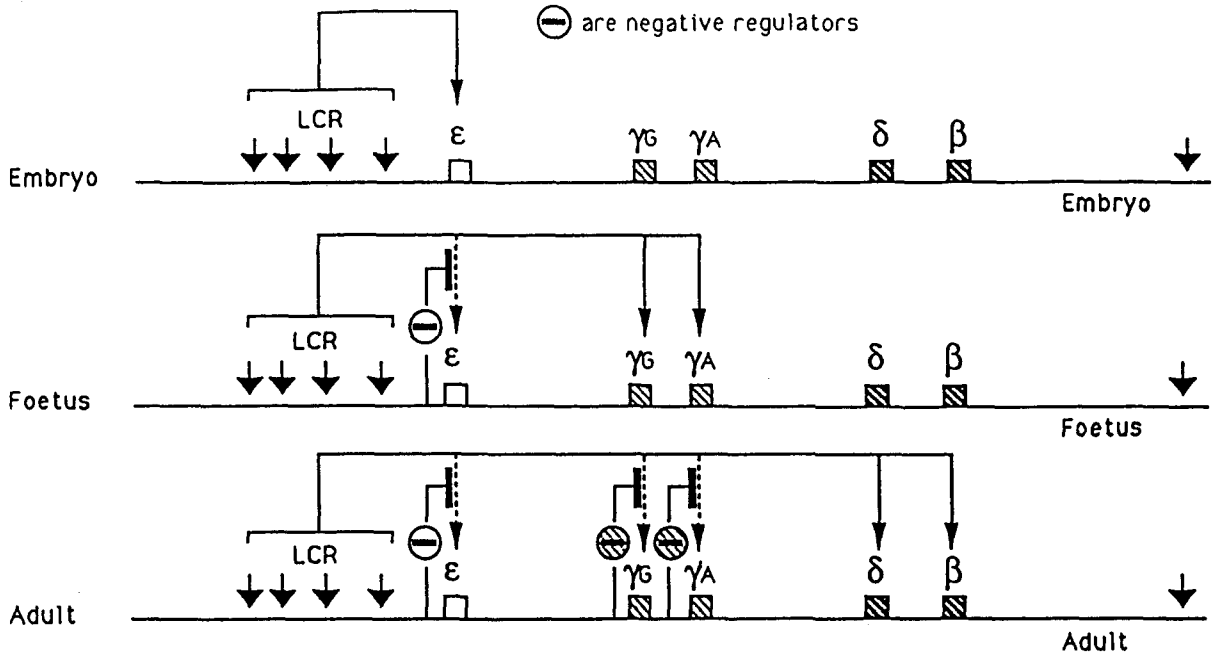

Fig. 9. Model for stage-specific regulation of the genes of the β-globin locus. *Solid lines* indicate activation of genes by the LCR. The symbol θ indicates stage-specific negative factors silencing the gene. The location of these is not accurate and there may be more than one factor for each gene

β-globin locus is a complex process which centres around developmentally specific suppressors and the polarity of the locus (Figs. 9, 10). The earliest gene to be activated, the ε-gene, is also the one closest to the LCR. The γ- and β-genes may be suppressed by competition with ε; alternatively, or in addition, the γ- and β-genes may bind embryonic stage-specific factors which keep their promoters suppressed. The ε-promoter is silenced in the foetal liver by one or more suppressor factors, negating its competitive ability (Fig. 9). As a result the γ-genes are expressed, and they in turn keep expression of the β-gene suppressed by competition. The γ-genes are switched off during the period around birth, again by stage-specific negative regulators, and as a consequence the β-gene is activated and expressed in the bone marrow. We propose that loop formation between regulatory elements is the crucial parameter to explain the suppression of the late genes by the early genes at early stages but not vice versa.

The frequency of interaction between the promoters and the LCR will depend on the effective volume in which these elements operate. This effect would be most pronounced if the LCR and the genes were all present on one structural chromatin loop several times the distance between the LCR and the genes. The fact that the LCR controls DNase hypersensitivity of the β-globin locus over at least 150-kb [19] suggests that the entire β-locus may be present on one very large chromatin loop. If we assume that to be the case, the frequency of interactions between any of the promoters with the LCR would be proportional to their effective concentration relative to the LCR (Fig. 10). On basis of ring closure probabilities with naked DNA, the effective concentration of two points on the DNA will be related to the volume of a sphere and will be proportional to the power of 3/2 of the distance. Applying the rule to the β-locus, the β-gene is twice as far as the Gγ-gene from the HS 2 enhancer of the LCR. Therefore, the β-gene occupies an approximately eight-fold larger volume relative to the HS-2 enhancer of the LCR than the Gγ-gene, which should give it a three-fold lower fre-

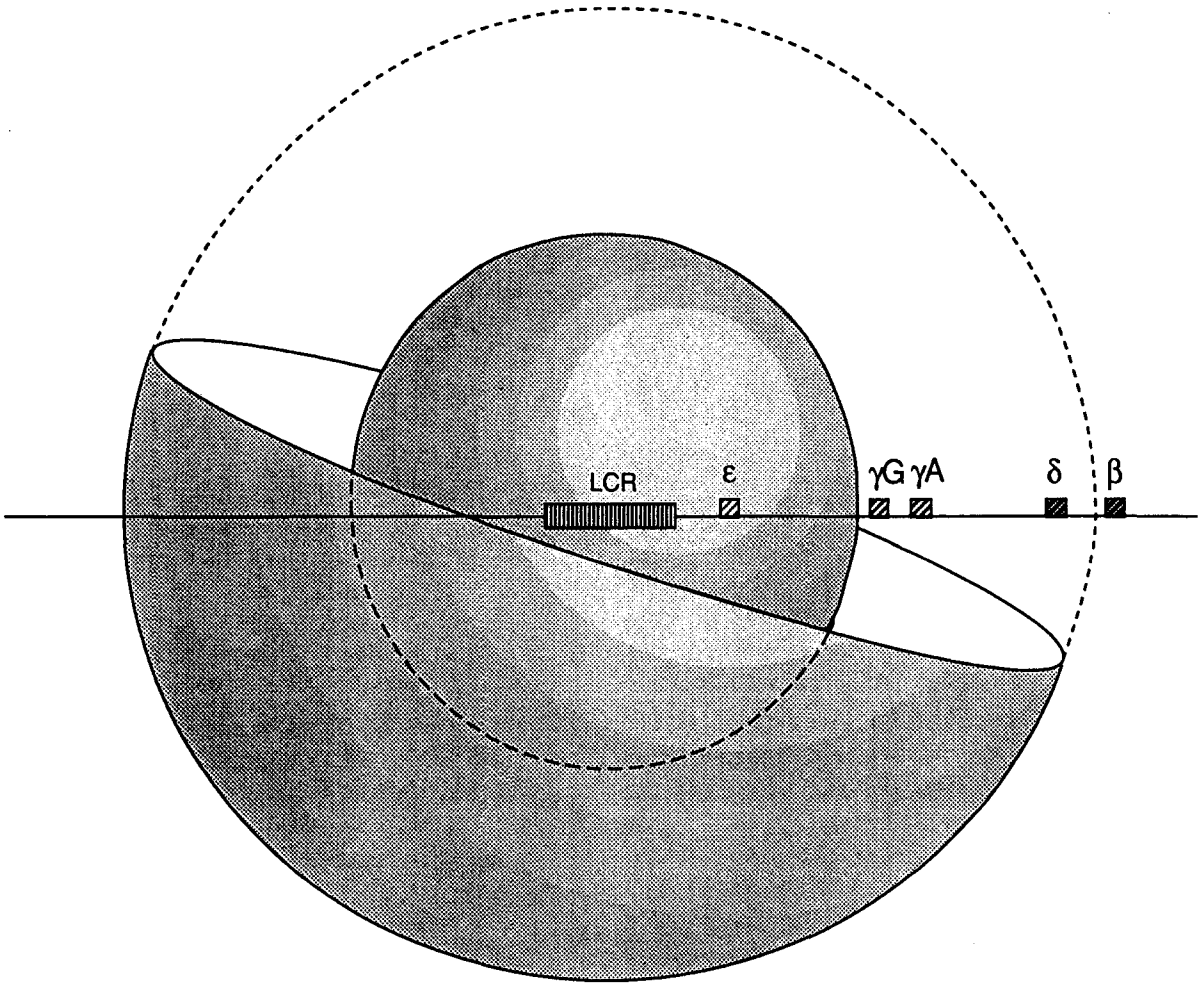

Fig. 10. Schematic representation of the relative volumes occupied by the Gγ- and β-genes relative to the LCR. For simplicity of presentation the LCR is shown as a fixed point in the centre of the sphere. Only half the β-globin gene outer sphere is shown

quency of interaction with the LCR (Fig. 10). This effect will work in favour of the proximal gene, decreasing the affinity differences required for competition, but it will work against the distal gene. Distal genes would be incapable of suppressing upstream genes under similar circumstances unless the downstream gene promoter increased its affinity by several orders of magnitude relative to the upstream gene. The transgenic mouse data on the expression of the β-globin gene at the embryonic and foetal/adult stages argue strongly against this possibility. Instead, the problem is solved by local suppression of the upstream promoters to allow expression from the downstream gene (Fig. 9). Experiments to substantiate or disprove this prediction are presently in progress.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Clinical Aspects

Bone Marrow Transplantation in Children with Acute Leukemia: A 1990 View

D. Pinkel [1]

Introduction

Since the first case report in 1959 of total body irradiation and bone marrow transplantation (BMT) in a child with acute leukemia, the popularity of this approach has steadily increased [1]. The introduction of human leukocyte antigen typing and mixed leukocyte cultures and improved methods of supportive care made BMT a successful way of treating certain immunodeficiency disorders, severe aplastic anemia, chronic myeloid leukemia, and certain other blood dyscrasias as well as acute leukemia [2]. The purpose of this essay is to examine critically the practice of myeloablation and marrow transplantation in children with acute leukemia.

Acute Myeloid Leukemia

The first generally accepted use of allogeneic BMT in children with leukemia was for those with acute myeloid leukemia (AML) in hematological remission after initial chemotherapy [2]. It was thought that relapse was almost inevitable in these patients, so the reports of apparently permanent remission after BMT convinced most hematologists that BMT was the treatment of choice if a histocompatible sibling donor were available. However, in the past 10 years it has become apparent that combination chemotherapy alone without BMT may be as effective as BMT. This is reflected in a 1989 statement of the International Bone Marrow Transplant Registry (IBMTR): "It is not known whether chemotherapy or bone marrow transplantation is the more effective treatment for acute myelogenous leukemia in first remission" [3].

A recent 6-year follow-up report from St. Jude Children's Research Hospital of a 1980–1984 study of therapy of AML describes no significant difference in 6-year continuous complete remission rates between BMT and chemotherapy [4]. Nine of 19 BMT patients remained in continuous complete remission for a median of 68 months and 13 of 42 chemotherapy patients for a median of 74 months (Fig. 1 a). Similar experience has been reported from the Johns Hopkins Oncology Center in young adults with AML [5]. When corrected for patient selection, their data indicate that the frequency of lengthy continuous complete remissions was similar for 41 patients treated with allogeneic BMT and 46 patients who received intensive chemotherapy without BMT (Fig. 1 b).

From January 1986 to February 1989 the Children's Cancer Study Group admitted 617 children with AML into a study in which those with histocompatible sibling donors underwent BMT after remission induction while the others received intensive chemotherapy for 3 months with or without subsequent maintenance chemotherapy [6]. The actuarial 2-year event-free survival from the time of BMT or intensive chemotherapy is not significantly different, 41% for

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

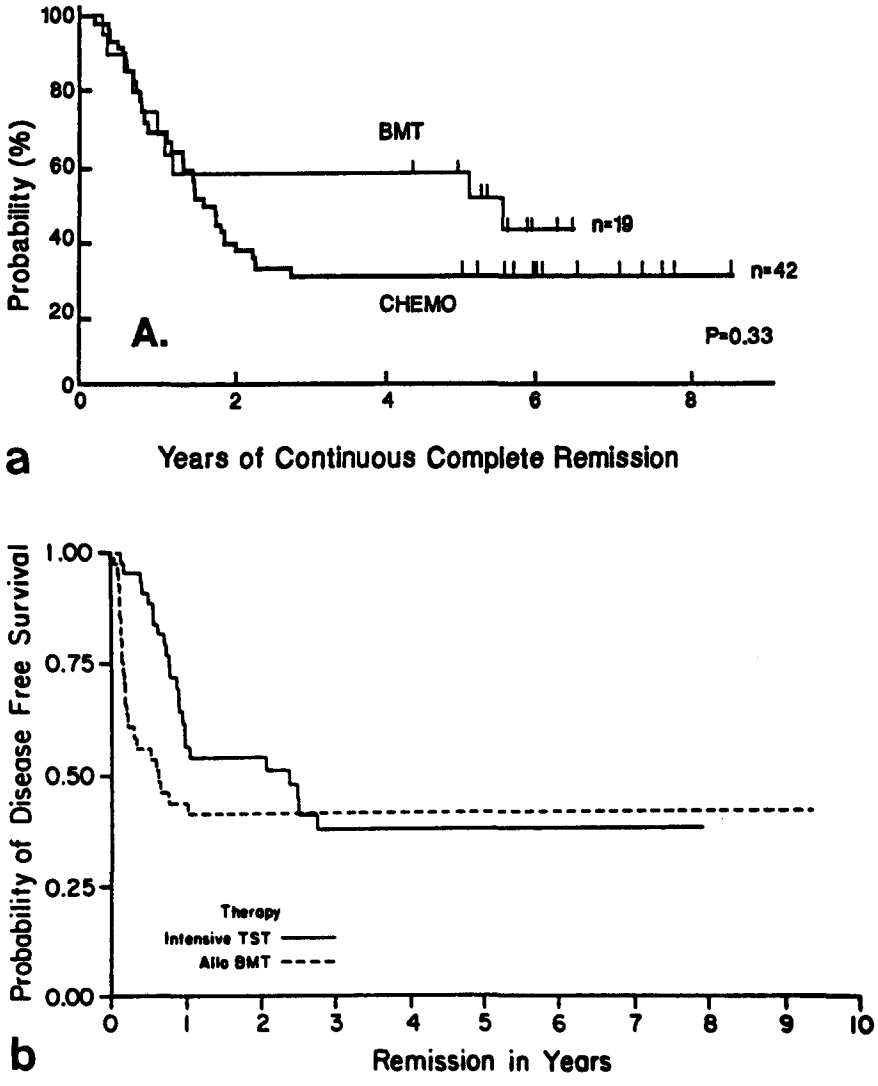


Fig. 1. **a** Duration of continuous complete remissions of children with AML in first remission treated with chemotherapy alone vs. chemotherapy (*chemo*), myeloablation and allogeneic marrow transplantation (BMT) in St. Jude study AML-80. Transplantation did not affect the probability of lengthy complete remission. (From [4]). **b** Duration of complete remissions in young adults treated with intensive timed sequential (*TST*) chemotherapy vs. myeloablation and allogeneic marrow transplantation (*Allo BMT*) at the Johns Hopkins Oncology Center. (From [5])

intensive chemotherapy and 50% for BMT ($p = 0.41$). It is possible that late deaths from chronic graft vs. host disease and its complications, secondary neoplasms, or late relapses might modify this outcome in favor of one or the other methods.

Acute Lymphoid Leukemia at High Risk of Relapse

BMT has been employed in first remission of acute lymphoid leukemia (ALL) with features considered to augur an unfavorable outcome. One report describes 50% disease-free survival of patients with "poor-risk" ALL in complete remission after BMT [7]. The median delay from diagnosis to BMT in these patients was 179 days. Since estimates of relapse risk in ALL are based on complete remission duration, this 6-month delay likely excluded those who were at "poorest risk." A similar report with a higher proportion of survivors, but shorter follow-up, is subject to the same criticism [8]. The achievement of re-

mission and the delay of BMT for 1–12 months again tends to exclude the patients with the greatest risk of unfavorable outcome.

Young adults have a higher risk of relapse of ALL than do children. Recently the IBMTR compared the remission experience of 484 young German adults with ALL who received intensive chemotherapy and 251 treated with allogeneic BMT during the same period [9]. Statistical corrections were applied for selection factors. The 5-year leukemia-free survival was similar for both groups.

Acute Lymphoid Leukemia in Second Remission

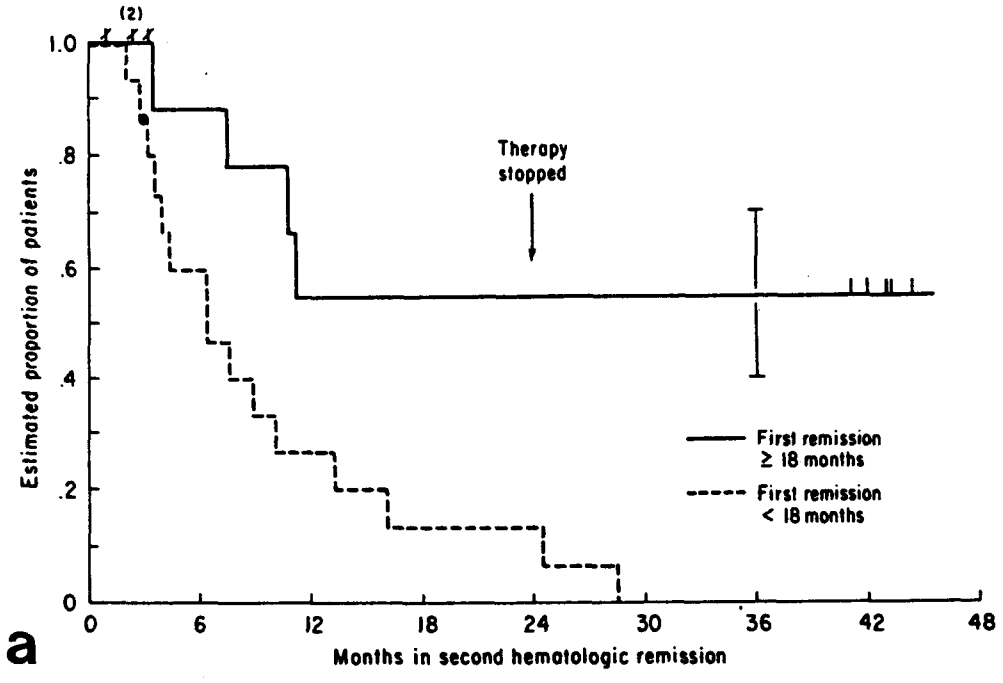
One of the early publications concerning allogeneic BMT is second remission of ALL concluded that "marrow transplantation offers the best chance of long term remission and potential cure after a child with ALL has had a relapse in the marrow" [10]. This was based on a non-random comparison in which 9 of 24 children survived in remission after BMT and only 1 of 21 after chemotherapy alone. However, scrutiny of the published data reveals that 11 of the 24 BMT patients had isolated extramedullary relapse, which has a more favorable response to treatment [11], rather than marrow relapse. This contrasted with 4 of 21 chemotherapy patients who had extramedullary relapse. The median duration of first remission, an important prognostic factor for second remission [12], was 25 months for BMT patients and 13 months for chemotherapy patients. Finally, the delay between remission induction and BMT ranged up to 17 months, thus tending to exclude patients with early relapse and, therefore, the worst prognosis. In retrospect, the data did not justify the conclusion.

A recent report comparing allogeneic BMT vs. chemotherapy without BMT in children with ALL in second remission attempts to address the problems of other such comparisons [13]. The patients who received chemotherapy alone had "risk factors" for relapse comparable to the BMT patients and had been in complete remission for 2–3 months prior to entry into the study. However, BMT was delayed up to 13 months and no description is given of the drug schedules and medical care of the chemotherapy patients. For these reasons the reader cannot be certain whether the superior outcome of BMT was related to the exclusion of patients with early relapse, the most reliable prognostic factor. Also, one is unable to assess whether medical care was comparable in the two groups and whether the chemotherapy alone patients received optimal drug therapy.

Bone Marrow Autografts in Acute Leukemia

There is a surge of interest in treating childhood acute leukemia with myeloablation and autografting of cryopreserved bone marrow obtained during hematological remission and subjected to "purging" with biological or chemical agents.

A recent report concludes that this may be a treatment option for children with ALL in second or subsequent remission whose first remissions were longer than 24 months [14]. Of 44 patients grafted, 15 were in continuous second remission 10–94 months; all 15 had initial remissions longer than 24 months. Children with T-cell ALL or B-precursor ALL without CD10 or CD9 surface antigens were excluded. Delays of 1–11 months between remission and autograft excluded other patients with more aggressive or resistant leukemia. The event-free survival was similar to that reported previously for a group of 28 children with ALL in second hematological remission treated with chemotherapy alone [12]. In both the autograft and the chemotherapy alone series those with brief initial remissions had short second remissions while those with long first remissions had longer and sometimes durable second remissions (Fig. 2). There is no evidence



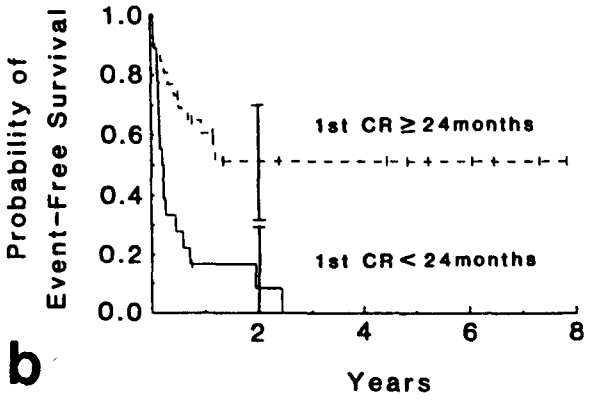


Fig. 2a, b. Duration of remission. **a** In 44 children with ALL in second or subsequent remission who received ablative chemotherapy and autografts of purged bone marrow. All patients with initial remissions of less than 24 months experienced relapse but one half of those with initial remissions longer than 24 months survived free of leukemia. (From [14]). **b** In 28 children with ALL in second hematological remission who received chemotherapy alone in Pediatric Oncology Group study 8201. All children with initial remissions of less than 18 months experienced relapses but one half of those with initial remissions longer than 18 months remained in complete remission after completion of treatment. Note the similarity with the bone marrow autograft results. (From [12])

that adding a marrow autograft procedure to chemotherapy changed the outlook for survival. In contrast to the chemotherapy only regimen, all long-term survivors of the autograft procedure had growth failure and 9 of 28 patients in remission 3 months after grafting experienced hemolytic-uremic syndrome.

A report from the IBMTR summarizes their view: "Whether autotransplants are equivalent or superior to other therapies... is uncertain, since prospective trials are not reported and data analysis is confounded by selection of subjects and time-censoring" [15].

Sequelae of BMT in Children with Acute Leukemia

From the earliest reports of curative approaches to children with acute leukemia pediatricians have been concerned about the quality of survival. Studies have focused on anthropometric and neuropsychological measurements of surviving children. Cure has been defined as not only eradication of leukemia but restoration of normal health and normal capacity for physical, intellectual, social, and emotional growth and development.

The need for weighing the value of each component of treatment against its ultimate risk to normal health, growth, and development of the children has been emphasized.

Children who survive leukemia have the potential for a much longer life than adult survivors and thus a longer period of risk for delayed effects such as second cancers or organ failures. In addition, the growing tissues and more rapidly replicating cell systems of children are more vulnerable to cytotoxic agents. For example, preschool children are more likely to experience neuropsychologic deficits after cranial irradiation than older children and adults [16]. Children's hearts are apparently more vulnerable to delayed anthracycline cardiomyopathy than adults [17]. For these reasons one must necessarily be concerned about the late effects of treatment of children.

There are relatively few descriptions of the delayed sequelae of BMT. Growth failure is universal in the Seattle series, probably as a result of total body irradiation [18]. Survival must therefore be considered dysfunctional despite the courage and vigor of the children, their families, and their physicians in overcoming the problem. This contrasts with the outcome of chemotherapy alone in which "catch-up" growth usually occurs after cessation of therapy in those children whose growth is slowed on treatment [19].

Although gonadal failure may follow treatment with alkylating agents, the majority of children with leukemia receive little or no drugs of this class and fertility is usually preserved [20]. In contrast, approximately 70% of BMT survivors experience gonadal failure [18, 21]. Other endocrine deficiencies, rare in chemotherapy survivors, are reported in about one third of BMT survivors.

Chronic graft vs. host disease occurs in approximately one third of children after allogeneic BMT [18, 21]. This can result in crippling organ failure as well as a continuous risk of life-threatening infection. Obstructive and restrictive pulmonary disease, often fatal, is another complication of BMT not seen in children with leukemia treated with chemotherapy alone [22].

Second malignant solid tumors 10–30 years later are among the delayed sequelae of childhood cancer. Some may be related to the first neoplasm but the greatest risk appears to arise from treatment with radiation therapy and alkylating agents [23]. The administration of total body irradiation and high dosages of alkylating agents such as busulfan and cyclophosphamide are customary methods of myeloablation in marrow transplant and autograft procedures. Given the long life expectancy of children cured of cancer and the carcinogenic effects of radiation and alkylating agents, it can be anticipated that children with leukemia treated with BMT or autografts will experience a very high incidence of malignant solid tumors as young adults.

In summary, available data indicate that the human "price of cure" is appreciably higher in children treated with BMT than with current chemotherapy regimens.

Discussion

The difficulties in comparing outcomes of alternative treatments of cancer are well known. Among them are patient selection, lack of randomization, enthusiasm for test therapy, differences in quality or level of medical care, misuse of survival curves, and failure to describe fully the sequelae of treatment so that its human cost can be compared to its benefits.

In the evaluation of reports of BMT in acute leukemia of children there are some specific problems [24]. First is the exclusion of potentially eligible patients. BMT is usually performed during hematological remission. Therefore, patients who fail to experience remission are excluded from the procedure. Because of delays between remission and the BMT procedure patients who experience relapse prior to BMT are also excluded. Since

failure to enter remission and early relapse tend to signify more resistant, more aggressive leukemia with poor prognosis for survival, these exclusions are highly selective for providing BMT candidates that have a relatively favorable outlook.

An example of this selective process was demonstrated in the Pediatric Oncology Group (POG) 8710 study of treatment of children with ALL in first hematological relapse [25]. Of 100 patients registered in the study, 74 had HLA-typing. Of 16 children found to have a fully matched sibling donor, only seven underwent BMT. The other nine children either failed to experience a second hematological remission or suffered another relapse before BMT could be performed. Thus, one half of the eligible patients, the half with the worst outlook for survival, were excluded from BMT.

The effect on apparent therapeutic outcome of excluding patients who have early relapses from BMT can be appreciated by consideration of expected failure rates for patients under 21 years of age during the first few months after remission induction of acute nonlymphoid leukemia (ANLL) [26]. Almost one fifth of patients experience relapse during the first 3 months of remission. Therefore, any intervention introduced after 3 months of remission will be followed by an apparently better relapse-free survival than no intervention because these early relapses are discounted. If a comparison is made between patients who receive the intervention and cohorts who do not, the relapse-free survival of those who do not receive the intervention will appear to be less because their number will include *all* the patients who experienced relapse in the first 3 months. In other words, the apparent result of a delayed intervention looks favorable for two reasons – exclusion of early relapse patients from the intervention group and their inclusion in the nonintervention group. An example is the initial comparison of BMT vs. chemotherapy for continuing remission of ALL in second hematological remission in the POG study 8303 [27]. A marginal superiority was noted for BMT with regard to remission duration. However, remission duration was measured from remission induction for the chemotherapy patients and from time of BMT, 3–28 weeks later, for BMT patients. Thus, early relapse patients reduced the apparent failure rate of BMT and increased the apparent failure rate of chemotherapy without BMT.

Conclusions

In determining the value of alternative therapeutic interventions in childhood acute leukemia, two questions need to be answered. Which treatment results in the higher cure rate, and what is the relative cost/benefit ratio of the treatments? At present there is no demonstration of superior survival of children treated with allogeneic BMT for ANLL in first remission, ALL with "unfavorable prognostic factors" in first remission, or ALL in second remission. There is also no demonstration of superior survival with bone marrow autografts. At the same time, the immediate toxicity and late sequelae of these procedures are clearly greater than with modern chemotherapy, especially current successful protocols that avoid or minimize use of radiation therapy, anthracyclines, and alkylating agents [28].

For these reasons BMT and autograft procedures in children with acute leukemia need to be reserved for experimental investigations in those leukemias and preleukemias that are clearly demonstrated to be usually fatal with current chemotherapy regimes. Secondly, the investigations should be collaborative and prospective with randomization for BMT immediately prior to myeloablation, optimal graft procedures and chemotherapy regimes, and comparable specialized medical care. Just as important, there must be complete accounting and description of the health and growth of survivors as well as meticulous data analysis and reporting.

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Proliferation-Inducing Effects of Recombinant Human Interleukin-7 and Interleukin-3 in B-Lineage Acute Lymphoblastic Leukemia *

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Introduction

Acute lymphoblastic leukemia (ALL) is a clonal disorder characterized by derangements of self-renewal and differentation of lymphoid precursor cells in the bone marrow. Corresponding to the inconsistent stimulatory effects of the recombinant hematopoietic growth factors studied to date on B-lineage ALL blasts in vitro [1], a reproducible culture assay that supports proliferation and maturation of ALL blasts has not yet been reported.

Recently, a new cytokine has been defined by its stimulatory effects on DNA synthesis in murine pre-B cells from Whitlock-Witte culture [2]. This stromal cell-derived cytokine, termed interleukin-7 (IL-7), has been purified and molecularly cloned, and the recombinant murine and human proteins are now available [3, 4]. Since IL-7 also stimulates murine pre-B cells from bone marrow [5], murine thymocytes, and, as comitogen, mature T cells [6] and induces proliferation of human T cells [7], we investigated whether IL-7 could stimulate DNA synthesis in B-lineage ALL blasts in suspension culture and also the capacity of IL-7 to induce blast cell maturation in vitro.

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Methods

Low-density peripheral blood ($n = 10$) or bone marrow ($n = 4$) cells were separated by Ficoll-Hypaque density centrifugation and were classified as common ALL (cALL; including pre-pre-B-lineage all and pre-B-lineage ALL; $n = 10$; HLA-DR+/CD 10+/CD 19+/sIg−) or B-lineage ALL ($n = 4$; HLA-DR+/CD 10− or +/CD 19+/sIg+) [8]. All samples were depleted of adherent cells and then incubated with OKT4, OKT8, and OKM1 and rabbit complement in order to eliminate mature myeloid and T-lymphoid cells. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM), 20% fetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin supplemented by IL-7 (50 U/ml, Immunex) or IL-3 (50 ng/ml, Behring) and incubated in 96-well plates at $1-2.5 \times 10^5$ cells per well (quadruplicate values). After 7 days of liquid culture, samples were pulsed with [^{3}H]thymidine (1 μCi/well) for 4 h and harvested on nitrocellulose filters. Thymidine uptake was defined by liquid scintillation counting. Cells of responsive samples were further characterized by four-parameter flow cytometry using a panel of monoclonal antibodies (moAb) and by immunogenotyping in order to monitor individual leukemic cell populations prior to and after suspension culture. All moAbs were from Becton and Dickinson (anti-CALLA CD10, Leu12 CD19, Leu16 CD20, anti-κ, anti-λ, anti-HLA-DR, LeuM9 CD33, anti-HPCA1 CD34), Coulter Clone (My7 CD13) and Medac (goat anti-mouse IgG for indirect

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

immunofluorescence staining). After culture, one sample was incubated with Leu 12 and propidium iodide (PI) in order to examine viability and CD 19 expression in different cell populations characterized by their light scatter properties. Southern blot analysis was performed as previously described [9]. *Eco*RI and *Hin*dIII digests were hybridized to a 2.4-kb *Sau* 3 a JH probe and *Bam*HI and *Hin*dIII digests to a 1.3-kb *Eco*RI Cμ, as well as Ck probe to demonstrate Ig gene rearrangements. To analyze configuration of T-cell receptor genes *Eco*RI, *Bam*Hi and *Hin*dIII digests were hybridized to a TCRβ probe and to a TCRγ probe, and *Hin*dIII- and *Bgl*II-digested DNA was hybridized to TCRδ probe $J\delta_{S16}$.

Results and Discussion

The stimulation indices (SI), defined as counts per minute (cpm) of the sample/cpm of control are given in Table 1. With an arbitrary cut-off of a SI > 5,4 of 10 cALL samples (5, 7, 8, 9) and 1 of 4 B-ALL samples (14) were stimulated by IL-7. IL-3 stimulated DNA synthesis in 5 of 9 cALL and 3 of 4 B-ALL samples. In two of the cALL samples responsive to IL-7, IL-7 was more potent than IL-3 (8, 9), while IL-3 was more effective than IL-7 in all B-ALL samples examined.

To further define the nature of proliferating cells and the maturation stage of the leukemic blasts, samples 6, 8, 9, and 14 were analyzed by four-parameter flow cytometry and Southern blot analysis prior to and after liquid culture. Table 2 summarizes the results of immunophenotyping; Fig. 1 presents the analysis of sample 14. Analysis gates were fitted to light scatter properties of PT-negative cells in order to gate preferentially viable cells after liquid culture. Sample 6, which was Ph^+, revealed a marked increase in the percentage of $CD33^+$ and $CD13^+$ cells at day 7, suggesting that non-lymphoid cell populations preferentially proliferated during suspension culture. In sample 8, a decrease in CD 34 and CD 19 expression and in the percentage of CD 10/CD 19 double-positive cells were detectable, while the percentage of $CD20^+$ cells was unchanged. Sample 9 revealed a decrease in $CD34^+$ cells, but CD 19 expression was unchanged and CD 10/CD 34 double-positive cells were detectable after 7 days of liquid culture. A net increase in $CD19^+$ cells (data not

Table 1. Stimulation indices of ALL blasts stimulated by IL-7 and IL-3

Sample	Diagnosis	IL-7 (50 U/ml)	IL-3 (50 ng/ml)
1	cALL	1.5	1.5
2	cALL	2.6	nd
3	cALL	3.3	0.6
4	cALL	1.3	1.8
5	cALL	5.1	6.7
6	cALL Ph	2.3	6.8
7	cALL	15.8	25.5
8	cALL	5.9	1.5
9	cALL	53.6	37.1
10	cALL	3.4	45.1
11	B-ALL	1.8	3.5
12	B-ALL	2.6	9.2
13	B-ALL	1.5	15.6
14	B-ALL	8.9	18.3

Mean SI of quadruplicate cultures (cpm sample/cpm control). Control cpm were < 850 in all cases examined.

Table 2. Surface marker analysis prior to and after suspension culture of ALL samples

Sample[a]	Antigen	% positive cells	
		Day 0	Day 7
6 cALL	CD 34	11	54
	CD 33	3	43
	CD 13	< 1	75
8 cALL	CD 34	24	9
	CD 19	69	45
	CD 20	36	41
	CD 10/CD 19	69	37
9 cALL	CD 34	96	75
	CD 19	68	64
	CD 20	6	4
	CD 10/CD 19	63	47
	CD 10/CD 34	92	42
14 B-ALL	CD 34	< 1	< 1
	CD 19	14	57
	CD 20	72	82
	sIgκ	36	< 1

[a] Samples 8, 9, and 14 were stimulated by IL-7 (50 U/ml) and sample 6 by IL-7 (50 U/ml) combined with IL-3 (50 ng/ml).

shown), combined with a lack of surface-bound Ig κ light chain, was found in sample 14, suggesting proliferation of B-lineage restricted cells during culture. The lack of surface-bound Ig light chain and the consistent pattern of Ig recombination prior to and after liquid culture (see below) suggest that leukemic transformation occurred at a maturation level preceding B-cell stage. Maturation induction, e.g., expression of surface-bound Ig after liquid culture, was not detectable in any of the examined cases.

As indicated in Table 3, the immunogenotype corresponded to the immunophenotype in the cases examined. In order to monitor individual leukemic cell populations, defined by specific molecular genetic markers, cells were analyzed prior to and after liquid culture. In cases 9 and 14, IL-7 and IL-3 induced almost exclusively proliferation of the leukemic cell clone as concluded from the consistent pattern of Ig recombination prior to and after liquid culture. In cases 6 and 8, nonleukemic cell proliferation associated with the generation of $CD33^+$ and $CD13^+$ cells (case 6) and detection of burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte-

Table 3. Immunogenotype of ALL samples

Sample	Phenotype	Rearrangement	Proliferative population[a]
6	cALL Ph	IgH, TCR δ	Nonleukemic
8	cALL	IgH, TCR γ, TCR δ	Nonleukemic
9	cALL	IgH, TCR γ, TCR δ	Leukemic
14	B-ALL	IgH, Ig κ	Leukemic

[a] Proliferation of the leukemic cell clone was concluded from the consistent pattern of Ig recombination observed prior to and after liquid culture.

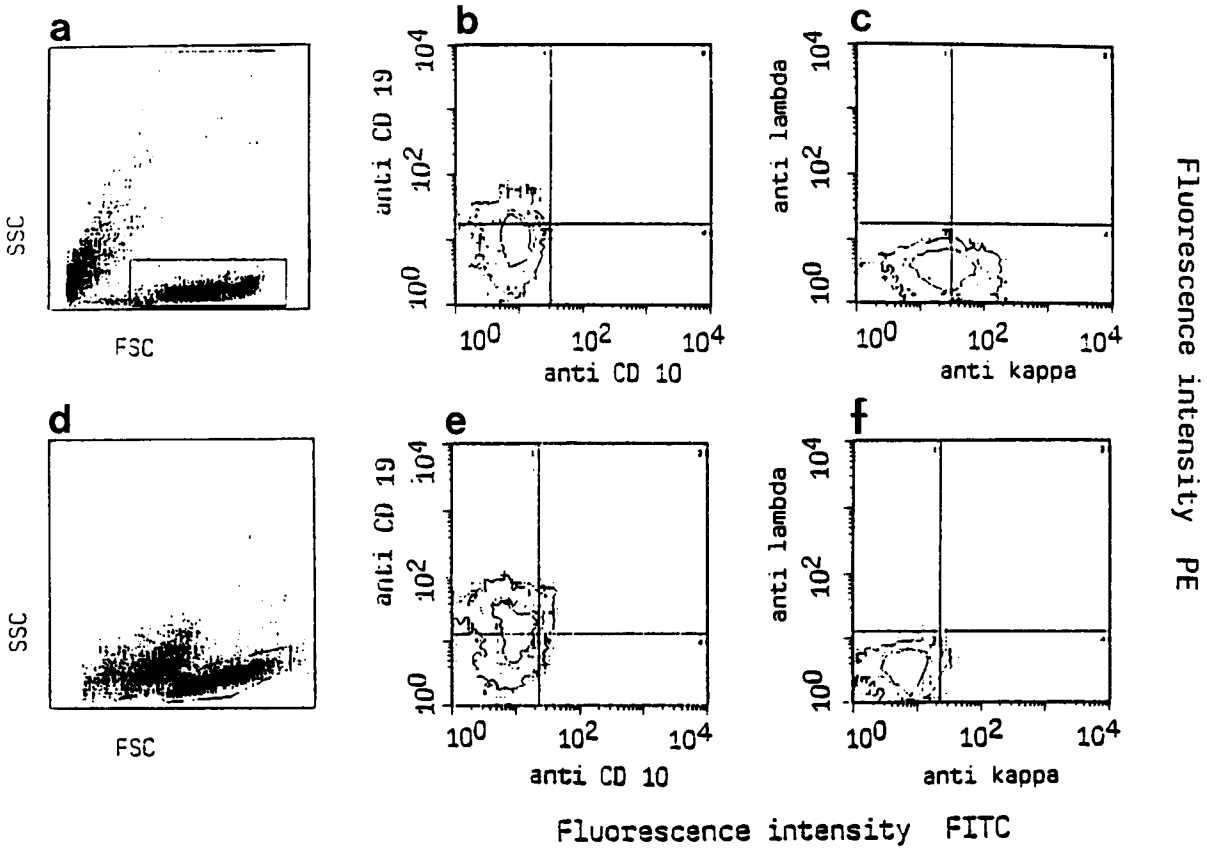

Fig. 1a–f. Four-parameter flow cytometry of sample 14 prior to and after 7 days of liquid culture. Cells were analyzed prior to (**a–c**) and after 7 days of liquid culture (**d–f**). **a**, **d** Dot plot diagrams of foreward (FSC) and sideward scatter (SSC) properties. Viability of gated cells was 93% (**a**) and 98% (**d**). **b**, **c**, **e**, **f** Contour graphs from two-color FACS analysis. Samples were stained as indicated. Quadrants were chosen corresponding to controls stained by unspecific MsIgG-FITC and MsIgG-PE

macrophage (CFU-GM) after liquid culture (case 8; data not shown) was observed when cultures were stimulated by IL-7 or IL-3. The detection of non-leukemic cells generated during suspension culture stimulated by IL-7 or IL-3 underlines the necessity to define exactly the nature of proliferating cells in responsive samples.

We conclude that IL-7 and IL-3 stimulate proliferation of leukemic cells in a subset of B-lineage restricted ALL without evidence of concurrent maturation induction. However, additional growth factors may be required to improve the in vitro culture of ALL blasts.

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Effects of Granulocyte Colony-Stimulating Factor in Children with Severe Congenital Neutropenia *

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Introduction

Severe congenital neutropenia (SCN; Kostmann's syndrome), a disorder of myelopoiesis, is characterized by an impairment of myeloid differentiation in bone marrow with absolute neutrophil counts (ANC) below 200/μl in blood of affected patients [1–6]. This disorder was first described by Kostmann [1, 2]. Patients with SCN experience frequent episodes of fever, pneumonitis, skin infections, perianal and liver abscesses, usually beginning in early infancy and often leading to fatal infections in spite of antibiotic therapy. Bone marrow morphological findings in these patients have been variable, but a maturation arrest of myelopoiesis at the promyelocyte stage is usually seen [1–6]. Several methods of therapy have been attempted in these patients including white cell transfusions, corticosteroids, vitamin B_6, lithium, androgens, and bone marrow transplantation (BMT). To date, only BMT has resulted in partial or complete correction of the neutropenia [7, 8]. The etiology of SCN is unknown. There is no evidence for serum inhibitors of myelopoiesis or antineutrophil antibodies in these patients.

Recently, granulocyte colony-stimulating factor (G-CSF) has been purified, molecularly cloned, and expressed as recombinant protein [9, 10]. It has been shown to be a potent stimulus for normal myeloid proliferation and differentiation in vitro [9, 10] and in vivo [11, 12]. Using bone marrow cells from SCN patients, colony-forming unit-granulocyte macrophage assays (CFU-GM) in the presence of recombinant human G-CSF (rhG-CSF) demonstrated predominantly monocyte/macrophage colonies. The growth of neutrophil colonies with G-CSF as growth factor was significantly diminished (own unpublished observation).

In a previous clinical trial we investigated the effects of granulocyte-macrophage-CSF (GM-CSF) in SCN patients [13] and because only one of seven patients showed any increase in circulating neutrophils we subsequently initiated a study with G-CSF. The objectives of this study were to determine the biological effectiveness of G-CSF in the treatment of SCN in order to design an optimal therapy for this fatal disease.

Methods

Patients

Thirty patients (14 girls, 16 boys; aged between 2 months and 21 years) with SCN were treated in a phase II clinical trial with recombinant human G-CSF (rhG-CSF). Seven patients had been pretreated with rhGM-CSF [13] up to 1–3 months prior to rhG-CSF. The diagnosis of SCN was established on the following basis:

1) absence of blood neutrophils (< 200/μl),

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

2) maturation arrest in the neutrophil lineage at the promyelocyte stage in bone marrow with normal cellularity, and
3) history of frequent severe bacterial infections starting during the first 12 months of life.

In general, these patients also experience compensatorily increased monocyte and eosinophil counts, and elevated immunoglobulin levels. The leukocyte counts prior to rhG-CSF treatment ranged between 3200 and 10.600/µl.

Treatment with rhG-CSF

All patients started their treatment with 3 µg/kg per day rhG-CSF subcutaneously (s.c.). The next doselevels were 5, 10, 20, 30, 40, and 60 µg/kg per day. If no response was observed by day 14 of any dose level, patients were moved to the next dose level. Two patients who did not respond to 60 µg/kg per day s.c. were treated with 120 µg/kg per day rhG-CSF continuous intravenously (cont. i.v.). Patients with complete responses (ANC > 1000/µl) at any dose level were eligible for enrollment into a maintenance treatment. rhG-CSF was provided by Amgen (Thousand Oaks, CA). It was expressed in *E. coli* and purified to homogeneity. The rhG-CSF has a specific activity of approximately 10^8 U/mg protein [10]. It was endotoxin-free as judged by the rabbit pyrogen test and by the limulus amebocyte lysate assay.

NFS-60 Proliferation Assay for the Measurement of G-CSF

The murine myeloblastic leukemia cell line, NFS-60 [14], was used to determine G-CSF levels in sera from patients with SCN. Serial dilutions of sera from SCN patients prior to rhG-CSF therapy and appropriate controls were incubated with NFS-60 cells (10^6/ml) for 48 h in 96-well flatbottom microtiter plates (Nunc, Roskilde, Denmark; 200 µl/well). Identical samples were also tested in the presence of the neutralizing anti-G-CSF antibody 74 A (4 µg/ml; Amgen, Thousand Oaks, USA). [^{3}H]Thymidine (0.5 µCi/well; Amersham-Buchler, Brunswick, FRG) was added for the last 4 h of culture. Cells were then lysed and DNA harvested on glass fiber strips. Incorporated radioactivity was measured in a liquid scintillation counter. Serial dilutions of rhG-CSF were used as standard, the concentrations of the samples were calculated by probit analysis from the standard curve and shown in picograms per milliliter.

Results

Serum Levels of G-CSF in SCN Patients Prior to rhG-CSF Therapy

Sera from six patients (numbers 4, 6, 9, 12, 16, 28) were investigated prior to rhG-CSF treatment for G-CSF activity using the NFS-60 proliferation assay. Sera from patients with SCN demonstrated significantly higher G-CSF levels than sera from controls (Table 1). From these proliferation data we calculated the amount of G-CSF for patients (between 150 and 670 pg/ml) and for controls (0–100 pg/ml). The addition of neutralizing antibody to these assays reduced the biologic activity of the G-CSF-containing sera by between 60% and 100% (Table 1).

Effects of rhG-CSF on Blood Cells

The effects of rhG-CSF on blood neutrophil counts are shown in Fig. 1 and in detail for patient 8 in Fig. 2. rhG-CSF administration led, in 29 of 30 patients, to an increase in ANC to levels above 1000/µl (Fig. 1). The dosage needed to achieve an ANC of 1000 was between 3 µg/kg per day and 60 µg/kg per day s.c. or 120 µg/kg per day cont. i.v. Patient 9 showed only a minor response even at the

Table 1. G-CSF concentration of serum samples from patient with SCN (proliferation of NFS-60 cells)

	G-CSF (pg/ml)		Inhibition by anti-G-CSF antibody[a] (%)
	Median	100% range	
Patients (6)	300	150–670	60–100
Controls (11)	25	0–100	n.d.

n.d., not done
[a] Monoclonal anti-G-CSF antibody 75A (IgG_2), 4 µg/ml

highest dose (120 µg/kg per day cont. i.v.), with an increase in ANC to only about 200/µl. The dosage required to maintain an ANC of above 1000/µl was different in each patient: Fifteen patients required rhG-CSF dosages between 1 and 8 µg/kg per day, 9 patients between 10 and 20 µg/kg per day, and 4 patients between 40 and 60 µg/kg per day. In one patient (number 5) the rhG-CSF maintenance dosage was reduced to 0.8 µg/kg per day because of a vasculitis, most likely due to high neutrophil counts [13]. Her neutrophil counts ranged between 500 and 1000/µl. Mean and standard deviation of all invidual measurements of neutrophil counts from all patients prior to therapy and within the time intervals 1–4, 5–12, 13–52, and 53–104 weeks of rhG-CSF treatment are shown in Fig. 1. Due to the oscillation of neutrophil counts observed in all patients (see also Fig. 2) the standard deviation at a given point in time is high. However, this oscillation did not affect the beneficial clinical responses. The neutrophils did show normal functions as judged by phagocytosis, intracellular killing of staphylococci, and reactive oxygen production [15]. The absolute eosinophil counts (AEoC) did not change significantly during rhG-CSF therapy in any patient. The absolute monocyte counts (AMC) increased two- to eight-fold in the majority of patients during rhG-CSF treatment. The most dramatic increase in AMC was seen in patient 3. However, he started the rhG-CSF treatment with an

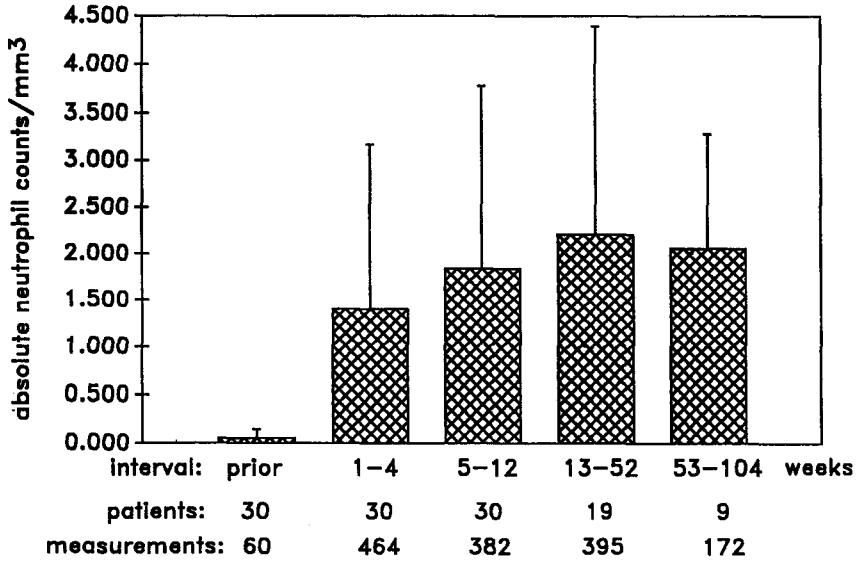

Fig. 1. Mean and standard deviation (SD) of all individual measurements of absolute neutrophil counts from all patients with severe congenital neutropenia prior to and within weeks 1–4, 5–12, 13–52, and 53–104 of rhG-CSF treatment

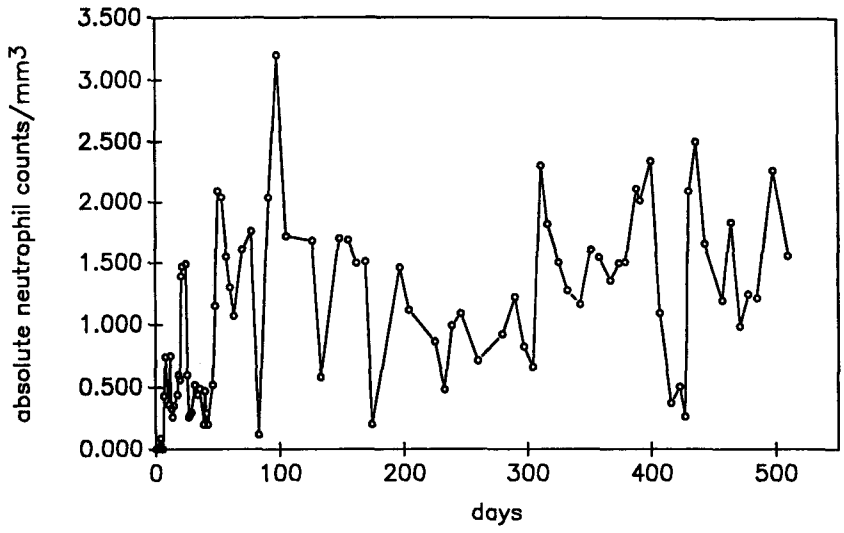

Fig. 2. Absolute neutrophil counts of patient 8 during maintenance treatment. The maintenance dosage of rhG-CSF was 10 μg/kg per day s.c.

already excessively high AMC (3438/μl), which increased further up to 24800/μl during the first 6 weeks of treatment.

The number of CFU-GMs, myeloblasts and promyelocytes in the bone marrow during rhG-CSF treatment did not change significantly during rhG-CSF maintenance treatment.

Clinical Responses

During rhG-CSF treatment, the peptostreptococcus-caused lung infiltrates in patient 1 dramatically resolved within 6 weeks of therapy. Prior to rhG-CSF treatment she received 6 weeks of i.v. antibiotics in a hospital setting. During the first 6 weeks of rhG-CSF treatment, her pulmonary situation resolved to a degree that the i.v. antibiotics could be replaced by prophylactic oral antibiotic therapy. This resolution appeared in association with the increase in neutrophils. Patient 11 suffered from a life-threatening severe lung abscess which had destroyed most of the normal lung tissue of the left lung. She did not respond to up to 60 μg/kg per day s.c., but responded to 120 μg/kg per day cont. i.v. with an increase in ANC to above 1000/μl. Her left lung could then be removed without complications. Interestingly enough, after the removal of the infected lung tissue, the rhG-CSF dosage could be reduced to 50 μg/kg per day s.c., maintaining an ANC above 1000/μl. In patient 12 a severe anal abscess and anal fistula which had persisted for about 1 year prior to rhG-CSF treatment in spite of surgical intervention and antibiotic treatment resolved within 3 months during rhG-CSF therapy. Patient 13 had suffered for more than 2 of the 3 years of his life from fungal liver abscesses. As soon as the neutrophils increased, the liver abscesses shrank and were not detectable anymore at a second-look laparotomy on day 90 of rhG-CSF treatment. No new severed bacterial infections have developed in these patients.

Adverse Events

The adverse events included necrotizing cutaneous vasculitis (patient 5), generalized vasculitis (patient 17), and mesangioproliferative glomerulonephritis (patient 22), all associated with a prompt increase in ANC and not with the dose of rhG-CSF. All three patients suffered from these side effects at the lowest dose of rhG-CSF (3 μg/kg per day). Patient 5 now receives rhG-CSF at a dose of 0.8 μg/kg per day. At this dose, she has ANC of 500–1000/μl without further

recurrence of the vasculitis. In patients 17 and 22, rhG-CSF was discontinued. Patient 17 developed acute monoblastic leukemia 6 months after dicontinuation of rhG-CSF therapy. Patient number 6 developed myelodysplasia 2 years after initiation of rhG-CSF treatment. Two patients suffered from mild hematuria and one patient from mild thrombocytopenia. In these three patients, rhG-CSF treatment could be continued without clinical problems.

Discussion

In this study, rhG-CSF induced an increase of blood neutrophils in 29 of 30 patients. The dose necessary to reach and maintain an ANC of above 1000/μl varied from patient to patient and ranged between 3 and 120 μg/kg per day. The neutrophils in the rhG-CSF-treated patients had normal functional activities as judged by in vitro functions and by clinical parameters. In four patients, there was resolution of severe bacterial infections (pneumonitis, lung abscess, liver abscess, anal abscess) resistant to i.v. antibiotic treatment prior to rhG-CSF therapy. The maintenance treatment did not lead to an exhaustion of myelopoiesis: 29 patients have now been treated for 12 months and longer. The ANC of all patients was stable during the maintenance treatment and no increases in the dosage were necessary for maintaining the ANC during long-term treatment. The number and severity of infections decreased significantly in all patients during rhG-CSF treatment as compared to a similar time period prior to therapy. Additional SCN patients have been treated with rhG-CSF by Bonilla et al. [16] and showed similar increases in ANC.

The hypotheses for the pathomechanism of the underlying disease include defective production of G-CSF, or defective response of neutrophil precursors to G-CSF or other hematopoietic growth factors. A defect of G-CSF production does not seem likely in light of new data which show that serum from these patients contain normal or elevated levels of G-CSF as judged by western blot analysis [17] and in vitro bioassays (Table 1). However, these endogenous G-CSF levels are not sufficient to induce maturation of neutrophil precursors in SCN patients. Therefore, the more attractive hypothesis for the genetic disposition affecting these patients involves a defective G-CSF response, either by reduced binding affinity of G-CSF to its receptor, low G-CSF receptor numbers, or defective intracellular signal transduction. Different mutations in molecules involved in the G-CSF response could explain the variations from patient to patient to achieve an ANC of 1000/μl, and the need for pharmacological dosing (3–120 μg/kg per day) to reach this low but adequate neutrophil level supports this hypothesis. These dosages would induce an ANC of 20000–100000/μl in other patients [12].

There were side effects from rhG-CSF treatment in these patients. Two patients experienced a vasculitis, and one a mesangioproliferative glomerulonephritis. Since these side effects were clearly associated with ANC of above 1000/μl and not with the dose of rhG-CSF, the relatively increased numbers of neutrophils have to be considered as the cause for these adverse events. The pathogenetic mechanisms of the vasculitis could be explained by infiltration of inflamed vessel walls with neutrophils and mononuclear cells and subsequent disruption of the small superficial cutaneous vessels. Deposits of immunoglobulins, complement components, or circulating unspecific immune complexes, all compensatively elevated in the blood of this patient, may have potentiated this process. The development of myelodysplasia and acute monoblastic leukemia is most likely due to the underlying disease suggesting that congenital neutropenia is a preleukemic state. This is supported by data published prior to G-CSF treatment [18].

These findings demonstrate that rhG-CSF is the most promising of all available treatments for SCN. The correction of neutropenia with resultant improvement of clinical status can dramatically change the high morbidity and therefore the quality of life in these patients. The risk of death from severe bacterial infections will most likely be diminished. These results show also the feasibility of maintenance treatment with rhG-CSF for up to 2 years without exhaustion of myelopoiesis, and the benefical effects of rhG-CSF in patients with SCN.

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Interleukin-1 Production in Patients with Nonlymphocytic Leukemia and Myelodysplastic Syndromes

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A common feature of all cases of myeloid leukemia is a block in normal maturation of blast cells which may be associated with the disturbance in the biochemical pathways of receptor cross-modulation or with the control of expression of the genes encoding various differentiation factors [9].

In some cases the leukemic cells can produce colony-stimulating factor (CSF) [6]. It should be noted that leukemic cells from many patients do not proliferate in vitro in the presence of CSF, but those that do not grow in vitro obviously may proliferate in vivo [9]. These findings suggest that there may be some other hematopoietic growth factors which participate in the regulation of leukemic cell proliferation. One of them may be interleukin-1 (IL-1).

IL-1 does not induce normal hematopoietic cell colonies but has been reported to act synergistically with the colony-inducing cytokines (IL-3, CSFs) [8, 12]. On the other hand, it had been revealed that IL-1 also participates in the regulation of leukemic myelopoiesis. IL-1 can induce differentiation in certain murine myeloid leukemic clones (SL, mouse r1) so that this is mediated by the endogeneous production of the differentiation-inducing protein MGI2 and is also associated with the production of granulocyte-macrophage CSF (GM-CSF) [5].

The present work was carried out to determine IL-1 production in patients with myelodysplastic syndrome (MDS) and nonlymphocytic leukemia (NLL). MDS represents a diverse group of disorders with mild, relatively benign form and disorders with impressive symptoms and rapid progression to overt leukemia. In all instances MDS is a typical clonal disease in which the abnormal hematopoietic cells are derived from the mutated stem cell and the malignant clone progressively replaces normal hematopoiesis. It is especially interesting to examine the role of IL-1 in the transformation of MDS to leukemia.

Materials and Methods

Patients with acute NLL and MDS were examined. The diagnosis of acute myeloid leukemia (AML), acute myelomonocytic leukemia (AMML), or MDS was made using FAB criteria following conventional cytochemical staining. All blood samples were obtained prior to therapeutic manipulations or 2–3 weeks after the last steroid dose or chemotherapy.

Mononuclear cells were prepared by Ficoll-Pack sedimentation. Adherent cells were cultured for 24 h in a CO_2 incubator in Dulbecco's medium supplemented with 5% fetal calf serum with or without lipopolysaccharide (LPS) from *Bacillus prodigiosum*. Spontaneous and LPS-induced IL-1 production was determined by conventional murine thymocyte comitogenic assay [7]. IL-1 concentration was calculated using a standard recombinant human IL-1 B preparation and expressed in units per milli-

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

meter and in units per million adherent cells.

Data were analyzed by nonparametric methods to avoid assumptions as to the distribution of the variables under study, using the Mann-Whitney test. χ^2 criteria were also used. Values are given as mean ± SEM.

Results

Spontaneous and Induced IL-1 Production in Acute Leukemia

Spontaneous IL-1 production was found in 5 of 14 examined patients with acute NLL (ANL) (Table 1). Various levels of spontaneous IL-1 production were also found in 3 of 18 healthy donors examined (30, 80 and 160 U/ml). Differences in spontaneous IL-1 production were found between the ANL patients and healthy donors (see Table 1). The induced IL-1 production in patients with ANL was 96.9 ± 29.5 U/ml and it did not differ from the rate in the control group. However, IL-1 production in patients with AML was similar to that in patients with AMML. Induced IL-1 production was not detectable in 5 of 14 patients with ANL but it was found in all healthy donors.

Figure 1 shows the relation between the patients' adherent cell counts and IL-1 production in U/ml culture media and IL-1 production per 10^6 adherent cells. It is clear that the decrease in IL-1 production in some patients was not caused by the decrease in adherent cell counts. In most patients there was a normal rate of IL-1 production and a rather high rate of production of IL-1 per cell. In two patients, the adequate IL-1 production was due to increased production of IL-1 per cell, accompanied by simultaneously decreased adherent cell count. In the other patient, IL-1 production was significantly higher, but it may be considered to be caused by increased adherent cell counts and by the cells enhanced ability to produce IL-1 in these patients. The data indicate that patients with acute leukemia represent a diverse group in relation to IL-1 production (Fig. 2).

Spontaneous and Induced IL-1 Production in Patients with MDS

Spontaneous IL-1 production was determined in 3 of 6 patients examined with refractory anemia (RA) without excess blasts (Table 1). In all patients with chronic myelomonocytic leukemia (CMML) spontaneous IL-1 production was found. Its level was significantly higher than in the control group. Induced IL-1 production in patients with RA did not differ from that in healthy donors (Table 2). Decreased induced IL-1 production were found in patients with RA with excess blasts (RAEB) and with RAEB in transformation (RAEBtr). Patients with CMML had greatly increased levels of both spontaneous and induced IL-1 production. The relation between the CMML patients' adherent cell counts

Table 1. Spontaneous IL-1 production (mean ± SEM) in patients with acute leukemia and MDS

Groups of patients	Number of patients	Patients with spontaneous IL-1 production	Spontaneous IL-1 production	
			(U/ml)	($U/10^6$ cells)
Healthy subjects	18	3	110 ± 33	1413 ± 420
ANL	14	4	2077 ± 1927	18270 ± 13843
RA	6	3	279 ± 148	2198 ± 1414
RAEB + RAEBtr	5	0	0	0
CMML	5	5	4232 ± 3872	ND

ND, no data.

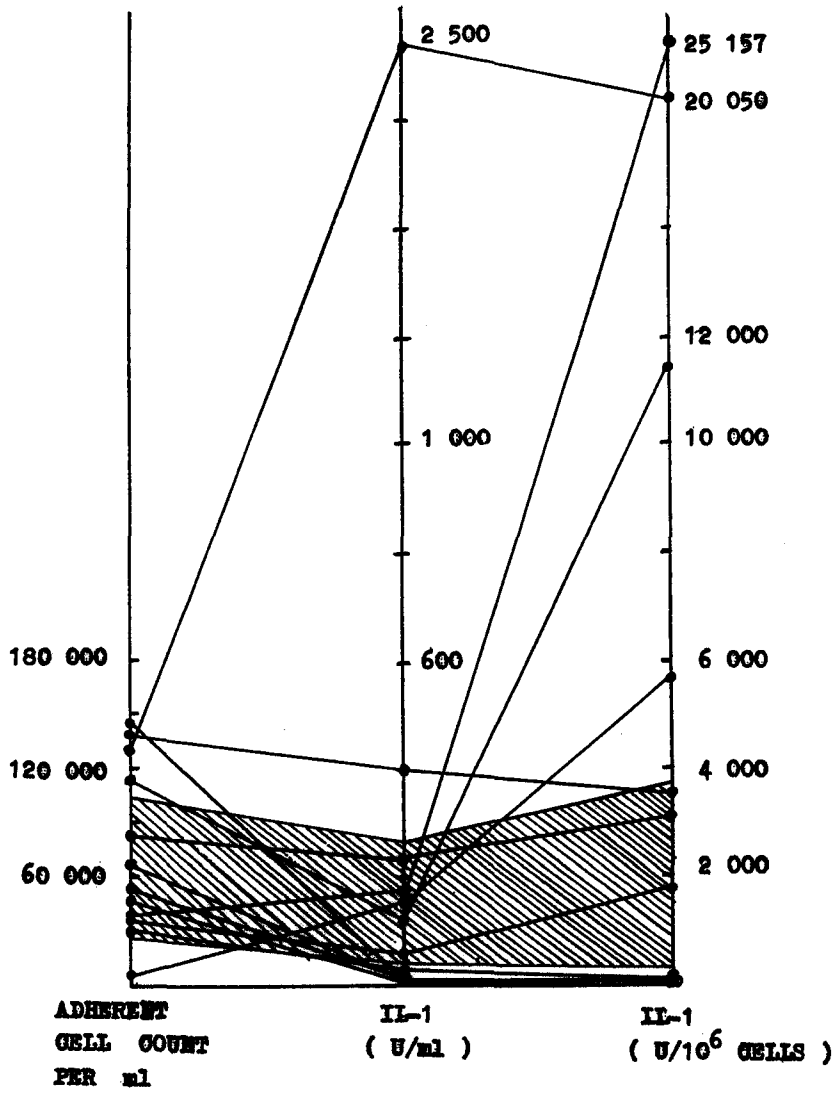

Fig. 1. Correlation between the adherent cell counts and IL-1 production in patients with acute leukemia

Table 2. Induced IL-1 production (mean ± SEM) in patients with acute leukemia and myelodysplastic syndromes

Groups of patients	Number of patients	Patients without detectable IL-1 production	LPS-induced IL-1 production		
			U/ml	$U/10^6$ cells	χ^2
Healthy subjects	15	0	123 ± 30	1721 ± 480	
ANL	14	5	102 ± 33	4251 ± 1908	8.28 ($\beta < 0.057$
AML	9	4	79 ± 33	4127 ± 827	
AMML	5	1	154 ± 86	4531 ± 1701	
RA	6	1	277 ± 92	2982 ± 993	
RAEB + RAEBtr	5	3	40 ± 25[a]	464 ± 354	5.76 ($\beta < 0.05$)
AML after myelodysplasia	5	2	118 ± 66	5368 ± 4022	
CMML	5	0	4756 ± 3528	10915 ± 4646	

[a] Analysis was performed by the nonparametric Mann-Whithney test, $\beta < 0.05$.

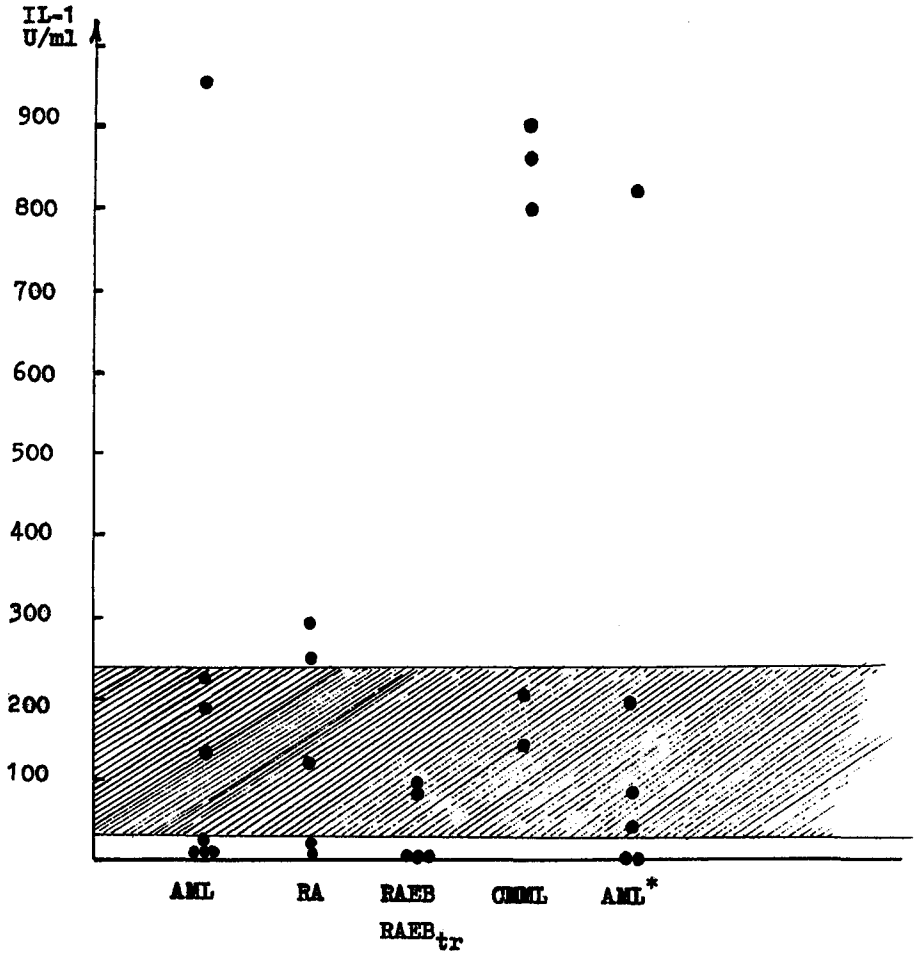

Fig. 2. Induced IL-1 production in patients with ANL and MDS. The *shaded area* represents the normal values (M + δ). In 27% of patients with MDS and 35% of patients with acute leukemia no IL-1 production was detectable.

and IL-1 production is presented in Fig. 3. Patients with CMML also represent a diverse group – 3 patients had a higher IL-1 production level than others. IL-1 production in patients with ANL differed significantly from that of CMML and CML patients. In some patients IL-1 production was not determined.

Survival was analyzed in patients with normal IL-1 production and those with decreased IL-1 production (Fig. 4). The mean value of IL-1 production in healthy donors was 122.7 ± 30.0 U/ml. A normal induced IL-1 production level was considered as one which was higher than the mean value in healthy donors minus SE, while a decreased level was considered to be lower than the mean value minus SE. No significant differences in survival of patients with normal or decreased IL-1 production levels were found.

Discussion

In this report we have demonstrated that IL-1 production by peripheral blood monocytes is not identical in patients with acute leukemia and in patients with MDS. In every group of patients there were significant differences in IL-1 production. The data imply that leukemia and MDS represent a diverse group of disorders with markedly different substrates of proliferation and different levels of cellular maturation. In all instances, MDS is a typical clonal disease in which the abnormal hematopoietic cells are derived from the same mutated stem cell but

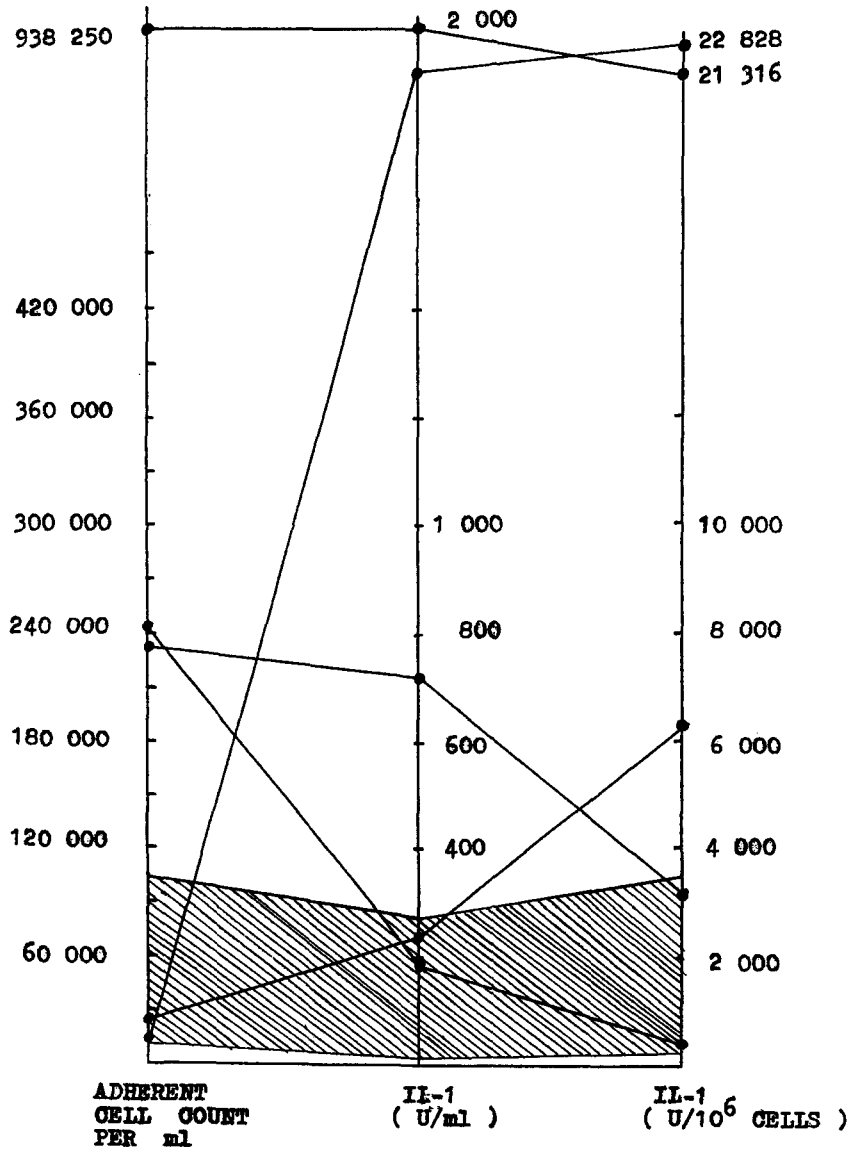

Fig. 3. Correlation between the adherent cell counts and IL-1 production in CMML patients

where clinical features and functional properties of abnormal cells may be different. Cells derived from the mutated clone often retain some form of maturation, leading to the peripheral appearence of abnormally differentiated cells with impaired function [9].

We have found two forms of disturbance of IL-1 production in patients with ANL and MDS. In the first case the IL-1 production was significantly increased; an especially high level was found in patients with CMML and in some patients with AMML. We suggest that the high IL-1 production was due to both an increase in monocytic cell counts in some patients and enhanced ability of individual cells to produce IL-1. Recently, it has been shown that monocytes of MDS patients may be abnormally differentiated cells [1]. Thus, the observed increase in IL-1 production in MDS patients may be due to enhanced IL-1 gene expression in these cells. The data obtained on the elevated induced IL-1 production in some patients agree with data from other authors. IL-1 may support proliferation of leukemia cells in patients with ANL [2–4, 10]. When freshly isolated adult T-cell leukemia (ATL) cells were cultured with recombinant or natural human IL-1a or IL-1b, the

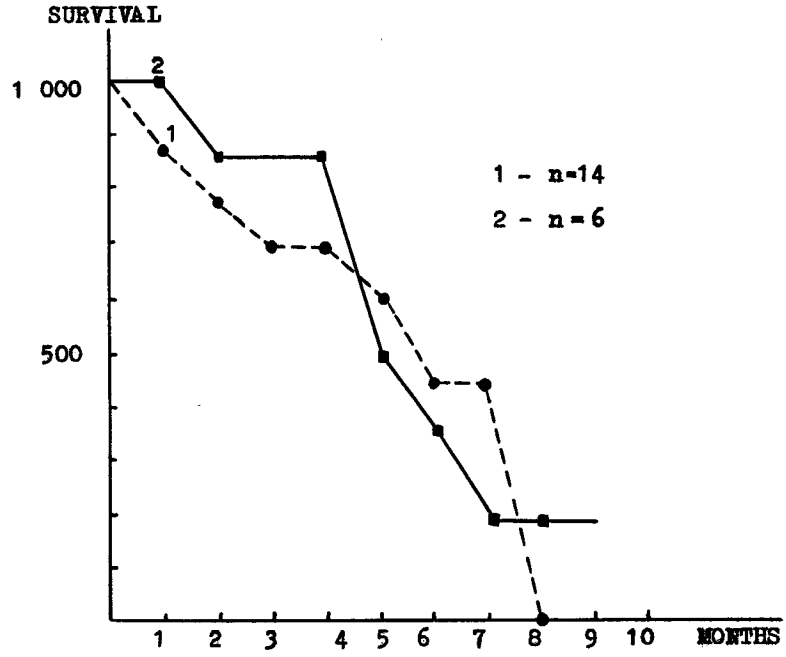

Fig. 4. Survival of patients with acute leukemia with various IL-1 production levels. *1* > M ± SE, *2* < M ± SE. No significant difference was revealed

growth of ATL cells was stimulated in a dose-dependent manner [11].

Regarding patients with MDS, a decreased induced IL-1 production was revealed in the group with RAEB plus RAEBtr, but in other groups there were no differences from the healthy donors. RAEB, RAEBtr, and overt leukemia may be considered as stages of one process, but the mechanism of transformation is not absolutely clear. It is possible that the decrease in IL-1 production in these patients may assist in the progression of disease. In the next stage of progression it is replaced by increased IL-1 production, which could support the leukemic proliferation.

On the other hand, in 27% of patients with MDS and in 35% of patients with ANL, no induced IL-1 production was detectable. What is the role of the lack of IL-1 production in the development of leukemia? This question remains unsolved, because even high IL-1 production does not guarantee the best survival.

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The Results of Treatment of Acute Lymphoblastic Leukemia Relapses in the Polish Children's Leukemia/Lymphoma Study Group

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Introduction

Within the past 15 years, improvement in the prognosis of childhood acute lymphoblastic leukemia (ALL) has been achieved [1]. In most studies, prolonged disease-free survival has ranged from 60% to 80% [2, 3]. Nevertheless, the quality of remission is unsatisfactory in about 30% of patients, resulting in recurrence of the disease [2, 3]. The chemotherapy method elaborated by the West German Study Group (BFM) seems to offer a new way for achieving a second long-term remission in relapsed ALL [4, 5].

The aim of this study was to evaluate the results of ALL relapse therapy in children treated in seven oncology centres of the Polish Children's Leukemia/Lymphoma Study Group.

Material and Methods

A total of 126 children (83 boys and 43 girls), aged between 6 months and 18 years (median 8.5 years), with a first relapse of ALL treated according to the BFM 1985 protocol during the years 1987–1990, were included in this study. The initial characteristics of the relapsed patients are given in Table 1.

The probability of event-free survival (EFS) in the children studied was calculated according to the Kaplan-Meier method [6].

Results

The median time from the date of obtaining the first complete remission (CR) to relapse was 24 months (3–34 months). For further analysis, the relapsed children were divided into two groups: early relapse when relapse occurred during therapy or within 6 months after completing treatment; and late relapse, when relapse occurred more that 6 months after the end of therapy. Early relapse was diagnosed in 83 patients and late relapse in 43 patients. Table 2 shows the type and time of the first ALL relapses in the children studied. A summary of the treatment response is shown in Table 3.

The EFS of the children with early and late ALL relapse treated according to the BFM protocol is shown in Fig. 1. The estimated EFS in the 30th month was 52% in the late relapse group and 14% in the early relapse group ($p = 0.02$).

The probability of EFS for the children in the early relapse group was better with an extramedullary localization than with bone marrow involvement (45% vs. 8%, $p = 0.02$). The difference in the late relapse group was not statistically significant (Figs. 2, 3).

Concluding Remarks

It was shown in our previous study that the BFM protocol produced an improve-

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Initial characteristics of the patients

	Patients		Age at initial diagnosis (years)		Age at first relapse	
	(*n*)	(%)	Mean	Range	Mean	Range
Boys	83	66	6	1–15	9	2–18
Girls	43	33	4	7/12–13	7	16/12–15

Duration of first complete remission: median, 38 months; range, 3–8 months.
Follow-up time after first relapse: median, 24 months; range, 3–34 months.

Table 2. Type and time of first ALL relapse in 126 children

Type	Early	Late	Total	
	(*n* = 83, 65%)	(*n* = 43, 35%)	(*n*)	(%)
Isolated (*n* = 104, 82,4%)				
Bone marrow	53	22	75	60
CNS	10	3	13	10
Testes	8	8	16	13
Total	71	33	104	
Mixed (*n* = 22, 17,6%)				
Bone marrow + CNS	5	4	9	8
Bone marrow + testes	2	5	7	8.5
Bone marrow + testes + CNS	1	–	1	1.3
CNS + testes	1	1	2	2.6
Other	3	–	3	3.9
Total	12	10	22	

Table 3. Summary of response to treatment of the first relapse (*n* = 126)

	Early relapses		Late relapses	
	n	%	n	%
Total number	83	100	43	100
Complete remision	56	67	36	84
No complete remision	27	33	7	16
death because of:				
ALL progression	23	28	6	14
infection	2	2	–	–
toxicity of therapy	2	2	1	2
Second relapse	40	48	16	37
Therapy related death in second complete remision	5	6	5	11
Still in second	11	13	15	35

Duration of complete remision: median, 20 months; range, 1–60 month.

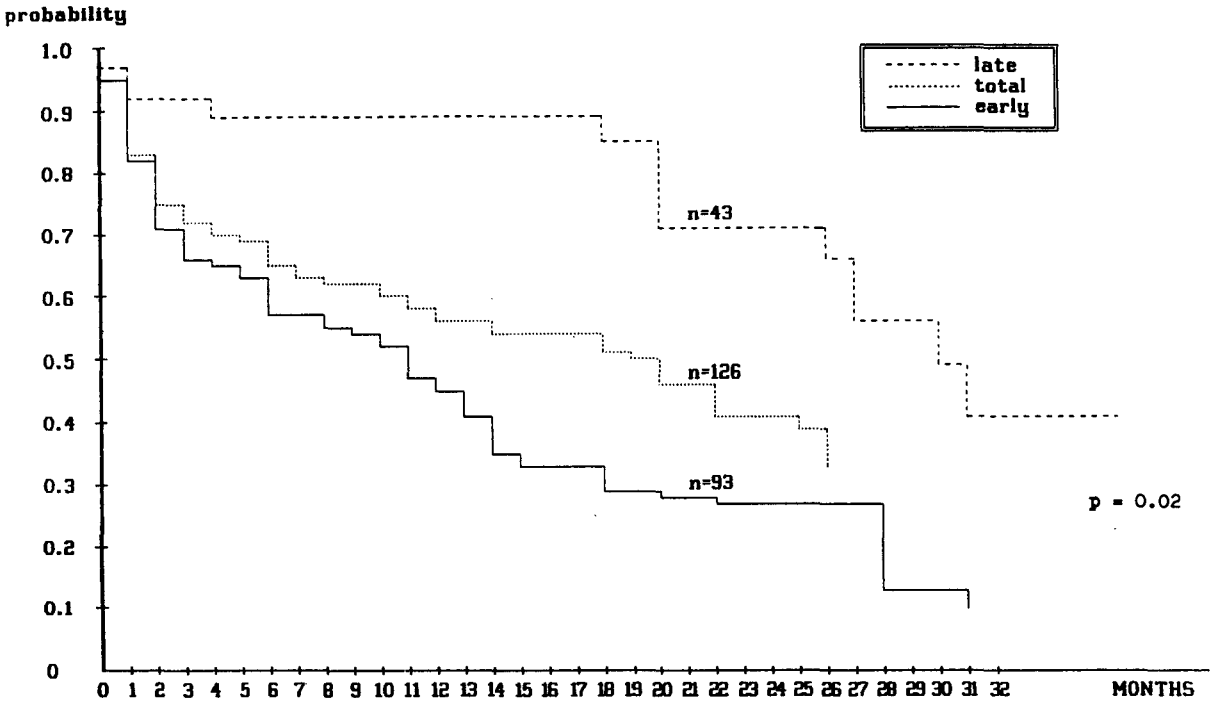

Fig. 1. Event-free survival of children with first relapse of ALL treated according to BFM protocol

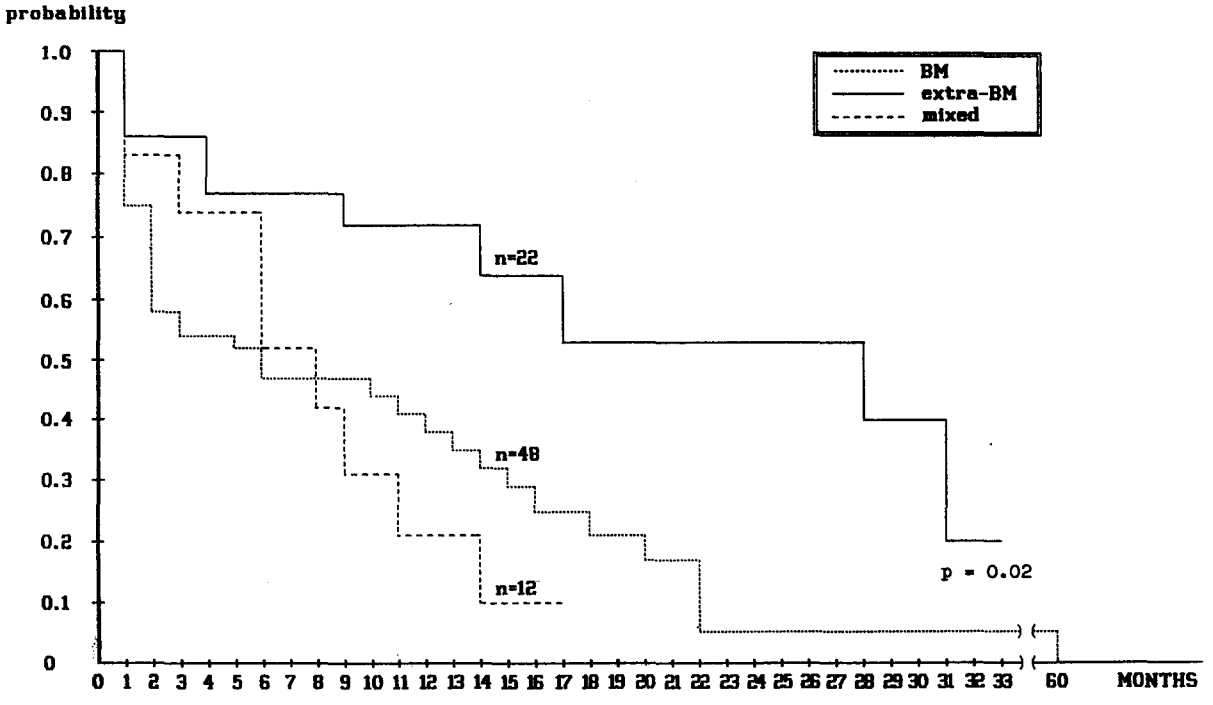

Fig. 2. Event-free survival of children with first early relapse with regard to type of relapse

ment of EFS in children with first relapse of ALL in comparison with chemotherapy previously used by the Polish Leukemia/Lymphoma Study Group [7].

On the basis of this work, we can conclude that the probability of a second complete remission in relapsed ALL children treated with the BFM 1985 protocol was better for the late relapse group ($p = 0.02$). The results achieved in early relapse with bone marrow involvement were not satisfactory, so some other

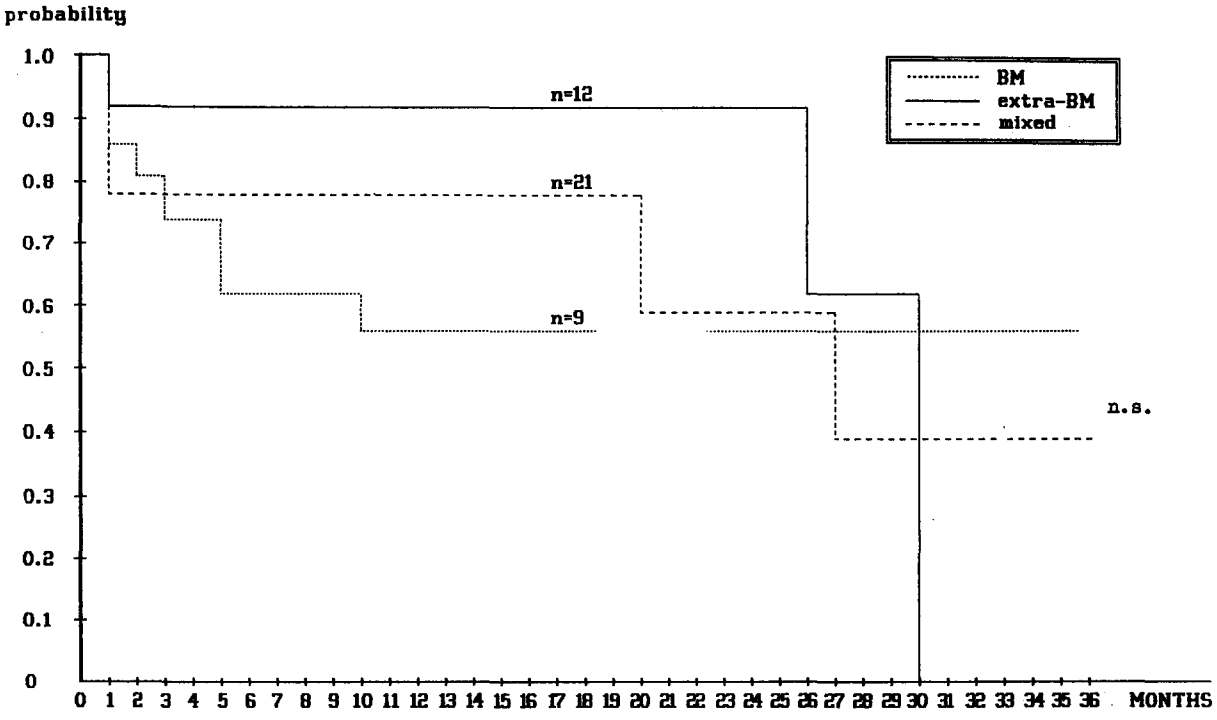

Fig. 3. Event-free survival of children with first late relapse with regard to type of relapse

methods of therapy should be proposed for these patients.

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Long-Term Follow-Up Study in Childhood Acute Lymphoblastic Leukemias

A. Petrakova [1], and J. Stary [2]

Summary

A long-term follow-up genetic study in 121 children with the diagnosis of acute lymphoblastic leukemia (ALL) is presented. The control group of 121 healthy children free of any cancer or precancer disorder in their medical history (matched by sex and age) was studied for statistical comparison. The medical history, familial history with special attention to cancer incidence and to mutagenic factors, dermatoglyphics, chromosome and nuclear DNA cytometric data at the time of ALL diagnosis, and late effects with special attention to secondary malignancies and reproduction of ALL survivors were examined. Finally, the prognosis in the ALL patients investigated was calculated. For this prognostic calculation, not only classical data (sex, age at the time of diagnosis, WBC count at the time of diagnosis etc.), but also our clinical and laboratory genetic data (familial cancer history, prenatal mutagenic exposure, occupational mutagenic exposures in parents were used. Using this complex model of prognostic calculation we divided our ALL patients more exactly into different prognostic groups.

These first results of our long-term follow-up genetic study helped us not only in differenting ALL patients according to prognosis, but also in providing genetic counselling in at-risk families. The initial experiences concerning reproduction of ALL long-term disease-free survivors are good.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Monoclonal Blast Cell Proliferation in Transient Myeloproliferative Disorder *

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Introduction

Transient myeloproliferative disorder is a hematological condition observed during the neonatal period in patients with Down syndrome [1]. This disorder mimics congenital leukemia and the blast cells were often reported to show characteristics compatible with those of megakaryoblasts [2] or pluripotent stem cells [3]. Although this disorder usually resolves gradually without antileukemic treatment, in some patients, leukemia develops after periods of spontaneous remission [4], raising questions about the benign origin of this disorder.

It is therefore unclear if transient myeloproliferative disorder is a leukemia, preleukemia, or transient dysplasia of myelopoiesis. This important issue might be resolved by analysis of the clonality of the blast cells of this disorder. We therefore studied the clonality of blast cells in transient myeloproliferative disorder using antigen receptor genes as well as X chromosome inactivation patterns.

Materials and Methods

Patient Samples. The patients studied included three newborn infants, all of whom had Down syndrome with standard trisomy 21 (Table 1). They all developed transient myeloproliferative disorder during the neonatal period with more blast cells in the peripheral blood than in the bone marrow, and were followed by spontaneous resolution without antileukemic treatment.

The follow-up period of these patients was from 72 days to 5 months. One out of three patients was alive at the time of this study (patient 3). The remaining two patients died while there was no evidence of leukemia. The clinical course of patient 2 was reported previously [5].

Cell Separation and Immunological Analysis. Heparinized peripheral blood was separated on a density gradient of Ficoll-Metrizoate (Lymphoprep, Nyegaard, Oslo, Norway). The interface of mononuclear cells was subjected to immunological and DNA analysis.

Reactivity of the blast cells with monoclonal antibodies against lineage-specific antibodies (Table 2) was assayed by an indirect immunofluorescence technique using a fluorescence-activated cell shorter as described before [6].

Southern Blot Analysis. High-molecular weight DNA was extracted from the mononuclear cells according to the

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Clinical data of three patients with transient abnormal myelopoiesis

Patient no.	Sex	Age of sampling	WBC (10^9/l)	Hb (g/dl)	Platelet (10^9/l)	Blast (%)		Follow-up period	SR	Outcome
						PB	BM			
1	F	2 d	50	16.8	254	47	8	72 d	+	Died of SIDS*
2	F	15 d	130	17.3	173	93	63	77 d	+	Died of hepatic failure*
3	F	3 d	12	13.5	16	24	12	5 m	+	Alive and well

d: day; *m*: month; *SR*: spontaneous remission; *SIDS*: sudden infant death syndrome; Hb, Hemoglobin; PB, peripheral blood; BM, bone marrow

* No evidence of leukemia at the time of death.

method described previously [6]. For the antigen receptor gene analysis, IgJH, Cβ, Jγ, Cδ, and Jδ2 probes were employed, with 6 µg of *Bam*HI-digested DNA samples. Clonal analysis with the X-linked phosphoglycerate kinase (PGK) probe [7] was performed as described by Vogelstein et al. [8].

Results

Immunological Analysis of Blast Cells. All of the samples of blast cells that we studied were positive for platelet associated antigens (CD41, CD42b, or KOR-P77 [9]). Two out of three samples also showed positivity for CD7 antigen. None of three samples expressed lymphoid lineage-associated antigens such as CD3, CD10 or CD19 (Table 2).

Antigen Receptor Gene Configuration of Blast Cells. None of the three samples of blast cells showed any rearrangements of the IgH or TCR loci using JH, TCRβ, γ, and δ probes (data not shown).

X Chromosome Inactivation Analysis of Blast Cells. 94%, 99%, and 82% of the peripheral mononuclear cells were blast cells after the density gradient cell separation in patients 1, 2, and 3 respectively. After an additional digestion by restriction enzyme *Hpa*II, DNA of the blast cells of the three patients showed complete loss of either the 1.05 kb or the 0.9 kb band, suggesting monoclonal expansion of the blast cells (Fig. 1, lanes 2, 6, and 8). In contrast, the mononuclear cells of patient 1 after spontaneous remission showed the retention of both of the alleles (Fig. 1, lane 4).

Discussion

Our initial study into the lineage and clonality of blast cells using the antigen receptor genes IgJH and TCRβ, γ, and δ showed no rearrangements of these loci in the blasts of any of the three patients. As

Table 2. Peripheral blood immunophenotyping

Antigens	Patient 1 (% of cells)	Patient 2 (% of cells)	Patient 3 (% of cells)
CD 3	–	NT	NT
CD 4	23.5	–	NT
CD 7	52.4	81.8	NT
CD 8	–	NT	NT
CD 10 (J5)	–	–	–
CD 13 (MY7)	–	–	NT
CD 14 (My4)	–	–	NT
CD 19 (B4)	–	–	–
CD 20 (B1)	–	NT	NT
CD 25	–	–	NT
CD 41	81.6	NT	19.3
CD 42b	73.2	NT	NT
KOR-P77	59.2	48.8	NT

Peripheral blood mononuclear cells were immunophenotyped by the method described in the materials and methods. Percentage of positive cells in fluorescent antibody screening is shown. *NT*: not tested; –, <15%.

all the patients demonstrate more than 82% of blast cells in the mononuclear cell fraction, our negative findings for clonal rearrangement cannot be attributed to the low sensitivity of this assay. Furthermore, as almost all of the leukemias with lymphoid characteristics show antigen receptor gene rearrangement [10], our results suggest that the proliferating blast cells are of nonlymphoid origin.

In order to study the clonality of blast cells, we employed the X chromosome-linked polymorphic gene, *PGK*. In patient 1, mononuclear cells were compared during blastic phase and remission phase, which was achieved without therapy, for sensitivity to *Hpa*II restriction enzyme digestion. As the results show, the absence at blastic phase, but the presence at remission phase of the allele identified at 1.05 kb indicates that mononuclear cells at blastic phase are of single clonal origin. Although we could not examine the samples at remission phase in patients 2 and

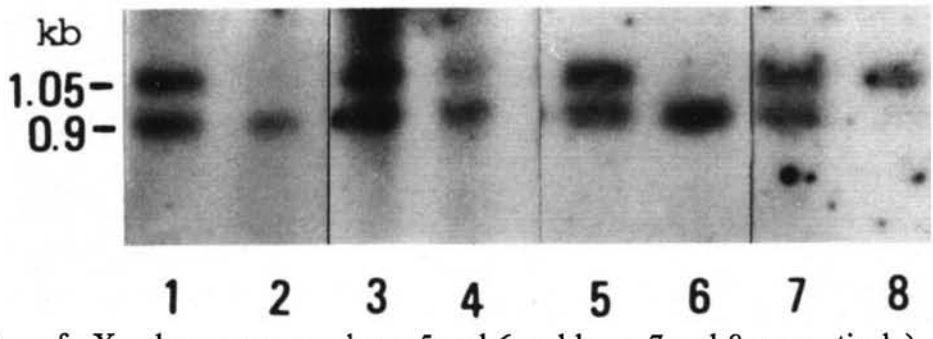

Fig. 1. PGK analysis of X chromosome inactivation patterns in three patients. An autoradiograph of a Southern hybridization experiment is shown, wherein a PGK gene probe was hybridized to DNA from mononuclear cells from patient 1 (initial presentation and after remission, lanes *1* and *2* and lanes *3* and *4*, respectively) and mononuclear cells from patients 2 and 3 (initial presentation, lanes *5* and *6* and lanes *7* and *8*, respectively). In lanes *1*, *3*, *5*, and *7* the DNA has been digested with restriction enzymes (*Bst*XI, *Pst*I) that reveal two polymorphic PGK alleles (1.05-kb and 0.9-kb bands). In lanes *2*, *4*, *6*, and *8* DNA has been digested with these enzymes and also with *Hpa*II, an enzyme that distinguishes active from inactive alleles through methylation differences.

3, the complete absence of one of two alleles indicates a monoclonal proliferation of blast cells. Altogether our results strongly suggest that monoclonal expansion of a progenitor cell with nonlymphoid characteristics occurs in this disorder with Down syndrome. Megakaryoblastic features characterized by expression of CD41 (patients 1 and 3), CD42b (patient 1), or KOR-P77 (patients 1 and 2) are of interest in the light of the recent recognition that acute leukemia in Down syndrome is much more frequently of a megakaryoblastic nature than previously recognized [11].

There are several possible explanations for blast cell proliferation in this disorder. Firstly, blast cells might proliferate in response to an increase in production of growth factors such as IL-3 and/or IL-6 [12]. Secondly, hemopoietic dysregulation, including that caused by defective immunological surveillance during the neonatal period, might be responsible for this disorder. Thirdly, transient myeloproliferative disorder could be a preleukemic state. Since in the former two interpretations polyclonal proliferation of blast cells is expected, our findings strongly support the third possibility. This idea is also supported by other reports that this is due to the result of a spontaneous resolution of a malignant clone [4, 13]. Alternatively this condition might be heterogeneous, some cases being benign reactive conditions with polyclonal myeloproliferation, while others are actually preleukemic conditions. Studies on more patients with a longer survival period will clarify whether there is any heterogeneity in clonality among patients with this disorder.

Acknowledgements. We are indebted to Drs. J. Singer-Sam, P. Leder, T. Rabbits, and T. W. Mak for providing the DNA probes, PGK, IgJH (λCH 28-6), TCRγ, and TCRβ and δ respectively. We also thank Dr. S. Nakazawa for immunophenotyping data from patient 1.

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The Comparison of the Expression of Activation Antigens on Peripheral Blood Mononuclear Cells in Chronic Lymphocytic Leukemia and in Hairy Cell Leukemia

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Introduction

Based on the results of the immunophenotypical analysis both B-cell chronic lymphocytic leukemia (B-CLL) and B-cell hairy cell leukemia (B-HCL) appear to be derived from activated lymphocytes [1–4]. However, CLL-B cells represent an earlier stage of differentiation than the cells of HCL. The data of the gene rearrangement studies also suggest that HCL derives from the clonal expansion of a cell at a later developmental stage than the CLL [5].

In this study the peripheral blood mononuclear (PBM) cells of patients with CLL and HCL were characterized by the presence of a variety of cell surface differentiation and activation antigens.

Patients and Methods

Twenty CLL patients were studied. Their age ranged from 42 to 79 years (mean: 63.0). The male to female ratio was 0.67:1. Five HCL patients in the leukemic phase of the disease were selected from the 24 investigated HCL patients (age 22–79 years, mean: 54.3). All patients were untreated for at least 3 months at the time of the study. Eight healthy age- and sex-matched persons served as controls. Indirect immunofluorescence was used to detect the differentiation and activation antigens on PBM cells.

T cell-associated antibodies were CD3 (T3), CD2 (T11), CD8 (T8), CD4 (T4) from Ortho Diagnostic System and (Raritan, New Jersey, USA), CD5 (Leu-1) from Becton-Dickinson (Mountain View, California, USA). B cell-associated antibodies were CD19 (B4) and CD20 (B1) from Coulter Corporation (Haleah, Florida, USA). Class II antigen was detected with HLA-DR from Becton-Dickinson. Anti-immunoglobulin M (IgM) antibody (Heintel Vienna, Austria) was used as directly conjugated with fluorescein isothiocyanate (FITC). RAB-1 monoclonal antibodies (moAbs) were used to detect the hairy cells [6]. The Immune Monitoring Kit of Becton-Dickinson was used to detect activated T cells. The monoclonal antibodies (moAbs) from the panel of the IVth International Workshop on Human Differentiation Antigens (CD25, CD30, CD40, CD69, CD70, CD39, CD71) were used to detect the activation antigens. Analysis of all samples was done using a FACSTAR flow cytometer (Becton-Dickinson).

Results

The cells of 20 CLL patients were found to be B-cell phenotype when studied with Abs directed against CD19, CD20, HLA-DR and surface immunoglobulin (sIg) antigens. Furthermore, significant percentage of the cells gave a positive reactions with moAbs to CD5 [7]. The

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

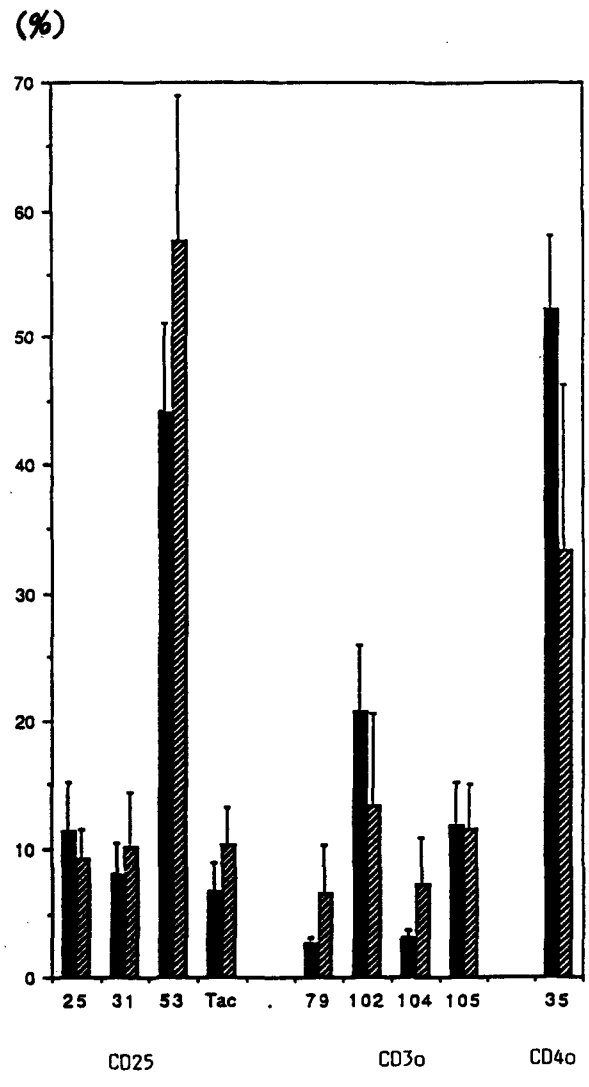

Fig. 1. The comparison of activation antigen expression on CLL (*black bars; n* = 20) and HCL (*hatched bars; n* = 5) cells I

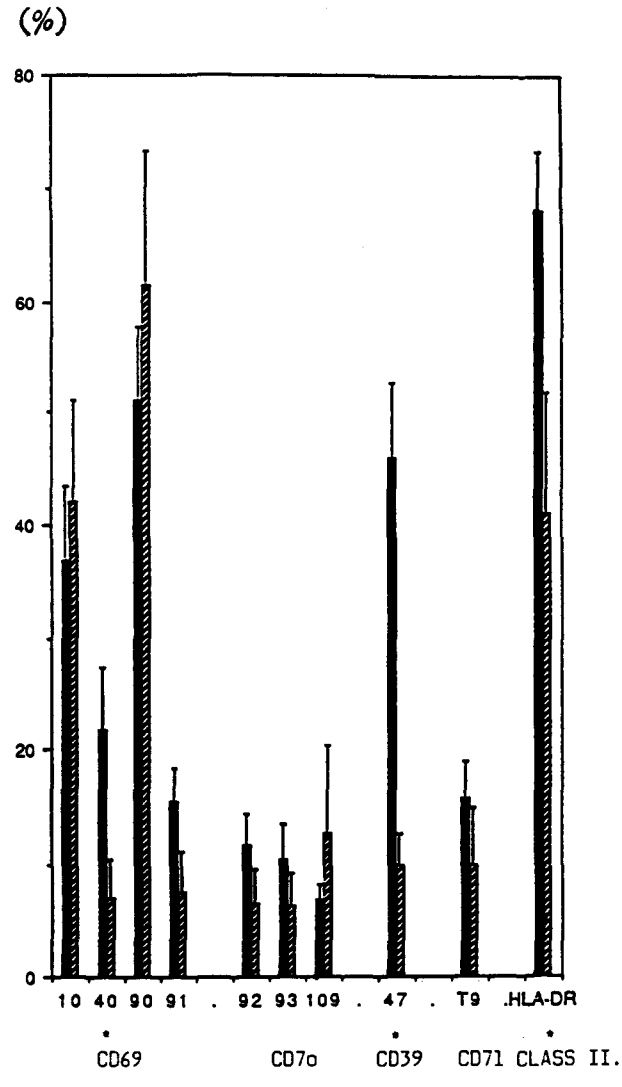

Fig. 2. The comparison of activation antigen expression on CLL (*black bars; n* = 20) and HCL (*hatched bars; n* = 5) cells II

PBM cells from 24 HCL patients showed similar antigen profile: CD19, CD20, HLA-DR and sIg positivity, but CD5 negativity. The moAbs to hairy cells RAB-1 showed a positive reaction both on CLL and HCL cells, but the expression of RAB-1 was found to be significantly higher in HCL than in CLL. Neither CLL nor the HCL cells expressed the CD21 antigen (C3d receptor, EBV receptor).

Investigating the presence of various activation antigens using 18 moAbs grouped into seven clusters, both CLL and HCL PBM cells were found to carry a significant proportion of activated cells when compared to the healthy controls. Comparing the CLL and HCL cells with each other, the activation stage of PBM cells in CLL and HCL differed only in two out of the 18 activation antigens (Figs. 1, 2). The expression of the CD39 cluster as well as the expression of a single antigen of the CD69 clusters were significantly higher in CLL than in HCL.

Discussion

Our data confirm and extent earlier suggestions that both CLL and HCL might be derived from subpopulations of activated lymphocytes. The CLL cells were characterized by the expression of CD25, CD30, CD39, CD40, CD69 and CD70 activation antigens, while the HCL cells

were bearing CD25, CD40 and CD69 activation antigens. The expression of RAB-1 antigen on CLL lymphocytes suggests that RAB-1 might be also an activation marker [8]. The interleukin (IL-2) receptor (CD25) and two early activation antigens, CD40 and CD69 [9], were found to be expressed in cells of both CLL and HCL while the CD25 antigen positivity was significantly higher in HCL than in CLL.

The antigens of the CD30 and CD70 clusters which are present on the cell lines that related to activated T and B lymphoma cells were found to be expressed only in CLL cells, suggesting that HCL cells represent a later stage of differentiation.

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Multiple Myeloma: Immunodeficient, Osteolytic, Renal, and Amyloid Syndromes

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Introduction

Multiple myeloma (MM) is a malignant clonal B-lymphoproliferative disease with typical immunodefficient, osteolytic, renal, amyloid syndromes. Disturbance in the immunologic system appears early and influences the outcome of MM [1]. Light chain isotype suppression (LCIS) [2] and imbalance in T-cells [3] are the factors determining the immunodefficiency syndrome. There are no convincing data about changes in natural killer (NK) activity in MM [4, 5]. Osteolysis is the second most frequently observed syndrome, and interleukin-1_β (IL-1_β), plays the main role in its development [6, 7]. IL-1_β probably is also responsible for chronic renal insufficiency (CRI) [7, 8], which is of great prognostic value im MM [9]. Amyloidosis rarely appears in MM patients (6%–15%) [10]. The aims of this study were to clarify the role of LCIS, to determine NK activity of peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) and IL-1 production by peripheral blood monocytes (PBM) in MM, and to assess any possible correlation between these data and the course of MM.

Material and Methods

140 patients with MM were investigated. Amyloidosis was diagnosed in 15 patients according to the typical pathology of skin specimens with Congo Red staining.

Clinical Classification. MM was classified according to previously described criteria [11].

Light Chain-Isotype Suppression. The presence of cell antigens was determined by an indirect immunofluorescence assay [12]. LCIS was determined from the ratio of kappa lymphocytes to lambda lymphocytes (kappa/lambda ratio). The normal range for the kappa/lambda ratio was determined by testing 30 blood donors, and was found to be 0.6–3.0. Patients with kappa myeloma were regarded as having LCIS if the kappa/lambda ratio was less than 0.67; patients with lambda myeloma and a kappa/lambda ratio of more than 3.0 were also regarded as having LCIS. The presence of LCIS was detected by anti-kappa and anti-lambda monoclonal antibodies, which were provided by the Central Research Institute of Roentgenology and Radiology, Leningrad, USSR.

NK Activity. Effector cells were obtained from heparinized peripheral blood and bone marrow by centrifugation over Ficoll-Hypaque. Bone marrow cells were preparated and cultured as described by Yoda et al. [13] with recombinant IL-2 200 U/ml (Institute of Organic Synthesis, Riga, Latvia, USSR). K-562 cells were used as target cells. Cytotoxity was measured in a standard 18-h [^{3}H]uridine microcytotoxicity assay [14]. NK activities (CTX%) were calculated using the following formula:

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

$$\text{CTX \%} = \left(1 - \frac{\text{experimental cpm}}{\text{control cpm}}\right) \times 100\%$$

Effector: target ratio = 25:1

IL-1 Activity. PBM were cultured in RPMI 1640 in the presence or absence of lipopolysaccharide (LPS; "Pyrogenal", USSR, 40 mg/ml). After 24 h incubation, supernatants were removed, filtered, and frozen at −20°.

Mice thymocytes (C3H/j) ($1 > 10^7$ cells/ml) were incubated in RPMI 1640, supplemented with 1% fetal calfserum (FCS), gentamycin (0.2 mg/ml) L-glutamine, and PGA (1 mg/ml; Difco), with various dilutions of the tested supernatants in a CO_2 incubator for 72 h. The level of cell proliferation was measured by the incorporation at 16 h of [^{3}H]thymidine added to thymocytes. One unit per milliliter of IL-1 activity was defined as the reciprocal of the dilution causing 50% of the maximal response [15, 16].

Statistics. Data were evaluated using Student's *t* test.

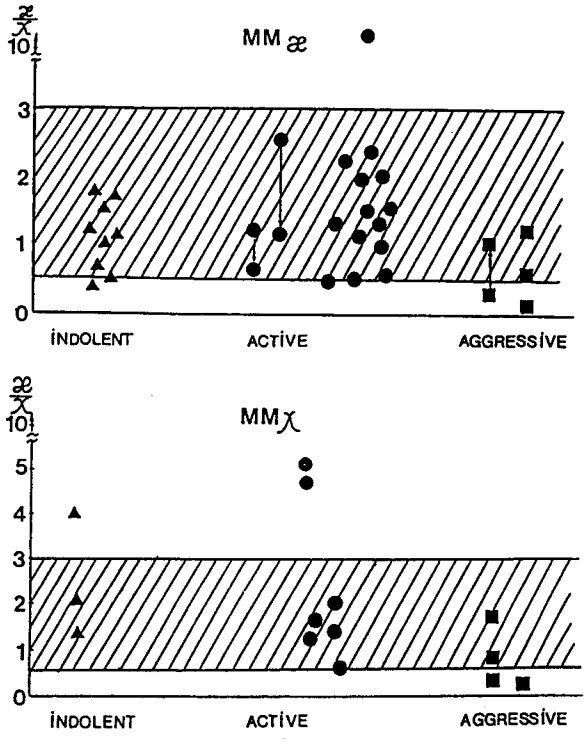

Fig. 1. The relationship between light chain isotype suppression (LCIS) and the course of disease in patients with kappa MM (*top*, $n = 29$) and with lambda MM (*bottom*, $n = 14$). Kappa MM patients with LCIS (kappa/lambda ratio < 0.67): 2 with indolent course (▲), 2 with active course (●), and 2 with aggressive course (■). Lambda MW patients with LCIS (kappa/lambda ratio > 3.0): 1 with indolent course, and 2 with active course (●)

Results

Immunodeficiency Syndrome. Some patients with indolent, active, or aggressive MM had LCIS (Fig. 1). There were no differences in NK activity of PBMC and BMMC in all of MM patients as compared to normal donors (Fig. 2). However, a significantly lower NK activity was found in active and aggressive MM than in normal donors (Fig. 3). The NK activity of BMMC increased after cultivation in media with IL-2 for 72 h (Fig. 4). Pyrogenal-stimulated IL-1 production by PBM showed a tendency to be higher in active and aggressive MM than in indolent MM and normal donors (Fig. 5).

Osteolytic Syndrome. Survival was higher in patients with no or minimal bone changes (osteoporosis) than in patients with moderate or extensive bone lesions (Fig. 6). Patients with indolent MM had mild osteoporosis and no bone lesions (Fig. 7). More than half of the patients with active and aggressive MM had moderate or extensive bond lesions. Pyrogenal-stimulated IL-1 production by PBM differed significantly between patients with no bone lesions or with osteoporosis and patients with moderate or extensive bone lesions (Fig. 8).

Renal Syndrome. Patients with CRI at presentation had shorter survival timer than patients without CRI (Fig. 9). No patient with indolent MM had CRI at presentation, whereas CRI was diagnosed in 4.7% of patients with active MM and in 44.4% of those with aggres-

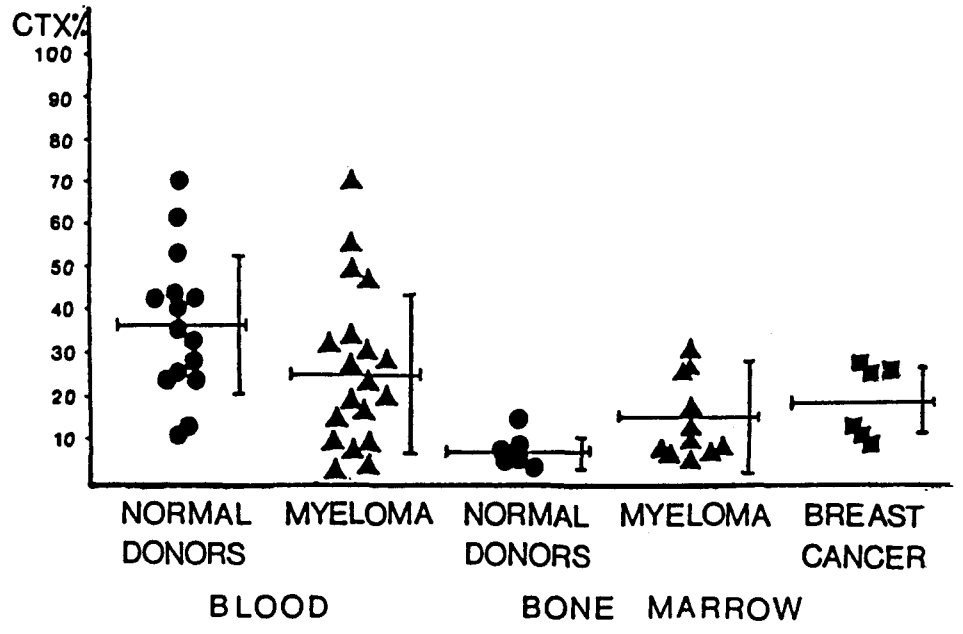

Fig. 2. NK activity (CTX%) of peripheral blood mononuclear cells (PBMC) and bone marrow cells in patients with MM and breast cancer.
Mean ± SD level of CTX% of PBMC: in MM patients, 24.9 ± 18.5 ($n = 19$); in normal donors, 36.2 ± 16.4 ($n = 15$) ($p > 0.05$). Mean CTX% in bone marrow: in MM patients, 17.5 ± 12.6 ($n = 11$); in normal donors 7.3 ± 4.1 ($n = 6$) ($p > 0.05$); in breast cancer patients, 17.95 ± 8.7 ($n = 6$) ($p < 0.05$ with respect to normal donors)

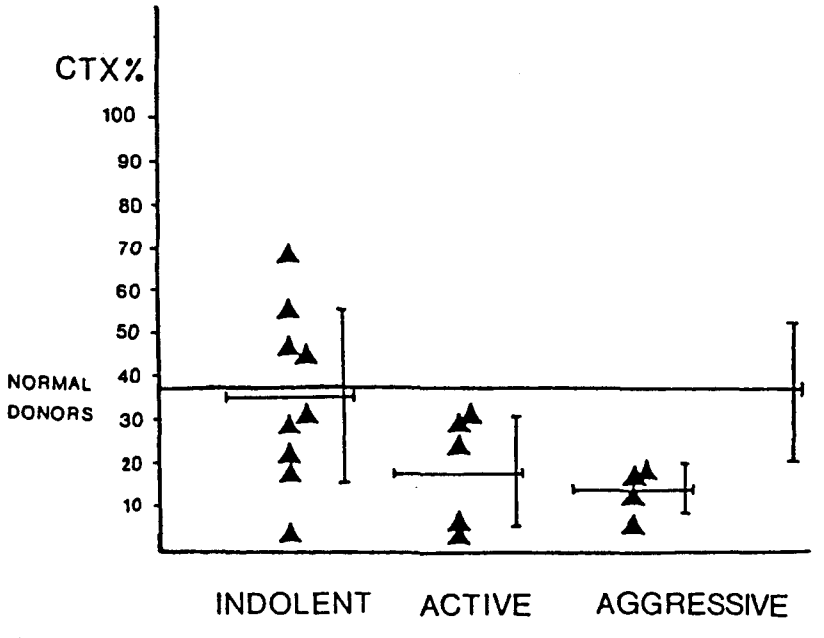

Fig. 3. NK activity of peripheral blood mononuclear cells (PBMC) in MM patients with different courses. Mean ± SD level of CTX%: in indolent course, 35.6 ± 20.0 ($n = 9$) ($p > 0.05$ with respect to normal donors); in active course 18.1 ± 13.4 ($n = 5$); in aggressive course, 13.8 ± 6.35 ($n = 4$) ($p < 0.05$ with respect to normal donors)

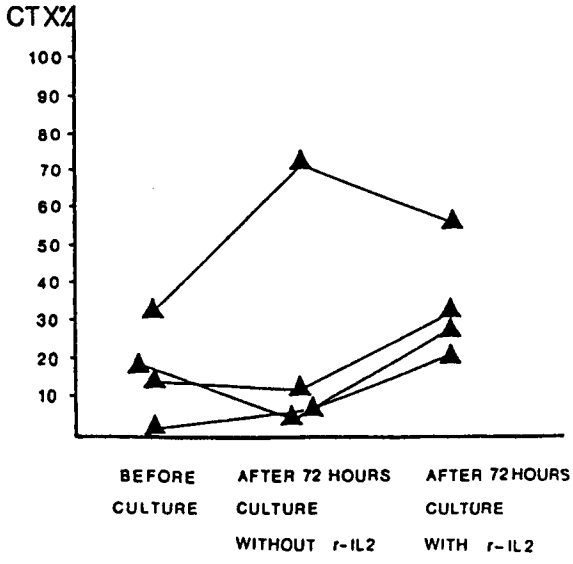

Fig. 4. Effects of recombinant IL-2 (*r-IL2*) on NK activity of bone marrow cells in MM. Bone marrow mononuclear cells were cultivated with IL-2 200 U/ml over 72 h

sive MM. The number of patients without monoclonal protein secretion was higher in aggressive MM (Fig. 10). Pyrogenal-stimulated IL-1 production by PBM was significantly higher in patients with CRI than in patients without CRI (Fig. 11).

Amyloid Syndrome. A difference was found in survival between active MM patients with amyloidosis compared to all MM patients (Fig. 12). None of patients with amyloidosis in indolent and active MM died during the period of observation. NK activity of PBMC in MM patients with amyloidosis was 41.2 $\pm$22.4% (mean$\pm$SD), which is significantly higher than in MM patients without amyloidosis (19.1$\pm$13.4%) ($p < 0.05$). There were no differences in pyrogenal-stimulated IL-1 production by PBM in MM patients with or without amyloidosis (Fig. 13).

Discussion

LCIS plays a certain role in development of immunodefficiency in MM, but it has also been shown to be a factor inhibiting tumor growth [1]. The development of LCIS is considered to be a favorable sign [17]. According to our data, this phenomenon is a rare one, and is more typical for indolent and active MM. It may be present in aggressive MM, and is probably not the basic factor in genesis of immunodefficiency syndrome and in suppression of tumor growth. There are many data about the antitumour effects

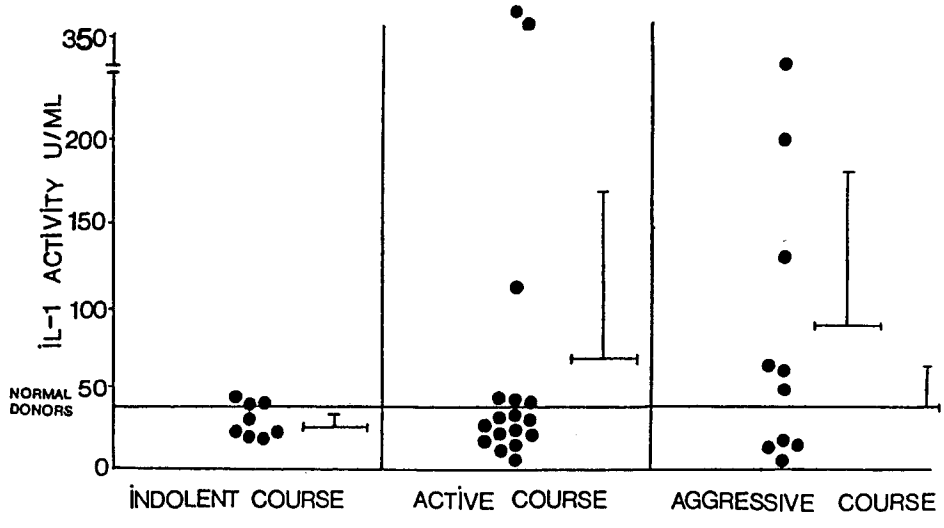

Fig. 5. Pyrogenal-stimulated production of IL-1 by peripheral blood monocytes (PBM) in MM patients with different types of course. Mean$\pm$SD level of IL-1 production by PBM: normal donors, 39.6$\pm$26.03 U/ml ($n = 11$); patients with indolent course, 27.05$\pm$8.2 U/ml ($n = 8$); patients with active course, 67.4$\pm$102.2 U/ml ($n = 17$), patients with aggressive course, 80.8$\pm$84.25 U/ml ($n = 10$) ($p > 0.05$)

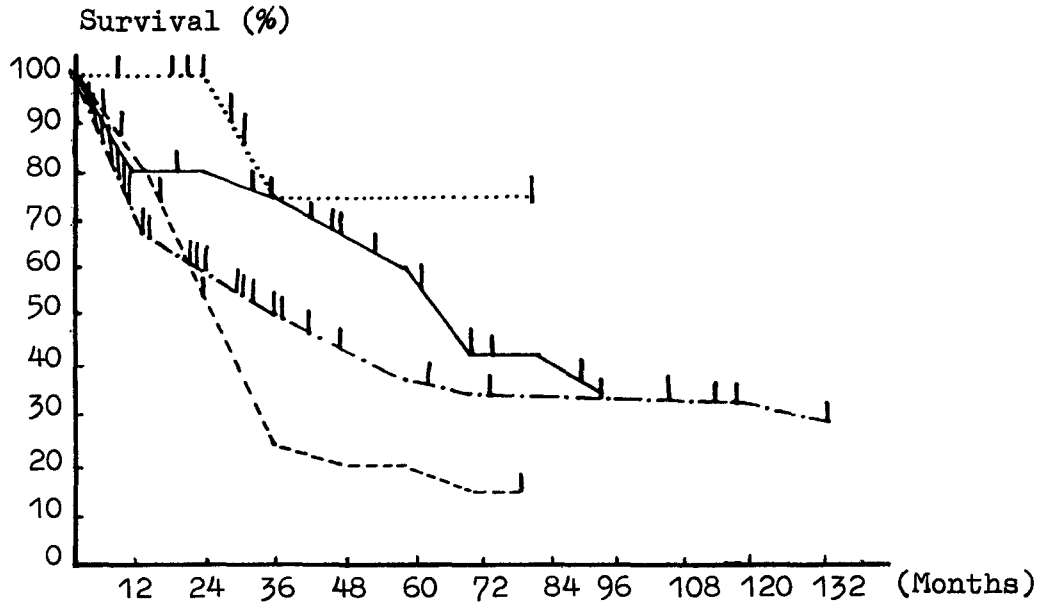

Fig. 6. Bone lesions and the survival of patients with MM. ..... no lesions ($n = 8$), —— minimal lesions (osteoporosis) ($n = 24$), median survival 62.7 months; –.–.– moderate lesions ($n = 71$), median survival 36 months; ---- extensive lesions ($n = 20$), median survival 24 months

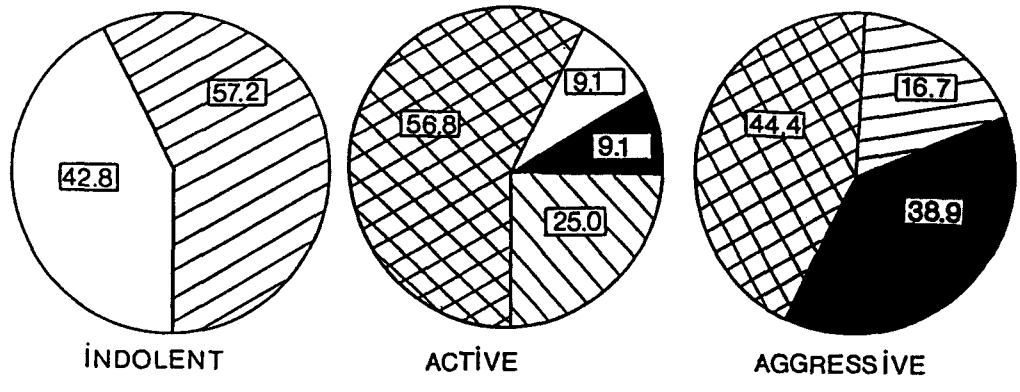

Fig. 7. Bone lesions in MM patients with different courses. ▭, no lesions; ▨, minimal lesions; (osteoporosis); ▩, moderate lesions; ■, extensive lesions

of NK-cells [18, 19]. As described previously [4, 5], there were no differences in NK activity of PBMC between MM patients and normal donors. In this study, NK activity of PBMC varied widely between MM patients and did not differ from that in normal donors. At the same time, NK activity of PBMC in active or aggressive MM was significantly lower than in normal donors. This shows that there is a decrease in natural antitumor resistance in MM patients with active and, especially, aggressive MM. The possibility of increasing NK activity of BMMC in culture with recombinant IL-2 enabled us to use this drug in MM treatment [20–22]. It was shown that IL-1 stimulates T-cells to synthesize IL-2 and receptors to IL-2 [22]. The increase in IL-1 production by PBM in active and aggressive MM may indicate the activation of antitumor mechanisms that are, however, still insufficient to stimulate IL-2 synthesis. The frequency of extensive bone lesions was higher in active and, particularly, in aggressive MM. There is also a positive correlation between the degree of bone lesions and the level of IL-1

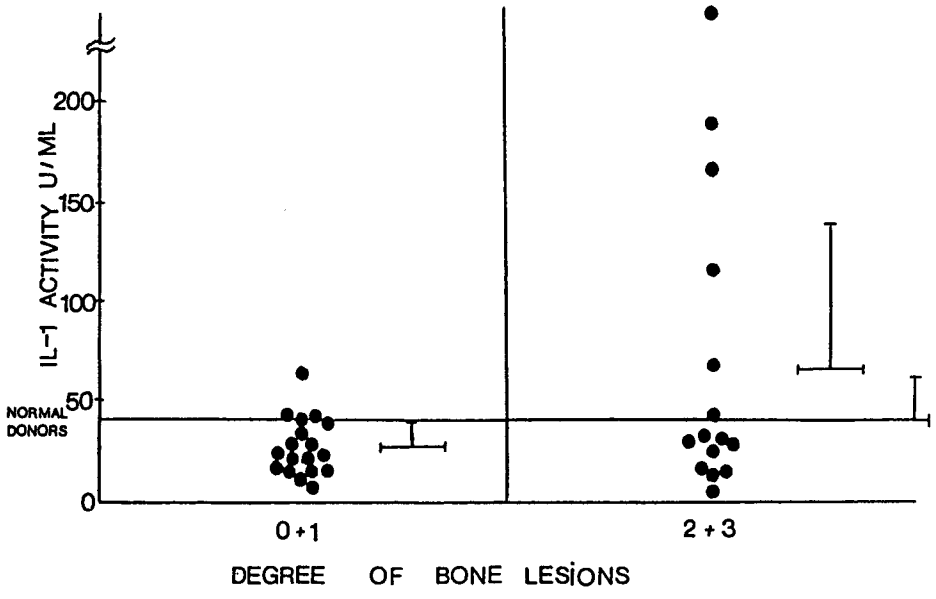

Fig. 8. Pyrogenal-stimulated IL-1 production by peripheral blood monocytes (PBM) in MM patients with different degrees of bone lesions: 0 no bone lesions; 1 osteoporosis; 2 moderate bone lesions; 3, extensive bone lesions. Mean $\pm$ SD IL-1 production by RBM; 0 + 1 patients, 27.9 $\pm$ 13.8 U/ml ($n = 18$); 2 + 3 patients, 67.1 $\pm$ 73.4 U/ml ($n = 15$) ($p = 0.05$)

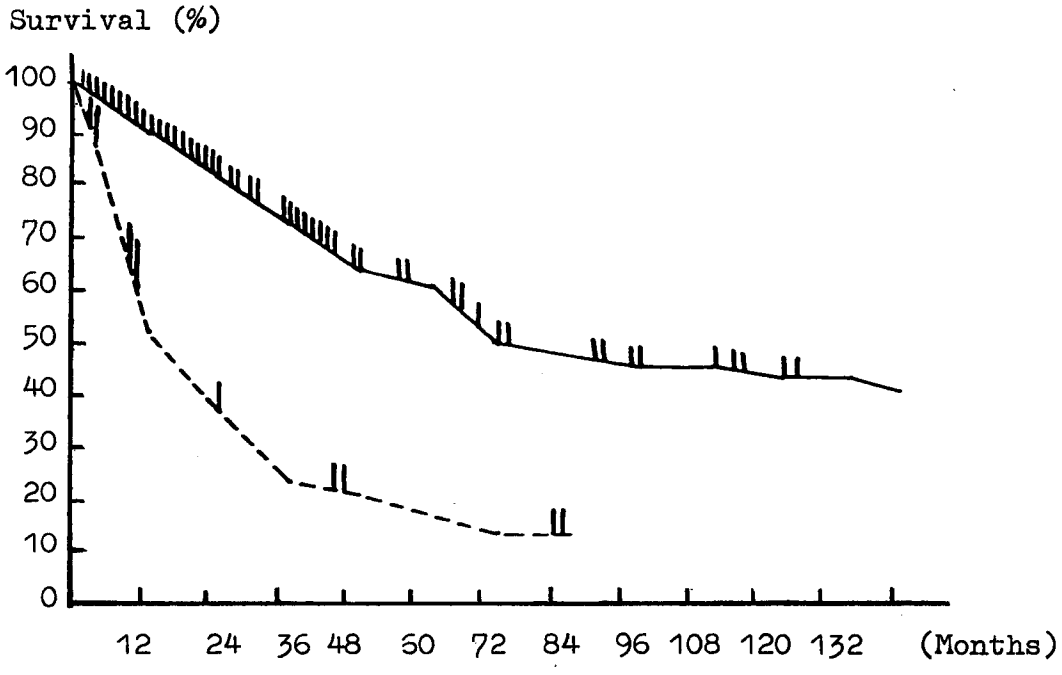

Fig. 9. Survival of MM patients with and without chronic renal insufficiency (CRI). ——, survival of MM patients without CRI ($n = 9$), median 72 months; ----- survival of MM patients with CRI ($n = 43$), median 14.4 months

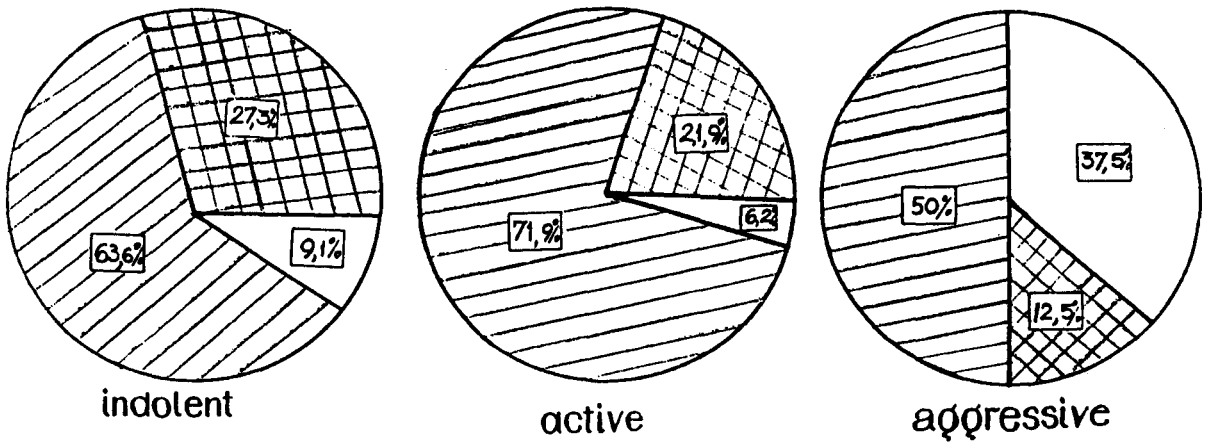

Fig. 10. Immunoglobulin light chain type in MM with different courses: ☐, nonsecreting; ▨, kappa chain; ▩, lambda chain

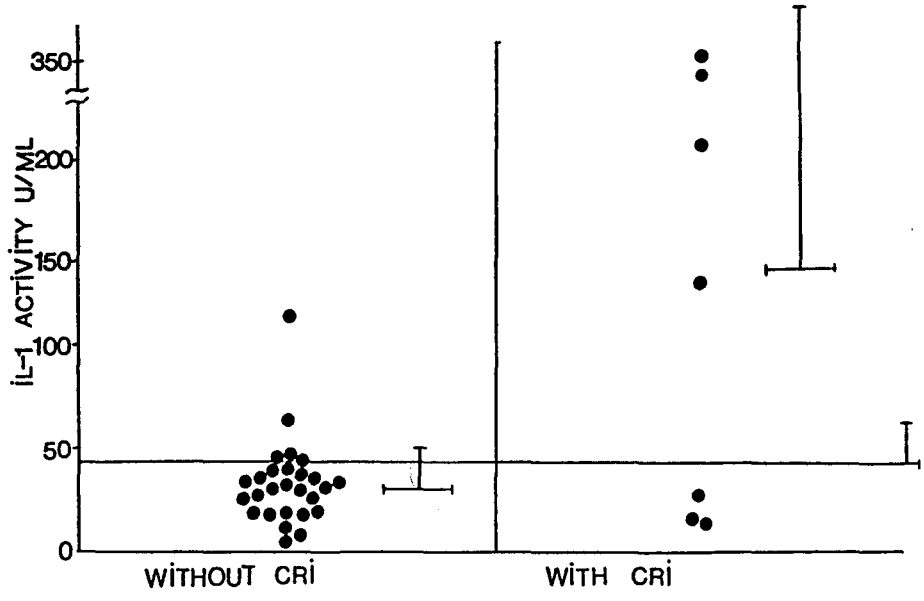

Fig. 11. Pyrogenal-stimulated production of IL-1 by peripheral blood monocytes in MM patients with and without chronic renal insufficiency (CRI). Mean ± SD IL-1 production by PBM in MM patients without CRI ($n = 27$), 32.3 ± 22.3 U/ml; in MM patients with CRI ($n = 7$), 141.5 ± 131.7 U/ml ($p < 0.01$)

production by PBM in MM patients. These data indicate that IL-1$_\beta$, produced leading role in the genesis of osteolytic syndrome in MM [6, 7]. In this study, monocytes, known to be precursors of osteoclasts, increased IL-1 production. IL-1 is probably an autocrine factor that stimulates osteoclast function Myeloma with CRI and nonsecretory myeloma are of a more aggressive type.

There is a positive correlation between the development of CRI and the level of IL-1 production by PBM in MM patients, and this may play a certain rol in the genesis of CRI [7, 8]. The increased survival of patients with active MM with amyloidosis in comparison with all active MM patients without amyloidosis may be the result of activation of antitumor immunity. NK activity of PBMC in MM patients with amyloidosis was significantly higher that in MM patients without amyloidosis. Perhaps that makes the MM more favorable. Further investigation to clarify the pathogenesis of MM clinical syndromes are necessary.

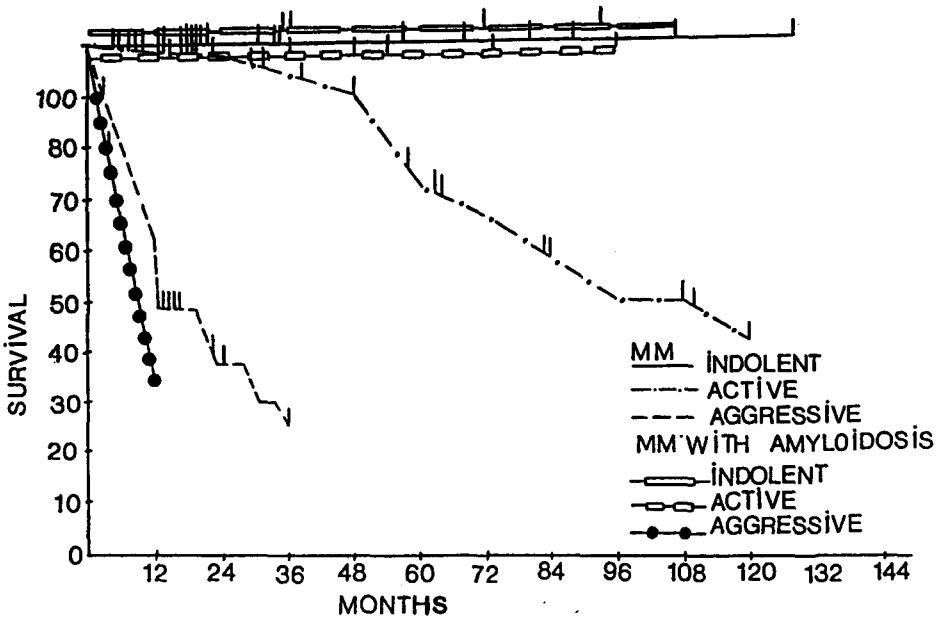

Fig. 12. Survival of patients with MM in different courses with and without amyloidosis. ——, indolent MM ($n = 11$); –.–.–, active MM ($n = 32$) (median survival 96 months); – – – –, aggressive MM ($n = 23$) (median survival 12 months); –□–, indolent MM with amyloidosis ($n = 5$); –□–□–□, active MM with amyloidosis ($n = 3$)

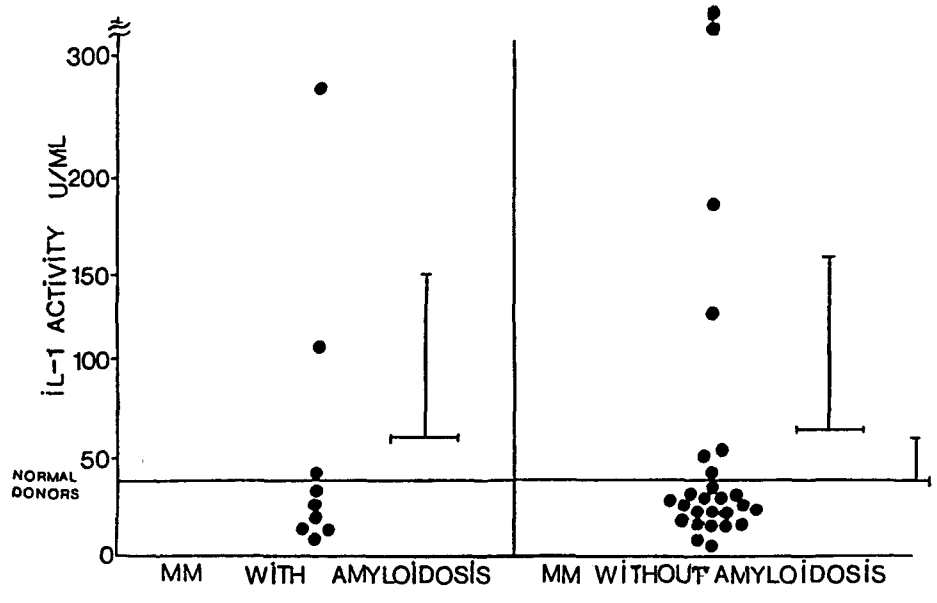

Fig. 13. Pyrogenal-stimulated production of IL-1 by peripheral blood monocytes in MM patients with and without amyloidosis. Mean ± SD IL-1 production by PBM; in MM patients with amyloidosis ($n = 9$), 62.9 ± 81.5 U/ml; in MM patients without amyloidosis ($n = 26$), 65.2 ± 88.3 U/ml ($p > 0.05$)

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Prognostic Factors in Patients with Multiple Myeloma

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Introduction

Multiple myeloma (MM) is a malignant clonal B-lymphoproliferative disease. Survival ranges from a few months to many years [7]. The determination of prognosis for the disease's course is important in therapeutic choice. At least two factors determine the course of MM:

1) the biology of myeloma cells and
2) the interrelation of malignant cells and host organism.

That is why the morphology of myeloma cells is one of the most important prognostic factors of the disease [4]. There are also other prognostic factors associated with both malignant cell characteristics and the status of the host's immunocompetent system [7]. Taking into account the data on the decrease in colony-forming units – granulocyte/macrophage (CFU-GM) in MM [2, 11], and the antitumor effect of tumor necrosis factor alpha (TNFα) [8, 9] and interleukin-2 (IL-2) [10], we investigated myeloma cell morphology, the level of serum β_2-microglobulin (Sβ_2-M) and CFU-GM, TNF production by peripheral blood monocytes, and IL-2 production by peripheral blood mononuclear cells in different types of MM course in order to determine their prognostic significance.

Materials and Methods

We investigated 101 patients with MM. Diagnosis was made according to previously described criteria [5].

Clinical Classification. It is impossible to predict individual prognosis in MM according to the stage of the disease [6] at the first presentation. That is why we classified our patients according to their life duration, which determined the type of the disease. This classification is a result of prospective investigation. We divided the patients into three groups: first – indolent myeloma, second – active myeloma, third – aggressive myeloma.

The diagnosis was made on the following criteria:

1) *Indolent myeloma.* Patients who presented with Kyle's criteria [7], patients who had been in remission for more than 5 years without chemotherapy, and patients with monoclonal gammopathy of undetermined significance who had been under investigation from 2 to 5 years (Fig. 1).
2) *Active myeloma.* Patients in whom first line chemotherapy was effective in achieving remission or the plateau stage. Median survival was 96 months.
3) *Aggressive myeloma.* Patients who had primary or secondary resistance to chemotherapy. Median Survival was 12 months.

Morphological Classification. The degree of myeloma cell maturation was determined according to the following criteria

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

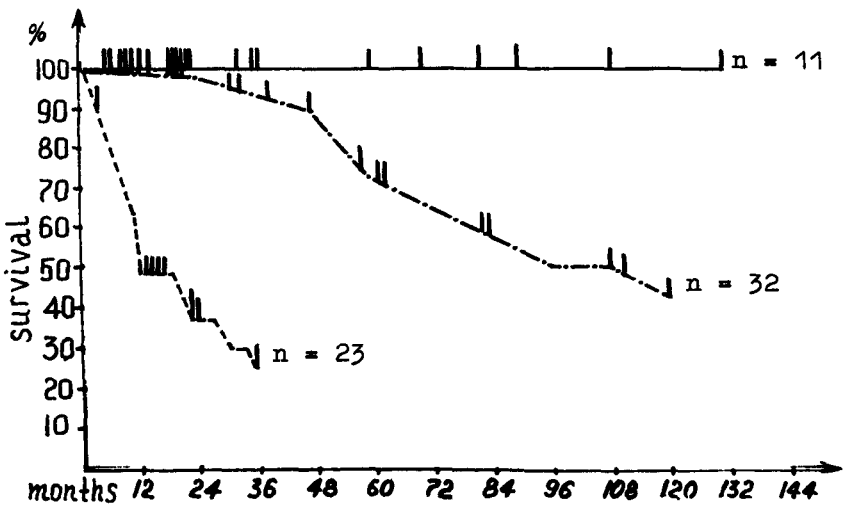

Fig. 1. Survival of patients with various courses of multiple myeloma. The *solid line* shows the indolent course; the *dash-dot line*, the active course, the *line of dashes*, the aggressive course

of the morphological classification system for MM [4]:

1) *mature* – comprising more than 50% mature plasma cells;
2) *immature* – comprising more than 50% proplasmacytes and mixed cell myeloma where none of the morphologically distinct plasma cell types exceed 50% (the proportion of lymphoplasmacytoid cells was always less than 50% of the bone marrow plasma cells);
3) *plasmablastic* – comprising more than 50% plasmablasts.

Serum β_2-Microglobulin. Sβ_2-M was measured by ^{125}I radioimmunoassay.

Colony-Forming Unit-Granulocyte/Macrophage. The cloning of hemopoietic cells was performed in a agar drop-liquid medium system [1].

Biological Activity of IL-2. The biological activity of IL-2 in conditioned media of peripheral blood mononuclear cells was assayed according to the method reported by Bockman and Ropo [3].

TNF$_\alpha$ Activity. The spontaneous and pyrogen-stimulated (50 µl/ml) production of TNF$_\alpha$ by peripheral blood monocytes was assayed by exploring the immunoenzyme kits (performed by the Institute of High Purified Preparations (HPP) Leningrad, USSR), based on monoclonal and polyclonal antibodies to TNF$_\alpha$. In order to form the calibration curve in each case TNF$_\alpha$ was used (made by NPO "Ferment", Tallin, USSR).

Results

It was revealed that mature plasma cells were the morphological substrate in indolent myeloma (Fig. 2) at the same time in active myeloma, mature cells predominated in 77% of the patients, whereby in the remaining 23% of the patients, the cells were immature. In aggressive myeloma, immature cells were the main morphological type in 56.3% of the patients, plasmablasts prevailed in 37.5% of the patients, and mature cells were in 6.2% of the patients.

We discovered that the level of Sβ_2-M correlated with the course of MM in most of the patients (Fig. 3). There was a significant difference between the level of Sβ_2-M in patients with indolent myeloma (3.3 ± 0.4 mg/l) and in healthy controls (1.7 ± 0.4 mg/l; $p < 0.05$). We revealed a significant difference between the level of

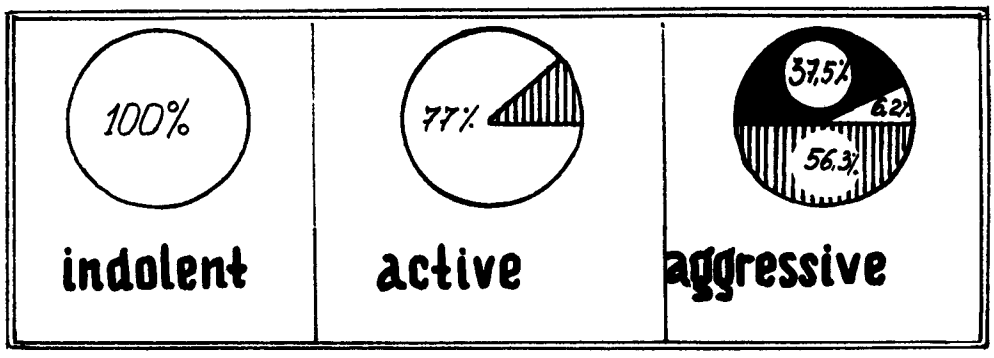

Fig. 2. Morphology and course of MM. The *white area* shows the percentage of mature cells; the *hatched area*, the immature cells; and the *black area*, the plasmoblastic cells

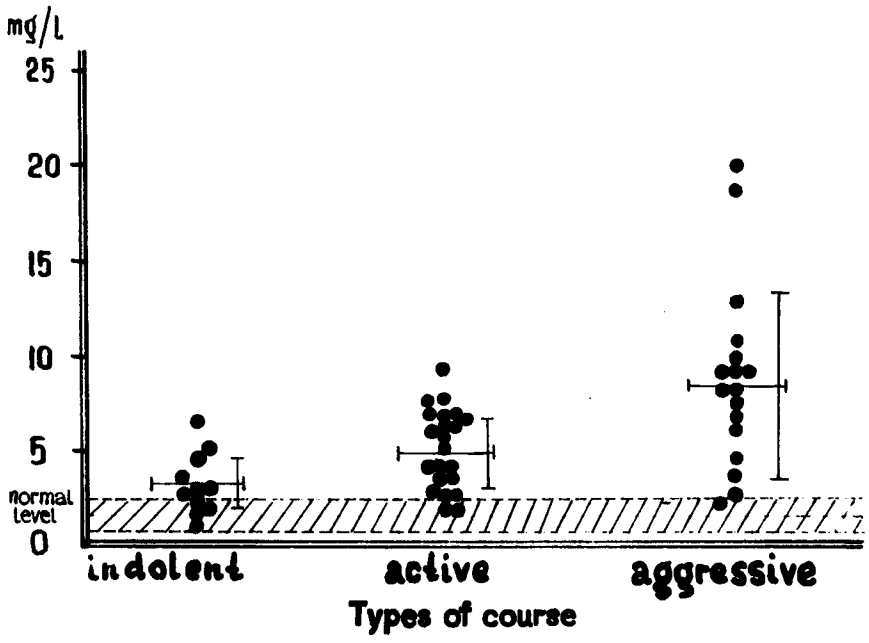

Fig. 3. Serum β_2-microglobulin and types of courses in patients with multiple myeloma

$S\beta_2$-M in patients with indolent and active myeloma (4.9 ± 0.4 mg/l; $p < 0.05$). There was also significant difference between the level of $S\beta_2$-M in patients with active and aggressive myeloma (8.6 ± 1.2 mg/l; $p < 0.05$) and a significant difference between patients with aggressive and indolent courses ($p < 0.01$; Fig. 4).

Studies on the clonogenic ability of CFU-GM in patients with an indolent course gave unusual results. In most patients with an indolent course, the number of CFU-GM was higher than in healthy controls (Fig. 5). The number of colony-forming cells in agar culture ranged from 30 to 300 per 10^5 bone marrow cells, whereas in normal bone marrow cells it ranged from 40 to 60 per 10^5 bone marrow cells. High levels of CFU-GM were also found in some patients with active myeloma as compared with healthy controls. The number of colony-forming cells in agar culture in active myeloma ranged from 42 to 420 per 10^5 bone marrow cells. In patients with aggressive myeloma the number of colony-forming cells in agar culture ranged from 0 to 25 per 10^5 bone marrow cells, which was much less than in patients with indolent and active myeloma and less than in normal controls.

There was an insignificant difference in IL-2 production between healthy con-

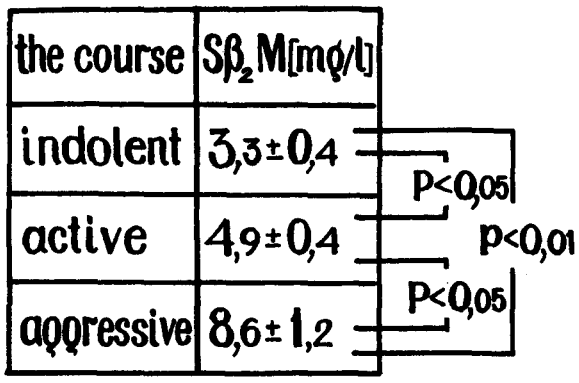

Fig. 4. Serum β_2-microglobulin and the course of multiple myeloma

trols (13.9 ± 1.2 units of activity; $p < 0.05$) and patients with indolent (12.9 ± 1.1 units of activity) (Figs. 6, 7) and active courses (11.9 ± 0.7 units of activity) and between the indolent and active courses. Then significant differences were found in the IL-2 production between patients with active and aggressive myeloma (8.6 ± 0.6 units of activity; $p < 0.01$) and also between aggressive and indolent myeloma and healthy controls ($p < 0.01$).

An insignificant difference between the pyrogen-stimulated production of TNF_α in patients with an indolent course (1.5 ± 0.3 ng/ml) and healthy controls (2.2 ± 0.38 ng/ml) was detected ($p > 0.05$; Fig. 8).

A significant difference was found between the pyrogen-stimulated production of TNF_α in patients with indolent and active courses (6.1 ± 1.1 ng/ml; $p < 0.01$). There was also a significant different in TNF_α production between patients with active myeloma and healthy controls ($p < 0.05$), and also in TNF_α production in patients with active and aggressive myeloma (2.0 ± 1.2 ng/ml; $p < 0.05$). There was an insignificant difference between TNF_α production in patients with aggressive and indolent courses and healthy controls ($p > 0.05$; Fig. 9).

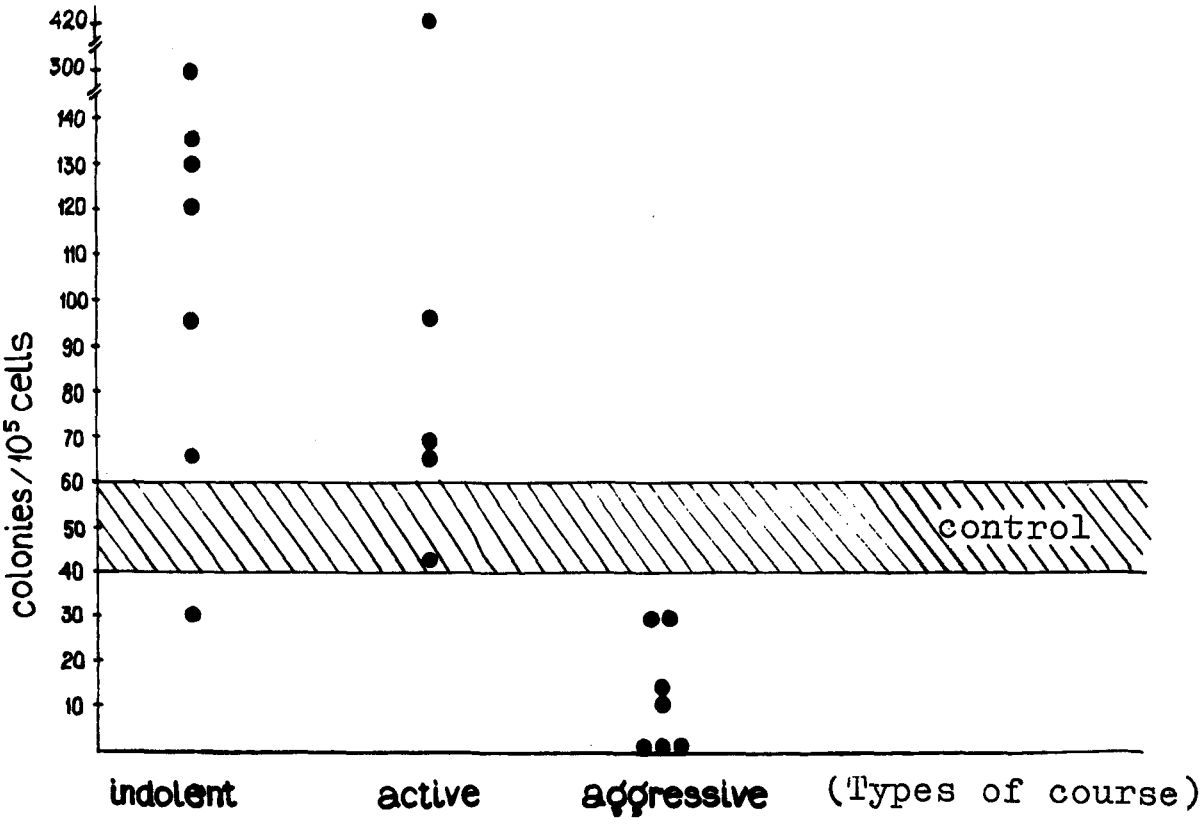

Fig. 5. Colony-forming unit granulocyte and macrophage of bone marrow and the multiple myeloma course

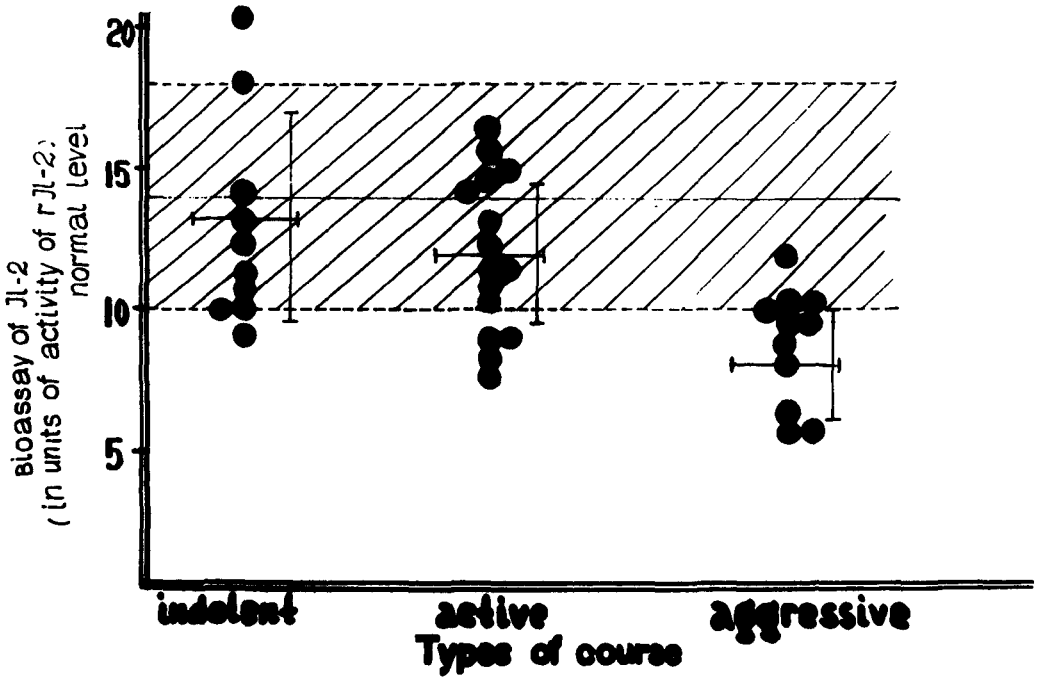

Fig. 6. Production of interleukin-2 by peripheral blood mononuclear cells in patients with different types of courses in multiple myeloma

the course	Jl-2un.of act.
indolent	3,3±0,4
active	4,9±0,4
aggressive	8,6±0,6

p<0,05
p<0,01
p<0,05

Fig. 7. Interleukin-2 and the course of multiple myeloma

Conclusion

Our data suggest that:

1) Morphology of myeloma cells is the most important prognostic factor which, without a doubt, can be easily reproduced, and is widely used in clinical practice.
2) The level of $S\beta_2$-M may be used to determine the MM course type.
3) CFU-GM probably may also be used as a prognostic factor. An increased or normal level of CFU-GM confirms the indolent or active course of MM, while a decreased level of CFU-GM reflects the aggression of MM.
4) Decreased production of IL-2 by peripheral blood monocytes is characteristic of an aggressive MM course.
5) TNF_α has a prognostic significance if it is used together with myeloma cell morphology as
   a) mature myeloma cells and normal production of TNF_α by peripheral blood monocytes are typical of an indolent course;
   b) mature or immature morphology of myeloma cells and increased production of TNF_α are typical of an active course; and
   c) immature or plasmablastic morphology and normal production of TNF_α are typical of an aggressive course.

The studies mentioned above were necessary in order to find out the role of these factors in MM pathogenesis.

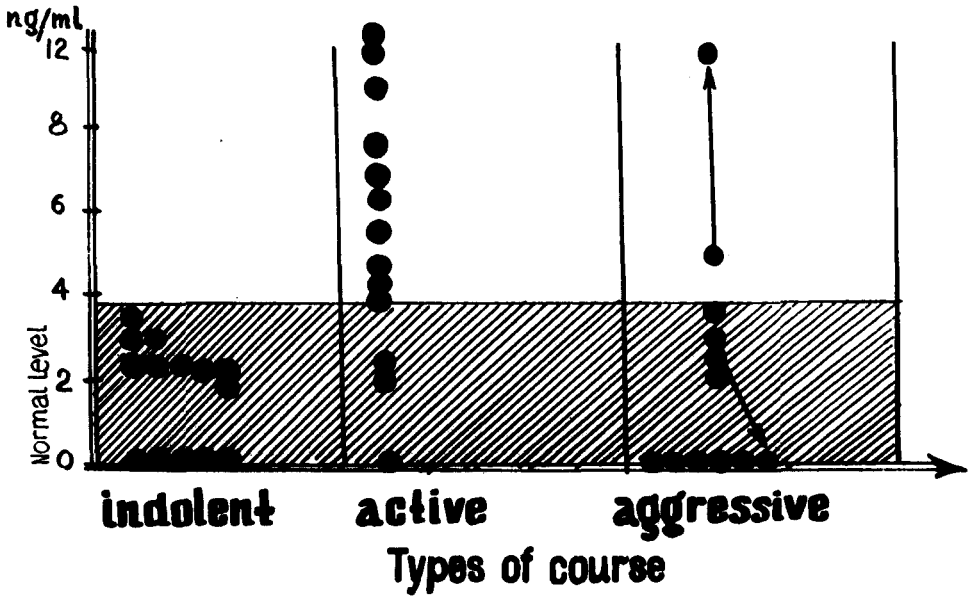

Fig. 8. Pyrogenal-stimulated tumor necrosis factor-alpha production by peripheral blood monocytes in patients with multiple myeloma

the course	TNF-α [ng/ml]
indolent	1,5±0,3
active	6,1±1,1
aggressive	2,0±1,2

P<0,01
P>0,05
P<0,05

Fig. 9. Tumor necrosis factor-alpha and the course of multiple myeloma

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Cytogenetic Disorders During Tumor Progression in Leukemia

A. G. Zedginidze [1]

The successes in studying the karyotype of malignant tumors as well as the achievements in molecular biology and molecular genetics have considerably deepened our knowledge concerning the role of chromosomal disorders in oncogenesis [1–4]. Recognition of clonal origin of leukemias [5] has stimulated the interest in studying the formation and development of cytogenetically marked leukemic clones during development of leukemias.

The purpose of this investigation was to analyze the pathogenetical and clinical significance of karyotypic peculiarities in different stages of the leukemic process.

Chromosomal analysis emplyoing the G-banding technique was carried out in 394 patients with preleukemia (PL), secondary leukemia, different stages of chronic myeloid leukemia (CML), and different variants of leukemia (AL).

The group with PL consisted of 95 patients with firm cytopenia and either hypo-, normo- or hypercellular bone marrow with erythropoietic disorders. Cytogenetic examinations revealed only normal diploid metaphases, but increased percentages of aneuploid and polyploid cells and structural aberrations, i.e., instability of the karyotype and clonal anomalies.

Karyotypic instability manifested both in quantitative and structural aberrations. Quantitative disorders were common for patients with bone marrow hypoplasia, whereas structural aberrations were more often observed during erythropoietic disorders (Table 1).

Both chromosomal and chromatid types of aberrations were found, including breaks, fragments, deletions, chromatid exchanges, marked chromosomes, and, sometimes plural aberrations.

We observed transformation into acute leukemia in 13 out of 88 patients (14.8%). It was found that in patients with karyotypic instability, the frequency of subsequent emergence of abnormal clones and leukemic processes is reliably higher (36.4%) than in patients with normal karyotype (1.8%).

These data suggest that karyotypic instability may provide a favorable background, or even be the first step, for the formation of leukemic clones. The discovery of abnormal clones may be interpreted as evidence of the leukemic process, even without clinical manifestations. During tumor progression, we observed an increase in the percentage of abnormal clones cells as well as the appearance of new clones. The majority of patients with leukemia that developed after preceding preleukemic disorders had clonal abnormalities.

The second group investigated included patients with secondary leukemia that developed as a complication of different neoplastic diseases, treated by chemo- or radiotherapy, 11 months to 8 years after diagnosis of the primary tumor. In 11 out of 13 such patients, abnormal clones were found. Quantitative and structural aberrations of chromosomes 7, 5, 3, and 17 were charac-

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Cytogenetic characteristics of patients with preleukemia

Investigated group	Karotype	Total no. of metaphases	Metaphases with quantitative abnormalities (%)				Metaphases with structural abnormalities (%)		
			Hypodipl. (range)	Hyperdipl. (range)	Polydipl. (range)	Common	Chromosomal type	Chromatid type	Multiple aberr.
Bone marrow hypoplasia ($n=51$)	Normal ($n=34$)	1320	7.4 (1–10)	0.15 (0–2.5)	2.6 (0–4)	0.4 (0–3.1)	–	0.4	–
	Unstable ($n=14$)	550	13.5 (7–20)	8.0 (3–20)	7.0 (0–10)	4.2 (0–20)	0.2	2.5	1.5
	Clonal abnormalities ($n=3$)	56	% of cells of abnormal clones; 48, 27, 43						
Erythropoietic disorders ($n=44$)	Normal ($n=21$)	630	7.1 (2–9)	0.5 (0–2.5)	2.3 (0–5)	0.95 (0–1.5)	–	0.95	–
	Unstable ($n=19$)	604	15.2 (5–34)	7.1 (0–13)	5.8 (0–10)	14.2 (0–40)	1.0	9.2	4.0
	Clonal abnormalities ($n=4$)	85	% of cells of abnormal clones: 75, 27, 16, 33						

Hypodipl: hypodiploidy; *hyperdipl:* hyperdiploidy; *polydipl:* polydiploidy; *aberr:* aberrations.

teristic of this group. Abnormal clones were often observed on the background of bone marrow hypoplasia and karyotypic instability.

The investigations carried out in different stages of CML enabled us to study chromosomal disorders regularly during tumor progression. In 95% of patients with the developed stage of CML, only t(9;22) was found, and neither additional quantitative nor structural aberrations were observed. Supplementary chromosomal abnormalities, both clonal (2PH, +8, +9, +21, i17q) and random (instability), were found in 48% of patients during the period preceding the blast crisis.

The rise of new clones, constituting the basis of blast crisis, was observed more often on the background of karyotypic instability and presented evidence of transformation into the new malignant stage. In the terminal stage of CML, the new abnormal clones were found in 68% of investigated cases.

Thus, our research has shown that karyotypic instability might be considered as a risk-factor of leukemic clone development, or as the first step in their formation. The appearance of abnormal clones can be regarded as a basic differential-diagnostic criterion for revealing early stages of acute leukemia.

The study of AL itself has shown the presence of abnormal clones with nonrandom chromosomal disorders in 58% of patients, 63% in acute lymphocytic leukemia (ALL) and 55.6% in acute nonlymphocytic leukemia (AnLL). In AnLL, mostly with hypo- or pseudodiploidy, chromosomes 5, 7, 8, and 21 were damaged most often, while for ALL, hyperdiploidy with rearrangement of chromosomes 6, 9, 8 and 21 was most common.

The correlation between normal and abnormal metaphases plays a certain role in course of disease and is one of the significant prognostic factors.

The discovery of chromosomal disorders preceding manifestation of AL, the nonrandom chromosome changes in abnormal clones formed during the preblastic stage of CML, the presence of some specific chromosome aberrations in AL, and the correlation between chromosomal disorders and the course of leukemia point out the important role of chromosomal disorders in leukemogenesis.

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New Cytogenetic Aspects of Myelodysplasia and Acute Leukemia

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The use of modern chromosomal banding techniques in preleukemia and leukemia has revealed the presence of nonrandom chromosome abnormalities, some of which have clear diagnostic and prognostic significance. Among these are translocations t(8;21), t(15;17), t(9;11), t(4;11), t(8;14), t(9;22); trisomies 4, 8, 12, 21, 1q; monosomies 5, 7, X, Y; deletions 5q-, 7q-, 9p-, 12p-, 20q- and some others [4]. According to our data nonrandom chromosome abnormalities were revealed in 35 of 47 patients (70%) with myelodysplastic syndromes and in 55 of 108 patients (51%) with acute leukemia.

The study has shown the pure noncomplicated chromosome changes in acute leukemia patients to occur infrequently, which is why the analysis of their role in pathogenesis and prognosis of leukemia is very difficult. Besides, most of them can disappear from the karyotype after cytostatic therapy. The majority of the above-mentioned chromosome abnormalities typical for acute leukemia are found at the stage of myelodysplasia. This evidence and the possibility of repeating investigations of karyotype make myelodysplasia a perfect object for studying many aspects of leukemia cytogenetics.

The accumulated information concerning the karyotypic abnormalities in patients with myelodysplastic syndromes is given in Table 1. A closer look at the material reveals a great variability of cytogenetic findings. The more frequent among them were monosomies and deletions of the long arms in chromosomes 5 and 7, followed by trisomies 8, 21 and 1q, as well as deletion 20q.

The best-known and most widespread anomaly is the 5q-anomaly, described by van den Berghe et al. [9]. The 5q-marker is formed as a result of interstitial or terminal deletion of a long arm of chromosome 5. It occurs in a quite characteristic clinicohematologic setting now termed the 5q- syndrome. This disorder typically develops in elderly female patients. Their anemia is macrocytic and is resistant to therapy. The other significant feature of the syndrome is a presence of normal or elevated platelets in spite of morphologically characteristic megakaryocyte abnormalities. Finally, it is worthwhile that the clinical course of 5q-syndrome is often mild. Transformation into acute leukemia is relatively rare, at least when 5q- is the only abnormality present at diagnosis. Among our patients it took place only once (patient no. 4), when karyotype at diagnosis was 47, XX, 5q-, + 21. On the other hand, growing experience shows that some patients with 5q- anomaly may reveal the nonclassical course of the disorder. For example, our first patient (49 years old, female), was treated at our clinic for 5 years. Initially she had only mild macrocytic anemia which was resistant to therapy and normal or slightly decreased platelet level. Later lymphocytic infiltration of stomach and moderate paraproteinemia had been revealed. However, serial cytogenetic studies did not show any additional nonrandom chromosome changes. For some time the patient was

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Results of cytogenetic analysis of bone marrow cells from patients with myelodysplasias

Case no.	Age/sex	FAB class	Karyotype	Survival without AL (months)
1.	49/F	RA	46, XX, 5q−(q13, q33)	61
2.	78/M	RA	46, XX, 5q−(q12, q337	30+
3.	72/M	RAEB	46, XY, 5q−(q13, q34)	6+
4.	50/F	RA/RAEB	47, XX, 5q−(q13, q33), +21	12
5.	54/M	RAEB	45, XY, −7	3
6.	54/M	RAEB	43, X, −Y, −5, 7q−(q22), −12	1
7.	65/M	RAEB	48, XY, 5q−(q13, q33), 7q−(q22), +8, +9, 12p−(p12), 13q−(q14)	3
8.	80/F	RAEBT	46, XX, −7, +8, inv(9)	4
9.	21/F	RAEB	45, XX, −5, −6, −7, +der(11), t(11p, ?), +22q−(q11)	10
10.	28/F	RSA	46, XX, t(3; 3) (q21; q26)	108+
11.	37/M	RA	45, XY, −7, t(3; 3) (q21; q26)	
12.	62/M	CMML	46, XY, 3q−(q13, 3q23)	23
13.	58/F	RAEB	43, X, −X, 1q+, −3, −5	4
14.	73/F	RAEBT	46, XX, 20q−(q11)	4
15.	49/M	RAEBT	47, XY, −6, +der(6), t(2; 6) (p12; q25), 20−(q11), +20	28
16.	56/M	RAEB	47, XY, +8	10+
17.	68/M	RSA	46, XY, 11q−(q22)	4+
18.	56/F	CMML	47, XX, +11	20+
19.	65/F	RA	46, XX, −14, +i(14q)	5?
20.	18/F	RA	46, XX, −17+der(17), t(1; 17) (q21; p11)	68
21.	28/M	RA	46, XY, −2, +der(2), t(1; 2) (q21; q36)	20
22.	72/F	RA	46, XY, −5, +8, −9, +der(9), t(1; 9) (q11; q32)	36
23.	65F	RAEBT	49−53, XX, −5, +8, +11, +13, +14, −16, +19, +22, +22, +Mar1, +Mar2, +DM	?
24.	52/M	RAEBT	55, XX, +1, +2, −5, +6, +9, +10, +11, +14, −16, +21, +22, Mar1, Mar2	2

AL, acute leukemia; *F*, female; *M*, male; + means the available; patients are alive; ?, no complete information available; *RA*, refractory anemia; *RAEBT*, refractory anemia with excess of blasts; *RSA*, refractory sideroblastic anemia; *CMML*, chronic myelo-monocytic leukemia.

treated with blood transfusions, desferal, and steroids. She died 60 months later from serious bowel infection complicated by collapse. There were no signs of leukemia or lymphoma on autopsy. The case may be considered as an example of a real involvement of B-lymphocyte precursors into the pathological process in patients with 5q− syndrome.

Other nonrandom karyotype changes of the patients with myelodysplasias were monosomy 7 or deletion 7q. They were revealed mainly in refractory anemias with or without blasts and were often combined with some other chromosome abnormalities, including monosomy 5 or deletion 5q. It is noteworthy that three of the patients (cases 5, 6, and 9) had

previous exposure to radiotherapy or cytotoxic drugs. According to our data, pure monosomy 7 was found only in a 54-year-old patient (patient no. 5) who had survived without leukemia for 3 months only. It should be kept in mind that the time of survival without acute leukemia in three other patients of this group (cases 6–8) did not exceed 4 months. It was longer only in two younger patients (nos. 9 and 11), who had additional numerical and structural chromosome rearrangements, including classical translocation t(3;3)(q21;q26). Of particular interest is the patient with t(3;3) associated with monosomy 7, who had no elevated platelet count in spite of the predisposing chromosome rearrangement. Furthermore, there were very few megakaryocytes in the patient's bone marrow. On the contrary, the presence in karyotype of translocation t(3;3)(q21;q26) alone which was revealed in a patient with acquired idiopathic sideroblastic anemia (no. 10) was associated with thrombocytosis and with prolonged (more than 9 years) survival without leukemia. It is worth noting that recently Caroll et al. [3] found the same translocation t(3;3)(q21;q26) in a similar patient with acquired idiopathic sideroblastic anemia and thrombocytosis.

The next nonrandom chromosome abnormality found in two patients with refractory anemia with excess blasts in transformation was deletion 20q. In one of the patients (no. 14) it was a sole rearrangement while in the other (no. 15) the karyotype was complicated by the other chromosome abnormalities. Yet the survival time was shorter in the first case which may be ascribed to her old age.

The results presented in Table 1 show part of the chromosome changes revealed to be associated with increasing cellular genetic material. First of all it concerns trisomies 8 or 11 as well as partial trisomy for the long arm of chromosome 1. The best-known chromosome anomaly among these is trisomy 8, which has been shown repeatedly to be connected with all famous types of myelodysplastic syndromes besides chronic myelomonocytic leukemia. As in our case 16, trisomy 8 may be a sole karyotypic change. However, its combinations with other chromosome abnormalities are more frequent (cases 8, 22, and 23).

The other common chromosome change in patients with myelodysplasia and leukemia [6] is a partial trisomy for 1q. The mechanisms through which trisomy 1q has arisen are translocations t(1;2), t(1;9), t(1;17). The latter took place in a young (18-year-old) woman who was under observation for 5 years [5]. She presented in 1984 with stomatitis, leukopenia and pseudo-Pelger-Huët anomaly. Bone marrow aspirate showed normal cellularity with clear morphologic dyshemopoiesis of all three lineages. Serial cytogenetic studies of the bone marrow cells revealed the cellular clone with t(1;17) and partial trisomy 1 which showed no changes until death. A diagnosis of myelodysplastic syndrome was made. The course of the disease was severe because of many infections which had not been properly controlled by antibiotics. She demonstrated neither clinical nor morphological features of leukemia. Autopsy confirmed the diagnosis of myelodysplasia. The case is interesting in several aspects. First, it demonstrates a new variant of partial trisomy of the long arm of chromosome 1 in a patient with myelodysplasia. Second, it shows that this translocation predisposes the formation of pseudo-Pelger-Huët anomaly. This conclusion is based on recent data illustrating close correlation between deletion 17p and the above-mentioned anomaly in chronic myelocytic leukemia [8]. As for survival time, it was comparatively long in a group of patients with additional genetic material. Thus, the prognosis of myelodysplasia patients, including leukemia risk, is apparently not influenced by the presence of clone with pure trisomy 1q and partial monosomy 17p. On the other hand, the presence of karyotypes with multiple abnormalities is concomitant with unfavor-

able prognosis, including an increased risk of leukemia (cases 24 and 25).

In general, the role of certain chromosome changes at the stage of transformation of myelodysplasia into acute leukemia depends on the character of the abnormality. Some of them, especially monosomy 7 or deletion 7q, are associated with particularly bad prognosis and frequent transformation into acute leukemia. In contrast, such numerical chromosome changes and structural arrangements as trisomy 8, deletion 5q, partial trisomy 1q and translocation t(3;3) can persist in the cells tested from 1 to 9 years. On the basis of these data the conclusion may be drawn that influence of the above-mentioned chromosome abnormalities on prognosis of myelodysplasia patients and on its transformation into acute leukemia is minimal.

Comparison of basic chromosome abnormalities in myelodysplasia and in acute nonlymphoblastic leukemia patients whos their similarity (Table 2). In general, all chromosome changes revealed in myelodysplastic syndromes are now found in leukemia too. On the other hand, some of the most important and constant acute nonlymphoblastic leukemia arrangements – t(9;11), t(15;17), t(9;22) and inv(16)(p13q22) – are not seen in myelodysplasia. This fact indicates that patients with the chromosome abnormality mentioned above pass through a very brief phase pf myelodysplasia, if at all.

Analysis of the cytogenetic changes uncommon to both diseases showed that the most frequent among them were t(9;11), t(9;22), and t(15;17). The first abnormality was associated with M5a subgroup of actue myeloid leukemia (AML) (Table 3). The breakpoints clustered to two regions: 11q22–q24 and the more proximal 11q14 [1]. In most observations including our series the arrangements of the long arm of chromosome 11 were interpreted as reciprocal translocation between 9 and 11 chromosomes. In general, chromosome abnormalities in patients with acute monoblastic leukemia

Table 2. Comparison of basic chromosome abnormalities in myelodysplasia and acute nonlymphoblastic leukemia patients

Chromosome abnormalities	Myelo-dysplasia	Acute leukemia
1q+	+	+
t (3; 3)	+	+
5q−/−5	+	+
7q−/−7	+	+
+8	+	+
+11	+	+
11q−	+	+
12p−	+	+
20q−	+	+
t (6; 9)	−	+
t (9; 11)	−	+
t (15; 17)	−	+
t (9; 22)	−	+
inv (16)	−	+

(M5 variant according to the French-American-British Working Group for Leukemia Classification (FAB) criteria) were more complex than in those with acute myelomonocytic leukemia (M4 variant). It concerned both the number of patients with normal karyotype and the character of chromosome changes. Thus, the group of the patients with M5 variant of AML revealed many nonrandom chromosome abnormalities and the absence of normal karyotypes. In contrast, the karyotype of the cells tested in 8 of 13 patients with M4 variant of AML was normal while the others revealed only moderate chromosome abnormality. A possible explanation for this difference may be the presence in the bone marrow from patients with acute myelomonocytic leukemia of certain granulocytic and/or erythroid elements with unchanged chromosomes.

Another frequent abnormality is the Ph′ chromosome. In our series it was found in ten patients with acute lymphoblastic leukemia. One of them (37-year-old woman) had karyotype, showing duplication of Ph′ chromosome and partial trisomy for the long arm of chromosome 1. The latter arised trough

Table 3. Comparison of nonrandom chromosome changes in patients with M4 and M5 variants of acute nonlymphoblastic leukemia

Karyotype	Number of cases	Type of leukemia	
		M4	M5
46, XX or 46, XY	8	8	–
46, XX, inv. (9)	1	1	–
46, XX, 9q−(q21; q22)	1	1	–
46, XY, t (8, 17) (q22; q23)	1	1	–
46, XY, t (8, 21) (q22; q22)	1	1	–
46, XY/46, XY, DM (7%)	1	1	–
46, XY, t (9; 11) (p22; q23)	1	–	1
46, XX, t (9; 11) (p22; q23)	1	–	1
46, XX, t (9; 11) (p22; q23) /the same +t (12; 18) (q13; p11)	1	–	1
47, XX, +8, t (9, 11) (p22; q24)	1	–	1
47, XY, −4, −5, +Mar1, +Mar2, +Mar3	1	–	1
43, XY, 5q−, −7, −12, −14, +der (7), t (7; 14), −15, −22, +der (22), t (18; 22)	1	–	1
48, XY, +11, +21	1	–	1
55, XX, +1, +2, −5, +6, +9, +10, +11, +14, −16, +21, +22, +Mar1, +Mar2	1	–	1

translocation of part of a long arm of chromosome 1 to the short arm of chromosome 14. Furthermore, the cells tested had lost one chromosome 16 and revealed additionally marker 19p+ which remained unrecognized. In spite of massive cytostatic and hormonal therapy which continued for 2 years, complete remission was not achieved and the patient expired. A similar situation occurred in six more patients with Ph′ positive acute lymphoblastic leukemia where it was impossible to control the expansion of leukemic clone by cytostatics. The current literature indicates that the average survival for Ph′ positive AML patients was about 10 months [7]. Similar prognostic features were also characteristic for Ph′ positive acute lymphoblastic leukemia in which the rate of remission was very slow [2].

Taken together, these data show that rearrangements of chromosomes found in myelodysplasia and acute leukemia play the significant role in pathogenesis and prognosis of these disorders. Furthermore, the breaks of such loci as 3q26, 11q22–24, 16q22, and 17p11 are related to the formation thrombocytosis, monocytosis, eosinophilia, and Pelger-Huët anomaly.

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Detection of Minimal Residual Leukemia by Polymerase Chain Reactions *

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Introduction

Disease recurrence following successful remission induction by polychemotherapy or bone marrow transplantation represents a major clinical problem in the treatment of leukemia patients. Sensitive methods for the identification of neoplastic cells escaping therapeutic interventions and potentially causing relapse are important for the monitoring of therapeutic effectiveness. The in vitro amplification of genomic or complementary deoxyribonucleic acid (cDNA) target sequences by polymerase chain reaction [1] has opened new avenues toward the detection of minimal residual leukemic cells at frequencies of 1:10000 to 1:1000000. In the following we will briefly summarize our recent experience with the application of polymerase chain reaction (PCR) strategies to patients with chronic myelocytic leukemia (CML) and acute lymphoblastic leukemia (ALL) in complete remission according to clinical and laboratory parameters.

Materials and Methods

Cell Samples

Since 1986, bone marrow (BM) or peripheral blood (PB) cell specimen obtained from 332 children with ALL have been referred to our laboratory for immunogenotype analysis as part of the prospective German multicenter BFM (Berlin-Frankfurt-Münster) ALL/NHL-1986 trial [2]. Details of molecular genetic and immunological data and their correlation to clinical features will be described elsewhere after completion of the trial. Clonospecific probes were prepared from 17 patients (eight T-ALL, nine cALL) exhibiting a suitable T cell receptor (TCR) δ gene rearrangement, and used for evaluation of the remission status by PCR. We also investigaged three pediatric Ph-positive ALL. The study of Ph-positive CML patients treated by allogeneic bone marrow transplantation (BMT) at the Department of Internal Medicine III, University of Ulm, was restricted to 40 cases being in complete remission at the time of initial PCR analysis according to clinical, hematological, cytogenetic, and Southern blot criteria [3].

Polymerase Chain Reaction

For the detection of Ph-positive leukemic cells we used PCR protocols described previously [4–6]. After the synthesis of cDNA by reverse transcriptase, messenger ribonucleic acid (mRNA) from the hybrid BCR/ABL or normal ABL alleles was amplified by a set of nested primers. As internal ABL control we amplified a fragment of 173 bp with oligomers complementary to ABL exon II and III sequences (Fig. 1). Rearranged BCR/ABL molecules were amplified by primers derived from either BCR exon I and ABL exon II to detect 271 bp fragments corre-

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Haematology and Blood Transfusion Vol. 35
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R. Neth et al. (Eds.)

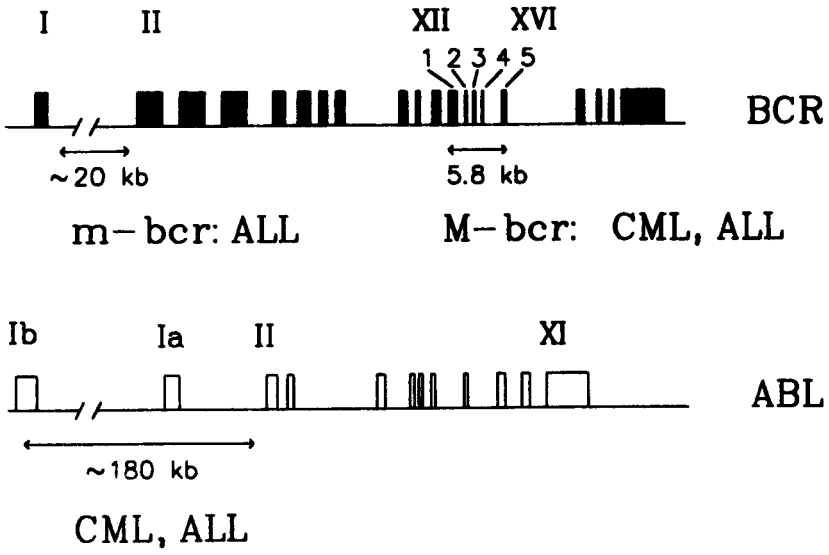

Fig. 1. The Ph translocation is molecularly characterized by a rearrangement of the BCR and ABL genes. The breakpoints on chromosome 22 are clustered within two limited areas (*M-bcr* and *m-bcr*), the breaks on chromosome 9 occur in the first ABL intron

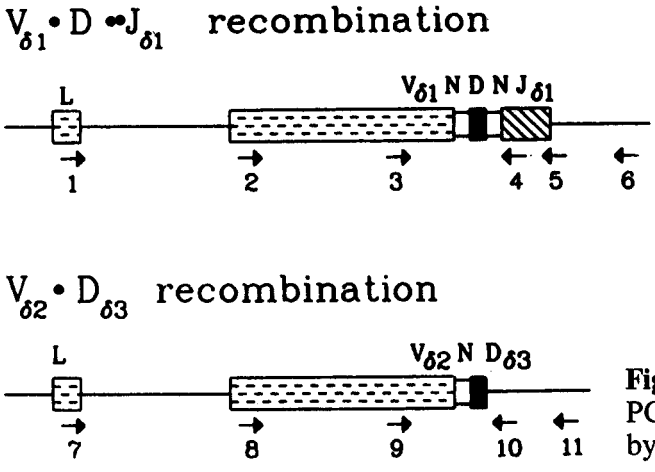

Fig. 2. Position of oligomers (*arrows*) used for PCR analysis of ALL patients characterized by $V_{\delta 1}DJ_{\delta 1}$ or $V_{\delta 2}D_{\delta 3}$ rearrangements

sponding to breaks within m-bcr, or M-bcr exon 1 + 2 and ABL exon II resulting in 395 or 320 bp fragments depending on the presence or absence of M-bcr exon 3 in the hybrid transcript (Fig. 1).

Isolation and hybridization of clono-specific TCRδ probes for the detection of residual ALL followed a PCR strategy recently described by us in detail [7]. The position of nested primers used for probe preparation (oligomers 1/6 + 3/4 or 7/11 + 9/10) and detection of residual leukemic cells (oligomers 1/6 + 2/5 or 7/11 + 8/11) is indicated in Fig. 2.

Results and Discussion

Ph-Positive Leukemias

The Ph translocation is based on a recombination of the BCR and ABL genes [8]. The breaks vary over a distance of more than 180 kb on chromosome 9, but cluster within two limited regions of the BCR gene on chromosome 9, called the major (M) and minor (m) breakpoint cluster region (bcr) (Fig. 1). In virtually all Ph-positive CML patients the breaks occur in introns between exon 2/3 or 3/4 of the M-bcr, while in Ph-positive ALL either of the two areas is involved. Despite dif-

Table 1. PCR analysis of 40 Ph-positive CML patients in CCR after BMT

Patients (*n*)	Clinical features	Initial PCR status	Follow-up
6	Long-term survivors (> 5 years)	all negative	CCR
6	T-cell depleted BMT, initial PCR 19–48 months after BMT	all positive	2 clinical relapses 1 genetic relapse
28	Initial PCR 1–37 months after BMT	11 positive	4 PCR negative 2 genetic relapses 3 clinical relapses
		17 negative	1 genetic relapse 1 clinical relapse

ferences at the genomic level, Ph-positive leukemias become accessible to PCR analysis because BCR exons are always spliced to ABL exon II [4, 5].

Since allogeneic BMT appears to be the only accepted curative therapy for CML resulting in the eradication of the malignant cell clone, we recently determined the remission status of 40 transplanted CML patients by PCR analysis. The result of this study is summarized in Table 1. All long-term survivors (5–9 years) showed no residual disease in at least two independent PCR and might be truly cured from CML. In contrast, all patients receiving a T-cell depleted marrow for prevention of graft-versus-host disease exhibited residual leukemic cells. Two of the patients experienced a clinical relapse within the following year; in a third patient, Ph-positive cells became visible again in Southern blot and cytogenetic analyses. These data might be reconciled with previous reports on a significant increase in clinical relapses after T-cell depleted BMT [9]. In 28 patients initial PCR analysis was performed 1 month to 3 years after BMT (Table 1, Fig. 3). Fifteen patients scored repeatedly PCR-negative during follow-up, while 2/17 cases became PCR-positive and relapsed either clinically or cytogenetically. Among the 11 cases with an initially PCR-positive result, five patients experienced clinical or cytogenetic relapses. Interestingly, four patients became PCR-negative during the following year.

Our data underline the importance of longitudinal PCR analysis in individual patients. Thus in some cases residual leukemic cells may be diluted out during the post-BMT period. However, patients may also become PCR-positive and relapse clinically, despite an initially negative result. We would like to emphasize that the precise clinical relevance of PCR analysis remains to be elucidated in prospective trials including more patients. Along this line it is remarkable that one of the repeatedly PCR-positive cases has been in otherwise complete remission for more than 4 years. Conflicting data as to the frequency of residual leukemia have thus far emerged from reports on PCR analysis in transplanted CML patients [6, 10–16]. These discrepancies could originate from various sources including differences between therapeutic regimen, cell samples, or technical problems. False positive reaction due to contamination with as few as one specific molecule is a major challenge that can only be overcome by rigorously following standardized precautions [17].

The PCR strategy discussed above is also applicable to a significant number of ALL patients. In adults the Ph translocation constitutes the most frequent cytogenetic abnormality, while it is found in only 5% of pediatric cases. Irrespective

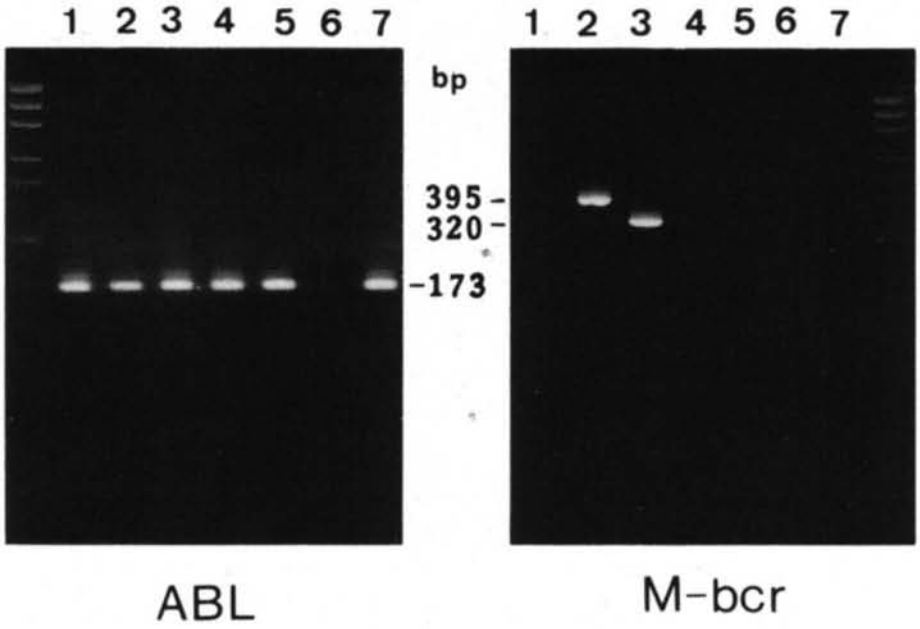

Fig. 3. PCR analysis of six Ph-positive CML patients in complete remission after BMT. Two equal volumes of BM cDNA were amplified by use of oligomers detecting either ABL or rearranged BCR-ABL fragments. All cases exhibit a positive ABL control fragment of 173 bp. Residual leukemic cells characterized by fragments of 395 bp or 320 bp are visible in two patients (*lanes 2 and 3*). *Lane 6* contains a negative control (water). Samples were run in agarose gels and visualized by ethidium bromide staining. *Hae*III digested ∅X174 DNA served as molecular weight marker

of age, however, Ph-positive ALL signals a very poor prognosis. PCR is an important alternative strategy to cytogenetic analysis for the rapid diagnosis and subclassification of Ph-positive leukemias and offers a useful approach for the detection of residual leukemic cells. Thus far we have monitored three children with Ph-positive ALL. Although the patients were otherwise at standard risk and achieved a complete clinical-hematological remission, the leukemic cell clone was never eradicated completely as indicated by PCR. All three patients relapsed 6–9 months after initial diagnosis. It appears to be necessary and worthwhile to extend respective investigations to more patients in multicenter trials.

ALL Patients Characterized by Distinct TCRδ Recombinations

Different PCR strategies based on the specific immunogenotype of ALL patients have been proposed for the detection of residual leukemic cell populations [7, 18, 19]. The approach developed in our laboratory takes into account the striking observation that the vast majority of all ALL patients show a TCRδ recombination and, moreover, a preferential use of specific TCRδ elements depending on the immunological subtype [7, 20–27]. In our series of 332 pediatric ALL cases analyzed prospectively for immunoglobulin and T-cell receptor arrangements, we observed a TCRδ recombination in 96% of T-ALL (58/60) and 81% of cALL patients (163/204). Southern blot analysis demonstrated a hybridization pattern predicting a $V_{\delta 1}DJ_{\delta 1}$ recombination in 25% of T-ALL and a $V_{\delta 2}D_{\delta 3}$ rearrangement in 57% of the cALL patients. This interpretation was confirmed for the 23 cases examined by PCR-directed sequence analysis [27].

Based on Southern blot data we prepared clonospecific probes from leukemia cell DNA of eight T-ALL characterized by a $V_{\delta 1}DJ_{\delta 1}$ rearrangement and nine cALL exhibiting a $V_{\delta 2}D_{\delta 3}$ recombination (Fig. 2). None of the probes showed cross-hybridization to DNA obtained from leukemic cells of the other ALL patients or from healthy probands, con-

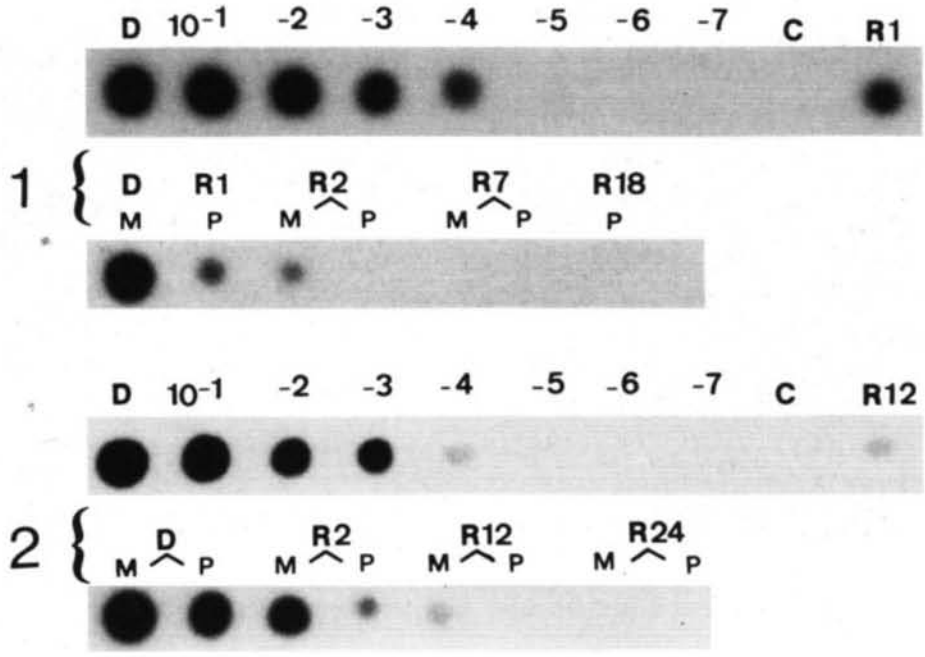

Fig. 4. Detection of minimal residual leukemia in two cALL patients characterized by $V_{\delta 2}D_{\delta 3}$ rearrangements. DNA obtained from leukemic cells at diagnosis (*D*) was diluted into peripheral blood cell DNA of a healthy control (*C*) at 10^{-1} to 10^{-7}. Upon amplification DNA fractions (2 ng) were spotted onto nylon membrane and hybridized to the clonospecific TCRδ probes derived from leukemic cells of each patient, thus establishing a detection limit at 1:10000. PCR analysis of bone marrow (*M*) and peripheral blood (*P*) DNA obtained during continuous complete remission (*R*) 1–24 months after initial diagnosis revealed significant differences in the decrease of residual leukemic cell populations between both patients

firming our previous experiences [7]. To determine the sensitivity of the clonospecific probes in detecting residual disease, we performed dilution experiments (Fig. 4). Following 70 PCR cycles primed by nested amplimers, 10^{-4} leukemic cells were detected in all cases. Due to greater junctional diversity, the sensitivity of clonospecific $V_{\delta 1}DJ_{\delta 1}$ probes was even higher and identified leukemia DNA when representing as little as 0.0001% of total DNA.

We next analyzed BM DNA samples of the 17 ALL patients during complete clinical-hematological remission; none of the samples revealed rearranged TCRδ fragments upon Southern blot analysis suggesting a frequency of residual leukemic cells, if any, below 1%. However, PCR demonstrated residual leukemia in a significant proportion of patients (Table 2). Remarkably, all eight patients investigated 3 weeks to 4 months after starting chemotherapy showed residual leukemic cells (range 10^{-2} to 10^{-5}), while the six patients studied more than 18 months after diagnosis scored negative. Longitudinal studies performed in seven cases indicated that residual neoplastic cells may persist, independent of known

Table 2. PCR analysis of 17 children with ALL in CCR using clonospecific TCR δ probes

Months after diagnosis	Children[a] (*n*)	PCR status of BM cells
1	5	all positive
2–6	9	6 positive 3 negative
7–12	6	3 positive 3 negative
13–23	4	1 positive 3 negative
>24	4	all negative

[a] Consecutive remission samples were analyzed from seven cases.

risk factors, for variable periods in different individuals (Fig. 4). Thus in some patients the leukemic cell clone is obviously eradicated after 3 months, while in other cases residual disease was demonstrated up to 14 months after diagnosis.

We would like to mention some possible limitations of the PCR approach. Thus PCR will also amplify DNA sequences of functionally irrelevant or dead cells and even residual DNA particles originating from leukemic cells. On the other hand, any subpopulation of leukemic cells characterized by further recombination of sequences comprised in a clonospecific probe would escape detection by PCR, despite a potential capacity to proliferate and cause clinical relapse.

Although still preliminary, our data suggest that PCR analysis offers a useful tool to elucidate the biological and clinical significance of residual disease in ALL patients. Ultimately this approach may be part of a rationale to adapt highly aggressive therapeutic regimen to the individual demand of a leukemia patient thereby preventing excess of therapy. For the time being any conclusion drawn from PCR analysis has carefully to consider possible technical pitfalls as well as clinical features and complementary laboratory data.

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Structural Changes in Blast Cell Chromatin and DNA in Patients with Acute Leukemia After Cytosar and Irradiation Treatment

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It is known that chromatin DNA is the main target for many drugs in the living cell. Drugs which bind with double helix DNA are divided into two classes:

1) those which directly interact with DNA and change its stability and conformation and
2) those which indirectly influence DNA.

Cytosar (or β-cytosine arabinoside) belongs to the first class of drugs. Influence of Cytosar on DNA and RNA is not restricted only to its binding with these structures. It also makes ruptures in double helix DNA, causing cell destruction [1].

Here we present microcalorimetrical results, showing how Cytosar and laser irradiation influence the structure stability of leukocyte chromatin in vivo and leukocyte DNA in solutions. We think that these results lead to a better understanding of how genetic material behaves in vivo under the influence of chemical and physical factors of cells.

A differential scanning microcalorimeter was used to study the complex biological systems [2]. Its sensitivity is 10^{-7} W, the measuring cell volume 0.2 cm^3, and heat rate 20 °C/h. Irradiation was carried out with two beams of helium-neon laser (30 mW) for 10–15 min. Twenty samples were studied. Cytosar was added so that each DNA nucleotide pair had 0.1 ± 0.01 of a molecule. Cells were incubated at 37 °C for 1 h.

In earlier works it was shown that the thermal denaturation process of cell suspension can be divided into three main temperature ranges: the first range 40°–65 °C with $T_d^1 = 57$ °C, the second 62°–75 °C with $T_d^2 = 73$ °C, and the third 75°–95 °C with $T_d^3 = 82$ °C. It was established that in the first and second ranges membranes, proteins, and ribonucleic protein (RNP) complex were denatured but in the third, the chromatin complex was denatured [3].

Figure 1 shows heat absorption curves of normal cells, leukocytes, and blast cells of human bone marrow. In the case of pathology, the chromatin complex in cell composition is more thermostabile than the norm. The temperature corresponding to the maximum blast cell chromatin denaturation peak is ~ 87 °C. This value is 7 °C higher than in the norm ($T_d = 80$ °C). Striking changes are observed in other parameters too: T_d increases by 4°–11 °C (in the norm $\Delta T_d = 7$ °C), melting enthalpy (Q_d) increases by 30%, i.e., 26.0 ± 3.0 kal/g DNA. As for thermostability of leukocyte RNP complex it is also more thermostabile than the norm by approx. 4 °C.

During incubation of the blast cells with the cytostatic drug Cytosar, we observe a shift of the chromatin peak maximum (Fig. 1, curve 3) to a temperature 3 °C lower in respect to leukocytes ($T_d = 84$ °C) and the peak narrowing to $\Delta T_d = 9.5$ °C. The analogical situation is observed for RNP complex too. After laser irradiation of blast cell suspension previously incubated with Cytosar for 12 min, the thermodynamic characteristics of chromatin denaturation and

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

RNP complex processes become more similar to the norm parameters.

From these results we can conclude that Cytosar and laser irradiation in used doses destabilize the tumor chromatin complex, bringing its thermodynamic parameters closer to the norm.

Figure 2 presents heat absorption curves of human bone marrow leukoblasts after laser irradiation for 12 and 15 min. In the first case (curve 1), chromatin T_d is shifted to a lower temperature by 7 °C for leukocytes but the width of the peak half height is narrowed by 3 °C. Q_d remains almost unchanged, i.e., 26.5 ± 3.0 kal/g DNA. We also observed other changes: In particular, a clear shoulder has appeared on the high temperature side at $T_d = 87$ °C. At 15 min irradiation $T_d = 75$ °C for leukocytes this value is 12 °C less; $\Delta T_d = 9°$ and $Q_d = 26.2 \pm 3.0$ kal/g DNA. In this case we again observe a high temperature shoulder at 87 °C.

From the results given above, it follows that after laser irradiation of intact blast suspension chromatin complex the thermostability decreases by 5°–12 °C, but after irradiation of suspension previously incubated with Cytosar, the denaturation temperature decreases by 7 °C (Fig. 1, curve 4). It points to the rough structural disturbances in chromatin complex. Consequently, laser irradiation influences the effect of cytostatic drug preparations on blast cell genome models in vitro.

It was interesting to find out what caused T_d decrease of leukocyte chromatin in cell composition after treatment with Cytosar and laser irradiation. Its decrease was probably connected with the decrease in DNA double helix thermostability as a result of its structure change or with change of the histone binding strength or histone proteins with leukocyte chromatin DNA.

As seen in Fig. 3, addition of Cytosar in leukocyte DNA solution causes a shift in the melting curve to higher temperatures (T_d), a broadening of the transition interval (ΔT_d) and an increase of the melting enthalpy (Q_d) by 1.5 °C, 2.5 °C,

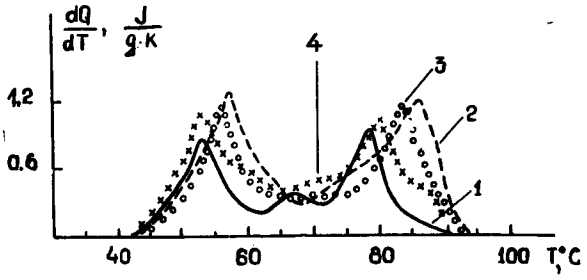

Fig. 1. Heat absorption curves of human bone marrow cells in Henks' solution (pH 7.2). *1*, Norm; *2*, leukocytes; *3*, blast cells treated with Cytosar ($r = 0.01$ mol cyt./bp DNA); *4*, blast cells treated with Cytosar after 12 min laser irradiation

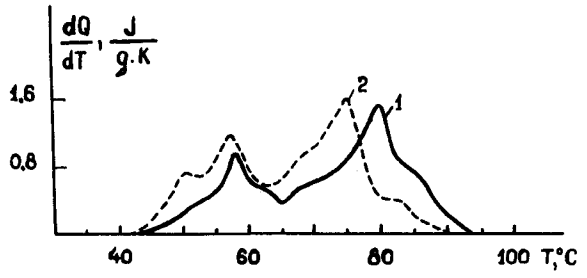

Fig. 2. Heat absorption curves of human bone marrow blast cells in Henks' solution (pH 7.2). *1*, after 12 min laser irradiation; *2*, after 15 min laser irradiation

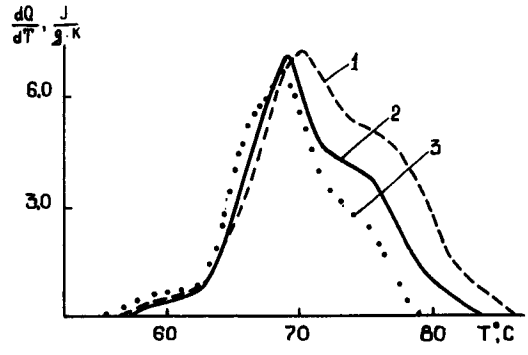

Fig. 3. Heat absorption curves of DNA dilute solutions. *1*, Norm; *2*, DNA + Cytosar ($r = 0.01$ mol.cyt/bp DNA); *3*, DNA + 12 min laser irradiation ($C_{DNA} = 0.12\%$, pH 7.2, 0.1 SSC)

4.5 kal/g DNA, accordingly. Besides this, a strong shift (by ~ 3 °C) to higher temperatures of a high temperature shoulder ($T_d \sim 77$ °C) by 1 °C in comparison with the main fraction ($T_d \sim 69$ °C) is clearly seen. We think that observed changes of the transition parameters (Q_d, ΔT_d, T_d)

and melting curve profile are connected with the fact that Cytosar weakly but specifically stabilizes GC pairs.

So we see that the influence of Cytosar on the DNA molecule is not the same in vivo and in vitro. Nearly the same situation is observed in the case of laser irradiation.

We conclude:

1) Cytosar in its metabolized form acquires new properties leading to disturbance of double helix DNA structure [1];
2) it influences genetic material through change of interaction between DNA, histones, and nonhistone proteins.

So these data show that study of DNA physicochemical properties alone is not always enough to understand DNA behavior in vivo. We can also affirm that the method of scanning microcalorimetry developed in the Institute of Physics of Georgian Academy of Sciences can serve as a convenient and cheap method but in some cases as an quick method for discovering drug influence on genetic material in vivo.

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Cell Biology

Animal Models of Normal and Leukemic Human Hematopoiesis

J. E. Dick [1]

Introduction

Over the last 40 years the hematopoietic system has provided many of the important paradigms that guide our understanding of stem cell function. Much of our knowledge of the regulation of the hematopoietic system is derived from experiments in the mouse; these studies have involved identification of various classes of progenitor cells, growth factors that stimulate growth and differentiation, and molecular events that underlie the abnormalities that occur in diseases such as leukemia. This information has derived largely from the development of in vivo transplantation assays for normal stem cells and the ability to establish and grow leukemic cells in vitro and in vivo [1]. In contrast, our understanding of the biology of the human hematopoietic system has suffered relative to that in the mouse because of the lack of similar assays for normal stem cells and leukemic cells. Normal and leukemic human cells often appear to have complex growth factor requirements that are not easy to provide in short- or long-term cultures. Furthermore, the difficulties in growing primary human leukemic cells in culture suggest that there are selective processes that may result in alterations of the properties of such cells over time and the resultant cell lines do not accurately reflect the original disease. In an attempt to develop in vivo animal models for human leukemic cells, a large body of literature has accumulated over the past 20 years on the growth of human tumor xenografts in immune-deficient *nude* mice [2]. However, the growth of human leukemic cells as an ascites or solid subcutaneous tumor in *nude* mice does not reflect the normal course of the disease in humans. In addition to leukemic cells, normal human hematopoietic cells have also been introduced into *nude* mice directly or in diffusion chambers. The transplantation of human bone marrow directly into mice generally yielded inconclusive results [3], while the implantation of diffusion chambers demonstrated the development of human progenitors for as long as 28 days in vivo although it was not possible to distinguish between persistence of progenitors and engraftment of stem cells [4].

The recently described approaches to engraft human cells into two novel strains of immune-deficient mice provide the foundation for in vivo assays to characterize the normal developmental program of human hematopoietic stem cells along the myeloid and lymphoid lineages [5, 6, 7] and to develop models of several human hematopoietic diseases. Already, important preliminary experiments have established the feasibility of such models for leukemia [8], cancer [9, 10, 11], infectious diseases [12, 13, 14], and autoimmunity [15, 16]. Here I will briefly review our attempts to develop animal models for normal and leukemic human hematopoiesis.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Successful engraftment of human cells into mice is dependent on the development of two particular strains of immune-deficient mice; *scid* and *bg/nu/xid* (abbreviated *bnx*). The *scid* mouse has already played an important role in the characterization of the murine lymphoid system because it is an ideal recipient for transplantation experiments designed to detect lymphoid reconstitution without the complications of lethal irradiation [17]. The *scid* mutation, carried on mouse chromosome 16, prevents the production of nature T and B cells; all other hematopoietic lineages including stem cells, myeloid progenitors, B cell precursors, and natural killer (NK) cells are normal [18]. The action of this gene product is not restricted to normal lymphoid development since *scid* mice have a generalized radiation repair defect that renders the animals at least two times more sensitive to the effects of γ-radiation [19]. In spite of these defects, homozygous *scid* mice have a normal life span and in all other respects appear normal although they are highly susceptible to infection.

The underlying defect and origin of the *bg/nu/xid* mouse is quite different to that of the *scid* mouse. It was constructed by combining three recessive mutations, *beige*, *nude*, and *xid*, to generate a potential recipient mouse for human tumor xenografts that was more immune-deficient then the parental *nude* mouse [20]. The *nude* mutation affects the development of the thymic epithelium, thereby preventing T cell differentiation resulting in an athymic mouse; all other lineages appear normal. It should be noted that some extrathymic processes lead to low levels of mature T cells especially in mice stimulated by exposure to antigens. The *bg* mice have a deficiency in cytotoxic T cells and NK cells [21]. The mutation is a lysosomal storage defect producing abnormally large cytoplasmic granules, so the effect on the immune system is indirect. The *xid* defect is an X chromosome linked gene that affects lymphokine activated killer (LAK) cells as well as B cell responses to certain thymus independent antigens [22]. Animals in which the *nude* and *xid* mutations are combined are very deficient in B cells that appear to be blocked in development at some point between pro-B cells and before cytoplasmic immunoglobulin expression [23]. In the particular outbred *bg/nu/xid* mouse used in our experiments, the *bg* mutation is not as active as if it was alone and the mice have only slightly reduced levels of NK cells, although they are deficient in LAK activity presumably by the action of the *xid* gene. Although the exact relationship between NK and LAK cells is unknown, both may display anti-tumor activity and appear to play an important role in host resistance to xenografting [24, 25]. It is important to stress that both *scid* and *bg/nu/xid* mice have NK cells and intact non-lymphoid resistance systems such as macrophages, therefore any environmental exposure to antigens stimulate these resistance mechanisms. We have found that animals with even mild subclinical infections are extremely resistant to xenografting [8].

In our initial experiments, human interleukin-3 (IL-3) and granulocyte macrophage–colony-stimulating factor (GM-CSF) were delivered to the animals by implanting an osmotic minipump subcutaneously. The animals were also given sublethal doses of radiation since syngeneic transplantation is faster and more complete if the recipient animals are conditioned with irradiation or chemotherapy prior to transplant. Animals were killed at various lengths of time after transplant and the bone marrow and spleen were analyzed by molecular techniques using human specific probes to determine whether human DNA was present; human cells comprised approximately 0.1%–1.0% of these tissues [6]. Since the goal of these experiments was to determine whether any of the earlier hematopoietic cell types had engrafted the mice, cells from these tissues were plated in in vitro progenitor assays that were selective for the growth of human

colony-forming unit–granulocyte macrophage (CFU-GM). Significant numbers of human progenitors were detected in the spleen and bone marrow. No human DNA or progenitors were detected in wild-type animals prepared the same way and only very low numbers of human progenitors could be detected in the hematopoietic tissues of *scid* mice transplanted with human bone marrow.

There did not appear to be any difference in the level or speed of engraftment in the presence or absence of exogenously added human growth factors. This applies only to the two factors tested and on the level of CFU-GM progenitors. It remains to be seen whether other combinations are more effective and whether the presence of these or other factors affect the differentiation of progenitors into mature cell types. There are several explanations for successful engraftment in the absence of exogenous human factors. The human cells could be secreting their own growth factors or the cells are responding to some cross-reactive murine factor or the murine hematopoietic microenvironment. We have recently found that some human factor-dependent myeloid leukemic cell lines proliferate in immune-deficient mice (C. Sirard and J. E. Dick, unpublished) and others have reported the stimulation of human lymphoid leukemic cell lines on murine stromal cells [26], lending credence to the idea that the murine environment is capable of stimulating human hematopoietic cells.

While these data clearly showed that human progenitors can engraft mice, the more important question was whether human stem cells had also engrafted. This is not an easy question to anwer because stem cells can only be identified by their function; this includes the ability to differentiate into all lineages, high self-renewal capacity, slow cell cycling, ability to engraft for long periods of time, etc. [1]. Two lines of evidence suggest that a cell type at least earlier than CFU-GM is responsible for maintaining the engrafted cells. Examination of the kinetics of engraftment indicated there was a rapid increase, of at least 40-fold, in the number of CFU-GM during the first 14 days of engraftment. CFU-GM have a very low self-renewal capacity and would not be able to generate such a large increase, suggesting that an earlier cell type is responsible for this large increase. Furthermore, human progenitors have been detected in animals 7 months after transplantation. Finally we have preliminary evidence that human bone marrow, highly enriched for earlier cell types, can engraft the mice. Taken together this suggests that some earlier cell type can engraft immune-deficient mice, although the exact nature of this cell type is unknown.

Gene transfer provides a powerful tool to more conclusively characterize the engrafting cell types. This stem cell marking technology has been powerfully applied to elucidate the stem cell hierarchy in the mouse [27] and a similar strategy in the human system should enable more precise identification of the human cell types responsible for engrafting the mice. Human bone marrow was infected with a retrovirus vector that contained the dominant selectable *neo* gene using the optimized conditions [31]. Infected and preselected cells were transplanted into immune-deficient mice. A large proportion of the human progenitors detected in the bone marrow for at least 4 months after transplantation contained the retrovirus. In combination with polymerase chain reaction (PCR) technology to clone out the virus integration site in small numbers of cells [29], these gene transfer experiments lay the foundation to determine whether the different progenitors engrafting the mice arise from a common progenitor. The engraftment of mice with both myeloid and lymphoid cells should permit the detection of a pluripotent human stem cell using these approaches.

Models of Human Leukemic Diseases

The growth of normal human hematopoietic cells in *scid* or *bg/nu/xid* mice suggested that these mice may also be useful recipients in which to grow human leukemic cells that often are difficult to establish in culture and to provide a system to study the growth of human leukemia in vivo. Non-T acute lymphoblastic leukemia (ALL) is the most prevalent childhood leukemia and is characterized by a pre-B cell phenotype [30]. Our initial studies have focussed on bone marrow taken directly from patients with non-T ALL and cell lines recently established from relapse patients. One such cell line (A-1) is Epstein–Barr virus (EBV) free, has a normal karyotype, and grows autonomously, producing an unidentified factor which augments its growth in semi-solid clonogenic assays and suspension cultures. Not only did A-1 grow in *scid* mice transplanted according to the same procedures as normal bone marrow but they showed a pattern of infiltration reminiscent of that observed in many children with ALL [8]. For the first month post-transplant, the A-1 cells could be detected only in the bone marrow, and only after an additional 8 weeks were large numbers (>75%) of leukemic cells present in the spleen and bone marrow. Small infiltrates were also present in the kidney and liver at this time. High numbers of A-1 cells were found in the peripheral circulation several weeks later, coinciding with widely disseminated leukemic infiltrates in many organs, including the brain. The animals began to die at 12 weeks after transplant. The widely disseminated growth particularly in the central nervous system is a feature of the terminal disease in children.

Cell lines with different growth factor requirements proliferate differently in *scid* mice. Another cell line (G-2) which responds to different growth factors than A-1 and grows slower in culture, proliferates extremely rapidly in mice, killing animals in 6 weeks at comparable cell doses to A-1. Limiting dilution experiments indicate as few as 100 G-2 cells will produce leukemic growth in *scid* mice. An additional observation has been the infiltration of G-2 into the thymic remnant present in *scid* mice and elevated levels of the CALLA antigen on the cells that infiltrated the thymus. CALLA is a differentiation antigen expressed on early human B cells and is a marker for non-T ALL. It was expressed on the cells originally obtained from the patient but was completely lost during in vitro cell culture. Independent clones and limiting dilution experiments all suggest that CALLA expression is being modulated in vivo as opposed to selection of rare positive cells in the population. The growth and modulation of differentiation antigens of non-T ALL cells in immune-deficient mice implies that they are responding to some murine growth factor or microenvironmental influence. Interestingly, Gluck et al. found that murine stromal cells can support the growth of factor dependent non-T ALL cell lines, lending further credence to this idea [26].

The ability to engraft bone marrow directly from patients with leukemia, either before or after treatment, into *scid* mice could be a valuable tool for predicting the clinical course of the disease, detecting residual leukemias, and for developing individualized therapeutic strategies. Toward this objective, bone marrow cells taken directly from patients at diagnosis with non-T ALL were injected into *scid* mice. Samples taken directly from patients at diagnosis grew little or not at all in the bone marrow and spleen of *scid* mice even after 8 months of observation. In contrast, all of the bone marrow samples taken from patients with recurrent disease in first or second relapse proliferated extensively into a widely disseminated leukemia in mice. Cells from patients who have relapsed several times and who are not responding well to therapy were the most aggressive in mice with the shortest latency. These data show that some biological parameters associated with poor clinical outcome,

such as rapid and widely disseminated proliferation, can be reproduced in SCID mice.

In addition to lymphoid cells, acute myeloblastic leukemia (AML) and chronic myelocytic leukemia (CML) cell lines also grow in immune-deficient mice (C. Sirard and J. E. Dick, unpublished). Some of the well-established lines such as K 562 which do grow in *nude* mice also grew very rapidly in *scid* mice. As noted earlier, one of the AML cell lines (MO7E) which is human growth factor (IL-3, GM-CSF) dependent grew extensively in the bone marrow of *scid* mice without an exogenously added factor.

The establishment of an in vivo model for human leukemia presents a unique system in which to address experimentally a number of biological questions governing the clinical outcome and the growth of leukemic cells in vivo. For example, the identification of leukemic cells in bone marrow usually dictates the course of chemotherapy; however, histological methods for detecting low numbers of residual cells are neither sensitive nor precise. Further refinement of the animal model could offer a sensitive method by which to study residual cells from patients undergoing chemotherapy. Furthermore, new chemotherapeutic and immunotherapeutic protocols, combinations of biological response modifiers, or new unconventional therapies that are difficult to develop and evaluate by human experimentation can be tested in an in vivo situation which mimics the progression of human leukemia. Using high-efficiency gene transfer technology, individual leukemic cells can be marked to follow the growth and development of clones during the multistage progression of the disease. Gene transfer in conjunction with this model system should allow the introduction of key growth regulatory genes, such as oncogenes or tumor suppressor genes, into normal human bone marrow, to determine how their aberrant expression affects normal hematopoiesis and leukemic transformation and progression. CML, in particular, is a good candidate for these gene transfer experiments because some of the genes involved in its etiology (e.g., *bcr/abl*) and progression (e.g., *p 53*) have been identified. It should be possible to directly test the role of these genes in the multistage progression of CML using gene transfer with the long-term goal of developing an animal model for CML.

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Colony Stimulating Factors: Regulation of Production *

M. Akashi [1,2] and H. P. Koeffler [1]

Introduction

Life span of mature blood cells is ephemeral, requiring hematopoiesis throughout life. A complex network of hematopoietic progenitor cells and cytokines maintain an enormous daily production of granulocytes, monocytes, erythrocytes, platelets, and lymphocytes. This population of hematopoietic cells must be able to respond rapidly to changing needs such as bleeding, infections, cancer, or exposure to cytotoxic agents. Colony stimulating factors (CSFs) are a family of glycoproteins that promote growth and differentiation of hematopoietic progenitor cells and also enhance the function of the mature blood cells. production of CSFs is under tight control since either their over- or underproduction will result in dysregulation of hematopoiesis. Proliferation of hematopoietic progenitor cells require the continuous presence of these factors.

A variety of cells including nonhematopoietic cells such as fibroblasts, endothelial cells, and smooth muscle cells are capable of producing many kinds of CSFs. This chapter will describe cells that make CSFs and the mechanisms involved in this production.

Mesenchymal Cells

Mesenchymal cells originate from either mesoderm or ectoderm. Three major cells that compose the mesenchymal tissues include fibroblasts, vascular endothelial cells, and smooth muscle cells. Fibroblasts provide the scaffolding required for cellular organization; this extracellular matrix is required for tissue cohesion [39, 40]. These cells are not functionally effete; they produce CSFs and a variety of cytokines. They play a major role in the response to tissue injury, being the primary cells involved in tissue repair. These cells respond to interleukin-1 (IL-1) and tumor necrosis factor (TNF) by proliferation, synthesis, and assembly of collagen [18]. Fibroblasts in the bone marrow may function as part of the microenvironment [15, 21].

Pluznik and Sachs originally showed that fibroblasts could produce CSF [36]; somewhat more recently endothelial cells and smooth muscle cells were found to be capable of stimulating granulopoiesis [25, 35]. Studies have not shown definitely that, in vivo, these cells constitutively make CSF. Our preliminary studies suggest that embryonic and adult lung fibroblasts produce very low amounts. Sustained myelopoiesis in long-term culture of bone marrow cells requires the presence of stromal cells composed of a complex network of cell types, including fibroblasts and endothelial cells [16].

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

These cells produce low levels of hematopoietic growth factors.

Physiological Stimulators of CSF Production

Bagby et al. [4, 5] initially noted that macrophages exposed to lipopolysaccharide produced factors that stimulated both endothelial cells and fibroblasts to produce CSFs. Several years later, we [31] found that TNF-a, one of the products of macrophages, was able to stimulate fibroblasts, endothelial cells, and smooth muscle cells to produce CSFs. At the same time, IL-1 was noted to increase synthesis of CSFs in the same cells [6, 8, 27, 45]. Further studies have shown that mesenchymal cells cultured with either TNF or IL-1b produced macrophage-CSF (M-CSF) [1, 24], as well as IL-1b and IL-6 [2, 28, 42]. We have noted that messenger ribonucleic acid (mRNA) for each of these growth factors is produced in a coordinate fashion after mesenchymal cells are stimulated by either TNF or IL-1b. Lymphotoxin, which is produced by activated lymphocytes and has peptide homology to TNF, can also stimulate mesenchymal cells to produce CSFs, although the potency of this cytokine may be less than TNF (Fig. 1) [1, 9].

TNF-a and IL-1b are made in abundant amounts by activated macrophages [17, 34]; lymphotoxin is mostly synthesized by activated lymphocytes. A number of conditions, including bacterial invasion, are known to stimulate these cells to synthesize TNF, IL-1, and lymphotoxin, which can enhance CSF production by mesenchymal cells. This inter-

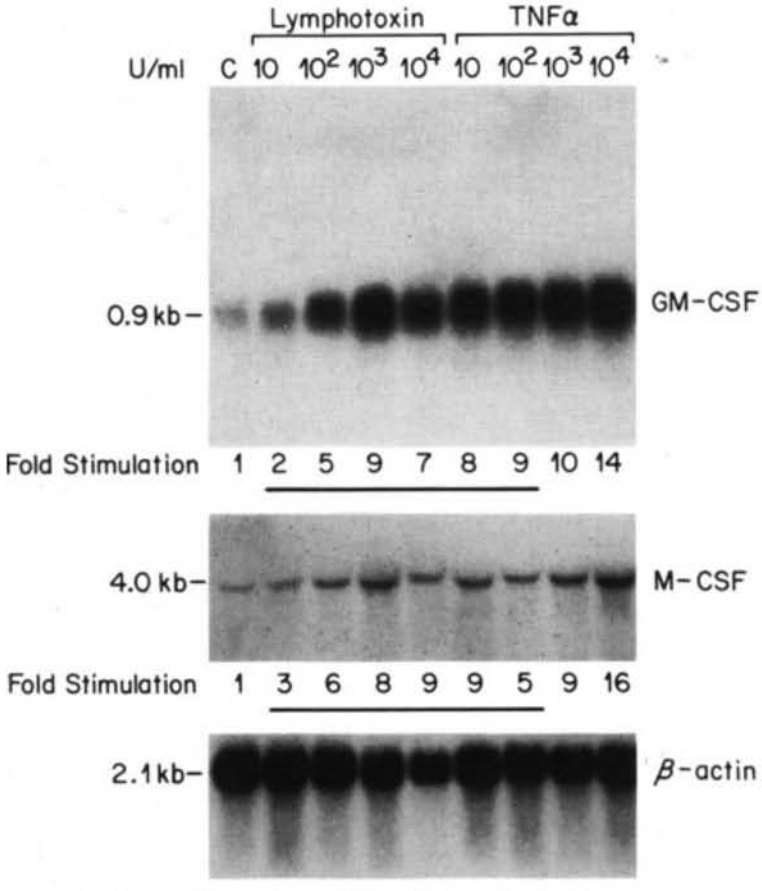

Fig. 1. Dose-dependent effect of lymphotoxin and TNF on levels of GM- and M-CSF mRNAs in human lung fibroblasts. Fibroblasts were cultured with lymphotoxin or TNF for 8 h. Cytoplasmic RNA (15 μg per lane) was prepared and analyzed by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was with ^{32}P-labeled GM-CSF and M-CSF cDNA and β-actin DNA. Fold stimulation of levels of CSF mRNAs as compared to levels in untreated cells was equalized for levels of β-actin

communication of cells results in a cascade of synthesis of cytokine in the region of inflammation, such as sites of bacterial and viral infections, rheumatoid arthritis, and some collagen vascular disorders. Steady state hematopoiesis in the bone marrow perhaps is in part regulated by the constant, short-range production of cytokines synthesized by mesenchymal cells, macrophages, and lymphocytes.

CSF: Regulation of Synthesis

Mesenchymal cells have detectable levels of CSF mRNA within 30–60 min of exposure to TNF [27]. This stimulation by TNF can occur in the absence of new protein synthesis [27]. Previous studies have shown that protein kinase C activators can increase accumulation of CSFs in fibroblasts (Fig. 2) [1, 27]. Depletion of protein kinase C activity by prolonged exposure of fibroblasts to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) blocks the accumulation of GM-CSF RNA by TPA but does not affect the accumulation of this RNA induced by TNF [41]. This result suggests that the effect of TNF is independent from protein kinase C activation.

Further studies of TNF showed that it can cause the alkalinization of mesenchymal cell; this probably occurs through stimulation of the Na^+/H^+ antiporter [43]. Amiloride blocks this alkalinization, but does not block accumulation of GM-CSF mRNA. Taken together, our experi-

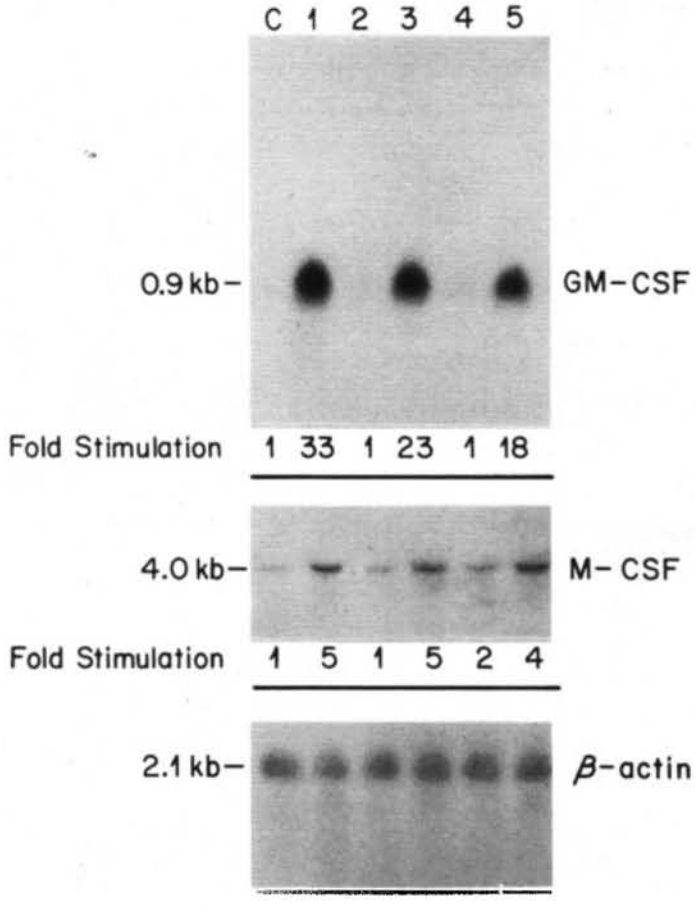

Fig. 2. Effect of various derivatives of phorbol ester on expression of GM- and M-CSF studied by northern analysis. TPA (*lane 1*), PDD (*lane 3*), and PDB (*lane 5*) are phorbol esters that are potent activators of protein kinase C; their derivatives, 4-*O*-methyl TPA (*lane 2*) and 4-alpha-PDD (*lane 4*) are unable to activate protein kinase C. Fibroblasts were exposed to each compound (50 nM) for 2 h and levels for CSFs mRNA were measured. Fold stimulation of levels of mRNA as compared to levels in untreated cells was calculated as described in Fig. 1. Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDD, phorbol 12,13-didecanoate; PDB, phorbol 12,13-dibutyrate

ments provide strong evidence that TNF does not mediate its action through either PKC or Na^+/H^+ antiporter.

Using an array of agonist and antagonist, we found that those agents that increase levels of intracellular Ca^{2+} and K^+ also increase levels of CSF mRNA [43]. Increase of K^+ levels may stimulate the Ca^{2+}/K^+ pump causing increased levels of cytosolic Ca^{2+}. In addition, we found that NaF in the presence of Al^{3+} is a potent stimulator of levels of CSF mRNA. This stimulation cannot be blocked by pertussis toxin suggesting that NaF/Al^{3+} may be enhancing the activity of G-binding proteins that are insensitive to the action of pertussis toxin. This observation is consistent with preliminary data suggesting that transformation of mesenchymal cells by transfection of activated H-*ras* can lead to their increased expression of GM-CSF mRNA.

Some tumors are able to synthesize CSF constitutively and patients with these tumors often have peripheral blood leukocytosis. We examined cell lines from tumors that produced CSFs; these tumors were associated with leukocytosis in the patients (H. Ross and H. P. Koeffler, in preparation). Cells of each expressed high levels GM-, G-, and M-CSF mRNAs as well as IL-1 and IL-6 mRNAs. Furthermore, the stability of mRNA coding for each of these growth factors was 10 to 20-fold greater than that in nontransformed cells. The tumors have well-defined oncogene alterations that may be closely associated with inappropriate stability of normally transiently expressed genes.

Monocytes/*Macrophages*

Macrophages are pivotal in inflammation and immunity. In the 1970's, monocytes/macrophages were found to produce CSF [12, 20]. Further studies have found that human monocytes/macrophages from many tissues produce predominantly G- and M-CSF, as well as IL-1, IL-6, and TNF, but synthesize very little GM-CSF. However, other studies found that human monocytes/macrophages accumulate GM-CSF when exposed to lipopolysaccharide, fetal calf serum, or thioglycollate, or when cells phagocytose and adhere in the presence of fibronectin [38]. Resting macrophages produce little CSF, but their synthesis of CSF markedly increase with activation after exposure to a variety of physiologically relevant agents including TNF, interferon-r (IFN-r), GM-CSF, IL-3, IL-1, and endotoxin. Besides producing M-CSF, IL-1, and TNF, these cells have receptors for cytokines, suggesting that under certain circumstances these cells might develop an autocrine stimulation which might foster inflammation. This inflammation may be either salutary (e.g., bacterial infections) or detrimental (e.g., rheumatoid arthritis). Nuclear run-on transcription assay and half-life studies showed that the induction of G- and M-CSF genes is due to mRNA stabilization [19].

Granulocytes

Granulocytes share a number of common properties with monocytes, including phagocytic activity, similar membrane receptors, and a common progenitor cell. They are relatively short-lived, nondividing cells, which often are considered to have little biosynthetic capacity. However, as early as 1948, granulocytes were known to release endogenous pyrogen [7]. Recent studies have demonstrated that granulocytes can be induced to accumulate mRNA coding for G- and M-CSF, IL-1, and TNF after exposure to GM-CSF [30]. These cells also produce a number of other proteins such as plasminogen activator [22], FOS [23], and IL-1 [29]. These findings suggest that neutrophils may be involved in the regulation of hematopoietic growth factors.

Lymphocytes

Cline and Golde [13] first showed that human lymphocytes in vitro produce sig-

nificant CSF; these cells especially synthesize large amounts when stimulated with lectin or antigenic stimulation. CSF can be synthesized by both $CD4^+$ and $CD8^+$ lymphocytes; the former are the most potent producers of cytokines. T lymphocytes can produce all the interleukins and GM-CSF. The cells lack the ability to secrete G- and M-CSF suggesting that transregulatory proteins may be different in mesenchymal cells and T lymphocytes, and those that regulate G- and M-CSF production possibly are different from those that control GM-CSF. A recent study showed that mRNA for M-CSF can accumulate in natural killer cells stimulated with IL-2 and CD16 ligands [14]. Only T lymphocytes secrete IL-3 in the human system [44].

The in vivo importance of production of CSFs by T lymphocytes is unclear. They are present in small but significant numbers in bone marrow, allowing them to interact closely with hematopoietic progenitor cells by releasing growth factors. A role of lymphocytes in regulating normal hematopoiesis is indirectly suggested by alternation of hematopoiesis with alternation of subsets of lymphocytes. Nevertheless, children with congenital deficiencies of T lymphocytes appear to have fairly normal myeloid hematopoiesis, suggesting that other sources of CSFs can compensate for a lack of T lymphocytes.

Comparison of Production of CSF by Mesenchymal Cells, T Lymphocytes, and Macrophages

In the resting state, both mesenchymal cells and macrophages transcribe cytokines, but do not accumulate these mRNAs (Table 1). With stimulation, cytokine mRNA accumulates in macrophages and mesenchymal cells as well as in T lymphocytes. Maximal mRNA accumulation occurs after 2–8 h of stimulation in all three cell types. The constellation of cytokines produced by each of these cells differs. For example, G- and M-CSF mRNA can be synthesized by mesenchymal cells and macrophage, but not by T lymphocytes; GM-CSF mRNA is produced predominantly by T lymphocytes and mesenchymal cells, but little is synthesized by human macrophages. Many of the same signals of CSF production are operative in two or three of the cell types including IL-1, TNF, agents that increase intracellular calcium levels, endotoxin, and stimulators of protein kinase C. T lymphocytes are unique for several reasons. Studies suggest that they require two signals for CSF production instead of one, such as lectin plus phorbol ester, or calcium ionophore plus phorbol ester. In contrast, only one is probably required for macrophages and mesenchymal cells. T lymphocytes are also unique in another manner; these cells can be stimulated by special antigens, in the presence of an antigen presenting cell, to produce CSFs.

CSF: Regulation of Gene

We constructed a promoter-reporter gene construct containing various regions of the GM-CSF gene 5' to the start site of transcription. These were transfected into fibroblasts and stimulated with either TNF or IL-1. These constructs showed no enhancement of reporter-gene activity. In contrast, protein kinase C activators markedly increased levels of the reporter-gene [33]. These are consistent with our notion that TNF and IL-1 do not have a major effect on transcription of CSF but modulate post-transcriptionally levels of CSFs. On the other hand, protein kinase C activators stimulate both transcription as well as stabilization of these CSF mRNAs in each of the cell types. Promoter sequences encompassed by -53 to the start site for transcription of the GM-CSF gene are required to stimulate transcription by protein kinase C activators in mensenchymal cells and lymphocytes [11].

RNA of most cytokines including GM-CSF have a short half-life. Stabilization

Table 1. Regulation of CSF

Variables	Mesenchymal cells	T lymphocytes	Macrophages
CSF produced in activated state	G-, M-, GM-CSF, IL-1, IL-6	GM-CSF, All interleukins	G-, M-CSF
CSF produced in resting state	Transcription, little accumulation	No transcription	Transcription, little accumulation
Maximal level of CSF mRNA after activation	4–8 h	4–8 h	2–8 h
Mechanism of enhanced accumulation of CSF mRNA			
Stimulator			
GM-CSF, IL-3	–	–	Stabilization
TNF, IL-1	Stabilization	–	Stabilization
PKC stimulator		Increased stability and increased transcription	
GM-CSF mRNA half-life			
Resting	< 0.25 h	–	?
Activated (TNF or mitogen)	0.6 h	0.6 h	?
TPA or CHX	> 4 h	> 2 h	?
Signal pathway for CSF synthesis	One signal	Two signal	?
Signals	TNF, IL-1	IL-1	TNF, IL-1
	PKC	PKC	PKC
	Ca^{2+}	Ca^{2+}	?
	NaF	?	?
		Specific antigens	
Critical region for GM-CSF expression	DNA sequences from −53 to +1		

CHX: cycloheximide; *PKC:* protein kinase C.

of short-lived mRNAs play a pivotal role in the accumulation of cytokines in each of the cell types. AU-rich sequences have been found in the 3' untranslated regions of most of the genes coding for these short-lived cytokines and oncogenes [10, 37]. We found that TNF, IL-1 b, phorbol diesters, NaF, and cycloheximide enhance expression of GM-CSF RNA through stabilization (Fig. 3) [1, 27, 41]. We transfected into fibroblasts constructs containing an AT-rich sequence from GM-CSF gene placed into the 3' untranslated region of the reported gene [3]. These transfected cells were stimulated with TPA, cycloheximide, NaF, TNF, or IL-1 b. TPA, NaF, and CHX required an AT-rich sequence for stabilization of the reporter gene. On the other hand, a reporter gene containing the AT-rich sequence does not respond to either TNF or IL-1 b. These experiments suggest that TNF and IL-1 b stabilize GM-CSF RNA independent of these AU sequences and that different mechanisms are used by various agents to stabilize GM-CSF RNA.

Conclusion

Hematopoietic cells are produced and destroyed continuously under precise control. This chapter begins to illustrate

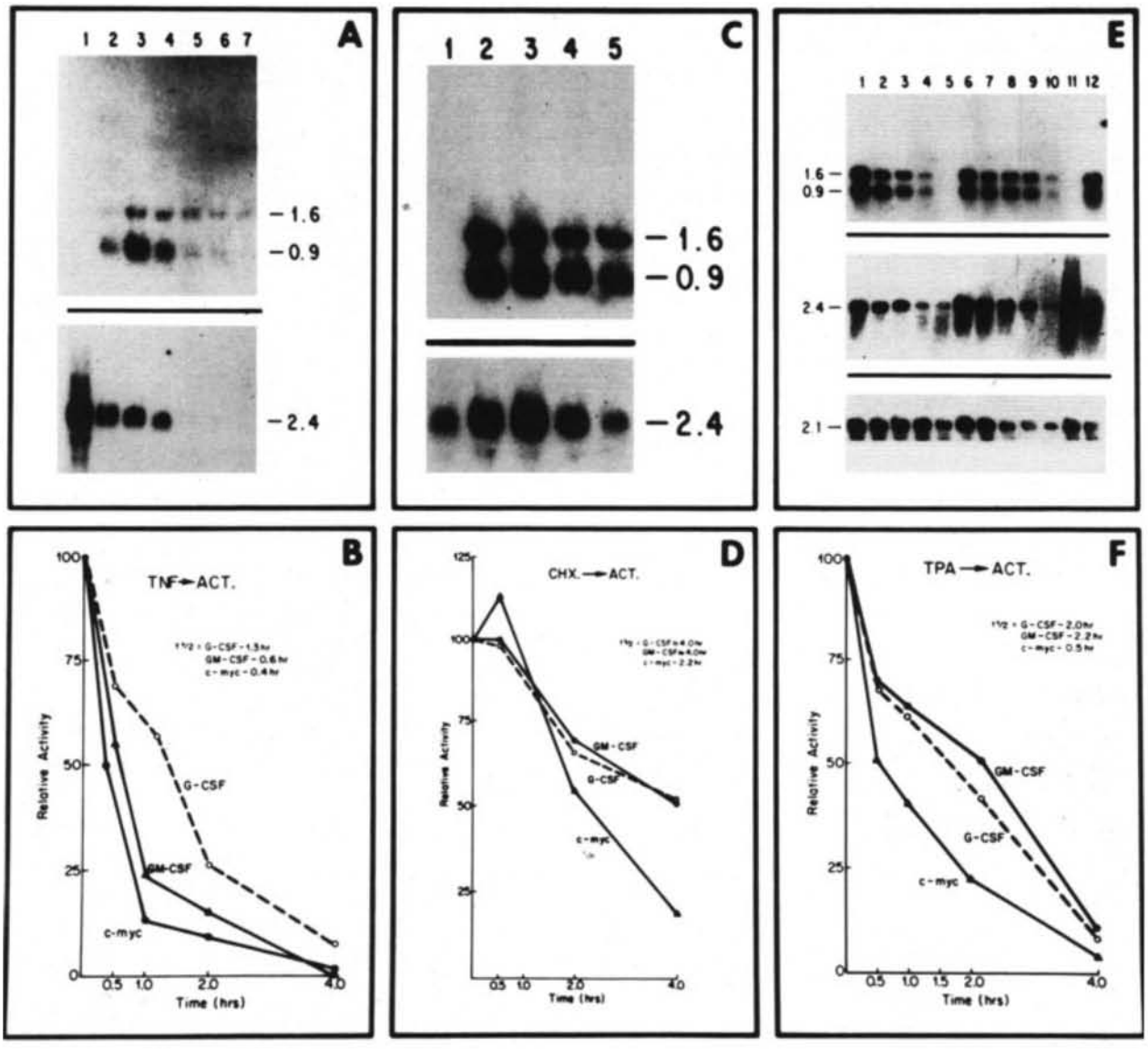

Fig. 3A–F. Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts and ability of TNF and TPA to stimulate accumulation of these mRNAs in absence of protein synthesis. **A, B** Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts exposed to TNF. Cells were cultured with TNF (25 ng/ml per 4 h) and then actinomycin D (ACT) (5 μg/ml) was added to the culture for various durations. Total RNA (10 μg per lane) was analyzed by RNA blotting. Hybridization was with GM-CSF cDNA (0.9 kb band of hybridization) and the G-CSF oligonucleotide (1.6 kb band) and with the *c-myc* (exon II) probe (2.4 kb). Lanes: *1*, HL-60 (negative control); *2*, control fibroblasts; *3*, fibroblasts exposed to TNF alone;*4*, *5*, *6*, and *7*, cells exposed to TNF and also to ACT for 0.5, 1, 2, and 4 h, respectively. Intensity of hybridization was determined by densitometory. **C, D** Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts exposed to CHX. Cells were cultured with CHX (20 μg/ml per 4 h) and then ACT was also added for various durations. RNA was analyzed and intensity of hybridization was determined by densitometory as described in **A** and **B**. Lanes: *1*, control fibroblasts; *2*, cells exposed to CHX alone; *3*, *4*, and *5*, CHX and ACT for 1, 2, and 4 h, respectively. **E, F** Ability of TNF and TPA to stimulate accumulation of G- and GM-CSF and *c-myc* mRNAs in absence of protein synthesis, and stability of steady state of these mRNAs in fibroblasts exposed to TPA. Lanes: *1*, pretreated with CHX (0.5 h) and then cultured with CHX and TPA for 4 h; *2*, pretreated with CHX (0.5 h) and then cultured with CHX and TNF for 4 h; *3*, CHX (4.5 h); *4*, TNF (4.5 h); *5*, untreated control cells; *6*, TPA (50 nM, 4 h); *7* to *10*, TPA (50 n*M*, 4 h) and ACT for 0.5, 1.0, 2.0, and 4 h, respectively; *11*, HL-60 cells; *12*, Lu-CSF-1 (positive control). Abbreviations: *CHX*: cycloheximide; *TPA*: 12-*O*-tetradecanoylphorbol 13-acetate

the complexity of cytokine-mediated communication pathways in induction of hematopoietic growth factors. Regulation of induction of hematopoietic growth factors reflects an integrated network of bioregulator molecules. Some act directly on the hematopoietic progenitor cells; other affect the accessory cell; and some have both direct and indirect affects on the hematopoietic cells. Many hematopoietic growth factor genes have been cloned and their products have been expressed in mammalian cells. Use of these clones has provided the opportunity to evaluate the regulation of expression of CSFs and the evaluation of their affects on target cells.

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Adhesive Interactions in the Regulation of Haemopoiesis

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Introduction

The role of stomal cells in the regulation of haemopoiesis has been amply demonstrated both *in vivo* and *in vitro*. In normal adult humans haemopoietic activity is restricted to parts of the skeleton, particularly the sternum and pelvis [1] and the success of clinical bone marrow transplantation shows that stem cells can seek out haemopoietic tissue. *In vitro*, the production of haemopoietic stem cells in the culture system described by Dexter et al. [2] and the formation of colonies of human blast cells on stromal feeder layers [3] provide convenient systems for investigating regulatory interactions between haemopoietic cells and the stromal cells of the haemopoietic microenvironment.

The distribution of haemopoiesis *in vivo*, the "homing" of transplanted cells and the close association between haemopoietic cells and stromal cells in *in vitro* culture systems suggest that specific binding interactions are involved in the microenvironmental regulation of haemopoiesis. Indeed, a capacity to bind to stroma by blast colony-forming cells (Bl-CFC) in the stromal feeder layer assay [3] is a prerequisite for detection in this culture system (see Methods). In contrast to normal haemopoietic stem cells, leukaemic stem cells are not restricted to the skeleton, circulate in the bloodstream and proliferate in extramedullary sites. For example, large numbers of circulating stem and progenitor cells are characteristic of the chronic phase of chronic myeloid leukaemia (CML) [4–7]. Thus, there may be a failure in leukaemia of the binding interactions that normally hold stem cells under the regulatory influence of the haemopoietic microenvironment.

Here, we will summarise information relevant to the stroma-mediated regulation of normal haemopoiesis and its dysregulation in leukaemia.

Materials and Methods

Normal bone marrow cells have been obtained, with informed consent, from donations of marrow for allogeneic transplantation and peripheral blood cells from chronic phase CML patients at presentation. The normal bone marrow cells and the CML blood cells were separated using Lymphoprep (Nyegaard, Oslo) to obtain the mononuclear cell fraction.

The Bl-CFC assay is set up in a series of stages. First, confluent stromal layers are grown in 35 mm petri dishes or in wells from normal marrow mononuclear cells (5×10^5/ml) in α-medium (GIBCO) supplemented with 10% fetal calf serum, 10% horse serum (GIBCO) and 2×10^{-6}/ml mononuclear cells are added to the stromal layers and incubated with them for 2 h. The stromal layers are then washed thoroughly to remove any cells that have not bound to the stroma, covered with a layer of 0.3% agar in α-medium supplemented with 15% fetal calf serum and incubated for

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

5–7 days. Some cells that bind to the stroma produce colonies of more than 20 blast cells that can be scored by inverted phase contrast microscopy.

Implications of Stroma-Dependent Blast Colony Formation for the Regulation of Haemopoiesis

Clearly, Bl-CFC can bind to stromal layers *in vitro* and form colonies and, since we believe that these progenitor cells represent an early stage in haemopoiesis [8], the assay can be used as a model to investigate further the microenvironmental regulation of stem cell activity. It is presumed that binding of Bl-CFC to stroma is mediated via a cell adhesion molecule (CAM) and that proliferation is stimulated by endogenous growth factors produced by stromal cells of by exogenous growth factors. As discussed by Gordon and Greaves [9] a regulatory unit can be envisaged wherein stem and progenitor cells bind to specific microenvironments, according to their stage of development and lineage of differentiation, which in addition to binding cells also sequester the appropriate growth factor so that it can be presented to the immobilised target cell.

The Cell Adhesion Mechanism

A CAM responsible for binding Bl-CFC to stroma has not been identified and studies in this area are difficult because there are no representative stromal or haemopoietic cell lines that can be exploited. Neither is binding inhibited by antibodies to any known CAMs [10]. However, we have made a number of studies of the functional properties of the Bl-CFC/stroma cell adhesion mechanism, showing it to be: heparan sulphate dependent [11]; calcium, magnesium and serum independent [10]; partially sensitive to trypsin, partially sensitive to phosphatidylinositol-specific phospholipase C (PI-PLC) [10]; tissue specific [12]; species nonspecific [13]; modulated during haemopoietic cell maturation [14]; regulated during development [12].

Sequestration of Growth Factors by the Stromal Microenvironment *In Vitro*

Either endogenously produced or exogenous growth factors could be responsible for stimulating progenitor proliferation in the Bl-CFC assay. There is evidence that both mechanisms could operate by binding to the extracellular matrix (in particular the glycosaminoglycan fraction) produced by the stromal cells in vitro. Examples of this evidence are that: endogenous growth factor can be extracted using salt [15]; haemopoietic growth factors bind to glycosaminoglycans; GM-CSF binds to hyaluronic acid, IL-3 binds to heparan sulphate (unpublished data); binding is selective, saturable and retains the biological activity of the growth factor [15]. The model for the microenvironmental regulation implies that different growth factors, as well as different progenitor cells, have different binding specificities and this has been confirmed using stromal layers [16] and glycosaminoglycan-coated sepharose beads (unpublished observations).

Binding Properties of Bl-CFC in CML

Large numbers of Bl-CFC circulate in chronic phase CML [7], indicating that their capacity to interact with stromal layers is defective. This idea has been explored further, and several differences between the binding properties of normal and CML Bl-CFC have emerged. These are:

- Bl-CFC circulate in CML, normal Bl-CFC do not [7]
- CML-Bl-CFC bind to stroma grown without methylprednisolone, normal Bl-CFC do not [17]
- CML-Bl-CFC bind transiently to stromal layers grown with methylpred-

nisolone, normal cells bind irreversibly [18]
- CML-Bl-CFC are insensitive to PI-PLC treatment, normal cells are partially sensitive to PI-PLC treatment

The finding that Bl-CFC in CML are insensitive to treatment with PI-PLC indicates that they lack a functional phosphatidylinositol-anchored component of the cell adhesion mechanism. In support of this idea, preliminary results have shown that normal PI-PLC-treated Bl-CFC (i.e., rendered deficient in the PI-anchored cell adhesion component to resemble CML cells) bind transiently to cultured stromal layers. Moreover, these treated cells, like CML cells, can interact with stromal layers grown without methylprednisolone.

Summary and Conclusions

The Bl-CFC assay system provides a method for investigating regulatory interactions between normal and abnormal haemopoietic cells and their stromal microenvironment. Normally, regulation may be achieved by specific binding interactions between haemopoietic cells and stroma together with the presentation of appropriate growth factors. In CML, early progenitor cells do not interact properly with stromal cells and this may be attributable to an absence of a functional PI-linked component of the cell adhesion mechanism. We propose that abnormal expression of CAMs in CML may contribute to the dysregulation and extramedullary distribution of stem cells in this disease.

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In Vitro Effect of GM-CSF and IFN-γ on the Establishment of Stromal Layer and Hemopoiesis in Human Dexter Culture

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Introduction

Recently, the phenomenon of communication and interaction between stromal cells and hemopoietic stem cells has become the focus of interest. The questions of what role colony-stimulating factors (CSF) play in this respect and whether it may be possible to manipulate this process are important for the development of new therapeutic strategies.

The aim of our study was to test the influence of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (100 IU/ml), alone or in combination with recombinant human interferon-γ (rhIFN-γ), (10 IU/ml) on the establishment of the stromal layer and on the proliferation and differentiation of hemopoietic cells in 10-day Dexter culture of normal bone marrow cells.

Materials and Methods

Light-density mononuclear cells were isolated from iliac bone marrow aspirates diluted [1:1 with Iscove's modified Dulbeco's medium (IMDM) containing preservative-free heparin (G. Richter, Hungary] by Ficoll-Visotrast gradient separation at a density of 1.077 g/ml.

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The cells were tested for colony-forming unit – granulocyte-monocyte (CFU-GM) growth, cytomorphological composition (staining according to Pappenheim), expression of HLA-DR and CD14 antigens [fluorescence activated cell sorting (FACS) analysis after incubation with monoclonal antibodies: L234 (HLA-DR) derived from hybridomas (ATCC, Rockeville, CA), and LeuM3 (CD14) (Becton and Dickinson, Heidelberg, FRG)].

The Dexter liquid culture [1–3] introduced for human bone marrow cells [7] was slightly modified [6]. Briefly, 5×10^5 cells/ml culture medium (70% IMDM, 10% horse serum, 10% fetal calf serum, 10% autologous bone marrow plasma, 10^{-6} M hydrocortisone sodium succinate) were incubated in 7.5% CO_2 at 37 °C. At the start of the culture 100 IU rhGM-CSF/ml (Behring, FRG) and 10 IU rhIFN-γ/ml (Boeringer, FRG) were added alone or in combination. After 10 days in Dexter liquid culture, stromal formation was evaluated by the expansion of the adherent cell layer (stromal grades 1–4 correspond to an area covered by adherent cells of 25%–100%). The establishment of active hemopoiesis was evaluated by the presence of hemopoietic islands. Adherent cells were removed from the culture dishes with a cell scraper. After having been washed twice, the whole cell population (adherent and nonadherent cells) was tested again for CFU-GM growth, cytomorphology, and expression of HLA-DR and CD14.

The estimation of CFU-GM counts was performed in a monolayer system

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

with soft agar, IMDM supplemented with 20% fetal calf serum, 20% mixed culture conditioned medium and $5 \times 10^{-5} M$ mercaptoethanol.

Results and Discussion

Exogenous rhGM-CSF caused an increase in both stromal grade (Fig. 1) and activity of hemopoiesis (number and size of hemopoietic islands). Cultures with 10 IU rhIFN-γ/ml showed almost the same degree of stromal formation as the control cultures. The composition of hemopoietic cells was influenced by the factors added (Fig. 2). rhIFN-γ (10 IU/ml) alone caused a switch in the differentiation of hemopoietic cells to monocytes and macrophages. There was an increase in the number of immature cells in cultures with rhGM-CSF, both alone and in combination with rhIFN-γ.

The CFU-GM derived from the Dexter cultures were more numerous than those from the controls without precultivation in suspension (Fig. 3). An additional increase in the CFU-GM count was seen in all Dexter cultures with rhGM-CSF, both alone and in combination with rhIFN-γ. This elevation in CFU-GM count was mainly due to an increase in the number of small compact aggregates. This is expressed by an elevated cluster/colony ratio (Fig. 3).

Our preliminary data seem to indicate that exogenous rhGM-CSF affects CFU-GM as well as stromal precursor cells in Dexter liquid culture.

GM-CSF added to a preestablished Dexter culture had no effect [4, 5, 12]. In our study, however, we examined the important early period of the process of establishing the microenvironment.

Exogenous rhGM-CSF accelerates the maturation of monocytes to macrophages and, therefore, shortens the time which these cells need to acquire the ability to function within the network of stromal cells.

Another humoral mediator for hemopoietic regulation is IFN-γ. It exerts an inhibitory action on CFU-GM growth [8, 9, 11] and is able to induce myeloid progenitor cells to differentiate towards the monocytic lineage [10]. In our cultures, there was no effect of rhIFN-γ

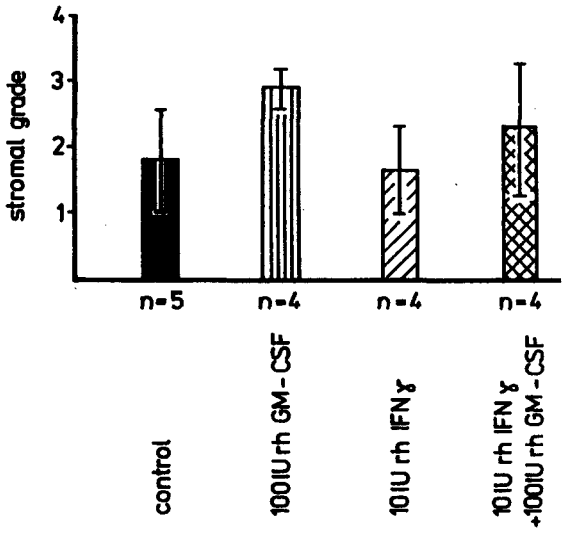

Fig. 1. Stromal formation of normal human bone marrow cells after 10 days in Dexter liquid culture

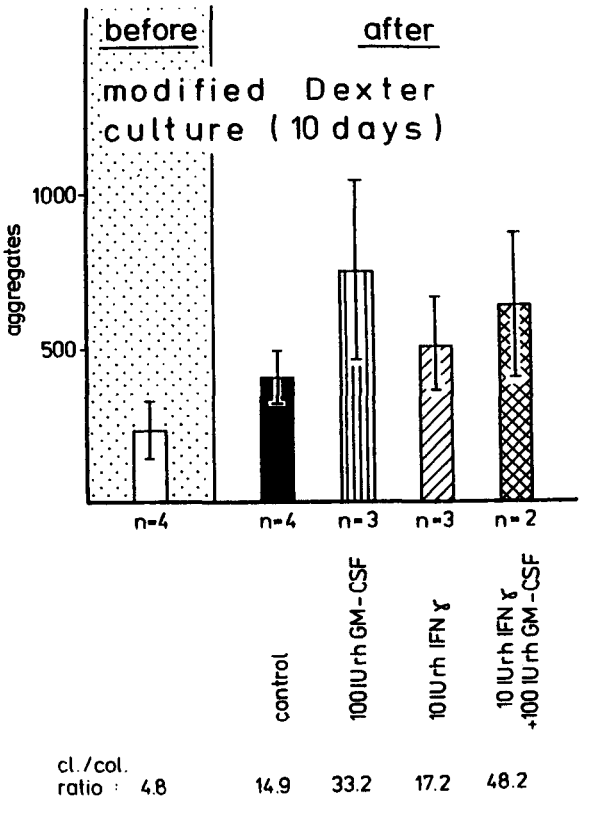

Fig. 2. CFU-GM counts and cluster/colony ratios derived from Dexter cultures after 10 days of cultivation

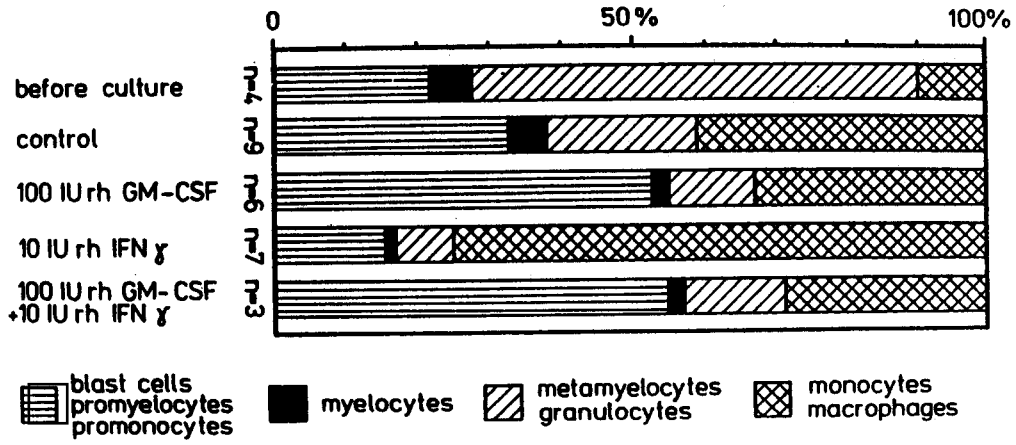

Fig. 3. Distribution of cytomorphologically differentiated subpopulations of hemopoietic cells derived from Dexter cultures after 10 days of cultivation

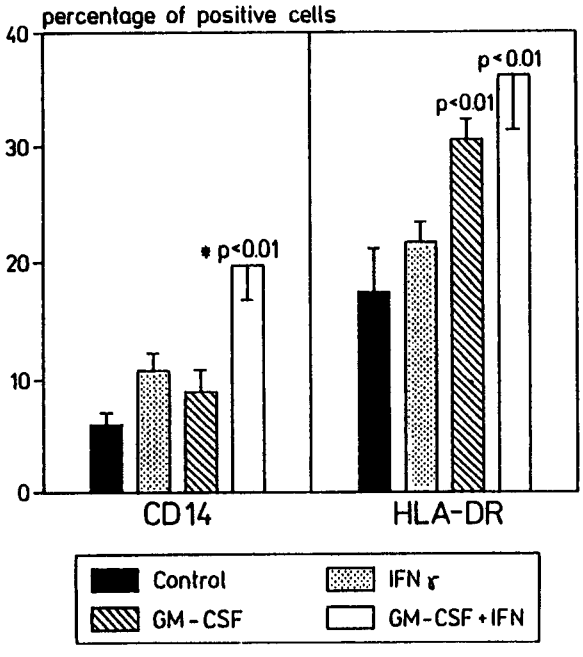

Fig. 4. The influence of rhGM-CSF and rhIFN-γ on the expression of the CD14 and DR antigen on bone marrow cells derived from 10-day Dexter liquid cultures

(10 IU/ml) on stromal formation and a minimal enhancing effect on the CFU-GM count. A combination of rhIFN-γ and rhGM-CSF had the same stimulatory effect on CFU-GM derived from Dexter cultures as rhGM-CSF alone. Simultaneously, an expansion of immature cells (blasts, promyelocytes, promonocytes) was observed, which correlated with elevated HLA-DR expression (activation marker). It is of special interest that further specification of the cells by FACS analysis revealed elevated expression of the monocytic marker CD14 after the combined application of rhGM-CSF and rhIFN-γ, but not after rhGM-CSF alone (Fig. 4).

We assume that the addition of both factors to the Dexter culture stimulates the establishment of marrow stroma and also the proliferation of hemopoiesis with a switch in the differentiation towards monoblasts.

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The Effect of Bone Marrow Fibroblast and Stromal Cell-Conditioned Media on Hemopoietic Cells in Culture

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Introduction

It is well known that fibroblasts [1] and stromal cells (Sc) are the main components of the bone marrow microenvironment [2–4]. The concept of short-range regulation of hemopoiesis by Sc is widely accepted [2, 4]. Meanwhile bone marrow fibroblasts and Sc are potent producers of long-range factors affecting hemopoiesis: stimulators [5–8], inhibitors, [9] and probably restrictins [1]. Many of the regulatory effects of Sc were shown to be due to the well-known hemopoietic growth factors [10–13]; some of them are anchored to extracellular matrix [14].

Stimulatory activity of bone marrow fibroblasts in semisolid cultures were thoroughly studied, but the results are controversial: absence of any stimulatory activity [7], the necessity of the presence of monocytes for stimulating the granulomonocyte colony formation [8], direct induction of granulomonocyte colony formation [5, 6]. It was also reported that fibroblasts but not their conditioned media (CM) could be potent inhibitors of hemopoietic cell proliferation [9].

In this paper we tried to study the effect of bone marrow fibroblast- and stromal cell-conditioned media (FCM, ScCM) in different hemopoietic disorders on proliferation of hemopoietic cells and cell lines in culture.

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Materials and Methods

Patients. Healthy individuals, patients with acute nonlymphocytic leukemia (ANLL), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), myelodysplastic syndrome (MDS), and leukemia-unrelated neutropenias were studied for their bone marrow FCM granulocyte-macrophage colony-stimulating (GMCSA) and inhibiting activity (Tables 1, 2) and for their bone marrow FCM and ScCM effects on blast cell growth (Fig. 1). Experiments were started before drug administration.

Target Cells. Nonadherent bone marrow cells were used as targets in semisolid bone marrow cultures. K-562 and HL-60 line cells and peripheral blood cells of patients with ANLL were used for [^{3}H]thymidine uptake.

Bone Marrow Fibroblasts. Bone marrow cells (5-IO $\times 10^6$) were cultured in Carrel flasks using medium 199 and human serum (20%). The medium was changed twice a week. Two-to-four-week cultures were treated by trypsine (0.125%) and recultured. FCM were collected from secondary confluent cultures, frozen, and then tested.

Bone Marrow Sc. Unprocessed bone marrow cells were cultured in 25-ml T-flasks containing McCoy's 5A medium, 12.5% of fetal calf serum (FCS) 12.5% of horse serum and 10^{-7} *M* hydrocortisone (Upjohn).

Assay for Stimulators in Agar Culture. FCM (0.75 ml) was incorporated in the

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

bottom layer of bilayer agar culture. Previously this system was used to study human blood serum colony stimulating activity (CSA) [15]. Human cord blood serum was used instead of FCS. Colonies were scored after 7 days of incubation at 37°C in humidified CO_2 (7%) atmosphere.

Assay for Inhibitors in Agar Culture. FCM activity was studied in three kinds of experiments: (a) addition of FCM to leucocyte feeder, (b) addition of FCM to leucocyte-conditioned medium in the bottom layer, and (c) comparison of stimulatory activity of FCM when nonadherent (NA) or nonfractioned (NF) bone marrow cells were used as targets. The amount of FCM incorporated was 0.75 ml. Optimal concentrations of leucocyte feeder cells and leucocyte-conditioned medium were used.

The Effect of FCM and ScCM on Hemopoietic Cell [^{3}H]Thymidine Uptake. Liquid culture was used in an experiment to asses the effect of the CM on hemopoietic cell [^{3}H]thymidine uptake. HL-60, K-562 line cells, Ficoll-Hypaque fractionated peripheral blood cells of patients with acute myelocytic leukemia (AML), and CML-BC (more than 95% of blasts) were cultured in RPMI medium (10% FCS) in 96-microwell plates. Cell concentration was $1 \times IO^4$ per well. [^{3}H]thymidine (1 μCi/ml) was added to the peripheral blood cells at the initiation of culture for 12 h, and to the cell line cells after 48 h of serum deprivation (2%).

Results and Discussion

FCM of healthy individuals appeared to stimulate GM colony formation by nonadherent bone marrow cells (Table 1).

Our success in revealing stimulatory activity may be due to the large amount of CM added to the bilayer agar culture. Another possibility is the influence of human serum on the production of hemopoietic growth factors. It was recently shown that serum compounds dramatically affect the secretion of regulatory molecules by the cells of bone marrow stroma [11].

FCM CSA varied on a large scale in different hemopoietic disorders. This may be the result either of the presence of hemopoietic cells in fibroblast culture or of the different number of fibroblast cells per culture. These possibilities were studied. Comparative cytogenetic studies were performed in bone marrow hemopoietic cells and in cultured fibroblasts in four CML patients. All mitotic hemopoietic cells were Ph-positive, whereas all fibroblast metaphases were Ph-negative (data not shown), although admixture of nondividing macrophages cannot be precluded.

Preliminary data have shown that confluent cultures in different hemopoietic disorders have nearly equal numbers of fibroblastic cells. Rough correlation is seen between FCM CSA and the amount of myeloid tissue. FCM CSA is much higher in CML or megaloblastic anemia than in AA.

The results of fibroblast inhibitory activity are shown in Table 2. Addition of CML or MDS FCM to LCM resulted in inhibition of colony formation. The effect seems to be due to the direct inhibition of granulomonocyte progenitor cells. Inhibition of colony formation was also noted when FCM was added to the feeder layer. MDS FCM revealed the most significant effect. By contrast, CML fibroblasts failed to reveal an inhibitory effect in this kind of experiment.

Lower colony-forming ability stimulated by feeder plus FCM in comparison to feeder alone may result from the depression of CSA release.

The calculated ratio of NF/NA bone marrow CFA stimulated by FCM shows that NA CFA is higher than NF CFA stimulated by the same conditioned media. There was no significant difference in NF and NA CFA stimulated by LCM. This effect of FCM could be explained neither by the direct inhibition of GM CFC nor by the depression of

Table 1. FCM CSA in different hemopoietic disorders (±SD)

Healthy individuals	ALL	AML	CML	AA	Megaloblastic anemia and hypersplenism
18±8	4±3*	28±15	29*±6	5±5*	25±15
n 17	*n* 9	*n* 11	*n* 12	*n* 4	*n* 5

n, number of patients studied; AA, aplastic anemia.
* Difference in FCM CSA between healthy individuals and patients; $p < 0.05$.

Table 2. Inhibitory activity of FCM in bilayer agar culture

Source of FCM	Assay method		
	$\frac{\text{FCM+Feeder}}{\text{Feeder}}$ (%)	$\frac{\text{FCM+LCM}}{\text{LCM}}$ (%)	$\frac{\text{NF+FCM}}{\text{NA+FCM}}$ (%)
Healthy individuals	120±29.8 ($n=9$)	90±20.8 ($n=17$)	61.7±4.2** ($n=7$)
CML	130.0±3 ($n=8$)	74.6±9.8* ($n=7$)	12.5±2.1** ++ (n=7)
MDS	16.0±4.0** ++	62.3±2.1*	44.2±1.1** +
Absence of FCM	100±5.1 ($n=42$)	100±4.1 ($n=35$)	–
Absence of FCM+LCM	–	–	107±12.1 ($n=13$)

*, ** Differences in the data when FCM is Present or absent: ($p<0.05$; $p<0.01$)
+, ++ Differences between FCM inhibitory activity of healthy individuals and patients with hemopoietic disorders: ($p<0.05$; $p<0.01$).

CSA release. Really, fibroblasts of healthy individuals did not express any inhibitory activity in the previous experiments (Table 2). The most reasonable explanation is the production of inhibitory molecules by mononuclear cells induced by FCM.

We further studied the effect of ScCM and FCM on [^{3}H]thymidine uptake by leukemic cells (Fig. 1). All kinds of ScCM and FCM appeared to inhibit the growth of AML and CML-BC blast cells. ScCM of healthy individuals and AML patients were potent inhibitors of [^{3}H]thymidine uptake by HL-cells whereas CML and CML-BC ScCM stimulated their proliferation. AML ScCM tended to be superior in their inhibitory activity towards all target cells in comparison to ScCM of the other groups of patients.

The effect of ScCM and FCM coincided in all groups studied with more or less quantitative differences. This is in agreement with the data on the common nature of fibroblasts and stromal cells [16]. In healthy individuals and in patients with CML, ScCM was as a rule comparable or inferior to that of FCM, with the exception of healthy individuals' effect of ScCM towards K-562 cells. In AML cells ScCM tended to be more active than FCM.

The difference between FCM and ScCM could not be explained by the presence of hydrocortisone in ScCM. It was shown that hydrocortisone in concentrations equal to its amount in the CM has an insignificant influence on target cell proliferation (less than 12%).

The problem of the heterogeneity of FCM and ScCM activities in different hemopoietic disorders arises. It is probably related to the heterogeneity of fibroblast and stromal cells. In fact, cell

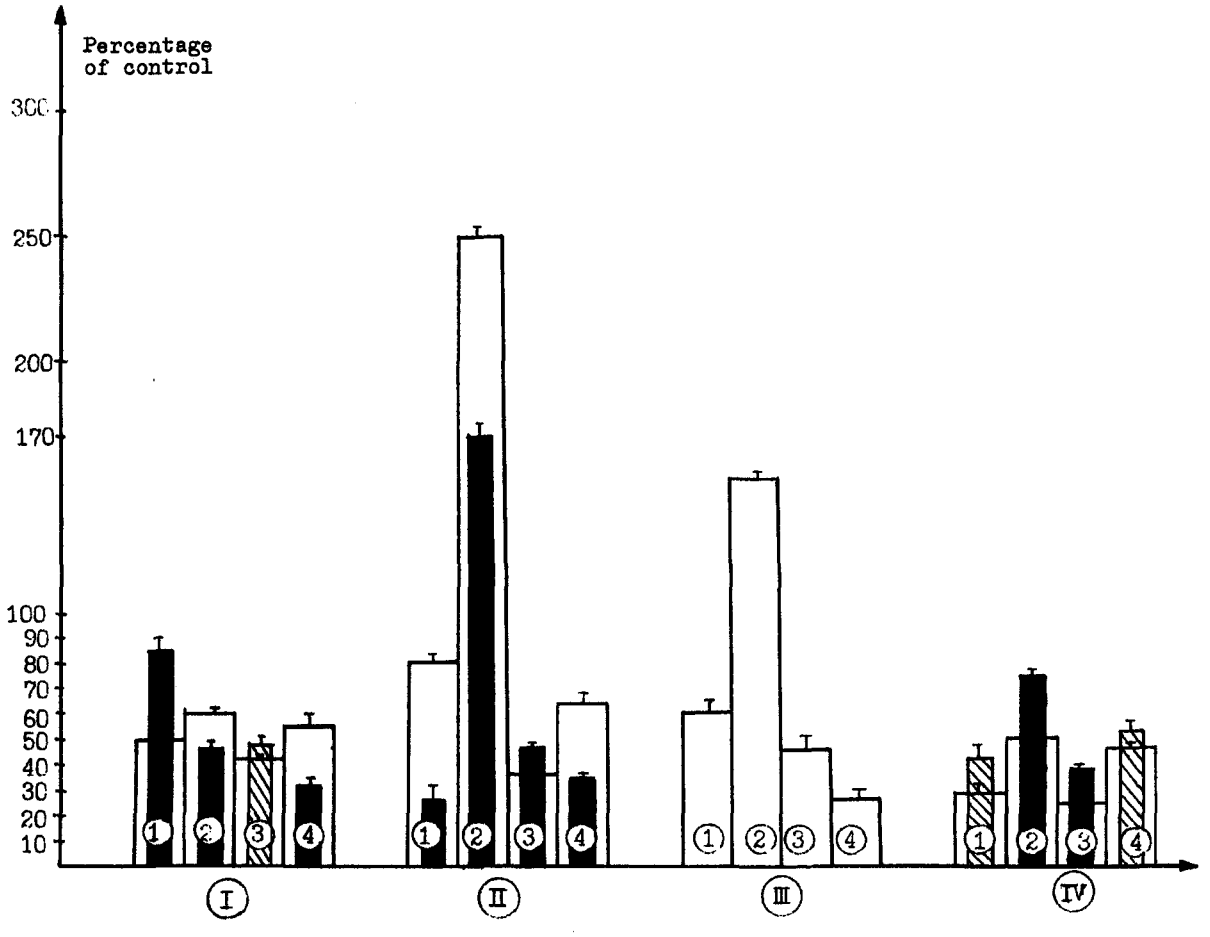

Fig. 1. Inhibiting activity of stromal cell- and fibroblast-conditioned media toward hemopoietic cells in liquid culture. *I–IV*, FCM and ScCM: *I*, healthy individuals ($n = 7$); *II*, patients with CML ($n = 5$); *III*, patients with CML-BC (myeloid) ($n = 5$); and *IV*, patients with AML ($n = 6$). *1–4*, target cells: *1*, K-562; *2*, HL-60; *3*, AML; *4*, CML-BD (myeloid). *Wide columns*, ScCM activity (± SD). *Narrow black and shaded columns*, FCM activity (± SD). *Black narrow columns*, FCM activity is statistically different from ScCM when the same target cells and the source of CM are used

lines derived from stromal cells are very diverse in their functions [17]; moreover, bone marrow fibroblasts in hemopoietic disorders differ in the amounts of receptors to glucocorticoids [18]. Therefore, differences in stimulating or inhibiting activity of FCM or ScCM may be due to the predominant proliferation of different types of stromal cells.

Finally, we have revealed significant variations in FCM and ScCM in the ability to stimulate and inhibit proliferation of hemopoietic cells in patients with hemopoietic disorders. When derived from a nonleukemic clone [19, 20], these cells probably modulate proliferation of normal and leukemic cells in order to prevent stroma-independent leukemic cell growth.

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Hemopoietic Stem Cells in Embryogenesis of the Mouse

N. L. Samoilina, A. L. Medvinsky, O. I. Gan, and T. E. Manakova [1]

Introduction

Erythropoietic islands located in the yolk sac (YS) mesoderm are known to be the first sites of hemopoiesis in the ontogeny of mice. Committed precursors colony-forming unit–granulocyte-macrophage (CFU-GM) and burst-forming unit–erythroid (BFU-E), as well as pluripotent progenitors (CFU_{mix}) were found in murine YS as early as 8 days of gestation; in embryos proper (bodies) and in the circulation, committed precursors appeared 1 day later than in YS [1–3]. As for CFU–spleen (CFU-S), there are few communications and they are contradictory. Moore and Metcalf [1] reported that CFU-S initially appeared in murine YS on day 8 of gestation, and they were evident in the embryonic circulation by day 10 of development. However, other investigators failed to find macroscopic spleen colonies after the injection of day-9 YS cells [4, 5]. After the cultivation of a day-9 YS in organ culture for 24–96 h, CFU-S were readily found [4]. In the present study we tested the accurate stages – in somite pair (SP) numbers – of embryonic development when CFU-S and CFU-GM appeared for the first time in the YS, circulation, embryos proper, and liver rudiments (LR). We have used the organ culture system to examine whether pre-CFU-S, capable of differentiating into CFU-S in vitro, exist in the 9-days YS and embryo proper.

Materials and Methods

C57Bl/6 females were mated with CBA males. The day on which the vaginal plugs were observed was designated as day 0 of gestation. SP were counted in 8 to 10-day embryos. Embryonic blood was collected after the separation of the YS from the bodies. Beginning with the 9th day of gestation, LR were removed from the bodies and investigated separately. For routine CFU-GM testing the tissues were minced and treated with 0.1% trypsin. For CFU-S testing tissues were minced and pipetted gently. Cell suspensions were injected i.v. to lethally irradiated (^{137}Cs, 12.5 Gy) CBF_1 mice; 7th-day macroscopic colonies were counted. The filter organ culture method [6] was used with some modifications. Briefly, whole 9-day embryos or separated embryos and YS were cultured on Millipore filters (HA, pore size 0.45 μm), supported by a stainless steel grid (37 °C, 5% CO_2 in air). After cultivation explants were tested for CFU-S content.

Results

CFU-GM were observed first in the 8-day YS (0 to 5-SP stage), and they were practically absent from the body at that time. Reliable numbers of CFU-GM in the embryo body appeared from day 9. The appearance of CFU-GM in the embryo proper generally coincided with the beginning of their detection in the circulation (13 to 16-SP stage). The maximum content of CFU-GM was observed on day 10 of development both in the YS and

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

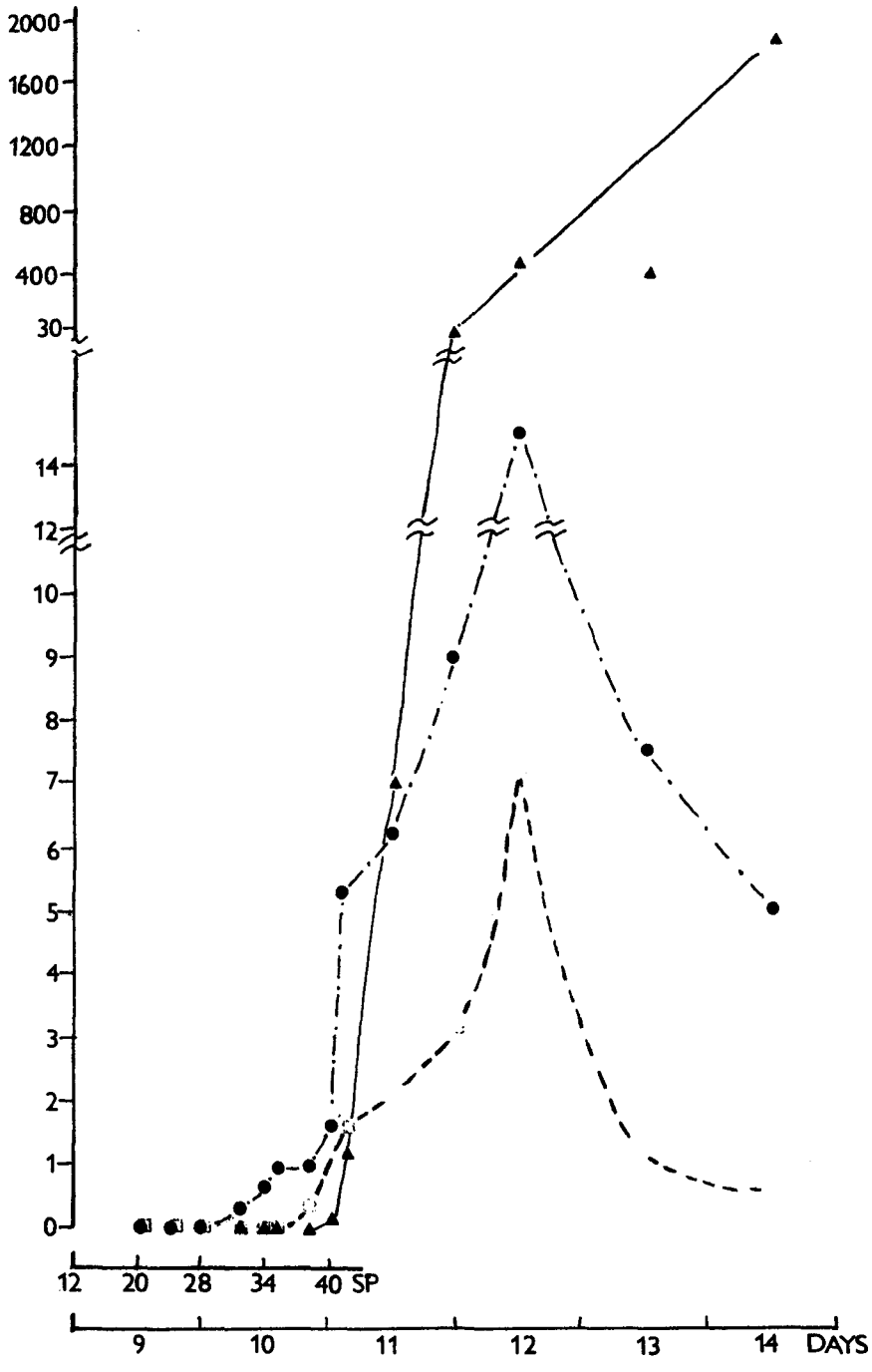

Fig. 1. CFU-S in embryonic tissues (per embryo) during 9th–14th days of development. *Abscissa*, Days of embryonic development and numbers of somite pairs (SP); *ordinate*, the number of CFU-S in the YS (□–□), the body (●—●), and the liver (▲—▲)

the body. It must be emphasized that the first CFU-GM which were found in the body of the embryo were not localized in the LR, where they appeared from approximately 25 to 26-SP stage (9th day). From the 11th day of development the liver became the main source of GM progenitors in embryos (data not shown).

CFU-S first appeared in the embryo proper, out of the LR, at the 30 to 33-SP stage on day 10 (Fig. 1). In the YS, CFU-S could be found beginning at the stage of 37–38 SP. In the circulation, CFU-S were observed from about the 37-SP stage. From the initial level of 0.24 CFU-S per 10^6 cells, their concentration increased to 1.69 per 10^6 cells by day 11, and reached a maximum on day 13 (3.5 per 10^6 cells). The detectable number of CFU-S in LR was found only at the end of the 10th day

Table 1. CFU-S in 9-say-old embryos after in vitro cultivation

Culture	Age of donor (SP)	Number of embryos	Colonies/spleens
Whole embryo	14–23	105	1/30
	24	43	3/13
	25–28	41	7/16[a]
	29–30	5	3
Yolk sac	23–24	9	0/1
	25–28	92	9/19[a]
	29–30	28	6/7
Body with liver rudiment	23–24	9	0/1
	25–28	56	5/22[a]
	29–30	7	2/5
Body without liver rudiment	25–28	62	13/18
	29–30	18	1/9
Liver rudiment	26–30	61	0/8
Irradiation only			1/96

[a] One large colony was selected for chromosome analysis. Donor origin (T6 chromosome) of CFU-S was confirmed.

of gestation ($\approx$ 40 SP). CFU-S number in the YS as well as in the embryo body attained a peak on day 12 and then decreased. From the 11th day, the CFU-S content in embryonic liver increased suddenly.

After 4 days in organ culture CFU-S were not detected in any tissues explanted earlier than the 25 to 28-SP stage, with the exception of the 24-SP whole embryo group in which quite a few colonies were found (Table 1). In the YS and the embryo body explanted at the stage of 25–28 SP or later, significant numbers of colonies were observed. Embryo bodies without LR were cultured in order to test whether CFU-S production in the embryo could be ascribed to the developing liver. The results indicate that CFU-S production in cultures of "the body without LR" was no less than in cultures of "the body with LR." No CFU-S were produced in isolated LR.

Discussion

We have failed, as others [4, 5], to support the data of Moore and Metcalf [1] that CFU-S first appear on the 8th day of murine development. We could not demonstrate any CFU-S in the YS or in the embryonic body till the 10th day of gestation. Moreover, CFU-S did not appear earlier in the YS than in the body. So we encountered the fact that CFU-S were absent from the hierarchy of hemopoietic progenitors in the 8 to 9-day YS, though committed precursors (CFU-GM) were present. Whether these two kinds of embryonic hemopoietic precursors have a common origin or *take* arise independendently is not clear now. One cannot rule out the possibility of a transitory hemopoiesis in the YS of an early (7–8 days) murine embryo such as takes place during development in birds [7]. It has been shown earlier [4] that the YS of a 9-day embryo contains pre-CFU-S which can differentiate into CFU-S after 1–4 days in organ culture. We have not only confirmed this observation but have also found that pre-CFU-S appeared both in the YS and embryos proper simultaneously in the late 9-day (25–29 SP) embryos.

In conclusion, the early (8–9 days) embryonic (YS) period of hemopoiesis

differs from adult and fetal hemopoiesis in that CFU-S are absent from the stem cell compartment. Before fetal liver hemopoiesis starts (the 10th day of development), there are two sites, the YS and the embryo body, from which CFU-S could migrate into the developing liver. According to the data obtained, one cannot prefer the YS or the body as the source of CFU-S origin since CFU-S as well as pre-CFU-S are detected in both sources simultaneously.

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Induction of Differentiation of the Human Promyelocytic Cell Line (HL-60) by Conditioned Medium of *Ceathea letifera*-Stimulated Mononuclear Cells *

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Introduction

Studies by some investigators have shown that human leukemic cell lines, primarily of myeloid lineage, retain their ability to differentiate in vitro when exposed to a variety of compounds such as differentiation-inducing factors present in conditioned medium (CM) including retinoic acid [1], phorbol diesters [2], dimethyl sulfoxide, *Clerodendron fragrans* [3], and when cocultured in CMs secreted from lectin-stimulated lymphocytes or Chinese herb-stimulated mononuclear cells [4]. Recently, it became apparent from other studies that interferon-γ (IFN-γ), colony-stimulating factor (CSF), and interleukin-2 (IL-2) have been identified in CM and have been found to express some of their effects by inducing differentiation. However, there is also an unidentified differentiation-inducing activity (DIA) distinct from the above well-known factors that has a similar effect [4].

There are only a few papers reporting CM of Chinese herb-stimulated mononuclear cells to have a capacity to induce differentiation of HL-60 cells. In the present study, we report another CM, called CL-CM, secreted from *Ceathea letifera*-stimulated mononuclear cells, which has the capacity to induce HL-60 cells to differentiate into mature cells.

Material and Methods

Conditioned Media. Human peripheral blood mononuclear cells, separated on a Ficoll-Hypaque gradient, were incubated in RPMI 1640 medium (10^6 cells/ml), either with or without 10% fetal bovine serum (FBS), supplemented with *Ceathea letifera* (1 mg/ml), phytohemagglutinin (PHA; 10 μg/ml), concanavalin A (Con A; 125 μg/ml), and pokeweed mitogen (PWM; 10 μg/ml), for 72 h at 37 °C in 5% CO_2 in air. Cell-free supernatants were collected and used as crude preparations denoted CL-CMF$^+$ (containing 10% FBS), CL-CMF$^-$ (serum-free), PHA-CMF$^+$, PHA-CMF$^-$, ConA-CMF$^+$, ConA-CMF$^-$, PWM-CMF$^+$, and PWM-CMF$^-$. The CL-CMF$^+$ and CL-CMF$^-$ media were shown to have the capacity to induce HL-60 cells to develop into mature monocytes.

Crude Extract of *Ceathea letifera*. The extract was dissolved in 50% ethanol at 40 °C, and concentrated to 1:20 at negative pressure in RPMI 1640. This Chinese herb used as a mitogen was cultured with normal mononuclear cells (10^6 cells/ml) in serum-free RPMI 1640 for 3 days. The resulting CM (called CL-CMF$^-$) was obtained and then cultured with HL-60 cells in RPMI containing 10% FBS for 5 days. The ratio of the volume of CM to

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

10% FBS-containing RPMI medium was 3 to 7. In addition, IFN-γ, TNF, and IL-2 (Genzyme, USA) were added to the CL-CM to examine the additive or synergistic effects on differentiation of HL-60 cells. Parameters which identified the differentiation of treated HL-60 cells and analysed the factors in CL-CM included the following:

(a) cell number;
(b) cell viability;
(c) phagocytosis and nitroblue tetrozolium test (NBT) [4];
(d) surface marker study – Mo1 and Mo2, using an indirect immunofluorescence method;
(e) cytochemistry test (POS, PAS, CES, and NES);
(f) the contents of IL-1, IL-2, TNF, and IFN-γ using ELISA kits;
(g) assay for granulocyte-macrophage colony-forming cells (CFU-GM) [5];
(h) sodium dodecylsulphate–polyacylamide gel electrophoresis (SDS-PAGE) [6].

Results

Viability and Cell Number. Table 1 shows that cells cultured in CL-CMF$^-$, IFN-γ, TNF, and IL-2 or in a combination with CL-CMF$^-$ with IFN-γ, TNF, or IL-2 showed no evidence of a decreased cell number in comparison with control on the 3rd and 5th days of culture; in addition, there was a slightly lower cell viability compared with controls on the 3rd and 5th days of culture.

Differentiation Effects. Following treatment with CL-CMF$^-$ for 5 days, about 50% of the HL-60 cells underwent a morphological change from promyelocytes to mature monocytoid cells. Cytochemical studies revealed that these cells were 22% positive for NES stain but negative for CES, POS, and PAS stains. These cells became phagocytic (38% $\pm$ 8.3%) (Table 2) but only 5% $\pm$ 25% positive for NBT. Correspondingly, 28% $\pm$ 6.4% and 10% $\pm$ 1.5% of the treated cells were positive for Mo1 and Mo2 antibodies, respectively (Table 2).

The biological response modifiers IFN-γ, TNF, and IL-2 (Genzyme, USA) were added to the CL-CMF$^-$ to examine the additive or synergistic effects on differentiation. Our results showed that a combination of CL-CMF$^-$ with TNF, IFN-γ, or IL-2 has an enhanced differentiation-inducing effect on HL-60 cells according to surface marker studies (Table 2).

TNF, IFN-γ, IL-1, and IL-2 Contents. ELISA test kits were used to detect the concentrations of TNF, IFN-γ, IL-1 and

Table 1. Cellularity (cell) and viability (viab) of HL-60 cells treated by various compounds

	Day 1		Day 3		Day 5	
	cell	viab	cell	viab	cell	viab
Control	53 $\pm$ 8	98 $\pm$ 2	90 $\pm$ 8	96 $\pm$ 1	172 $\pm$ 12	94 $\pm$ 1
CL–CM	45 $\pm$ 5	97 $\pm$ 2	82 $\pm$ 8	95 $\pm$ 3	138 $\pm$ 10	93 $\pm$ 2
TNF	45 $\pm$ 7	94 $\pm$ 1	99 $\pm$ 4	89 $\pm$ 2	124 $\pm$ 11	84 $\pm$ 2
IFN-γ	51 $\pm$ 6	93 $\pm$ 3	76 $\pm$ 10	90 $\pm$ 4	127 $\pm$ 13	88 $\pm$ 3
IL-2	50 $\pm$ 5	96 $\pm$ 2	93 $\pm$ 8	95 $\pm$ 2	166 $\pm$ 20	90 $\pm$ 1
CL–CM + TNF	43 $\pm$ 4	92 $\pm$ 2	92 $\pm$ 6	87 $\pm$ 3	112 $\pm$ 15	84 $\pm$ 4
CL–CM + IFN-γ	40 $\pm$ 3	95 $\pm$ 2	74 $\pm$ 7	88 $\pm$ 4	110 $\pm$ 14	82 $\pm$ 5
CL–CM + IL-2	51 $\pm$ 5	95 $\pm$ 2	83 $\pm$ 2	93 $\pm$ 3	115 $\pm$ 17	90 $\pm$ 4

Values are (10^4 cells/ml) expressed as mean $\pm$SD, $n = 5$.
CL–CM: Ceathea letifera-conditioned medium.

Table 2. Surface markers Mo1 and Mo2 and phagocytosis of HL-60 cells treated by various compounds

	Day 1			Day 3			Day 5		
	Mo1	Mo2	Phago	Mo1	Mo2	Phago	Mo1	Mo2	Phago
Control	0±0	0±0	2±1	2±1	1±1	3±1	3±1	1±1	4±1
CL−CM	12±3	3±1	20±7	22±5	7±2	25±4	28±6	10±1	38±8
TNF	10±2	2±1	17±3	17±2	7±2	24±6	25±4	12±3	52±8
INF-γ	12±2	7±2	18±4	22±7	13±4	43±7	31±6	20±3	62±5
IL-2	5±1	2±1	6±1	6±3	3±1	13±2	8±2	4±1	24±6
CL−CM+TNF	14±4	5±8	25±5	28±6	12±2	36±1	36±4	17±4	51±7
CL−CM+IFN-γ	16±3	10±3	20±4	27±6	16±4	48±1	44±8	23±6	65±1
CL−CM+IL-2	14±4	4±1	24±6	21±5	10±3	29±7	27±8	15±3	40±6

Values are expressed as percentages, mean ± SD, $n = 5$.
Phago: Phagocytosis.

IL-2 in CL-CM, PHA-CM, ConA-CM, and PWM-CM. The results are shown in Table 3. In the CL-CMF$^+$ which contained 10% FBS in CM, IL-1 at 1320 pg/ml, TNF at 300 mg/mm, and IL-2 at 0.05 L/ml were detected, but no IFN-γ was found. In contrast, in CL-CMF$^-$ which did not contain FBS in CM, only IL-1 at 110 pg/mg was detectable.

Effect of CL-CMF$^-$ on GM-CSF. Table 4 shows the average of three separate experiments in which the effect of CL-CMF$^-$ on granulocyte-macrophage colony formation was tested. On day 7, only a few colony and cluster formations were induced by CL-CMF$^-$ or in the control group, whereas the formation of many colonies was noted when placental CM was added as a colony-stimulating factor.

The Results of SDS-PAGE. Figure 1 shows the 10% SDS-PAGE profile of serum-free CMs using the reducing method. CL-CMF$^-$ (lane 1), RPMI

Fig. 1. 10% SDS-PAGE profile of serum-free CMs using reducing method. *1*, CL-CMF$^-$; *2*, RPMI media; *3*, *Ceathea letifera*

Table 3. Components in the conditioned media with different stimulant

		IFN-γ (U/ml)	TNF (pg/ml)	IL-2 (U/ml)	IL-1 (pg/ml)
PHA	+	0	300	0.05	ND
	−	7	0	0	ND
ConA	+	30	970	0.05	ND
	−	38	710	0	ND
PWN	+	32	750	0	ND
	−	65	225	0	ND
CL	+	0	300	0.05	1320
	−	0	0	0	110

ND: not detectable.

Table 4. Colony-stimulating activity

	CSF (placenta)	CL−CMF$^-$
No. of CFU−GM	86/167	0/24

Data are expressed as colonies versus clusters.
CSF: colony-stimulating factor.

media (lane 2), and *Ceathea letifera* (lane 3), were prepared and analyzed on SDS-PAGE. Lane 1 shows a wide homogeneous band, mainly at 50−60 kDa. There are no such bands in lanes 2 and 3.

Discussion

Traditionally, the Chinese herb *Ceathea letifera* was widely used for treatment of hemorrhage, infection, and diarrhea and also as an antidote for some poisons. The main components have been analyzed and consist of adianton, aspidinol, fernene, filicin, hopene-diploptene, hopanol-29, neritoiol, and tannic acid. To our knowledge, this is the first report describing CM of *Ceathera letifera*-stimulated mononuclear cells to have the capacity to induce differentiation of HL-60 cells into mature cells.

Based on the cytochemical and surface marker studies described here, we can conclude that CL-CM can promote the differentiation of the HL-60 cell line along the monocytic pathway.

We were also interested in the differentiation-inducing activity of CL-CMF$^-$. These data show that components of CL-CMF$^-$ are distinct from well-known differentiation-inducing factors described by others [7–9]. Firstly, no IFN-γ, TNF, IL-2, or CSF was found using ELISA test kits and GM-CSF assay in CL-CM; secondly, combination of CL-CMF$^-$ with TNF, IFN-γ or IL-2 has an enhanced differentiation-inducing effect on HL-60 cells by Mo1 and Mo2 surface marker study; and thirdly, the protein has a molecular weight of 50−60 kDa, as determined using SDS-PAGE, which is different from the CMs of other mitogens such as PHA, ConA-stimulated mononuclear cells, which contained TNF, IFN-γ, and IL-2 (Table 3) [7–9]. Although the nature of this protein remains to be determined, CL-CMF$^-$ is obviously an important candidate for further studies.

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Molecular Mechanisms in Myeloid Lineage Development *

L. R. Rohrschneider [1]

Recent work from my laboratory has described a new mechanism for controlling the expression of one hematopoietic colony-stimulating factor (CSF) receptor by the interaction of another CSF with its receptor [1]. This type of transmodulation occurs at the level of mRNA stability and has interesting implications for hematopoietic cell development. It is particularly relevant to mechanisms that determine myeloid lineage restriction and its potential involvement in this process will be presented and analyzed.

The development of eight distinct mature blood cell types occurs from a single stem cell population and represents a challenging problem in understanding the mechanisms that control both normal and abnormal growth and differentiation. As far as we now know, the development of the cells along the hematopoietic cell lineages is controlled by a set of glycoprotein factors called CSFs [2]. Some CSFs such as interleukin-3 (IL-3, also called multi-CSF) and granulocyte-macrophage CSF (GM-CSF) have the potential to stimulate development of several mature cell types, whereas macrophage CSF (M-CSF) and granulocyte CSF (G-CSF) are more restricted in their actions even though they do have some effects on early hematopoietic cell development in combinations with other CSFs [3, 4].

Our efforts to understand the mechanisms that control hematopoietic cell development have focused on the segment of the myeloid pathway that leads to mature macrophage and granulocytic cells (Fig. 1). These two cell populations arise from a common bipotential progenitor cell. The type of mature cell that develops from the bipotential progenitor depends on the concentration and type of CSF that stimulates this cell [1, 5–7]. M-CSF and G-CSF will stimulate, respectively, the formation of macrophages and granulocytes almost exclusively. IL-3 and GM-CSF stimulate formation of both macrophages and granulocytes. GM-CSF in particular exhibits an interesting preferential stimulation of macrophages at very low concentrations [1, 6, 7], but favors the formation of granulocytes at higher concentrations of the growth factor. These results indicate there are interesting effects of CSF on determining the ultimate fate of a bipotential progenitor cell; however, at present there are no indications as to how this occurs.

To study the mechanisms that influence lineage restriction we developed a cell line that expressed three of the four CSF receptors and responded to the three CSFs. The parental cell line, FDC-P1, normally has receptors for GM-CSF and IL-3 [8, 9], and a further subclone expressing M-CSF receptors was selected by forced growth with M-CSF as the sole growth factor. This cell line was called FD/MAC. Individual clones of FD/MAC cells grew with either IL-3, GM-CSF, or M-CSF as the only growth

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

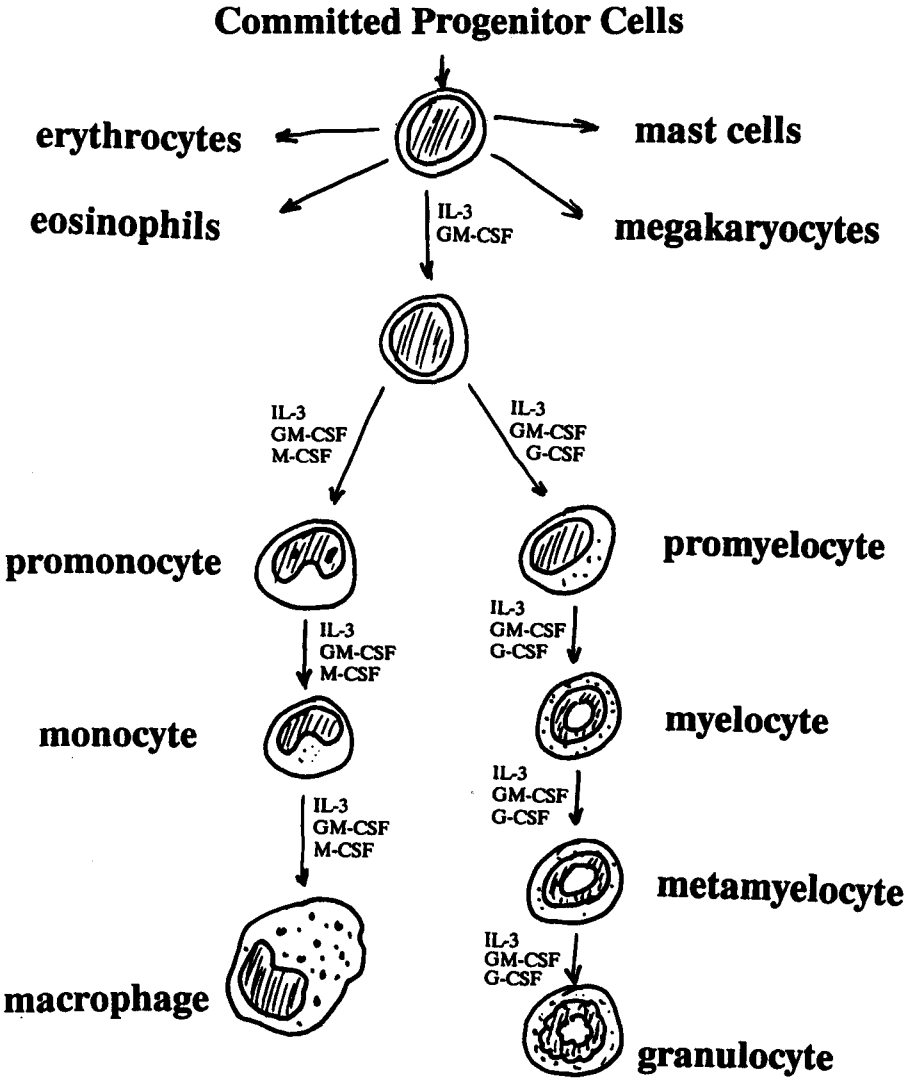

Fig. 1. Developmental scheme for the macrophage and granulocytic cell lineages. A bipotential progenitor cell can develop into either a macrophage or a granulocyte and this decision is influenced by a set of four colony stimulating factors (IL-3, GM-CSF, M-CSF, and G-CSF)

factor, and therefore each cell must express receptors for each of these CSFs. GM-CSF and IL-3 stimulated only growth in the FD/MAC cells, but M-CSF induced both growth and differentiation, as we have previously demonstrated [10].

An early observation with the FD/MAC cell line was that M-CSF receptor protein was lost after stimulation with either GM-CSF or IL-3. Factor-switching experiments demonstrated that the loss was due to the dominant actions of the stimulated GM-CSF and IL-3 receptors because removal of the FD/MAC cells from any growth factor caused expression of the M-CSF receptor (even if they had been grown in GM-CSF), while switching to growth on GM-CSF or IL-3 caused loss of the M-CSF receptor (even if they had been grown in M-CSF). The interaction of GM-CSF (or IL-3) with its receptor therefore induced some positive signal that was directly responsible for the loss of the M-CSF receptor.

The point at which this regulation occurred was found to be at the level of M-CSF receptor mRNA stability ([1], see also B. C. Gliniak, L. S. Park, and L. R. Rohrschneider, Mol Biol Cell 3, in press). Transcription, as measured by nuclear run-on assays, of the M-CSF receptor gene (c-*fms*) was not affected by the CSF in which the cells were grown, but

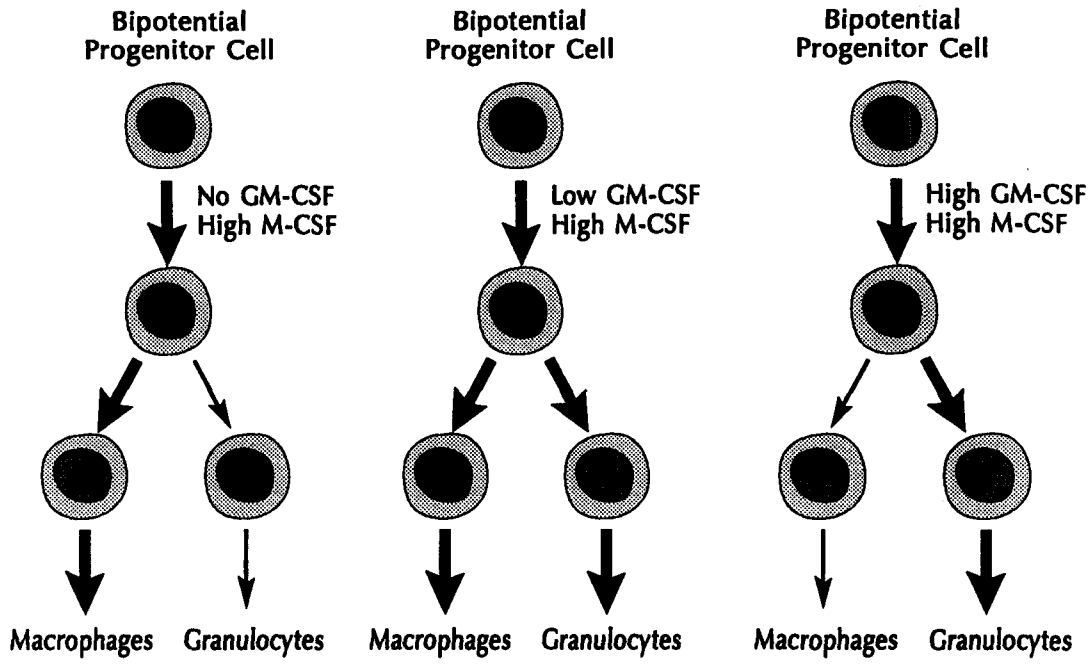

Fig. 2. Summary of the results of agar colony assays on mouse bone marrow cells grown in the presence of combinations of M-CSF and GM-CSF. The *darker arrows* indicate the favored pathways in development of a bipotential progenitor cell to mature macrophages or granulocytes grown with the growth factors indicated

Northern analyses of M-CSF receptor mRNA showed dramatic reductions (50- to 100-fold) when cells were grown in the presence of GM-CSF or IL-3. Further studies demonstrated that the reduction in M-CSF receptor mRNA caused by GM-CSF or IL-3 was dominant and concentration dependent. Low concentrations of GM-CSF had little effect on the M-CSF receptor mRNA whereas higher concentrations completely abolished M-CSF receptor mRNA. When GM-CSF was added to cells already growing in M-CSF, there was a loss of M-CSF receptor mRNA. These results indicate that the dominant effect on M-CSF receptor expression occurs posttranscriptionally.

Preliminary studies with inhibitors of transcription and protein synthesis indicate that the likely control point is right at the level determining the mRNA stability (Gliniak, Park, and Rohrschneider, Mol Biol Cell 3, in press). Experiments with actinomycin D and cyclohexamide used singly or together indicated the presence of a rapidly turning over protein (probably an RNase) that regulates the degradation of the M-CSF receptor mRNA. So far, we have no direct proof for the existence of a stabilizing factor for the M-CSF receptor mRNA reported in other cell types [11].

The above results indicate that the concentration of GM-CSF can have a dramatic influence on the expression, and therefore function, of the receptor for M-CSF. This effect was detected in a cell line and therefore, to obtain some evidence that a similar effect occurs in hematopoietic cell development, we examined the influence of combinations of M-CSF and GM-CSF on the development of murine bone marrow cells in agar assays [1]. A diagrammatic summary of the results is presented in Fig. 2. M-CSF stimulated the formation of macrophage colonies exclusively, whereas addition of even small amounts of GM-CSF caused a shift to production of granulocytic colonies and especially mixed colonies containing both granulocytes and macrophages. With increasing amounts of GM-CSF added to a fixed M-CSF concentration the shift toward more granulocytic colonies was more apparent and the

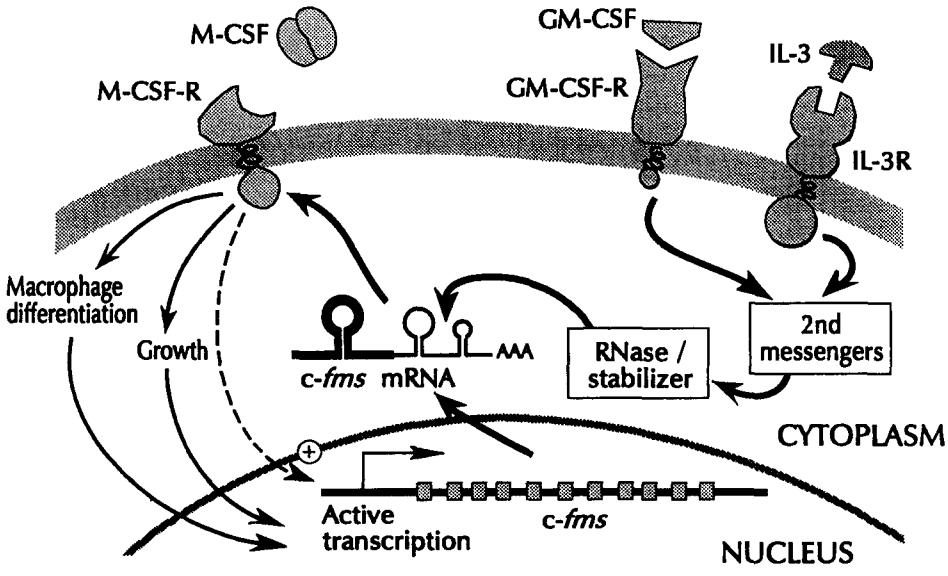

Fig. 3. Current model for how activated GM-CSF and IL-3 receptors (GM-CSF-R and IL-3R) cause destabilization of the mRNA for the M-CSF (c-*fms*) receptor (M-CSF-R). The rationale for the model is discussed in the text

mixed colonies were also numerous. The colony assays, although complex in nature because of the different cell types involved, did support the notion that GM-CSF can act in a dominant fashion to suppress development along the macrophage pathway.

The diagram in Fig. 3 illustrates our present concept for the regulation of M-CSF receptor mRNA by the interaction of GM-CSF and IL-3 with their respective receptors. The binding of M-CSF to its receptor stimulates both a differentiation and growth response, and in addition there is some evidence from the nuclear run-on studies for a small autoactivation of c-*fms* transcription [1]. GM-CSF and IL-3 also stimulate growth but send additional signals that control c-*fms* mRNA stability. Presumably some type of second messenger system is involved in sending a signal that affects c-*fms* mRNA stability. This signal ultimately can either inactivate a factor that stabilizes the mRNA or activate a factor such as an RNase that degrades the mRNA. Preliminary results suggest the latter case may be more correct, and that activation could occur through increased transcription of the factor or posttranslational modification(s). The GM-CSF (or IL-3)-induced signal for degradation of c-*fms* mRNA is dominant, and the amount of degrading activity is dependent on the GM-CSF concentration.

The dominant actions of GM-CSF on suppressing expression of the receptor for M-CSF and negatively influencing the development of mature macrophages suggests that this mechanism could help to explain lineage restriction. The decision of a bipotential progenitor cell to restrict itself to one defined lineage could be determined by the relative concentrations of growth factors encountered by that cell at the time the lineage selection is made. If the cell is in an environment rich in GM-CSF, then the M-CSF receptor will not be expressed and that cell will most likely proceed down the granulocytic pathway. If, however, the environment lacks GM-CSF then the M-CSF receptor will be expressed and that cell has the opportunity to become a macrophage. This mechanism has the advantage that a cell can respond quite rapidly to fluctuations in CSF concentration and shift lineages by controlling expression of receptors through regulation of mRNA stability. Activating or inactivating transcription machinery is not necessary.

This proposed mechanism would also suggest a slightly different purpose for

GM-CSF and its receptor. Previously, GM-CSF has been envisioned as a growth factor that merely stimulated growth and development toward mature cells. The role of GM-CSF in regulating the expression of the M-CSF receptor would now suggest an additional role of the GM-CSF receptor in determining the lineage that a bipotential progenitor cell will select. The concentration of GM-CSF may serve as a switch between alternate lineages.

Hematopoiesis may be viewed as a developmental system, and many parallels exist with other developmental model systems. The proposed role for GM-CSF in controlling lineage development by negative regulation has a counterpart in many other developmental pathways [12–18]. In these systems cell contact or signals from one developing cell inhibit development of another cell lineage, or cause that cell to select an alternate lineage. In some cases these effects are known to occur through cell surface receptors.

The new results presented here and elsewhere [1] provide a different look at hematopoiesis that may help explain the molecular details of this process. There are, however, many more molecular questions unanswered and further experiments are required to confirm the mechanisms presented here and to fill in the missing details.

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Proteoglycans in Cellular Recognition and Secretory Functions in the Haemopoietic System

M. Ranson and J. T. Gallagher [1]

Introduction

Proteoglycans (PGs) are synthesised by all nucleated haemopoietic cells and are also present in the α-granules of platelets [65]. Their sulphated polysaccharide chains, the glycosaminoglycans (GAGs), have been recognised for many years as prominent components of secretory granules of mast cells and basophils, and the unusually high degree of sulphation of these GAGs is responsible for the pronounced metachromasia of the granular structures [31, 133]. Earlier studies also detected GAGs in lysosomes or prelysosomes of neutrophils [94]. The core protein of the major PG of secretory granules has been cloned and sequenced and appears to be quite widely distributed in the haemopoietic system. Recent immunological studies have also identified three distinct proteoglycan species in lymphocytes, and two of these, syndecan and the Hermes antigen, are expressed on the cell surface whilst the other, the invariant chain (Ii), is found inside the cells transiently in association with the class II antigens. In this review, we describe the structures and functions of the PGs in haemopoietic cells with particular emphasis on cells of the lymphoid lineage.

The Glycosaminoglycans (GAGs)

GAGs are linear polysaccharides largely composed of repeating disaccharide units [39, 73, 100]. The major GAG produced by most haemopoietic cells is chondroitin sulphate, the notable exception being the connective tissue mast cell which synthesises mainly heparin [65]. Dermatan sulphate and heparan sulphate have a more restricted distribution than chondroitin sulphate, dermatan sulphate being clearly identified only in activated mucosal mast cells [68, 133] while heparan sulphate is found mainly in cells of the lymphoid lineage where expression may be developmentally regulated [114]. The foregoing GAGs are all synthesised directly on core proteins by sequential addition of monosaccharide units. Serine residues within specific peptide sequences or "sequons" are sites of protein glycanation [34].

Several types of sequon have been identified, but a common feature is that serine is always adjacent to glycine [18], and the Ser-Gly dipeptide appears to be the minimum requirement for recognition of the protein by a xylosyl transferase which transfers xylose to serine, priming the protein for the synthesis of a protein-linkage sequence upon which the disaccharide repeating units of the GAG are assembled (Fig. 1).

Chondroitin and Dermatan Sulphate

In chondroitin sulphate the disaccharide repeat is *N*-acetylogalactosamine (GalNAc) and glucuronic acid (GlcA):

GalNAc β 1,4 G1cA
|
S

This is ester sulphated (denoted S) at C4 (type A) or C6 (type C) of GalNAc.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

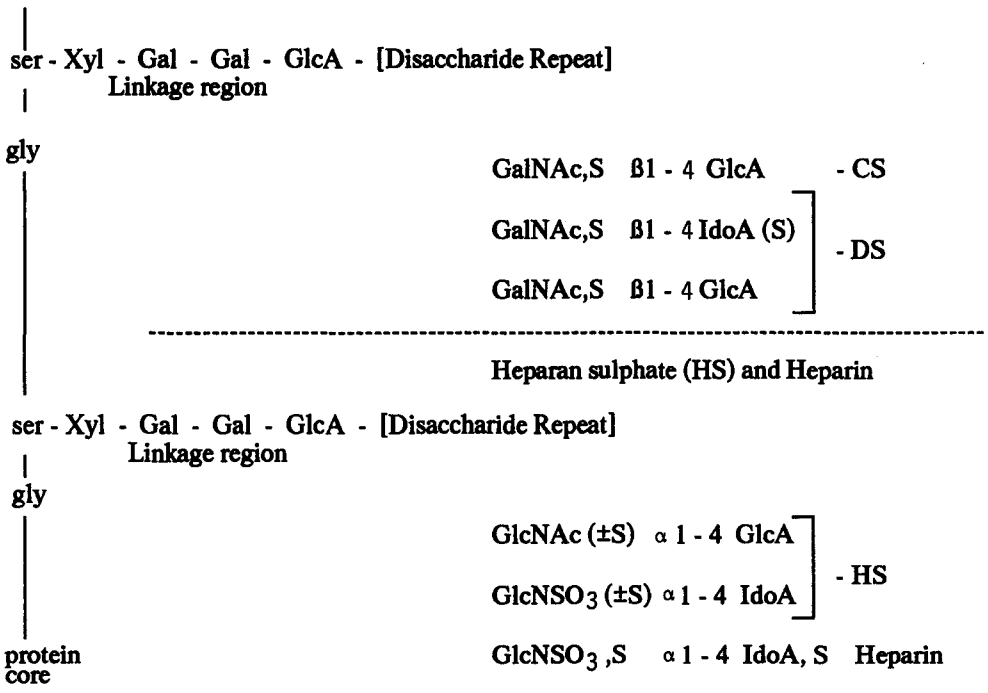

Fig. 1. Glycosaminoglycans in covalent linkage to protein. Sulphated glycosaminoglycans (*GAGs*) are commonly linked to protein by a tetrasaccharide linkage region in which a terminal xylose forms on O-glycosidic linkage to serine in a Ser-Gly sequence. The GAG chains contain from 50 to 200 disaccharide units. The chondroitin sulphate disaccharide repeat is N-acetylgalactosamine (*GalNAc*) and glucuronic acid (*GlcA*) with sulphation (indicated by *S*) at C4 or C6 of GalNac. Dermatan sulphate contains a variable proportion of disaccharides in which GlcA has been converted to iduronic acid (*IdoA*), and the latter may be sulphated at C2. In heparan sulphate (*HS*) and heparin a glucosamine hexuronate repeat comprises the polysaccharide backbone. HS contains approximately equal proportions of N-acetylated (*GlcNAc*) and N-sulphated ($GlcNSO_3$) disaccharides, whilst in heparin the amino sugar is mainly in the form of $GlcNSO_3$. In HS, N-acetylated and N-sulphated disaccharides occur mainly in separate sequences. The N-sulphated domains are enriched in IdoA residues and ester-linked (O)-sulphate groups. O-sulphation of HS chains is variable and is indicated by ($\pm S$). Heparin consists predominantly of trisulphated disaccharides of the type shown, in which O-sulphates are at C6 of $GlcNSO_3$ and C2 of IdoA (see text for details)

Chondroitin sulphate chains often consist of mixed disaccharide isomers, though haemopoietic cells commonly contain chains that are almost entirely sulphated at C4. Some chondroitin sulphate disaccharides are sulphated at both C4 and C6, and such disulphated units, called Di-E type disaccharides, are synthesised by mast cells, activated macrophages, eosinophils and human lymphocytes.

Dermatan sulphate is synthesised by polymer level epimerisation of a variable proportion of the GlcA units to the C5 epimer, iduronic acid (IdoA). In dermatan sulphate the GalNAc is mainly sulphated at C4 but the iduronate residue can also be sulphated at C2. Dermatan sulphate disaccharides may occur as disulphated units, called di-B type structures: (GalNAc, 4S β1, 4 IdoA, 2S).

Heparan Sulphate and Heparin

In heparan sulphate and heparin the disaccharide repeat units are rather complex due to variations in sulphation pattern and, in heparan sulphate, to the presence of extended nonsulphated

regions [37, 149]. The amino sugar component of heparan sulphate and heparin is glucosamine, which may be N-acetylated (GlcNAc) or N-sulphated ($GlcNSO_3$). Heparan sulphate contains about equal amounts of these two derivatives, GlcNAc always being in association with glucuronate whereas $GlcNSO_3$ is mainly linked to iduronate:

GlcNAc α 1,4 GlcA
|
(6S)

$GlcNSO_3$ α 1,4 IdoA
|
(6S) (2S)

In the heparan sulphate chain, these two basic types of disaccharide tend to be segregated into N-acetylated or N-sulphated sequences of variable length rather than being uniformly distributed [147, 148]. The major locations of the ester linked (O)-sulphates (indicated in parentheses above) are C6 of the amino sugars and C2 of iduronate. The degree of O-sulphation varies in heparan sulphates from different cell types [37], suggesting that these polysaccharides may have very specific roles to play in recognition events at the cell surface. O-sulphations are largely confined to the N-sulphated regions of heparan sulphate, creating domains of high charge density.

Rare but important isomers in heparan sulphate are 2-sulphated GlcA (GlcA, 2S) and 3-sulphated $GlcNSO_3$ ($GlcNSO_3$, 3S). GlcA, 2S is enriched in a heparan sulphate fraction found in the nucleus of a hepatoma cell line and its nuclear concentration has been correlated with the arrest of cell division [51, 52]. $GlcNSO_3$, 3S is an essential component of the anti-thrombin III pentasaccharide binding sequence in heparin and heparan sulphate which is largely responsible for the anticoagulant properties of the polysaccharides [74]. The sequence is:

$$-GlcNAc-GlcA-GlcNSO_3-IdoA-GlcNSO_3\,\alpha$$
$$\quad\;|\qquad\quad\;|\quad\;|\quad\;|$$
$$\quad 6S\qquad\quad 3S\quad 2S\quad 6S$$

The discovery of this sequence suggests that other important protein binding sequences may be present in heparan sulphate and heparin. The heparan sulphates display a broad repertoire of protein interactions which include growth factors, enzymes, extracellular matrix macromolecules and membrane receptors [36]. In the context of haemopoiesis, it is especially significant that heparan sulphate from inductive stromal cells binds the growth factors interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The growth factor – polysaccharide complexes are mitogenically active [108], suggesting a possible role for heparan sulphate on stromal cells in sequestration and presentation of growth factors in bone marrow or lymphoid organs [35, 42].

Heparin is the most highly sulphated of mammalian GAGs, and the main disaccharide unit is the trisulphated derivative [74]: $GlcNSO_3$, 6S α 1,4 IdoA, 2S. Only 10%–20% of the disaccharides in heparin are N-acetylated and, in contrast to heparan sulphate, the molecule has a relatively uniform level of sulphation [37].

Proteoglycans in the Haemopoietic System

Secretory Granule Proteoglycan – Serglycin

Serglycin is the major PG in the secretory granules of mast cells, basophils, natural killer (NK) cells, eosinophils and platelets [8, 9] (for review see [65]). The 20-kDa protein core is characterised by the presence of contiguous Ser-Gly repeats (24 repeats in the rat, 10 in mouse and 9 in human) and each serine is a potential glycanation site, resulting in the GAG chains being clustered in a short peptide region. This region is highly resistant to

proteolytic cleavage. The protein core is glycanated with different polysaccharides, including heparin in connective tissue mast cells, chondroitin sulphate enriched in E-type disaccharides in mucosal mast cells and basophils and chondroitin-4-sulphate in platelets. These data indicate that the protein core of serglycin is not the major determinant of the GAG substitution pattern. The PG may play an important role in complexation and packaging of basic proteases, cytolysins and histamines in the secretory granule matrix [133].

In the rat, a single gene encodes the peptide core of the *extracellular* serglycin of the yolk sac tumour cell and the core protein of the *secretory granule* serglycin of basophilic leukaemia cells [18, 142]. The transcription start site that has been predicted for the serglycin of the rat yolk sac tumour mRNA is ~ 220 nucleolides 5′ to the start site for transcription of the rat basophilic leukaemia cell serglycin [8]. It is possible that the eventual cellular destination of these PGs could be regulated by untranslated sequences within the 5′ regions of the transcripts, by their binding to specific subclasses of ribosomes which function to target proteins to different areas within the cell. By itself, the final translated core protein does not appear to dictate the eventual location of the PG. Alternatively, the expression of two differently sized serglycin mRNAs could result from the use of two distinct promoters, and may indicate that the serglycin gene is controlled by different tissue-specific regulatory sequences [18].

Invariant Chain

The class II antigens are major histocompatibility encoded molecules on the surfaces of lymphocytes, macrophages and monocytes. They are heterodimers composed of two transmembrane polymorphic glycoproteins, the α- and β-chains, and they play a central role in immune regulation by mediating the presentation of proteolytically processed foreign antigens at the cell surface. Class II molecules are often found in association with a third, nonpolymorphic protein called the invariant chain (Ii) [61]. The Ii can be substituted with a single chondroitin sulphate chain (Ii-CS) of variable length [17, 85, 116, 117], and the Ii-CS component appears to be tightly bound to the class II antigens since it can be coimmunoprecipitated with antibodies to the α- or β-subunits [115]. The function of the glycanated variant of Ii is inknown. Indeed, the function of Ii itself is not firmly resolved. The majority of Ii molecules are present as an intracellular pool of free protein monomers lacking condroitin sulphate substitution [81, 82, 116, 117]. However, the Ii-CS variant seems to be almost entirely bound to class II and the trimeric complex is principally located inside the cells, probably in a post-Golgi vesicle since chondroitin sulphate biosynthesis occurs exclusively in Golgi membranes. Ii may be essential for efficient transfer of class II to sites of intracellular processing of antigen [63]. The chondroitin sulphate chain may in some way facilitate this transfer and perhaps also prevent class II from associating with normal cellular proteins [111]. Class II dissociates from Ii-CS immediately before binding to antigen, the exchange apparently occurring in an acidic endosome (for further details see [65]).

Syndecan

Syndecan (Fig. 2) is a transmembrane PG first identified in cultured mouse mammary epithelial cells [101]. It is widely expressed in adult epithelia and in condensed embryonic mesenchyme and is believed to play a central role in binding epithelial cells to structural proteins of the interstitial matrix [118]. These binding properties are determined by the heparan sulphate chains. The core protein of syndecan (32 kDa) contains a short C-terminal cytoplasmic region, a hydrophobic segment and five potential glycanation sites in the N-terminal ectodomain [119]. An interesting feature of the syndecan structure is the hybrid char-

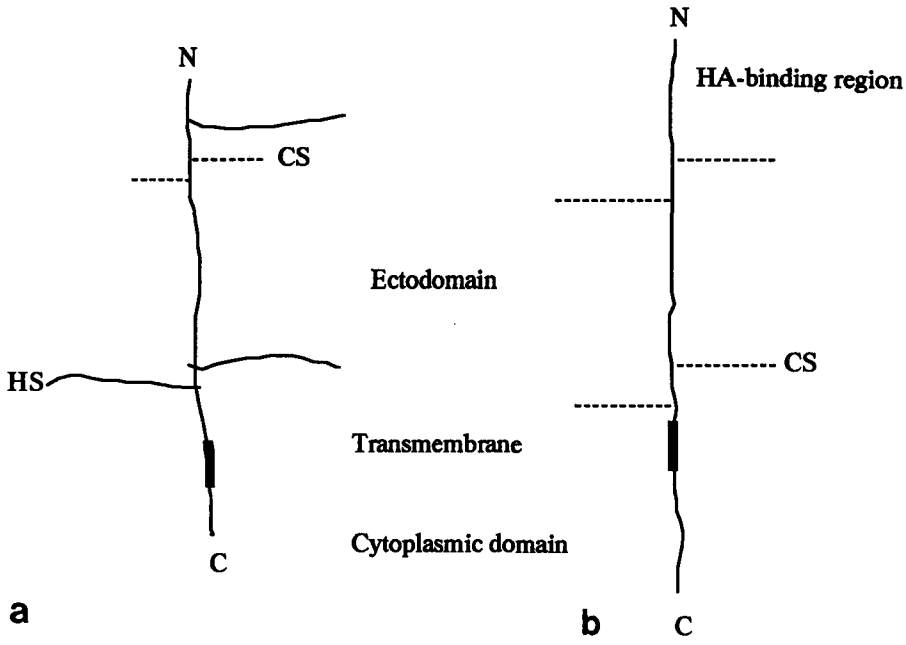

Fig. 2a, b. Transmembrane proteoglycans of the lymphocyte cell surface: **a** syndecan, **b** Hermes or CD44. Syndecan has a 32-kDa core protein substituted with heparan sulphate (*HS*) chains (*solid line*) on murine B lymphocytes. It is also expressed on epithelial cells as a hybrid proteoglycan containing both heparan sulphate and chondroitin sulphate (*CS*) chains. Hermes (CD_W44) is a lymphocyte homing receptor that participates in binding to molecular determinants (addressins) on high endothelial venules and to hyaluronic acid in the pericellular matrix. It may be synthesised as a glycoprotein with extensive N- and O-glycosylation or as proteoglycan in which the core protein (37 kDa) is further subsituted with glycosaminoglycan chains of the Cs type. Distinct functions are likely to be associated with the glycanated variant.
Syndecan and Hermes are transmembrane proteins, which hydrophobic peptide regions and relatively short cytoplasmic domains

acter of the glycanation profile, with both heparan sulphate and chondroitin sulphate chains present in the same protein. Heparan sulphate is the principal GAG but the number of GAG chains and the chain length can vary according to the tissue of origin [118]. Recently, syndecan was detected on the surfaces of murine B lymphocytes exclusively as a heparan sulphate PG [114]. Syndecan expression is closely linked to B cell differentiation. It is present on 40% of B cell precursors and on the majority of mature plasma cells. This pattern of expression fits well with the requirements for extracellular matrix interactions during B cell development.

Hermes – CD44

Hermes (or CD44) is a cell surface glycoprotein (85–95 kDa) involved in the recognition of high endothelial venules (HEV) by T and B lymphocytes [53, 54]. It is sometimes referred to as a homing receptor and appears to play a role in lymphocyte binding to peripheral, mucosal and synovial HEV [15]. Different determinants on the glycoprotein may be involved in specific molecular recognition. Hermes is also found on other haemopoietic cells and epithelial cells, including carcinomas [130]. Variations in glycosylation may influence the binding activity of the molecule. The glycoprotein is heavily N- and O-glycosylated and, interestingly, a variant of Hermes expressed on lymphocytes contains chondroitin sulphate chains [55] which increase the apparent molecular mass to 180–200 kDa (Fig. 2). There are four Ser-Gly dipeptides in the protein sequence that are potential glycanation sites. It is not known whether the gly-

canated variant of Hermes has a specific role to play in lymphocyte homing.

In common with the other lymphocyte surface PGs, Hermes is a transmembrane protein and the N-terminal region in the ectodomain is homologous with the hyaluronic acid binding region present in the major PG of cartilage [41, 130]. Evidence has recently been presented which indicates that Hermes is a major cell surface receptor for hyaluronic acid [5]. This property is unlikely to directly determine HEV homing activity but may be required for transendothelial migration and subsequent cell movement within the lymph node matrix.

Proteoglycans of the Lymphoid Lineage

Syndecan and the glycanated variants of Hermes and Ii were identified in lymphocytes using specific antibodies. The majority of studies of lymphocytes have utilised metabolic radiolabelling to detect PGs and the contribution of these defined components to the overall PG content of the cells in unknown. PGs are normally labelled in their polysaccharide chains using [^{3}H]glucosamine and [^{35}S]sulphate as biosynthetic precursors.

T Lymphocytes

Murine T lymphocytes synthesise both chondroitin and heparan sulphate in vitro [12, 21, 46, 107, 154] and synthesis is enhanced by activation with concanavalin A, phytohaemagglutinin, IL-1 or IL-2, independently of cell proliferation [12, 46].

The PGs of a number of murine T cell lymphoma cell lines have also been studied. The lines EL-4 and RDM-4 incorporated [^{35}S]sulphate into PGs of M_r 100–150 kDa which contain GAG chains of 10–20 kDa. The secretory products were exclusively PG, whereas cell extracts contained both intact PG and free GAG chains [106, 154]. However, in sharp contrast to the normal uncloned murine T cells, heparan sulphate has been found as the major GAG species in all cellular compartments in these T cell lymphomas cultured in vitro.

The murine T lymphoma cell line Eb and its highly metastatic variant Esb have been studied for several parameters which may play a role in determining their different metastatic potential. The Esb variant constitutively expresses high levels of a β-glucuronidase which can degrade heparan sulphate chains and may be important for cell migration across basement membranes in which heparan sulphates are important structural elements [11, 48, 153]. Esb cells synthesize no detectable heparan sulphate. However, both the synthesis and the secretion of chondroitin sulphate is enhanced when compared to the nonmetastasising counterpart [125]. Whether these proteoglycan differences have any direct bearing on metastatic properties is unclear and further work is needed to clarify this point.

Mitogen-stimulated normal human T cells expanded in vitro by phytohaemagglutinin and IL-2 produce chondroitin-4-sulphate PG which is rapidly secreted into the medium [24, 140]. The molecular masses of the PG and GAG chains have been estimated to be 130 kDa and 25 kDa, respectively. Whilst the core protein has not been characterised or sequenced, the T cell chondroitin sulphate PG is largely resistant to proteolysis, suggesting that the core may have a domain of repeating Ser-Gly residues characteristic of the serglycin type.

Of the intracellular chondroitin sulphate of human T cells, a significant proportion (40%–50%) was found as free GAG chains [140]. These chains were not normally secretory products – a feature which has been noted for human B cells (Ranson, unpublished observations), monocytes [63] and multipotential stem cells [86]. A limited study of human T cell PGs by Levitt and Ho [70] did not identify a specific intracellular pool of GAGs but noted the presence of small quantities of

heparan sulphate in both the secreted and cell-associated fractions.

Human T cell subsets cloned by limiting dilution and expanded in vitro by phytohaemagglutinin, IL-2 and irradiated B lymphoblastoid and peripheral blood mononuclear cells have also been analysed [139]. CD4-positive (T helper) cells were found to be more active in the secretion of PG and contained a lower proportion of intracellular free GAG chains than T8-positive (T suppressor) cells following in vitro labelling (reviewed in [65]). How these differences in metabolism relate to the distinct functions of these T cell subsets is unknown. Evidence has been presented to suggest that T cell PGs can stimulate proliferation of murine B cells [71, 72].

B Lymphocytes

A brief biochemical analysis of B cell PGs has been reported [70] with both human B-lymphoblastoid cell lines and peripheral blood B cells being found to synthesise chondroitin and heparan sulphate PGs which were constitutively secreted in vitro.

Recent work in our laboratory on neoplastic and normal human B cells cultured in vitro has shown the principal PG synthesised by these cells to be chondroitin sulphate. Heparan sulphate has also been identified, and comprised between 2% and 20% of the total radiolabelled GAG material. An analysis of centrocytic non-Hodgkin's lymphoma cells revealed the presence of both intact heparan sulphate PG ($\sim$ 28 kDa) and intact GAG chains ($\sim$ 8 kDa) in secreted material. Heparan sulphate PGs of varying sizes ($<$ 30 kDa to 150 kDa) have similarly been noted in murine T cells and T lymphoma cells lines [46, 105, 154].

The presence of heparan sulphate species in human B cells is of considerable interest since these molecules may have important roles in lymphocyte function. Mention has already been made of the involvement of heparan sulphate in the binding of several growth factors and in the control of cell adhesion. They could therefore be important in the regulation of lymphoma growth and disease dissemination. Further work to investigate both the structure and the function of lymphocyte heparan sulphate is clearly indicated. It will be of special interest to know whether the heparan sulphate is associated with syndecan, which has so far only been studied in murine B cells.

The major PGs of human B lymphocytes are chondroitin sulphates, and these are significant secretory products [70] (Ranson and Gallagher, unpublished). Our own work indicates that the majority of malignant phenotypes and tonsillar B cells appear to synthesise chondroitin sulphates in which a proportion of the disaccharides are disulphated (type E). In some human B cell lymphomas, distinct chondroitin sulphate species can be identified on the basis of differing chain length and sulphation. This contrasts with the homopolymeric condroitin-4-sulphate seen in normal human T cells [140], though whether this distinction is maintained for neoplastic T cell is unclear.

In common with a number of other studies of lymphocytes and monocytes, cell-associated radiolabelled material in neoplastic and normal human B lymphocytes consisted mainly of free chondroitin sulphate GAG chains that are usually retained by the cell. The function of this intracellular pool remains elusive.

Natural Killer Cells

Natural killer (NK) cells comprise a heterogeneous population of CD3-negative lymphoid cells that manifest cytotoxic activity against certain tumours and virally transformed cells. In addition, they are thought to play a role in haemopoietic differentiation and B lymphocyte function [49].

The secretory granules of these cells contain condroitin-4-sulphate together

with cytotoxic effectors such as pore-forming protein (perforin/cytolysin), protease and factors which resemble tumour necrosis factor and lymphotoxin [16, 56, 80, 121, 145, 156]. The PGs contribute to the efficient concentration of these effectors in the secretory granule and probably act to stabilise the proteolytic activities of the enzymes within the granules [146]. By analogy to mast cell heparin, the NK granule PGs may also alter the ability of these enzymes to cleave some substrates following exocytosis [40, 144]. It has been suggested that chondroitin sulphate may serve to protect the NK cells from cytolytic effectors such as perforin [24], perhaps by inhibiting perforin polymerisation.

Whilst NK cell PGs have been suggested as candidate mediators of cytotoxicity [156], work using β-xylosides, which produced a reduction in PG synthesis by 50%, showed this to have no significant effect on the cytotoxic activity of cultured NK cells and cytotoxic T cells [24]. Cytotoxicity assays using murine NK cells and a variety of target cells have also shown that exogenous chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate at concentrations of up to 1 mg/ml have no effect on NK cytotoxicity as assessed by in vitro models [155]. However, in this assay system, exogenous heparin at low concentrations was found to be inhibitory, and analyses of several heparin types suggested that there was a positive correlation between increasing negative charge density and inhibition of cytotoxicity. Perhaps heparin inhibits the activity of the cytotoxic mediators or prevents NK cell activation, since similar suggestions have been proposed in lymphocytes for mast cell-derived heparin [33, 123]. It is interesting to note that, in contrast to heparin's inhibitory effects on NK cells, it has been found to be inactive for cytotoxic T cell-mediated cell lysis, emphasising the fact that cytotoxicity can clearly be achieved by multiple mechanisms [156].

Granulocytes

The staining characteristics of the intracellular granules of mature granulocytes (or polymorphonuclear cells) enables their classification into three broad populations: neutrophils, basophils and eosinophils. Most of the studies on granulocyte PGs have been performed on peripheral blood neutrophils using biochemical, autoradiographic and histochemical methods. Early work demonstrated that the intracellular granules contained chondroitin sulphate in association with basic proteins [93–96]. Chondroitin-4-sulphate was subsequently demonstrated in primary granules (lysosomes), with smaller amounts in secondary lysosomal granules [98, 141]. Chondroitin sulphate is a major secretory component of human basophils [84]. More recent studies have also identified both heparan sulphate and dematan sulphate in cell-associated and released material [72, 90], indicating that greater diversity in PG synthesis may be possible than had been suggested by earlier studies.

The PGs synthesised in vitro by peripheral blood neutrophils are primarily retained within the cells, being released into the medium upon adhesion and when neutrophils are exposed to microorganisms that stimulate phagocytosis [47, 72, 142]. In comparison to normal mature neutrophils, immature malignant human myeloid cells exhibit a greater rate of chondroitin sulphate and hyaluronic acid biosynthesis [87, 92].

The HL-60 cell line, derived from a patient with promyelocytic leukaemia, readily differentiates in vitro to more mature granulocytic cells with agents such as retinoic acid and dimethylsulphoxide [25]. On the other hand, phorbol esters and heparan sulphate-containing fractions of bone marrow lead to the development of some phenotypic features usually associated with macrophages [77, 79, 113]. Irrespective of the differentiation-induction agent, HL-60 cells synthesise only chondroitin-4-sulphate,

though there is a notable reduction in the rate of synthesis upon differentiation [14, 77, 78]. HL-60 cells produce the serglycin type of PG [89, 137], and similar gene products are to be found in PGs from other polymorphonuclear cells, namely, the rat basophilic leukaemia-1 cell line [8] and human eosinophils [112].

Pulse-chase experiments with the mouse myeloid stem cell line FDCP-mix suggest that the synthesised chondroitin sulphate is metabolised as two distinct pools: a short half-life pool representing PG which is destined for secretion, and material destined for intracellular degradation to free chondroitin sulphate chains and some smaller fragments [86]. A similar secretion and degradation pathway has been elucidated for the human monocytoid cell line, M1 [76].

Mast Cells

Mast cells are distinctive in having intense metachromatic cytoplasmic granules (due to the attachment of highly sulphated GAGs to the serglycin protein), a capacity to synthesise and store histamine, and in having high affinity receptors for IgE (for reviews see [31, 134]). Distinct subpopulations of mast cell exist in vivo which differ both in their anatomical site and their biochemical features (Table 1). To date, two main populations have been clearly defined: mucosal mast cells and connective tissue mast cells. Mucosal mast cells can change their phenotype to connective tissue mast cells when placed in the appropriate environment, and vice versa [57, 88, 128].

Mucosal Mast Cells

Mucosal mast cells are not normally present in sufficient numbers in tissues to allow the isolation and characterisation of their proteoglycans, but the use of helminth-infected rats has enabled the analysis of mucosal mast cells following in vivo labelling with [^{35}S]sulphate [32, 133]. The isolated ^{35}S-labelled macromolecules contain highly sulphated chondroitin sulphate chains enriched in type E and type B disaccharides [68, 133]. Mucosal mast cells derived from human colon also synthesise chondroitin sulphate E rather than heparin [30].

In mice, a similar mucosal mast cell population can be derived from murine bone marrow (so called "bone marrow-derived mast cells") [102, 104]. In isolation these mast cells preferentially synthesise chondroitin sulphate E [103, 136], but in coculture with fibroblasts they become histochemically and morphologically similar to connective tissue mast cells [27]. Analogous phenotypic changes

Table 1. Differences between rat mucosal and connective tissue mast cells

Property	Mucosal mast cell	Connective tissue mast cell
Histochemistry	Safranin negative	Safranin positive
Gramules	Few, small	Many, large
Life span	Short ($t_{1/2}$ 40 days)	Long ($t_{1/2}$ > 6 months)
T cell factor dependence	Yes	No
Proteoglycans	Chondroitin-4-sulphate Chondroitin-4,6-disulphate (type E) Chondroitin sulphate (type B)	Heparin
Histamine content	Low	High
Serine protease	Protease II	Protease I

can be induced by injecting bone marrow-derived mast cells into the peritoneal cavity of mast cell-deficient W/W^v mice [97].

Connective Tissue Mast Cells

The secretory granules which contain PG are a prominent morphological feature of connective tissue mast cells. In rodents these cells synthesise heparin proteoglycans of 750–900 kDa containing 10–12 GAG chains of 70–80 kDa [109, 157].

Although [^{35}S]sulphate is incorporated into heparin by connective tissue mast cells, small quantities of *unlabelled* chondroitin sulphate E can also be detected [58]. This may represent residual chondroitin sulphate synthesised during an earlier (mucosal mast cell-like) stage of development. However, it is clear that connective tissue mast cells retain a latent capacity for chondroitin sulphate E synthesis since they produce this GAG in the presence of β-xylosides [134, 135].

Although the mediators produced and released by mast cells have been well characterised, the precise biological functions of mast cells remain unclear in many instances. However, roles for mast cells in immediate hypersensitivity reactions and in host defence against parasitic infections have been well established [83, 122]. The highly acidic nature of the PG components of the secretory granule probably help to maintain charge neutrality with histamine and basic proteins and may facilitate the osmotic swelling of the secretory granule prior to it fusing with the plasma membrane [124]. PGs may continue to exert regulatory influences following mast cell degranulation. PG-protease complexes have been detected on the mast cell surface, and this will limit the diffusion of discharged enzymes and regulate their activities [4, 123, 126, 144].

Additional interactions of mast cell-derived heparin with complement components may occur, heparin having been found to inhibit the activity of late complement components in vitro [146] and to inhibit complement-mediated inflammation and cell lysis in experimental models [28, 29]. The proteolytic activity of the secretory granule enzyme chymase which degrades the complement component C3a is also promoted by heparin [40]. Since mast cell heparin is a potent anticoagulant, a role in regulating local coagulation in areas of inflammation has been proposed [75].

The common occurrence of mast cells at sites of tumour neovascularisation and on the mitogenic effects of mast cell heparin on some cultured endothelial and fibroblast cell lines has led to suggestions that mast cell heparin is involved in tumour angiogenesis [59, 110]. The release of a heparan sulphate-degrading endoglycosidase during mast cell degranulation may also facilitate the release of heparan sulphate-bound bFGF, an angiogenic factor, from the extracellular matrix [13].

Platelets

Chondroitin sulphate PGs have been reported primarily in platelet α-granules with small amounts on the cell surface [10, 132]. The α-granules contain several bioactive molecules including serotonin, fibrinogen, platelet factor 4, ADP, thrombospondin and platelet derived growth factor. Whilst complexes between platelet proteoglycan and platelet factor 4 have been investigated [10, 50, 69], and suggestions made that the PG enables an efficient packaging of platelet factor 4 in the α-granule, it is likely that the chondroitin sulphate interacts with more than one component of the α-granule [120].

The core protein sequence has been reported for human platelet PG [2, 99], and the N-terminal sequence is highly homologous to serglycin. Interestingly, a proposed site for proteolytic scission of the serglycin protein was shown to have been cleaved in a proportion of the platelet PG extract [99]. This raises the possibility that processing of the platelet core protein may occur inside the α-granule or

following its release from the cell. Such a modification may conceivably alter the functional properties of the PG and further studies are required to assess whether this processing step has biological importance.

The chondroitin sulphate PG isolated from serum is very similar to the PG isolated from platelets [91] and it has been mooted that platelet-derived chondroitin sulphate PG may function as a natural inhibitor of the complement factor C1q [3, 91, 127]. Plasma membrane chondroitin-4-sulphate on human platelets has also been demonstrated to effectively shield distinct platelet activation receptors and to participate in membrane calcium flux [131, 132]. The protein core of the membrane-associated PG has not been identified.

Monocytes and Macrophages

Monocytes and macrophages form part of the phagocytic system and are important for antigen processing and presentation, enzyme secretion and tumour cell lysis. The cells are both highly secretory as well as motile [1]. The extensive secretory repertoire of these cells includes enzymes, complement components, clotting factors, growth factors and PGs [63]. Freshly isolated human peripheral blood monocytes synthesise and secrete chondroitin-4-sulphate PGs. After 5 days of culture on plastic the cells develop macrophage-like morphology and begin to secrete more highly sulphated chondroitin sulphates which contain about 20% of the E-type disaccharides [66]. End-differentiated macrophages derived from human peritoneal fluid also secrete these chondroitin sulphate E-containing proteoglycans in vitro [62]. The synthesis of this species of chondroitin sulphate appears closely coupled to monocyte/macrophage differentiation [64, 66, 150]. Plastic surfaces and phorbol esters induce this switch in chain sulphation, whilst interferon-γ and lipopolysaccharide are ineffective [151], implying that the nature of the differentiation signal may be important in the modulation of PG sulphation. Monocyte differentiation is inhibited by culture on fibronectin substrata and under these conditions the cells fail to synthesise type E disaccharides [67].

The major fate of synthesised PGs in monocytes cultured in vitro is secretion via a constitutive pathway, but the functions of the secreted materials remain to be elucidated. It is also unknown whether this pathway is active in vivo and whether it can be modulated.

Similarly, the role of cell surface PGs in monocyte function has not been extensively investigated although the presence of cell surface receptors for GAGs and other sulphated polysaccharides has been suggested [23]. Levels of cell surface GAGs and hyaluronic acid are increased upon macrophage adhesion or phagocytosis [22, 45, 60], and since these cells display adhesive interactions with other cells and with matrix components, cell surface PGs may be important elements in monocyte and macrophage function.

Summary

The most prevalent and best characterised PGs in the haemopoietic system are the serglycins. They are found in immunosecretory cells and bind to molecules such as serine proteases, histamine, platelet factor 4, and perforin to facilitate concentrated packaging of these effectors in storage vesicles. There is also evidence to suggest that the serglycins have roles in regulating the activities of these effectors following their release from the cell [6, 7, 126, 138, 146].

The biochemical properties of PGs suggests that they are suited to roles in regulating cell adhesion and cell migration. A range of anionic polysaccharides including GAGs are capable of modulating lymphocyte migration in vivo and lymphocyte adhesion in vitro [19, 20, 26]. In lymphoid malignancy, studies in murine lymphoma cells have shown that

the potential of the cells for PG secretion correlates with their metastatic potential [125].

Cell surface PGs in haemopoietic cells such as syndecan and the glycanated variant of CD-44 have recently been demonstrated and further examples will certainly follow. It is likely that this field of research will lead to a clearer understanding of the roles of PGs in haemopoietic cell development. The potential of PGs to bind, and present in active form, several haemopoietic growth factors has already been documented [43, 108], and additional examples of PGs sequestering fibroblast growth factor, transforming growth factor-β and neuronal growth factors have been cited [34].

PG-mediated cell adhesion may also be a crucial requirement for progenitor cell growth [38, 42, 44]. Whilst much attention has focused on heparan sulphates, recent work on membrane-associated chondroitin sulphates suggests that these polysaccharides may be important for the binding of haemopoietic progenitors to stromal cells [143] and for haemopoietic cell proliferation in bone marrow [129]. Although some of the adhesive interactions are likely to be relatively nonspecific, they may have some therapeutic potential in, for example, assisting haemopoietic recovery from chemotherapy, inhibiting tumour dissemination, or facilitating stem cell engraftment following bone marrow transplantation. Research into the biochemistry of haemopoietic PGs and GAGs should not only provide new understanding of basic science, but hopefully provide novel therapeutic approaches for the treatment of human disease states.

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Modification of the In Vitro Replication of the Human Immunodeficiency Virus HIV-1 by TPSg, a Polysaccharide Fraction Isolated from the Cupressaceae *Thuja occidentalis* L. (Arborvitae)*, **

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Introduction

The acquired immune deficiency syndrome (AIDS) is caused by an infection with the human immunodeficiency virus (HIV-1) [2–8]. CD4-positive lymphocytes were shown to be one major target in HIV-1 infections [9–10]. Apart of $CD4^+$ cell depletion, the functional impairment of the T-cell system also plays an important role in the progress of this disease [11, 12, 13].

Two distinct approaches to controlling HIV-1 infections have been explored so far, specifically, inhibition of the reverse transcriptase and inhibition of HIV-1 replication.

For the first approach, inhibition of the virus replication, 3′-azido-3′-deoxythymidine (AZT) [15] and its nucleoside analogues [16, 17], suramin and its derivatives [18], phosphonoformic acid [19], and antimoniotungstate [20] have been used. Inhibition of virus replication was demonstrated on the other hand using interferon-α [21, 22], AL721 [23], D-penicillamine [24], amphotericin analogues [25], dextrane sulfate [36], chondroitine sulfate [36, 42], Avarone [27], Avarol [27], and synthetic oligonucleotides [26].

The need to obtain an effective principle for the treatment of AIDS prompted the search for selective and nontoxic anti-HIV-1 agents even in medicinal plants. Some extracts with anti-HIV-1 properties have been isolated from medicinal plants of Chinese folk remedies [46], for instance, *Altherantera philoxeroides* [44], *Viola yedoensis* [45], and the chemically partially defined prunellin isolated from *Prunella vulgaris* [43]. Most of these extracts and partially purified substances have shown in vitro anti-HIV-1 properties accompanied by some cytotoxic activities [43–46]. Lai et al. have reported a dose-dependent modification of the viral replication of HIV-1-infected CR10, CEM, and U937 cells by two defined extracts (PC6 and PC7) from the Japanese white pine (*Pinus parvifloria* Sieb. et Zucc.) [49]. In previous studies, extracts from *Thuja occidentalis* L. (Arborvitae), another plant in the cedar/pine family, were shown to be in vitro inhibitors of plant pathogenic viruses and human herpes simplex viruses (HSV-1 strain) [34, 35].

In the present paper we are dealing with a new substance, the g fraction of

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

thujapolysaccharides (TPSg), and its ability to modify HIV-1 replication in both human MT-2 and MT-4 cells as measured by determination of reverse transcriptase (RT) activity, cell growth (both MT-4 cell system), and the expression of HIV-1-specific proteins by indirect immunofluorescence (MT-2 cell system).

Materials and Methods

Virus and Cell Lines

The HIV-1 strain $HTLV_{IIIb}$ used for the MT-2 experiments was obtained from culture supernatants of virus-producing H9 cells, as previously described [4].

MT-2 cells were maintained in RPMI 1640 (Gibco, Eggenstein, FRG) containing 15% fetal calf serum. MT-2 is a HTLV-1-preinfected human T-cell leukemia line and has been shown to be highly susceptible to infections with HIV-1 [28, 47].

MT-2 cells have been used as target cell lines for in vitro HIV-1 infection experiments using indirect immunofluorescence assays [39].

H9 cells used as the HIV-1 source for the MT-2 experiments were also maintained in RPMI 1640 (Gibco, Eggenstein, FRG) containing 15% fetal calf serum. This cell line was a kind gift from M. Popovic (NCI, Bethesda, Maryland, USA).

MT-4 cells were kept in Click-RPMI medium (Biochrom, Berlin, FRG) containing 10% (v/v) complement-inactivated fetal bovine serum (Seromed, Berlin, FRG) and antibiotics.

MT-4 cells are highly susceptible to in vitro HIV-1 infections [39], too. For the in vitro infection experiments with MT-4 cells, the $HTLV_{IIIb}$ strain of HIV-1 was used. HIV-1 has been generated on Jurkat cells as described in detail elsewhere [41]. Jurkat cells were also grown in RPMI 1640 medium (Gibco, Karlsruhe, FRG) with the supplements described above.

Virus Titration

For virus titration on MT-2 cells, cell-free supernatants were harvested from HIV-1-infected H9 cells. The virus titration was performed by indirect immunofluorescence. The quantitative determination of the infectious capability of the HIV-1 stocks was performed according to the method described by Kaerber et al. [31]. The HIV-1 preparations for the MT-2 experiments were shown to have a titer of 1×10^7 $TCID_{50}$/ml. In the MT-4 system, a final infectious activity of 100 $TCID_{50}$ for each well was used.

Indirect Immunofluorescence

For immunofluorescence experiments, both freshly HIV-1-infected and noninfected MT-2 cells were used and incubated for 12 days at 37 °C. For preparing the cell smears, HIV-1-infected and noninfected MT-2 cells were contrifuged for 10 min at 250 g. The supernatants were removed and the sediments resuspended in phosphate-buffered saline (PBS). Cell smears were performed on 10-well multitest slides (Flow Lab., Meckenheim, FRG). The slides are air dried and fixed for 10 min in acetone at −20 °C. A standardized HIV-1-positive human serum was used as reagent.

Cell smears of HIV-1-infected and noninfected MT-2 cells were incubated for 60 min in a moist chamber at 37 °C with titrated serum of an AIDS patient (25 µl/well; dilution 1:20) [29, 30]. HIV-1-positive cells were visualized after incubation with FITC-conjugated goat anti-human immunoglobulin G (AHS Deutschland, Bereich Merz and Dade, Munich, FRG) for 30 min (25 µl/well; dilution 1:200).

As negative controls, sera of noninfected human individuals were used. The specific reaction was determined by fluorescence microscopical evaluation.

Determination of RT activity

Uninfected MT-4 cells or MT-4 cells infected with HIV-1 were treated with

various concentrations of TPSg and incubated for 5 days under standard conditions. For the RT inhibition assay, HIV-1 was harvested from infected Jurkat cells by centrifugation. The virus was then suspended in PBS at pH 7.2 and mixed with the same amount of ultrapure glycerol (Serva, Heidelberg, FRG). Different final concentrations of TPSg were examined in 50 m*M* Tris-HCl pH 7.8, 5 m*M* dithiothreitol (DTT), 25 m*M* Mg^{2+}, 30 m*M* KCl, 6% Triton X-100, 1 µg polyrC:oligodG, 9 µ*M* dGTP, 1 µCi [^{32}P]dGTP and lysed HIV-1. The RT assay was performed according to the procedure described previously [40, 42].

The influence of TPSg on virus production in infected MT-4 cells was monitored by RT activity in culture supernatants. The virus was prepared from the supernatants by centrifugation as described above and the RT assay was performed as shown.

[^{3}H]Thymidine Incorporation

[^{3}H]Thymidine incorporation experiments were performed according to standard procedures to measure HIV-1-specific cytopathic effects on MT-4 cells [39]. The MT-4 assay was performed in 96-well microtiter plates as described previously [42]. 3×10^4 MT-4 cells/well were incubated with TPSg at 625 µg/ml, 62.5 µg/ml, 6.25 µg/ml and 625 ng/ml final concentrations, with or without HIV-1.

The concentration of the infectious particles used was 100 $TCID_{50}$ for each well. Fresh Click-RPMI medium was added to each well 3 days after set-up. 5 days after infection, 0.1 µCi [^{3}H]thymidine (Amersham-Buchler, Brunswick, FRG; specific activity 185 GBq/mmol) was added to the cultures. The cells were harvested 20 h later on glass fiber filters (Whatman GFC, UK) using a Scatron cell harvester and dried. After addition of scintillation cocktail (PPO, POPOP, and toluene; Roth, Karlsruhe FRG) filters were counted in a β-liquid scintillation counter.

The results were expressed as the arithmetic mean in counts per minutes of triplicate determinations.

As an alternative to the determination of the cellular DNA synthesis in the MT-4 assay, the cell growth was measured on day 5 after infection. Cell viability was assayed microscopically in a hematocytometer by trypan blue exclusion experiments and the RT activity was measured in the supernatant of the cultures.

Preparation of TPSg

Thujapolysaccharides, g-fraction (TPSg), from the Cupressaceae *Thuja occidentalis* L. (Arborvitae) was prepared as described in detail elsewhere (EPO 315182). TPSg was stored up to use lyophilized at −20 °C. TPSg was reconstituted in the appropriate cell culture media and was sterilized directly before use using 0.2 µm filter systems (Sartorius, FRG).

Results

The anti-HIV-1 activity and cytotoxicity of the polysaccharide fraction TPSg was examined in MT-2 and MT-4 cell culture systems. The ability of TPSg to inhibit the HIV-1-specific RT was also examined. Finally, the 50% inhibitory concentration (IC_{50}) of TPSg on MT-4 cells was determined.

Protection of HIV-1-Dependent Cytopathic Effects by TPSg

TPSg inhibited HIV-1-dependent cell death at final concentrations of 625 µg/ml (Fig. 1). At this concentration TPSg was shown to be completely nontoxic for MT-4 cells, which had not been infected with HIV-1 (Figs. 1, 2).

This result was confirmed by comparing the cell growth of TPSg-treated infected and noninfected MT-4 cells (Fig. 2). These experiments were performed in triplicate and repeated three times.

Inhibition of HIV-1 Expression by TPSg

HIV-1-specific viral antigen expression was measured by indirect immuno-

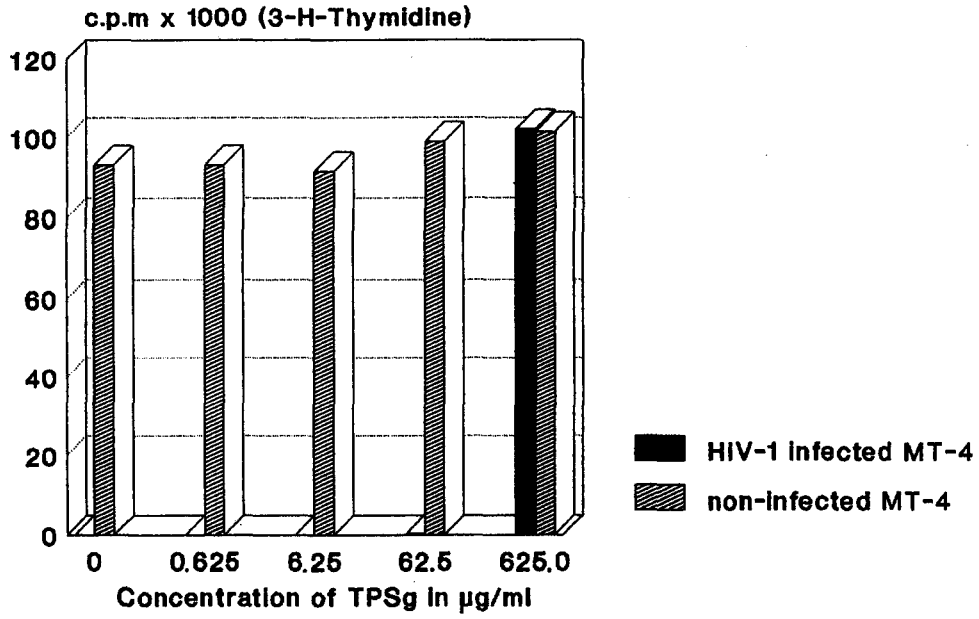

Fig. 1. Anti-HIV-1 activity of TPSg in the MT-4 cell assay. The anti-HIV-1 activity of various concentrations of TPSg is expressed as the [^{3}H]thymidine incorporation into HIV-1-infected and noninfected MT-4 cells (median of three experiments). The cells were treated with final concentrations of 625 ng/ml to 625 µg/ml

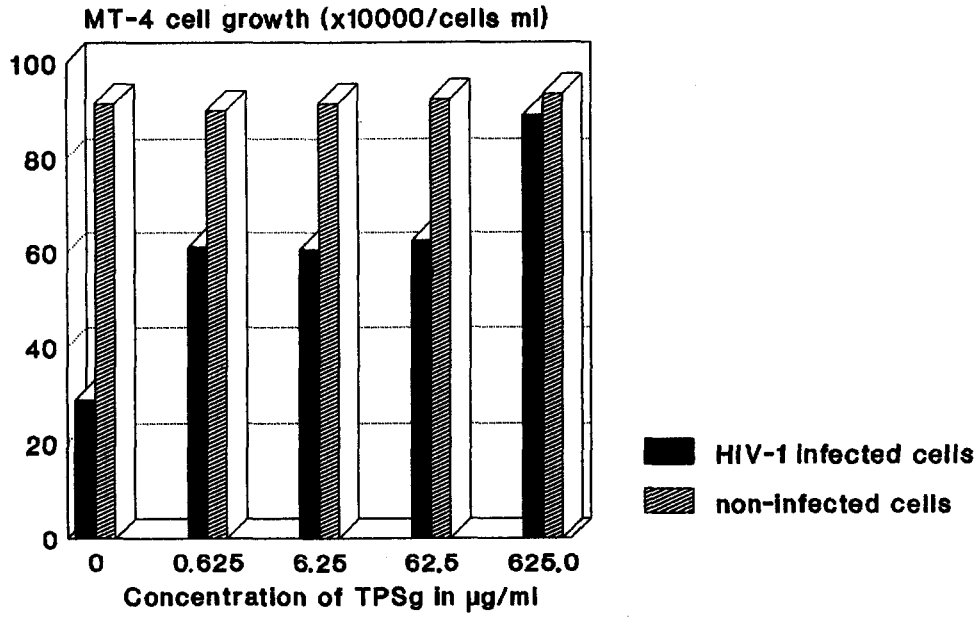

Fig. 2. Effect of TPSg on growth of MT-4 cells. The numbers of noninfected and HIV-1-infected cells were examined (media of three experiments). The cells were treated with TPSg at final concentrations of 625 ng/ml to 625 µg/ml

fluorescence. The inhibitory effect of TPSg was tested on freshly HIV-1-infected MT-2 cells.

TPSg was shown to inhibit HIV-1-specific antigen expression on freshly infected MT-2 cells in a dose-dependent manner (Figs. 3, 4). TPSg did not alter viral antigen expression at a concentration of 0.625 µg/ml (99.6% ± 0.5%). A significant reduction in HIV-1 antigens measured by immunofluorescence was observed at a concentration of 6.25 µg/ml 69.8% ± 10.8% of HIV-1 infected MT-2 cells expressed HIV-1-specific antigens).

Only 0.4% of all HIV-1-infected MT-2 cells counted (200 cells/slide) were shown

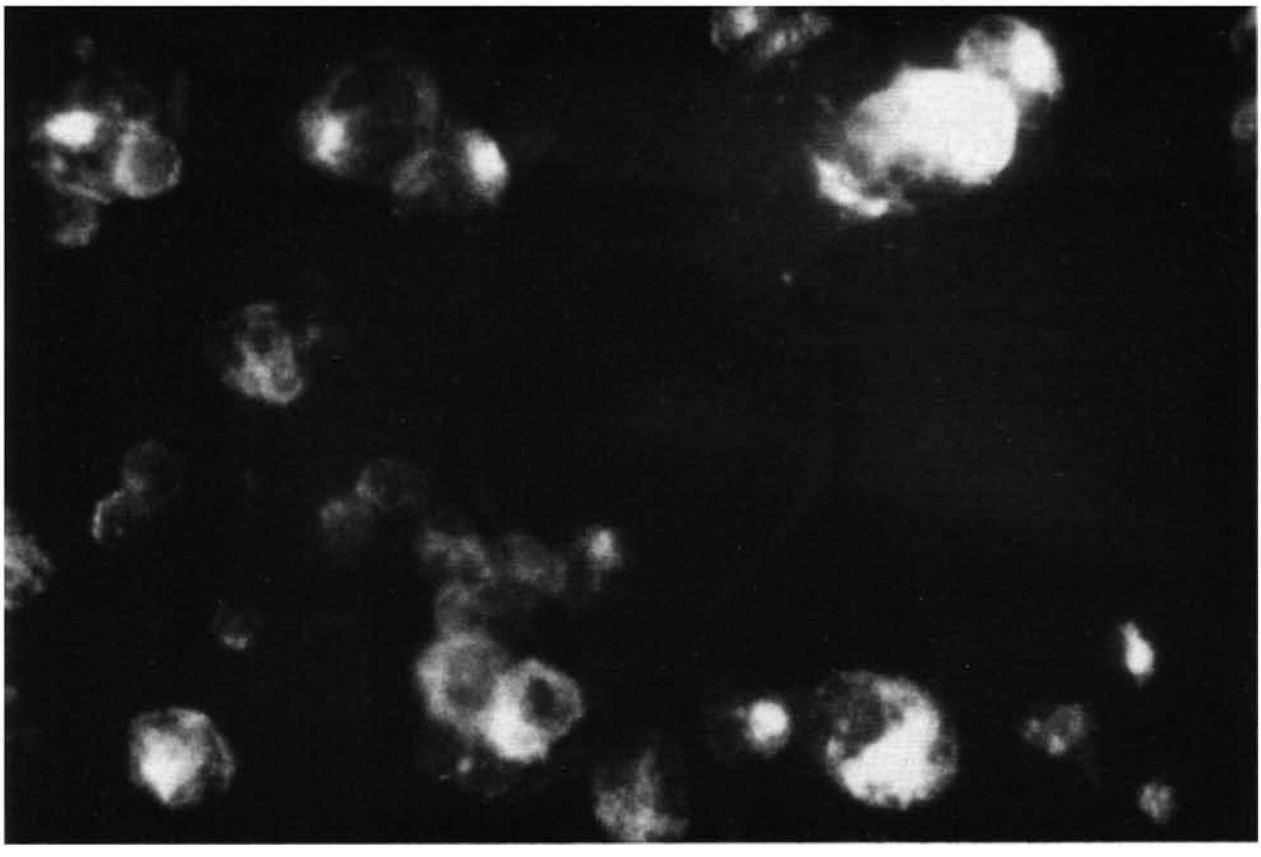

Fig. 3. Indirect immunofluorescence of freshly HIV-1-infected MT-2 cells. The cells were prepared as described in "Material and Methods." They were labelled with an anti-serum against HIV-1 and FITC-conjugated goat anti-human IgG. This micrograph shows the non-TPSg-treated freshly HIV-1-infected MT-2 cells after 5 days of incubation. × 500

to express HIV-1-specific antigens at final concentrations of 62.5 μg/ml, and an inhibition of 99.94% ± 0.08% of HIV-1 expression was measured at the final TPSg concentrations of 625 μg/ml.

Inhibition of RT Activity by TPSg

As an additional approach, HIV-1 replication was determined by measuring RT activity in the supernatants of HIV-1-infected MT-4 cells 5 days after infection. In uninfected MT-4 cells, no RT activity was detected in the culture medium after an incubation period of 5 days. In contrast to HIV-1-infected MT-4 cells not treated with TPSg, no RT-dependent dGMP incorporation was found in supernatants of infected MT-4 cells treated with final concentrations of TPSg of up to 62.5 μg/ml (Fig. 5).

In addition, the inhibition of RT activity was measured with disrupted HIV-1. TPSg was found to be active against the enzyme (Fig. 6) with a IC_{50} of 300 μg/ml.

Discussion

Several authors have reported antiretroviral activities of plant extracts, for example, extracts of *Prunella vulgaris* [43], *Alternanthera philoxeroides* [44], *Viola yedoensis* [45], *Gerardia savaglia* [49] and some Chinese medicinal herbs [46, 50, 51]. Lai et al. have reported a dose-dependent modification of the viral replication of HIV-1-infected CR10, CEM, and U937 cells by two defined extracts (PC6 and PC7) of the Japanese white pine (*Pinus parvifloria* Sieb. et Zucc.), a plant of the pine family [49]. Previously, extracts of *Thuja occidentalis* L., another plant belonging to the cedar/pine family, were shown to inhibit the cytolytic activity of herpes simplex virus

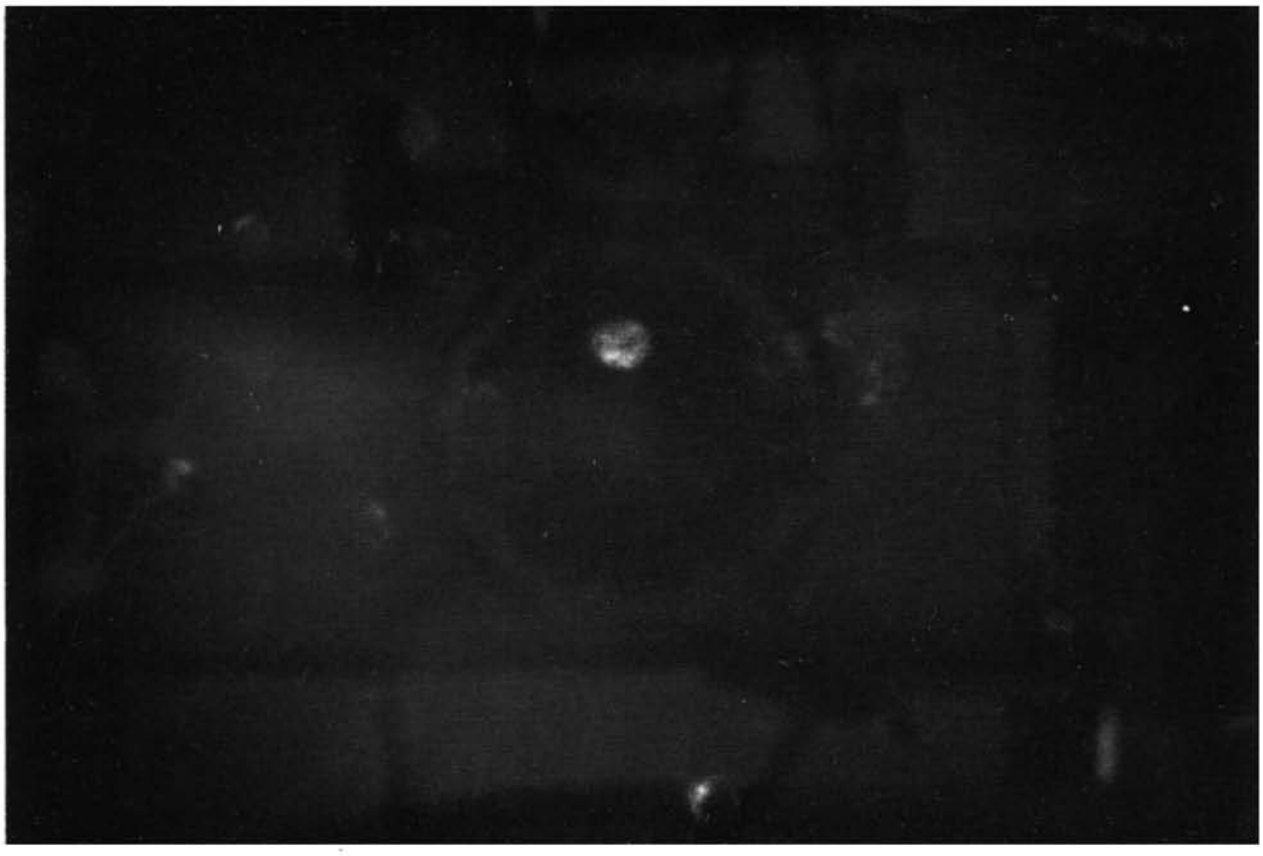

Fig. 4. Indirect immunofluorescence of freshly HIV-1-infected MT-2 cells treated with TPSg. The cells were prepared as described in "Material and Methods." They were labelled with an antiserum against HIV-1 and FITC-conjugated goat anti-human IgG. This micrograph shows freshly HIV-1-infected MT-2 cells treated with TPSg 625 µg/ml after 5 days of incubation. × 500

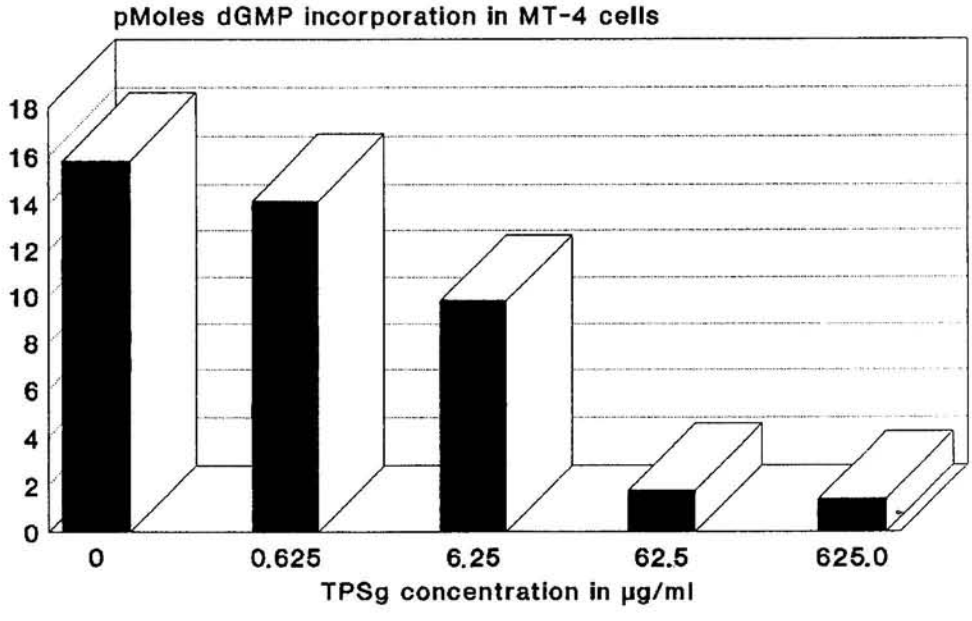

Fig. 5. RT activity in the supernatant of HIV-1-infected MT-4 cells treated with different concentrations of TPSg (median of three experiments). The cells were treated with final concentrations of TPSg of 625 ng/ml to 625 µg/ml. The RT activity is expressed in picomoles dGMP incorporated into DNA

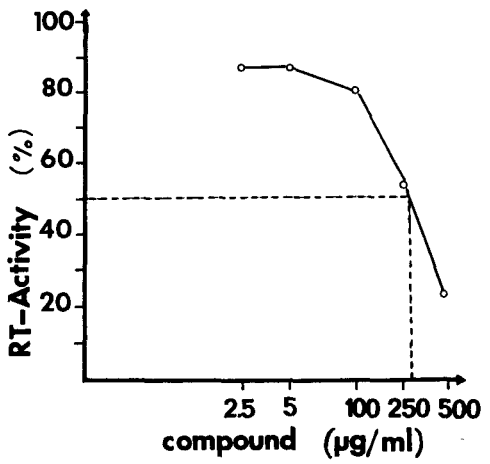

Fig. 6. Inhibition of the RT activity of an HIV-1 lysate expressed in %. The 50% inhibitory dose of TPSg (ID_{50}) is extrapolated from the curve

type 1 and some plant pathogenic viruses in vitro [34, 35].

TPSg, a high molecular weigth polysaccharide fraction isolated from *Thuja occidentalis*, was shown to be a compound with "immunomodulatory" properties. This compound was demonstrated to induce the proliferation of T-cells (CD^{4+}) of the human peripheral blood [1, 37, 48]. Furthermore, TPSg was shown to induce a different pattern of cytokines such as interleukin-1, interleukin-2, and interferon-γ [32].

In the BALB/c system, TPSg was found to cause a modification in terms of upregulation of natural killer cell activity against YAC-1 target cells [43].

These findings indicated possible antiviral properties of this compound. Hence, in this preliminary study, we have evaluated the antiretroviral potential of this compound.

TPSg was found to inhibit the HIV-1-dependent cell death of HIV-1-infected MT-4 cells at concentrations of 625 µg/ml. Additionally, it was shown to block the expression of HIV-1-specific proteins in freshly HIV-1-infected MT-2 cells in a dose-dependent manner, as judged by a 99.94% (99.6%) inhibition of the HIV-1-mediated specific immunofluorescences at a final concentrations of 625 µg/ml (62.5 µg/ml).

TPSg completely blocks HIV-1 release into the culture supernatant at concentrations up to 62.5 µg/ml, as demonstrated by the lack of RT activity in the supernatants of HIV-1-infected MT-4 cells. Furthermore, TPSg blocks the RT of disrupted virus particles with an IC_{50} of 300 µg/ml.

In the present paper, TPSg was demonstrated to be a compound with an inhibitory effect on both HIV-1 entry and HIV-1 absorption in both MT-2 and MT-4 cells. Even at high concentrations, it was shown to be nontoxic for MT-4 (Fig. 2) and MT-2 (data not shown) cells. Furthermore, it was demonstrated to be nontoxic for primary human leukocyte cultures (PBL), even at high concentrations [33].

In comparison with most of the plant extracts described above, TPSg therefore shows promising antiviral and immunomodulating properties.

Since TPSg is only a partially purified natural product, isolation of the active principle(s) is required. This work is in progress. First hints in this direction were given by Hans et al. [38], who described the monosaccharide composition of sprouts and wood of the Arborvitae.

Future investigations concerning this compound must rule out the possibility of its inducing autoimmune diseases and must show a lack of toxicity in vivo and mutagenicity in vitro. The present study might be a hint to further and more detailed investigations of the anti-HIV-1 properties of this compound. Whether TPSg might be of use in the therapy of primary and secondary immune deficiencies must be elucidated in further and more detailed investigations.

The present study exemplifies the necessity of synergy of pharmacognostic research with molecular biology, clinical research, and immunology, to obtain new substances with significant immunomodulatory and antiviral properties.

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Molecular Genetics

Molecular Cloning and Expression of CD34: A Haemopoietic Progenitor-Associated Cell Surface Glycoprotein

M. F. Greaves, J. Brown, L. Fina, D. Robertson, D. Delia, and H. V. Molgaard [1]

Introduction

The CD34 group of monoclonal antibodies recognize a 105–120 kDa cell surface glycoprotein which is selectively expressed by human myeloid and lymphoid progenitor cells probably including the haemopoietic stem cell [1–3] (for a recent review see [4]). CD34 antibodies therefore offer a prospect of using highly enriched human stem cells for analysis of their regulation [5–7], gene transfection and/or transplantation [8]. The CD34 antigen is also expressed on vascular endothelial cells [9].

The restricted pattern of expression of CD34 in haemopoiesis suggests that it may have a significant function in the earliest stages of blood cell differentiation in the bone marrow. However, neither biochemical characterization of the antigen nor functional studies using monoclonal antibodies have so far identified its function. The protein sequence of the antigen has been deduced from a complementary deoxyribonucleic acid (cDNA) clone (D. Simmons and B. Seed, personal communication) and is in agreement with N-terminal amino acid sequence derived from the purified protein [10]. The sequence predicts that the CD34 antigen is a type 1 transmembrane protein with a protein backbone molecular weight of 39 kDa and with no close homology to any other protein in the data bases. The protein is extensively glycosylated with both N- and O-linked carbohydrate, and the epitopes recognized by most of the monoclonal antibodies are dependent on the presence of the carbohydrate including sialic acid residues [10, 11]. The gene encoding CD34 has been mapped to the long arm (q) of human chromosome 1 [12, 13].

We report here characterization of the CD34 gene and its pattern of expression in murine and human cells.

Cloning of Murine CD34

We have shown previously that human CD34 cDNA hybridizes under stringent conditions to DNA from three different rodent species [12], indicating some degree of conservation of the gene. This has allowed the isolation of a murine CD34 cDNA and genomic coding regions containing the complete coding sequence. The strategy used for cloning murine CD34 is illustrated in Fig. 1 and details are given in Brown et al. [14].

The murine gene is organized in eight exons in 22 kb of DNA. The first exon lies in a GC and CpG rich island. The sequence of the gene and the cDNA predict a 382 amino acid long protein containing a N-terminal signal peptide and one transmembrane region 73 amino acids from the carboxyl terminus. The extracellular part of the protein contains a 140 amino acid long N-terminal region 40% of whose residues are serine or threonine – potential attachment sites for O-linked carbohydrate as well as five potential attachment sites for N-linked carbohy-

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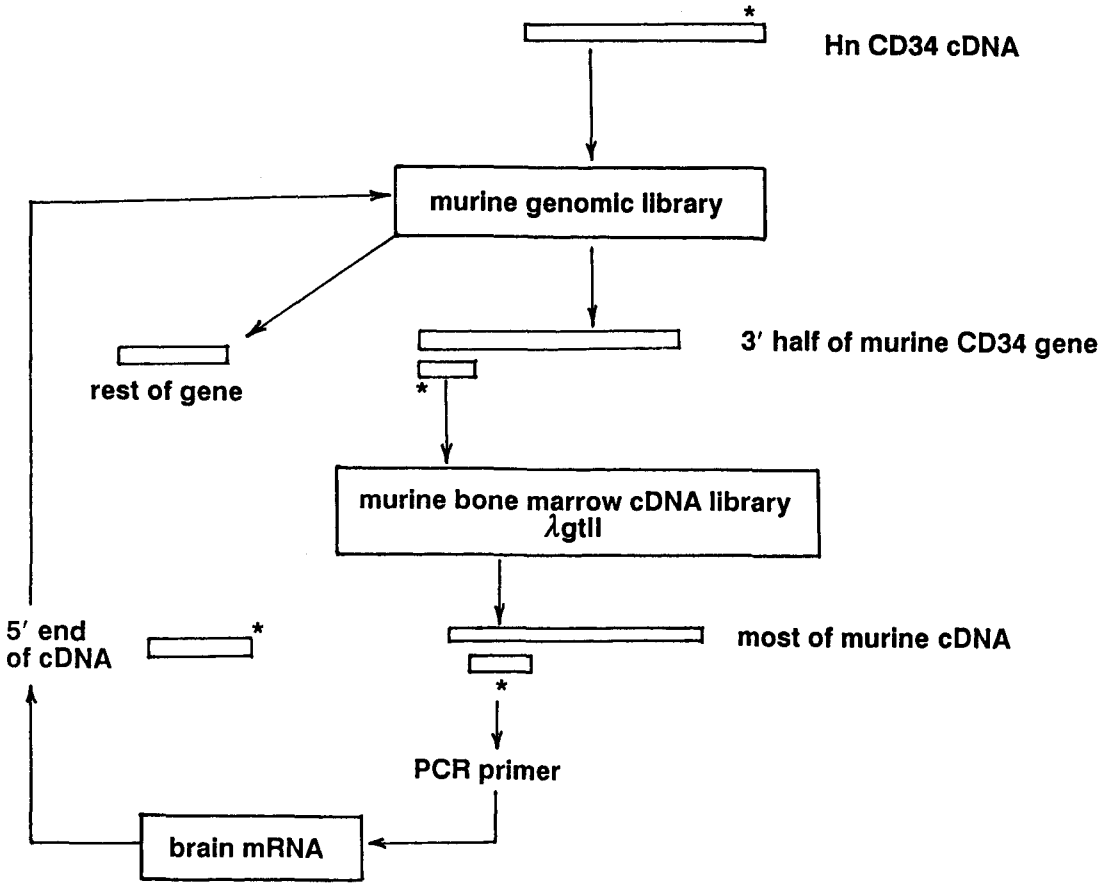

Fig. 1. Strategy used to isolate murine genomic and cDNA clones. The mouse gene and cDNA homologous to human CD34 were isolated by a combination of genomic DNA and cDNA library screening and the PCR. Initially a murine genomic DNA library was screened at low stringency with the human CD34 cDNA. This allowed the isolation of the 3′ half of the murine gene. The most 5′ coding region of this murine genomic DNA was used to screen a murine bone marrow cDNA library and lead to the isolation of cDNA clones which encoded most of the coding region of the murine CD34. The coding region at the 5′ end of the cDNA was isolated by the PCR technique using a primer complementary to a sequence near the 5′ end of the cDNA and a primer complementary to a polyA tail added to the first strand cDNA. The murine cDNA clones were then used to complete the isolation of the murine gene

drate. Proximal to the extracellular membrane, there is a 79 amino acid long cysteine rich region. The homology with the human sequence is highest in the intracellular domain (90% amino acid identity) and lowest in the N-terminal region (43% amino acid identity) (Fig. 2). The protein is not homologous with any other proteins currently in the data bases.

Expression of CD34

Since CD34 antibodies may recognize carbohydrate-dependent epitopes [4, 10], it is not clear whether the pattern of CD34 antigen expression in haemopoietic cells reflects activity of the CD34 gene itself or whether alterations in glycosylation determine the expression and availability of antigenic epitopes. The protein product encoded by the CD34 gene might, therefore, be more widespread in its expression than anticipated from antibody studies. A comparison of CD34 mRNA and antigen expression of a panel of different haemopoietic cell types (Table 1) indicates that there is, in fact, a good correlation between presence of mRNA and accessible epitopes, confirming therefore that the CD34 gene

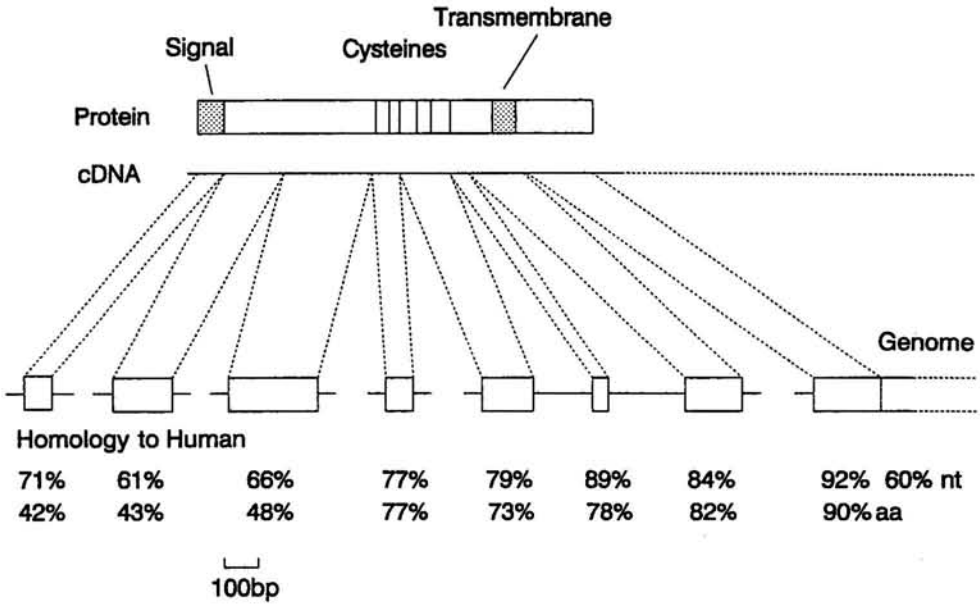

Fig. 2. Schematic diagram of murine CD34 protein, cDNA and exon organization. DNA and protein sequence homology with the human CD34 sequence (D. Simmons and B. Seed, personal communication) for each murine exon is shown in relation to a schematic diagram of the murine protein. The isolated and sequenced cDNA is represented by a *solid line*. The *broken line* represents the 3′ end of the cDNA deduced from the genomic sequence. (From [14])

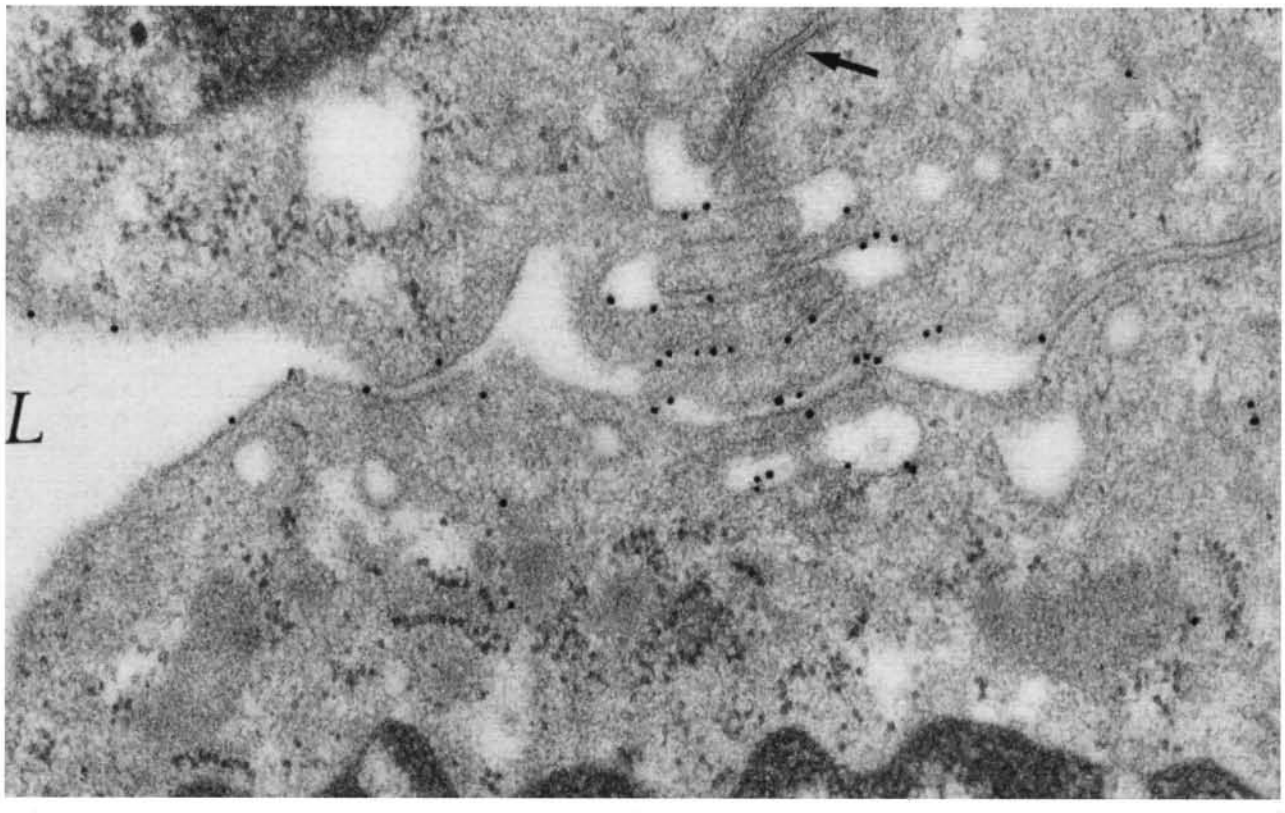

Fig. 3. Ultrastructural localization of CD34 antigen. Electron micrographs of endothelial cells. The sections have been reacted with a CD34 monoclonal antibody (QBEND-10) localized with colloidal gold markers. The figure shows a region of opposing membranes of two endothelial cells from human umbilical artery. Some colloidal gold is present on the lumenal membrane (*L*) of the cells, but the majority of the gold markers are located on the lateral membranes of the cells, where they form complex interdigitations. Colloidal gold is not present on the membrane in areas of cell junctions, such as tight junctions (*arrow*). Original magnification, ×77 500. (From [9])

Table 1. Co-expression of CD34 mRNA and antigen in human heamopoietic cell lines

Cell type	Line[a]	Antigen[b] (%)	mRNA
Multi-lineage progenitors	KG1	>90	+++
	KG1a/b[c]	>90	+++
	K562	–	–
Myeloid precursors	HL-60	–	–
	EM1/EM2	–	NT
	U-937	–	–
B precursors	REH	–	–
	NALM-6	–	–
	RS4:11	–	–
	TOM-1	~10–15	+
	KM3	–	–
	P3	–	–
	NALM-1	–	–
Mature B	B85	–	–
T precursors	JM	~10–15	+
	CEM	–	–
	PEER	–	–
	MOLT13	~10–15	NT
	T-ALL (FE)	~10–15	NT

[a] All derived from leukaemic cells except B85, which is an EBV transformed normal B cell.
[b] % cells stained by immunofluorescence labelling with monoclonal CD34 antibodies and flow cytometry assessment, or stained by alkaline phosphatase labelled antibodies and assessed by light microscopy.
[c] Sub-lines of KG1 co-expressing some early lymphoid markers [17] (S. Watt and M. F. Greaves, unpublished observations).
NT: not tested.

itself, at least at the level of stable mRNA, has a highly restricted pattern of expression in early haemopoiesis. Expression of the CD34 gene is not, however, limited to haemopoietic cells.

All seven of a set of CD34 monoclonal antibodies also recognized human vascular endothelium (details in [9]). Capillaries of most tissues were CD34 positive, as were umbilical artery and, to a lesser extent, vein, but not the endothelium of most large vessels and the endothelium of placental sinuses. Angioblastoma cells and parafollicular mesenchymal cells in fetal skin were also CD34 positive, as were some stromal elements. An ~ 110 kDa protein can be identified by western blot analysis with CD34 antibodies in detergent extracts of freshly isolated umbilical vessel endothelial cells, and CD34 mRNA is present in cultured umbilical vein cells as well as other tissues rich in vascular endothelium (breast, placenta) [9]. These data indicate that the binding of CD34 antibodies to vascular endothelium is to the CD34 gene product, and not to cross-reactive epitopes. Electron microscopy of umbilical artery, breast, and kidney capillary vessels revealed that in all three sites CD34 molecules are concentrated on membrane processes, many of which interdigitate between adjacent endothelial cells. However, well-established endothelial cell contacts with tight junctions are CD34 negative [9].

Although no antibodies to murine CD34 are as yet available, the availability of murine CD34 cDNA allowed us to investigate the pattern of gene expression

in mouse cell types and tissues in comparison with the data available from studies on human cells (details in ref [14]).

The results of probing northern blots of mouse tissue mRNAs show that the murine homologue of the CD34 gene is expressed as a 2.5–2.7 kb mRNA, identical in mobility to the human mRNA [9], both in haemopoietic tissues (liver, spleen, bone marrow, and thymus) and in two non-haemopoietic tissues (brain and testis). Comparison of the intensities of bands suggests that CD34 is expressed at about a 100-fold lower level than actin in the mouse tissues analyzed, except in brain where the level of CD34 mRNA is only about tenfold lower than actin and where a minor higher molecular weight (3.7 kb) RNA is also seen. The presence of a variety of cell types in these tissues prevents identification of the actual cell lineages expressing CD34. An analysis of the expression of CD34 in haemopoietic cell lines revealed a selective pattern of expression similar to that in human cells. Among the haemopoietic cell lines tested so far, CD34 expression was detected only in progenitor cells lines [15]: A4, Clone 17, and 416B. Two other progenitor cell lines, Clone 15 and LyD9 did not have detectable levels of CD34. CD34 mRNA could also not be detected in the B lineage precursor cell line 18–8 and was not detectably expressed in cell lines with a more mature phenotype, including EL4 (a T cell), WEHI 3B (myelomonocytic cell), WEHI 274 (monocyte/macrophage) and NS1 (plasma cell/myeloma). Of the non-haemopoietic cell lines tested, CD34 expression was not detected in C3H 10T1/2 an embryonic cell line, but very high levels of CD34, approximately equal to actin, were detected in three other embryonic fibroblast lines: NIH 3T3, Swiss 3T3 and Swiss 3T6 but not in a fibroblast line derived from lung tissue (line CMT 64/61).

The high level expression of CD34 in certain murine fibroblasts accords with recent ultra-thin section electron microscopic evidence for CD34 antibody binding to stromal fibroblasts in vivo; established cell lines of human fibroblasts are, however, CD34 negative (M. F. Greaves, D. Robertson and S. Pegram, unpublished observations).

Conclusions

These studies have indicated that the CD34 gene and protein are so far unique with no strong homologies to other cloned genes. The gene itself is relatively highly conserved, though asymmetrically, between murine and human DNA, and although its expression may be restricted to stem cells and progenitor cells in haemopoiesis, it is expressed in other cell types, including vascular endothelial cells and certain fibroblasts. This then implies a function that is not restricted to haemopoiesis, and a clue as to what this might be comes from the ultra-structural studies revealing that CD34 protein may be concentrated on the surface of interdigitating membranes of adherent vascular endothelial cells. Additional studies have shown that CD34 molecules are localised on abluminal endothelial processes occurring at the tips of vascular sprouts during angiogenesis [16]. These observations clearly imply a role for CD34 in cell-cell and/or cell-matrix interaction. This possibility is now being pursued by attempting to isolate a ligand from cells or stromal matrices that will bind to purified CD34 molecules.

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Molecular Genetics of the Human GM-CSF Receptor

N. M. Gough [1]

Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a glycoprotein of 23000 daltons which stimulates the proliferation, differentiation and functional activation of granulocytes, macrophages and eosinophils. Molecular clones encoding murine and human GM-CSF have been isolated and recombinant protein tested in vivo in various animal models. GM-CSF is also in advanced clinical trials to assess its efficacy as an agent for enhancing haemopoietic recovery and function in immunocompromised patients (for review, see [10]).

The biological activities of GM-CSF are transduced via specific cell-surface receptors. In both murine and human systems, autoradiographic analyses have indicated that GM-CSF receptors are present in low numbers (a few hundred per cell) on cells within the granulocyte/macrophage series. In addition, GM-CSF receptor have also been detected in various non-haemopoietic cells, including endothelial cells, small cell lung carcinoma cell lines, simian COS cells and placental cells (see [8], and references therein). Receptors of both high ($K_D \sim 30\,pM$) and low ($K_D \sim 1-3\,nM$) affinity have been detected [3, 4, 7, 18, 19, 24]. Chemical cross-linking studies have revealed multiple molecular species that can be cross-linked to radiolabelled GM-CSF [3, 4, 18, 24]. In murine systems, molecules of 180000, 130000, 70000 and 51000 daltons have been described, while in the human system, the reported molecular weights are 135000, 100000 and 80000. In some cases the higher molecular weight species have been correlated with high-affinity binding and the lower molecular weigth species with low-affinity binding [3].

We have recently cloned a complementary deoxyribonucleic acid (cDNA) encoding a human GM-CSF receptor with low binding affinity [8], and our recent biological, biochemical and molecular genetic studies on this receptor cDNA are summarized in this report.

Results and Discussion

Cloning of a Low-Affinity Human GM-CSF Receptor

A human GM-CSF receptor cDNA was cloned [8] by a direct expression strategy using a cDNA library prepared from placental RNA in the COS cell expressing vector πH3M (obtained from Dr. Brian Seed, Massachusetts General Hospital). The procedure we adopted utilized the extraordinary sensitivity and specificity of microscopic cell autoradiography to identify pools of cDNA that contained clones which could transfer the capacity to bind human GM-CSF to COS cells. Two independent cDNA clones, with identical coding regions, were isolated from approximately 5×10^6 clones screened (in 500 pools). The protein encoded by these cDNAs comprised an N-terminal hydrophobic leader sequence of

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

22 amino acids, an extracellular domain of 297 amino acids, a single transmembrane segment of 27 non-polar residues and a short intracellular domain of 54 amino acids. The arrangement of four of the 11 cysteine residues in the extracellular domain as well as several other stretches of conserved amino acid sequence, notably the sequence motif Trp-Ser-X-Trp-Ser, identify the GM-CSF receptor as a member of a newly defined class of receptors [8], currently including those for growth hormone, prolactin, interleukins-2 (β chain), 3, 4, 6, 7, erythropoietin and granulocyte CSF. This cloned receptor does not bear any sequence homology with the protein tyrosine kinase family of receptors.

When introduced into COS cells, the cloned GM-CSF receptor cDNA directed the expression of large numbers (up to a million per cell) of receptors with the same low binding affinity ($K_D = 6$ nM) as found on placental tissue, and displaying a molecular weigth of about 85000 daltons [8].

Signal Transduction via the Low-Affinity Receptor

In order to determine whether the cloned low-affinity receptor could transduce a proliferative signal in haemopoietic cells, the receptor cDNA was introduced into murine FDC-P1 cells via a retroviral vector [15]. FDC-P1 cells are absolutely dependent upon murine GM-CSF or IL-3 for proliferation in vitro and express high-affinity murine GM-CSF receptors. FDC-P1 cells do not proliferate in response to human GM-CSF, which does not bind to murine receptors, even at very high concentrations.

After introduction of the human GM-CSF receptor, FDC-P1 cells displayed the same single class of low-affinity binding as seen on placental membranes and COS cells, and were able to proliferate in response to human GM-CSF [15]. The dose-response curve for human GM-CSF was, however, shifted to a 500 times higher dose than for murine GM-CSF, presumably reflecting the lower binding affinity of the human receptor.

Thus it was concluded that the cloned low-affinity receptor is able to deliver a proliferative signal in murine haemopoietic cells, and therefore, although there is no cross-species reactivity of murine and human GM-CSF, the human receptor is capable of interacting with the distal elements of the murine mitotic signalling pathway. Whether this receptor subunit is capable of transmitting a signal to induce differentiation remains to be determined.

Cross-Species Divergence of the GM-CSF Receptor Gene

The human GM-CSF receptor is encoded by a unique gene extending over about 50 kb of DNA (N. M. Gough, in preparation). In order to ascertain whether the human GM-CSF receptor cDNA could detect a murine homologue, Southern blots of murine and human genomic DNA were probed with various hGM-R cDNA probes at a range of stringencies. For example, in Fig. 1 at a stringency of 60 °C in 6 × SSC or above, no murine homologue was detected, and below this stringency (e.g. 55° C 6 × SSC) the level of background hybridization was too high for any cross-hybridization to be evident. In no experiments where hybridization conditions around this region were evaluated was any cross-hybridization to a murine homologue detected. A similar situation pertains for the ligand GM-CSF, in which no cross-hybridization between murine and human genes is perceptible on genomic Southerns (Fig. 2). These sequences display some 50% nucleotide sequence homology and cloned cDNAs clearly can cross-hybridize, albeit inefficiently, at reduced stringency (Fig. 3). Assuming that the rate of divergence of a hormone is restrained by the rate of divergence of its receptor, it would be likely that the murine and human GM-CSF receptors

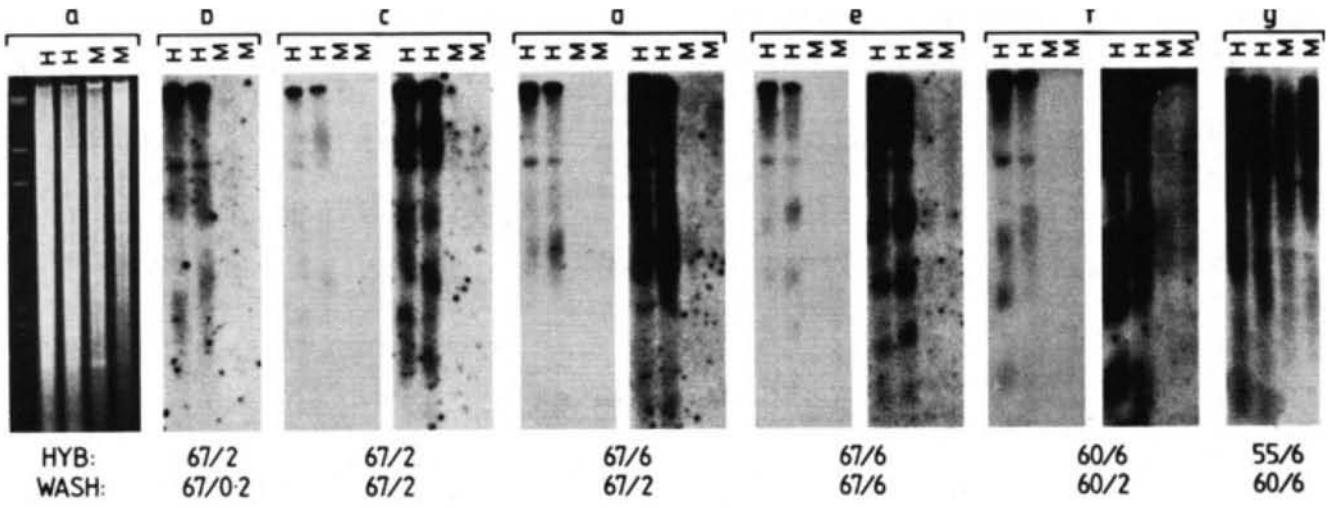

Fig. 1. Hybridization of human GM-CSF receptor cDNA to murine and human genomic DNA. DNA from peripheral blood of two normal human individuals (*H*) or two murine tissues (*M*) was digested with *Eco*RI and electrophoresed on 0.8% agarose gels. After transfer to nitrocellulose the DNA was hybridized with a probe corresponding to the region of the GM-CSF receptor cDNA clone pGMR138 located 5′ of the *Eco*RI site [8], under a variety of conditions of hybridization and washing (temperature and concentration of SSC given below the autoradiographs). General conditions for hybridization are described in [8]

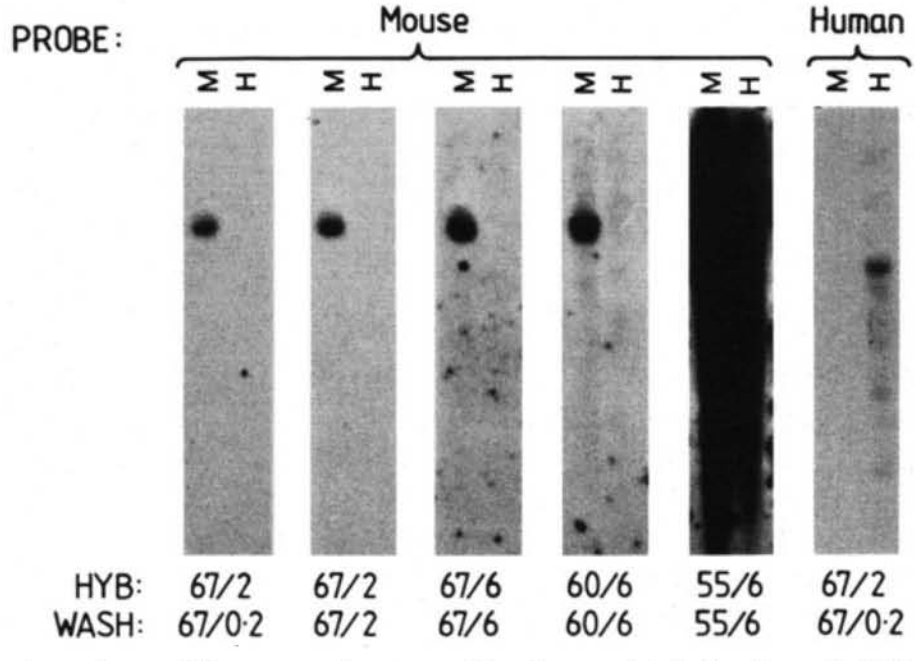

Fig. 2. Hybridization of murine and human GM-CSF cDNAs to murine and human genomic DNA. Normal human peripheral blood DNA (*H*) or murine DNA (*M*) was digested with *Hin*dIII and electrophoresed on 0.8% agarose gels. After transfer to nitrocellulose the DNA was hybridized with a murine (*left hand panels*) or human (*right hand panel*) GM-CSF cDNA probe, under a variety of conditions of hybridization and washing (temperature and concentration of SSC given below the autoradiographs). General conditions for hybridization are described in [8]

would have a similar degree of homology as for GM-CSF (approximately 50%) and would therefore not be detectable on genomic Southerns. Portions of the receptor which interact with the downstream signalling pathway have however presumably been conserved, since this human receptor is able to transduce a proliferative signal in murine haemopoietic cells (above).

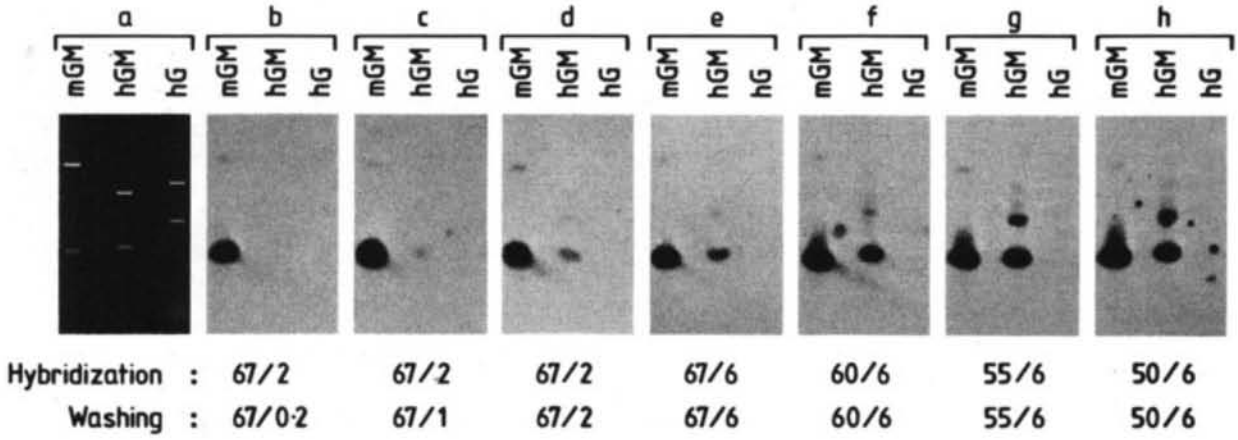

Fig. 3. Cross-hybridization of murine and human GM-CSF cDNAs. Plasmid DNA corresponding to a murine GM-CSF (*mGM*), human GM-CSF (*hGM*) and human G-CSF (*hG*) cDNA clones were digested to liberate the cDNA insert and electrophoresed on 1% agarose gels. After transfer to nitrocellulose the DNA was hybridized with a murine GM-CSF cDNA probe under a variety of conditions of hybridization and washing (temperature and concentration of SSC given below the autoradiographs). General conditions for hybridization are described in [8]

Pseudoautosomal Localization of the GM-CSF Receptor Gene

The human GM-CSF receptor gene was localized to the tip of the X and to the short arm of the Y chromosome by analysis of a panel of mouse-human somatic cell hybrids, RFLP analysis and in situ hybridization [11]. Localization of the GM-R gene to the tip of the sex chromosomes was consistent with it being within the pseudoautosomal region (PAR) [5]. This was formally proven by demonstration of exchange of alleles of the gene between the X and Y chromosomes during male meiosis. In three three-generation families studied, three exchanges of the GM-R gene between the sex chromosomes were found amongst 14 informative offspring, formally proving that GM-R locus is within the PAR. These data allowed a tentative location of the GM-R gene within the PAR to be deduced (see Fig. 4). The frequency of recombination of the GM-CSF receptor locus (20%) is clearly higher than for the MIC2 locus (2.5%), which has been mapped close to the PAR boundary, suggesting that the GM-R gene maps distal to this locus. The recombination frequency of the GM-R gene is similar to the frequencies observed for several anonymous pseudoautosomal loci, and the GM-R gene can be tentatively mapped between the loci DXYS15 and DXYS17 (Fig. 4). This localization is subject to more refined physical mapping studies, by pulsed field gel electrophoresis for example.

It has been long hypothesized that mammalian sex chromosomes have sequences in common which pair during male meiosis and allow exchange between the X and Y chromosomes (for review, see [5]). The existence of such a pseudoautosomal region was more recently directly demonstrated, and maps of the human PAR (a region of about 2500 kb) determined [5]. There is a great deal of interest in the nature of genes within this region and several candidates have been proposed, including genes associated with schizophrenia, cerebral dominance and Turner and Klinefelter syndromes [5]. MIC2, which maps just within the PAR, encodes a cell surface molecule implicated in T cell adhesion processes. The human GM-R gene is the first example of a gene of known function to be mapped to this region.

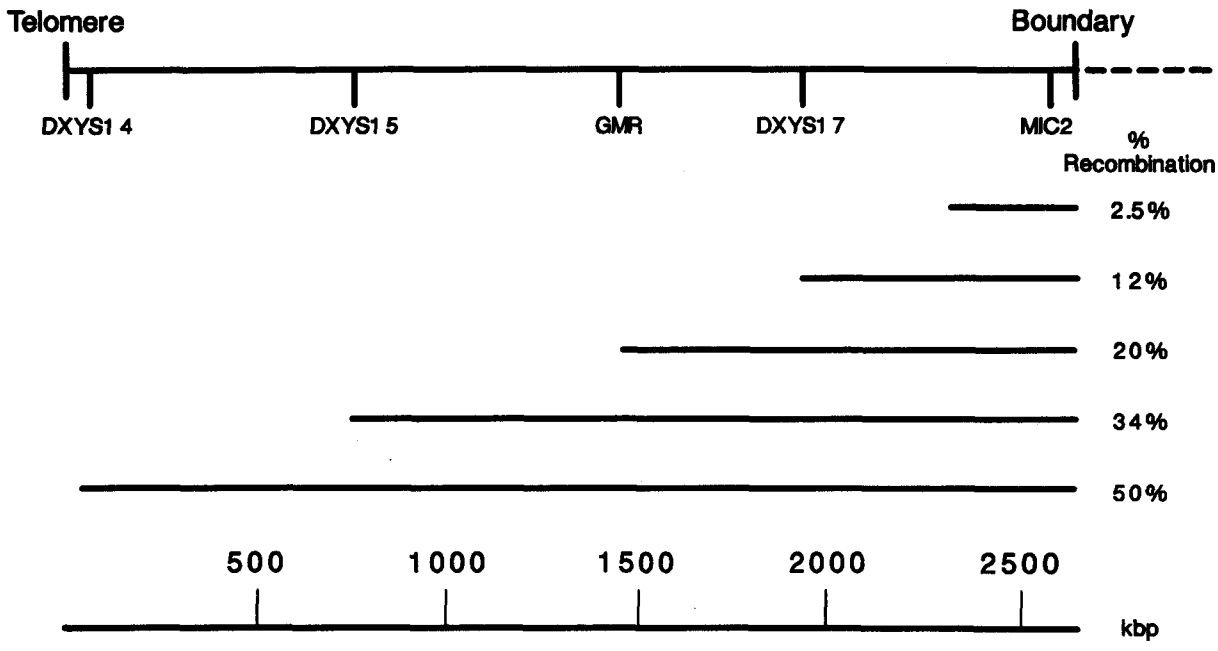

Fig. 4. Genetic map of the human pseudoautosomal region and localization of the human GM-CSF receptor gene. Recombination frequencies with respect to sexual phenotype are given on the *right*. Physical distance is given at the *bottom* in kb. (Data from [5])

Does the GM-CSF Receptor Gene Function as a Recessive Oncogene?

Loss of one or other of the sex chromosomes is frequent in certain acute myloid leukaemias, raising the possibility that loss of the GM-R gene may be involved in leukemogenesis. Loss of either the X or the Y chromosome is evident in 25% of acute myoblastic leukemias (AMLs) of the M2 subtype, compared with only 1%–6% in other AML subtypes [16] (Fig. 5a). Note that the X chromosome is lost from leukaemic cells of female patients with a very similar frequency to loss of the Y chromosome from leukaemias of males (Fig. 5b). The actual loss of the PAR (and hence the GM-R locus) may be significantly higher than 25%, since minor terminal chromosome deletions, which would be missed by standard karyotyping, have been described [20]. With the exception of chromosome 7, no other chromosome is lost to any significant extent in M2 AMLs [16]. It is important to note that loss of either the X or the Y chromosome occurs in 60%–65% of the subset of M2 AMLs characterized by the 8:21 chromosomal translocation (Table 1). Consistent chromosome loss is suggestive of the involvement of a "recessive oncogene" [21] in the genesis of M2 AML. Assuming that a single recessive oncogene is involved, then it is likely to be within the pseudoautosomal region, since if it were localized within a portion of the X chromosome not shared with Y (the majority of X) then similar loss of Y would not be predicted, and vice versa. That both chromosomes are involved implicates a gene shared between them, and hence most likely with the PAR. Thus, as has been described for the *Rb* gene implicated in the genesis of familial retinoblastoma [21, 25], it might be envisaged that gross cytological deletion of one allele of the putative oncogene would be associated with inactivation of the other allele. Although the PAR undoubtedly contains many genes that could be implicated, the GM-CSF receptor gene has the appropriate characteristics to be involved in the genesis of M2 AMLs. Loss or inactivation of both copies of this gene in a myeloid progenitor would generate a clone of cells unable to respond to GM-CSF, and hence possibly with a relatively

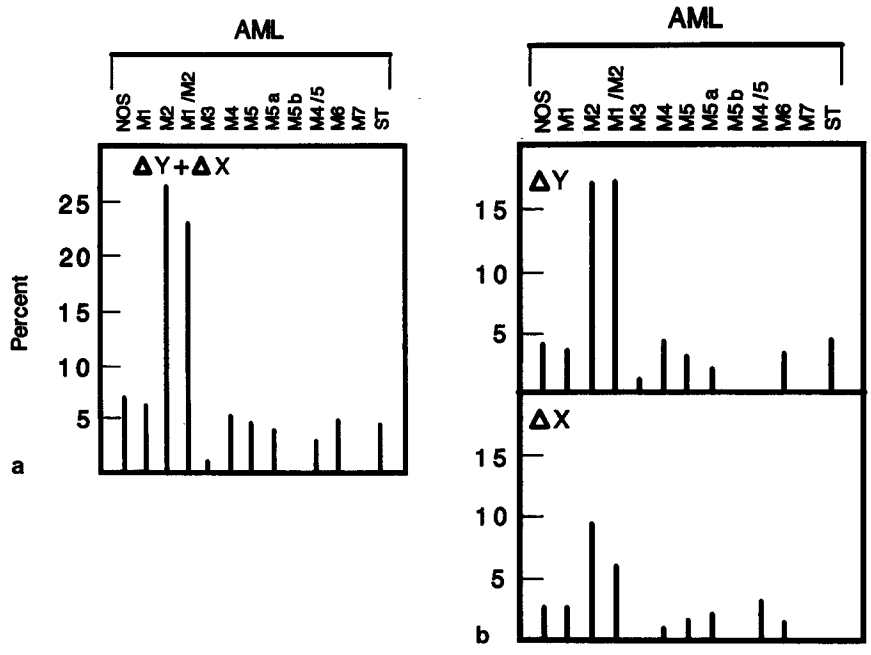

Fig. 5a, b. Loss of sex chromosomes in AML. The percentage of cases within each subtype of AML in which loss of either the X or the Y chromosome has been reported is plotted. The data for loss of either X or Y is compiled. The data are presented separately. (Data from [16])

undifferentiated phenotype, similar to that displayed by M2 AMLs. It is noteworthy that although most AMLs are responsive to GM-CSF, a significant subfraction are not [1, 2, 6, 17, 22, 23]. However, further studies are required to compare in more detail AML subtype, karyotype and GM-CSF receptor status. A second manner in which the GM-CSF receptor gene could function as an oncogene is in a semi-dominant fashion, analogous to the p 53 oncogene [13]. Thus an activating mutation on one allele may only be able to manifest itself in the absence of a wild type receptor, which might frequently be achieved by gross karyotypic deletion.

Table 1. Correlation of sex chromosome loss with t(8; 21) in M2 AML

Karyotype	Number of cases	
	M2 ($n=543$)	M1/M2 ($n=209$)
-X or -Y	25	14
-X or -Y/t(8; 21)	133	33
t(8; 21)	83	19

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Structural Organization of the Cytokine Gene Cluster on Human Chromosome 5

E. I. Frolova, D. V. Smirnov, G. M. Dolganov, I. A. Mazo, N. S. Bystrov, and I. V. Severtsova [1]

Introduction

Cytokines make up a family of glycoprotein growth factors that have been shown to support clonal proliferation of hematopoietic progenitor cells [1]. Interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (CSF-2) stimulate differentiation and proliferation of cell progenitors along multiple (myeloid and erythroid) pathways [2–4]. Interleukin-4 (IL-4) can affect B cell, T cell, mast cell and macrophage functions [5]. Interleukin-5 (IL-5) is a more specific cytokine, acting on later stages of eosinophil differentiation [6].

The human genes for CSF-2, IL-3, IL-4, and IL-5 have been cloned, sequenced [3, 7–9], and mapped by in situ hybridization to human chromosome 5 at bands q23–31 [10–15], a region that is frequently deleted in patients with myeloid disorders [del(5q)] and acute myeloid leukemia (AML) [16, 17]. A close genomic linkage of human IL-3 and CSF-2 genes was reported [18, 19]. The distance between the genes was found to be only 10.5 kilobases (kb). The physical linkage of the IL-4 and IL-5 genes to within 240–310 kb was also demonstrated by long-range mapping, using pulse-field gel electrophoresis (PFGE) [15, 20].

Close linkage between the IL-3 and CSF-2 genes and between IL-4 and IL-5 genes, together with the similar gene structure, regulation, and biological activities of the four genes [21], suggests that they may have been derived from a common ancestral gene(s) and might be a part of a gene cluster related to the putative antioncogene that is involved in the development of AML and therapy-related acute nonlymphocytic leukemia.

We report the molecular cloning and characterization of three regions of the long arm of human chromosome 5 that contain the CSF-2, IL-3, IL-4, and IL-5 genes; we also studied the physical organization of these genes using PFGE and hybridization probes derived from chromosome walking.

Results and Discussion

To isolate genomic DNA clones containing the genes for human CSF-2, IL-3, IL-4, and IL-5, phage and cosmid genomic libraries of 1.5×10^6 and 2×10^6 clones, respectively, were prepared from human leukocyte DNA. The libraries were probed with synthetic oligonucleotides from the published sequences.

Three cosmids and eight phage clones identified with the IL-3 and CSF-2 probes at the first step of the genomic walk cover 70 kb (Fig. 1) [22]. To continue the cosmid walk in both directions, we set out to isolate single copy fragments devoid of repetitive sequences from both extremities of the 70-kb region. We subcloned the end fragments of cosmids cos-2 and cos-C by digestion with restriction enzymes that have a unique site in the polylinker region of the cosmid vector, followed by circularization [23]. On the basis of primary structure of the insert DNA of these clones, oligonucleotide

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

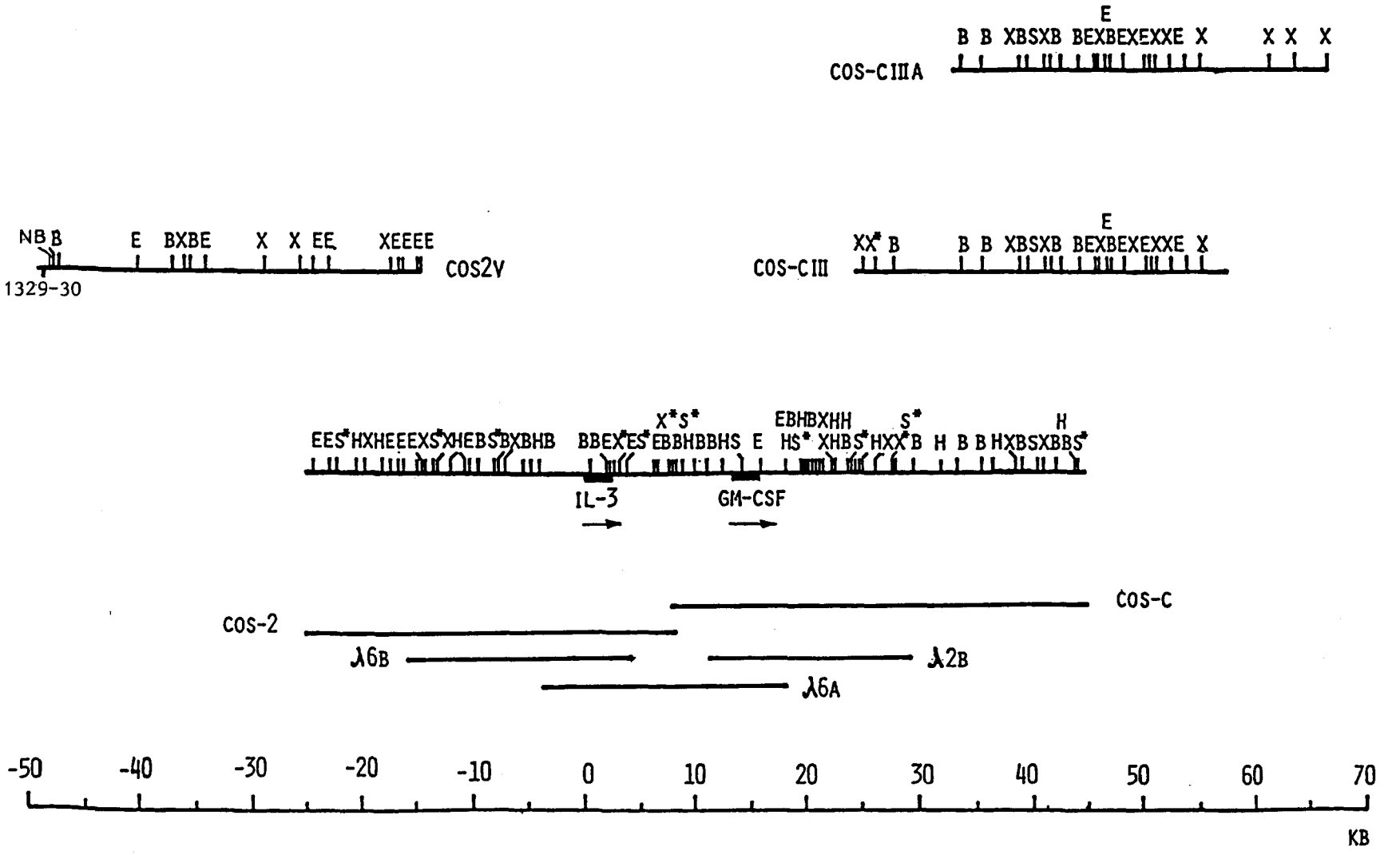

Fig. 1. Maps of the cloned 70-kb region that includes the genes for IL-3 and CSF-2 and cosmid clones that represent a 125-kb region: *E*, *Eco* RI; *X*, *Xba* I; *B*, *Bam* HI; *H*, *Hin* dIII; *S*, *Sal* I; *S**, *X**, *Xho* I; *N*, *Not* I; *Sf*, *Sfi* I; *B**, *Bss* HII. Cosmid and phage overlap. Localization of oligonucleotide probe 1329-30 is shown

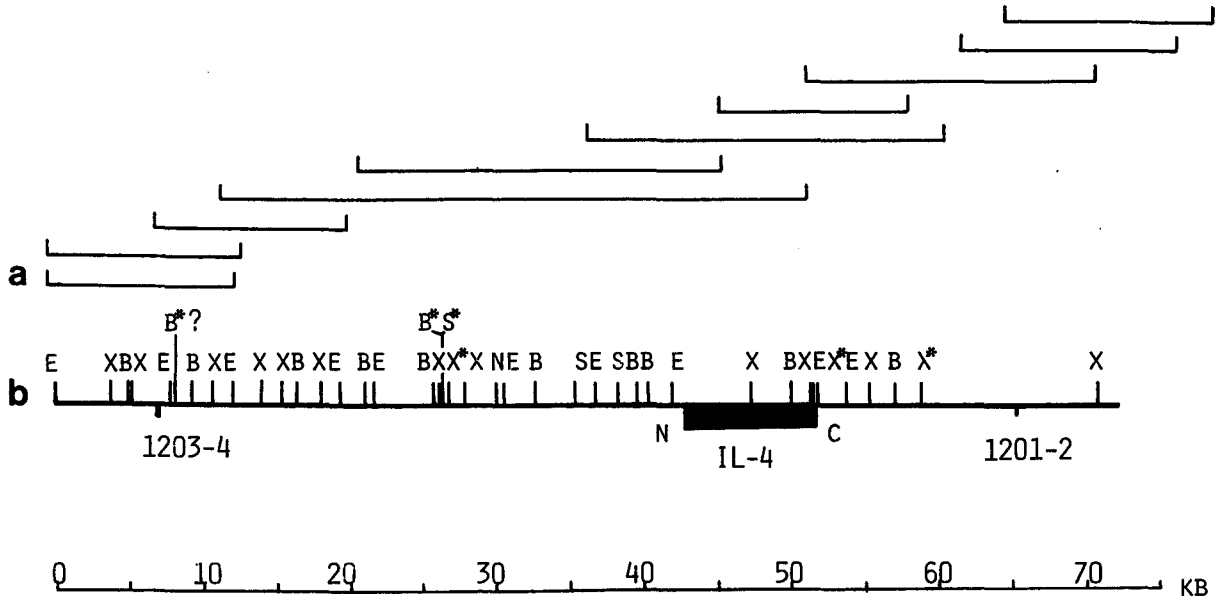

Fig. 2a,b. Map of the cloned 75-kb region that includes the IL-4 gene: *E*, *Eco*I; *X*, *Xba*I; *B*, *Bam*HI; *X**, *Xho*I; *S*, *Sfi*I; *B**, *Bss*HII; *S**, *Sac*II. Cosmid and phage overlap. Localization of oligonucleotide probes 1201-2 and 1203-4 is shown

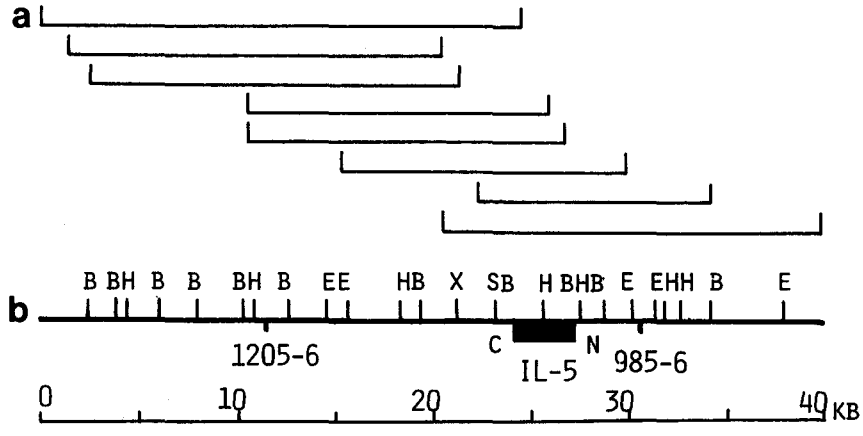

Fig. 3a,b. Map of the cloned 40-kb region that includes the IL-5 gene: *E*, *Eco*I; *X*, *Xho*I; *B*, *Bam*HI; *H*, *Hin*dIII; *S*, *Sal*I. Cosmid and phage overlap. Localization of oligonucleotide probes 1205-6 and 985-6 is shown

probes were synthesized. They were used to hybridize the cosmid library, yielding cosmids cos-2V, cos-CIII, and cos-CIIIA (Fig. 1). In total, the cosmid and phage walk comprised at least eight overlapping clones covering a 125-kb region around the IL-3 and CSF-2 genes.

Analogous genomic walks were performed around the IL-4 and IL-5 genes. More then six overlapping phage and cosmid clones represented the 75-kb IL-4 gene region (Fig. 2). A 40-kb region of the IL-5 gene was covered by two cosmid and at least three phage clones (Fig. 3). Further walking in both directions from the IL-5 gene was blocked by the presence of repetitive sequences.

We chose to analyze the sites for the rare-cutting restriction enzymes in three cloned regions. These enzymes usually have one or two CpGs in their recognition sequences and are methylation sensitive. The dinucleotide CpG, which is frequently methylated in a tissue-specific fashion, is underrepresent in bulk mammalian DNA by a factor of 5. Unmethylated restriction sites for the rare-cutting enzymes are often found clustered in

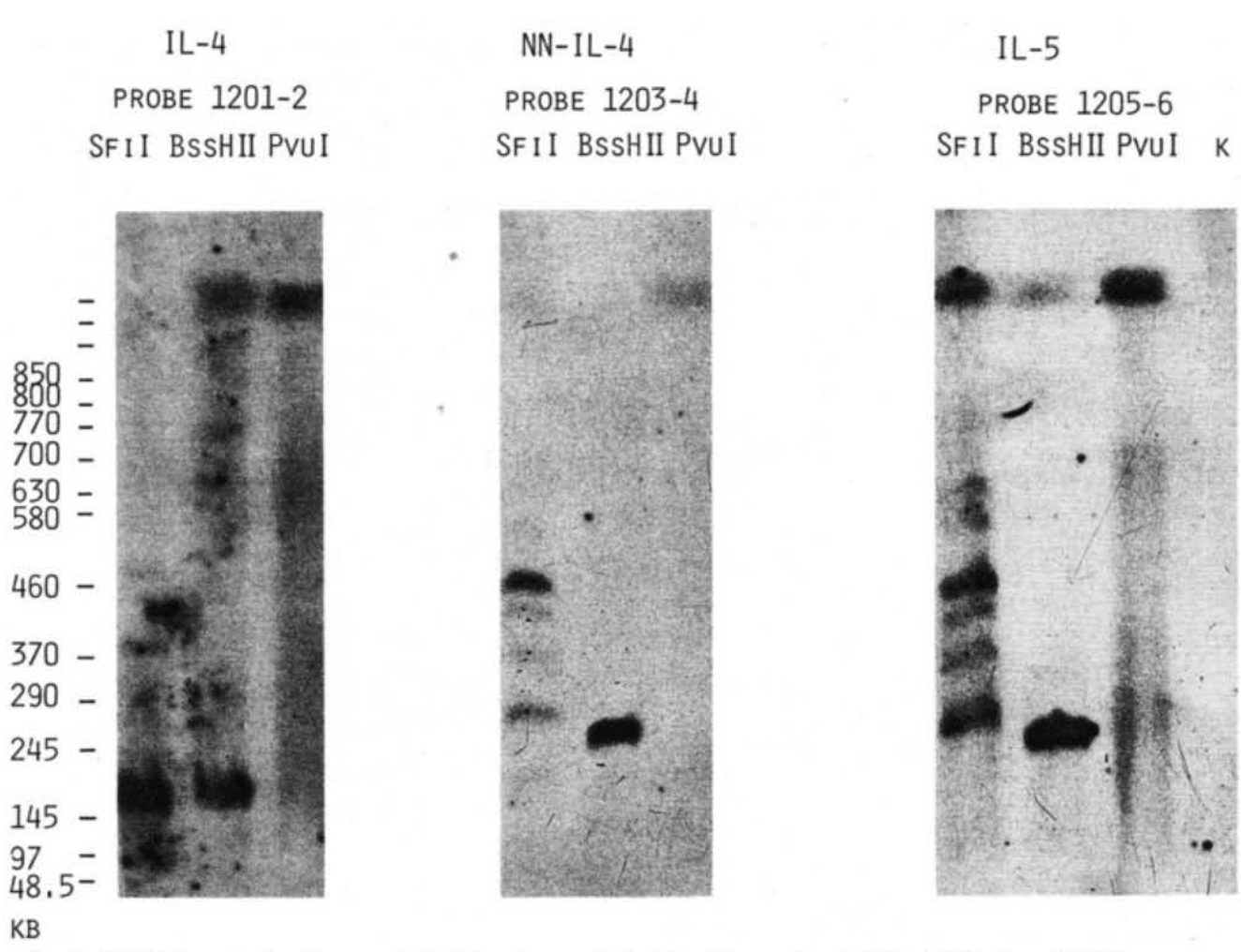

Fig. 4. PFGE analysis of genomic DNA using *Sac*II, *Mlu*I, and *Nae*I. Filter was sequentially hybridized to probes 1201-2, 1203-4, and 1205-6. The sizes of the markers are indicated

"CpG-rich islands," GC-rich regions of 1–2 kb where CpG is found at close to the frequency expected from local base composition and where methylation is suppressed. These islands are frequently found at, and mark, the 5′ ends of genes [24–26]. We mapped *Bss*HII, *Not*I, *Sfi*I, and *Sac* II restriction sites to trace possible CpG-rich islands.

As shown in Figs. 1 and 2, within the IL-3–CSF-2 region and the IL-4 region we found single restriction sites for *Bss*HII and *Sac*II. Only one *Not*I restriction site was present around 50 kb upstream from the IL-3–CSF-2 gene cluster (Fig. 1); this CpG-rich island may correspond to an unidentified gene because it lies adjacent to unique sequences conserved in evolution (data not shown).

To generate a large-scale map of the regions around the IL-3–CSF-2 and IL-4–IL-5 gene clusters we used rare-cutting restriction enzymes and PFGE. Several probes from each region derived from walking and partial sequencing were used for hybridization (Figs. 1–3). The results of several hybridization experiments are shown in Figs. 4–6; the sizes of the restriction fragments that hybridized to different probes are given in Tables 1 and 2. Our data confirmed physical linkage of the IL-4 and IL-5 genes shown earlier by hybridization with other probes [15, 20]: *Bss*HII, *Sfi*I, and *Nae*I digests revealed the same bands with probes 1203-4 and 1205-6 (Figs. 4, 5). Based on the length of the *Bss*HII and *Sac*II fragments (Table 2), we estimated the distance between the two genes as 240 kb. Genomic walks performed in the regions of the IL-4 and IL-5 genes allowed us to determine the orientation of both genes by using for hybridization the probes specific for the 5′ and 3′ ends of the genes separately (Figs. 4–6; Tables 1, 2). The results of these experiments allowed us to place the

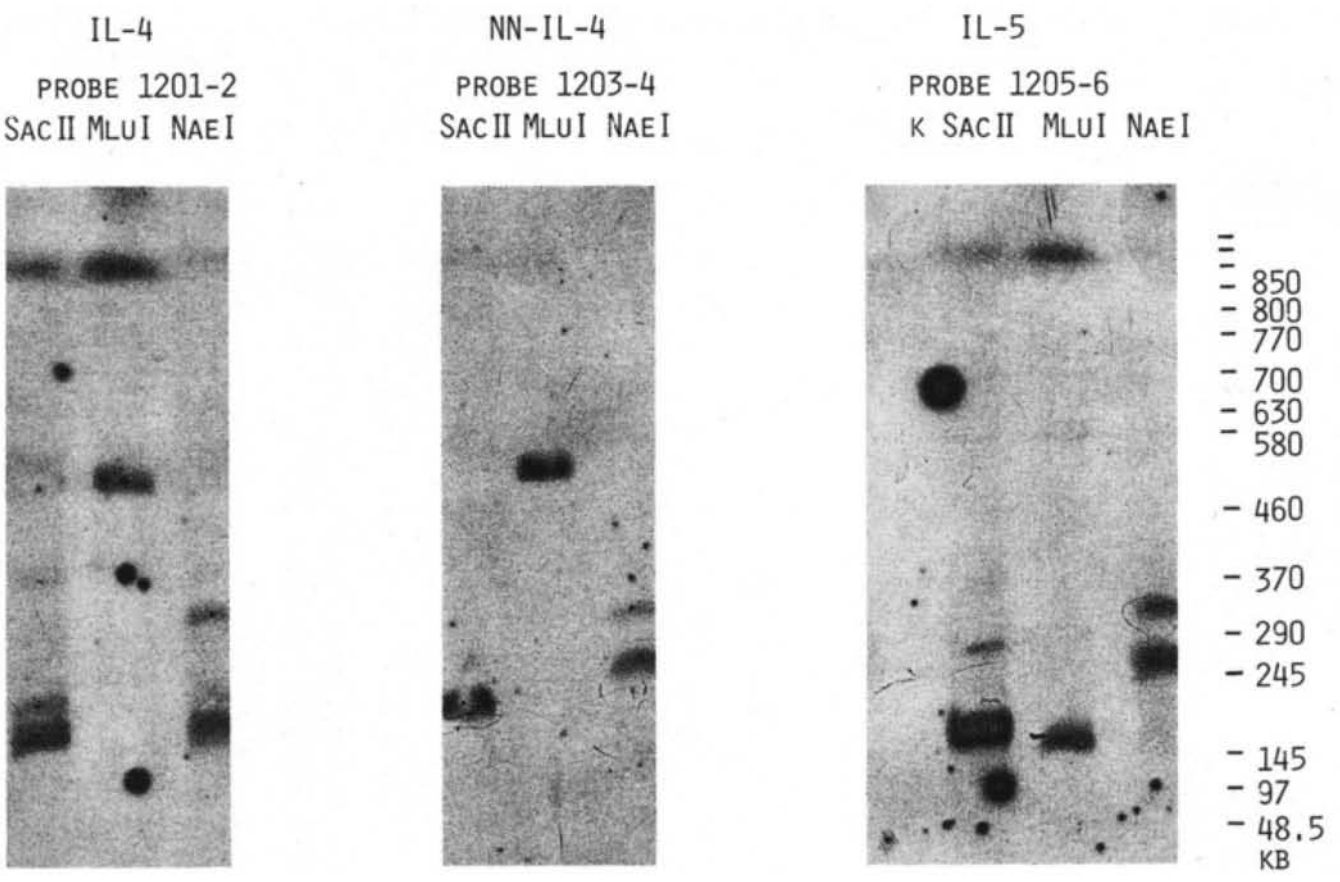

Fig. 5. PFGE analysis of genomic DNA using *Sfi*I, *Bss*HII, and *Pvu*I. Filter was sequentially hybridized to probes 1201-2, 1203-4, and 1205-6. The sizes of the markers are indicated

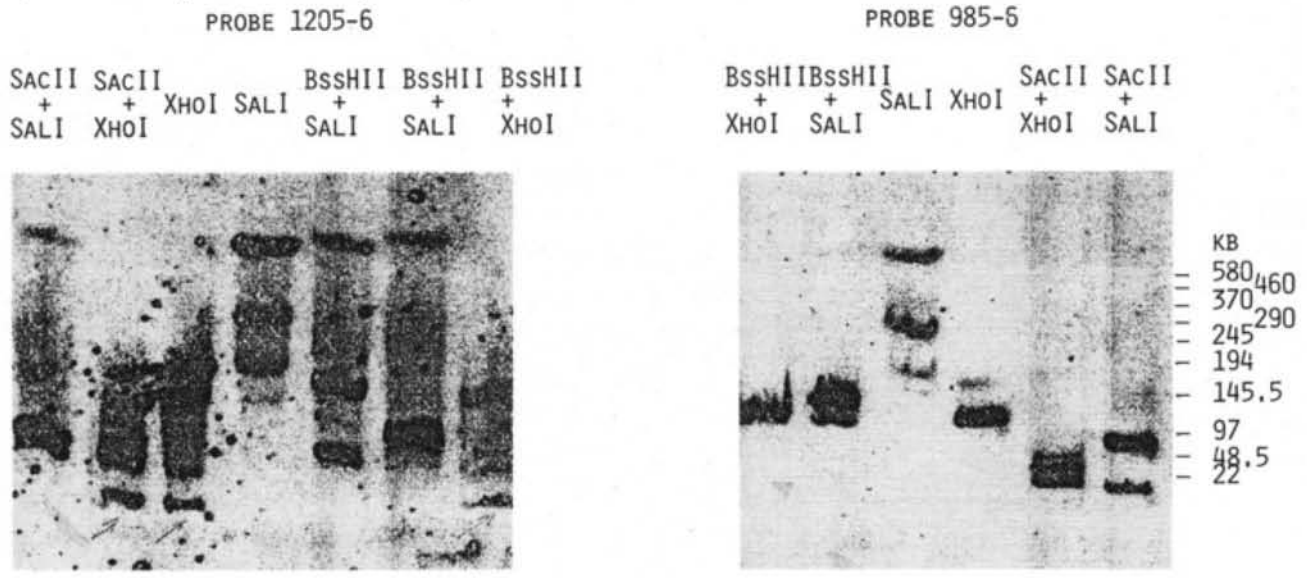

Fig. 6. PFGE analysis of genomic DNA using *Sal*I, *Xho*I, *Bss*HII + *Xho*I, *Bss*HII + *Sal*I, *Sac*II + *Xho*I, *Sac*II + *Sal*I. Filter was hybridized to probes 1205-6 and 985-6. The sizes of the markers are indicated

genes on a long-range restriction map of this cluster in head to head orientation (Fig. 8).

To test a possible linkage between the IL-4–IL-5 and IL-3–CSF-2 gene clusters we used for hybridization the probe 1329-30 mapped 50 kb upstreme from the 5′-end of the IL-3 gene (Fig. 1). No common bands were detected in *Not*I digests with this probe and IL-4-specific probe 1201-2 (Fig. 7) and we were unable to demonstrate physical linkage of the two gene

Table 1. Sizes in kilobase of restriction fragments obtained from complete and partial digestions

Fragment	Probe			
	1201–2	1203–4	1205–6	1329–30
Bss HII				
complete	180	245	245	275
Mlu I				
complete	550	550	155	>900
Nae I				
partial	200	260	260	
	340	340	340	300
Not I				
complete	345	345	345	950
Sac II				
complete	185	200	175	310
partial	225		265	
	415			
Sfi I				
complete	160	260	260	145
partial		350	350	
		410	410	
		450	450	

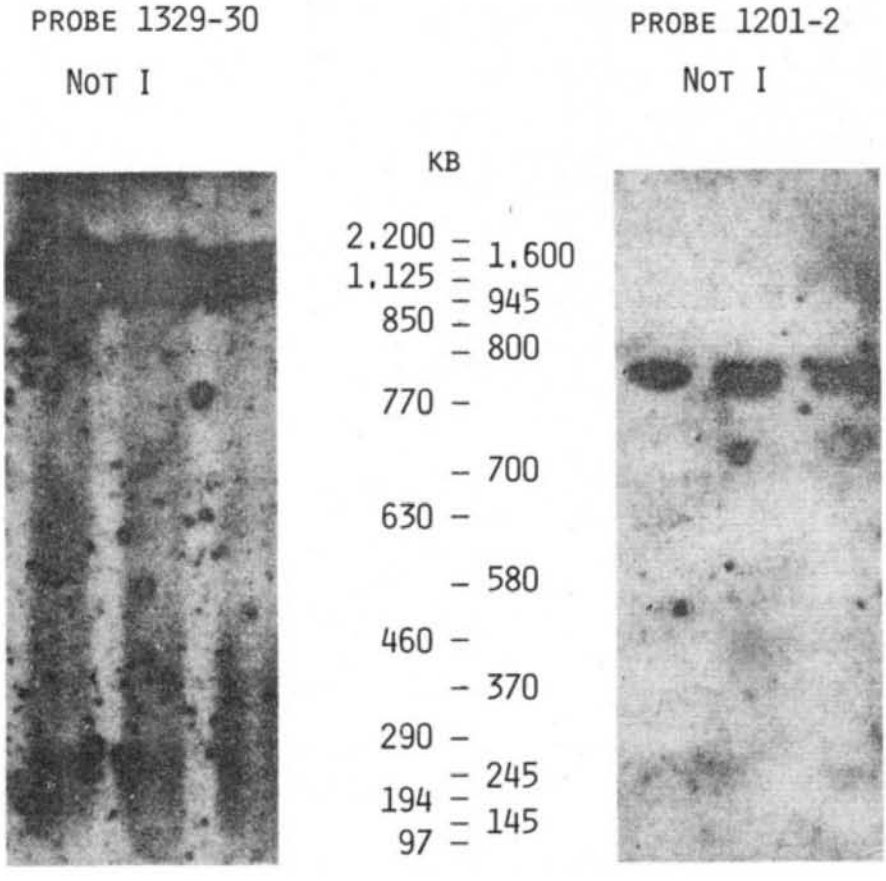

Fig. 7. PFGE analysis of genomic DNA using *Not*I. Filter was hybridized to probes 1329-30 and 1201-2. The sizes of the markers are indicated

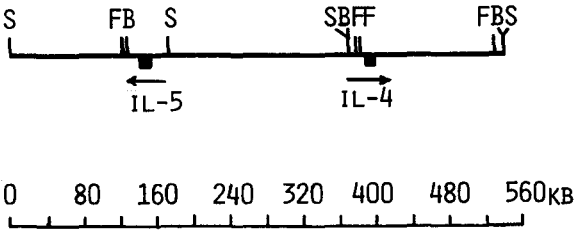

Fig. 8. Long-range physical map around IL-4 and IL-5 genes: *S*, *Sac*II; *F*, *Sfi*I; *B*, *Bss*HII. *Arrows* show the orientation of the genes from the 5′ end to the 3′ end

clusters. The data obtained by Huebner et al. [27] on somatic cell hybrids and clinical samples from patients with acquired deletions suggested the following order of these four genes on the long arm of chromosome 5: cen–(IL-4–IL-5)–IL-3–CSF-2–qter. Based on this order of the genes and on the length of *Not*I fragments (Table 1), we propose that the distance separating the IL-3 and IL-4–IL-5 genes was not less than 1050 kb.

Because of the close linkage of the four related genes, particularly the very close linkage of the IL-3 and CSF-2 genes, it might be suggested that they may have coordinate regulation during T-lymphocyte gene expression, and (or) that they diverged from a common ancestral gene producing a cluster of hematopoietic genes on chromosome 5. It

Table 2. Sizes in kilobases of restriction fragments obtained from complete and double digestions

Fragment	Probe	
	985–6	1205–6
*Sal*I		
partial	160	130
	195	195
	290	290
	> 600	> 600
Bss HII + *Sal*I		
complete	–	40
partial	130	–
	150	150
Sac II + *Sal*I		
complete	19	45
partial	65	65
*Xho*I		
complete	–	10
partial	130	22
	170	130
Bss HII + *Xho*I		
complete	–	10
partial	–	22
	130	140
Sac II + *Xho*I		
complete	21	10
partial	44	44
	55	55
Sac II + *Bss* HII		
complete	60	

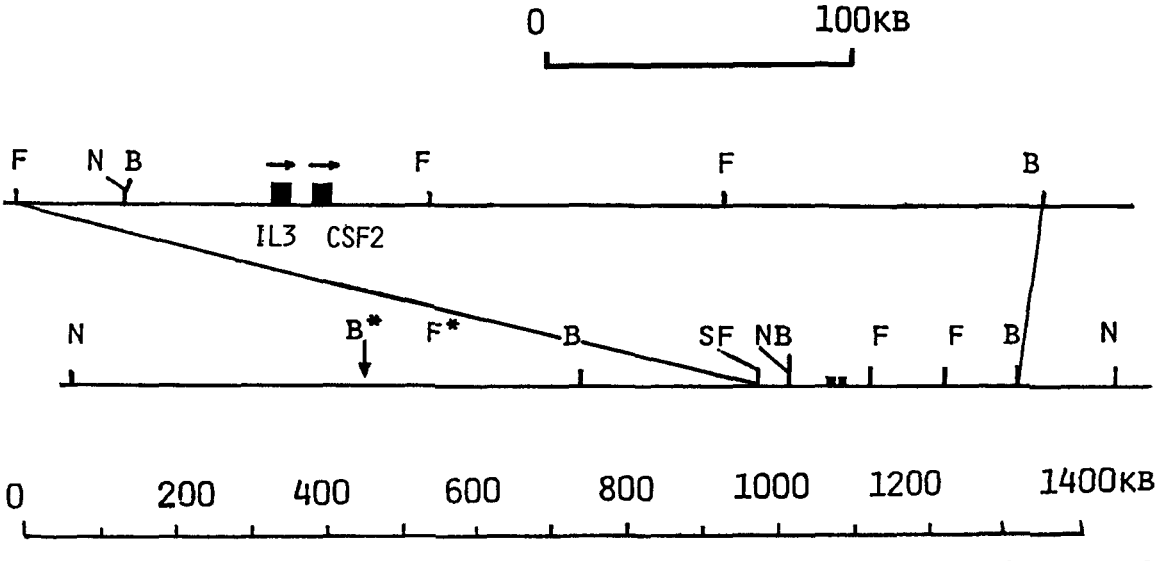

Fig. 9. Long-range physical map around IL-3 and CSF-2 genes: *F*, *Sfi*I; *B*, *Bss*HII; *N*, *Not*I. *Arrows* show the orientation of the genes from the 5′ end to the 3′ end

was shown that in the mouse genome the IL-3 and CSF-2 genes are also closely physically linked – they are only 14 kb apart and have the same orientation as in the human genome [15]. The conservation of linkage relationships supports both suggestions, and further study of the DNA between and surrounding the four genes is important in order to determine whether any other genes related to regulation of hematopoiesis are localized in the same cluster of genes.

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Genes Encoding Tumor Necrosis Factors: Genome Organization, Polymorphism, and Expression

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Introduction

Tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) are two related cytokines sharing a broad spectrum of activities [1–4]. In particular, TNF-α is one of the principal mediators of inflammation [2]. TNFs are also involved in the control of hematopoiesis. TNF-α stimulates production of hematopoietic growth factors by fibroblasts, macrophages, and endothelial cells [5, 6]. On the other hand, the same cytokine inhibits colony formation by hematopoietic progenitor cells in vitro [7, 8]. In vivo, TNF-α stimulates formation of spleen colonies and exhibits an overall protective effect in sublethally irradiated mice [9, 10].

We have previously cloned human, mouse, and rabbit genes coding for TNF-α and TNF-β [11–13]. The two TNF genes are tandemly arranged within 7 kb of genomic DNA and map inside major histocompatibility complexes in mice and humans [14, 15]. They apparently evolved from a common ancestor.

TNF-α and -β are differentially expressed. Therefore, TNF loci offer an interesting model system with which to study tissue-specific regulation of gene expression. Here we review our recent findings on the genome organization, polymorphism, and expression of human, mouse, and rabbit TNF genes.

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Materials and Methods

DNA Clones and Probes. Original phage clones 15 and 11 containing human TNF locus were described [11]. DNA of cosmid clone 031 A [16] was generously provided by T. Spies (Harvard University, Cambridge, MA, USA). Further subcloning was done into pGEM3/4 vectors (Promega Biotech, Madison, WI, USA). DNA samples from peripheral blood leukocytes of unrelated blood donors were kindly provided by P. M. Chumakov (Engelhardt Institute of Molecular Biology, Moscow, USSR).

Oligonucleotides. $(TC)_9$ and $(TC)_{13}$ and oligonucleotides used as polymerase chain reaction (PCR) and sequencing primers (see below) were purchased through Nauka, Inc. (Moscow, USSR); $(AC)_{15}$ was a gift from A. Edwards (Institute of Molecular Genetics, Baylor College of Medicine, Houston, TX, USA). Universal sequencing primers specific for pGEM3/4, 5′-TCACTATAGGGAGACCG-3′ and 5′-GTGACACTATAGAATAC-3′ were generously provided by V. N. Dobrynin (Shemyakin Institute of Bioorganic Chemistry, Moscow, USSR).

The following oligonucleotides flanking the $(AC/TG)_k$ and $(TC/AG)_n$ repeats were used as primers for PCR and sequencing:

IR1 5′-
GCACTCCAGC*CTAG*GCCACA
GA-3′ (*Sty*I site is underlined)

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

IR2 5′-
GCC*TCTAGA*TTTCATCCAGC
CACA-3′ (*Xba*I site is underlined)
IR4 5′-
CCTCTCTCCCCTGCAACACACA
-3′
IR5 5′-
GTGTGTGTTGCAGGGGAGAG
AG-3′

DNA Sequencing. The dideoxy termination method as modified for supercoiled double stranded templates [17] was used. Universal synthetic oligonucleotides specific for pGEM3/4 as well as gene-specific oligonucleotides (see above) were used as sequencing primers. Most of the sequencing was done using Sequenase (USB Corporation, Cleveland, OH, USA) following the protocol provided by the manufacturer.

Hybridization. Southern hybridizations with kinased oligonucleotides were performed as described [18], except that carrier DNA was excluded from prehybridization and hybridization buffers. Washing was done in 2 × SSC twice at room temperature for 20 min and twice at hybridization temperature for 1–2 min. The latter temperature for these salt conditions was calculated according to Itakura et al. [see 18].

PCR Conditions. PCR [19] was carried out in a volume of 10 µl with 100 ng of genomic DNA template. Conditions for PCR were the following: 5 min at 95 °C and 30 cycles with 30 s at 92 °C (denaturation), 60 s at 55 °C (annealing), and 30 s at 72 °C (elongation). The elongation step in the last cycle was extended to 5 min at 65 °C. Unlabeled PCR products were analyzed on 2% agarose minigels in TBE [18]. Labeling of PCR products was performed either by addition of end-labeled primers to the amplification mixture or by filling the ends of amplified DNA with Klenow fragments of DNA polymerase *E. coli* in the presence of radioactive dNTPs, after digestion with *Sty*I (for primer IR1) or *Xba*I (for primer IR4). Radiolabeled amplification products were analyzed on 5% polyacrylamide sequencing gels. Microsatellites containing polymorphic fragments were sized using sequencing markers, compared to those from cosmid clone 031A, amplified, and processed in parallel (clone 031A microsatellites were sequenced).

Gel Retardation Analysis. Nuclear extracts were prepared from bone marrow-derived macrophages before and 4 h after lipopolysaccharide (LPS) activation, from the RAW264 macrophage-like cell line, and from the RPMI-6410t human B lymphoblastoid cell line according to recently published procedure [20]. Synthetic oligonucleotides were kinased, gel purified, and used for binding. Complexes were analyzed on 5% native gels as described [18, 20].

Results and Discussion

Genome Organization and Polymorphism of the TNF Genes

Novel Sequence Polymorphism in the Human TNF Locus

TNF-α and TNF-β genes are tandemly arranged, linked to the major histocompatibility complex (MHC), and map centromeric to HLA-B (or H-2D) and telomeric to class III genes. Since both cytokines encoded by these genes are potent immunomodulators and since some MHC-linked autoimmune diseases are characterized by altered levels of their production or inducibility [21, 22], genetic variability in the TNF locus may be related to the functional polymorphism of MHC.

So far, based on restriction fragment length polymorphism (RFLP) data, low degrees of genetic polymorphism in the human TNF locus have been reported [23, 24]. In the course of hybridization analysis with oligonucleotide probes, we found, and mapped 3.5 kb upstream to the TNF-β gene, tandem AC/TG and TC/AG dinucleotide repeates (otherwise known as microsatellites; Fig. 1).

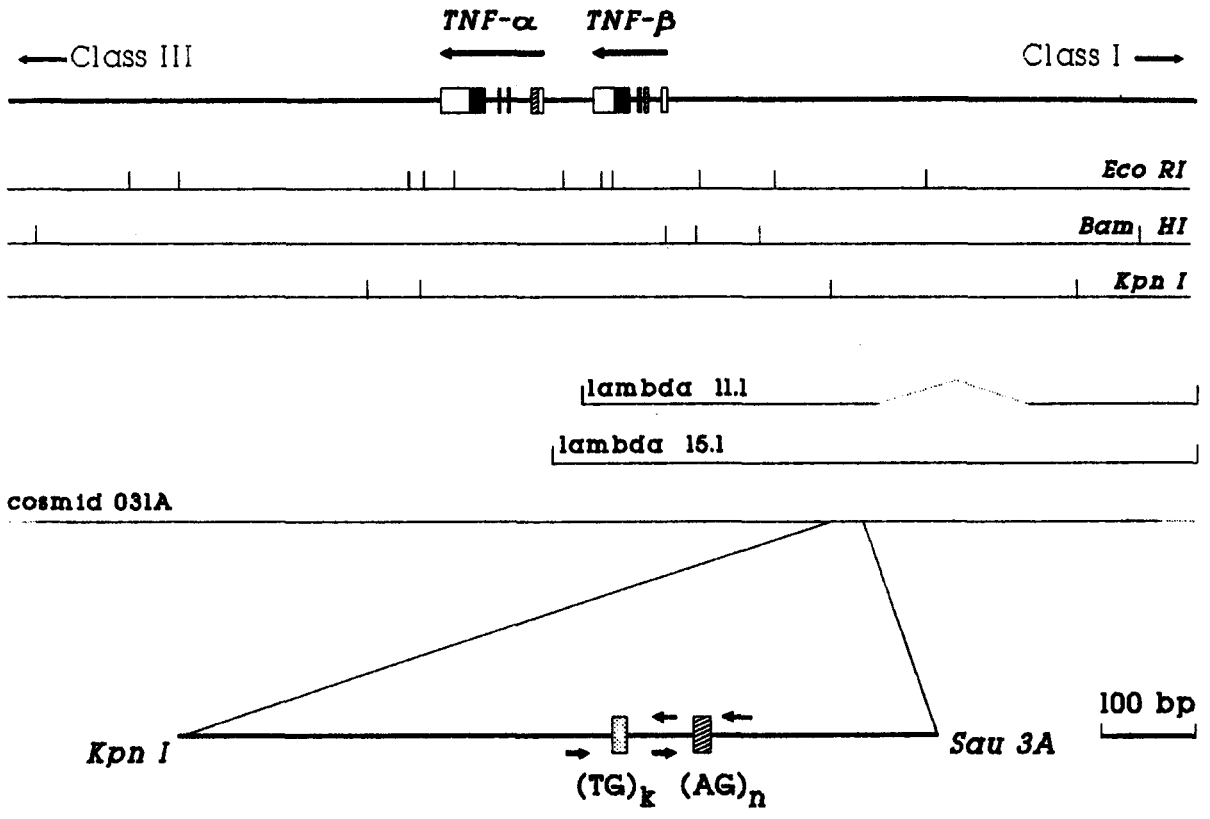

Fig. 1. Localization of polymorphic microsatellites (AC/TG and TC/AG repeats) in the human TNF locus. *Arrows* in the *lower center* correspond to positions of PCR primers. (Map of the TNF locus and restriction maps of phage and cosmid clones are taken from [11, 16])

To obtain DNA sequence information from this putative polymorphic region, the *Kpn*I–*Sau*3A 820-bp fragment derived from cosmid 031A was subcloned to pGEM4 and sequenced (Fig. 1). In addition to microsatellites, the sequence of the *Kpn*I–*Sau*3A fragment contained 200 bp with 73% homology to the human Alu-family interspersed repeat (clone BLUR7).

Since microsatellites found in many genes have been shown to contain various numbers of repeats [25], we measured the size of "TNF-linked" microsatellites in DNA samples from blood donors, using a PCR-based technique. Several oligonucleotide primers based on the sequence of 820-bp *Kpn*I–*Sau*3A fragment were designed to make PCR products across dinucleotide repeats with the expected fragment length of 100–200 nucleotides.

Initial PCR reactions were performed using primers IR2 and IR4 to amplify AC/TG-containing microsatellites from phage clones 11 and 15, cosmid 031A, and human B cell line RPMI-6410t. After cleavage with *Xba*I and labeling with Klenow polymerase, PCR products were analyzed on denaturing gels using an appropriate sequence ladder as a marker (Fig. 2). In this experiment we found that the AC/TG repeat was indeed polymorphic in length: clone 031A (from which *Kpn*I–*Sau*3A fragment was originally cloned) contained 13 AC/TG repeats, whereas both phage clones contained 15 repeats. RPMI-6410t cells were heterozygous and contained two alleles with ten and eight repeats (Table 1).

We then investigated whether the oligonucleotides we designed had unique sequences and could therefore define a novel sequence tagged site (STS) in the human genome. After that, we developed a protocol for genomic PCR analysis of DNA polymorphism in this particular human leukocyte antigen (HLA) region.

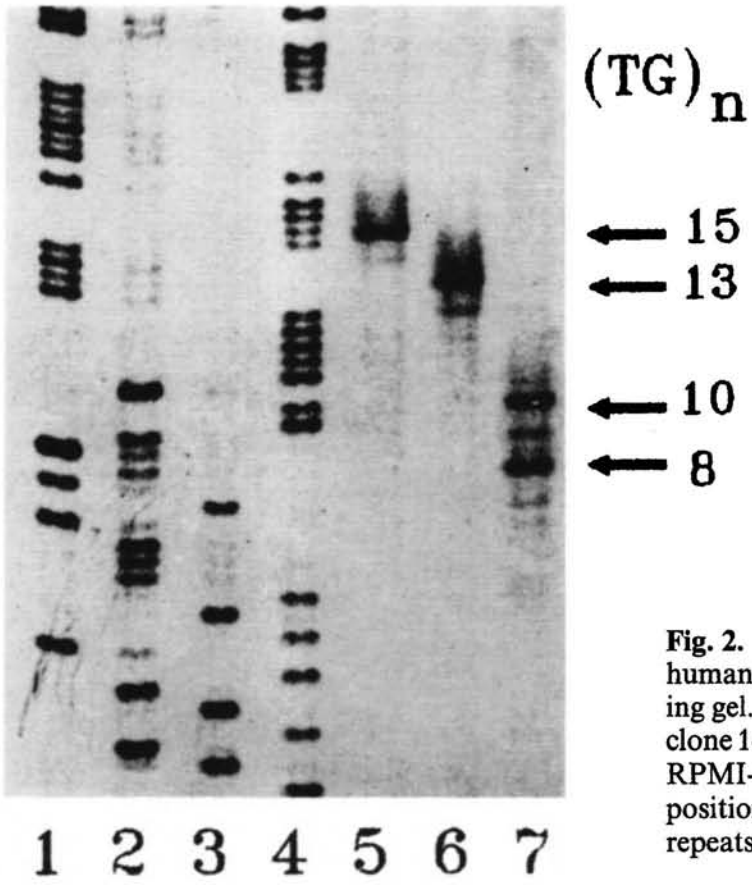

Fig. 2. Microsatellite polymorphism in the human TNF locus as determined on sequencing gel. Lanes *1–4*, sequencing marker; lane *5*, clone 14; lane *6*, clone 031 A; lane *7*, DNA from RPMI-6410t cell line. The *arrows* indicate the positions of specified numbers of AC/TG repeats

Table 1. AC/TG and TC/AG microsatellite length polymorphism in the human TNF locus

Source of DNA	$(AC)_n$	$(TC)_k$
Gosmid 031A	13	10
γ15.1	15	ND
γ11.1	15	ND
1[a]	12	10
2	12/11	11
3	16/11	11
4	18/14	11
5	15/12	10/8.5[b]
6	16/7	10/8.5
7	10/9	9.5[b]
8	17/10	9.5
9	15/11	9.5
10	16/7	10/8.5
RPMI-6410t	10/8	ND

[a] DNA samples 1–10 correspond to unrelated blood donors.

[b] Lengths 8.5 and 9.5 were calculated from the fragment size. Additional sequencing analysis was not performed.

ND: No data.

The protocol is based on two rounds of PCR amplification: first with primers IR1–IR2 across both microsatellites; and second with IR1 and kinased IR5, or with IR2 and kinased IR4 (for separate amplifications across TC/AG or AC/TG, respectively).

PCR products, after the first round of amplification of DNA from blood donors, ran as single (for homozygous donors) or double bands on agarose gels, with an apparent length of 215 bp (Fig. 3). Variations in the length of amplified fragments indicated the polymorphism of "TNF-linked" microsatellites. For the second round, IR4 primer (for AC/TG analysis) or IR5 primer (for TC/AG) labeled by polynucleotide kinase were added. PCR products after the second amplification were directly analyzed on sequencing gel. The results of some of those experiments are summarized in Table 1 and indicate that AC/TG microsatellite length in the human TNF locus can vary from 6 to 18 repeats, while the TC/AG repeat is less polymorphic and occurs from 8 to 11 times. The fact

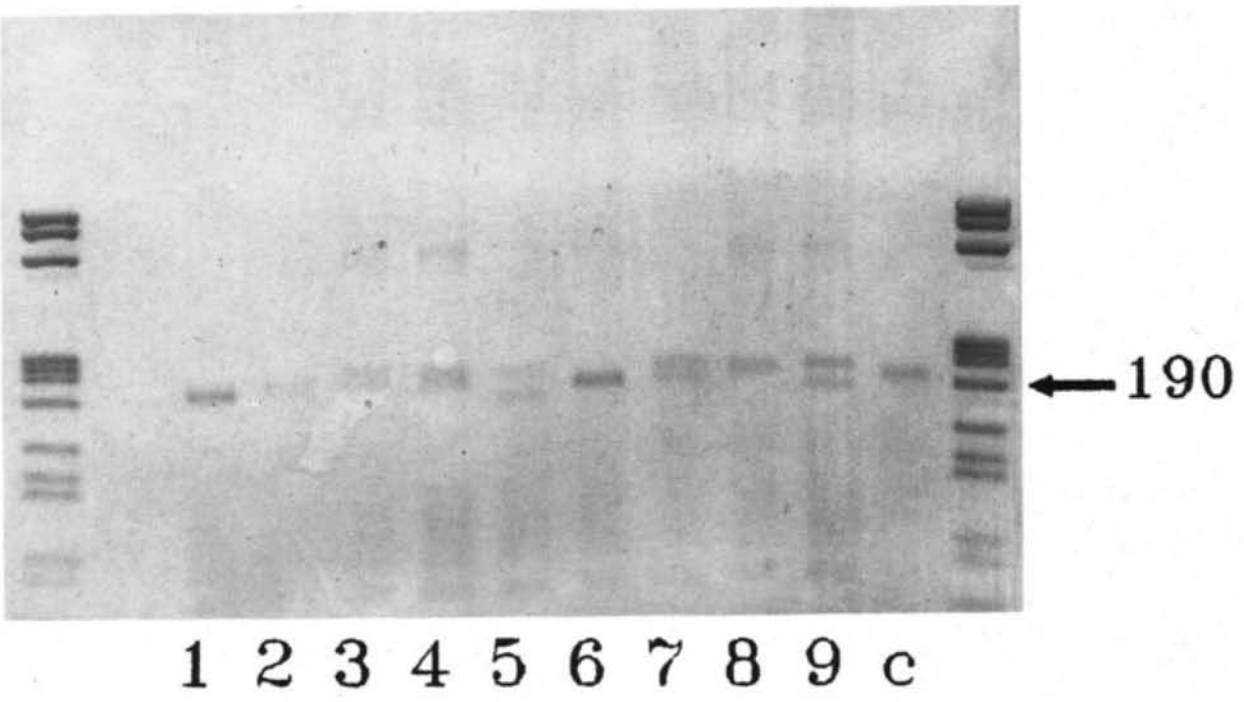

Fig. 3. Detection of microsatellite polymorphism in the human TNF locus by agarose gel electrophoresis. DNA samples were obtained from blood donors (lanes *1–9*) and from cosmid clone 031A (lane *c*)

that both microsatellites are polymorphic (with their combination giving additional variability) indicated that PCR assay of microsatellite lengths in the human TNF locus can be informative for disease-association studies. Similar analysis has been recently performed on the mouse TNF-α gene, where the AC/TG microsatellite located in the promoter region (lacking in homologous position in the human TNF locus) has shown five distinct alleles [26].

Our characterization of novel polymorphic DNA sequences closely linked to the human TNF genes might provide a new experimental tool for the analysis of genetic variability of TNF genes. In view of close linkage between TNF and HLA, it would be of interest now to investigate associations between distinct "TNF alleles," MHC haplotypes, and certain HLA-linked diseases.

Structure of the Rabbit TNF Locus and Homology to Human and Mouse TNF Loci

We have previously isolated a rabbit genomic clone containing TNF genes [13]. We recently sequenced 3.5 kb of DNA including the entire lymphotoxin (TNF-β) gene and the upstream region of TNF-α gene [27]. Our sequence overlaps with that published earlier by Ito et al. [28] and makes 6.5 kb of the rabbit TNF locus available for computer analysis. Several findings are due to this comparative study of human, mouse, and rabbit TNF loci.

First of all, by comparing gene structures for human, mouse, and rabbit TNF-β genes we were able to derive amino acid sequences of rabbit lymphotoxin for which protein or cDNA sequences are lacking [27].

Second, we noted that putative polymorphic microsatellites are lacking in homologous positions in the three TNF loci (see above). We also find that the enhancer sequence which apparently plays an important role in transcriptional regulation of the mouse TNF-α gene is not conserved (site 3, according to [29]). This observation prompted us to search for additional putative regulatory sequences in the human and rabbit TNF loci (see below).

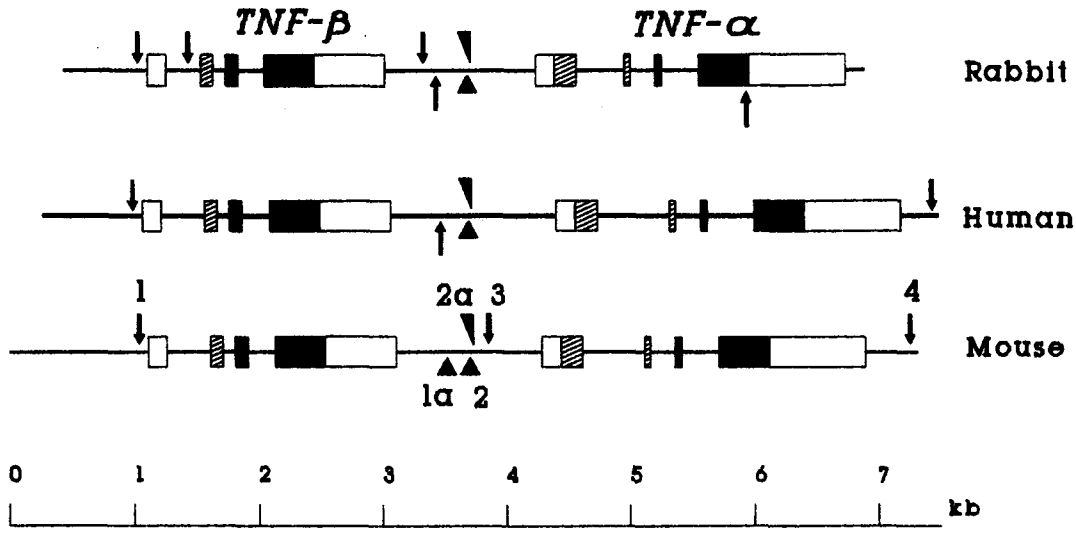

Fig. 4. Localization of κB and κB-related sites in the aligned sequences of the rabbit, human, and mouse TNF loci. *Downward arrows* correspond to sequences in direct orientations, *upward arrows* indicate κB and related sites in reverse orientations

Regulation of Expression of TNF Genes

Multiple κB and κB-related Sites in TNF Loci and Their Role in the Regulation of Transcription

In collaborative study with the group led by C. V. Jongeneel (Ludwig Institute for Cancer Research, Lausanne, Switzerland) we recently presented evidence that κB-related sequences play an essential role in transcriptional activation of the TNF-α gene in mouse macrophages [29]. The role of necrosis factor (NF)-κB or related factors was also suspected in the control of TNF-β expression in T and B lymphocytes, since the κB enhancer sequence is a highly conserved feature of the upstream region of the TNF-β gene (Figs. 4, 5).

Computer analysis of human, mouse, and rabbit TNF sequences (Fig. 4) revealed a number of κB sites [30] located in direct and reverse orientations in the upstream regions, in the intron (rabbit TNF-β gene), and also downstream to mouse and human TNF-α genes (the corresponding portion of the rabbit sequence is not yet available). In particular, we found the previously overlooked symmetrical NF-κB/H2TF1 binding site [31] closely linked to another κB-related sequence (Figs. 5,6). The latter sequence might be a binding site for recently described NF-GMa [32], although it weekly binds NF-κB (Fig. 6). Interestingly, these two binding sites, which both correspond to conserved features of the three TNF loci (Fig. 4), are separated by three turns of double helix (32 bp), placing the two recognition sequences on the same side of the DNA molecule. Whether factors bind cooperatively and make direct protein-protein contacts or, alternatively, whether they interfere, is currently under study.

Analysis of κB DNA – Protein Interactions: Possible Role of Flanking Regions

In view of the variety of κB enhancer-like sequences found in TNF loci we initiated comparative studies of their functional properties in vitro and in vivo. To this end, a collection of synthetic κB sites containing a consensus [30] of 10–11 nucleotides with various natural or artificial flanking sequences was assayed for in vitro binding to NF-κB containing nuclear extracts from mouse macrophages or the human lymphoblastoid cell line RPMI-6410t. Using gel retardation assay we found that the nonconserved κB site 3 from the mouse TNF gene and the conserved κB site from TNF-β gene, both

1	CTTCTAAGCCCTGGGGGCTTCCCCAAGCCCCAGCCC
1a	CCCCGGTCTTCCAAGGATTCCCCTCCCCCACCCTCC
2	GCTTGTGAGGTCCGTGAATTCCCAGGGCTGAGT
2a	TCATTCCCTCTGGGGCTGCCCCATACTCATCCCA
3	AGAACTCAAACAGGGGGCTTTCCCTCCTCAATATCA
4	TGCCCTGGGGCATGGGAATTTCCCACTCTGGGAATT
Consensus	GGGRNTYYCC

Fig. 5. Nucleotide sequence of several κB-related sites with their natural flanks from the mouse TNF locus (consensus nucleotides are *underlined*). Numbering corresponds to that on Fig. 4. In the case of site *1a*, the complementary sequence is shown. Sequences of sites *2* and *2a* are adjacent (see text for details)

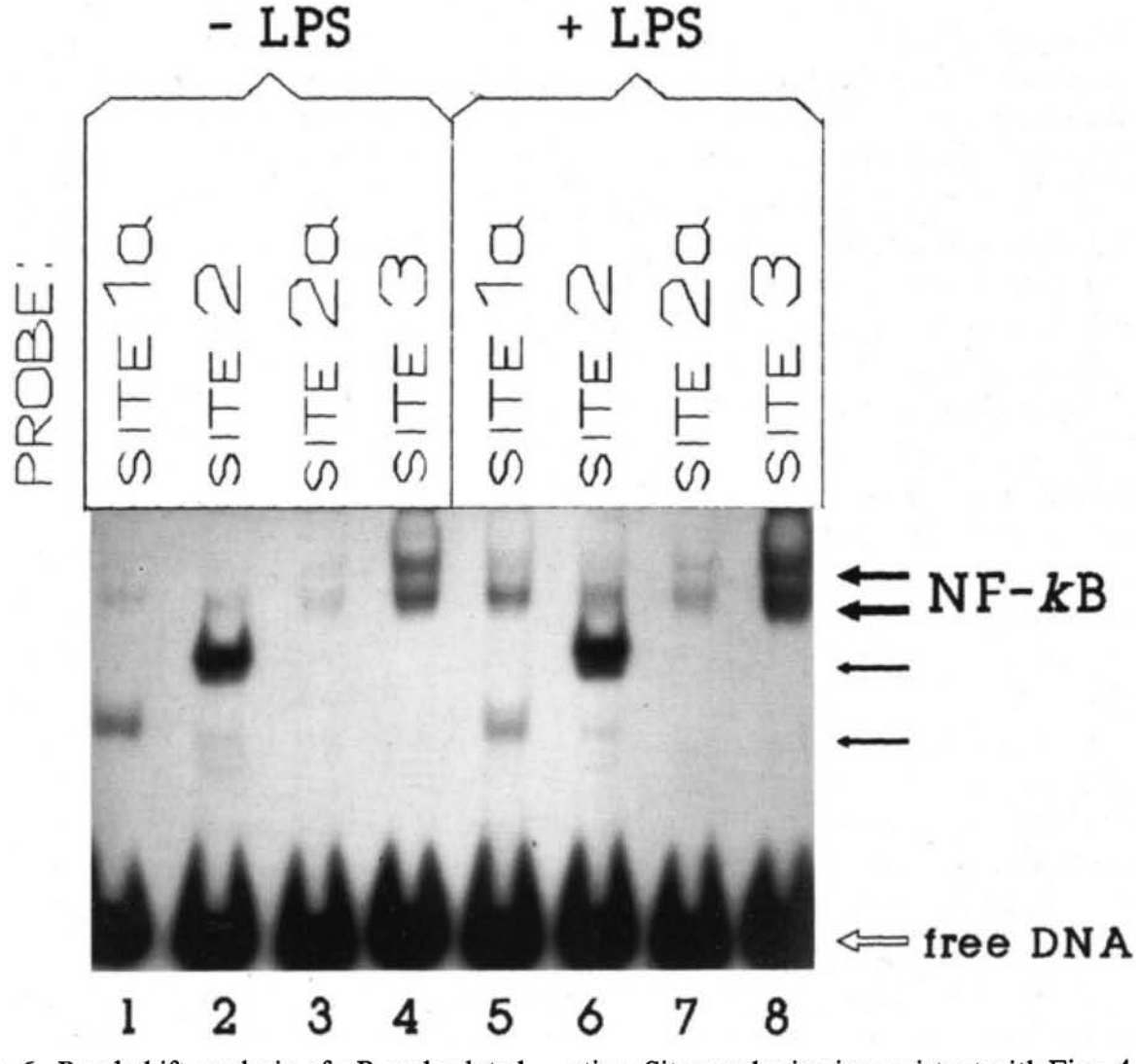

Fig. 6. Band shift analysis of κB and related sites from the mouse TNF-α gene with extracts from mouse bone marrow-derived macrophages before (−) and after (+) *LPS* activation. Site numbering is consistent with Figs. 4 and 5. *Site 1a* (promoter of TNF-β) has affinity similar to that of *site 3*

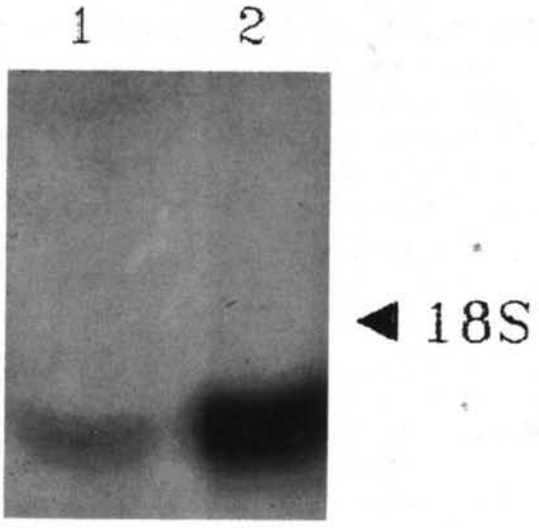

Fig. 7. Northern analysis of TNF-β mRNA in RPMI-6410t cells before (*1*) and after (*2*) PMA activation

with their natural flanks, were the best specific in vitro NF-κB binders (see Fig. 6 for examples). Attachment of the flanks from site 3 to some of the poorer binding κB sites resulted in improved binding. These preliminary results implied the role for DNA sequences immediately flanking the consensus 10–11 nucleotides in κB- and NF-κB interactions. Since one feature of site 3 with its natural flanks resembled that found in the "ideal nucleosome" [33], our current hypothesis is that wrapping of DNA around a protein complex facilitates affinity of binding. We recently cloned NF-κB site 3 into pBend2 vector [34] and prepared a set of labeled fragments with various locations of the target sequences. Band shift analysis confirmed that the κB sequence, which itself had no intrinsic bend, could be bent once bound to NF-κB.

Expression of TNF Locus in a Human Lymphoblastoid Cell Line RPMI-6410t

We have shown previously that the human lymphoblastoid cell line RPMI-6410t secretes an autocrine growth activity which can be neutralized by antibodies raised against purified recombinant human TNF-β [35]. Cytotoxicity neutralization assay with antibodies specific for purified human TNF-α or TNF-β showed the secretion of significant amounts of TNF-β, but little or no TNF-α. The constitutive level of TNF-β expression was enhanced up to a hundred fold after phorbol myristate acetate (PMA) treatment, reaching a maximum 3–4 days after the beginning of stimulation [35].

Northern (Fig. 7) and nuclear run-on (Fig. 8) analysis showed that both TNF

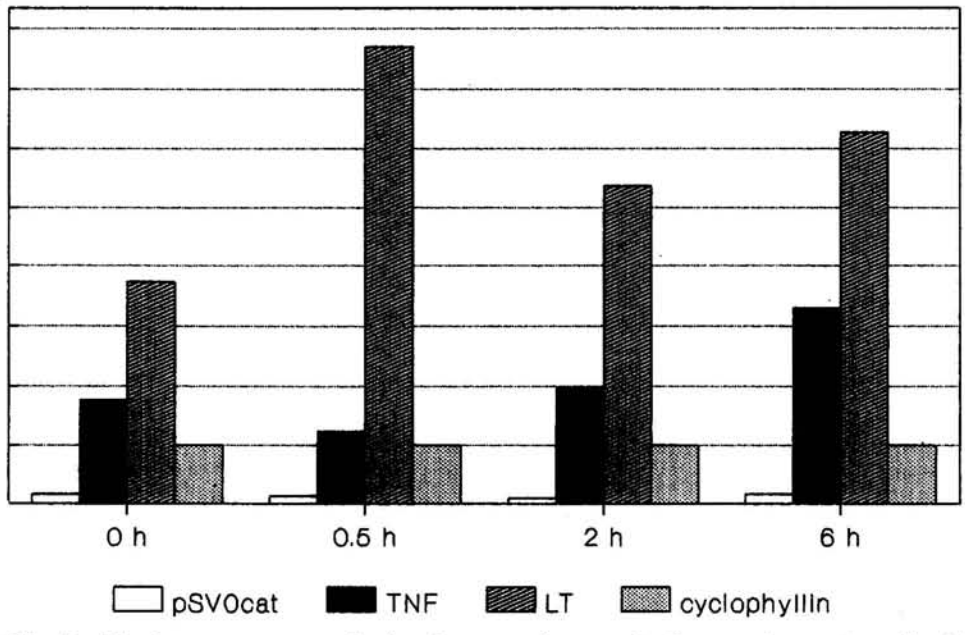

Fig. 8. Nuclear run-on analysis of expression of TNF-α (*TNF*) and TNF-β (*LT*) genes in RPMI-6410t cells. Experimental details are similar to those described in [36]. Signals are normalized against cyclophyllin gene transcription

genes were transcribed, that TNF-β mRNA was more abundant, and that its level was significantly enhanced upon PMA treatment (TNF-α mRNA level remained the same; data not shown). In contrast to mouse CTL clones [36], where the TNF-α gene was always transcribed more actively (in spite of the absence of protein expression of TNF-α), transcription of TNF-β in RPMI-6410t seemed to be more active. Additionally, nuclear run-on assay showed significant constitutive level of TNF-β and TNF-α transcription which was only slightly changed upon PMA activation for various time intervals (Fig. 8). These data implied that regulation of TNF-β expression in the RPMI-6410t cell line occurred primarily on a posttranscriptional level.

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Molecular Characterization of the Mouse Interleukin-3 Receptor

A. Miyajima [1]

Introduction

Interleukin-3 (IL-3), also known as a multi-colony stimulating factor, is a potent hemopoietic growth factor which acts on the multi-potential hemopoietic stem cells as well as committed progenitor cells such as erythroblasts, eosinophils, megakaryocytes, mast cells, and pro-B cells [1]. Whereas IL-3 is capable of regulating proliferation and differentiation of various hemopoietic cell lineages, production of IL-3 is limited to antigen-stimulated T cells and activated mast cells and so far there is no evidence indicating that IL-3 is produced in normal bone marrow. Therefore, the major role of IL-3 may be in antigen-induced hemopoiesis rather than normal hemopoiesis in the bone marrow [2].

IL-3 manifests its multiple biological activities through specific cell surface receptors and signal transduction pathways in target cells. Information about the structure and function of the receptor should contribute to an understanding of the multiple biological activities of IL-3. Here we describe the biochemical characterization and molecular cloning of the mouse IL-3 receptor gene.

The IL-3 Receptor and Signal Transduction

Previous reports indicated that IL-3 bound only to a single class of high affinity receptor. However, our binding data clearly shows the presence of two distinct binding sites on various IL-3 dependent cells: high affinity ($Kd \sim 100\ pM$) and low affinity ($Kd \sim 10\ nM$) binding sites. In addition, two binding sites have also been distinguished by dissociation kinetics. The dissociation rate from the low affinity site ($T_{1/2} = 4$ min) is much faster than that from the high affinity site ($T_{1/2} = 4$ h) [3]. Cross-linking experiments using ^{125}I-labeled IL-3 have revealed the presence of 140, 120, and 70 kDa proteins. The interrelation between these proteins and two distinct binding sites was unknown.

While the structure of the IL-3 receptor is unclear, it has been well established that IL-3 induces rapid tyrosine phosphorylation of a specific set of proteins including 140, 95, 90, 70, and 55 kDa proteins [4, 5]. Evidence indicates that the 140 kDa IL-3 binding protein is tyrosine phosphorylated [6]. In addition, it is known that, in IL-3 dependent cells, the IL-3 requirement is abrogated by oncogenes having a tyrosine kinase. In particular, *v-abl* carrying a temperature-sensitive tyrosine kinase abrogates the IL-3 requirement in a temperature dependent manner (Fig. 1 A, B) [7, 8], suggesting the involvement of tyrosine phosphorylation in the IL-3 signal transduction pathway. These results indicate that the IL-3 receptor is directly or indirectly coupled to a tyrosine kinase.

If the IL-3 receptor is linked to a signal transduction system similar to the growth factor receptors containing a tyrosine kinase, such as the epidermal growth factor (EGF) receptor, the requirement

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Haematology and Blood Transfusion Vol. 35
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R. Neth et al. (Eds.)

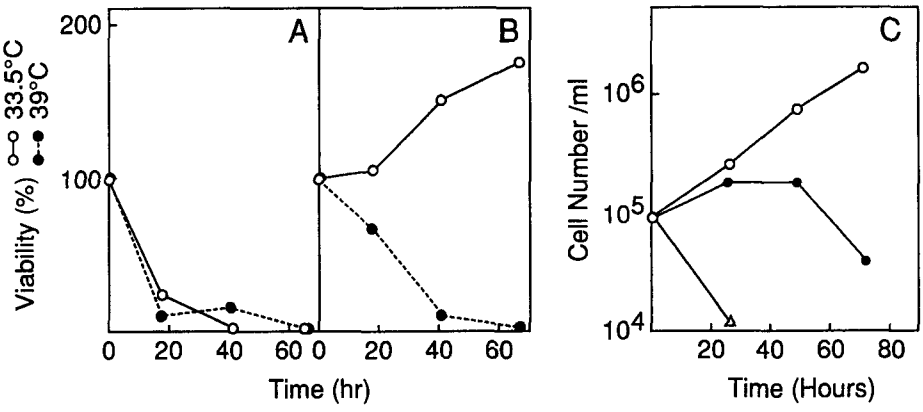

Fig. 1A–C. Conditional abrogation of IL-3 requirement from IC2 cells. **A, B**: Cell viability after depletion of IL-3 at 39 °C (*dashed line*) and 33.5 °C (*solid line*). **A** IC2 parental cells. **B** IC2 cells infected with *v-abl* containing a temperature-sensitive tyrosine kinase. **C** IC2 cells transfected with the human EGF receptor cDNA were cultured in IL-3 medium. Exponentially growing cells were washed, resuspended in medium in the absence (△) or presence of IL-3 (○) or EGF (●) and viable cell numbers were counted

for IL-3 might be substituted by those growth factors in IL-3 dependent cells. To examine this possibility we expressed the human EGF receptor complementary deoxyribonucleic acid (cDNA) in an IL-3 dependent mouse immature mast cell line, IC2 [9]. The cells expressing the EGF receptor proliferated continuously in response to IL-3, whereas EGF only transiently sustained cell viability (Fig. 1C). Both IL-3 and EGF maintained the level of c-*myc* RNA which is necessary for maintenance of cell viability [8]. This indicates that the signal transduction pathways of the IL-3 receptor and the EGF receptor overlap only partially and that IL-3 apparently induces an additional signal(s), not induced by EGF, which leads to long-term proliferation [9].

Molecular Cloning of the IL-3 Receptor

To better understand the structure and function of the IL-3 receptor, we cloned the IL-3 receptor cDNA by using the anti-Aic2 antibody [10]. This antibody was raised against an IL-3 dependent cell line, IC2, and partially inhibits IL-3 binding. A cDNA library from an IL-3 dependent mast cell line, MC/9, was made in the SV40-based mammalian expression vector and was introduced into COS7 cells. After 3 days, COS7 cells expressing the Aic2 antigen were collected by panning using the anti-Aic2 antibody. Plasmid DNA was recovered from the COS 7 cells into *E. coli*. After three cycles of this enrichment, individual plasmids were analyzed and we obtained two different plasmids (AIC2A and AIC2B). Both plasmids expressed the Aic2 antigen on COS7 cells equally well. However, only AIC2A cDNA conferred IL-3 binding following transfection in COS7 cells (Fig. 2) [10, 11].

The AIC2A cDNA encodes a protein of 878 amino acids composed of a signal sequence, an extracellular domain, a transmembrane domain, and a cytoplasmic domain of 22, 417, 26, and 413 amino acid residues, respectively. Fibroblasts transfected with the AIC2A cDNA specifically bind IL-3 and the binding is not competed with by other cytokines, including GM-CSF. IL-3 binds to the AIC2A protein with low affinity ($Kd \sim 10$ nM) and dissociates rapidly ($T_{1/2} \sim 3$ min at 15 °C). These binding characteristics are identical to those of the low affinity binding site in IL-3 responsive cells. Cross-linking of ^{125}I-labeled IL-3 to the AIC2A transfectants has revealed similar protein as to the IL-3 dependent MC/9 cells, suggesting that

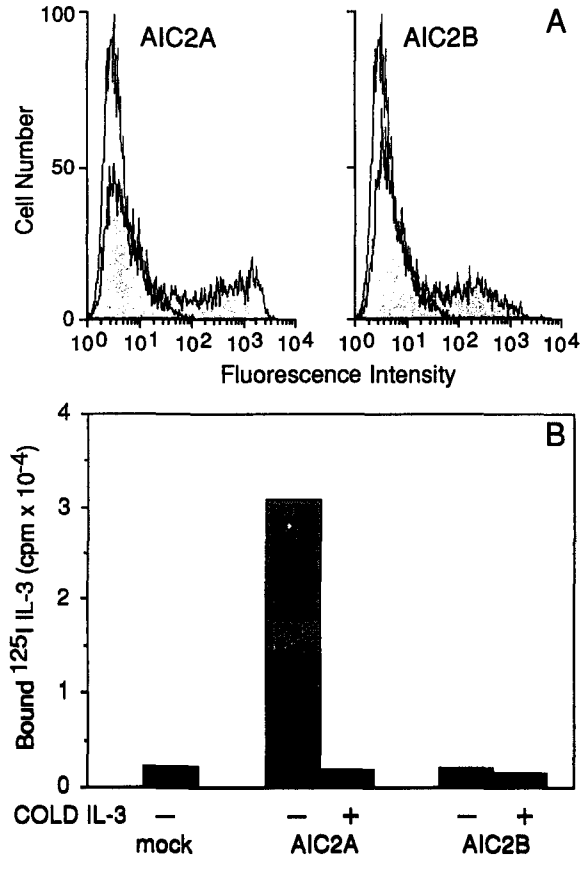

Fig. 2A,B. Expression of AIC2A and AIC2B cDNA in COS7 cells. **A** Anti-Aic2 antibody staining of COS7 cells transfected with AIC2 cDNAs. **B** IL-3 binding to COS7 cells transfected with AIC2 cDNAs

AIC2A is a major IL-3 binding protein [10].

The cytoplasmic domain does not contain any consensus sequence for kinases, phosphatases, or nucleotide binding proteins. Moreover, the Aic2 antigen expressed on L cells is neither down regulated nor induced to phosphorylate by IL-3 [10]. These results suggest that the high affinity IL-3 receptor is composed of multiple subunits and that the AIC2A protein is a major binding protein. This possibility is further supported by the isolation of a high affinity IL-3 receptor from IL-3 depentent cells [19]. Since the high affinity receptor has a tight association with IL-3, cells preincubated with biotin-IL-3 were washed to remove IL-3 bound to the low affinity sites. The biotin-IL-3-receptor complex was then isolated by streptavidin-agarose from a detergent extract. Proteins eluted from the agarose beads by acid were analyzed by SDS gel electrophoresis. We detected a major protein of 120 kDa and additional proteins of 70 and 50 kDa. Western blotting using the antibody against an AIC2A peptide revealed that the 120 kDa protein is the AIC2A protein. In addition, the 120 kDa protein was also recognized by an anti-phosphotyrosine antibody. These results indicate that the 120 kDa AIC2A protein is a major IL-3 binding component of the high affinity IL-3 receptor and that the AIC2A protein can be tyrosine phosphorylated [19]. Additional proteins found in the acid eluate from the agarose beads may involve in formation of the high affinity receptor.

AIC2B, an IL-3 Receptor-Like Molecule

A second cDNA clone, AIC2B, encodes another Aic2 antigen composed of 896 amino acids. Despite its unusually high degree of sequence homology (91% identity) to the AIC2A protein, the AIC2B protein does not bind IL-3 [11]. Nor did we find any specific binding of the AIC2B protein to other cytokines including IL-2, IL-4, IL-5, IL-9, GM-CSF, and erythropoietin.

Amino acid substitutions, deletions, and insertions are dispersed throughout the entire proteins, suggesting that two proteins are encoded by two distinct genes. This is supported by genomic Southern analysis using oligonucleotide probes specific for either AIC2A or AIC2B cDNA [11]. Moreover, cloning of two distinct genomic genes has confirmed the presence of two AIC2 genes. Genetic analysis has localized the AIC2 gene on mouse chromosome 15 and no other locus has been found, suggesting that the two genes are closely linked. In addition, the exon-intron structure of these two genes is very similar. These results indicate that the AIC2A and AIC2B genes were created by gene duplication [20].

Table 1. Expression of the AIC2 RNA

Cell lines	Cell types	AIC2A	AIC2B
IC2	Mast	++	+++
MC/9	Mast	+++	++++
PT18	Mast	++++	+++++
FDCP2	Myeloid	+	++
FDCP2(−)	Myeloid	−	−
NFS60	Myeloid	+	++
M1	Macrophage	+/−	+/−
P388	Macrophage	++	+++++
J174	Macophage	+/−	+
B5B3C4	Pre-B	+	++
BCL-1	B	−	+
CH12	B	+	++++
CH44	B	+	+++
CH32	B	−	+
K23Tr	T	−	−
HT2	T	−	−
D10	T	−	−
ALC8	Stromal	−	−
30E	Stromal	−	−
30R.7	Stromal	−	−
L	Fibroblast	−	−

RNA prepared from various cells were used to evaluate the expression of the AIC2 RNA using the SI protection assays.

Since two genes are so similar, the transcripts of each gene cannot be distinguished by northern analysis. We have developed S1 nuclease protection assays to study the expression of the AIC2A and AIC2B genes. Among various cell lines examined, the AIC2 RNA was detected in all the IL-3 responsive cells and in some IL-3 non-responsive cells. We did not find any AIC2 RNA in T cells, stromal cells, and fibroblasts. Interestingly, IL-3 non-responsive FDCP2(−) cells derived from IL-3 dependent FDCP2 cells do not express any IL-3 binding site nor AIC2A and AIC2B RNA (Table 1). These results suggest that both AIC2 genes are regulated under the same mechanism [11]. Although, the AIC2B protein does not bind any cytokine by itself, there remains the possibility that AIC2B is a component of a cytokine receptor. Recent evidence suggests this possibility [12].

Cytokine Receptor Gene Family

Comparison of the amino acid sequences of the external domains of the AIC2A and AIC2B proteins with other cytokine receptors (IL-2 receptor β chain, IL-4, IL-6, IL-7, GM-CSF, G-CSF, erythropoietin receptors) has revealed significant structural homologies in a stretch of about 200 amino acids (Fig. 3). Two highly conserved motifs are present: one at the N-terminal half of these segments contains four conserved cysteine residues and the other at just upstream of the transmembrane domains contains a Trp-Ser-X-Trp-Ser (WSXWS) motif. The AIC2A and AIC2B proteins have two such segments of the conserved sequences [10, 11]. In addition to the cytokine receptors, growth hormone and prolactin receptors have similar structural motifs [13]. The C-terminal half of the conserved motif of the cytokine receptor family has homology with the type III domain of fibronectin [14].

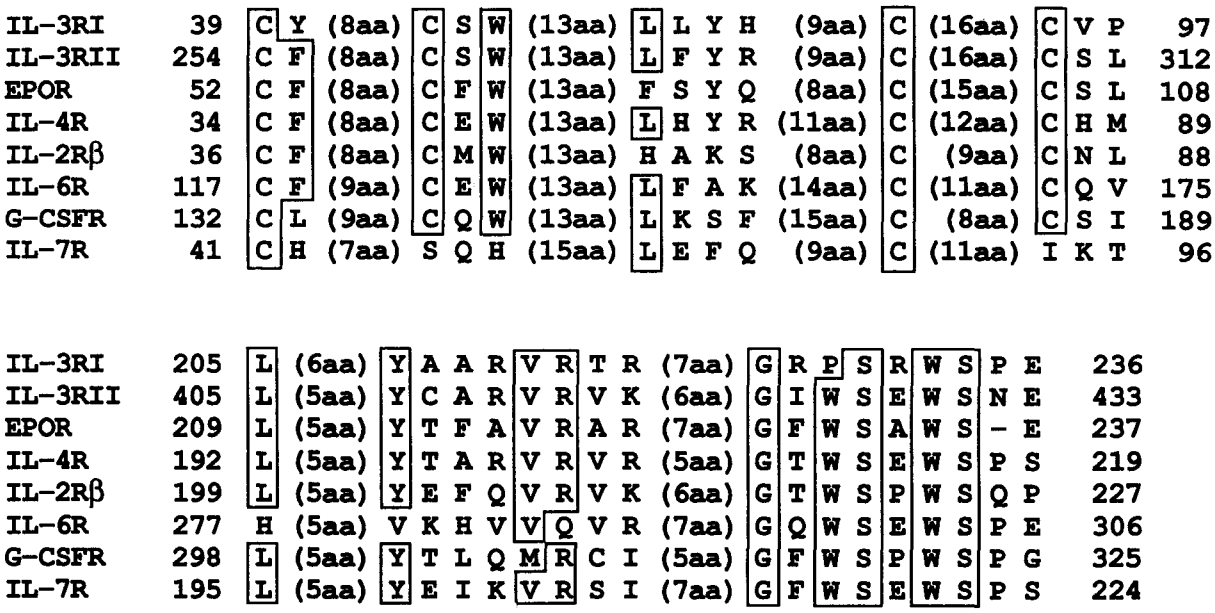

Fig. 3. Common motif of mouse cytokine receptor family. Amino acid sequence comparison of the external domains. Two homologous regions are shown

Another interesting feature of the cytokine receptors is multi-subunit structure. It is well known that the high affinity IL-2 receptor is composed of multiple subunits [15]. The high affinity IL-6 receptor is also composed of at least two subunits [16]. As described above, the high affinity IL-3 receptor requires multiple proteins. Similarly, the GM-CSF receptor cloned by Gearing et al. [17] has a low affinity. We recently isolated a cDNA encoding a second component of the GM-CSF receptor which confers high affinity GM-CSF binding with the low affinity receptor [12]. Interestingly, whereas several cytokines, whose receptor belongs to the cytokine rceptor family, clearly induce protein tyrosine phosphorylation [5, 18], none of those cytokine receptors has a kinase consensus sequence in the cytoplasmic domains nor kinase activity. Therefore functional receptors require additional components, possibly including protein kinase.

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Signal Transduction Through Foreign Growth Factor Receptors and Oncogenes Transfected into Interleukin-3-Dependent Hematopoietic Cells

J. H. Pierce [1]

Signal transduction mediated by growth factor interaction with specific membrane receptors is of critical importance in the regulation of normal cell growth and differentiation. Moreover, increasing evidence indicates that aberrations in these pathways are important in the neoplastic process. Factor-dependent hematopoietic cell lines provide a potentially important means of dissecting differences in growth factor regulatory pathways. In many cases, these lines can either proliferate or differentiate in response to several different hematopoietic cytokines. One such line, 32D, is strictly dependent on interleukin-3 (IL-3) for growth, possesses a normal diploid karyotype, displays an immature myeloid phenotype, and is nontumorigenic in nude mice. Granulocytic colony-stimulating factor (G-CSF) induces terminal neutrophilic differentiation of 32D cells when IL-3 is withdrawn from the culture. 32D cells do not express receptors for macrophage-CSF (CSF-1). Moreover, these cells are devoid of growth factor receptors which regulate cells of connective tissue origin, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors.

The molecular cloning of tyrosine kinase-containing growth factor receptor genes has made it feasible to investigate the ability of these specific receptors to couple with intracellular components needed to evoke a functional response in hematopoietic cells which do not normally express these growth factor receptors. We have introduced expression vectors for several tyrosine kinase-containing growth factor receptors and oncogenes into the 32D cell line to investigate signaling pathways through which they may couple.

An expression vector for the EGF receptor (EGFR) was introduced into the 32D myeloid cell line which is devoid of EGFRs and absolutely dependent on IL-3 for growth [1]. Expression of the EGFR allowed these cells to utilize EGF for transduction of a mitogenic signal (Table 1). When the transfected cells were propagated in EGF, they exhibited a more mature myeloid phenotype than was observed under conditions of IL-3-directed growth. Moreover, exposure to EGF led to a rapid stimulation of phosphoinositide (PI) metabolism, while IL-3 had no detectable effect on PI turnover. Although the transfected cells exhibited high levels of functional EGFRs, they remained nontumorigenic. In contrast, transfection of v-*erb*-B, an amino-terminal-truncated form of the EGFR, not only abrogated the IL-3 growth factor requirement of 32D cells (Table 1), but caused them to become tumorigenic in nude mice. These results showed that a naive hematopoietic cell expresses all of the intracellular components of the EGF-signaling pathway necessary to evoke a mitogenic response and sustain continuous proliferation.

Distinct genes encode α and β PDGF receptors that differ in their abilities to be triggered by three dimeric forms of the

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Mitogenic response and cloning efficiency of 32D transfectants

Cell line	Treatment	SI of ^{3}[H]TdR incorporation	(%) Cloning efficiency
32D	IL-3 + serum	234.5	35.5
	EGF + serum	1.0	< 0.1
	Serum	1.0	< 0.1
32D-v-*erb* B	IL-3 + serum	225.5	28.0
	EGF + serum	231.0	30.5
	Serum	228.0	28.5
32D-EGFR	IL-3 + serum	215.5	27.5
	EGF + serum	185.5	25.0
	Serum	1.0	< 0.1

The cell proliferation assay was performed as described [1]. Results are expressed as a stimulation index. Data are the mean of duplicate samples. The cloning efficiency was established by plating cells at various concentrations in growth medium supplemented with 15% FCS and 0.48% sea plaque agarose. IL-3 (50 U/ml) or EGF (100 mg/ml) was included when specified. Visible colonies were scored at 12 days after plating.

SI: stimulation index; *cpm ^{3}[H] TdR* incorporation with growth factor and/or serum/*cpm ^{3}[H]* incorporation with serum.
FCS: fetal calf serum.

PDGF molecule. We show that PDGF receptor mitogenic function can be reconstituted in IL-3-dependent 32D cells by introduction of expression vectors for either α or β PDGF receptor cDNAs into this naive hematopoietic cell line [2]. Thus, each receptor is independently capable of coupling with mitogenic signal transduction pathways inherently present in these cells. Activation of either receptor also resulted in chemotaxis, alterations in inositol lipid metabolism, and mobilization of intracellular Ca^{2+}. The magnitude of these functional responses correlated well with the binding properties of different PDGF isoforms to each receptor. Thus, availability of specific PDGF isoforms and relative expression of each PDGF receptor gene product are major determinants of the spectrum of known PDGF responses.

The c-*fms* proto-oncogene encodes the receptor for CSF-1. Expression vectors containing either normal or oncogenic point-mutated human c-*fms* genes were transfected into IL-3-dependent 32D cells in order to determine the effects of CSF-1 signaling in this murine clonal myeloid progenitor cell line [3]. CSF-1 was shown to trigger proliferation in association with monocyte differentiation of the 32D-c-*fms* cells. Monocytic differentiation was reversible upon removal of CSF-1, implying that CSF-1 was required for maintenance of the monocyte phenotype but was not sufficient to induce irrevocable commitment to differentiation. Human CSF-1 was also shown to be a potent chemoattractant for 32D-c-*fms* cells, suggesting that CSF-1 may serve to recruit monocytes from the circulation to tissue sites of inflammation or injury. Although c-*fms* did not release 32D cells from factor dependence, point-mutated c-*fms* [S301, F969] was able to abrogate their IL-3 requirement and induce tumorigenicity. IL-3-independent 32D-c-*fms* [S301, F969] cells also displayed a mature monocyte phenotype, implying that differentiation did not interfere with progression of these cells to the malignant state. All of these findings demonstrate that a single growth factor receptor can specifically couple with multiple intracellular signaling pathways and play a critical role in modulating cell proliferation, differentiation, and cell migration.

Proliferation and maturation of hematopoietic cells is a complex but orderly process involving growth factor-controlled programs that lead to self-renewal or terminal differentiation. Although many of the genes encoding growth factors and several of their receptors have been cloned, the intracellular mechanisms of action of these signaling pathways are not well understood. The recent cloning of IL-3 and G-CSF receptors has revealed that these genes do not contain tyrosine kinase-like domains. However, it has been demonstrated that at least IL-3 does stimulate the tyrosine phosphorylation of specific intracellular substrates in murine cell lines.

The fact that 32D is a clonal myeloid precursor cell line has raised the question as to whether signals transduced by all activated receptor tyrosine kinases might interact with the same specific intracellular substrates. After introduction of the tyrosine kinase receptors into 32D cells, they showed evidence of proliferation as well as partial myeloid maturation in response to their respective ligands. Since IL-3 only induces proliferation in this system and G-CSF strictly triggers terminal differentiation of these cells to mature neutrophilic granulocytes, it may now be possible to correlate specific alterations in second messenger systems or tyrosine-phosphorylated substrates involved in regulating specific proliferation and/or differentiation pathways within the myeloid lineage.

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Genetic Analysis of Ion Transport

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Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder, and di Sant' Agnese et al. [3] found that the sweat of CF patients contains an excess of sodium and chloride ions. Defects in the regulation of chloride ion transport have been documented in CF epithelial cells [5, 10, 14, 16]. The chloride channel normally responds to β-adrenergic agents, but CF cells are defective in this response [6, 9, 14]. It has been proposed that the CF defect involves a pathway whereby cAMP regulates ion transport.

The symptoms of CF patients are heterogeneous between and within families [13]. Although most individuals are diagnosed by the time they reach the age of ten, a few remain undiagnosed until adulthood [2, 15]. Approximately 15% of CF patients do not require supplemental pancreatic enzymes and are designated as pancreatic sufficient (PS) [7]. PS is typically concordant within families, suggesting that PS patients may have less severe mutations in the CF gene. However, the heterogeneity within families suggests that additional genetic and environmental factors contribute to the severity of the disease.

The molecular cloning of the CF gene has provided additional research strategies to further understand the disease, and the regulation of ion transport in secretory cells. The gene encodes a 170 kDa polypeptide that is a member of a superfamily of membrane-bound active transport molecules [4, 11, 12]. A three-nucleotide deletion in a putative ATP binding domain has been found in 70% of CF chromosomes; this alteration removes a phenylalanine codon at position 508 (ΔF508). To further understand the relationship between mutations in the gene and the phenotype of patients, we have examined a group of patients who do not contain the common mutation on both chromosomes.

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Methods and Results

To identify mutations in the CF gene, specific regions were amplified by the polymerase chain reaction (PCR) and assayed for single-stranded conformation polymorphisms (SSCPs). This newly described method allows the rapid screening of samples for the presence of genetic variation [8]. SSCPs are detected by denaturing the DNA and resolving it on nondenaturing acrylamide gels. Each strand of the DNA fragment can potentially form a unique conformation (and

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Table 1. Detection of mutations using the SSCP technique

Exon	PCR product size (bp)	Intron or exon	Number of patients tested	Number of mutation per exon
1	180	E	150	
2	340	I	150	
3	310	I	50	
4	210	E	150	3
5	400	I	150	
6A	330	I	150	2
6A	420	I	150	
7	240	E	150	3
8	330	I	25	
9	560	I	150	
10	190	E	150	1
11	425	I	150	
12	420	I	75	
13	720	E	150	2
14A	500	I	150	1
14B	–	–	–	
15	480	I	–	
16	–	–	–	
17A	–	–	–	
17B	–	–	–	
18	100	E	75	
19	250	E	150	1
20	470	I	–	
21	480	I	–	
22	170	E	50	
23	–	–	–	
24	200	E	150	

E: primers fully contained within the exon; *I:* primers from flanking intron sequence.

have a distinct mobility), and any mutation within that segment can potentially affect the mobility. We screened 150 CF patients who have at least one chromosome that does not contain the common (F508) mutation (Table 1). Primers were chosen to individually amplify coding regions of the gene. Each patient that displayed an aberrantly migrating fragment on an SSCP gel was chosen for the subsequent direct sequence analysis.

Alterations were classified as CF mutations based on the following criteria: (1) The alteration shifts the reading frame and causes premature termination of the protein; (2) An amino acid is replaced with a dissimilar residue, and this alteration does not occur on a large number of normal chromosomes with the same haplotype. Eleven separate CF mutations have been identified, eight of which are frameshift or nonsense mutations and three that replace amino acids (Table 2). Each of the frameshift mutations has been found in only a single family, whereas two of the three-point mutations are found in multiple families. The phenotypes of the patients are summarized in Table 2. All individuals that we have examined that are homozygous for the ΔF508 mutation are pancreatic insufficient (PI) and have moderate to severe disease (data not shown). Most of the patients with the frameshift mutations are homozygous for the absence of the common mutation, and therefore must contain an additional, unidentified mutation. These patients are clinically het-

Table 2. CFTR mutations

Name	Introduced change	Exon	ΔF508[a] status	Number of individuals	Nationality	Pancreatic status
444delA	Term	4	+/+	1	Afr Am	PI
G460C	Asp/His	4	+/−	1	Caucasian	PI
G482A	Arg/His	4	+/−	6	Caucasian	PS
1154insTC	Term	7	+/−	1	Caucasian	PI
G1172C	Arg/Pro	7	+/−	5	Ger/Fr	PS
1213delT	Term	7	+/+	1	It/It	PS
1677delTA	Term	10	+/+	1	Russian	—
2522insC	Term	13	+/+	1	Italian	PI
2566insAT	Term	13	+/+	1	Fr/It	PI
C2683T	Term	14A	+/+	1	Caucasian	PI
3821delT	Term	19	+/+	1	Russian	PS

Mutations are named by nucleotide number, according to Riordan et al. [11].
CFTR: CF transmembrane conductance regulator; *Term:* termination;
Afr Am: Afro-American; *Ger:* German; *Fr:* French; *It:* Italian.
[a] + refers to wild-type (absence of F508).

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1063        TTCTTCTCAGGGTTCTTTGTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGA
            F  F  S  G  F  F  V  V  F  L  S  V  L  P  Y  A  L  I  K  G
LRO         ..............................................................
COW         ..............G...............................................
SHEEP       ..............A.....................T.....GC.....................
WHALE       ..............A.....................T.....GC.....................
MOUSE       ..............T.....................G.....AC.....................
FROG        ..............A.....T.....T.....T.....GC.T.....................

1123        ATCATCCTCCGGAAAATATTCACCACCATCTCATTCTGCATTGTTCTGCGCATGGCGGTC
            I  I  L  R  K  I  F  T  T  I  S  F  C  I  V  L  R  M  A  V
LRO         ..........A.........A.....................................A...
COW         ...G......A..............................................
SHEEP       ........T..A.........A.....................C.............A..T
WHALE       ........T..A.........A.....................................A..T
MOUSE       ...G......A.........A.....................C...T.........A...
FROG        ...GG...T..A.........A.....................C.............A..

1183        ACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAACAAA
            T  R  Q  F  P  W  A  V  Q  T  W  Y  D  S  L  G  A  I  N  K
LRO         .....A.......................
COW         ............................
SHEEP       ........G..C.............T.
WHALE       ........G..C.....A.......T.
MOUSE       ..........C.................
FROG        ..........C.............T.
```

Fig. 1. Sequence of a portion of the CFTR transmembrane region from exon 7. The nucleotide sequence is shown from humans, as well as other vertebrate species, with numbering of the human sequence as in Riordan et al. [11]. The *underlined* codon is arginine 347, which was found mutated in several CF patients (mutation G1172C, Table 2). *LRO*: lion tamarin; *WHALE*: humpback whale; *FROG*: *Xenopus*; *MOUSE:* Balb/c

erogeneous; they typically have moderate to severe disease. The majority of the patients with CF point mutations have ΔF508 on their other chromosome, are diagnosed as PS, and have mild disease. Therefore, the type of mutation at the CF locus appears to play an important role in the clinical presentation of the patient.

To further explore the role of the missense mutations in the function of the CF Transmembrane conductance regulator (CFTR) we have amplified the region surrounding these mutations from DNA from a variety of species. The sequence of the species obtained is displayed in Fig. 1. All of the residues that we have found mutated are conserved in all of the species examined. Overall, these regions of the gene show a high degree of conservation, suggesting that alterations in the transmembrane domain are poorly tolerated.

Discussion

Because the most common mutation accounts for only 70% of CF chromosomes [4], a large proportion of CF patients (40%–50%) are compound heterozygotes, i.e., they have two different mutations in the gene. Thus there is a large number of possible genotypes found in CF patients. This appears to account, in part, for the variation observed in the phenotype of patients. However, within families affected individuals can show differences in sweat chloride levels and severity, demonstrating that additional genetic and/or environmental factors contribute to these phenotypes.

The clearest correlation between the patient's genotype and phenotype is seen in the pancreas. All patients we have observed that are homozygous for the ΔF508 deletion are PI. However, even in these patients, genetically identical at the CF locus, there is considerable variation in clinical outcome. This variation is expressed in the age of diagnosis, pulmonary function, and sweat chloride value. In the lungs of CF patients, damage is principally caused by bacterial infection. These infections are believed to be secondary to the abnormal mucus present in patients. Furthermore, immune function genes such as the human lymphocyte antigens (HLA) and/or the T cell receptor locus could play a role in the susceptibility and/or response to bacterial infection.

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Virology

The Molecular Biology of the Myeloproliferative Leukemia Virus

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Introduction

Most acutely transforming leukemogenic or sarcomagenic retroviruses have transduced in their genomes altered cellular genes named oncogenes [1]. These viruses are usually defective in replication as a result of the deletion of structural genes, and they require a helper virus for propagation. Isolation of new acutely transforming retroviruses, comprehensive studies of their physiopathological processes, and molecular analysis of their genomes remain, therefore, powerful means for the identification of key genes involved in the regulation of cell growth, differentiation, or development.

The purpose of this paper is to summarize the data that we have accumulated over the past few years on a recently isolated acute leukemogenic murine retrovirus named myeloproliferative leukemia virus (MPLV).

Isolation of the MPLV

We isolated the MPLV in 1985 at the Curie Institute (Orsay, France) during a research program designed to evaluate the in vivo transforming properties of different Friend helper viruses (F-MuLV). While several clonal F-MuLV isolates have the capacity to induce a rapid erythroblastosis in newborn-inoculated NIH Swiss or BALB/c mice [2], DBA/2 mice were found to be resistant to this early erythroleukemia [3]. Nevertheless, they developed various types of hematopoietic malignancies after a latent interval of 7–12 months [4–6]. In general, these leukemias (either myelogenous, lymphoid, or erythroid) were associated with a more or less severe anemia.

Out of 238 DBA/2 mice inoculated at birth with F-MuLV clone 57 [7], one mouse developed, after 7 months of infection, an hepatosplenomegaly unusually accompanied with a polycythemia. Cell-free extract prepared from the original leukemic spleen or supernatant medium from an in vitro permanent cell line derived from the leukemic spleen cells caused an explosive leukemia upon inoculation into adult mice of most strains, including C57Bl strains. The disease was characterized by hepatosplenomegaly, polycythemia, pronounced myelemia but no thymus or lymph node involvement, and death within 1–3 months. Spleen and liver were extensively infiltrated with maturing precursor cells belonging to the granulocytic, erythroblastic and megakaryocytic lineages. Typically, the blood of severely diseased animals was also massively invaded by morphologically normal polymorphonuclears, erythroblasts, and platelets.

Several hematopoietic lineages were obviously involved in this disease, hence our name for the virus isolate, "myeloproliferative leukemia virus" [8].

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Genetic Analysis of MPLV Isolate

Virologic studies of MPLV by Penciolelli et al. [9] demonstrated that this highly leukemogenic virus isolate contained two dissociable retroviral genomes: one was the parental replication-competent F-MuLV 57, and the second was a new replication-defective component now designated as MPLV. A comparison of viral RNA species expressed in F-MuLV alone or F-MuLV + MPLV-producing cells by Northern blot analysis showed that MPLV was 0.8 kb shorter than F-MuLV and that a deletion had probably occurred in the MPLV *env* gene. This was further confirmed by the establishment of the MPLV restriction endonuclease map which was compared with that of F-MuLV [10]. From their data, these investigators concluded that the MPLV-defective genome

(a) was derived from F-MuLV,
(b) had conserved the F-MuLV *gag* and *pol* regions, and
(c) was deleted and rearranged in its *env* region [9].

Although MPLV does not transform fibroblasts in culture, its isolation free of replicating F-MuLV in nonproducer cells was feasible since the MPLV titer in the original isolate was approximately equivalent to that of F-MuLV. By the technique of limiting dilution and single-cell cloning, nonproducer cells containing MPLV were derived from *Mus dunni* fibroblasts [9]. Supernatant medium from these nonproducer cells did not cause any disease in inoculated mice demonstrating the defectiveness of MPLV. However, when superinfected with a variety of replicating helper viruses, supernatants reproduced the same acute myeloproliferative syndrome as caused by the original isolate. These experiments provided circumstantial evidence that the helper-dependent MPLV genome contained the genetic information necessary for the observed pathological processes.

Genomic Composition of MPLV

In an attempt to define the origin and nature of the genetic sequences contained in the MPLV-rearranged *env* region, Souyri et al. [11] derived cDNA probes which were nonhomologous to sequences contained in F-MuLV. Two probes were found to be MPLV specific, in that they hybridized to RNA of MPLV-containing nonproducer cells but did not hybridize to RNA of ecotropic MuLVs nor to RNA of amphotropic or xenotropic murine viruses. This indicated that, in contrast to Friend spleen focus-forming viruses (SFFV), MPLV did not result from a recombination between F-MuLV and a portion of the *env* gene of murine xenotropic virus [12, 13].

A full-length biologically active MPLV provirus was molecularly cloned from a genomic library of a nonproducer *Mus dunni* clone [11]. Sequence analysis revealed that the MPLV *env* gene contains a large open reading frame which could code for a polypeptide of 284 amino acids. This protein would contain 64 amino acids derived from the amino terminus of the F-MuLV gp70, including the signal peptide, 36 amino acids from a central region of the F-MuLV *env* gene, and 184 amino acids that are specific to MPLV (Fig. 1). A hydrophobicity plot of the amino acids sequence revealed that, in addition to the 34 hydrophobic amino acids of the gp70 signal peptide, the MPLV-specific domain contained a stretch of 22 uncharged amino acids. Thus, the putative MPLV *env* product presents the features of a transmembrane protein comprising an extracellular domain of 143 amino acids, a single transmembrane domain of 22 amino acids, and a cytoplasmic domain of 119 amino acids without consensus sequence for kinase activity [14]. Computer analysis of the deduced amino acid sequence revealed that the MPLV-specific sequence did not correspond to any known genes.

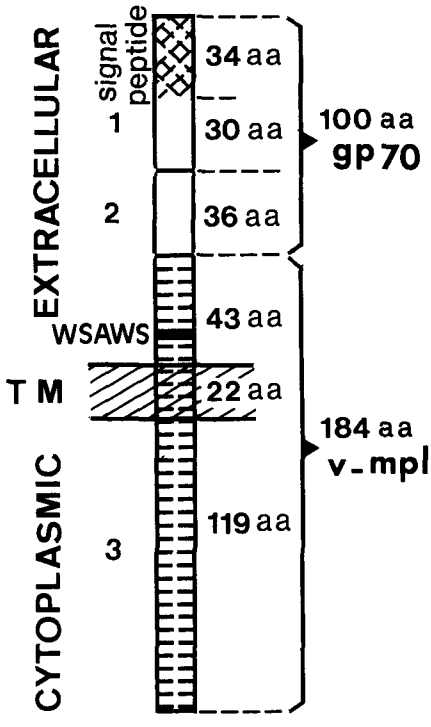

Fig. 1. Schematic representation of the putative MPLV *env* product. The ENV-*mpl* fusion protein consists of three fragments: part *1* derives from the NH_2-terminal region of the F-MuLV envelope gene (gp70) and contains a signal peptide; part *2* derives from a central part of the F-MuLV gp 70; part *3* corresponds to a transduced cellular sequence, most probably truncated at its N-terminus. The product encoded by this rearranged gene has the features of a single membrane-spanning domain (TM). The extra cellular domain of v-*mpl* possesses the amino acid sequence WSAWS highly conserved in the hematopoietin receptor superfamily, while the cytoplasmic domain does not contain consensus sequence for know catalytic activity

MPLV Has Transduced a Novel Oncogene

Since nonviral sequences found in the genome of acutely transforming retroviruses derive from cellular genes and are conserved phylogenetically, we looked for the presence of MPLV-specific sequences in genomic DNA from different mammals. Under stringent hybridization conditions, discrete bands were revealed in DNAs from mouse, rat, mink, dog, cow, and human. In addition, MPLV-specific probes recognized a 3.0-kb mRNA in spleen and bone marrow from adult mice and in fetal liver cells, but not in nonhematopoietic tissues [11]. Thus, taking into consideration the biological properties of MPLV, the cellular origin of the sequence contained in its *env* gene, the conservation in the genome of mammals and the expression in normal hematopoietic tissues, we concluded that MPLV had transduced a novel oncogene which was designated as v-*mpl*. By in situ hybridization and genetic analysis studies, chromosomal localization of the c-*mpl* proto-oncogene was assigned to mouse chromosome 4 (Vigon et al., unpublished data) and to human chromosome 1p34 [15].

Leukemogenic Properties of MPLV

In vivo studies by Wendling and coworkers have indicated that MPLV induced a rapid suppression of growth factor requirements for in vitro colony formation of a large spectrum of committed as well as multipotential progenitor cells [16, 17]. The primary manifestation of viral infection was a switch to erythropoietin (EPO) independence of the colony forming unit–erythroid (CFU-E) population which was complete in the spleen after 6 days of infection. A possible stimulating effect of EPO present or secreted in the culture medium was ruled out by the addition of neutralizing anti-EPO antibodies to the culture system. The effects of MPLV infection on the early and primitive erythroid progenitor cells (BFU-E) was assessed in methylcellulose serum-free cultures. It was found that well hemoglobinized pure and mixed erythroid colonies developed without the addition of interleukin-3 or EPO. Moreover, while a majority of colonies contained erythroblasts mixed with megakaryocytes, about 12% revealed three or more lineages of differentiation [16]. Further in vivo studies have documented that MPLV infection also induced the spontaneous colony formation of myeloid progenitors, i.e., granulocyte macrophage colony-forming cell (GM-CFC) granulocyte (G)-CFC, mega-

karyocyte (Meg)-CFC, and mixed CFC, probably as a result of direct infection of these progenitors and not as a consequence of a paracrine secretion of soluble colony stimulating factors by the accessory cells [17]. These observations supported the conclusion that MPLV acts on various progenitors, inducing their proliferation and terminal differentiation independently of signals normally provided by colony stimulating factors, interleukins, EPO, or any conditioned medium.

However, formal proof that MPLV can transform hematopoietic target cells in the absence of coinfection with a replicating MuLV was not provided by these experiments. We addressed this question by producing helper-free MPLV stocks using the packaging psi-CRE cell line that produces a high titer of infectious, nonreplicating particles but does not yield helper virus [18]. When adult ICFW mice were intravenously given helper-free preparations of MPLV, more than 90% of the mice were healthy 2 months after inoculation. Nevertheless, we observed that MPLV induced a mild but transient spleen enlargement with the appearance of colonies well visible on the spleen surface on days 5, 10, and 15 after inoculation. Histologically, colonies were composed of erythroblasts, or erythroblasts, granulocytes, and megakaryocytes clustered together in the splenic red pulp. On day 25 and thereafter, these colonies disappeared, leaving spleens with a normal aspect. In contrast, when helper-free preparations of MPLV were injected into mice pretreated with the aplastic drug 5-fluorouracil (5-FU, 150 mg/kg body weigth, 4 days before virus inoculation), all animals developed a typical MPLV syndrome and died from overt leukemia within 2 months (Wendling et al., unpublished data).

Together these data indicate that

(a) the MPLV component is primarily responsible for the myeloproliferative effects of the viral complex,
(b) expression of MPLV in erythroid and myeloid progenitors abolishes their growth factor requirement for in vitro colony formation, and
(c) induction of leukemia occurs in 5-FU-pretreated mice, suggesting that stable infection of cycling primitive progenitors is critical for leukemia development.

In Vitro Transformation Properties of MPLV

An area of current research in our laboratory is related to the ability of a helper-free preparation of MPLV to transform hematopoietic cells in vitro. A 2-h incubation of bone marrow cells enriched in highly dividing primitive progenitors by treatment of mice with 5-FU was sufficient to induce autonomous colony formation of about 30% of the colony-forming cells present in the preparation. Cytologically, half of these spontaneous colonies were composed of either granulocytes, megakaryocytes, or erythrocytes, while the remainders were mixed colonies of which about 20% contained three or more lineages of differentiation. Upon replating, the multilineage colonies produced secondary and tertiary mixed colonies, suggesting self-renewal [11].

The question of whether or not transformation of hematopoietic progenitors would lead to the generation of immortalized cell lines was then investigated. When marrow cells were cultured in liquid medium, it was observed that rapidly dividing nonadherent cell populations were produced in MPLV-infected cultures. After 10 to 12 days, these nonadherent populations could be transferred into fresh flasks devoid of stromal feeder layers. Cells continued to proliferate and generated permanent suspension cultures containing polymorphonuclears, megakaryocytes and erythroblasts. Upon continuous passages, the majority of the cell lines evolved towards a more restricted phenotype which remained stable over several months. Diverse immortalized

megakaryocytic, myelomonocytic, erythroblastic, or mastocytic cell lines retaining the ability to differentiate could easily be obtained. Since these permanent cell lines evolved from a multipotential to a more restricted phenotype, we investigated whether they were polyclonal or monoclonal by studying proviral-cell DNA junctions. Cultures were polyclonal 5 days after initiation. However, after 3 weeks and at a time where all cultures displayed a multipotential phenotype, one or a few major proliferating clones were detected in each cell line. Interestingly, the same clones were still found after 3 months of continuous passages when the cell lines appeared to be restricted in their differentiation potential [11]. Thus, it seems likely that MPLV induces the clonal outgrowth of a single or few transformed, probably multipotential, stem cells (clonal selection), the full differentiation capabilities of which being lost along with continuous culturing (clonal evolution).

The obtaining of immortalized in vitro cell lines raised the question of whether cells were tumorigenic. To approach this problem, 2×10^6 cells were subcutaneously grafted into either syngeneic or nude mice. Upon repeated assays, none of the cell lines developed tumor nodules at the site of inoculation when cells from cultures less than 4 months old were grafted. After prolonged passages (more than 7 months), 60% of the cell lines produced hematopoietic subcutaneous tumoral nodules, suggesting that additional genetic events must have occurred to reach a full malignant state.

Summary and Current Knowledge

The myeloproliferative leukemia virus isolate consists of two distinct viral components: a replicating F-MuLV and a helper-dependent MPLV. MPLV accounts for the rapid in vivo and in vitro transformation of a broad spectrum of multipotential, myeloid, and erythroid progenitors which acquire growth factor-independent proliferation and differentiation. By sequence analysis of a biologically active clone, MPLV has been shown to be an *env* recombinant virus containing sequences derived from the F-MuLV *env* gene and additional nonviral cellular sequences. These nonviral sequences are conserved in various mammals and are expressed in hemopoietic tissues from normal mice. MPLV was thus generated by transduction of an oncogene (v-*mpl*) in the envelope region of an F-MuLV genome. v-*mpl* does not correspond to any known gene, but the putative MPLV *env* fusion product has the features of a transmembrane protein with the N-terminal signal sequence of the F-MuLV gp70 directing the polypeptide across the membrane and a single transmembrane domain. Interestingly, the extracellular domain of v-*mpl* possesses, 13 amino acids upstream to the membrane-spanning domain, the amino acid sequence WSXWS, highly conserved in all cytokine receptors that make up the hematopoietin receptor superfamily [19]. In addition, a significant number of conserved amino acids were found when the extracellular domain of v-*mpl* was aligned with that of the IL-2β, IL-3, IL-4, IL-6, IL-7, GM-CSF, G-CSF, and EPO receptors [11]. Since the N-terminal part of the fusion protein consists of F-MuLV-derived sequences, it is not yet known whether the c-*mpl* proto-oncogene product would contain the highly conserved cysteine residues characteristically found in the ligand-binding domain of each of these receptors [19]. Nevertheless, with regard to the general features of v-*mpl*, it is tempting to speculate that MPLV has transduced a truncated form of a putative cytokine receptor. Cloning of the proto-oncogene cDNA is currently underway in our laboratory to allow further comparison.

A major focus of future research will be to understand the mechanism by which this viral oncogene can short-circuit the growth-regulatory signals delivered by the binding of various hematopoietic growth factors to their specific receptors.

This requires further studies on the mechanism of signal transduction by MPLV and by other receptors of the same family.

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Raf Function Is Required for Proliferation of NIH|3T3 Cells and Transformation by Nonnuclear Oncogenes

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Studies of oncogenes have established that their cellular homologs are part of a kinase cascade mechanism that regulates the translation of exogenous growth factor stimulation into a mitogenic gene response [reviewed in 1]. The cellular homolog of the v-*raf* oncogene [2], c-*raf*-1 [3], encodes a mitogen-regulated serine/threonine-specific protein kinase, whose specific activity is enhanced by a large number of growth factors [4–13]. Moreover, treatment of NIH/3T3 fibroblasts with platelet-derived growth factor (PDGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes translocation of a fraction of Raf-1 from the cytosol to the nucleus [12]. A similar fraction of v-Raf is found associated with the nucleus also in the absence of stimulation (W. B. Anderson and U. R. Rapp, unpublished results). These observations led to the speculation that Raf-1 serves as a shuttle enzyme which converts peripheral signals into a transcriptional response that results in mitogenesis [14]. Consequently, a key step in transformation can be envisioned to uncouple Raf-1 kinase from mitogen regulation, either through constitutive stimulation by upstream signal transducers or mutational activation of *raf* itself. This hypothesis was tested in NIH/3T3 fibroblasts using three complementary approaches to interfere with the function of normal and transforming versions of Raf-1. These strategies included expression of c-*raf*-1 antisense RNA or kinase inactive c-*raf*-1 mutants, as well as the analysis of a cellular mutant phenotype that can suppress v-*raf* transformation.

The first two approaches were used to determine whether Raf-1 is an essential component in receptor signaling mediated by serum growth factors. Serum was chosen for its pleiotropic action supplying both factors necessary for viability and long-term proliferation of fibroblasts. The major mitogen in serum is PDGF, which was previously shown to regulate Raf-1 kinase activity [4, 11]. As many growth factors also stimulate protein kinase C (PKC) [reviewed in 15], another serine/threonine kinase, activation of PKC by the tumor promoter TPA was examined as well. Different portions of mouse and human c-*raf*-1 cDNAs were expressed in both sense and antisense orientation using the retroviral expression vector pMNC (from Dr. B. Seed). This vector contains Moloney murine/leukemia virus (MoMuLV) long terminal repeats (LTRs), a neomycin resistance gene, and a human cytomegaly virus promoter that drives transcription of inserted sequences. As an initial screening assay these constructs were transfected into NIH/3T3 cells and the number of neomycin-resistant (neo^R) colonies was scored. Antisense constructs consistently yielded approximately twofold less

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colonies than the corresponding sense construct or the vector control plasmid indicating that expression of *raf* antisense RNA interferes with viability and/or proliferation of NIH/3T3 cells cultivated in the presence of 10% fetal calf serum. Based on our previous speculation that the Raf-1 regulatory domain interacts with activating ligands [14, 16], we reasoned that Raf protein inhibition might also be achieved through a competitive mechanism. Indeed, expression of a truncated Raf-1 protein, which encompasses most of the regulatory domain, reduced colony yield about fourfold. Expression of a mutant Raf-1 protein, cRaf301 (plasmid p301-1), that carriers an inactivating point mutation in the ATP binding site (lysine^{375} to tryptophan) was even more efficient, decreasing colony yield about sevenfold. This type of inhibition therefore most likely involves competition for activating ligands as well as Raf kinase substrates. Efforts to establish stable cell lines from the Raf inhibition experiments with NIH/3T3 cells repeatedly failed. The majority of cell clones died out and others lost expression of the transfected *raf* sequences after being kept in culture for six to ten passages.

Therefore, we turned to *raf* transformed cell lines where two parameters, morphological reversion and inhibition of proliferation, were studied. Transfection with the mutant *craf*301 expression plasmid efficiently reduced neo^R colony yield and moreover induced complete or partial reversion of the transformed phenotype in 25% or 35% of neo^R colonies, respectively. In both respects, expression of *raf* antisense RNA was less potent. It, however, yielded a range of cell clones with different morphologies that were stable enough to permit biochemical analysis. *raf*-transformed cells synthesize DNA even when serum-starved. The ability of morphological revertants to replicate DNA constitutively or in response to serum growth factors or TPA was reduced or eliminated in direct proportion to reduction in Raf protein levels (Fig. 1). In GMS-8/2, a cell clone that expresses low levels of v-Raf and Raf-1 proteins, constitutive DNA synthesis was decreased in starved cells, but still could be stimulated by TPA or serum. Further reduction of Raf protein levels in clone GMS-8/3 resulted in a substantial decline in serum and an almost complete loss of constitutive and TPA-inducible DNA replication. We conclude that, at least in NIH/3T3 cells, Raf-1 is an essential component of signal transduction pathways used by serum growth factors and PKC.

Given the strong evidence that Ras proteins are crucial regulators of signals arising at the cell membrane, this study was extended to *ras*-transformed cells. v-Ki-*ras*-transformed NIH/3T3 cells were transfected with the p301 plasmid series (Table 1). Judged by the neo^R colony yield, proliferation was impaired to a similar degree as seen in NIH/3T3 cells. Morphological reversion of *ras*-transformed cells, however, was about twofold less efficient than in *raf*-transformed cells. This difference could be due to secondary events that occured after transformation or else indicate that *ras*-induced transformation is only partially dependent on Raf-1. To test the effect of *raf* inhibition on the initiation of *ras* transformation a constant amount of v-Ha-*ras* (pSV2neo/*ras* [17], from Dr. T. Shih) plasmid was cotransfected with an equal or 4:1 molar excess of the p301 vectors (Table 2). Although the neomycin resistance marker of pMNC-based plasmids accounts for a background of flat neo^R colonies that presumably do not express pSV2neo/*ras*, it is clearly evident that transfection with p301 vectors causes an increase in morphologically reverted colonies at the expense of transformed colonies. The inhibition is dose-dependent and higher with the *craf*301 sense construct (p301-1). Transfected in a fourfold excess p301-1 almost completely interferes with the establishment of *ras* transformation indicating that Raf-1 function is necessary for *ras*-mediated proliferation as well as initiation of transformation.

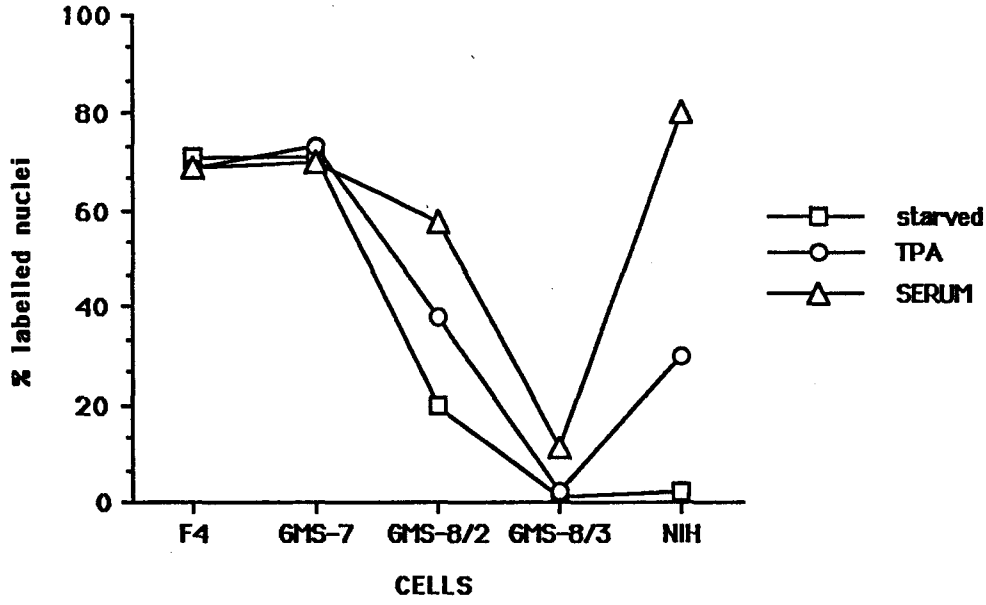

Fig. 1. Mitogen responsiveness of Raf-depleted cells. A 3611-MSV (v-*raf*)-transformed NIH/3T3 cell line, F4, was transfected with neo^R pMNC plasmids expressing *raf* antisense RNA targeted at the kinase domain (GMS-8) or a corresponding sense RNA (GMS-7). The analysis of neomycin-selected clones demonstrated that morphological reversion correlated strictly with down-regulation of v-raf and endogenous Raf-1 protein levels by antisense RNA, whereas expression of sense RNA showed neither effect. GMS-8/2 and GMS-8/3 are flat antisense induced revertant clones which, as judged by western blotting, express very low or undetectable levels of Raf proteins, respectively. GMS-7 represents a pool of ten clones transfected with the corresponding sense control plasmid. Selected clones were tested for mitogen responsiveness. DNA synthesis induced by serum or TPA in serum-starved cells is depicted as the number of nuclei incorporating [^{3}H] thymidine

The requirement of Raf function for transformation was more extensively studied in CHP25 cells. CHP25 is a flat revertant cell line that was generated from v-*raf*-transformed NIH/3T3 fibroblasts by selection with 4-*cis*-hydroxyproline, an amino acid analog which is significantly more toxic for fast growing, transformed cells than for normal NIH/3T3 cells [18, 19]. CHP25 cells express a functional v-*raf* oncogene, but are nontumorigenic, and do not form colonies in soft agar indicating that the revertant cell phenotype is due to acti-

Table 1. *raf* inhibition blocks *ras* mediated proliferation

Plasmids	Yield of neo^R colonies	Morphology of neo^R colonies		
		Flat (%)	Intermediate (%)	Transformed (%)
pMNC	197	0	0	100
pMNC301-2	120	2	15	83
pMNC301-1	58	15	15	70

V-Ki-*ras* transformed cells were transfected with the indicated plasmid DNAs, and neo^R colonies were microscopically examined for morphological reversion. pMNC301 are pMNC vectors which express the point-mutated c*raf*301 cDNA in either sense (p301-1) or antisense (p301-2) orientation. Percentages are calculated based on examination of a total of ≥ 200 colonies per transfection.

Table 2. *raf* inhibition blocks *ras* transformation

Plasmids	Inhibition	Morphology of neo^R colonies		
	(%)	Flat (%)	Intermediate (%)	Transformed (%)
Ratio 1:1				
ras + pMNC	0	27	17	56
ras + p301-2	53	28	46	26
ras + p301-1	61	46	32	22
Ratio 1:4				
ras + pMNC	0	33	23	44
ras + p301-2	61	48	35	17
ras + p301-1	84	67	25	7

NIH/3T3 cells were co-transfected with v-Ha-*ras* (pSV2 noe/ras) and p301.

vation of a cellular suppressor function. The variant phenotype presumably resulted from a single mutation, since retransformants could readily be isolated from cultures growing at high cell densities. CHP25 cells are resistant to transformation by *sis*, *ras*, and tyrosine kinase as well as serine/threonine kinase family oncogenes suggesting that Raf functions downstream of most peripheral signal transducers. In contrast to v-*raf*-transformed cells, in which the endogenous Raf-1 protein kinase is constitutively activated, v-Raf in CHP25 cells did not activate endogenous Raf-1 kinase. Since mitogen regulation of Raf-1 kinase in CHP25 cells remained intact CHP25 cells seem to be blocked at the level of Raf-1 substrate phosphorylation. Consistent with this interpretation, CHP25 cells show specific alterations of early gene induction. The serum induction of c-*fos* and *jun*D as well as the serum and TPA induction of *jun*B and *egr*-1 are almost completely abolished. These data demonstrate that Raf-1 signaling is essential for transformation of NIH/3T3 cells by peripheral oncogenes and for regulation of a subset of early-response genes by TPA and serum growth factors. The availability of a permanent cell line, CHP25, that is blocked at the level of Raf function should greatly facilitate the dissection of this pleiotropic *raf*-dependent gene response pattern as well as help to identify raf substrates which mediate *raf*-regulated mitogenesis.

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erb-B*[a]: An "Ignition Spark" for the *Xiphophorus* Melanoma Machinery?

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Introduction

Neoplasia is not limited to human beings, or to mammals, but can develop in all taxonomic groups of the recent Eumetazoa and even in multicellular plants. It therefore appears to be inherent to the multicellular organization of life [1]. The oncogenes that are associated with human cancer are also distributed throughout the animal kingdom [2–9]. Moreover, tumor-suppressor genes [10] that may control the expression of oncogenes and the manifestation of a tumor phenotype have been identified in humans and were also detected in the invertebrate *Drosophila melanogaster* and lower vertebrates of the genus *Xiphophorus* [11–17]. According to one current concept, carcinogenesis is a multistep process that includes activation of one or more "dominant acting oncogenes" and the inactivation of tumor-suppressor genes [18, 19]. The lower vertebrate genus *Xiphophorus* offers the possibility to study both the activation of oncogenes and the inactivation of tumor-suppressor genes.

Members of the genus *Xiphophorus*, teleost fish, inhabit freshwaters of the Atlantic drainage systems of Central America [20]. Eighteen species have evolved [21–24], comprising innumerable races and populations which, besides their meristic characters, display a population-specific homogeneous coloration [25–27] composed of melanophores, pterinophores, and purinophores [28–30]. In addition to the homogeneous pigmentation, certain populations exhibit black spot patterns which are composed of giant melanophores [31]. Comparative histological, ultrastructural, and biochemical studies have shown that the giant pigment cells are actually neoplastically transformed pigment cells (Tr melanophores) which in the purebred fish are restrained from proliferation by terminal differentiation [30].

Xiphophorus collected from wild populations in their natural habitat and bred in closed stocks in the laboratory are almost completely insusceptible to neoplasia, i.e., are insensitive to mutagenic carcinogens and tumor promoters, whereas certain hybrids between different populations develop neoplasms spontaneously or after treatment with carcinogens [32–35].

The ability of certain purebreds to form spots in distinct compartments of the body and the capability of certain hybrids to develop melanoma spontaneously or after treatment with carcinogens is coded by a sex chromosomal gene complex, which is accessory in the genome; this complex harbors a Mendelian factor which appears as an oncogene and which was arbitrarily symbolized "tumor gene" *Tu* [13, 32–34, 36]. This complex consists of (a) the pterinophore locus (*Ptr*) which is responsible for pterinophore differentiation, (b) the compartment-specific loci (R_{Co}) which restrict both pterinophore and Tr melanophore differentiation to a distinct part of the body, and (c) the melanophore

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locus (*Mel-Tu*) which, if impaired to *Mel-Tu'*, neoplastically transforms the melanin-containing pigment cells [13, 32, 37]. Both R_{Co} and *Mel-Tu*, which together form the so-called *Tu* complex, are impaired in the accessory *Tu* complex and are therefore oncogenic. The activation of the oncogenic potential of the *Tu* complex may occur in the soma (induced melanoma) or in the germline (spontaneous melanoma in the progeny) [13] by mutation. The activity of the *Tu* complex is regulated by the nonlinked tumor-suppressor gene *Diff*, which if present in the homozygous state, restrains the transformed pigment cells from proliferation by terminal differentiation [13–15, 37].

Comparative studies on the inheritance of restriction fragments with polymorphic lengths in fish with and without the accessory *Tu* complex revealed that *Mel-Tu'* contains a v-*erb*-B-related gene [7, 40–44] that is well known in humans as c-*erb*-B [38, 39]. This gene was named x-*erb*-B* (x-*egfr*-B) by our group [40–42] and X*mrk* by others [43]; in the following pages it will be referred to as x-*erb*-B*. Cloning and subsequent sequencing of the fragments with polymorphic length and a complete cDNA showed that x-*erb*-B* is closely related to the human c-*erb*-B-1 (h-*egf-r*) gene and that it encodes a receptor tyrosine kinase which apparently is a member of the epidermal growth factor receptor (EGF-R) family [9, 40–43, 45]. Southern blot analyses showed that x-*erb*-B* is not only part of the accessory *Mel-Tu'* locus, but also of a locus that is indispensable in the fish; this locus probably harbors the indispensable *Tu* complex, which appears to be inherent in the genome of *Xiphophorus* irrespective of whether these animals have inherited the susceptibility to melanoma [7, 9, 40]. The indispensable, x-*erb*-B* was named x-*erb*-B*^{i}; the x-*erb*-B* which is part of the oncogenic *Mel-Tu'* is accessory in the genome of the fish and was designated x-*erb*-B*^{a} [9, 41]. The sequences of the x-*erb*-B* fragments of polymorphic length showed that there are some differences between x-*erb*-B*^{i}, the X-chromosomal x-*erb*-B*^{a}, and the Y-chromosomal x-*erb*-B*^{a}, respectively (Zechel, Schleenbecker unpublished data and [6–9, 45]). It appears that – at least in some cases – x-*erb*-B*^{i} is also located on the *Mel-Tu'*-carrying sex chromosomes and that x-*erb*-B*^{a} evolved from it by gene duplication [46]. Some species of *Xiphophorus* (e.g., *X. helleri*) have not evolved sex chromosomes, and in these cases x-*erb*-B*^{i} may be located on the corresponding chromosome [7].

It appears that x-*erb*-B* is not the EGF-R gene of the fish but is a slightly different gene [9, 40]. The gene encoding the xiphophorine EGF-R has been partly cloned and sequenced; it was named c-*erb*-B (= x-*egfr*-A, x-*egf-r*) [7, 40–42]. The xiphophorine c-*erb*-B is apparently not part of the *Mel-Tu'* locus and is not inherited in parallel with spots and melanoma [9]; therefore, it appears to be of minor importance for the formation of melanoma.

In this paper we show that c-*erb*-B may be of some relevance for the manifestation of the tumor phenotype. However, since x-*erb*-B**a* is located within the accessory *Mel-Tu'* it appears to be the most likely candidate to act as an "ignition spark" for the *Xiphophorus* melanoma machinery. To verify the importance of x-*erb*-B* in melanoma formation, we studied the inheritance of x-*erb*-B*^{i} and x-*erb*-B*^{a} in fish mutants insusceptible to melanoma or susceptible to inducible and spontaneous melanoma. We show that the indispensable x-*erb*-B*^{i} is expressed in nontumorous tissues of fish harboring and lacking the accessory *Mel-Tu'*. Furthermore, we describe the expression of x-*erb*-B*^{i} and the overexpression of x-*erb*-B*^{a} in different melanomas. The complex relation between the inheritance of x-*erb*-B*, pp60^{x-src} activity, and inositol-lipid turnover are discussed, as well as the possibility of cooperation between x-*erb*-B* and other genes.

Results and Discussion

The x-erb-B^{*i}, *x-erb-B*^{*a}, *and c-erb-B Genes*

Inheritance

To verify the importance of x-*erb*-B^{*a} for melanoma formation we studied the inheritance of all three v-*erb*-B-related genes, namely x-*erb*-B^{*i}, x-*erb*-B^{*a}, and c-*erb*-B (= x-*egf-r*) in fish harboring and lacking the accessory *Tu* complex. We shall recapitulate here that some of the *Tu* complex-mediated spot (Tr melanophore) patterns of the purebreds [13, 21–29] give rise to a melanoma following appropriate interpopulation or interspecific hybridization. Depending on the specificity of the inherited *Tu* complex, melanoma formation may not be inducible [47], or melanoma formation may occur spontaneously or after induction with promoters and initiators [33]. Studies on polymorphic lengths of restriction fragments (RFLP analyses) showed that the x-*erb*-B^{*a} genes map to the *Tu* complexes which are terminally located on the X- and Y(Z)-chromosomes of *X. maculatus* and *X. variatus* [48]. More specifically, this was shown by the detection of *Eco*R1 fragments specific for the x-*erb*-B^{*a} of the respective *Tu* complex: fragments of 4.9 kilobases (kb) and 11.0 kb were assigned to the *Tu* complex of a certain X-chromosome of *X. maculatus* and *X. variatus*, respectively; the *Tu* complexes of a certain *X. maculatus* Y- and Z-chromosome harbor a 6.7-kb fragment. The x-*erb*-B^{*i} and the c-*erb*-B (x-*egf-r*) are not located within the respective loci.

We studied the inheritance of x-*erb*-B*-(x-*erb*-B^{*i}- and x-*erb*-B^{*a}-) and c-*erb*-B-(x-*egf-r*-)specific restriction fragments in purebred *X. xiphidium* and *X. andersi*, as well as in different purebreds and hybrids harboring and lacking aberration in the *Tu*-complexes.

Figure 1 (A, B, left) shows that Southern analyses with a v-*erb*-B-specific probe

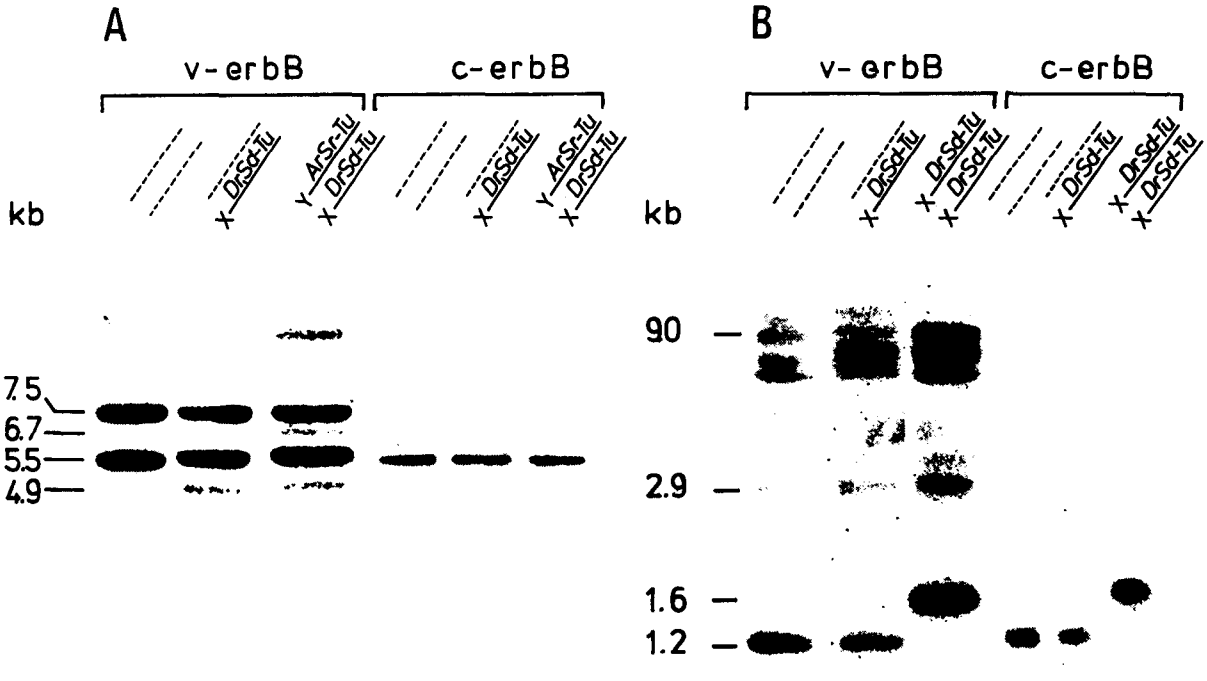

Fig. 1 A, B. Restriction fragments homologous to both v-*erb*-B and the xiphophorine EGF receptor gene c-*erb*-B (= x-*egf-r*). Hybridization against **A** *Eco*R1 and **B** *Hin*dIII-digested genomic DNA from *X. helleri* from Rio Lancetilla (---/---), *X. maculatus* from Rio Jamapa (male, X *Dr Sd-Tu*/Y *Ar Sr-Tu;* female X *Dr Sd-Tu*/X *Dr Sd-Tu*), and a BC hybrid *mac/hell* (X *Dr Sd-Tu*/---). Filters were probed with v-*erb*-B under stringent conditions (40% formamide; 1 × SCC/1% SDS, 65 °C) and with c-*erb*-B under high-stringency conditions (50% formamide; 0.1 × SSC/1% SDS, 68 °C)

Table 1. Presence of x-*erb* B*-specific *Eco* R1 fragments in purebreds and hybrids with and without aberrant sex chromosomes

Species (river provenance)/ interspecific hybrid	m, f / F_1/BC	Phenotypically detected chromosome aberration (sex, *Pter*, *Tu* complex)		x-*erb* B* fragments (kb)	No. of fish analyzed[a]
X. maculatus (Rio Jamapa)	f	X *F Dr Sd-Tu* / X *F Dr Sd-Tu*	–	7.5, 4.9	16
X. maculatus (Rio Jamapa)	m	Y *M Ar Sr-Tu* / X *F Dr Sd-Tu*	–	7.5, 6.7, 4.9	22
X. helleri (Rio Lancetilla)	f	———— / ————[b]	–	7.5	82
X. helleri (Rio Lancetilla)	m	———— / ————[b]	–	7.5	147
X. helleri (Rio Lancetilla)	f	*Db-Tu* / *Db-Tu*	–	7.5	2
X. helleri (Rio Lancetilla)	m	*Db-Tu* / *Db-Tu*	–	7.5	2
mac/hell	BC_n	X *F Dr Sd-Tu* / ————	–	7.5, 4.9	22
	BC_n	X *F Dr Sd-Tu'* / ————	*Sd-Tu* mutation	7.5, 4.9	5
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	7.5, 4.9	32
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	7.5	1
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	12.5, 7.5	4
	BC_n	X *F Dr* ——— / ————	*Sd-Tu* deletion	13.0, 7.5	10
	BC_6	X *F Dr* ——— / ————	*Li-Tu* deletion	7.5, 4.9	15
mac/hell	BC_n	*Sd-Tu* / ————	*Sd-Tu* translocation	7.5, 4.9	10
	BC_6	*Sd-Tu* / ————	*Sd-Tu* translocation	7.5, 4.9	6
mac/hell	BC_n	Y *M Ar Sr-Tu* / ————	–	7.5, 6.7	8
	BC_n	X *F Dr Ar Sr-Tu* / ————	*Ar Sr-Tu* translocation	7.5, 6.7	9
	BC_n	X *F Dr ar* ——— / ————	*Sr-Tu* deletion	7.5	11
	BC_1	Y *M* ——— / ————	*Ar Sr-Tu* deletion	7.5	4
	BC_1	*Sr-Tu* / ————	*Sr-Tu* translocation	7.5, 6.7	2
X. variatus (Rio Panuco)	f	X *F Ye Li-Tu* / X *F Ye Li-Tu*	–	11.0, 7.5	8
X. variatus (Rio Panuco)	m	Y *M Or Pu-Tu* / X *F Ye Li-Tu*	–	11.0, 7.5	16
var/hell	BC_n	X *F Ye Li-Tu* / ————	–	11.0, 7.5	4
var/mac	F_1	X *F Ye Li-Tu* / X *F Dr Sd-Tu'*	*Sd-Tu* mutation	11.0, 7.5, 4.9	5
var/mac/hell	BC_5	X *F Dr Li-Tu* / ————	*Li-Tu* translocation	7.5, 4.9	15
X. maculatus (belize River)	f	W *F* ——— / Z *M Br Ni-Tu*	–	7.5, 6.7	9
X. maculatus (Belize River)	m	Z *M Br Ni-Tu* / Z *M Br Ni-Tu*	–	7.5, 6.7	13
X. maculatus (domestic stock)	f	W *F C''-Tu* / Z *M Br N'-Tu*	*Ni-Tu* mutation	14.0, 7.5, 4.9	1
X. maculatus (domestic stock)	m	Z *M Br N'-Tu* / Z *M Br N'-Tu*	*Ni-Tu* mutation	7.5, 4.9	1
mac/hell	F_1	W *F C''-Tu* / ————	*Ni-Tu* mutation	14.0, 7.5	3
	F_1	Z *M Br N'-Tu* / ————	*Ni-Tu* mutation	7.5, 4.9	2

Table 1 (continued)

Species (river provenance)/ interspecific hybrid	m, f / F_1/BC	Phenotypically detected chromosome aberration (sex, *Pter*, *Tu* complex)		x-*erb* B* fragments (kb)	No. of fish analyzed[a]
X. maculatus (Belize River)	f	W *F* / Z *M Br Ne-Tu*	–	7.5, 6.7	4
X. maculatus (Belize River)	m	Z *M Br Ne-Tu* / Z *M Br Ne-Tu*	–	7.5, 6.7	9
mac/hell	BC_3	Z *M Br Ne-Tu* / ----------	–	7.5, 6.7	5
	BC_3	Z *M Br Ne-Tu* / ----------	–	8.5, 7.5, 6.7	1

[a] All fish were analyzed individually;

[b] *X. helleri* has not evolved sex chromosomes; dashes indicate the corresponding chromosome; m, male; f, female; F_1, hybrid; BC_n, highly backcrossed individuals (higher than BC_7); sex, sex locus; *M*, *F*, sex-determining region; *Ptr* = pterinophore pattern: *Dr*, dorsal red; *Ar*, anal red; *Ye*, yellow; *Or*, orange; *Br*, brown. *Tu* complex, Tr melanophore pattern: *Sd*, spotted dorsal; *Sr*, stripe sided; *Db*, dabbed; *Sd'*, spotted dorsal, modified; *Li*, lineatus; *Pu*, punctatus; *Ni*, nigra; *N'*, patched nigra; *Ne*, nigra extended; *C''*, phenotypically similar to crescent' (*C'*).

revealed a differing distribution of several v-*erb*-B homologous (A) *Eco*R1 and (B) *Hin*dIII fragments in the spotted *X. maculatus* from Rio Jamapa (male, Y *Ar Sr-Tu*/X *Dr Sd-Tu;* female, X *Dr Sd-Tu*/X *Dr Sd-Tu*), the nonspotted *X. helleri* from Rio Lancetilla (---/---; the accessory *Tu* complex and x-*erb*-B*[a] are lacking), and the melanoma-bearing interspecific hybrid (X *Dr Sd-Tu*/---) (symbols, see Table 1); *X. helleri* was used as a suitable recurrent parent to generate these BC hybrids. Besides the *Eco*R1 fragments of 4.9 kb and 6.7 kb that map to the sex chromosomal *Tu* complexes *Sd-Tu* and *Sr-Tu*, respectively, Fig. 1 A (section v-*erb*-B) shows two v-*erb*-B-homologous *Eco*R1 fragments of 5.5 kb and 7.5 kb that are present in all fish of the genus *Xiphophorus* analyzed to date, irrespective of whether these animals harbor accessory *Tu* complexes. Hybridization of the v-*erb*-B-specific probe against DNA preparations of *X. xiphidium* from Rio Soto la Marina reveals accessory *Eco*R1 fragments of 2.6 and/or 2.8 kb, one of which is always present in F_1 hybrids of *X. xiphidium*/*X. andersi;* these fragments, therefore, may also represent parts of a x-*erb*-B*[a] (not shown). As mentioned above, *X. helleri*, in general, neither harbors an accessory *Tu* complex nor any of the x-*erb*-B*[a]-specific *Eco*R1 fragments. As an exception, a certain race of *X. helleri* may develop a spot pattern called "dabbed" (*Db-Tu*) which, however, does not give rise to melanoma formation following interspecific hybridization [47], thus far we could not assign any accessory x-*erb*-B*[a]-specific *Eco*R1 fragment to the *Db-Tu* (Table 1). Provided the presence of an accessory *Eco*R1 fragment, that is to say the presence of a certain x-*erb*-B*[a], is a prerequisite for the susceptibility to melanoma, then the interspecific hybrids carrying the *Db-Tu* are insusceptible to neoplasia, possibly because they do not harbor any of the accessory x-*erb*-B* gene fragments that we mentioned in the last paragraph. It remains unclear whether a

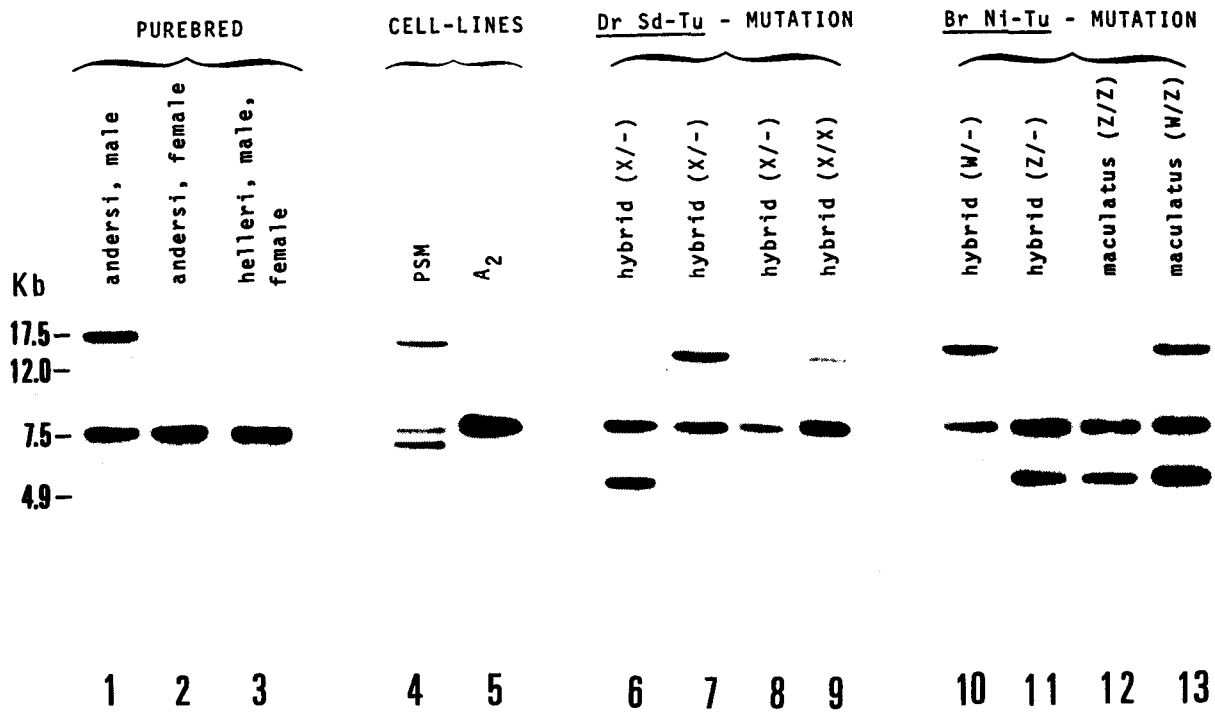

Fig. 2. Inheritance of x-*erb*-B*^{i} and x-*erb*-B*^{a} in the purebreds *X. andersi* (Rio Atoyac) and *X. helleri* (Rio Lancetilla), in the cell lines PSM and A_2, and in purebreds and/or hybrids that display mutations of the X-chromosomal *Dr Sd-Tu* and the Z-chromosomal *Br Ni-Tu*, respectively (Southern blot analysis). The 7.5-kb *Eco*R1 fragment is indispensable (x-*erb*-B*^{i}), while the *Eco*R1 fragments of 17.5, 16, 14, 13, 12.5, 6.7 and 4.9 kb are accessory (x-*erb*-B*^{a}). Filters were probed with x-*erb*-B*-specific probes under high stringency conditions (50% formamide; 0.1 × SSC/1% SDS, 68 °C)

mutation in x-*erb*-B*^{i} may cause the formation of the dabbed spot pattern.

Figure 1 (A, B, right) shows that hybridization of the c-*erb*-B- (x-*egf-r*-) specific fragment against *Eco*R1-digested DNA led to the detection of a single 5.5-kb band; this is true for all genotypes analyzed [7, 9]. In contrast, this fragment detects *Hin*dIII fragments of polymorphic length (RFLP). This RFLP is not inherited in parallel with the X-chromosome and the tumor phenotype: The 1.6-kb band of *X. maculatus* undergoes a successive substitution for the 1.2-kb band of *X. helleri* in the hybrids during the process of introgressive hybridization.

When hybridized against *Eco*R1-digested genomic DNA, the cloned 4.9-kb and 6.7-kb *x-erb*-B*^{a}-specific fragments detect themselves and generally a 7.5-kb band [9, 40]. The 7.5-kb band shows the presence of the indispensable x-*erb*-B*^{i} (Fig. 2, lane 3). Hybridization of these probes against genomic DNA from *X. andersi* from Rio Atoyac [23] revealed the presence of the indispensable 7.5-kb *Eco*R1 fragment in females (Fig. 2, lane 2); besides this fragment, the probe detected an additional *Eco*R1 fragment of 17.5 kb in the male (Fig. 2, land 1).

In Southern hybridizations of the *x-erb*-B*-specific probes against *Hin*dIII-digested DNA (data shown in [7]), the presence of the X-chromosomal x-*erb*-B*^{a} is detectable by the polymorphic length of the *Hin*dIII restriction fragments. The length of the *Sd-Tu*-specific *Hin*dIII fragment is 7.2 kb. The presence of the indispensable x-*erb*-B* as well as the presence of the Y-chromosomal x-*erb*-B*^{a} is marked by a 9.2-kb band.

To answer the question whether inactivation or loss of the above-mentioned x-*erb*-B*^{a} fragments and x-*erb*-B*^{a} genes will result in loss of the capability to form melanoma spontaneously or after induction with carcino-

gens, we bred mutants showing phenotypically detectable mutations of the sex chromosomal gene complex including *Ptr* and the *Tu* complex. Several of the mutants used were genetically and phenogenetically analyzed in 1973 [32, 40]. Since then, more new mutants have been isolated and studied (for photographs, see [7, 32, 34]; A. Anders, unpublished data). The mutations studied in this work concern the X-chromosome and the Y-chromosome of *X. maculatus* from Rio Jamapa, and the Z-chromosome of *X. maculatus* from Belize River. We would like to point out here that the gene complexes of interest are arranged in an uniform order on the respective chromosomes with (a) the sex-determining region proximal to the centromer, followed by (b) the *Ptr* loci (*Dr*, *Ar*, *Br*) and (c) the accessory *Tu* complexes (*Sd-Tu*, *Sr-Tu*, *Ni-Tu*, *Ne-Tu*) (symbols see Table 1). The pterinophore loci serve as phenotypically detectable markers in purebreds and hybrids with and without aberrant sex chromosomes; the activity of the accessory *Tu* complex in question is monitored by the formation of spots in the purebred and the susceptibility to melanoma in the hybrids.

The X-chromosome of *X. maculatus* from Rio Jamapa shows the following gross constitution: X *F Dr Sd-Tu* (*F*, female; *Dr*, dorsal red; *Sd-Tu*, Tr melanophores in the dorsal fin). Hybrids (*mac/hell*) carrying this chromosome show a dorsal reddish coloration and develop melanoma spontaneously in the dorsal fin; the presence of x-*erb*-B*^{a} in the *Sd-Tu* region is disclosed by the 4.9-kb *Eco*R1 fragment (Table 1). The 4.9-kb fragment is also present in the mutant *Sd'-Tu*, which shows an enlarged spot pattern in the purebred and a melanoma which may cover large parts of the dorsal part of the body as well as parts of the fins and the mouth.

We isolated five types of hybrids with aberrant X-chromosomes; these hybrids appeared to be "loss of function" mutants, since they lost the ability to form dorsal melanomas (may be referred to as *Sd-Tu* deletion); the *Dr* locus mediates a dorsal reddish coloration of the body and thereby serves as a marker for the presence of the X-chromosome. One type of mutant lost both the ability to form a melanoma and the x-*erb*-B*^{a}-specific 4.9-kb band (Fig. 2, lane 8). Two types of mutant retained the 4.9-kb fragment (Fig. 2, lane 6). Two other types of mutant lost the 4.9-kb band but gained a 12.5-kb and 13-kb fragment, respectively (Fig. 2, lanes 9 and 7; Table 1). These data show that loss of function of the *Sd-Tu* locus in the different reddish mutants is caused by different events. In three cases the loss of function appears to involve gross structural changes in the x-*erb*-B*^{a} locus. Molecular analysis of a comparable mutant with a 12-kb *Eco*R1 fragment showed that the loss of function mutation may be due to a insertion [43]. Since the 4.9-kb and the 6.7-kb x-*erb*-B*-specific probes used for the present analysis cannot detect the complete x-*erb*-B*^{a} gene [7], and since the Southern blot hybridization cannot monitor minor changes in a gene (e.g., point mutations), it remains unclear whether the mutants with the "intact" 4.9-kb fragment show a mutation of the x-*erb*-B*^{a} locus. Since these nonspotted animals develop melanoma with low frequency following treatment with carcinogens [40], it appears that the oncogenic potential of the *Sd-Tu* locus, and possibly of the respective x-*erb*-B*^{a} locus, may have been inactivated in this mutant by a point-mutation or other small structural changes.

Besides the *Sd-Tu* "deletion," we studied two mutants which phenotypically exhibit a translocation of the *Sd-Tu* locus onto an autosome of *X. helleri*. The mutations occurred independently and had different influences on the sex determination processes in the hybrids; both mutants, however, lost their reddish coloration but retained the ability to develop a dorsal melanoma and the x-*erb*-B*^{a}-specific 4.9-kb band (Table 1).

The Y-chromosome of *X. maculatus* from Rio Jamapa shows the following

gross constitution: Y *M Ar Sr-Tu* (*M*, male; *Ar*, anal red; *Sr-Tu*, stripe sided, Tr melanophores on the body side). Hybrids (*mac/hell*) carrying this chromosome show a reddish coloration of the whole body and may develop a clonal melanoma after treatment with tumor-initiating agents [13, 33, 35]; the presence of x-*erb*-B*^{a} in the *Sr-Tu* region is disclosed by a 6.7-kb *Eco*R1 fragment (Fig. 1).

We studied four types of mutational changes at the *Ar Sr-Tu* locus: (a) two types of change which appear to be due to a translocation of the terminal region of the Y-chromosome onto the X-chromosome in *X. maculatus* and onto an autosome in *X. helleri*, and (b) two types of change that apparently involve a "loss of function" or a deletion of the *Sr-Tu* locus. The results are summarized in Table 1. The type of mutant which gained the X/Y-chimeric chromosome (X *F Dr Ar Sr-Tu*) lost both the ability to develop melanoma spontaneously and the *Sd-Tu*-specific 4.9-kb *Eco*R1 fragment, but gained the sensitivity to initiators and the 6.7-kb *Eco*R1 fragment. Mutation of X *F Dr Ar Sr-Tu* (may be referred to as *Sr-Tu* deletion) in a BC hybrid resulted in progeny that showed an intense reddish coloration but lacked the ability to develop a melanoma spontaneously or after treatment with carcinogens; these animals neither harbor the 4.9-kb nor the 6.7-kb *Eco*R1 fragment (Table 1). Another type of *Sr-Tu* deletion was observed in an *X. maculatus* purebred: The animals lost the reddish coloration of the anal fin and the Tr melanophore pattern "stripe sided"; interspecific hybridization of these mutants (*mac/hell*) resulted in an F_1 generation that was free of Tr melanophores when it inherited the Y-chromosome; both the purebreds and the hybrids that carry the aberrant Y-chromosome only harbor the indispensable 7.5-kb fragment. The second translocation event was also observed in the purebred *X. maculatus:* the animals lost the reddish coloration of the anal fin but retained the spot pattern "stripe sided" and the 6.7-kb *Eco*R1 fragment. The respective BC hybrids (*mac/hell*) lack the reddish coloration, but show, besides intense green and blue stripes, an enlarged *Sr-Tu* spot pattern; these animals possess the 6.7-kb *Eco*R1 fragment. The hybrids are now being tested for their sensitivity to carcinogens. The investigation of the accessory *Tu* complex of the *X. maculatus* Y-chromosome strongly suggests that the presence of a functional x-*erb*-B*^{a} copy is a prerequisite for the capability to develop melanoma after treatment with tumor-initiating agents.

The Z-chromosome of *X. maculatus* from Belize River (male, ZZ; female, WZ) shows the following gross constitution: Z *M Br Ni-Tu* or Z *M Br Ne-Tu* (*M*, male; *Br*, brown; *Ni-Tu*, large black spots on the body side, nigra [47]; *Ne-Tu*, body side is almost completely black, nigra extended). The accessory *Tu* complexes *Ni-Tu* and *Ne-Tu*, respectively, harbor an x-*erb*-B*^{a} that was diclosed by a 6.7-kb *Eco*R1 fragment [40]. The W-chromosome does not harbor an x-*erb*-B*^{a} that can be detected by the polymorphic length of *Eco*R1 fragments (Table 1). Hybrids (*mac/hell*) carrying one of the mentioned Z-chromosomes show a brownish coloration of the body and may spontaneously develop melanoma.

We shall firstly describe a new type of *Ni-Tu* mutant and secondly discuss an aberrant *Eco*R1 fragment pattern in one female with *Ne-Tu;* the results are summarized in Table 1. The mutation of the *Ni-Tu* locus resulted in a changed Tr melanophore pattern in the purebred *X. maculatus:* the animals show a modified spot pattern on the body side and a new pattern in the caudal fin. The aberrant *Ni-Tu* was named *N'-Tu* (patched nigra), while the new spot-pattern was designated *C''-Tu*, because of its similarity to the pattern *C'-Tu* [47]. Hybridization of the x-*erb*-B*-specific probe against *Eco*R1-digested DNA of the female (WZ) reveals bands of 14 kb, 7.5 kb (x-*erb*-B*^{i}), and 4.9 kb (Fig. 2, lane 13). The male (ZZ) shows bands of 7.5 kb and 4.9 kb (Fig. 2, lane 12). Hybrids (*mac/*

hell) with the pattern *C″-Tu* inherited the 14-kb and the 7.5-kb bands, while hybrids with *N′-Tu* inherited the 7.5-kb and the 4.9-kb bands (Fig. 2, lanes 10 and 11). These data support the idea that the 4.9-kb fragment can be assigned to the Z-chromosomal *N′-Tu*, while the 14-kb fragment is probably part of the W-chromosomal *C″-Tu*. We assume that a crossover between the W- and Z-chromosomes changed the Z-chromosomal *Ni-Tu*-region, including the x-*erb*-B*^{a}, and, furthermore, generated an accessory W-chromosmal x-*erb*-B*^{a} that is detectable by the polymorphism of restriction fragment lengths. We wonder whether the changes of the W- and Z-chromosomal x-*erb*-B*^{a} imply changes in the susceptibility of the mutant to neoplasms. Investigation of the susceptibility of (highly backcrossed) hybrids to neoplasms on the one hand, and the molecular structure of the x-*erb*-B*^{a}-fragments on the other hand, will give some information of both the structural changes of the W- and Z-chromosomes and their relevance for melanoma formation.

The "*Ne-Tu* mutant" was detected when we analyzed the x-*erb*-B*-specific *Eco*R1 fragments of BC hybrids harboring the Z *M Br Ne-Tu* chromosome of *X. maculatus* (Belize River); the hybrid did not exhibit changes of the phenotype. Hybridization of the x-*erb*-B*-specific probe against digested DNA disclosed the expected 6.7-kb and 7.5-kb fragments and an additional one of 8.5 kb (Table 1). This additional band may possibly indicate a point mutation in one allele (generation and/or deletion of an *Eco*R1 site) and/or duplication of an x-*erb*-B* gene.

Expression in a Melanoma Cell Line, the Melanoma, and Non-melanomatous Cell Lines

Preparations of total RNA and polyA^{+}-selected RNA were subjected to northern blot analyses using rat β-actin [49], *Drosophila* β_1-tubulin [50], and *Xiphophorus* rRNA [51, 52] as a control for the amount of RNA applied to the nylon membrane; moreover, the filters were stained with methylene blue and the stain was scanned with a densitometer. To calculate the size of a detected mRNA, we coseparated a commercially available size marker (RNA ladder, BRL) and/or human 28 *S* and 18 *S* rRNA on the formaldehyde agarose gels, and utilized *Xiphophorus* 28 *S* rRNA (3.3 kb [51]) and 18 *S* rRNA (1.8 kb [51]) as internal size markers.

Melanoma Cell Line PSM. The PSM cell line was derived from a hereditary melanoma of a hybrid exhibiting a Tr melanophore pattern on the body side [54]. This cell line, which was immortalized during subculturing, exhibits tyrosinase activity [54] and is able to form foci in the culture dish and colonies in soft agar ([55], H. Schäfer-Pfeiffer, K. Krüger, C. Zechel, unpublished data). Hybridization of the x-*erb*-B*-specific probes against digested DNA revealed *Eco*R1 fragments of 16 kb, 7.5 kb, and 6.7 kb (Fig. 2, lane 4), and *Hin*dIII fragments of 9.2 kb and 4.8 kb [7]. The fragments of 7.5 kb and 6.7 kb probably represent x-*erb*-B*^{i} and the Y-chromosomal x-*erb*-B*^{a}, respectively, while the 16-kb fragment may be the result of a rearrangement of a x-*erb*-B* gene.

Both types of gene, the x-*erb*-B* (x-*erb*-B*^{i} and x-*erb*-B*^{a}) and the c-*erb*-B (x-*egf-r*), are expressed in PSM cells [7, 40, 56]. Two x-*erb*-B*-specific transcripts (4.6 kb and 4.0 kb) were detected; the 4.6-kb transcript is a mRNA of low abundance while the 4.0-kb mRNA is highly abundant (Table 2; Fig. 3). Northern hybridization against varying amounts of polyA^{+}-selected RNA proved the mRNA character of the 4.6-kb and 4.0-kb transcripts (smaller RNA species, see p. 223). The 4.6-kb mRNA probably represents the transcript of the x-*erb*-B*^{i} gene, while the 4.0-kb mRNA is probably transcribed from the x-*erb*-B*^{a} locus disclosed by the 6.7-kb Southern band; thus far, we have not been able to assign any detectable transcript to the locus disclosed by the 16-kb Southern fragment. Our studies on the expression of x-*erb*-B* genes in

Table 2. Expression of c-*erb* B (x-*egf-r*) and x-*erb* B* in xiphophorine cell lines, embryos, juvenile fish, tissues of the adult fish, and melanomas

Origin of RNA	c-*erb* B (=x-*egf-r*) 4.5-kb mRNA	Indispensable x-*erb* B* 4.6-kb mRNA	Accessory x-*erb* B*	
			4.0-kb mRNA	8.0-kb mRNA
Cell lines				
A2	+(+)	++(+)	0	0
PSM	++ [a]	+	++++	0
Sd' Sr'	n.t.	0	0	0
Golden	n.t.	0	0	0
Embryo, stage				
0	+++	+++	0	0
1–4	++	++	0	0
5–9	++	+(+)	0	0
10–12	++	+(+)	0	0
13–14	++	+(+)	0	0
15–16	++	+(+)	0	0
17–20	++	++	0	0
21–24	++	+(+)	0	0
25–26	++	+(+)	0	0
Juvenile fish, day after birth				
1–10	++	++	0	0
Adult fish, tissue				
Brain	+	+	0	0
Eye	+	+	0	0
Fin	n.t.	+	0	0
Gill	+++	++(+)	0	0
Heart	+	0	0	0
Kidney	+++		0	0
Liver	0	0	0	0
Muscle	0	0	0	0
Skin	n.t.	+	0	0
Spleen	+(+)	0	0	0
Testis	+	+	0	0
Melanoma				
Spontaneous				
benign	0–+	+	+–++	0
malignant	+–++	+	++–++++	0
Promoted				
benign	+–++	+	+–++	0
malignant	++	+	++–++++	0
malignant [b]	+	(+)	0	+–+++
Initiated				
benign	0–+	+	+–++	0
malignant	+	+	++–+++	0

o, not detectable; +, detectable (low expression); ++, expression; +++, high expression; ++++, very high expression; ( ), intermediate; n.t., not tested.

[a] mRNA of 4.3 kb.

[b] Fish with the X-chromosomal *Li-Tu* express a x-*erb* B*-specific RNA of 8.0 kb.

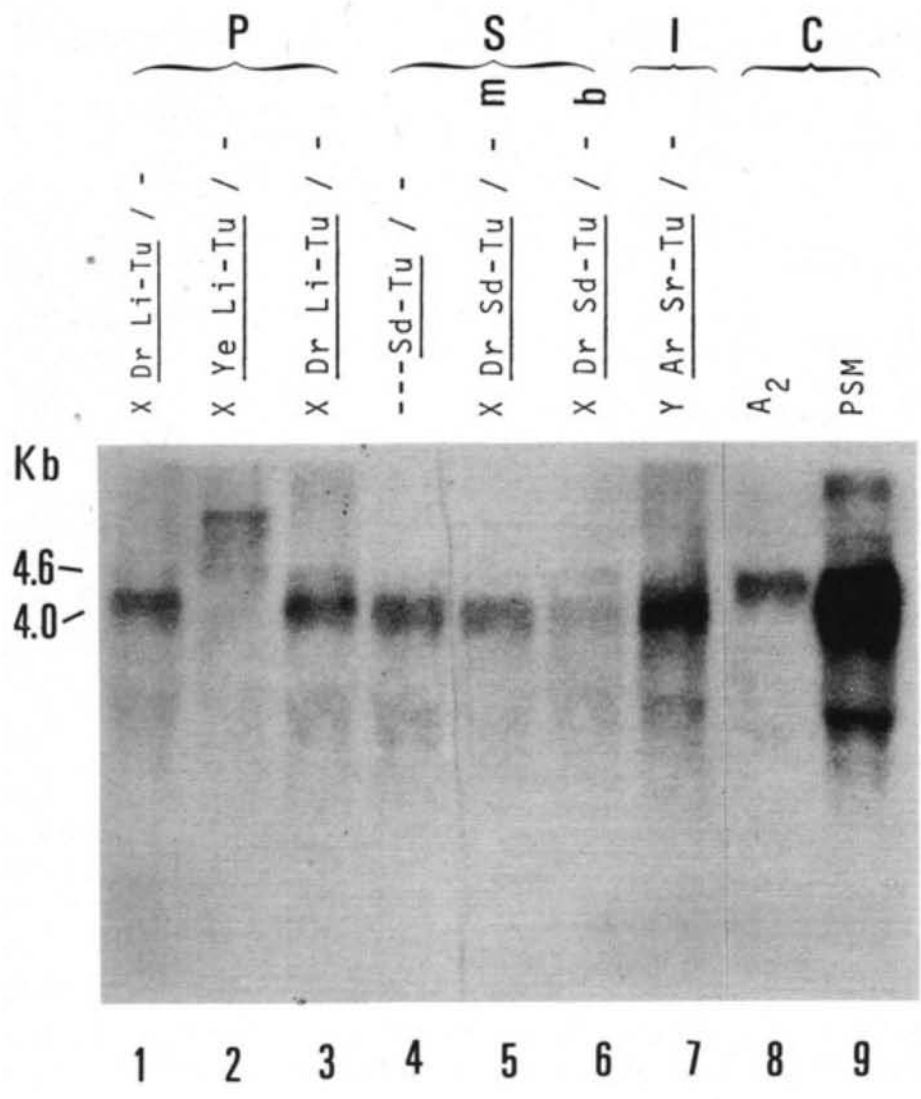

Fig. 3. Expression of x-*erb*-B* genes in: *P*, promoted melanomas; *S*, spontaneous melanomas; *I*, initiated melanomas; and *C*, cell lines (*m*, malignant melanoma; *b*, benign melanoma). RNA filters were probed with x-*erb*-B*-specific probes under high stringency conditions (50% formamaide; 1 × SSC/1% SDS, 68 °C). For symbols, see Table 1

transformed and nontransformed tissues and studies on the transcriptional activation [45] support the idea that x-*erb*-B* mRNAs of different size are transcribed from distinct genes, namely x-*erb*-B*^{i} and x-*erb*-B*^{a}. The mRNA transcribed from the c-*erb*-B (x-*egf-r*) locus is 4.3 kb in size (Fig. 4).

Cell Line A_2. The A_2 cell line [57] was derived from embryos of *X. xiphidium*, and harbors the oncogenic Y-chromosomal *Tu* complex *F1-Tu* [45]. A_2 cells are immortalized, but do not form colonies in soft agar [55]. Hybridization of the x-*erb*-B* specific probes against digested DNA revealed an *Eco*R1 fragment of 7.5 kb (Fig. 2, lane 5) and *Hin*dIII fragments of 9.2 kb and 4.8 kb [7]. Hybridization against polyA^+-selected RNA and total RNA revealed a x-*erb*-B*-specific transcript of 4.6 kb (Fig. 3). Expression of a single c-*erb*-B-specific transcript of 4.5 kb was clearly detected in polyA^+-selected RNA [7].

Cell Lines Sd′Sr′ and Golden. Both cell lines were established from epithelial tissues of adult fish [58], and show an epithelial cell-like shape. They were subcultured approx. 30 times and cannot therefore yet be referred to as immortalized.

Sd′Sr′ cells were derived from a *X. maculatus* male (Y *Ar Sr′-Tu*/X *Dr Sd′-Tu*) which is homozygous for the tumor suppressor *Diff* and therefore terminally differentiates the Tr melanophores in the

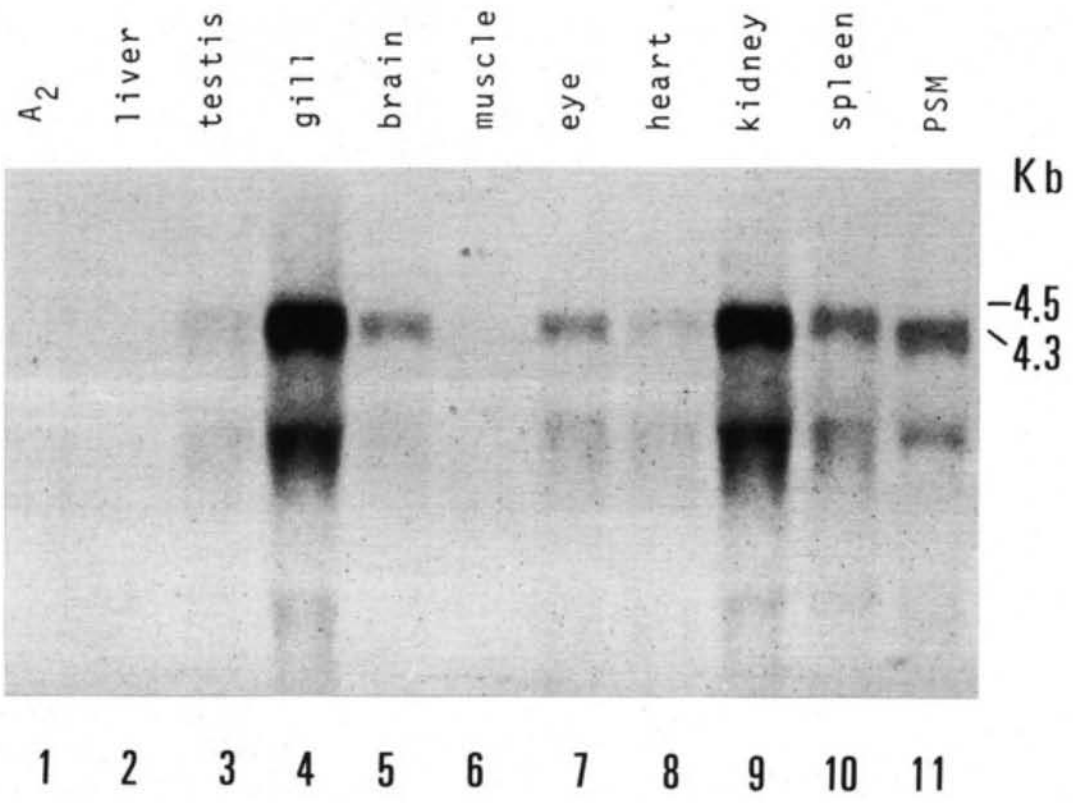

Fig. 4. Expression of the xiphophorine EGF receptor gene c-*erb*-B (= x-*egf-r*) in the cell lines A_2 and PSM, and in tissues of the adult nontumorous fish. Conditions, see Fig. 3

dorsal fin and on the body side. Sd'Sr' cells show the expected x-*erb*-B*-specific Southern fragments of 7.5 kb, 6.7 kb, and 4.9 kb (not shown) but do not express any detectable x-*erb*-B^{*i} or x-*erb*-B^{*a} transcripts. It must be shown by further studies whether the lack of x-*erb*-B* expression (x-*erb*-B^{*i} and x-*erb*-B^{*a}) in the Sd'Sr' cell is due to the activity of *Diff*, or whether the x-*erb*-B* loci are kept silent in these epithelial cell-like cells by any other factor.

Golden cells [58] were isolated from a BC hybrid that carries the "golden" gene (see [8, 59]) but lacks an oncogenic *Tu* complex. The Golden cell does not possess any of the "polymorphic" x-*erb*-B^{*a} fragments and does not show detectable amounts of x-*erb*-B* transcripts (not shown).

Spontaneous Melanoma. Following appropriate interpopulational or interspecific hybridization, melanoma develops spontaneously in hybrids harboring the chromosome X *Dr Sd-Tu* or the chimeric chromosome ---*Sd-Tu* (phenotypically detected translocation of *Sd-Tu* onto an *X. helleri* autosome). Presence of the *Diff* gene results in development of a benign melanoma, while absence of *Diff* allows development of a malignant melanoma [13, 32]. The hybrids possess the x-*erb*-B^{*i} and the X-chromosomal x-*erb*-B^{*a} (Table 1). The x-*erb*-B*-specific probes detect mRNAs of 4.6 kb and 4.0 kb in both the benign and the malignant melanoma of the hybrid X *Dr Sd-Tu*/– (Fig. 3, lanes 5 and 6) and ---*Sd-Tu*/– (Fig. 3, lane 4), respectively. The amount of 4.0 kb mRNA is high in melanoma, the highest level of mRNA being detected in the malignant melanoma (Fig. 3; Table 2). We suppose that the 4.0-kb mRNA is transcribed from the x-*erb*-B^{*a}-locus, while the 4.6-kb mRNA, which is detectable in a low amount in the melanoma, may be transcribed from the x-*erb*-B^{*i} locus (see expression in PSM cells). The c-*erb*-B- (x-*egf-r*-)specific probe generally detects a mRNA of 4.5 kb in the spontaneous melanoma (not shown); the amount of mRNA detected in the malignant melanoma exceeds that detected in the benign melanoma (Table 2).

Promoted Melanoma. Amongst others things [8, 35], hybrids carrying the chromosome X *Ye Li-Tu* or the chimeric chromosome X *Dr Li-Tu* (*Li-Tu* translocation onto a *X. maculatus* X-chromosome; Table 1) are sensitive to tumor-promoting agents such as steroids [35, 60] or ultraviolet radiation [61]. Treatment of the hybrid X *Dr Li-Tu* (4.9-kb x-*erb*-B* fragment) with promoters resulted in development of a fast-growing malignant melanoma, which expresses the 4.6-kb and overexpresses the 4.0-kb x-*erb*-B*-specific mRNA (Fig. 3, lanes 1 and 3; Table 2). The malignant melanoma of the X *Ye Li-Tu*-hybrid (11-kb x-*erb*-B*^{a} fragment) expresses mRNAs of 4.6 kb and 8 kb (Fig. 3, lane 2). Our data indicate that the 8-kb mRNA is specifically transcribed from the *Li-Tu* locus of the *X. variatus* X-chromosome (Table 2; [52]). The x-*erb*-B- (x-*egf-r*-) specific probe detects a mRNA of 4.5 kb in hybrids with a promoted melanoma, the expression detected in a benign melanoma being lower than that in a malignant melanoma (Table 2).

Initiated Melanoma. Up to 20% [37] of the hybrids carrying the *X. maculatus* chromosome Y *Ar Sr-Tu* (6.7-kb x-*erb*-B*^{a} fragment) or the chimeric chromosome X *Dr Ar Sr-Tu* (*Ar Sr-Tu* translocation onto the *X. maculatus* X-chromosome; 6.7-kb fragment; Table 1) develop a clonal melanoma after treatment with tumor-initiating agents such as X-rays [63], *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), and others [33, 35–37]. The clonal melanoma expresses two mRNAs of 4.6 kb and 4.0 kb (Fig. 3, lane 7), the 4.0-kb mRNA probably being transcribed from the *Sr-Tu* locus. The highest amount of 4.0 kb mRNA was detected in fast-growing malignant melanomas. In any initiated melanoma, however, the amount of 4.0-kb mRNA greatly exceeds that of the 4.6-kb mRNA. As we have shown for the spontaneous melanoma and the promoted melanoma, in the initiated melanoma the amount of the 4.0-kb transcript is also positively related to the degree of malignancy (Table 2). Expression of the c-*erb*-B- (x-*egf-r*-)specific 4.5-kb mRNA was detectable in almost all of the initiated melanomas (Table 2).

In summary, the northern blot analyses revealed x-*erb*-B* specific transcripts of 8 kb, 4.6 kb, and 4.0 kb, and c-*erb*-B- (x-*egf-r*-)specific transcripts of 4.5 kb and 4.3 kb in preparations of total RNA and poly A^{+}-selected RNA. The results reveal that x-*erb*-B*^{a} is expressed or overexpressed in the melanoma and the melanoma cell line PSM. It appears that the mRNAs of 4.0 kb and 8 kb are probably specifically transcribed from the x-*erb*-B*^{a} genes in the melanoma; presumably, the 4.0-kb mRNA is specific for certain *X. maculatus* x-*erb*-B*^{a}, while the 8.0-kb mRNA is transcribed from the *X. variatus Li-Tu* locus. The 4.6-kb mRNA is probably transcribed from the x-*erb*-B*^{i} locus. The xiphophorine c-*erb*-B gene is also expressed in almost all of the melanomas analyzed and may be important for the manifestation of the tumor phenotype. To learn about the function of x-*erb*-B* (x-*erb*-B*^{i} and x-*erb*-B*^{a}) and c-*erb*-B in nontumorous tissues and to verify that the transcripts of 4.0 kb and 8 kb are tumor-specific, we studied the expression of both types of v-*erb*-B-related gene in nontumorous tissues of fish with and without accessory *Tu* complexes.

Expression in Nontumorous Tissues of Fish With and Without Accessory Tu Complex

Preparations of total RNA were subjected to northern blot analyses using the controls described previously (p. 221). Poly A^{+}-selected RNA of stage 21 embryos (stage according to [59]) and kidneys were analyzed to verify the mRNA character of the transcripts detected in embryonic or adult tissues.

Nontumorous Tissues of Fish Without Accessory *Tu* Complex. The embryos and juvenile and adult fish subjected to northern analyses contained the genetic in-

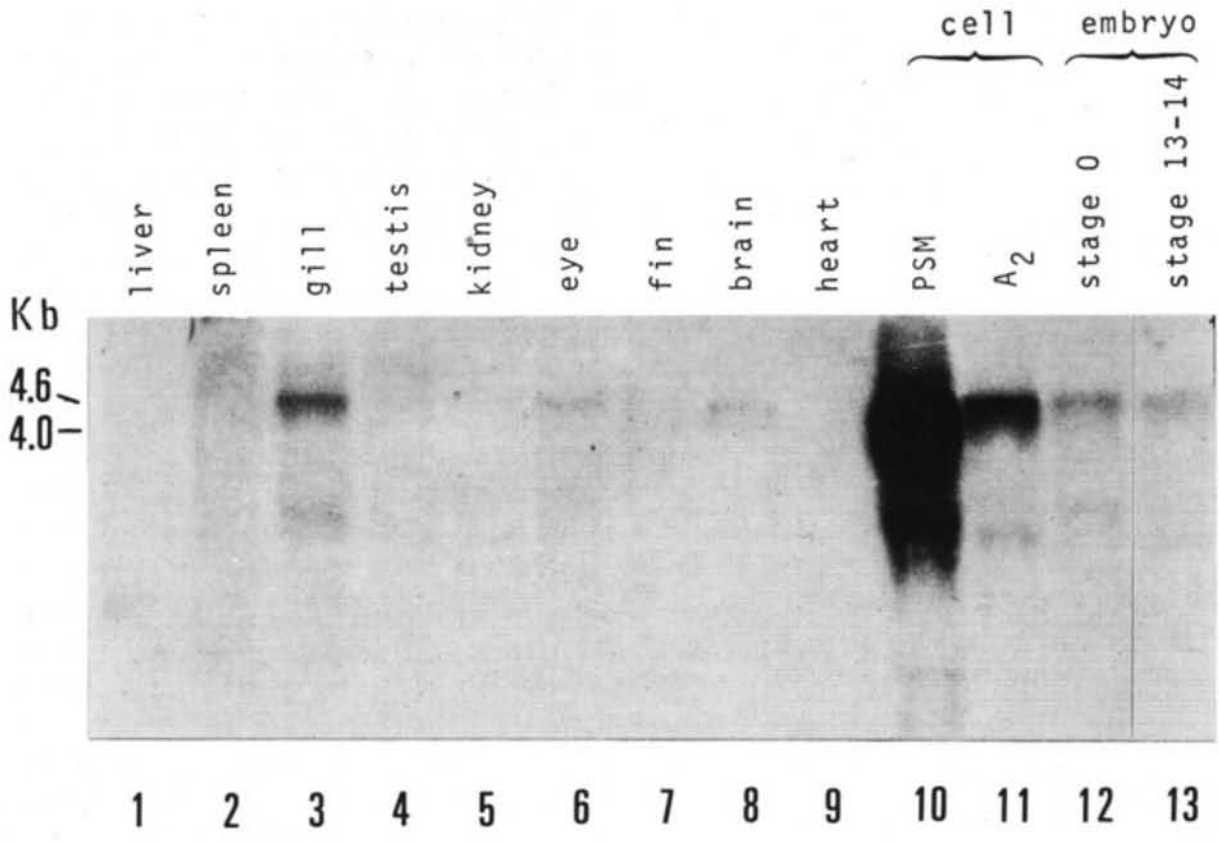

Fig. 5. Expression of x-*erb*-B* genes in tissues of the adult nontumorous fish, in PSM and the A_2 cells, and in embryos (stage 0, maternal RNA; stage 13–14, early organogenesis) of nontumorous fish. Conditions, see Fig. 3

formation of *X. helleri* from Rio Lancetilla which possesses x-*erb*-B*^{i} but lacks x-*erb*-B*^{a} (Table 1). In the embryo (Table 2; stages according to [59]), the x-*erb*-B*-specific probes detect only one type of mRNA, namely the 4.6-kb mRNA (Fig. 5, lanes 12 and 13), which – in the case of the melanoma – we suspected to be transcribed from the x-*erb*-B*^{i} locus (Table 2). The 4.6-kb transcript is stored in the unfertilized egg (stage 0). The amount of x-*erb*-B*^{i} mRNA decreases during early blastula formation (stages 1–4), slightly increases when invagination of the neural keel takes place (stages 5 and 6), and stays almost constant up to organogenesis. The amount of x-*erb*-B*^{i} transcript is enhanced during late organogenesis (stages 17–20), slightly decreases during late embryogenesis (stages 20–26), and stays almost constant during the first 10 days after birth (Table 2). In the adult fish (Table 2, Fig. 5), a considerable amount of the x-*erb*-B*^{i}-transcript is detectable only in the gills; expression of x-*erb*-B*^{i} is low in the eye and brain, and very low, but detectable, in testes, fins, and skin. Hybridization against polyA^{+}-selected RNA revealed that the 4.6-kb mRNA is the only mRNA expressed in nontumorous tissues of *Xiphophorus*. RNA species of larger or smaller size (e.g., RNA of approx. 3 kb) which were observed in preparations of total RNA of, for example, the gills (Figs. 5, 6) are not detectable in preparations of polyA^{+}-selected RNA and therefore may be considered as a x-*erb*-B*^{i}-precursor RNA, specific degradation products, or something else.

Our results clearly reveal that the expression of x-*erb*-B*^{i} is important for embryogenesis and for some tissues in the adult fish. The expression of x-*erb*-B*^{i} in the embryo and in the gills, both of which contain proliferating cells, indicate that x-*erb*-B*^{i} may be important for the regulation of proliferation processes. Since x-*erb*-B* codes for a protein of the EGF-R family and is expressed in proliferating nontumorous or tumorous cells, x-*erb*-B*^{i} and x-*erb*-B*^{a}, respectively, may be

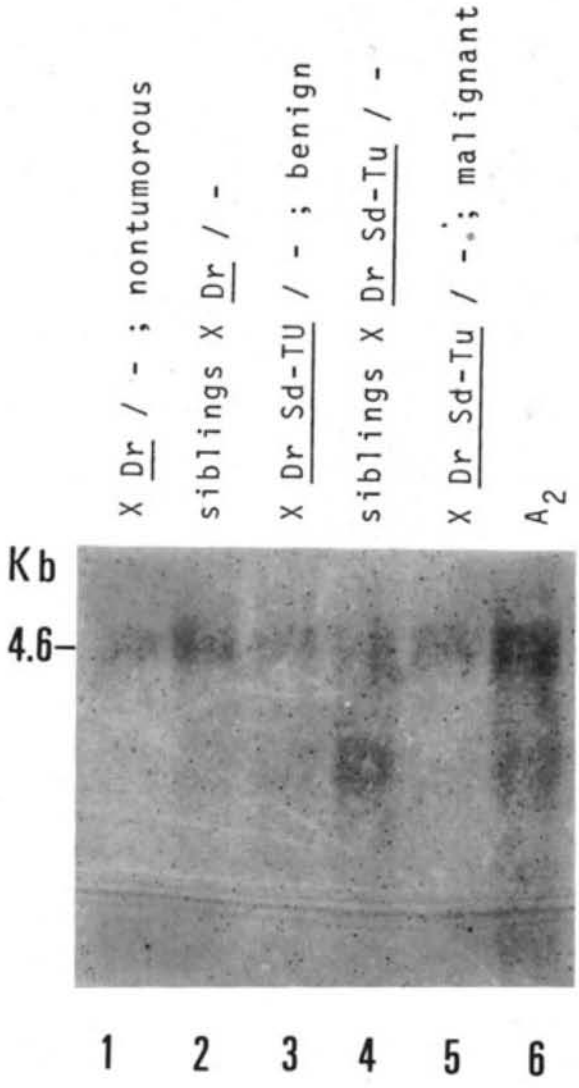

Fig. 6. Expression of x-*erb*-B* genes in the gills of hybrids that have inherited the X-chromosomal 4.9-kb x-*erb*-B*[a] fragment and in the gills of siblings from the same crossing that have not inherited the X chromosomal fragment. *1*, X *Dr*/–, reddish tumorfree hybrid with an aberrant X chromosome; *2*, nonreddish siblings of X *Dr*/– (the aberrant X chromosome is lacking); *3*, X *Dr-Sd-Tu*/–, reddish hybrid with a benign dorsal melanoma; *4*, tumorfree siblings of X *Dr Sd-Tu*/–, benign/malignant (X chromosome is lacking); *5*, X *Dr-Sd-Tu*/–, reddish hybrid with a malignant dorsal melanoma. Conditions, see Fig. 3; symbols, see Table 1

involved in a mitosis-stimulating pathway comparable to that described for the human EGF-R [64].

The EGF-R gene (c-*erb*-B) of *Xiphophorus* is differentially expressed during embryogenesis (Table 2), with the lowest amount of 4.5-kb transcript during early organogenesis (stages 13 and 14) and the highest expression detected in the unfertilized egg (stage 0). The expression of c-*erb*-B stays at an almost constant level during late embryogenesis (stages 20–25), birth (stage 26), and for the first 10 days after birth (Table 2). In the adult fish, c-*erb*-B is highly expressed in the gills and in the kidney (Fig. 4; Table 2). Expression was also detected in spleen, brain, and the eyes, while low expression was observed in testis and heart. The size of the c-*erb*-B transcript detected in embryonic and adult tissues, as well as the size of those transcripts observed in the melanomas and the A_2 cells, was 4.5 kb. The 4.3-kb c-*erb*-B transcript was only detected in the PSM cells and may disclose a modified EGF-R gene that is specific for PSM cells. Smaller RNA species (approx. 3 kb; Fig. 4) were observed in some preparations of total RNA; since these RNA species were not detected in polyA^+-selected RNA, they probably represent precursors of the mRNA, or specific degradation products (small RNA species in Fig. 4), or something else.

The EGF-R gene of *Xiphophorus* appears to be important for the development of the embryo and for some tissues in the adult fish, especially the gills and the kidneys. Possible, c-*erb*-B is functionally involved in stimulating mitosis in the course of the regeneration of the epithelial cells of both the gills and the kidneys. Expression of the human EGF-R gene was observed in nontumorous renal tissues, human renal cancer, and a number of other human epithelial malignancies [65].

Nontumorous Tissues of Fish With Accessory *Tu* Complex. To verify that the 4.0-kb mRNA is a tumor-specific transcript of x-*erb*-B*[a], we analyzed nontumorus tissues of hybrids that possess a normal or aberrant *X. maculatus* X-chromosome, or lack such a chromosome. Since our experiments showed that the x-*erb*-B*[i]-specific mRNA is well detectable in the gills, we studied the expression of x-*erb*-B* in the gills of hybrids with X *Dr Sd-Tu*, X *Dr* (phenotypically

detected *Sd-Tu* deletion) and the *Dr Sd-Tu* and *Dr*-free siblings of these hybrids. Hybrids with X *Dr Sd-Tu* possess the indispensable 7.5-kb x-*erb*-B*^{i} fragment and the 4.9-kb x-*erb*-B*^{a} fragment. We detected only one type of RNA, namely the 4.6-kb x-*erb*-B*^{i}-specific transcript, in total RNA from hybrids with benign melanoma (presence of *Diff*), hybrids with malignant melanoma (absence of *Diff*), and the Tr melanophore-free siblings of these hybrids (Fig. 6, lanes 3–5). A transcript of the same size was detected in the gills of the hybrids with X *Dr*, which possess the 7.5-kb and the 4.9-kb x-*erb*-B* fragments, as well as in the gills of their X *Dr*-free siblings (Fig. 6, lanes 1 and 2). The amount of 4.6-kb mRNA was almost the same in all five cases.

The data described in the preceding paragraphs indicate that the x-*erb-B*^{*a} locus is transcribed tumor specifically, while the indispensable x-*erb*-B*^{i} locus is transcribed in nontumorous tissues of fish harboring and lacking accessory *Tu* complexes. The regulation, that is to say the activation or inactivation, of both the "proto-oncogenic" x-*erb*-B*^{i} and the onocogenic x-*erb*-B*^{a} needs further investigation.

X-erb-B^{*a} *and Other Genes: Possible Cooperation?*

Nothing is known about the key genes critical in starting the molecular and biochemical machinery that initiates a tumor. Fortunately, from studies of the polymorphic length of restriction fragments of xiphophorine oncogenes, we can discriminate the parental origin of a certain oncogene in a particular hybrid [8]. We shall concentrate on hybrids that have inherited the *X. maculatus* X-chromosome or Y-chromosome, which in the hybrid (*mac/hell*) mediates the formation of a melanoma spontaneously or after induction with tumor-initiating agents, respectively. In our highly backcrossed hybrids bearing a melanoma or sensitive to initiators, the sex chromosome with the accessory *Tu* complex is the only chromosome that originates from *X. maculatus;* 47 chromosomes originate from *X. helleri.* The molecular and the biochemical machinery which causes the development of the melanoma in a BC hybrid is, therefore, mainly run by *X. helleri*-derived genes, while the signal for starting the machinery must be created and transmitted by the *X. maculatus* sex chromosome. Since this gene must be located in the region of the accessory *Tu* complex, it appears that the signal for the initiation of the processes preceding the morphological realization of the melanomas probably comes from the x-*erb*-B*^{a}. Besides x-*erb*-B*^{a} [40] and x-*erb*-B*^{i} [46], none of the 14 oncogenes that we detected in the genome of *Xiphophorus* could be assigned to the sex chromosomes and the accessory *Tu* complexes [8].

A possible way in which x-*erb*-B*^{a} could initiate the processes leading to melanoma development has not yet been investigated. So far, we have found that x-*erb*-B*^{a} is expressed and overexpressed in melanomas that developed spontaneously or after induction with carcinogens; the highest amount of x-*erb*-B*^{a} transcripts was observed in spontaneous and promoted malignant melanomas of hybrids carrying a certain *X. maculatus* X-chromosome [62].

The machinery necessary for melanoma formation remains poorly understood, but probably involves activity of several *X. helleri*-derived oncogenes and their products. We showed that the c-*erb*-B (x-*egf-r*) is expressed in almost all melanomas and, therefore, may be of some relevance for the melanoma machinery. Another gene, namely the xiphophorine *src* gene, x-*src*, is also somehow involved in melanoma formation. It was shown earlier that x-*src* is probably of importance for the melanoma machinery and may be related to the initial events: Biochemical studies, especially immunoprecipitation assays, showed that the xiphophorine counterpart to the Rous sarcoma virus oncogene product, namely

the pp60^{x-src} kinase, is active in several healthy tissues of *Xiphophorus*, particularly in brain tissues [2, 3, 34]. In hybrids that inherited the *Tu* complex *Sd-Tu* and, therefore, develop melanoma spontaneously, the pp60^{x-src} activity in the brain was three times higher than that in the brain of their tumorfree siblings (*Sd-Tu* is lacking). Moreover, the activity in the melanoma was elevated up to three times above that of the already elevated activity in the brain of the melanoma-bearing fish [8, 66]. Depending on the genetically determined degree of malignancy, the pp60^{x-src} activity of a particular fish may be increased in parallel in both the melanoma and the brain [2, 66].

The turnover of inositol lipids [67, 68] was recently found to correlate with the inheritance of accessory *Tu* complexes containing x-*erb*-B*^{a} on the one hand and with the pp60^{x-src} activity on the other hand [8, 41, 69, 70]. Phosphoinositide turnover and pp60^{x-src} kinase activity were found consistently elevated in the spontaneous melanoma and the healthy brain of hybrids carrying the *Tu* complex *Sd-Tu* before and after the onset of the malignant melanoma [70]. Predictions can be made about whether a certain individual that harbors an accessory x-*erb*-B*^{a}-containing *Tu* complex is prone to develop a spontaneous malignant melanoma. Hybrids that have inherited the *Tu* complex *Sr-Tu*, and therefore require carcinogen-induced somatic mutations of particular regulatory genes of this *Tu* complex for initiation of a melanoma, show standard levels of phosphoinositide turnover and pp60^{x-src} activity in the brain; only malignant melanomas induced in these genotypes show elevated inositol incorporation [69] and elevated pp60^{x-src} activity [66]. The malignant melanoma of these animals cannot be predicted by the inositol lipid turnover and pp60^{x-src} kinase activity. The complex correlation between the inositol lipid turnover and pp60^{x-src} activity and the genetically determined melanoma formation, respectively, indicate that the inositol lipids and the x-*src* are probably involved in the processes preceding the morphological realization of the melanoma. This correlation is intimately coupled with the inheritance of x-*erb*-B*^{a}. Further studies are required to show whether the activity of x-*erb*-B*^{a} or other genes could regulate inositol lipid turnover and influence pp60^{x-src} activity in the melanoma.

Northern blot analyses showed that, besides x-*erb*-B*, c-*erb*-B, and x-*src*, the xiphophorine *ras* gene (x-*ras*) is expressed in the melanoma [8, 56]; the x-*ras* protein could possibly be related to a putative signal transduction pathway that causes phophatidyl inositol-1,4-biphosphate degradation. Such a signal transduction pathway could also be influenced by the x-*erb*-B*, the c-*erb*-B (x-*egf-r*), or other gene products. Transcripts of the putative x-*sis* gene (x-*pdgf*) that codes for the platelet-derived growth factor (PDGF) of the fish [6] were detected in several melanomas [62], while the putative x-*pdgf-r* gene that encodes the xiphophorine PDGF receptor [6, 71] is expressed in all tumors analyzed [62]. We assume that x-*sis* and x-*pdgf-r* may mediate tumor growth by an autocrine or paracrine mechanism; furthermore, via its receptor, PDGF could have the opportunity to regulate a signal transduction pathway with some relevance for the tumor machinery. mRNAs of genes related to the human *erb*-A [7, 9] (see also [72]) are poorly detectable in melanomas [62].

x-*erb*-B*^{a} is the most likely candidate to initiate the complex of processes preceding the morphological realization of the melanoma. However, generation of transgenic fish [73, 74] which specifically develop melanomas following induction of a transfected x-*erb*-B*^{a} gene are needed to prove this hypothesis. The process of melanoma initiation may require collaboration of x-*erb*-B*^{a}, inositol lipid turnover, and pp60^{x-src} activity, while the melanoma machinery may involve the genes x-*erb*-B*^{i}, c-*erb*-B (x-*egf-r*), x-*ras*, x-*pdgf*, and x-*pdgf-r*.

Summary and Conclusions

The polymorphic length of certain restriction fragments so far have revealed only a certain v-*erb*-B-related gene that is inherited along with the accessory oncogenic *Tu* complexes which mediate the development of melanomas in *Xiphophorus* after appropriate interpopulational or interspecific hybridization. This gene, named x-*erb*-B*, codes for a tyrosine receptor kinase that belongs to the EGF-receptor family. Two types of x-*erb*-B* gene, namely x-*erb*-B*^{i} and x-*erb*-B*^{a}, exist in the genome of a melanoma-bearing hybrid. One of them, namely x-*erb*-B*^{i}, is indispensable in the genome of the fish and the other, namely x-*erb*-B*^{a}, is accessory in the genome of the fish. The latter is part of the melanoma-determining oncogenic gene complex and exists at least in three allelic forms. These forms can be assigned to the *Tu* complexes that are responsible for the development of the (a) spontaneous, (b) initiated, and (c) promoted melanoma, respectively. Studies of the inheritance and expression of x-*erb*-B*^{a} in mutants susceptible or insusceptible to melanoma indicated that x-*erb*-B*^{a} seems to act as the "ignition spark" of the *Xiphophorus* melanoma machinery. In particular, the expression data support the hypothesis that x-*erb*-B*^{a} is specifically activated in the tumor: x-*erb*-B*^{a} is highly expressed or overexpressed in the melanoma, the amount of x-*erb*-B*^{a}-specific mRNA (4.0 kb) being positively related to the malignancy of the melanoma. Expression of the indispensable x-*erb*-B*^{i} (4.6-kb mRNA) is also detectable, but comparatively low in the melanoma. x-*erb*-B*^{i} is probably involved in the regulation of the proliferation of embryonic tissues and epithelial tissues in the adult fish. In nontumorous tissues, e.g., the gills, of both fish harboring and lacking accessory *Tu* complexes, only the x-*erb*-B*^{i}-specific mRNA is detectable, the amount of 4.6-kb transcript detected in the tumor fish and in the tumor-free siblings being almost the same. The activation of the "proto-oncogenic" x-*erb*-B*^{i} probably occurred by gene duplication and mutation and resulted in the generation of the oncogenic x-*erb*-B*^{a}. This gene's oncogenicity is presumably suppressed by the *Diff* gene in the purebred; in the hybrid however, where crossing-related impairment of the *Diff* gene took place, the inheritance and expression of x-*erb*-B*^{a} is associated with the susceptibility to melanoma.

The c-*erb*-B (x-*egf-r*) gene of *Xiphophorus* which codes for the xiphophorine EGF receptor is probably involved in signal transduction processes in embryonic tissues and in epithelial tissues in the adult. Besides being expressed in healthy tissues, c-*erb*-B is expressed in almost all spontaneous or induced melanomas analyzed. The expression detected in the malignant melanoma exceeds that in the benign melanoma. A transcript of aberrant size, 4.3 kb instead of 4.5 kb, was only observed in the melanoma cell line PSM. Since, thus far, amplification or rearrangement of the c-*erb*-B gene has not been observed in fish susceptible to melanoma, we conclude that the xiphophorine EGF receptor gene is not involved in the initiation of the key processes preceding melanoma. The c-*erb*-B gene, however, may be important for the manifestation of the tumor phenotype by taking part in the melanoma machinery.

Besides x-*erb*-B*^{i}, x-*erb*-B*^{a}, and c-*erb*-B, several oncogenes are expressed in the melanoma. Expression of the x-*src* gene, that is to say pp60^{x-src} activity and phosphatidylinositol turnover, are elevated in healthy tissues of hybrids susceptible to spontaneous melanoma and in the melanoma itself; the elevation in the melanoma exceeds that in the nontumorous tissues of the fish. The elevation of both pp60^{x-src} activity and inositol lipid turnover are in parallel with the degree of malignancy of the tumor and the inheritance of a certain x-*erb*-B*^{a}.

We conclude that the signal for initiation of the key processes preceding melanoma, including the elevation of inositol

lipid turnover and pp60^{x-src} activity, is associated with the inheritance and expression of the accessory x-*erb*-B*[a].

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Analysis of Human Retroviral Regulatory Proteins Tax and Tat

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Introduction

The human retroviruses HTLV-I (human T-cell leukemia virus) and HIV-1 (human immunodeficiency virus) code, in addition to a complete set of replicatory genes (*gag*, *pol*, and *env*), for several regulatory genes including the *trans*-activators *tax* and *tat*, respectively, both of which lead to high expression of viral gene products. The molecular mechanism underlying these events is, however, different for the two proteins.

The Tax protein interacts with a Tax acceptor region (TAR) located in the HTLV-I long erminal repeat (LTR) upstream of the initiation site of RNA synthesis. The TAR harbors three 21-base-pair (bp) repeats, designated Tax response elements (TREs), which contain DNA sequence motifs recognized by a family of proteins such as AP-1, Jun, and cAMP response-element binding protein (CREB). The regions between the three 21-bp repeats comprise 27 and 79 nucleotides and may contribute to Tax function. Tax also trans-activates other cellular genes, presumably by an indirect mechanism. Previous analyses have indicated that Tax binds to DNA in a nonspecific fashion [1] and that several cellular proteins with molecular weights of 32, 36–42, 50, and 110 kDa bind to a single 21-bp repeat on a DNA affinity column [2].

The Tat protein of HIV-1 interacts with a Tat acceptor region (TAR; nucleotides +1 to +80) downstream of the RNA polymerase initiation site. Therefore, it is distinct from all known transcriptional activators. The Tat protein does not bind to this DNA but to the corresponding RNA instead, which can form a hairpin loop structure. Whether the Tat protein binds to this RNA directly or binding is mediated by cellular factors has been the subject of controversy. The Tat protein consists of a characteristic cysteine-rich domain and a highly basic domain with a cluster of six arginines and two lysines [3].

Materials and Methods

Prokaryotic Expression of Tat. For construction of a *Tat*-expressing vector, pEx31B, a derivate of pPLC24 [4] carrying a polylinker between the *Bam* HI and *Hind*III sites, was used. In this vector, the first coding exon of *Tat* was cloned by ligating the *Taq*I-*Ava*II fragment of clone BH10 (nucleotides 5392 to 5706) [5] into the *Bgl* II site of the polylinker. For this purpose, the ends of the fragment were converted to *Bgl*II-sites using linkers (Biolabs, Beverly, USA). In order to express the MS2-Tat fusion protein, the λPL promoter of pEX31B is induced by temperature shift of the growing bacteria at an OD_{600} of 1–1.5 from 28° to 42 °C for 2.5 h.

Cells and Antisera. C81-66-45 cells [6], Jurkat cells, and Tat-expressing Jurkat cells [7] were cultured in RPMI-1640 with 10% fetal calf serum (FCS). Preparation

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of CREB-specific peptide antiserum (W39 peptide [8]) was performed essentially as described previously [1]. Antiserum against MS2-Tat was raised in rabbits against gel-eluted MS2-Tat fusion protein (Fig. 2A). Antiserum against the carboxy terminus of Tat was raised against a synthetic carboxy-terminal Tat peptide with the sequence Ser-Gln-Pro-Arg-Gly-Asp-Pro-Thr-Gly-Pro-Lys-Glu containing an additional cysteine residue at its amino terminus. The peptide was coupled to keyhole limpet hemocyanin (KLH) via the cysteine, as described previously [1]. Antiserum against β-Gal-Tat was raised in rabbits against a purified β-Gal-Tat fusion protein.

Indirect Immunoprecipitation. Indirect immunoprecipitation was carried out as described previously [1]. Metabolic labeling of C81-66-45 cells with [^{35}S]methionine was performed as described [1]. HIV-1/H9 cells were labeled metabolically with [^{35}S]cysteine (500 μCi ml^{-1} per 10^7 cells) for 90 min using cysteine-free medium and dialyzed calf serum.

Immunoaffinity Purification of Tax and CREB Proteins. The Tax protein was isolated from [^{35}S]methionine-labeled C81-66-45 cells according to a previously published procedure [1], the only difference being that the immunoaffinity column was eluted with 6 *M* guanidine HCl containing β-mercaptoethanol 0.1% and bovine serum albumin (BSA) 0.1 mg/ml. The eluate was subsequently dialyzed against 50 m*M* Tris HCl, pH 7.5, 2 m*M* EDTA, 8% glycerol, 2 m*M* dithiothreitol (DTT). For storage, proteins were adjusted to 30% glycerol and 10 m*M* DTT and BSA was supplemented to 0.1 mg/ml. The immunoaffinity column consisted of peptide-specific immunoglobulin against the carboxy terminus of the Tax protein [1]. The CREB protein was purified from 2×10^8 C81-66-45 cells by immunoaffinity chromatography using a CREB-specific peptide antibody (W39 peptide [8]) and processed as described for the purified Tax protein. Purification of CREB-specific immunoglobulins and the immunoaffinity column set up were essentially as described previously [1].

Nuclear Extract Preparation and DNA Mobility Shift Assay. Nuclear extracts from C81-66-45 cells were prepared and depleted from the Tax protein as described previously [2]. DNA mobility shift analysis were performed as described [2], and binding reactions with nuclear extracts contained 0.5 μg poly[d(I-C)] for precompetition of nonspecific binding as previously described [2]. Shift assays in the presence of specific immunoglobulin were performed as follows: nuclear extract or purified protein was preincubated with excess of specific antibody for 15 min before gel shift buffer and, in the case of nuclear extracts, poly[d(I-C)] was added. The binding reaction was initiated by addition of the ^{32}P-labeled DNA probe.

In Vitro Transcription. The TAR RNA (+1 to 200) was transcribed in vitro from a SP6-TAR construct. For radioactive labeling, [α^{32}P]UTP was added during the RNA synthesis.

Protein Preparation and RNA Mobility Shift Assay. Nuclear extracts (5×10^8 cells) were prepared to a final concentration of 0.5–1 μg/μl as described previously [2], except that the extraction buffer contained 500 m*M* NaCl. MS2-Tat protein (0.5 μg/μl) was isolated according to [9]. The MS2-Tat protein partially precipitates during renaturation. Both the soluble and insoluble material were tested for RNA binding. RNA mobility shift assays were performed as described previously [2] in the presence of RNasin and poly(I-C) instead of poly[d(I-C)] for nonspecific competition.

Results and Discussion

Analysis of Tax-TAR DNA Interaction

The Tax protein was isolated by immunoaffinity chromatography from metabolically labeled C81-66-45 cells, an HTLV-I immortalized human T lymphocyte line which produces Tax but no other viral proteins [1] (Fig. 1 A). Alternatively, nuclear extracts were prepared from C81-66-45 cells as described previously [2] and the Tax protein removed from the extract by use of the identical Tax-specific immunoaffinity column. Presence or absence of Tax protein form the total nuclear extract and the Tax-depleted extract was confirmed by indirect immunoprecipitation analysis using extracts from [^{35}S]methionine-labeled cells and Tax-specific antiserum (Fig. 1 A).

To analyze TRE DNA-protein interactions, a gel electrophoresis DNA mobility shift assay was used. A DNA oligonucleotide of 26 bp corresponding to the 5′-TRE, spanning nucleotides − 251 to − 231 of the HTLV-I LTR, was synthesized and radioactively labeled. This ^{32}P-labeled oligonucleotide was analyzed for DNA-protein interaction with purified proteins and nuclear extracts by separating DNA-protein complexes from unbound oligonucleotide on nondenaturing polyacrylamide gels (Fig. 1 B). The affinity-purified Tax protein (IA-Tax) was unable to bind directly to the TRE DNA and both the Tax-containing and the Tax-depleted nuclear extracts gave rise to identical DNA-protein complexes, irrespective of the presence or absence of Tax protein. The HTLV-I LTR is responsive to cyclic AMP and all three of the 21-bp TREs contain the sequence motif TGACG, which is homologous to the half-site binding sequences of the CREB/ATF binding site TGACGTCA and the Jun/AP-1 binding site TGACTCA.

To determine whether to CREB protein is involved in the complex formed by nuclear extracts and TRE oligonucleotide, CREB-specific immunoglobulin was included in the gel shift assay. This resulted in an additional CREB-specific gel shift complex (marked by the arrowhead in Fig. 1 B). As expected, Tax-specific immunoglobulin did not affect the DNA-protein complex. To confirm both direct binding of CREB protein to the TRE DNA oligonucleotide and the specificity of the additional intermediate formed with CREB-specific immunoglobulins, CREB was purified by immunoaffinity chromatography as described in Materials and Methods. The affinity-purified CREB protein (IA-CREB) gave rise to a specific gel shift complex when analyzed with the TRE DNA oligonucleotide. This had the same migration properties as the complex formed with total nuclear extract proteins and was shifted up when CREB-specific immunoglobulins were added to the binding reaction (Fig. 1 B). The specificity of the DNA-protein complex formation has been confirmed by specific and non-specific competition analyses and mutation of the CREB binding motif in the 21-bp TRE ([2], and data no shown).

Analysis of Tat-TAR RNA Interaction

In order to analyze the effect of the Tat protein on TAR RNA, the Tat protein was expressed as a recombinant protein in *Escherichia coli*. The first of the two coding exons, comprising 72 out of 86 amino acids, was expressed as MS2-Tat fusion protein, which is under the control of a λPL promoter and inducible by temperature shift [4] (Fig. 2 A). The MS2-Tat protein was sliced out of the gel, electroeluted, and used as antigen (Fig. 2 A) for the production of polyvalent antibodies. The serum precipitates the authentic Tat protein from HIV-1-infected H9 cells labeled metabolically with [^{35}S]cysteine for 90 min (Fig. 2 B). The result shows two bands, one corresponding to both exons and one to one exon only. The presence of the Rev

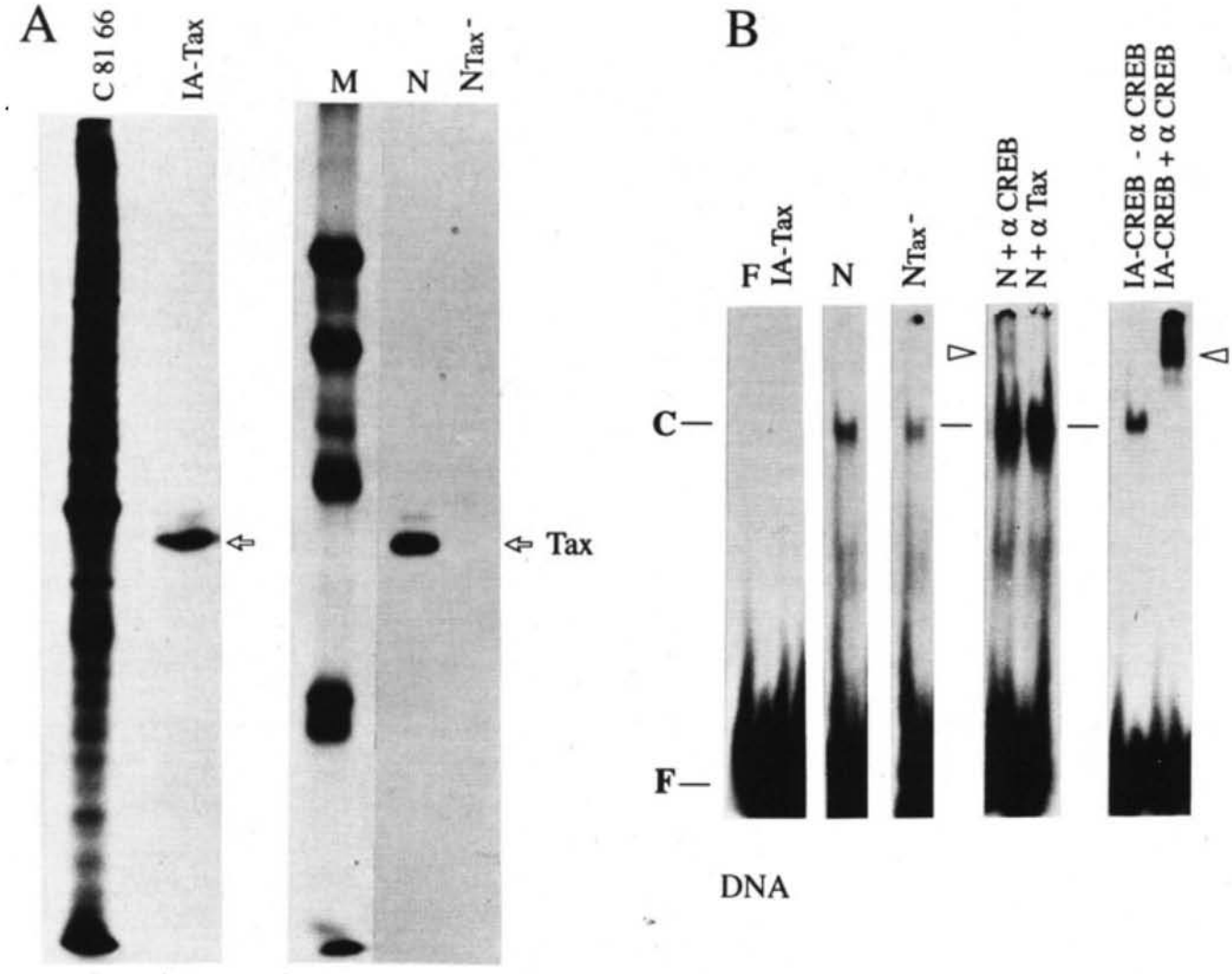

Fig. 1. **A** *Left*, Immunoaffinity column purification of the 40-kDa, Tax protein from [^{35}S]methionine-labeled C81-66-45 cells. Aliquots of the total cellular lysate and the purified Tax protein, designated *C8166* and *IA-Tax* respectively, were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *Right*, Indirect immunoprecipitation analysis of nuclear extracts. A Tax-specific immunoaffinity column was used to deplete nuclear extracts from [^{35}S]methionine-labeled C81-66-45 cells of Tax protein. The total nuclear extract and the Tax-depleted extract, designated *N* and N_{Tax-} respectively, were treated for indirect immunoprecipitation with Tax-specific antiserum. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *M* indicates ^{14}C-labeled marker proteins, from top to bottom: 92, 68, 53, 45 and 30 kDa. **B** DNA mobility shift analysis, using a ^{32}P-labeled synthetic DNA oligonucleotide of 26 bp which contains the 5′ 21-bp enhancer motif of the HTLV-I LTR and purified proteins or nuclear extracts. *IA-Tax*, affinity-purified Tax protein; *N*, Tax-containing nuclear extracts N_{Tax-}, Tax-depleted nuclear extracts; $N+\alpha CREB$, $N+\alpha Tax$, Tax-containing extracts in the presence of affinity-purified immunoglobulin specific for the cyclic AMP-responsive element binding protein (CREB) and the Tax protein respectively; $IA\text{-}CREB-/+\alpha CREB$, immunoaffinity purified CREB protein in the absence or presence of CREB-specific immunoglobulins; *F*, uncomplexed DNA oligonucleotide; *C*, specific DNA-protein complexes; *Arrowheads*, CREB-DNA complexes shifted by CREB-specific immunoglobulins

protein in the infected cells partially inhibits splicing and thereby leads to synthesis of the smaller Tat protein. An antiserum against a β-Gal-Tat fusion protein, patient serum, and carboxy-terminal synthetic peptide antiserum are shown as controls. The last one allows precipitation of the complete Tat protein, not of the protein corresponding only to the first coding exon (Fig. 2 B).

To study the RNA-Tat interaction, an *in vitro* transcribed RNA corresponding to the TAR RNA region was synthesized. It was labeled radioactively and used for

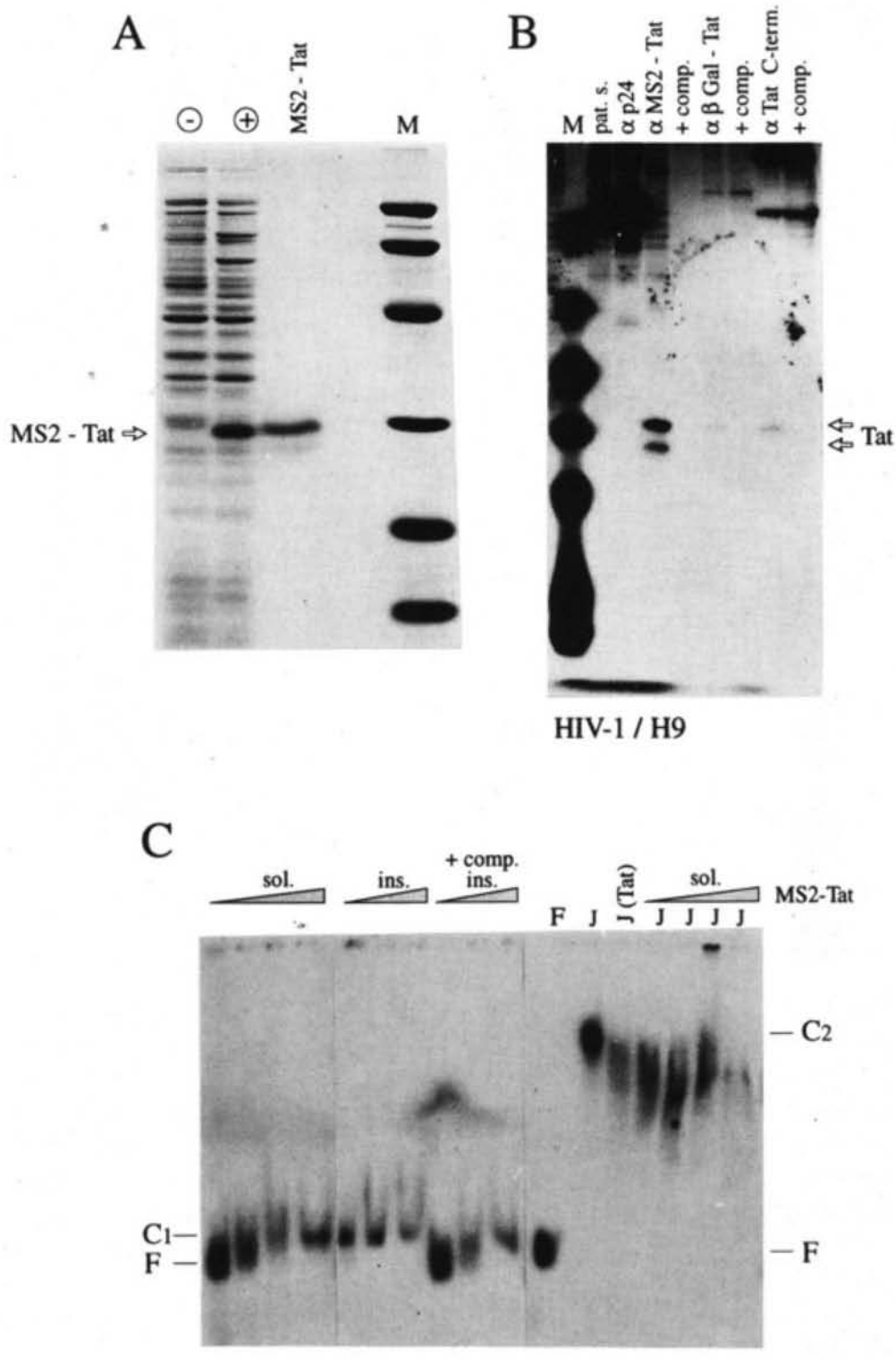

Fig. 2. **A** Expression of MS2-Tat fusion protein in *E. coli.* −, and +, bacterial lysates without and with induction of MS2-Tat (see *arrow*); *MS2-Tat*, isolated protein used as antigen; *M*, marker proteins, from top to bottom: 92.5, 66.2, 45, 31, 21.5, and 14.4 kDa. **B** Indirect immunoprecipitation of Tat protein from [^{35}S]cysteine metabolically labeled HIV-1-infected H9 cells (5×10^6). The sera used were: patient serum (*pat.s.*), antiserum (α) against p24, MS2-Tat, β-Gal-Tat, and a carboxy-terminal Tat peptide serum (*C-term*); *+ comp.*, competition with the respective antigen. *M*, ^{14}C-labeled marker proteins, from top to bottom: 43, 25.7, 18.4, 14.3, 6.2 and 3 kDa. **C** RNA-protein interaction was analyzed by gel shift analysis using increasing concentrations of soluble (*sol.*) and insoluble (*ins.*) MS2-Tat proteins (ranging from about 1 to 10 µg); *+ comp.*, competition with poly(I-C) (2 µg); *J*, *J*(Tat), nuclear extracts of Jurkat or Tat-expressing Jurkat cells (1 µg each); *F*, free RNA; C_1, C_2, protein-RNA complexes

gel shift analysis using purified Tat protein. This was recovered from gel slices, electroeluted, denatured by guanidine HCl, and renatured by dilution, which leads to some soluble Tat protein and some insoluble precipitates. Increasing concentrations of both proteins were analyzed in RNA mobility shift assays and gave rise to shift effects (Fig. 2C). The interaction was competed with the non-specific competitor poly(I-C), and resulted in some resistance of the RNA-Tat complex (Fig. 2C). The MS2-Tat-RNA interaction was not due to the MS2 moiety, which was excluded by using MS2-Orf as control (not shown). The TAR RNA also binds to cellular factors, as evidenced by using nuclear extracts from Jurkat cells which give rise to a gel shift band (Fig. 2C, right). This band was shifted down when the extract from Tat-expressing Jurkat cells was used. Also, addition of increasing amounts of purified soluble Tat protein gave rise to an increased migration velocity, suggesting some effect of the Tat protein on cellular proteins binding to the TAR RNA.

In summary, our data indicate that the Tax protein interacts with its TAR DNA element in concert with other factors, one of them being the CREB protein. The exact mechanism needs to be elucidated. The Tat protein, in contrast, binds to the TAR RNA, not DNA. Again, several host factors can bind to this region as well. The amount of Tat protein required for RNA shifts indicates that its binding is extremely inefficient, which may be due to the high degree of insolubility of the Tat protein or the need for additional host cell factors.

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Human Neuroblastoma: Paradigm for a Tumor with Oncogene Amplification and Loss of a Putative Tumor Suppressor Gene

M. Schwab [1]

Introduction

Human neuroblastoma cells often carry nonrandom chromosomal abnormalities signaling genetic alterations. Quite frequent are "double minutes" (DMs) and homogeneously staining regions (HSRs), both cytogenetic manifestations of amplified DNA, and chromosome 1p deletions indicating loss of genetic information. With the identification of amplified N-*myc* and the demonstration of a consensus deletion spanning the chromosome 1p36.1–2 region it now appears likely that both amplification of a cellular oncogene and loss of a tumor suppressor gene play important roles in neuroblastoma (Fig. 1).

N-*myc* Amplification

Biology of Amplified N-myc

N-*myc* was the first amplified oncogene that turned out to be of clinical significance due to its association with aggressively growing tumor phenotypes (for a review, see [1]). N-*myc* was originally identified when human neuroblastoma cells showing DMs or HSRs were analyzed with various oncogene probes [2, 3]. These surveys quickly established that, with few exceptions, cultured neuroblastoma cell lines carry the gene N-*myc* in an amplified form. At the same time neuroblastoma tumors were also found to carry amplified N-*myc* [3]. The initial surveys suggested that N-*myc* amplification was specific for neuroblastoma. It turned out later that N-*myc* amplification can be seen in small-cell lung cancer, retinoblastoma, and astrocytoma, although at much lower incidence. As a common feature, all these tumors have neural qualities. Until now N-*myc* has been the only gene, however, found amplified in neuroblastomas.

The oncogenic potential of enhanced expression of N-*myc* as the consequence of amplification has been addressed in various experimental systems. Enhanced expression, resulting from introduction of an N-*myc* expression vector, can assist mutationally activated H-*ras* in tumorigenic conversion of primary rat embryo cells [4], converts established cells of the rat [5] and of humans [6] to tumorigenicity, and rescues primary rat embryo cells from senescence [7]. Furthermore, N-*myc* has frequently been found activated by proviral insertion in murine leukemia virus (MuLV)-induced T cell lymphomas [8], and is involved in tumorigenesis in transgenic mice [9, 10]. These results clearly attest to the capacity of high N-*myc* expression to modulate the growth of cells, and it appears reasonable, therefore, to suggest that enhanced expression consequent on amplification contributes to tumorigenesis. The available evidence suggests that the nucleotide sequence of N-*myc* in neuroblastoma cells is unaltered compared to that of normal cells [11]. Consistent with this result, the biological activities of N-*myc* derived from normal or from neuroblastoma cells have not been found to differ [4, 7].

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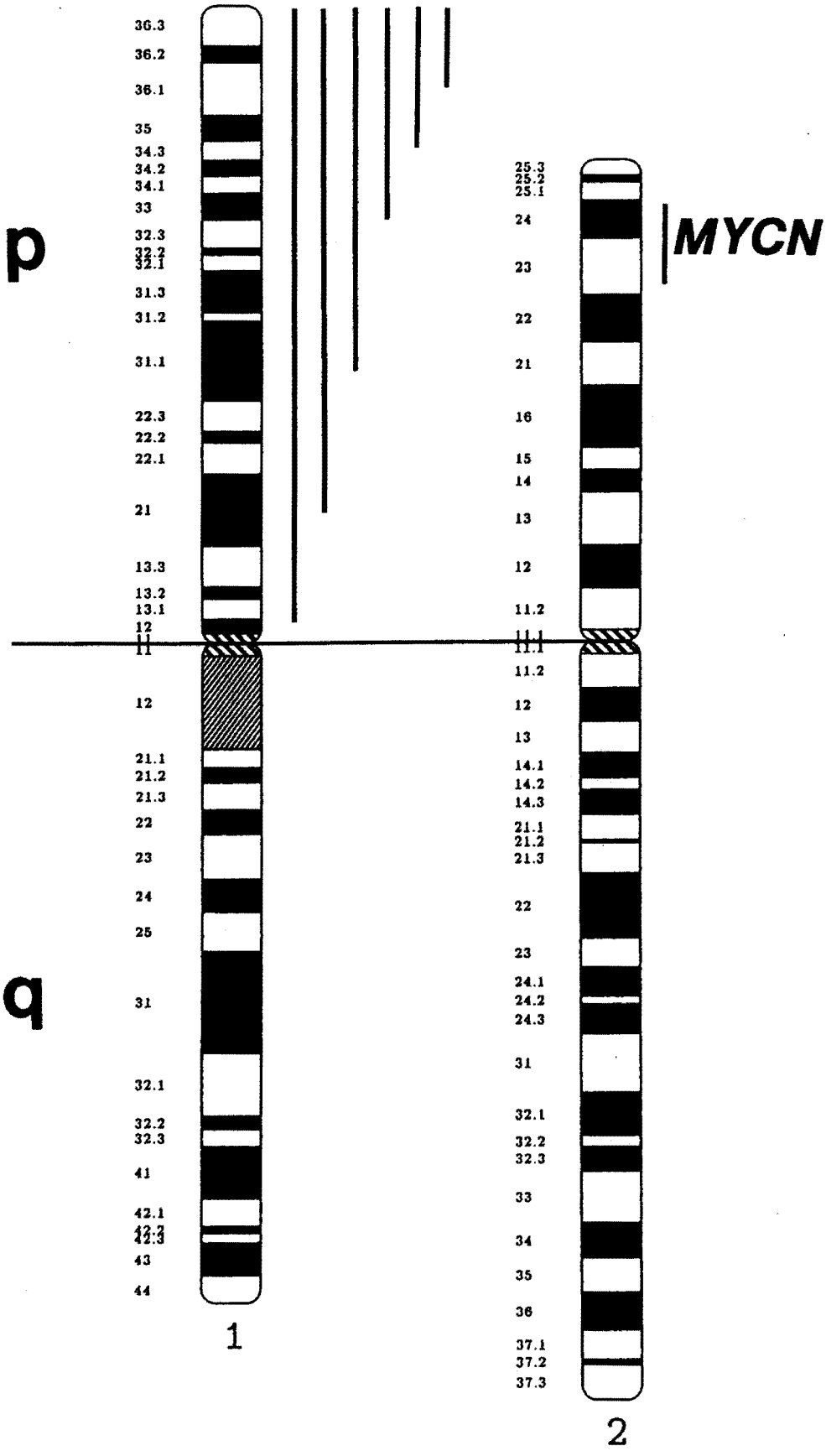

Fig. 1. Genes involved in human neuroblastoma. Loss of genetic material, possibly a tumor suppressor gene, from chromosome 1p36.1–2 and amplification of the oncogene *MYCN* from chromosome *2* seem to contribute in at least many cases to neuroblastoma. The *vertical lines* to the *right* of chromosome 1p indicate chromosomal deletions that vary in length in tumors of different patients. The region near the end of chromosome 1 has been found deleted in at least 90% of cases analyzed

Structural Arrangement of Amplified N-myc

There is little data available about the structure and the size of the amplified DNA encompassing cellular oncogenes, and what is known comes mostly from studies of N-*myc* amplification in neuroblastomas. By using random amplified probes isolated from flow-sorted chromosomes of the neuroblastoma line IMR-32 [12], it was observed that the amplified DNA encompassing N-*myc* differs when a series of neuroblastomas is analyzed [13, 14]. DNA amplified in IMR-32 was amplified to a lower degree or not at all in other neuroblastomas. The gene N-*myc* was amplified in all cases carrying amplified DNA, however. A similar observation was made by Zehnbauer et al. [15], who used a set of random probes from neuroblastoma line NGP. These authors also found that the amplification units in NGP cells and 12 different primary neuroblastoma tumors were similar over a contiguous region of at least 140 kb encompassing N-*myc*. In line with this, the gene encoding ornithine decarboxylase, which is linked to N-*myc*, has been found coamplified with N-*myc* in only one of six tumors [16].

The size of the amplified DNA containing N-*myc* and *myc* genes has been analyzed by employing denaturation and reassociation of DNA in agarose gels [17]. This approach involved cutting DNA with a restriction endonuclease, size fractionation through agarose gels, alkali denaturation, partial reassociation, and subsequent treatment with the single-strand-specific nuclease S1. Amplified sequences in tumor cells, due to their relative higher concentration, reassociate at a higher rate than their single-copy counterparts in normal cells and therefore become S1 nuclease-resistant when single-copy sequences are still sensitive. If the DNA is radioactively labeled, under suitable conditions the autoradiographs of the gels will reveal a banding pattern in cases of DNA amplification. This approach has indicated that the size of the amplified DNA encompassing N-*myc* ranges in different tumors from 290 to 430 kb, and that the DNA containing the *myc* units ranges in size from 90 to 300 kb [18].

A direct determination of the size and the structure of the amplified DNA in human neuroblastoma cells has been done by pulsed field gel electrophoresis, which is capable of fractionating DNA fragments in the size range from one hundred to several thousand kilobare pairs. The analysis was facilitated by the finding that the 5′-region of N-*myc* is in a CpG island and has recognition sequences for several rare cutting enzymes [19]. This situation made it possible to map the DNA encompassing N-*myc* over a distance of more than 1000 kb. By employing suitable N-*myc* probes derived from the 5′- and 3′-regions of recognition sites for rare cutting restriction endonucleases within N-*myc* the amplified DNA was found in many cases arranged in precise head-to-tail units. These units varied among different neuroblastomas and ranged from about 100 to 800 kb in size [19]. The precise and ordered head-to-tail arrangement is stable over long periods of time and does not change upon establishment of tumor cells into *in vitro* culture or during passages of tumor cells through athymic mice. There are principally two mechanisms that might be involved in initiation of amplification. Amplification may start with unscheduled replication of DNA encompassing N-*myc*, which maps to chromosome 2p23–24 [20] (Fig. 2A). Alternatively, amplification may start with loop formation and excision of the DNA (Fig. 2B). In either event, extrachromosomal DNA molecules that integrate into a distant chromosomal region and *in situ* amplification appear to result (Fig. 2) (for a detailed discussion see [1]). The amplified N-*myc* copies map in most instances to HSRs that are localized on different chromosomes in cells derived from different tumors. The direct repeat structure that in neuroblastomas is stable over many cellular divisions appears to be

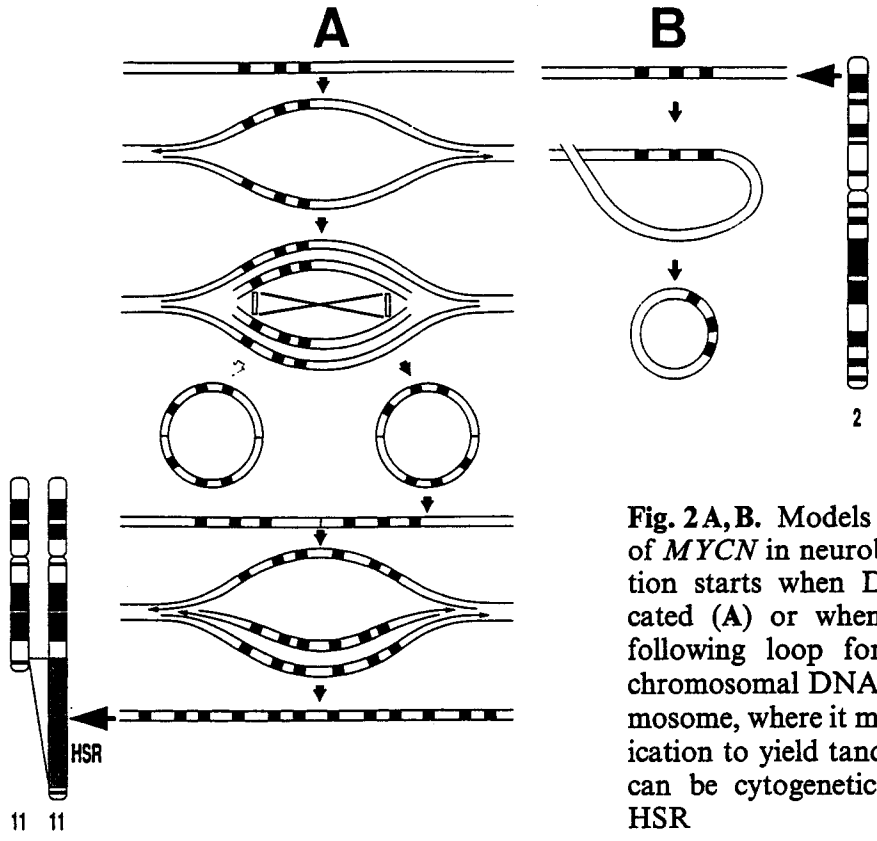

Fig. 2A,B. Models illustrating amplification of *MYCN* in neuroblastoma cells. Amplification starts when DNA becomes extrareplicated (**A**) or when DNA becomes excised following loop formation (**B**). The extrachromosomal DNA is integrated into a chromosome, where it may undergo in situ amplification to yield tandem DNA amplicons that can be cytogenetically demonstrated as an HSR

different from that of amplified DNA associated with drug resistance, where head-to-head arrangements with inverted repeats predominate and where high instability of the amplified DNA structure is observed [21]. It is not clear if these differences result from fundamental differences not clear if these differences result from fundamental differences of the mechanisms by which DNA in drug-resistant cells and in tumor cells become amplified, or if they are the consequence of exposure to cytotoxic drugs. It will be interesting to find out what the structure of the DNA encompassing other cellular oncogenes might be.

Clinical Significance of N-myc Amplification

An important prognostic variable for patients with neuroblastomas in the clinical stage. Patients with disease stages I and II have a good prognosis with 75%–90% 2-year disease-free survival, while patients with stages III and IV have a poor prognosis, with 10%–30% 2-year survival. Surveys of over 400 neuroblastomas revealed that a strong correlation exists between N-*myc* amplification and stage III and IV [22–25]. A number of patients with stage I or II have been identified carrying amplification. In all instances, these tumors, which on the basis of conventional diagnostic possibilities were of good prognosis, progressed later and turned out to be fatal. A peculiar stage IV-s characterized by frequent spontaneous regression rarely shows amplification (7%). Studies of three patients with N-*myc* amplification have been published [26–28]; all tumors progressed later. These observations clearly show that N-*myc* amplification is a reliable prognostic parameter for poor prognosis in patients with low stage of IV-s tumors.

The prognosis of patients over 1 year, mainly diagnosed with stage III or IV tumors, is particularly poor, and metastases occur predominantly in the bone, orbita, and distant lymph nodes. More than 50% of the patients over 1 year carried amplification of *myc*, while amplification was rarely seen in patients

below 1 year of age [24]. Altogether, N-*myc* amplification is associated with a higher malignant phenotype of neuroblastoma.

Current therapeutic strategies for treatment of neuroblastoma depend on the prognosis for survival which is evaluated in the basis of tumor stage, on the degree at which the tumor can be removed surgically and one the basis of genomic analyses of the tumor cells. The pilot study of the German Neuroblastoma Study Group advises treatment of patients according to protocols that are specific for each of four risk groups. Risk group A includes patients with a localized tumor that can be surgically removed to at least 90% (prognosis is 90%–100% for survival of patients). Risk group B includes patients with a localized tumor that extends beyond the area of the organ of origin and usually cannot be removed completely (prognosis 65%–80%). Risk group C includes patients that carry a metastatic tumor, or a localized tumor that cannot be removed after four cycles of chemotherapy (prognosis 20%–30%). Risk group D includes only patients with a IV-s tumor that frequently shows spontaneous regression (prognosis 75%–80%). Patients that on the basis of conventional parameters would be included in risk groups A and B are transferred to risk group C if there is N-*myc* amplification. Patients included in risk group C receive the most intensive therapeutic treatment. It remains to be seen if the same is advisable for patients of risk group D.

Chromosome 1p Deletion

Deletions of chromosome 1 were first described in 1975 [29]. Even though the breakpoints of the deletion were found to vary, the portion of the chromosome distal to band p32 seemed to be most consistently deleted. In all cases, the deletions appeared to involve only one chromosome. From cytogenetic analyses, the portion deleted appears to be lost from the genome, which means that the cells are monosomic for this genetic material [30].

At this point, the nature of the genetic material deleted and its significance to tumorigenesis can only be speculated upon. There is a good possibility that the deletion involves genetic information essential for normal differentiation of certain neutral cells. Loss could result in abnormal differentiation and could be a factor contributing to tumorigenesis. In a similar way, lack of genetic information identified by cytogenetic analysis in specific regions of other chromosomes seems to contribute to other types of tumors, in particular Wilm's tumor, retinoblastoma, lung cancer and certain forms of colon cancer (for a review see [31]). The general idea is that the presence of this genetic information supresses tumorigenesis and its lack allows tumors to develop. Genes behaving as suppressors of tumorigenesis have been termed "tumor suppressor genes," although their functions and the mechanisms by which they act are obscure as yet.

To define the role that loss of genetic information from 1p36 might have in neuroblastoma, we set out to determine a consensus deletion. Our approach depended on first generating a panel of DNA probes closely positioned to each other, and recently we reported on the generation of a microclone library specific for the very distal part of chromosome 1p [32]. The dense distribution of probes proved advantageous for the present study in different respects. Firstly, small deletions could be detected with a higher probability. Secondly, it provided the ability to detect allelic loss even in cases where adjacent loci were not informative due to homozygosity. Thirdly, it was necessary to define the borders of deleted regions within narrow limits and to determine a small region commonly deleted in different tumors.

Applying this strategy we were able to discover allelic deletions in a high proportion of tumors (90%) analyzed (Fig. 3). With respect to the cytogenetic data of

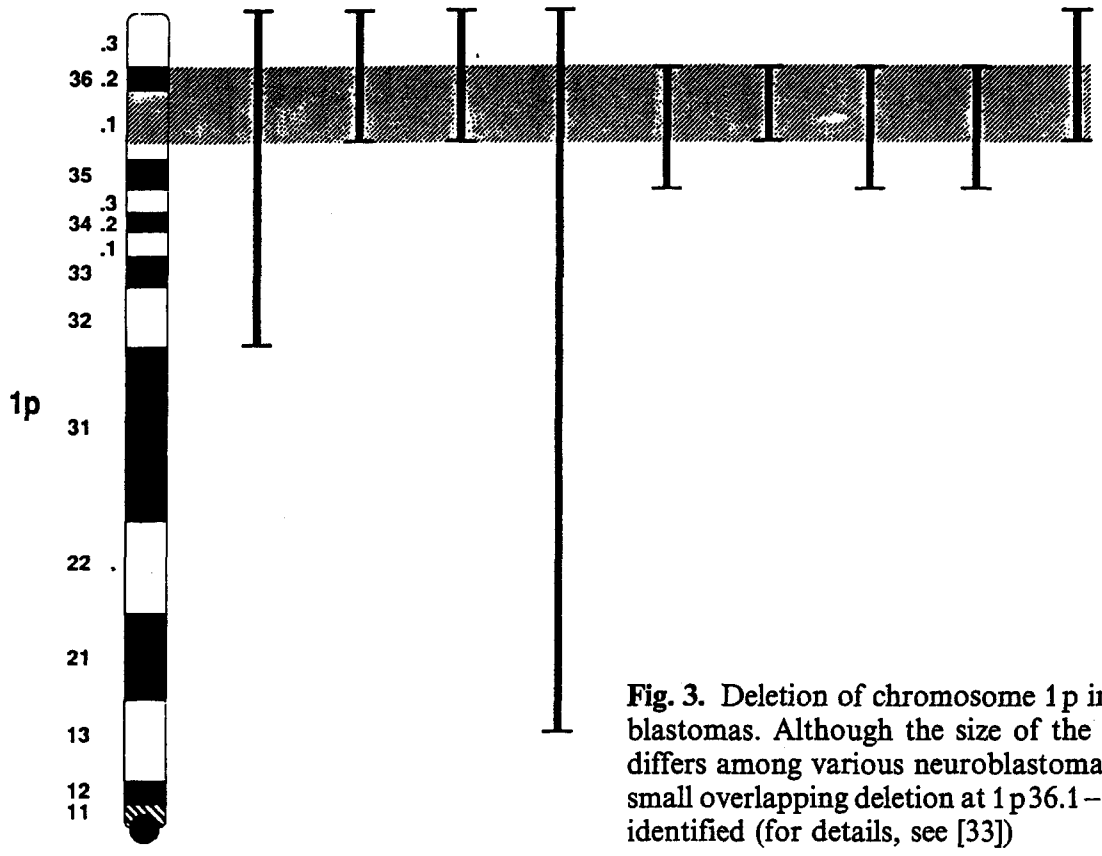

Fig. 3. Deletion of chromosome 1p in neuroblastomas. Although the size of the deletion differs among various neuroblastomas a very small overlapping deletion at 1p36.1–2 can be identified (for details, see [33])

neuroblastomas, our results revealed that distal 1p material was indeed lost from the genome of the tumor cells [33]. In another study of chromosomal deletions in neuroblastomas [34] loss of heterozygosity was also detected in 1p36, though the frequency described was lower (28%) than indicated by cytogenetic analyses. The higher overall frequency of loss of genetic material from the 1p region among stage III and IV tumors as compared to the frequency found by Fong et al. (28% vs. 90%) [34] is probably due to the fact that our microclone probes are from within the consensus deletion and are located closer to the genetic information related to neuroblastoma. The fraction of tumors displaying allelic loss with our probes exceeded that showing cytogenetically detectable rearrangements. This is not surprising, because the number of tumors displaying loss on the DNA level in the present study was bound to exceed that showing microscopically visible deletions. We suspect that small deletions are likely to remain undetectable upon microscopic investigation.

We found only one neuroblastoma that did not display any allelic loss. A deletion could probably not be detected in this case because two probes which resided in the area commonly lost in other tumors were not informative. Hence, it is still possible that in this particular case an as yet unidentified deletion is present. Alternatively, we must take into account that the tumor sample might have contained a high proportion of normal tissue, thus obscuring any loss of alleles. Yet another explanation may be that in this tumor the DNA in the consensus regions is altered by a subtle deletion or point mutation not detectable with our probes. Among the neuroblastomas analyzed we found evidence for interstitial deletions in four cases. Through these a consensus deletion could be defined which was commonly lost from the tumors. On this basis we have located a consensus deletion to the subbands 1p36.1–2. We are well aware of the limited resolution of *in situ* hybrid-

ization and of the uncertainty for an exact relative localization of DNA probes. We are in the process of defining the exact linkage relationship of these markers by long-range restriction mapping.

Even considering this uncertainty, a rough estimate can be made as to the size of the deleted DNA segment. The entire 1p36 band comprises about 0.7% of the total haploid chromosome length (for values of relative chromosome lengths, see [35]). Predicting an even distribution of the DNA along the chromosomes, 1p36 would contain about 20 Mbp (megabase pairs) of DNA (0.7% of the total haploid DNA content of 3×10^9). 1p36.1–2 represents roughly half of the 1p36 band. Hence the genomic region included in the consensus deletion would span about 10 Mbp of DNA. Since we can fall back on a large number of specific microcloned probes, we are in the process of establishing a long-range restriction map of this region with pulsed-field electrophoresis. This long-range map will provide information on the presence and location of CpG-rich islands, which often signify the 5′-regions of genes [36], and therefore will be a tool for the identification of coding DNA sequences in this region.

Recently, Suzuki et al. [37] reported on loss of heterozygosity in neuroblastomas using probes specific for various chromosomes. With a N-*myc*-specific probe they found only two out of 12 tumors to have lost alleles from 1p. This is in agreement with our data, assigning a putative consensus deletion more distal than the N-*myc* locus. In one tumor with an extended deletion we also found allelic loss involving the N-*myc*-region.

A significant correlation between the disease stage of neuroblastomas and N-*myc* amplification has been established in the past (for a review, see [38]). For the time being no data are available that elucidate the question of whether amplification of N-*myc* and loss of DNA sequences from chromosome 1 occur in association with each other or whether one of the mutational events precedes the other in neuroblastoma tumorigenesis. Our results do not support the idea of a correlation between these two events as has been proposed by Fong et al. [34].

At present, the molecular studies performed on human neuroblastoma indicate that the development of tumors may be a result of various genetic alterations involving amplification of the N-*myc* oncogene [38, 39], as well as loss of genetic information from 1p36 [33, 34] and 14q [37]. So far no evaluation of these mutational events can be made with respect to their function or to a hierarchy of events in the initiation and progression of human neuroblastoma.

Conclusions

Central issues in cancer research are (a) how genetic alterations contribute to tumorigenesis, (b) how specific genetic alterations can be turned into diagnostic tools to provide information on how to optimize existing therapeutic regimens, and (c) how to open up avenues for causal therapeutic strategies. During the past decade much has been learned about genetic alterations in tumor cells. The activation of the oncogenic potential of cellular genes can take different routes among which mutational alteration, translocation and amplification predominate (for a review, see [40]). In particular, amplification has found its way to practical clinical use due to its association with more aggressively growing types of human cancer. N-*myc* amplification in neuroblastoma has provided a paradigm for the prognostic significance of oncogene alteration, and at the same time has represented the clinical debut of oncogene research. The full significance of oncogene amplification as a predictor for poor prognosis became clear with the identification of amplified *erb*-B2 in aggressively growing breast cancers [41]. The state of the art is that amplified cellular oncogenes define cancer patients which have poor prognosis and may require specific therapeutic regimen.

Of great interest could be the identification of a gene that is commonly deleted in neuroblastomas from the chromosome 1p region. What will be the normal function of this putative tumor suppressor gene? And once the function is known, can this information be exploited to revert the malignant phenotype? The answer to these questions is not trivial, but the project is worth being pursued under this perspective.

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Activation of Gene Expression by Human Herpes Virus 6

M. E. M. Campbell and S. McCorkindale[1]

Introduction

Human herpes virus type 6 (HHV-6) was first detected by Salahuddin et al. [6] and has been isolated from patients with lymphoproliferative and immunosuppressive conditions. HHV-6 is the causative agent of exanthem subitum and the majority of the adult population has been infected with this virus.

HHV-6 is able to grow in lymphoblastoid cell lines. Previous studies have shown that coinfection with human immunodeficiency virus (HIV) leads to accelerated cell death [5] and that infection with HHV-6 increases expression from the HIV long terminal repeats (LTR).

We set out to study the effect of HHV-6 infection on transcription directed by the human T-cell leukaemia virus type 1 (HTLV-1) LTR as there is the potential for these two viruses to interact in vivo as both have similar cell tropisms.

Activation of HTLV-1 LTR CAT by HHV-6

In order to determine if infection with HHV-6 could increase expression of an HTLV-1 LTR CAT construct, transfection experiments were carried out. HIV LTR CAT and HTLV-1 LTR CAT were introduced into J. Jhan cells (a T-lymphocyte cell line) using the diethylaminoethyl (DEAE) dextran transfection method. Following transfection, media were added to the cells from uninfected or HHV-6-infected cultures. The cells were incubated for 2 days at 37 °C then harvested and the CAT activity assayed. An exceptionally marked increase in CAT activity was observed from both the HTLV-1 LTR and HIV LTR CAT constructs (Fig. 1).

Transcription mediated by the HTLV-1 LTR is upregulated by tax, a 40-kDa protein encoded by HTLV-1. To examine the effect of coactivation with tax and HHV-6, cells were transfected with HTLV-1 LTR CAT and either mock-infected, infected with HHV-6 or cotransfected with a plasmid carrying the HTLV-1 *tax* gene. The results (Fig. 2) show that when both methods of activation are used together a very strong response is observed which is greater than the sum of the individual activations. This result shows that tax and HHV-6 act synergistically and implies they have different mechanisms of activation.

Activation of Gene Expression During HHV-6 Infection Does Not Require Specific Promoter Regulatory Sequences

In order to study the sequences required for HHV-6 activation we constructed a deletion mutant of HTLV-1 LTR CAT by removing sequences upstream of 55 bp from the initiation site. The resultant plasmid pH2 CAT contains the TATA box but lacks upstream regulators. When pH2 CAT was introduced into cells its expression was increased by 41.8-fold by

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

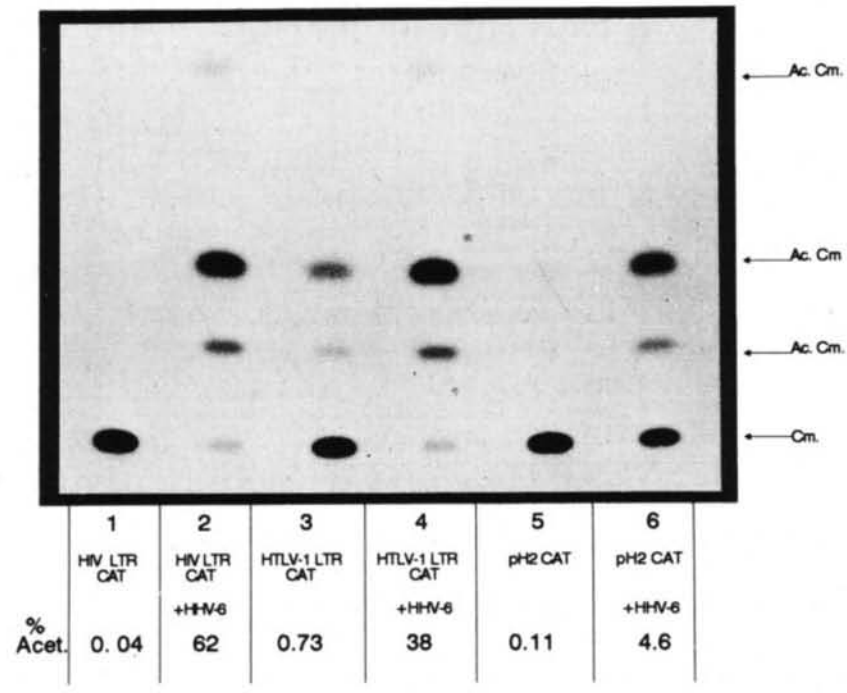

Fig. 1. Activation of promoter CAT constructs in transfected J. Jhan cells. Plasmids used and HHV-6 infections are indicated. The percentage acetylation of chloramphenicol/μg protein per hour of assay is shown (*Ac.Cm:* acetylated chloramphenicol; *Cm:* chloramphenicol)

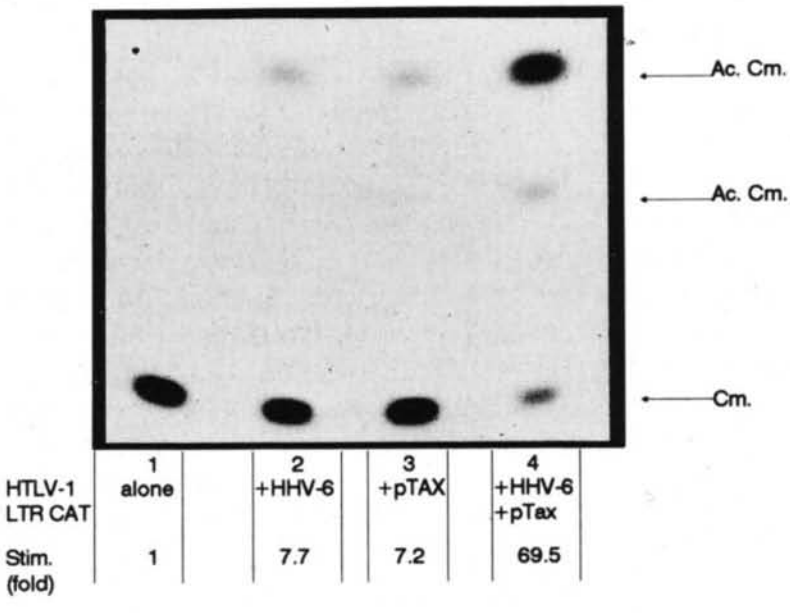

Fig. 2. CAT assays showing induction of HTLV-1 LTR CAT with HHV-6 and tax. Average-fold increase in CAT activity (for three experiments) compared to cells transfected with HTLV-1 LTR CAT alone is shown

HHV-6 infection, while in the same set of experiments HTLV-1 LTR CAT was stimulated by 52.0-fold (Fig. 1), although basal levels of pH2 CAT expression were lower. This result showed that upstream regulators are not required for HHV-6 activation.

A more detailed investigation of herpesvirus promoter elements required for activation by HHV-6 was carried out by utilizing a series of fine deletion mutants of the HSV-1 glycoprotein D gene [2] (Fig. 3). The constructs were transfected into J. Jhan cells and infected with HHV-

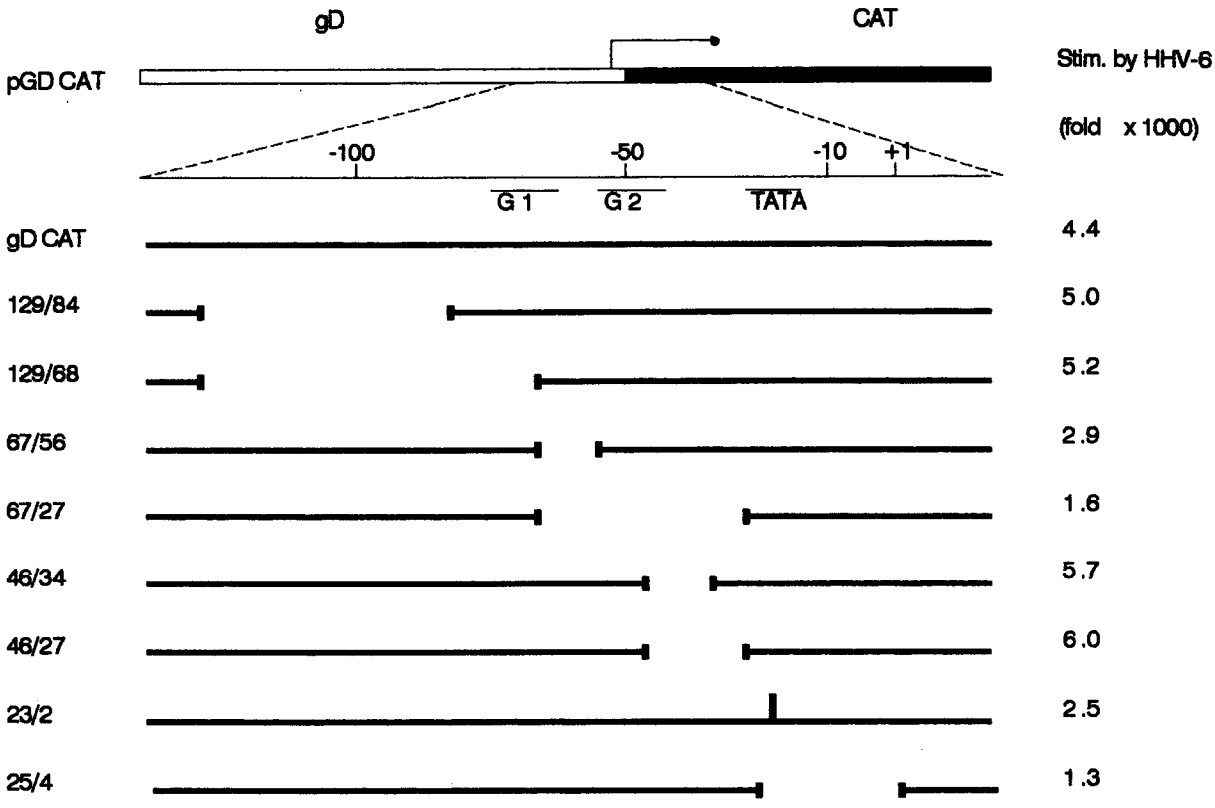

Fig. 3. Deletion mutants of HSV-1 pgD CAT. Promoter sequences of the gD gene are shown, indicating positions of the TATA box and GA-rich sequences (*G1* and *G2*). Stimulation (*stim*) in CAT activity due to HHV-6 infection is shown

6, or mock-infected. The level of activation of all pgDCAT constructs is very high (Fig. 3) and although some variation is observed this difference is small compared to the total level of induction. These results show that no essential promoter sequences are required for activation.

HHV-6 Infection Does Not Increase Levels of HTLV-1 mRNA

The C8166 cell line contains integrated defective HTLV-1 proviruses, one of which is able to synthesize the doubly spliced form of mRNA which encodes tax. Later species of RNA and infectious virus are not produced. To study the effect of HHV-6 on HTLV-1 RNA levels cytoplasmic RNA was isolated from cells which had been infected for 2 days with HHV-6 or from uninfected cells and was analysed by S1 nuclease mapping. A probe which spanned the mRNA start site was hybridized to the RNA and electrophoresed on a denaturing gel following S1 digestion. Both samples of C8166 RNA (Fig. 4, lanes 2 and 3) gave rise to a hybrid band of 58 bp which corresponds to correctly initiated HTLV-1 mRNA. The intensity of this band was not greater in the HHV-6 infected sample showing that infection did not affect levels of accumulated HTLV-1 mRNA.

HHV-6 Activation is Dependent On Gene Construct

We examined the effect of HHV-6 infection on the expression of different constructs under the regulation of the HSV-1 thymidine kinase (TK) promoter to determine if the nature of the reporter gene played any role in HHV-6 activation. The TK CAT constructs, like all other CAT constructs tested were strong-

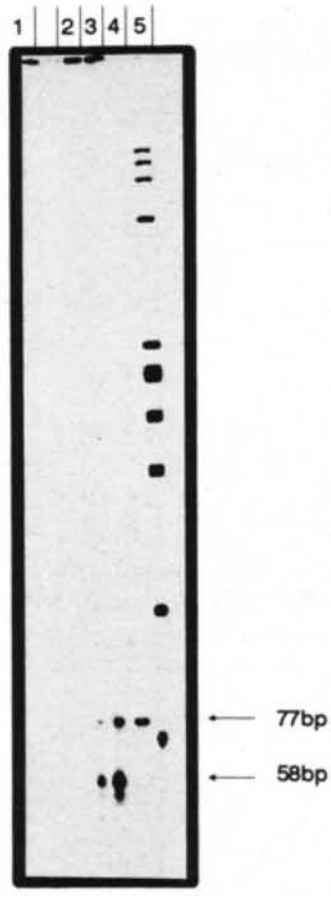

Fig. 4. S1 analysis of cytoplasmic RNA samples following electrophoresis on an 8% denaturing polyacrylamide gel. Samples used for S1 analysis were: J. Jhan RNA (lane *1*), HHV-6-infected C8166 RNA (lane *2*), mock-infected C8166 RNA (lane *3*). Markers were 77 bp probe (lane *4*) and *Hae*II-digested phi X DNA (lane *5*)

ly induced by infection (by 160-fold) (Table 1). However, the growth hormone (GH) gene controlled by the TK promoter, pTKGH, was only induced by 1.3-fold, showing this construct to have a very low, if any, response to infection. A similar level of activation was observed when both plasmids were cotransfected with a plasmid encoding the pseudorabies virus transactivator (pPRV IE), showing pTKGH can be efficiently expressed. These results reveal that induction of gene expression by HHV-6 is dependent on the recorder gene used. Expression of the CAT gene is strongly increased while very little change in GH levels is observed.

Conclusions

Our results show that activation by HHV-6 depends on the recorder gene used and that the role of the promoter or enhancer sequences are, at most, of minor importance. Induction of gene expression by HHV-6 is very likely to occur post transcription since any transcriptional effect would be expected to be independent of the reporter gene used.

The data we have obtained differ from that of Ensoli et al. [1] who studied activation of the HIV LTR by HHV-6. These workers found induction to occur at the level of transcription and that particular sequences within the HIV LTR were responsible. They also noted that an

Table 1. Activation of different reporter gene constructs by HHV-6

Plasmid[a]	CAT activity[b]	GH concentration (ng/ml)	Activation[c]
pTKCAT	0.12	–	–
pTKCAT + HHV-6	19.30	–	160.8
pTKCAT + pPRV IE	2.47	–	20.6
pTKGH	–	–	–
pTKGH + HHV-6	–	8.7	1.3
pTKGH + pPRV IE	–	106.6	14.6

[a] Plasmids were transfected into J. Jhan cells.
[b] Percentage acetylation per microgram of protein per hour of assay.
[c] Fold increase in activity due to HHV-6 infection.

HTLV-1 LTR CAT construct was unresponsive to HHV-6. The differences between our results and those of Ensoli et al. [1] are likely to stem from differences in the activity or expression of the transactivating polypeptides between the two strains of virus.

Our results, however, bear a strong similarity to those obtained by Kenney et al. [3, 4] who studied the BMLF1 gene, which encodes an immediate early transactivator of the Epstein Barr virus (EBV). These workers find activation of all CAT constructs by BMLF1, including those with no recognizable eukaryotic promoter, and they observe no increase in CAT mRNA levels. Most significantly they find that only CAT constructs and not GH constructs are induced by BMLF1. The similarity between these results and those obtained for HHV-6 suggests that the HHV-6 transactivator may be a homologue of BMLF1.

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Expression of S71-Related Sequences in Human Cells

C. Leib-Mösch[1], M. Bachmann[1], E.-M. Geigl[2], R. Brack-Werner[2], T. Werner[3], V. Erfle[2], and R. Hehlmann[1]

Introduction

The presence of C-type retrovirus-related proteins in normal or neoplastic human tissues has been described in several studies (for review see [1]). Antigens related to structural proteins of the simian sarcoma-associated virus (SSAV)/gibbon ape leukemia virus (GaLV) primate retrovirus group have been demonstrated in human leukemic cells [2] and placenta [3]. We have previously reported the isolation of a 70-kDa protein from human leukemic sera that cross-reacts with the capsid proteins (CA) of SSAV and baboon endogenous virus (BaEV) [4]. Furthermore, proteins related to the SSAV envelope gp70 protein (SU) seem to be of value in indicating the prognosis of patients with acute leukemia and chronic myelogenous leukemia (CML) in blast crisis [5]. In search of the origin of these proteins, we have identified about 25–35 SSAV-related sequences in the human genome by low stringency hybridization. One of these sequences, S71, was molecularly cloned and further analyzed [6]. S71 is a truncated retroviral element with the genomic organization 5-gag-SNRS-pol-LTR-3′ [7, 8]. SNRS represents a region of 1130 bp in S71 that consists of nonretroviral sequences. Here, we report our studies on the expression of S71-related sequences in human cell lines and tissues.

Material and Methods

The human leukemia cell line K562 was provided by Dr. Ziegler, University of Munich, FRG. The human placenta complementary deoxyribonucleic acid (cDNA) library in λgt11 was purchased from Clontech, Palo Alto, California, USA. Total cellular ribonucleic acid (RNA) was prepared from tissues and cell lines by the method of Chirgwin et al. [9]. The polyadenylated RNA fraction was purified from total cellular RNA by chromatography on oligo(dT)-cellulose columns. For Northern analysis, polyadenylated RNA was glyoxylated, separated by electrophoresis in agarose gels, and transferred to nylon filters (Zetaprobe, BioRad, Richmond California). Hybridization was carried out at 50 °C in 50% formamide, 1.5 × SSPE (1 × SSPE = 0.18 *M* NaCl, 0.01 *M* Na_2HPO_4, 0.001 *M* EDTA), 1% SDS, 0.5% powdered milk, yeast RNA 0.2 mg/ml, salmon sperm DNA 0.5 mg/ml, and ^{32}P-labeled DNA 1–2 × 10^6 cpm/ml. Filters were washed at 60 °C in 0.1 × SSC (1 × SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate), 1% SDS. Hybridization of Southern blots and phage filter lifts was carried out as described previously [6]. DNA fragments from isolated cDNA clones were subcloned in pUC120. Dideoxy sequencing of double-stranded plasmid DNA was carried out as described [8].

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Results and Discussion

By low-stringency hybridization of human DNA with specific probes of the long terminal repeat (LTR), *gag*, and *pol* regions of S71 we have found S71 to belong to a family of related sequences comprising about 15–20 copies per haploid genome (data not shown). To study expression of these retroviral elements we have screened several human cell lines by Northern blot analysis, using a 3-kb fragment of S71 that contained the 3′ part of SNRS, *pol*, and LTR. The human leukemia cell line K562, derived from a patient with CML in blast crisis, showed a prominent band of 2.9 kb as well as two minor bands of 3.6 and 2.5 kb (Fig. 1, lane 1). These bands could not be detected in normal human peripheral blood cells (lane 2) and other human cell lines (T-cell lymphomas, KE37, H9, HUT78; monocytes, U937; breast carcinoma, T47D; amnion cells, AMA; data not shown).

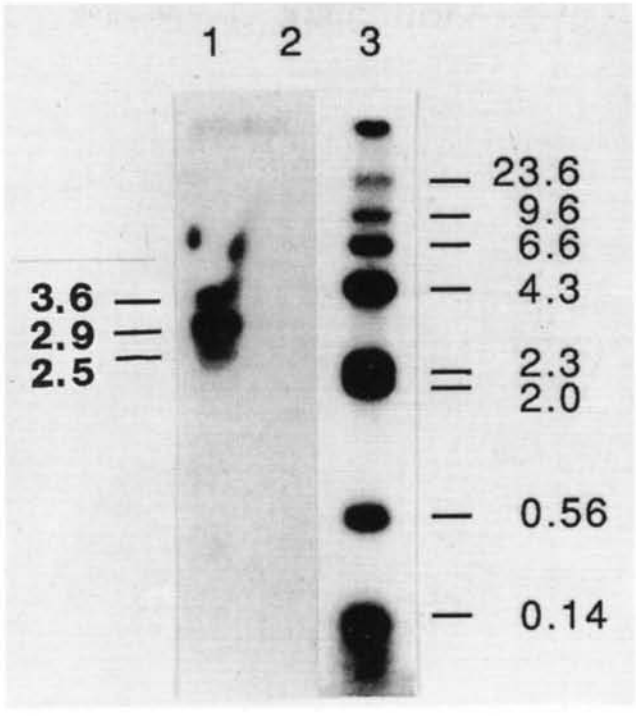

Fig. 1. Expression of S71-related sequences in human leukemic cells. mRNA isolated from human K562 cells (*1*) and normal peripheral blood cells (*2*) were hybridized with a 3-kb S71 SNRS-*pol*-LTR fragment as described in "Material and Methods." *3*, Size marker

High-level expression of human endogenous retroviral elements is often found in placenta, and fetal tissues (for reviews see [1, 10, 11]). Therefore, we screened a human placenta cDNA library under low-stringency hybridization conditions using a recombinant full-length S71 genome as hybridization probe. We isolated a cDNA clone 1.1 kb in length, Pl124, that hybridized specifically only with the S71 LTR, but not with S71 *gag*, *pol*, and SNRS sequences (data not shown). Sequence analysis revealed that a 350-bp stretch of Pl124 shows about 76% homology to the 3′ end of S71 (Fig. 2). The homologous region comprises half of the U3 sequences and is responsible for the strong hybridization signal obtained with S71 LTR. The remaining parts of Pl124 appear to be of nonretroviral origin. A search of the EMBL database revealed that the flanking sequences are not homologous to any known cellular or retroviral sequence. Immediately 3′ of the S71U3-related region, however, sequence elements were detected which resemble transcription signals like CAAT box and TATAA box. Although this region shows no obvious similarity with the U3 region of any known endogenous LTRs, it may have been introduced by a recombination event with another as yet unknown retroviral element. Singular, unrelated subregions within the U3 region of endogenous retroviral LTRs have also been observed in another family of human C-type elements [12]. During cDNA cloning, Pl124 may have lost its original 5-terminus. It is therefore not yet clear whether this sequence is transcribed from a 5′-LTR or a nonretroviral external promoter, as has been shown for other LTR containing retroelements [13].

For further characterization of the nonretroviral sequences of Pl124, we hybridized Southern blots of human genomic DNA with a Pl124 probe under high-stringency conditions (Fig. 3). After digestion with various restriction enzymes, the hybridization patterns of human DNA (lanes 1–5 and 7–11) showed only two or three prominent bands, indicating that the human genome

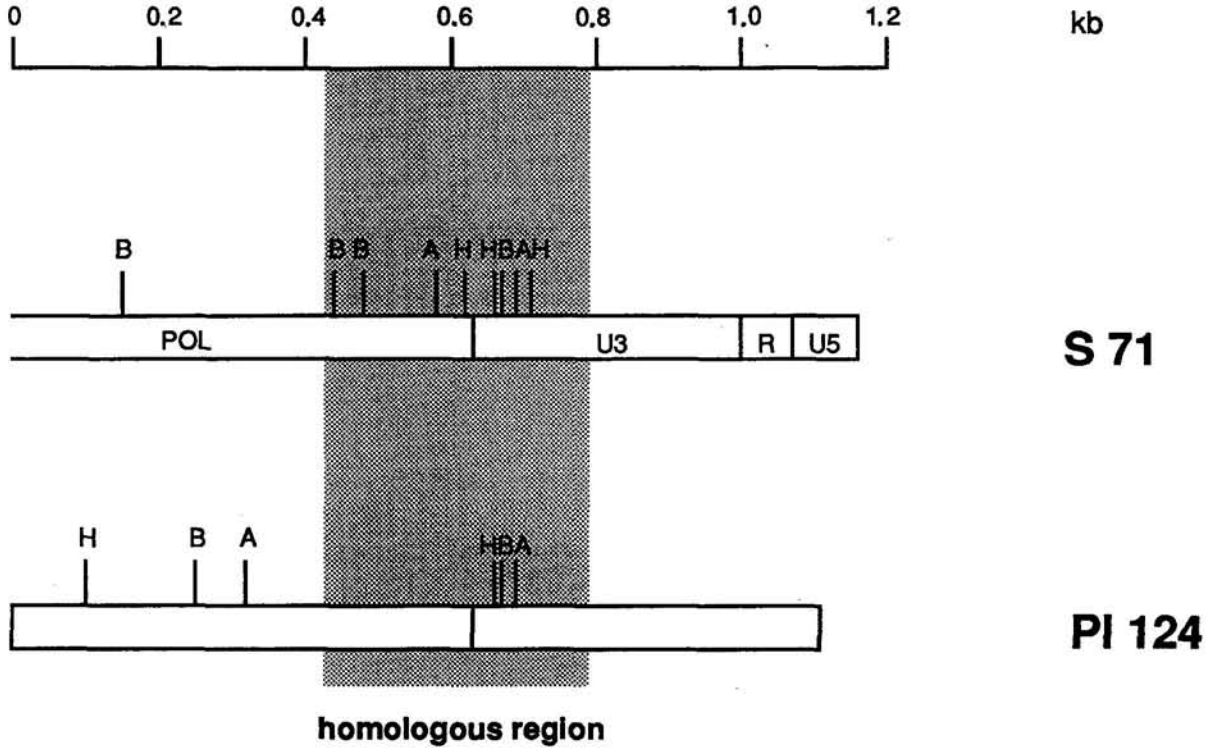

Fig. 2. Restriction map of Pl124 in comparison to the corresponding region of S71. *A, Ava* I; *B, Bam* HI; *H, Hae* I

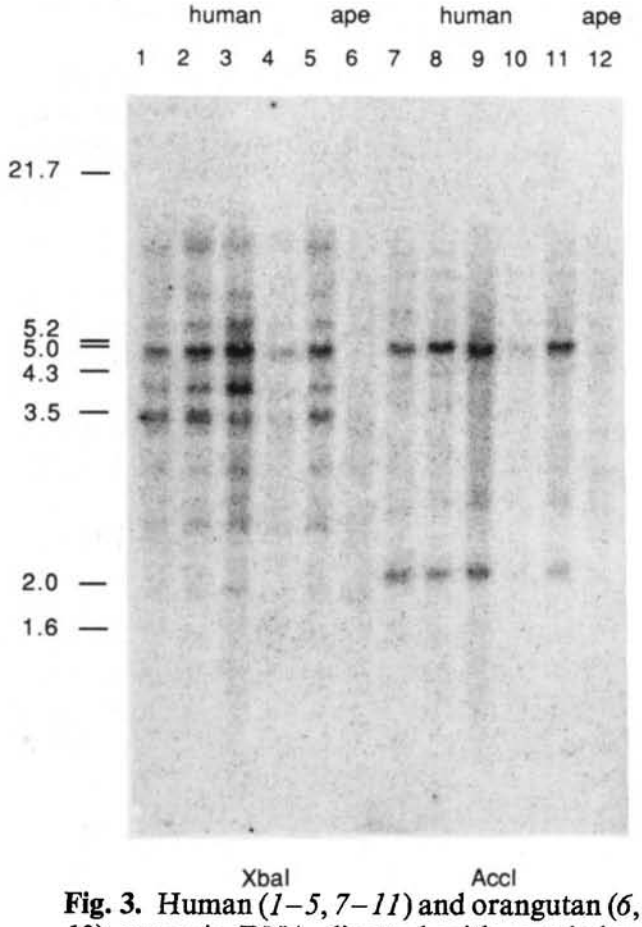

Fig. 3. Human (*1–5, 7–11*) and orangutan (*6, 12*) genomic DNA digested with restriction enzymes *Xba*I or *Acc* I and hybridized to a full-length Pl124 probe under high-stringency conditions

contains only one or a few Pl124 sequences. The appearance of numerous weakly hybridizing fragments is probably due to other S71 LTR-related sequences, including the original genomic S71 LTR, because a similar hybridzation pattern is observed using S71 LTR as hybridization probe (data not shown). Comparative hybridization of orangutan genomic DNA with the Pl124 probe (Fig. 3, lanes 6 and 12) yielded only very weak bands, indicating that orangutan DNA contains only distantly related copies of the Pl124 sequence.

Analysis of expression of several human retroviral elements has shown that transcripts containing retroviral and cellular sequences can be generated by a readthrough mechanism, possibly combined with subsequent splicing [14, 15]. Alternatively, recombination between retroviral and cellular sequences may also occur at the DNA level, as indicated for the SNRS sequence in S71 [8]. Both affect the expression of cellular genes and may therefore be involved in pathogenic events. Further examination of the nature

of retrovirus-related transcripts detected in human neoplastic cells should shed light on the role of endogenous retroviral elements in oncogenesis.

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Selective Integration of Rous Sarcoma Virus Genome in High- and Low-Metastatic Transformed Cell Lines

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Previously, different hamster cell lines transformed in vitro by Rous sarcoma virus (RSV) (SR-D strain) were established [1]. These cells displayed different levels of spontaneous metastatic activity in syngenic animals. The structure of RSV provirus in these cells has been analyzed. All cell lines contained integrated RSV genome without any indication of provirus rearrangement. The comparative analysis of restriction maps of the integrated provirus in the genomes of these cells showed that in most of the highly metastatic cell lines the exogenous RSV proviruses were localized within structurally similar loci of host DNA. For the analysis and sequencing of the flanking region downstream from the integrated provirus, the method of inverted polymerase chain reaction (PCR) with oligonucleotide primers to certain long terminal repeats (LTR) and *src* regions of RSV was used. We analyzed the primary structure of cellular DNA for a number of different highly metastatic cell lines. It was found that the flanking sequences of cellular DNA are similar in at least three cell lines. This common fragment was used as a probe for the analysis of normal DNA and a unique region in the hamster genome was found. Homologous sequences have been also identified in the human genome. Computer sequence analysis did not show any consistent homology of this locus with any known genes.

Previously it has been shown that RSV-transformed hamster cells in vitro have two discrete characteristics essential for in vivo selection by the effectors of the host's natural resistance. These two biochemically different characteristics, i.e., resistance to hydrogen peroxide (H_2O_2) and ability to secrete prostoglandin E (PGE) in contact with natural killer (NK) cells, macrophages, and neutrophils could be utilized as cell type markers. We tried to change the biological properties of high- and low-metastatic variants of these cells using various transfection protocols with a transforming oncogene. The low-metastasis cells (HET-SR) were transfected with pSVcN-*ras* containing the N-*ras* oncogene linked with the *neo* gene [2]. As a control in these experiments the plasmid carrying only the *neo* gene was used and G-418-resistant clones were selected. We analyzed the structure of integrated sequences, showing that the integrated N-*ras* oncogene is transcribed in transfected cells. Using MAb259 against the protein $p21^{ras}$ the processing of this protein was observed in transfected cells.

In work performed recently, it was found that the same clones lost resistance to H_2O_2 and the ability to secrete PGE (Deichman et al., 1986). The loss of these characteristic properties correlated with induction of N-*ras* expression and drastically reduced expression of RSV-specific RNA. In particular, the amount of *src* mRNA decreased more than five- to sevenfold as compared with nontransfected and *neo*-transfected cells. At the same time, in these cells the highest levels of tyrosine-specific protein kinase activity was observed in an immune complex of $pp60^{src}$ with MAb327. We were also

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

able to show that the specific activity of pp60^{src} was changed. The molecular mechanism of v-*src* and N-*ras* gene interactions in these cells is rather unique and needs to be investigated in further details.

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Molecular Mechanism of Alteration of H-Ras I Oncogene in Human Breast Carcinomas: G to T Transversion in 12th Codon of the Undeleted Allele in the Case of the Loss of the Other Gene Allele

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Introduction

It is widely accepted that tumor development and progression are due to illegitimate activation of cellular oncogenes by point mutation, retroviral insertion, chromosomal translocation and amplification or deletion of the gene [1–3]. Nonrandom deletions of chromosomal regions 13q14 and 11p13 have been detected in retinoblastoma [1] and Wilm's tumor [2, 3]. It has been proposed that these rare childhood cancers result from the deletion of dominant-acting genes, permitting the expression of tumorigenic recessive alleles [1]. Moreover, restriction fragment length polymorphism (RFLP) analysis has demonstrated loss of H-*rasI* oncogene allele (chromosome 11p15) in primary bladder, breast, ovarian, and lung carcinomas [4–7].

On the other hand, another important mechanism of activation of *ras* oncogene (including H-*rasI*) have been shown in 10–15% of certain types of human tumors, which involved a point mutation, causing an alteration at amino acid positions 12, 13 or 61 of the *ras* gene product $p21^{ras}$ [8].

The study discusses the possible suppressive action of the wild-type H-*rasI* allele on the mutant one in breast cancer.

Material and Methods

The RFLP of H-*rasI* oncogene was analyzed in 76 primary breast carcinomas as described [7]. H-*rasI* sequence spanning 145 base pairs across codon 12 was amplified in vitro by *Thermus thermophylus* DNA polymerase [9]. Subsequent *MspI* digestion allowed us to detect the mutation in "hot spot" due to the loss of the restriction site for *MspI* in the case of substitution in the 12th codon of H-*rasI* [10].

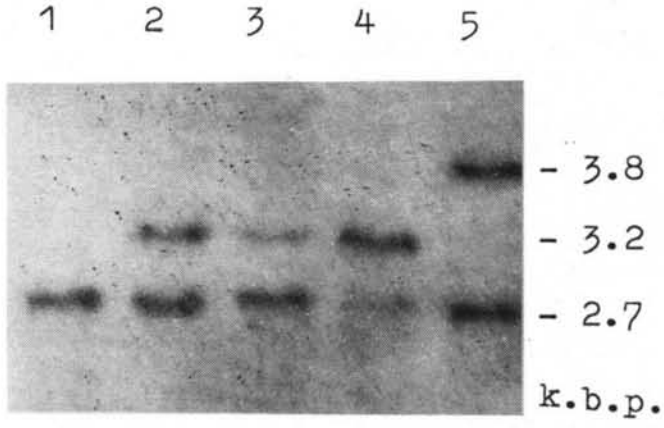

Fig. 1. Deletions of one of H-*rasI* allele in breast carcinomas (BC), identified by means of PvuII restriction of DNA samples, Southern blotting and hybridization with 6,6-fragment of pEJ [11]. Samples of DNA were derived from (*1*) BC12 – genotype A1/A1; (*2*) leukocytes of BC9 – constitutive genotype A1/A2; (*3*) BC9 – A1/A2, deletion of A2 allele; (*4*) BC5 – A1/A2, deletion of A1 allele; and (*5*) BC31 – genotype A1/A3. Slight hybridization signals at the place of lost A2 (*3*) and A1 (*4*) alleles are due to the contamination of the tumors by normal cells. In BC107 and BC109 the same deletions were detected as in BC9 (*3*)

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

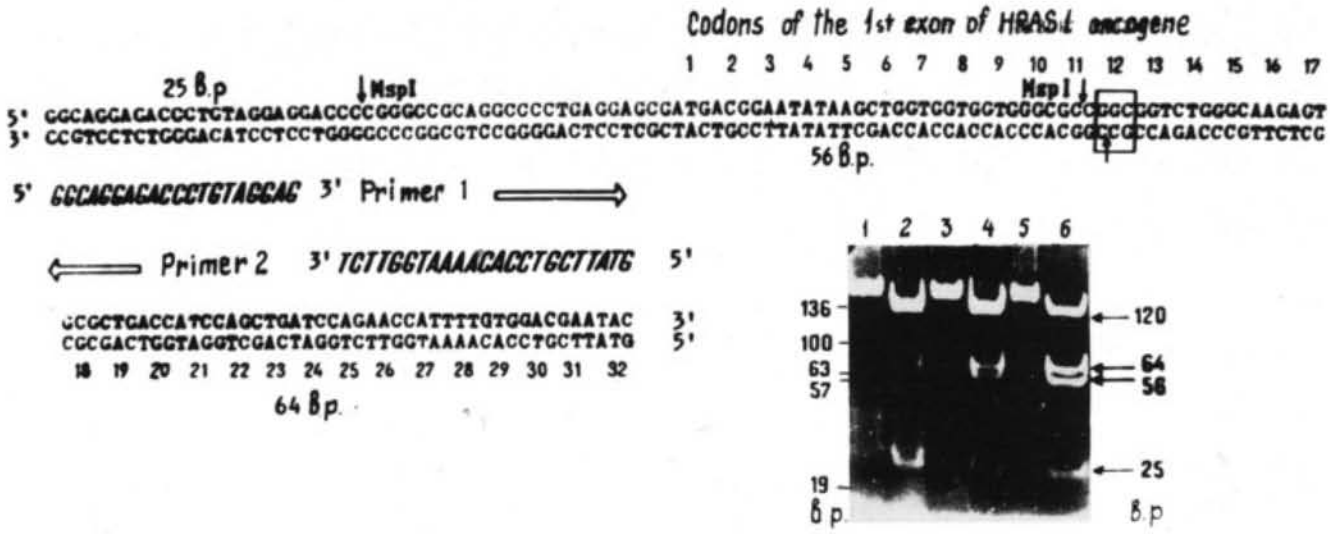

Fig. 2. Detection of H-*rasI* mutation in codon 12. The 145 base pair (bp) DNA sequence across 12th codon (in the *frame*) was amplified in vitro using the oligonucleotide primers (*large open arrows* indicate the 5′ 3′ orientation of the primers [9]. *MspI* digestion of the amplified sequences result in three fragments (25, 56, and 64 bp) in the case of wild-type allele, and two fragments (25 and 120 bp) in the case of substitution in codon 12 that altered *msp* site of restriction (*vertical arrows*). The *photograph* demonstrates the products of amplification of BC5 (*1*), BC9 (*3*), and BC107, BC109 (not shown) DNA samples and *MspI* restriction (*3*, *4*, and *6*, respectively). *Arrows on the right* of the *photograph* indicate bands corresponding to mutant and wild-type alleles. *Alu* fragments of pBR322 DNA (standard) are pointed out on the *left of the photo*

Results and Discussion

Restriction analyses of 76 DNA samples from primary breast carcinomas revealed deletions of one of the H-*rasI* allele in ten out of 41 (25%) heterozygous patients (Fig. 1). Enzymatic amplification and Msp restriction showed the presence of point mutation in the undeleted H-*rasI* allele in four out of ten carcinomas with the allelic loss (Fig. 2). All four mutations identified were the G-to-T transversion in the second position of codon 12 (Fig. 3).

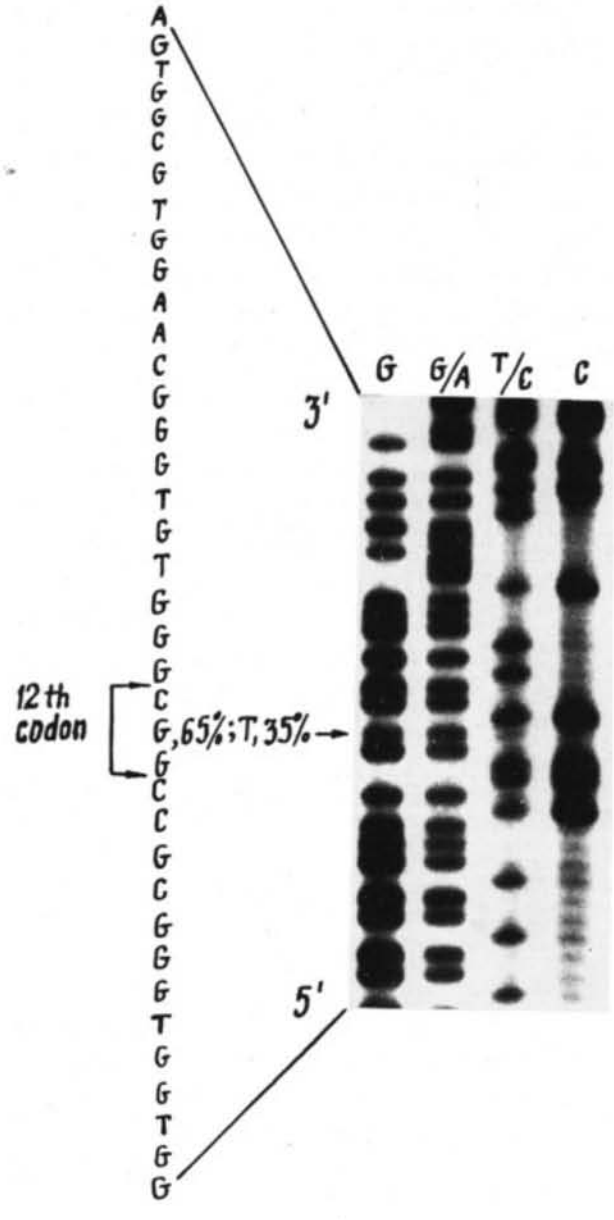

Fig. 3. Nucleotide sequence of the H-*rasI* oncogene fragment, containing the 12th codon with G-to-T transversion. The sequence was determined by means of a 8% polyacriamide sequencing gel and the Maxam and Gilbert procedure [12]. *Arrows* indicate the codon 12 and the mutated band

The loss of intact H-*rasI* allele and consequently its product – $p21^{ras}$ – normally involved in transport of mitogenic signal in the cell, might potentiate transforming activity of the oncoprotein, coded by the mutant allele. The deletion of wild-type allele of H-*rasI* oncogene is likely to unmask the mutant one.

Nevertheless it is possible that another cellular constraint of growth is present on chromosome 11p13-p15, and that the loss of this suppressor locus leads to activation of normally repressed class of genes.

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Immunology

Natural Killer Cell Mediated Defence Directed Selectively Against Target Cells Lacking MHC Class I Gene Products *

H.-G. Ljunggren, P. Höglund, C. Öhlén, and K. Kärre [1]

Introduction

Natural killer (NK) cells represent an important subset of lymphocytes. These cells fulfil crucial cytotoxic as well as regulatory functions of the immune system. Compromised NK cell activity has been found to be associated with the development of several diseases, including cancer, AIDS and virus infections. Intact NK cell activity appears to play an important role in health. More recent data suggests that NK cells may be involved in the pathogenisis of some human diseases and serve as an early predictor for susceptibility to disease [1]. In transplantation, NK cells participate in rejection of allogeneic bone marrow cells [2].

The molecular basis for the ability of NK cells to discriminate between normal and aberrant cells is not known in detail [3–5]. The aim of the present overview is to discuss studies of how target cell major histocompatibility complex (MHC) class I expression affects NK–target interactions and ultimately target susceptibility to NK cell mediated lysis.

The rationale for current studies is a hypothesis which has provided one testable model for self – non-self discrimination by NK cells [4, 5]. This hypothesis was originally based on (a) the patterns common to the various cytotoxic reactions attributed to NK cells and (b) a comparative analysis of the different strategies employed by vertebrates and invertebrates in order to distinguish between self and non-self. This hypothesis, presented as the "missing self" hypothesis [3], provided testable predictions for the investigation of the influence of the MHC class I gene products by NK cells. Briefly, it was suggested that NK cells could kill certain target cells because the latter express reduced levels of MHC class I molecules. This model underlies the work presented below. However, before going into the missing self hypothesis, present knowledge of NK cells and in particular their cytotoxic activity is briefly recapitulated.

Natural Killer Cells

The ability to kill certain tumour cell lines in vitro, without prior immunization or sensitization, was the first attribute of NK cells to be identified (reviewed in [6]). While this reaction was investigated in detail in the late 1970s several new insights as to their morphology, cell surface marker expression, tissue expression and action have become evident during the last 10 years (for reviews see e.g. [7–9]).

Morphologically, most NK cells are large granular lymphocytes (LGL). They are characterized by their intracytoplasmatic azurophilic granules and a high cytoplasm to nucleus ratio. In man,

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LGL comprise 2%–5% of peripheral blood lymphocytes. Early on, NK cells were found in the spleen peritoneal exudate and blood whereas they were scarcely found in lymph nodes, bone marrow, thoracic duct and the thymus. Later, NK cells were also isolated from the liver, tonsils, the epithelial lining of the upper respiratory tract and the lung interstitium [7–9].

NK cells commonly express certain cell surface markers, defined by monoclonal antibodies against CD 16 (the FcγIII receptor for IgG) and NKH-1 in humans and NK-1.1/2.1 in mice [7–9]. Additional cell surface markers, some of which define subsets of NK cells, have recently been described [10]. NK cells are of bone marrow origin [6], but their exact lineage is uncertain. They share certain cell surface markers with T cells and also share some characteristics with monocytes. There is now a general belief that mature NK cells are distinct from T lymphocytes [6–10].

The T-cell antigen receptor is not involved in NK cell recognition or cytotoxicity [7–9]. NK cells do not express CD3. There is no rearrangement of alpha-, beta-, gamma-, or delta-T cell receptor (TCR) genes and no synthesis of functional TCR messenger ribonucleic acid (mRNA) (even though nonfunctional beta and gamma TCR transcripts may be detected). However, CD3 positive (alpha-beta or gamma-delta) T cells may express, particularly upon activation, MHC nonrestricted cytolytic activity against target cells that are also sensitive to NK cells. According to a new definition proposed at the 5th International Natural Killer Cell Workshop (Hilton Head, S.C., 1988) these cells should *not* be termed NK cells but rather T cells displaying "NK-like" activity or "non-MHC-requiring" cytolysis [7, 8].

NK cells also respond to various lymphokines and interferons by elevated cytotoxic activity. They have been reported to produce different regulatory lymphokines themselves, e.g. IL-1, IL-2, IL-4, IL-5, interferons and colony-stimulating factors. Through such mediators (and maybe yet other unknown factors) NK cells are involved in regulation of haematopoesis and lymphocyte functions [7–9].

Natural resistance to infectious agents may be one of the more important functions for NK cells in vivo [11, 12]. Particularly during virus infections, high levels of IFN (primarily alpha or beta), are induced in lymphoid organs. Interferons activate NK cells to a higher level of cytotoxicity and stimulate their blastogenesis and proliferation in vivo. Biron et al. [12] recently described a patient with a complete and persistent absence of NK cells but otherwise normal immune functions. This patient first presented with an overwhelming varicella virus infection requiring treatment with acyclovir and later a life-threatening cytomegalovirus (CMV) infection.

Graft Rejection Mediated by NK Cells

Soon after the discovery of NK cells it became clear that these cells could kill certain tumour cell lines in vitro in spite of the fact that they expressed no or only low amounts of MHC class I molecules [13, 14]. This was a significant difference from cytotoxic T cells, which require the presence of MHC molecules to specifically kill target cells [15]. Further, NK cells were shown to be the effectors in rejection of small numbers of certain transplanted tumour cells [6, 16], in the prevention of metastasis [17] and in bone marrow graft rejection [2, 18]. However, NK cells seem to have no or only little impact on established larger cancers. NK cells are not found within tumours; there is no clonal expansion, but rather a systemic suppression of NK activity [1].

A peculiar rejection mechanism, now attributed to NK cells, is the F_1-hybrid resistance [2, 19, 20]. This phenomenon has been instrumental in recognizing the in vivo activity of NK cells and it is of particular importance for the following

discussion. Hybrid resistance was first reported in 1958 when Snell [21] observed that homozygous lymphomas grew better in the strain of origin than in F_1 hybrids obtained by crossing this strain with another strain. This F_1 hybrid effect violated a principle in tissue transplantation. According to the genetic rules of histocompatibility, an F_1 hybrid should accept grafts from either of its parents. The most extensive genetic analysis of the F_1 hybrid effect was carried out by Cudkowicz [in 20] who studied rejection of normal bone marrow cells in F_1 hosts. They explained the phenomenon on the basis of a positive recognition of hypothetical recessive Hh genes expressed in the parental (and on the graft) but suppressed in the F_1 animal [20]. Snell [22] later offered an alternative interpretation in which F_1 hybrid resistance was seen as a result of a combined match and mismatch rather than a complete match between host effector cells and transplanted cells. This theory originated before NK cells were known to be the effector mechanism in hybrid resistance but nevertheless formed the basis for the missing self hypothesis (developed below), providing an alternative explanation to the Hh model. Critical experiments demonstrating that NK cells were the effector mechanism in F_1 hybrid resistance were published in 1977 by Kiessling et al. [2]. Before that, Kiessling et al. [23] and Petranyi et al. [24] had reported a correlation between NK activity in vitro and tumour resistance in vivo among F_1 hybrids. Klein et al. [25] extended these conclusions and mapped F_1 hybrid resistance to the H-2 gene complex in several different tumour combinations. Carlson et al. [26] observed a rapid elimination of intravenously injected leukaemia cells whenever these were "mismatched", i.e. H-2K or D products were not present in relation to the host. This elimination occurred in nude mice but not in NK depleted mice.

Allogeneic lymphocyte cytotoxicity is a term used for the rapid destruction of intravenously injected allogeneic lymphocytes by unsensitized hosts. Allogeneic lymphocyte cytotoxicity has been reported in several mammalian species and it has been studied most extensively in rats [27]. It is mediated by NK cells and is in certain aspects related to F_1 hybrid resistance.

Strategies for Self–Non-self Discrimination: the Missing Self Hypothesis

Multicellular organisms need defence systems against destruction of their tissues by foreign invaders as well as against altered endogenous cells. A prerequisite for such a defence reaction is recognition of the potential threat. An organism should be able to discriminate between "self", i.e. everything constituting an integral part of a given individual, and the rest. This recognition could, in theory, be positive or negative [28]. In positive recognition, the organism actively recognizes "non-self" and reacts against it. In negative recognition, there is an active recognition of self and the reaction is triggered only as a consequence of the failure to recognize self. It is well known that higher vertebrates have evolved defence systems based on positive recognition. This is mediated by T and B cells which have clonally distributed receptors generated partly by a random process. The receptor repertoire is then somatically selected for the ability to positively identify "foreignness" either directly (B cells) or in the context of molecules of the MHC (T cells).

The missing self hypothesis is based on the second, negative type of recognition. It was suggested that NK cells kill certain targets because they fail to express adequate levels of self MHC class I gene products [3–5] (Table 1). This hypothesis originated from the observations that NK cells mediate rejection of allogeneic lymphoma and bone marrow grafts ($H\text{-}2^{a/a}$ rejects $H\text{-}2^{b/b}$) and also, in contrast to cytotoxic T lymphocyte (CTL), F_1-hybrid anti-parental resistance. In the

Table 1. Triggering signals for T cells and NK cells

Cell type	Strategy for self – non-self recognition	Signal
T cell	"Positive" recognition	Presence of a foreign antigen presented in the context of class I molecules of the MHC (triggering signal)
NK cell	"Negative" recognition	Presence of "self" class I molecules of the MHC (inhibitory signal)[a]

[a] Note that this does not exclude other (MHC class I independent) triggering signals as well (see [3]).

allogeneic as well as in the F_1 hybrid anti-parental situation, the graft "fails" to express at least one H-2 class I allele of the host. This failure to express a complete set of self MHC class I molecule by the target was postulated to be sufficient to cause elimination by NK cells [4, 5].

Transposed to an autologous situation, the hypothesis predicted that absence or reduced expression of self MHC class I products (whether caused by mutation, transformation, arrest in differentiation or virus infection) could be sufficient to make a cell recognized and rejected by NK cells [4, 5]. Conversely, an induction of self MHC class I molecules on such a target cell should be sufficient to prevent rejection mediated by NK cells. These predictions have been tested in vivo and the results will briefly be discussed below.

Immunological Defence Against H-2 Class I Deficient Cells in Vivo

In order to test the missing self hypothesis we chose to work with the well-characterized C57Bl/6 derived RBL-5 and EL-4 murine lymphoma cell lines (H-2^b haplotype). These lymphomas express high levels of H-2 and are highly malignant in the syngeneic host. Our experimental approach was to test the prediction that selection for loss of H-2 class I expression should be accompanied by an increased sensitivity to natural resistance in vivo and in vitro [4, 5].

As a starting point, H-2 deficient variant lines were selected from the RBL-5 and EL-4 lymphomas [5, 29]. Titrated doses of wild type and variant cells were inoculated either subcutaneously [5, 29], intravenously or intraperitoneally [30] in small groups of mice, age matched and usually littermates, in several independent experiments and tumorigeneicity was scored. This strategy minimized the risk that random fluctuations in the quality of the respective cell suspensions would be responsible for differences in the ability to form tumours.

In line with the prediction of the hypothesis, the H-2 class I deficient RBL-5 and EL-4 variants were rejected in syngeneic C57BL/6 mice after a small tumour inoculum (10^3 to 10^5 cells), whereas mutagenized but non-selected, H-2 positive, wild type lines were highly tumorigenic in the corresponding doses [5, 29]. The H-2 deficient cells required a 10^3 to 10^4-fold higher dose than the H-2 positive cells to induce more than 50% tumour take irrespective if the cells were inoculated subcutaneously, intravenously or intraperitoneally [5, 29, 30].

The rejection of the H-2 deficient lines showed several characteristics of an NK mediated response, including thymus independence and no requirement for pre-immunization [5, 29]. The resistance was weakened (but not totally abrogated) by 400 rad irradiation [29], and it was sufficient to remove asialo-GM_1 or NK1.1 positive cells (NK cells) from the animal

to abrogate the rejection ([29], unpublished results). Experiments comparing the distribution and survival of isotope prelabeled variant and wild type cells indicated that a rapid elimination of the former took place within 24 h after intravenous injection. These differences in rapid elimination of tumour cells were abolished in NK depleted mice [29]. The above mentioned pattern was observed in all organs studied with one exception – the brain (discussed in detail in [30–32]).

One possible explanation for the differential rejection patterns of the H-2 positive and H-2 deficient lymphoma cell lines in the syngeneic B6 mice was a difference in an afferent arm of a NK dependent host response [33]. In this scenario the H-2 deficient cells would recruit NK effector cells which would kill H-2 positive and deficient cells equally well without discriminating between them. However, the differential rejection pattern remained when H-2 positive and deficient cells were inoculated into the same animals, whether in different flanks [29] or mixed in the same inoculum [33]. H-2 deficient cells were selectively eliminated even when they were present in a 10-fold excess compared to H-2 positive cells in the same inoculum [33]. These results suggested that the NK dependent response against H-2 deficient cells was selective in an efferent (effector) arm of the response [33].

In recent experiments we have demonstrated that it is possible to restore the tumorigenicity of β2-microglobulin (β2m) negative EL-4 cell lines by transfection of β2m (R. Glas et al., to be published). This indicates that β2m may act as a tumour growth promoting gene when the host defence is dominated by NK cells.

The "missing self" hypothesis predicted that it should be possible to obtain NK mediated rejection of a H-2 positive target provided that the host carried one (or several) extra MHC class I allele(s) in relation to the target. F_1 hybrid resistance and allogeneic lymphocyte rejection were postulated to be examples of this [4, 5]. To directly test this concept, still within the RBL-5 model, we used the transgenic strain D8 generated by Bieberich et al. [34]. The D8 strain was produced by introducing an 8.0-kb genomic fragment containing the H-2D^d gene and 2.5 Kb 5′ and 2.0 Kb 3′ flanking sequences into B6 zygotes. The transgene product was expressed in different tissues in the same way as the endogenous H-2^b haplotype products, without alterations in the expression of the latter. Tumour growth was followed after subcutaneous inoculation of graded doses of RBL-5 lymphoma cells in D8 and B6 control mice [35]. The D8 strain was more resistant to subcutaneous challenge of "previously syngeneic" RBL-5 lymphoma cells than B6 controls. The direct role of the H-2D^d gene in this resistance was investigated by the use of (D8XB6)F_1 crosses and (D8XB6)XB6 backcrosses. The latter showed cosegregation with regard to the D^d expression and lymphoma resistance, both of which were inherited in a pattern consistent with control by a single dominant gene [35]. The resistance to RBL-5 (or other B6 derived lymphomas) in the D8 strain could be abrogated by pretreating mice with anti-asialo GM_1 antiserum or anti-NK1.1 mAb, indicating that NK cells were necessary for the rejection [35] Subsequent studies have shown that the elimination of RBL-5 in D8 is a rapid event taking place within 24 h (P. Höglund et al., J Exp Med, in press). In thus entirely resembles the elimination of RBL-5 H-2 deficient variant cells in B6 mice. A similar pattern was seen when the D8 strain was grafted with B6 bone marrow. The D8 recipients had acquired an ability to reject bone marrow from C57BL/6 donors but not from D8 donors and this rejection was dependent on the presence of NK cells in the host [18].

Recessive Hh antigens have been mapped to the D region of the H-2 complex, although rejection did not require expression of the dominant D locus product of the graft (reviewed in [20]). Our data do not address the role of putative Hh antigens at the tumour/graft

level. However, no transcripts have been detected from the flanking sequences of the D^d gene carried by the construct (G. Jay, unpublished observations). It is therefore concluded that the D^d gene itself is responsible for the resistance at the level of the host. These results are consistent with the predictions of the missing self hypothesis [3–5].

In conclusion, the results are in line with an NK cell mediated rejection of small tumour or bone marrow grafts providing that the graft lacks (or expresses greatly reduced levels) of at least one MHC class I allele of the host.

MHC Expression and Tumorigenicity – a Re-evaluation

The results obtained with the H-2 deficient lymphoma grafts in vivo predicted that if the critical immunological host–tumour interaction is dominated by NK cells rather than T cells in a given system, up-regulation of MHC class I expression would make tumour cells more malignant because they would survive interactions with NK effectors.

This has been observed in studies with different sublines of the mouse B16 melanoma, pioneered by Taniguchi et al. [37]. H-2 positive melanoma cells gave rise to a high number of metastatic lung colonies, whereas the H-2 low or deficient melanoma cells gave no or only few colonies after intravenous inoculation [37]. Subsequent studies showed that the non-metastatic H-2 deficient sublines acquired metastatic capacity if they were pretreated with inteferon (IFN), known to enhance H-2 class I expression, or if the mice were pretreated with anti-asialo GM1 serum, known to deplete NK cells [38].

In contrast to the result reviewed above, there are several reports in which decreased expression of MHC class I molecules are associated with enhanced tumour growth (reviewed in [39, 40]). The apparently conflicting results may depend on the antigenicity of the tumour and/or the experimental protocol used [3, 41]. This can be illustrated with the RBL-5 lymphoma, for which opposite results with regard to tumorigenicity were obtained when H-2 positive and deficient variants of this tumour were compared in two different experimental situations (Table 2):

1) A small subcutaneous inoculum in untreated mice led to growth of H-2 positive cells and NK dependent elimination of H-2 deficient cells, and
2) A large subcutaneous inoculum in preimmunized mice resulted in T cell dependent elimination of H-2 positive cells and growth of H-2 deficient cells which overrode the limited non-adaptive NK response.

Had the tumours only been tested under the latter conditions, we would erroneously have concluded that loss of class I

Table 2. Influence of experimental protocol on NK versus T cell mediated rejection of RBL-5 H-2 positive and H-2 deficient sublines in B6 mice[a]

RBL-5 tumour dose	H-2 expression	Pretreatment of the host	Progressive tumour growth
Low	High	–	Yes
Low	Low	–	No
High	High	–	Yes
Hig	Low	–	Yes
High	High	Preimmunization[b]	No
High	Low	Preimmunization	Yes

[a] Data based on [5, 29, 33] and H. G. Ljunggren et al. (unpublished observations).
[b] Preimmunized with RBL-5 (resembling grafting with a highly immunogenic tumour).

molecules is only associated with enhanced tumorigenicity of the tumour.

Thus, there is no obligatory association between reduced H-2 class I expression and increased malignancy. The effect of MHC class I expression on rejection or escape from immunological rejection will depend on the dominant host–tumour interaction. Such interactions may vary in different phases of tumour progression and under different experimental conditions [3, 41].

Tests for the effect of MHC modulation on tumour growth or immunotherapy therefore require careful experimental design to cover the action of different effector mechanisms in vivo. Since T cell responses, once elicited, would play a dominant role in the final outcome of tumour growth, the effect of H-2 changes on the NK defence could rather easily be missed in studies with relatively large tumour grafts and immunogenic tumours. This possibility can be controlled in any system, by using small grafts or short-term assays where rapid rejection of grafted cells is monitored, either in conventional transplantation assays or in tests of the survival of radiolabelled cells [29]. Additional controls can be obtained by the use of mice with either genetically inherited immunodeficiencies (e.g. nude mice or SCID mice) or mice where different subsets of the host immune defence experimentally has been depleted (e.g. irradiated, thymectomized, anti-NK1.1 treated, anti-asialo GM_1 treated, anti-L3T4 treated or anti-Ly2 treated mice).

NK Sensitivity of MHC Class I Deficient Cells In Vitro

A detailed review of the in vitro NK sensitivity of MHC class I deficient cells has recently been published [3]. A brief summary is given below.

The MHC class I deficient RBL-5 and EL-4 murine lymphoma cell lines, used in the in vivo studies described above, showed enhanced NK sensitivity in vitro compared to their wild type counterparts [3, 5, 33]. The association between high NK sensitivity and reduced MHC class I expression in murine models is not confined to lymphoma or melanoma variants derived by mutagenization and selection in vitro. Fibrosarcoma clones with constitutively low H-2 expression derived by cloning without selection were sensitive to NK mediated lysis, while clones (from the same primary tumour) with high levels of H-2 expression were resistant [42].

Increased NK sensitivity of human MHC class I deficient variants was first reported by Harel-Bellan et al. [43] and Storkus et al. [44]. They analysed T-LCL, B-LCL and B and T cell hybrid cell lines. The latter group analysed three different sets of cloned cell lines with corresponding variants that differed in their relative HLA class I expression. While sensitivity correlated with reduced class I expression it did not correlate with class II expression or transferring receptor expression [44]. In one of two more recent confirmatory studies on NK sensitivity of HLA deficient variants [45, 46], IL-2 activated effectors gave the same MHC class I related pattern as fresh NK cells while allospecific CTL lines showed an opposite pattern [45]. The latter study also showed that an intermediate HLA class I expressor (Haplotype loss) variant was moderately NK sensitive (compare to the relatively resistant wild type line), whereas a weak HLA class I expressor (with an additional down regulation of the remaining haplotype) was highly sensitive [45].

Enhanced NK sensitivity of murine and human MHC class I deficient variant lines does not correlate with a single molecular defect (see [47]). Rather, it appears that increased NK sensitivity can result from different defects in the MHC class I biosynthesis with the common denominator that cell surface expression of class I molecules was impaired.

In order to directly study whether MHC class I gene products can affect NK sensitivity it is essential to study cell lines

transfected with different genes with the purpose to specifically restore the HLA/H-2 class I expression. The first studies in this direction did not support a role for MHC class I gene products. The murine line 1 carcinoma express little if any H-2D^d in vitro [48]. Transfection of H-2D^p into line 1 led to a constitutive and dimethyl sulphoxide (DMSO) inducible expression of H-2D^p but this expression had no influence on NK sensitivity. Transfection of H-2K^b into a hepatoma gave a less conclusive result (reduced NK sensitivity in 4/9 experiments, no effect in 5/9 experiments) [49]. The authors further concluded that there was no difference in tumour formation between wild type and transfected cells, and thus that H-2K^b did not affect either NK susceptibility or tumorigenicity. The latter conclusion was based on an i.m. inoculation of 10^7 cells [49], as opposed to low dose inocula used to study rejection of H-2 deficient cells as reviewed in the present overview (Table 2).

However, when Quillet et al. [50] transfected the human β2m gene into the β2m negative human Burkitt lymphoma cell line Daudi, this led to the establishment of a line permanently expressing HLA-A10, -A11 and B-17 class I molecules. This transfected line showed a reduced sensitivity to both NK and lymphokine activated killer (LAK) cell lysis as compared to the HLA class I negative wild type cell line [50]. Transfection of the human HLA class I deficient lymphoblastoid B cell line C1R with HLA-A3, HLA-B7 and HLA-Bw58 also led to a reduction in NK sensitivity [51]. Although there was no significant variation among the HLA-A3, -A7 and -Bw58 alleles, HLA-A2 appeared unable to protect from NK cell lysis [51]. Comparison of amino acid sequence suggests a restricted number of residues which may be relevant to the protective effect. The protection did not extend to H-2D^p or K^b transfected C1R cells, nor was it seen when IL-2 stimulated NK effector cells were used [51]. Another B cell lymphoblastoid cell line, 0.221, with a selective loss of HLA class I expression was transfected with HLA-A1, HLA-A2, HLA-B8, HLA-B5 or an HLA-C gene [46]. Expression of MHC class I genes reduced NK cell sensitivity, with a general tendency for HLA-B genes to have the most prominent effect.

Transfection of human melanoma cells with c-*myc* is associated with a specific down regulation of class I expression which is most prominent for the B locus products [52]. This is associated with an increased sensitivity to NK cell lysis [53]. Interestingly, this increased NK sensitivity can be overcome by super-transfection of HLA-B7 and B27 genes to the melanoma cells which restores the original NK resistant phenotype (P. Schrier, personal communication). The increased NK sensitivity of melanomas with a c-*myc* induced HLA-B suppression as well as some of the above-mentioned transfection studies suggested that selective down regulation of certain allelic or locus specific products (and not necessarily all class I molecules) might be sufficient to induce NK sensitivity. This is interesting in relation to two papers describing the failure to induce a significant NK resistance upon transfection with HLA-A2 [54, 55].

There are now two murine analogues to the rescue of HLA in β2m transfected Daudi cells. Sturmhöfel and Hämmerling [56] selected an H-2 class I deficient line (S3) from the murine EL-4 cell line. This variant was found to have a defect in β2m expression and was highly sensitive to NK cell lysis. Transfection of the S3 line with the β2m clone restored cell surface H-2 expression and resulted in a considerable decrease in NK sensitivity. Transfection of class II genes had no effect. Blocking of class I molecules with Fab fragments against class I molecules increased NK sensitivity of EL-4 to the level of the S3 variant [56]. The second example is the transfection of the β2m deficient YAC-1 variant A.H-2- with β2m. This restored the YAC-1 phenotype with respect to inducible class I expression and a concomitant protection

from NK cell lysis after treatment with IFN-gamma [57]. The YAC-1 lymphoma has also recently been transfected with H-2K^b under control of the human metallothionein IIA promoter. These transfected H-2K^b positive sublines showed a reduced sensitivity to murine NK cell lysis [58].

At this stage it can be concluded that expression of certain MHC class I molecules can reduce NK sensitivity in several targets. Different molecular models for how MHC class I molecules can protect certain target cells as well as interpretations of the systems where MHC class I genes do not have this effect on NK sensitivity has recently been discussed (see [3]).

General Conclusions and Future Prospects

In the present overview we have discussed an NK cell defence against MHC class I deficient cells. The data in support of this concept were obtained with murine MHC class I deficient variant cell lines and their corresponding wild type cell lines in in vivo and in vitro studies. Using MHC class I transgenic mice, we have demonstrated that a deficiency of a single host MHC class I allelic product on a tumour cell graft is sufficient to cause rejection. β2m, one of the three subunits of an MHC class I molecule [59, 60], is the first identified molecule that contributes to the IFN-gamma mediated protection from NK cell lysis [57]. This was suggested, although formally not proven, to be mediated through the increased cell surface expression of class I molecules of the MHC. The general concept of a surveillance system geared to detect "missing self" explains some conflicting results regarding the relationship between MHC class I expression, tumorigenicity and metastasis (see [3]). The concept can also explain F_1-hybrid resistance and rapid elimination of allogeneic lymphocytes by NK cells. Rapid elimination of allogeneic lymphocytes, transferred during for example pregnancy or sexual contacts where the transferred cells may cause tissue damage by graft versus host or by transmitting infectious agents such as HIV, may be an important defence action by NK cells. From a more general immunological point of view the present results have challenged the notion that discrimination between self and non-self is the only strategy for immunological elimination of aberrant cells in mammals [3–5]. In relation to clinical bone marrow transplantation, the present defence system may be taken into consideration in discussions of host versus graft reactions [18].

The analysis of models where MHC class I molecules clearly do affect NK sensitivity must now focus on detailed molecular events. Experimental strategies for this analysis has recently been described [3]. The mechanism by which an NK cell recognizes a target cell, deficient in self MHC class I expression is unknown. A detailed analysis as to which parts of the MHC class I molecule contribute to the inhibit ion of NK cell lysis must be undertaken. Such an analysis may lead to insights into the recognition event. Other studies must focus on defining receptor-like structures on NK cells. In an "effector inhibition" model [3] certain specific questions could be asked. Does one of the NK receptors resemble a T cell receptor due to a possible MHC class I binding ability? Is such a receptor primed to recognize only presence of self MHC class I molecules, and does it trigger the lytic machinery if appropriate class I molecules are not recognized? It would also be of interest to know when during ontogeny NK cells learn to discriminate between self and non-self. Do NK cells themselves need to express self MHC class I in order to recognize absence of self MHC class I? Are quantitative levels of MHC class I on the target monitored relative to the MHC class I expression on the NK cell itself? The ultimate model for such and related studies may be β2m deficient mice as recently described in the literature [61, 62].

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Tumor Rejection Antigens and Immune Surveillance

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The existence of specific tumor rejection antigens was first demonstrated with chemically induced mouse sarcomas: each tumor was found to express a different antigen [1]. Similar findings were made with ultraviolet-induced tumors [2]. Later, the generality of the existence of tumor rejection antigens was questioned when spontaneous mouse tumors were found to be completely incapable of eliciting an immune rejection response [3]. However, further experiments demonstrated that even these tumors express weak transplantation antigens that are potential targets for immune rejection by the syngeneic host [4].

But what is the molecular nature of tumor rejection antigens? And what is the relation between their appearance and the tumoral transformation process? These questions are still unanswered because these antigens, which elicit strong T-cell mediated immune responses, do not stimulate B cells to produce antibodies. It has therefore been impossible to isolate the antigenic molecules by immunoprecipitation. Recently, we have developed a gene transfection approach aimed at identifying directly the genes that code for this type of antigen. It was applied to "tum$^-$" transplantation antigens, which arise on mouse tumor cells when they are treated with mutagenic agents, and to a tumor rejection antigen present on mouse mastocytoma P815.

Tum$^-$ Antigens

In *vitro* mutagen treatment of mouse tumor cells generates at high frequency stable immunogenic variants that are rejected by syngeneic mice [5]. Because of their failure to form tumors, these variants were named "tum$^-$" as opposed to the original "tum$^+$" cell, which produces progressive tumors. This phenomenon has been observed on a large number of mouse tumor cell lines of various types [6]. Most tum$^-$ variants express new transplantation antigens not found on the original tum$^+$ cell. The existence of these tum$^-$ antigens was first demonstrated by transplantation experiments [7]

We have studied a series of tum$^-$ variants derived from mastocytoma P815, a tumor induced in a DBA/2 mouse with methylcholanthrene. From clonal tum$^+$ line P1, we obtained more than 30 different tum$^-$ variants, which rarely produced progressive tumors even when they were injected at very large doses. When restimulated in vitro, spleen cells of mice that had rejected these variants produced cytolytic T cells (CTL) that lysed preferentially the immunizing tum$^-$ variant [8]. From these lymphocytes, we were able to isolate stable CTL clones [9]. Some of these appeared to be directed against a tumor rejection antigen of P815: they lysed P1 and all P815-derived cells but not syngeneic control tumors. Others lysed the immunizing tum$^-$ variant, but neither the original tum$^+$ cell

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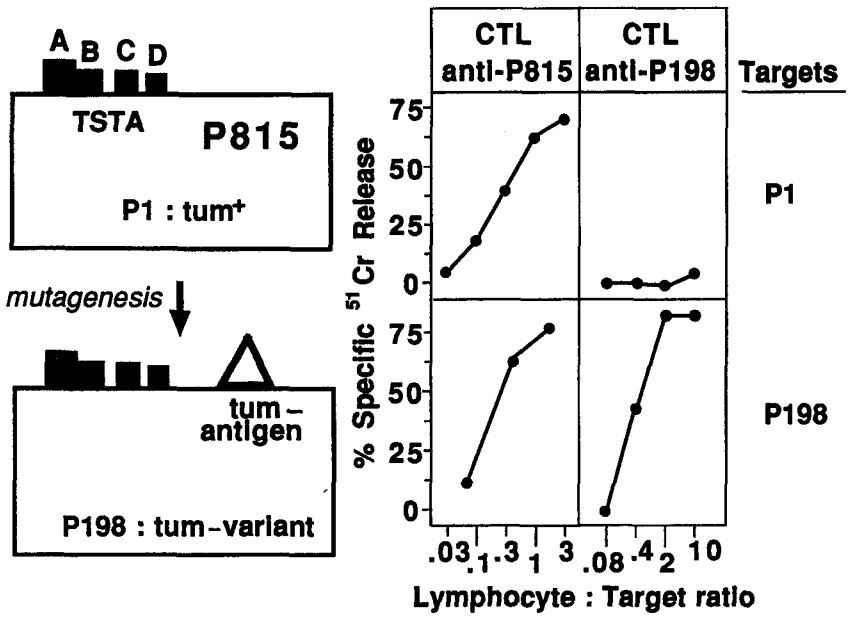

Fig. 1. Tumor rejection antigens and tum$^-$ antigen present on the original P815 line P1 and on tum$^-$ variant P198. Lysis by CTL clones directed against a tumor rejection antigen (anti-P815) or a tum$^-$ antigen (anti-P198)

nor the other tum$^-$ variants. They therefore defined new tum$^-$ antigens specific for each variant (Fig. 1). These antigens displayed considerable diversity: no antigen was found twice among 15 tum$^-$ variants that were analyzed. By in vitro immunoselection with anti-tum$^-$ CTL clones it was possible to demonstrate that some tum$^-$ variants carry several tum$^-$ antigens [10]. These experiments also demonstrated that the tum$^-$ antigens defined by CTL are relevant to the rejection of the variants, as shown by the correlation between the loss of these antigens and the reversal of the tum$^-$ phenotype [10, 11].

To find an explanation that could reconcile the remarkably high frequency of tum$^-$ variants with their stability and to understand the source of their diversity, it appeared essential to identify the antigenic molecules. We failed in our attempts to obtain antibodies directed against tum$^-$ antigens. Therefore, we undertook to clone directly the relevant genes on the basis of their ability to produce the antigens recognized by the anti-tum$^-$ CTL.

Cloning of Genes Encoding Tum$^-$ Antigens

The procedure that we developed for the cloning of the gene coding for tum$^-$ antigen P91A is based on gene transfection. It involves the use of a highly transfectable P815 cell line called P1. HTR [12] and the detection of antigen-expressing transfectants by their ability to stimulate CTLs [13]. By transfecting P1. HTR cells with a cosmid library prepared with the DNA of a cell expressing tum$^-$ antigen P91A, we obtained transfectants expressing this antigen at a frequency of 1 per 28000 [14]. By direct encapsidation of the DNA of these transfectants into lambda phage heads, we obtained a cosmid capable of transferring the expression of the antigen. An 800-bp restriction fragment from this cosmid was found to transfer the expression of the antigen. This fragment was then used to identify cosmids containing either the normal or the antigenic allele of the entire P91A gene as well as complementary (c)DNA clones of the homologous messenger RNA.

The procedure that led to the isolation of tum$^-$ gene P91A was applied with

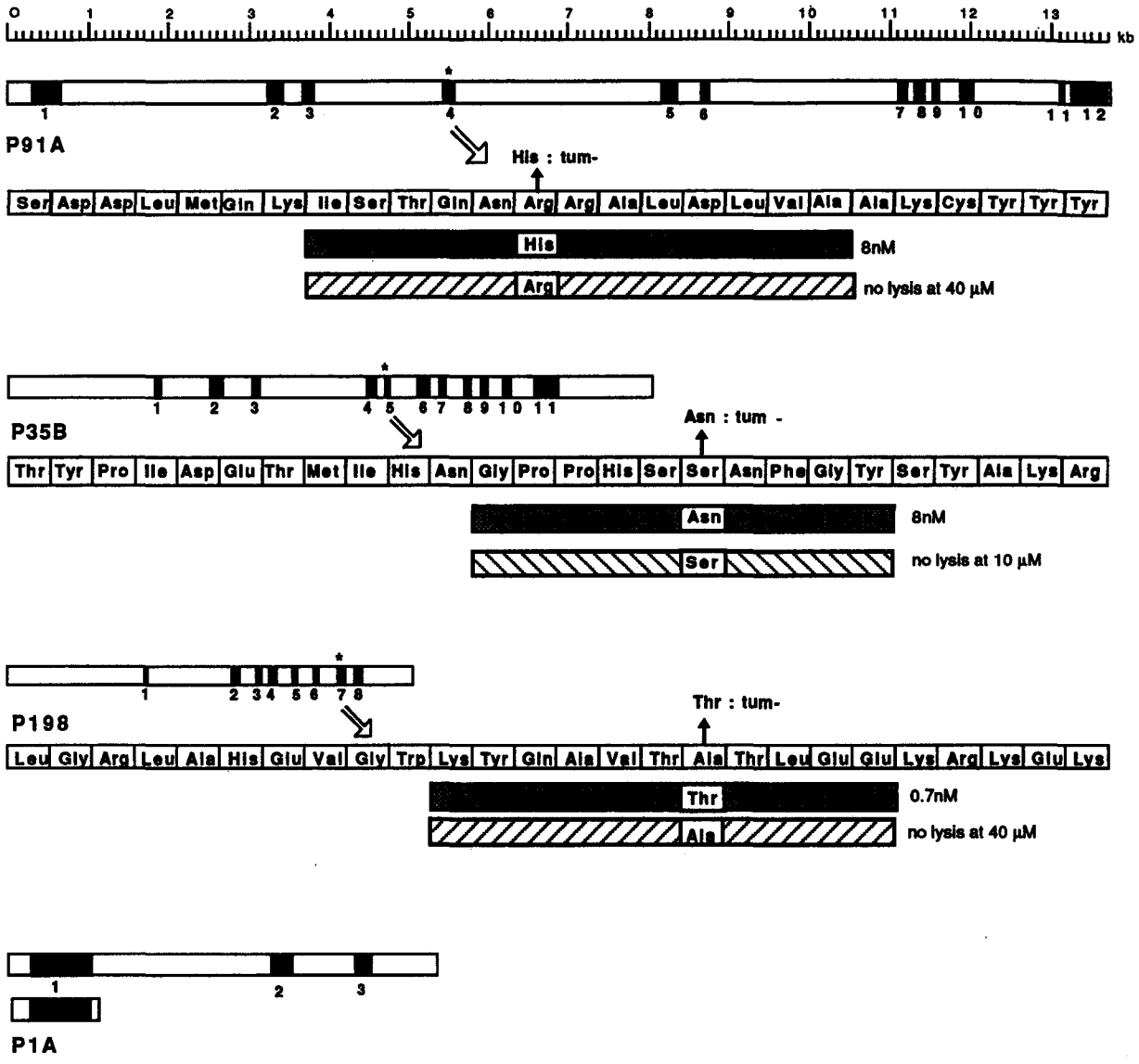

Fig. 2. Structure of genes P91A, P35B, P198, and P1A and antigenic peptides. *Dark regions* represent exons. The exon containing the tum$^-$ mutation is marked by an *asterisk*. Sections of the proteins located around the mutated amino acid are indicated. Synthetic peptides corresponding to the mutant and normal sequences of the genes are represented by *boxes*. They were tested for their ability to render P1. HTR cells susceptible to lysis by anti-tum$^-$ CTL. The concentration indicated to the right of each peptide provided 50% of the lysis obtained at saturating concentration of peptide. For P1A, the box indicates the subgenic fragment capable of transferring the expression of antigens P1A and P1B. The antigenic peptides for P1A and P1B are not yet identified

success to the cloning of tum$^-$ genes P35B and P198, which encode antigens expressed by other tum$^-$ variants derived from P815 [15, 16].

Tum$^-$ Mutations

Northern blots probed with the 800-bp fragment of gene P91A revealed a single messenger RNA species of 2.2 kb. The band was of equal intensity for tum$^-$ variant P91 and for P1, which does not express the antigen. The expression of antigen P91A is therefore not due to the activation of a silent gene.

The structure of gene P91A is shown in Fig. 2. It comprises 12 exons spread over 14 kb [17]. It does not show any similarity with Ig, T cell receptor or MHC genes. The complete sequence was obtained. It is unrelated to any sequence presently recorded in the main data banks.

A sequence comparison of the normal and tum$^-$ alleles of gene P91A indicated that they differ by a point mutation in the exon which is present in the transfecting 800-bp fragment (Fig. 2). This tum$^-$ mutation is a G to A transition that changes an arginine into a histidine in the main open reading frame of the gene [14]. This mutation appears to be the only difference distinguishing the normal from the antigenic allele.

The study of the tum$^-$ alleles of genes P35B and P198 also revealed that they differ from the normal alleles by a point mutation in an exon (Fig. 2). The general structures and the sequences of the three tum$^-$ genes isolated so far are completely unrelated.

Antigenic Peptides

The main open reading frame of gene P91A encodes a protein of 60 kDa, which does not have a typical N-terminal signal sequence [17]. In vitro translation experiments suggest that the two potential N-glycosylation sites present in the sequence are unused (Godelaine, Amar-Costesec, De Plaen, Beaufay, unpublished results). Antigen P91A is therefore unlikely to be borne by a membrane protein. This is however hardly surprising, considering the recent demonstration that CTL can recognize influenza antigens corresponding to endogenous proteins remaining inside the cell and considering the observation that CTL recognize small peptides that bind to surface class I MHC molecules [18–20]. On the basis of this evidence, we examined whether we could also identify a small peptide that would trigger the lysis of P815 cells by anti-P91A CTL. In our search for this peptide we were guided by the location of the tum$^-$ mutation. A short peptide (Fig. 2) corresponding to the mutant sequence induced the lysis of P1 by anti-P91A CTL. Transfection and peptide studies with H-2k fibroblasts, which expressed also either Kd, Dd or Ld, demonstrated that antigen P91A is associative with Ld. Antigenic peptides corresponding to the sequence surrounding the tum$^-$ mutation were also obtained for genes P35B and P198. They associate with Dd and Kd respectively.

Studies with P91A peptides enabled us to understand the role of the tum$^-$ mutation. A priori, the mutation could influence either the production of the antigenic peptide or its ability to associate with the Ld molecule (i.e., the aggretope) or also the epitope presented to T cells by the peptide-MHC complex. Having the antigenic P91A peptide, we prepared the homologous peptide corresponding to the normal allele of the gene. This normal peptide did not induce lysis by anti-P91A CTL, nor did it compete with the mutant peptide. Moreover, we found that the mutant peptide competed effectively to prevent a cytomegalovirus-derived peptide from inducing lysis by CTL directed against a Ld-associative cytomegalovirus antigen. The normal peptide did not compete [17] and we concluded that it does not bind to Ld. This indicates that the P91A tum$^-$ mutation generates the aggretope of the antigen, but does not exclude that it also influences the epitope. For antigen P198, the effect of the mutation appears to be different: here a new epitope is introduced on a normal peptide that is already capable of binding to the Kd presenting molecule.

Cloning of the Gene Encoding a Mouse Tumor Rejection Antigen

We have applied the same cloning procedure to the isolation of the gene coding for a tumor rejection antigen expressed by tumor P815 [21]. As opposed to the tum$^-$ antigens, these antigens are present on all P815 cells, whether they are mutagenized or not. The study of antigen-loss variants enabled us to identify four distinct antigens recognized by different syngeneic CTL clones. They were called P1A, B, C, and D (Fig. 1) [22]. Antigens P1A and P1B thus defined *in vitro* are

relevant *in vivo*, because P815 tumor cells that progressed in mice after nearly complete initial rejection were found to have lost the expression of one or both these antigens. Antigens P1A and P1B showed linkage, since several antigen-loss variants for P1A were found to have lost P1B concurrently.

For the transfection of antigen P1A, we used as recipient cell a $P1A^{-}$ B^{-} antigen-loss variant selected from line P1. HTR with an anti-P1A CTL clone. Transfectants expressing both antigens P1A and P1B were obtained with the genomic DNA of P1. HTR. This confirmed the close link between these two antigens. Transfectants were then obtained with a cosmid library made with the DNA of a genomic transfectant. By directly packaging the DNA of one of these cosmid transfectants, we obtained a cosmid that was able to transfect both antigens P1A and P1B.

The structure and the complete sequence of gene P1A were then obtained (Fig. 2). They proved completely different from those of the tum^{-} genes and of any known gene reported in data banks.

Transfection studies in H2-k fibroblasts previously transfected with either Kd, Dd, or Ld demonstrated that both P1A and P1B were presented to the CTL by the Ld molecule.

We compared the sequence of this gene, cloned from tumor cells, to the sequence of the equivalent gene cloned from normal cells of the same mouse strain. From a genomic library made with the DNA of normal DBA/2 mouse kidney we isolated the gene homologous to gene P1A. The analysis of this gene revealed that its sequence was identical to the sequence of the tumoral gene. To confirm this, we transfected this normal gene and found that it transferred the expression of antigens P1A and P1B as efficiently as the gene cloned from P815 cells (Fig. 3). The antigenicity is therefore not the result of a mutation in the tumoral gene, and P1A and P1B are presumably two different peptides derived from the same protein.

The tumor specificity of antigens P1A and P1B can nevertheless be partially explained by the pattern of expression of the gene. Northern blot analysis revealed

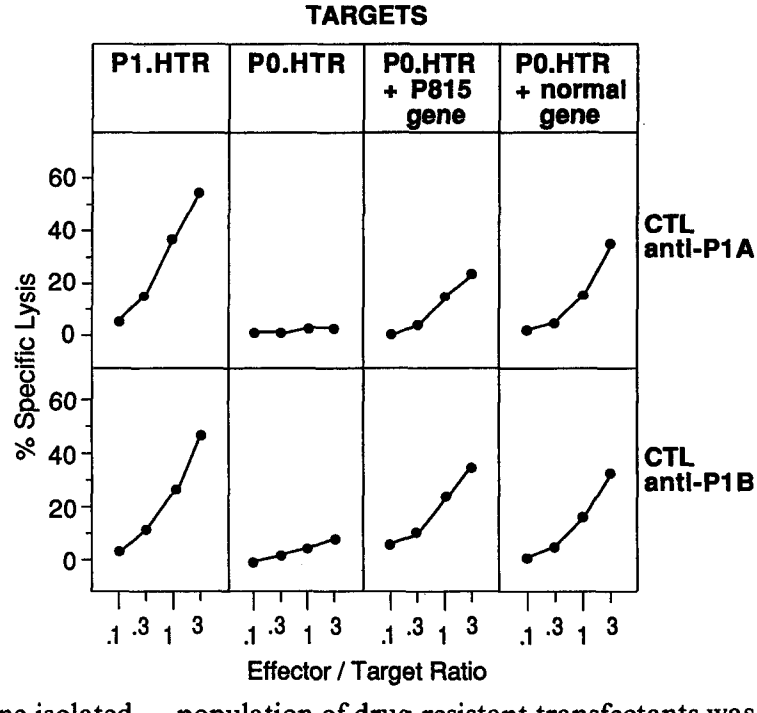

Fig. 3. Transfection of the P1A gene isolated from normal cells. The P1A gene isolated from a genomic library from normal DBA/2 mouse kidney was transfected in P0. HTR cells. The population of drug-resistant transfectants was tested with the anti-P1A and anti-P1B CTL clones

that the gene was silent in most normal tissues. However, one mast cell line (L138.8A) was found to strongly express messenger (m)RNA for P1A. This cell line, derived by Hültner et al. [28] from bone marrow of BALB/c mice, is cultivated in medium supplemented with interleukin 3. It grew as a permanent line and became spontaneously tumorigenic. Because BALB/c mice and DBA/2 mice express the same H2 haplotype, we were able to confirm the expression of gene P1A in L138.8A cells by lysis with the anti-P1A and anti-P1B CTL clones: we observed a significant lysis. Other nontransformed mast cell lines on the other hand were negative for P1A expression, so that we do not known whether the expression of the gene is associated to the mast cell lineage at a given stage of its differentiation, or whether it is related to the tumoral transformation. We failed to identify other tumor cell lines expressing mRNA for P1A.

Immune Surveillance, Tolerance, and Tumor Rejection Antigens

A first conclusion based on the results obtained with the tum$^-$ antigens is that mutations throughout all the mammalian genome generate at high efficiency antigenic peptides recognized by T cells, and that this mechanism could account for the presence of specific tumor rejection antigens on carcinogen-induced tumors.

However, the study of antigen P1A clearly showed that gene P1A is identical to its normal counterpart. The apparent tumor specificity of antigen P1A seems to be due to a specific regulation of the transcription of the gene rather than to a mutation generating an antigenic peptide. We now have to understand how the immune system may be sensitized against normal peptides to which is should be tolerant, and this without generating an obvious autoimmune pathology. Several hypotheses can be suggested. If the gene encodes an embryonic or oncofetal protein, then the antigen might have disappeared before the establishment of tolerance. If it codes for a differentiation or activation antigen, we can imagine that it is expressed very transiently by a small number of cells, so that tolerance does not develop and that an immune reaction directed against this antigen does not impair normal differentiation or activation. Lastly, if tolerance is actually present for P1A, then it must have been broken, and the simultaneous presence on the P815 tumor of other antigens like C and D may be important in that respect. These antigens could indeed be the result of a mutation and therefore be strongly immunogenic like tum$^-$ antigens. They could possibly trigger an immune response that would facilitate a response against P1A [4].

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Glycophosphatidylinositol-Anchored Membrane Proteins as Coreceptors in T-Cell Activation *

P. J. Robinson [1]

Successful destruction of tumours or virally infected cells by the immune system is thought to be dependent on induction of an effective T-cell response against endogenous antigens of the target cell, presented in association with major histocompatibility complex (MHC) antigens. The response of T lymphocytes confronted with this type of foreign antigen involves a complex array of molecular interactions between the T cell and stimulator cell, many of which occur at the cell membrane. Whilst the specificity of the response is determined by interaction of the antigen receptor complex on the T cell (TcR/CD3) with processed antigenic fragments presented by class I or class II (MHC) molecules, its magnitude is determined by a number of additional coreceptor/ligand interactions [1]. Originally, these additional molecules were loosely classified as either adhesion molecules or signaling structures. Nowadays, however, this classification has become more diffuse as several "accessory" molecules appear to have dual functions [2], and a large volume of literature is now devoted to their identification and functional characterisation.

Monoclonal antibodies generated by immunising rats with mouse or human lymphocytes have been important tools for studying coreceptors and their ligands. Panels of hybridomas screened for their ability to induce proliferation of T lymphocytes routinely contain antibodies reactive with components of the TcR/CD3 complex, but there are clearly a number of other molecules on T cells which have signal-transducing function, including CD2, CD28 and the interleukin receptors [3]. Although the *in vitro* mitogenic effects of these antibodies suggest the existence of alternative, antigen-independent, routes of T-cell activation, it is more likely that, *in vivo*, the function of these additional signaling molecules is to modify the effects of signals delivered by the TcR/CD3 complex.

Curiously, many hybridomas which induce proliferation of T cells bind to cell surface proteins which are linked to the cell membrane through glycophosphatidylinositol (GPI) anchors. The prototype GPI-linked signaling molecule is Thy-1, a differentiation antigen and a marker of the T-cell lineage in mice [4]. In other mammals Thy-1 homologues are found mainly on cells of the nervous system. Antibodies directed against Thy-1 cause proliferation of mouse T lymphocytes, in most cases giving optimal responses in the presence of costimulators such as phorbol myristate acetate (PMA), and anti-immunoglobulin antibodies [5]. Other GPI-linked signaling molecules, such as Ly-6 and 5′-nucleotidase are apparently unrelated to Thy-1 or to each other at the protein level, suggesting that the anchor is critical for signal-transducing capability [6]. A series of transgenic mouse strains carrying either transmembrane or GPI-linked derivatives of Qa-2 and H-2D^b MHC class I molecules were constructed to test this hypothesis. The

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Haematology and Blood Transfusion Vol. 35
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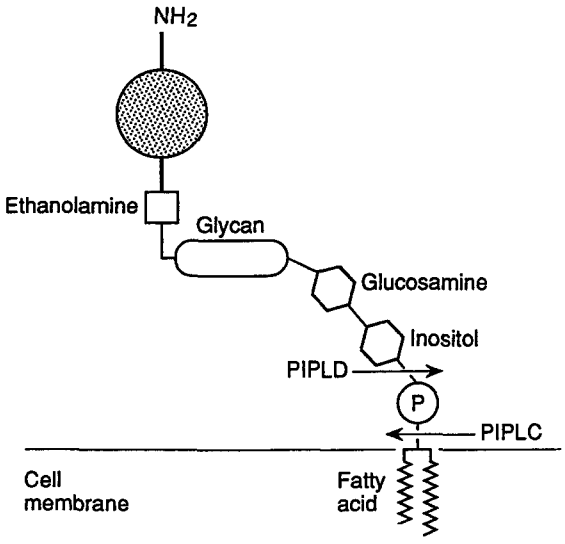

Fig. 1. Composition of a typical GPI membrane anchor. GPI anchors are found in yeast and in all higher eukaryotes so far examined. In some species, GPI appears to be the most common method of membrane anchoring. The anchor core is highly conserved and anchors from trypanosomes and mammalian cells differ only in their carbohydrate side chains and in the composition of the lipids. GPI anchors can be cleaved by phospholipases C and D at the positions shown (*arrows*), causing removal of the lipids and generation of water-soluble protein molecules

results show that GPI-linked class I polypeptides are effective signaling structures, but that the corresponding transmembrane forms are not [7]. As observed for Thy-1, optimal cell proliferation requires PMA and anti-immunoglobulin antibodies, indicating that these molecules cannot function independently to induce cell division.

Structural analysis of GPI anchors in diverse eukaryotes has provided few clues to the physiological function of this type of membrane anchor (Fig. 1). GPI-anchored molecules, however, possess a number of physiochemical characteristics not shared by other signal-transducing molecules which may be relevant to their function in T-cell responses [8]. Firstly, the majority of GPI anchors are sensitive to cleavage by phospholipases C and D. Treatment of cells with phospholipase C releases GPI-linked protein molecules from cell membranes in water-soluble form. Phospholipases specific for the phosphatidylinositol linkage (PIPLC and PIPLD) have been isolated both from bacteria and from eukaryotic cells. Enzymes of similar specificity may operate *in vivo* to prevent or to interrupt cell signaling events by detaching GPI-linked signaling molecules or ligands from the responding cell. In this context it is interesting that some populations of T lymphocytes, in particular those which have been previously activated by antigen or mitogens, possess GPI anchors which are resistant to PIPLC [9]. We have also found that a number of cloned antigen-specific mouse T-lymphocyte cell lines have PIPLC-resistant anchors (Robinson and Spencer, unpublished). If release of GPI-linked proteins by PIPLC-like enzymes has a limiting effect on T-cell proliferative responses, it is possible that cells with resistant anchors may have a selective growth advantage. It is therefore of interest to elucidate the molecular basis for this resistance.

A second property of GPI-linked molecules, and one which distinguishes them from other signaling molecules, is the absence of a direct interaction with cytoplasmic components. The fact that the fatty acid anchoring moiety of GPI is similar in size and composition to other membrane phospholipids indicates that it associates with only the outer face of the lipid bilayer. One manifestation of this is that the lateral mobility of GPI-linked molecules within the lipid bilayer is generally substantially greater than that of transmembrane molecules [10]. It also poses a dilemma in explaining how the binding of antibodies to GPI-linked proteins is able to induce intracellular biochemical changes. Two mechanisms have been proposed to resolve this paradox. Firstly, GPI-linked molecules may interact with additional, presumably transmembrane, proteins which transmit signals to the cytoplasm of the cell. Such hypothetical transducer molecules are likely to associate with structural motifs on the anchor, thus enabling a whole

range of structurally unrelated protein molecules to couple to a single signal transduction pathway [6]. Secondly, binding of antibodies or ligands may induce internalisation and subsequent degradation of the anchor, releasing potentially stimulatory fragments [11]. The main evidence for the first hypothesis is that, although a high proportion of molecules of any one GPI-linked species exhibits relatively high lateral mobility in membranes, there is invariably a proportion of low mobility [10]. This suggests that mobility can be restricted by interactions with other membrane components which are in turn associated with cytoskeletal elements. The main argument for the second hypothesis is that antibodies specific for GPI-linked proteins which are stimulatory in soluble form do not function when immobilised to plastic plates [12]. Immobilisation may thus prevent signaling by blocking internalisation of immune complexes. Antibodies against GPI-linked molecules, immobilised together with submitogenic amounts of TcR/CD3-specific antibodies, have been found to be highly mitogenic for T cells, suggesting that this may closely mimic a physiological situation in which the GPI pathway can amplify an antigen-specific stimulus [12].

Future research in this area must concentrate not only on the mechanism of GPI-mediated signaling, but on determining the role played by this group of molecules in cellular immunity. This will require identification of the ligands for GPI-linked molecules. Only one example of the natural ligand for a GPI-anchored protein is known to date. This is CD2, which binds specifically to the GPI-linked lymphocyte function-associated antigen LFA-3 [13]. CD2 is itself a signaling molecule on human T lymphocytes and a homologue is also known in mice. One approach towards identifying new ligands is to determine their distribution using water-soluble, PIPLC-cleaved, receptor molecules as probes to stain tissue sections. This strategy could be adapted for cloning the corresponding genes by screening cDNA expression libraries produced using episomally replicating vector systems [14]. Until more information is available on ligands for GPI-linked proteins it may be possible to investigate physiological responses using antibodies against GPI-linked molecules attached to the surface of stimulator cells. This can be achieved using antibodies derivatized with esters of palmitic acid [15]. The fatty acid chains can insert into membranes of the stimulatory cell and thus present the antibody molecules to T cells in a form closely resembling a normal membrane protein. This type of "surrogate" ligand more closely resembles a physiological situation than does the present approach of adding free soluble antibodies to cell cultures. There are examples of proteins which naturally use fatty acids, directly attached to the polypeptide backbone, as a means of anchoring in membranes ($p56^{lck}$) [16].

In summary, GPI-linked T-lymphocyte surface proteins are potent signal transducing molecules and are likely to be influential in controlling cellular immune responses. This represents the first clear physiological role for this group of proteins on mammalian cells and points towards a much wider application of this signaling pathway in biological systems.

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The Molecular Mechanism of Muramyl Peptides' Biological Activity

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Muramyl peptides (MPs) are known to influence greatly the immune response [1]. They are active as adjuvants, induce nonspecific resistance to certain viral and bacterial infections, and in some cases are active against various tumors. Many studies attempting to elucidate the mechanism of their biological activity have been carried out. However, the molecular basis of MP's effects on the immune system is still unclear.

The immune network includes at least three circuits: idiotype–antiidiotype interactions [2], the cytokine network [3], and the regulation of receptor expression on immunocompetent cells. MPs are known to induce cytokine production, and so clearly interfere with the cytokine cascade [4]. They also influence idiotype network, as they increase production of immunoglobulin. Little if anything is known about their effect on the expression of cell membrane molecules [5]. We approached this subject by studying oligosaccharide-containing MPs synthesized in our institute by T. Andronova, E. Makarov, and V. Ivanov.

Macrophages are known to be the primary target for MPs [1]. As these cells function as antigen-presenting cells, we evaluated the effect of *N*-acetylglucosaminyl-β 1-4-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP, Fig. 1) and its analogs on major histocompatibility complex (MHC) class II antigen expression, as these glycoproteins are crucial for recognition of antigens by T-cells [6]. BALB/c mice peritoneal macrophages were used. The number of Ia-positive cells was estimated by flow cytometry after labelling cells with biotinylated anti-Ia monoclonal antibodies and fluorescein isothiocyanate (FITC)-labelled avidin.

To summarize briefly the results obtained [7], it was found that:

1) Incubation of macrophages with GMDP in vitro caused a dose-dependent increase in the Ia-positive cell number and fluorescence intensity. Ia expression peaked at 18 h and persisted for at least the next 30 h.
2) This effect was also observed in vivo upon injection of GMDP into the peritoneal cavity of mice.
3) Biologically active MPs, with few exceptions, also possessed Ia-inducing activity; nonactive compounds were always inactive in this assay.
4) MPs directly affected macrophage. This was evident from the increase in Ia-antigen expression on myelomonocytic leukemia cells (WEHI-3). The effect could not be attributed to intermediate formation of tumor necrosis factor α (TNF-α), the known Ia inducer, as we failed to find TNF-α in WEHI-3 culture medium. At present, the involvement of other interleukins cannot be excluded.
5) The observed effect was not species specific: not only murine peritoneal macrophages but human monocytes as well could be induced to express MHC class II (HLA-DR) antigens, though the magnitude of the effect was lower.

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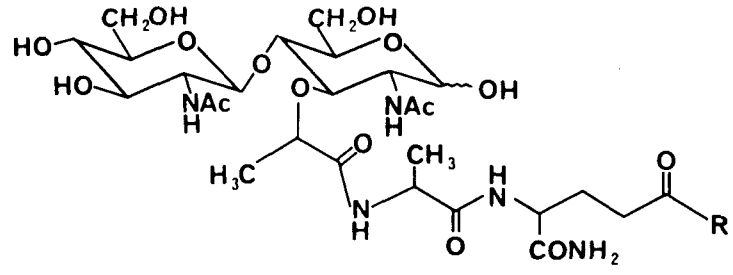
Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)



GMDP: R=OH

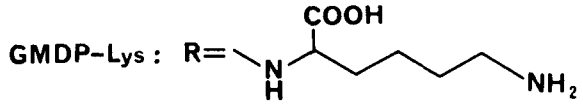


Fig. 1. *N*-Acetylglucosaminyl-β1-4-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (*GMDP*) and GMDP-Lys

The maximal HLA-DR expression was observed at 0.1–1 μg GMDP/ml.

6) Finally, besides MHC class II antigens, the expression of interleukin-2 (IL-2) receptors was induced by GMDP. Taking in account that IL-2 was shown to increase the cytotoxicity of macrophages against tumor targets, we assume that this mechanism might be involved in the antitumor activity of GMDP.

It was shown previously that MPs have no direct cytotoxic effect on tumor cells; rather, they kill tumor cells by activating the immune system [8]. We assumed that there might be another effect, namely an increase in expression of tumor-associated antigens and MHC antigens on tumor cells, resulting in their being better recognized by immunocompetent cells. Human lung adenocarcinoma cells (RL-4) and colon adenocarcinoma cells (WiDr) were used as tumor targets. These cells are known to express various levels of carcinoembrionic antigen (CEA) as tumor-associated marker. The expression of CEA and RL-4 cells was monitored by labelling the cells with FITC-anti-CEA monoclonal antibodies followed by flow cytometric enumeration of labelled cells.

RL-4 cells cultured without stimulant expressed only minute amounts of CEA. Incubation of RL-4 cells (3×10^5) with GMDP resulted in a dose-dependent increase in CEA expression with maximal expression at 10 μg/ml (Fig. 2). The number of CEA-positive cells peaked at 18–24 h. An additive effect was observed upon combined treatment of RL-4 cells with GMDP and interferon-γ (IFN-γ).

The treatment of RL-4 cells with GMDP also resulted in increased expression of HLA-DR antigens.

For WiDr cells an increase in CEA-expression after 48 h incubation was observed as well. In this case, flow cytometry could not be used due to clumping of cells. The CEA expression by WiDr cells was monitored by cell enzyme-linked immunosorbent assay (ELISA).

Whether the above-mentioned effects have functional implications regarding the recognition of tumor cells by the immune system remains to be studied.

Another goal of our study was to identify the MP-binding molecules on responding cells, as the existence and location of specific cellular MP receptors were controversial [9, 10, 11]. To address this question we used two approaches.

The first approach was based on direct staining of MP-binding cells with FITC-labelled GMDP-Lys: the FITC group was attached to the ε-amino group of lysine (see Fig. 1). Fluorescence-activated cell sorting (FACS) analysis of intact murine peritoneal macrophages and WEHI-3 cells showed no specific binding, but when the cell membrane was first fixed with paraformaldehyde and permeabilized with β-octylglucoside and then treated with GMDP-Lys-FITC,

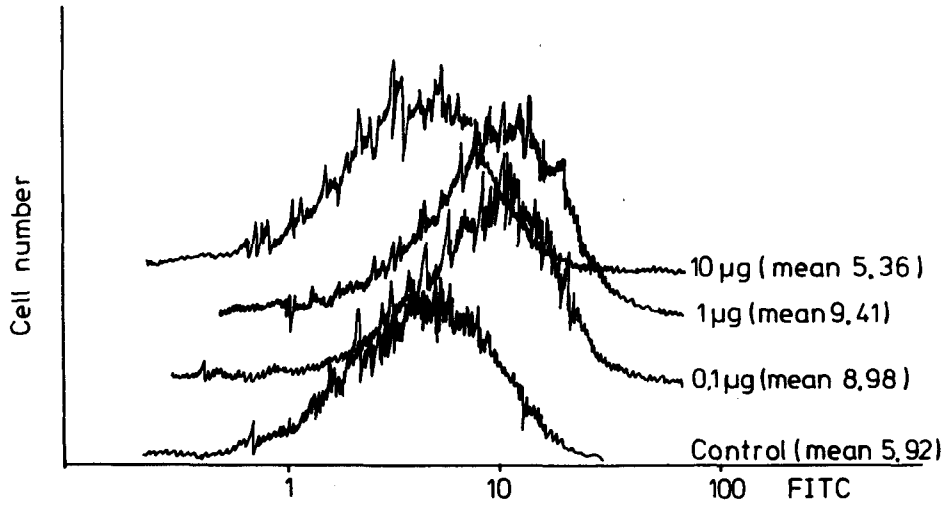


Fig. 2. Effect of incubation with GMDP on CEA expression by RL-4 cells

binding to macrophages and WEHI-3 cells was observed. This binding could be inhibited effectively by GMDP or GMDP-Lys. In contrast, the tripeptide Ala-Gln-Lys was rather ineffective as an inhibitor.

Thus, the GMDP-binding molecule seems to be located inside macrophages. The number of specific binding sites on WEHI-3 cells estimated by comparison with fluorescein-labelled beads of known molecular composition was in the range of 6×10^4, though the total number of binding sites was much higher (2.2×10^5). These values are lower than those obtained for rabbit bronchoalveolar lavage cells [12].

The second approach was based on evaluation of fluorescence polarization of GMDP-Lys-FITC upon binding to cells. Similarly to FACS analysis, only for permeabilized cells was specific binding observed, detected by a change in fluorescence polarization. The ability of GMDP and GMDP-Lys to compete with fluorescent congeners for binding sites suggested specificity of binding. The number of specific binding sites per cell calculated from these data was in the range of $4-5 \times 10^4$ and agreed reasonably well with data obtained by FACS analysis. The Scatchard plot suggested the presence of two populations of binding sites with K_d 2×10^{-8} M and 5×10^{-7} M (Fig. 3).

The above findings are consistent with intracellular MP-binding molecules. Thus, to display immunomodulatory activity MP must be internalized by macrophage. The binding of GMDP to a receptor molecule results in initiation of biosynthesis or enhanced biosynthesis of certain proteins, including cytokines and cell membrane molecules. The changes in

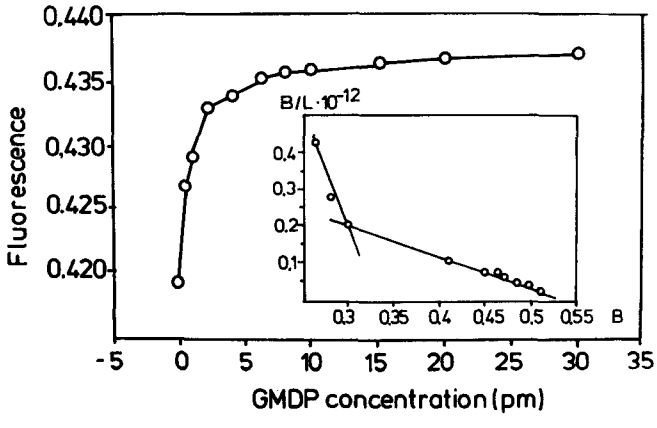


Fig. 3. Binding of GMDP

surface antigen expression influence the magnitude of the immune response.

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A Novel Nuclear Factor Binds to the NF-κB Motif in the Interleukin-2 Enhancer

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Introduction

An important aspect of T-lymphocyte physiology is the necessity for exquisitely tight control of interleukin-2 (IL-2) gene expression. IL-2 is produced only by T cells with extremely transient kinetics [1] (S. Kang, M. Lenardo, unpublished results). Moreover, in nontransformed T cells, significant transcription of the IL-2 gene appears to occur only in the presence of T-cell receptor signaling coupled with an as yet ill-defined "costimulatory signal" present on the surface of competent antigen presenting cells [2, 3]. Further evidence of fine control in IL-2 production is found in the TH_1/TH_2 subdivision of T cells in the murine system: after signaling through apparently the same T cell receptor apparatus, the TH_1 phenotype produces a lymphokine profile which includes IL-2 while the TH_2 phenotype does not [4].

Transcription of the IL-2 gene appears to be controlled in a T cell-specific manner by multiple *cis*-acting elements lying within 300 nucleotides upstream of the transcriptional start site [5]. Although the elements controlling the IL-2 gene have been vigorously investigated, the basis of the cell specificity remains unclear. Most of the activating nuclear factors which bind to these control elements, NF-κB, AP-1, and the octamer-binding proteins, are either not T-cell restricted or are ubiquitous. Only NF-AT appears to be T-cell specific; however, deletion of the NF-AT binding site does not eliminate the inducibility of the IL-2 promoter activity in T cells [6, 7]. We are studying these various enhancer elements to define the molecular basis of the tight regulation of IL-2 gene transcription.

Nearly all detailed studies of gene regulation have utilized transformed cell lines as their model system. Although transformed cells have clearly been informative, they have important limitations. Most significantly, they divide indefinitely without a stimulus. In addition, oncogene products in these cells may by themselves, or by affecting various cellular pathways, perturb normal gene regulation. Perhaps for these reasons, many interesting phenomena of normal cells cannot be reproduced with transformed cells. We have therefore undertaken to study IL-2 gene regulation in murine T-helper lymphocyte clones.

Using murine $CD4^+$ TH_1 cells, we have identified a novel nuclear factor which binds to a site in the IL-2 enhancer shown previously to interact with the well-characterized nuclear factor NF-κB [8, 9]. This factor also appears to bind with lower affinity to other NF-κB sites. Interestingly, the apparent regulation of this factor in normal T cells would suggest that it acts to repress IL-2 gene transcription. In addition, this factor appears to be regulated aberrantly in transformed cell lines.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

A Novel Factor Binds to the κB Motif

The IL-2 enhancer contains a site at −207 to −196 which has been shown by Lenardo et al. to bind purified NF-κB [9]. However, the site is unusual compared to other known NF-κB binding sites in that it contains a purine at the end of 3′ half-site. The IL-2 site has been shown by Serfling et al. [10] to be a "T-cell element" because it is a functionally active enhancer element only in T cells. The IL-2 site also resembles the "cytokine consensus" sequences defined by Shibuya et al. [11]. Cytokine consensus sites are found in numerous lymphokine genes, but appear to bind a variety of nuclear factors depending on fine sequence differences. For example, the "cytokine consensus site" in granulocyte macrophage–colony stimulating factor (GM-CSF) binds and is regulated by a factor NF-GMa [12].

NF-κB sites	κ-light chain:	GGGACTTTCC
	β-interferon:	GGGAAATTCC
IL-2 site		GGGATTTCACC
"Cytokine consensus"	GM-CSF:	GAGATTCCAC
	IL-3:	GAGATTCCAC

Nuclear proteins from the T-cell clone A.E7 [2] were assayed using a electrophoretic mobility shift assay (EMSA) [7]. Using an oligo containing the NF-κB site in the κ-light chain enhancer, we detected a small amount of constitutive NF-κB in these T cells, with marked induction of binding activity upon antigenic stimulation (Fig. 1). Unexpectedly, we also detected a faster migrating band in the unstimulated extracts which seemed to disappear in the stimulated extracts. We designate this factor as NF-CYT1. Thus, two deoxyribonucleic acid (DNA) binding complexes of apparently different molecular size and distinct responses to antigenic stimulation were detectable with the κB site from the κ-light chain enhancer.

NF-CYT1 Displays Distinct Binding Affinities from that of NF-κB

To determine the relative apparent affinities of the two factors for the motifs in the IL-2 gene and the κ-light chain enhancer, cross competition studies were carried out with resting A.E7 extract. Figure 2 shows that NF-CYT1 has an approximately 6-fold higher affinity for the site in the IL-2 gene than for the site in the κ-light chain enhancer (lanes 1–9). Reciprocally, NF-κB appears to have an approximately 6-fold higher affinity for the site in the κ-light chain enhancer than for the site in the IL-2 enhancer (lanes 10–18). The HIV LTR κB site and the IL2R κB site appear to bind NF-CYT1 and NF-κB with apparent affinities similar to the κ-light chain enhancer (data not shown). The apparent affinity differences between NF-κB and NF-CYT1 make it unlikely that the NF-CYT1 complex is a degradation product of NF-κB.

NF-CYT1 is Distinct from NF-GMa

NF-GMa is a tumor necrosis factor (TNF)-inducible nuclear factor thought to contribute to the transcriptional regulation of both IL-3 and GM-CSF [12]. NF-GMa binds to sites in the IL-3 and GM-CSF enhancers which bear resemblance to the IL-2 site (see above). Therefore, we tested the possibility that this factor also binds to the IL-2 site. Figure 3 shows that the complex bound by the IL-2 oligo can be competed with the IL-2 oligo but not with either the IL-3 or the GM-CSF oligos. The complex bound by the GM-CSF oligo is slower migrating

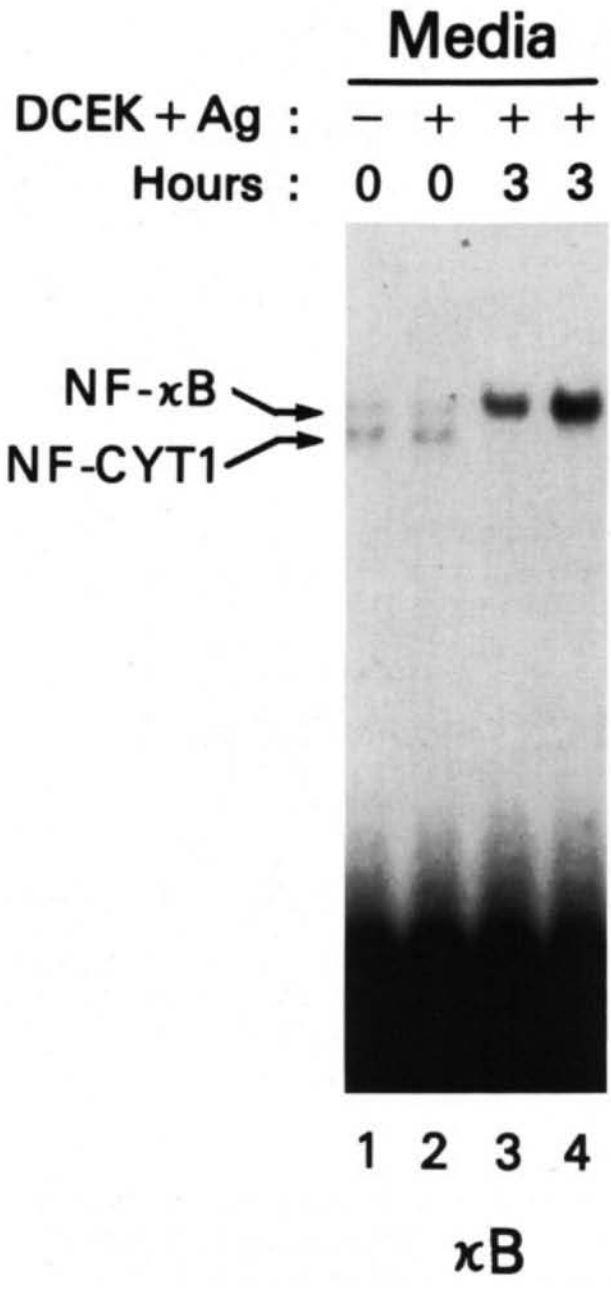

Fig. 1. EMSA using the κB site from the κ-light chain enhancer used as radiolabelled probe. *Lanes 1*, and *2*, unstimulated A.E7 extract (lane has antigen presenting cells but no antigen (denoted DCEK); *lanes 3* and *4*, extracts from A.E7 stimulated for 3 h with 10 μ*M* pigeon cytochrome C (frag. 81–104) and antigen presenting cells. Gel conditions essentially as described in Staudt et al. [13]

than that bound by the IL-2 oligo; further, it is competed for by the GM-CSF and IL-3 oligos (lanes 11–14), but not by the IL-2 oligo (lanes 15–17). Thus, NF-CYT1 has different binding characteristics from NF-GMa.

Loss of NF-CYT1 Binding Activity is Correlated with IL-2 Production

To explore the regulation of NF-CYT1 more fully, we analyzed nuclear extracts from cells which do and do not make IL-2 (Fig. 4). In the A.E7 clone which is an IL-2 producer, a dramatic decrease in NF-CYT1 activity (the lower band in lanes 1–4) can be seen with antigenic stimulation. The decrease is seen as early as 1.5 h after antigenic stimulation, and appears to plateau at 3 h. Note that the reference lane (lane 1) is not a 0-h time point, but a 4.5-h time point after incubation with antigen presenting cells, without antigen. We have reproducibly observed that binding activity of NF-CYT1 diminishes only in the presence of antigen (S. Kang and M. Lenardo, unpublished results). Also note that the level of NF-κB (the light upper complex in lanes 1–4) in the 4.5-h/no antigen lane appears to be at least as high as at 4.5 h with antigen. In contrast to NF-CYT1, which requires antigen for modulation, we have found that NF-κB can be induced in the presence of antigen presenting cells alone (S. Kang and M. Lenardo, unpublished results). This finding underscores the differential regulation of NF-CYT1 and NF-κB.

The D10 T-cell clone is a murine $CD4^+$ lymphocyte of the Th2 phenotype: it produces IL-4, IL-5, and IL-6, but not IL-2 or gamma-interferon [4]. We stimulated the D10 clone with concanavalin A (sufficient for full induction of lymphokine activity in these cells) and prepared nuclear extracts at the times indicated (lanes 12–15). NF-CYT1 activity in these cells does not decrease with stimulation but rather appears to increase.

Other non-IL-2 producing cells were tested for NF-CYT1 binding activity. Figure 4 shows that significant NF-CYT1 binding activity is found in BJAB cells (a human B-cell lymphoma), phorbol ester (PMA) stimulated HELA cells (a human cervical carcinoma), and DCEK cells (a mouse fibroblast line). The bands of higher mobility than NF-

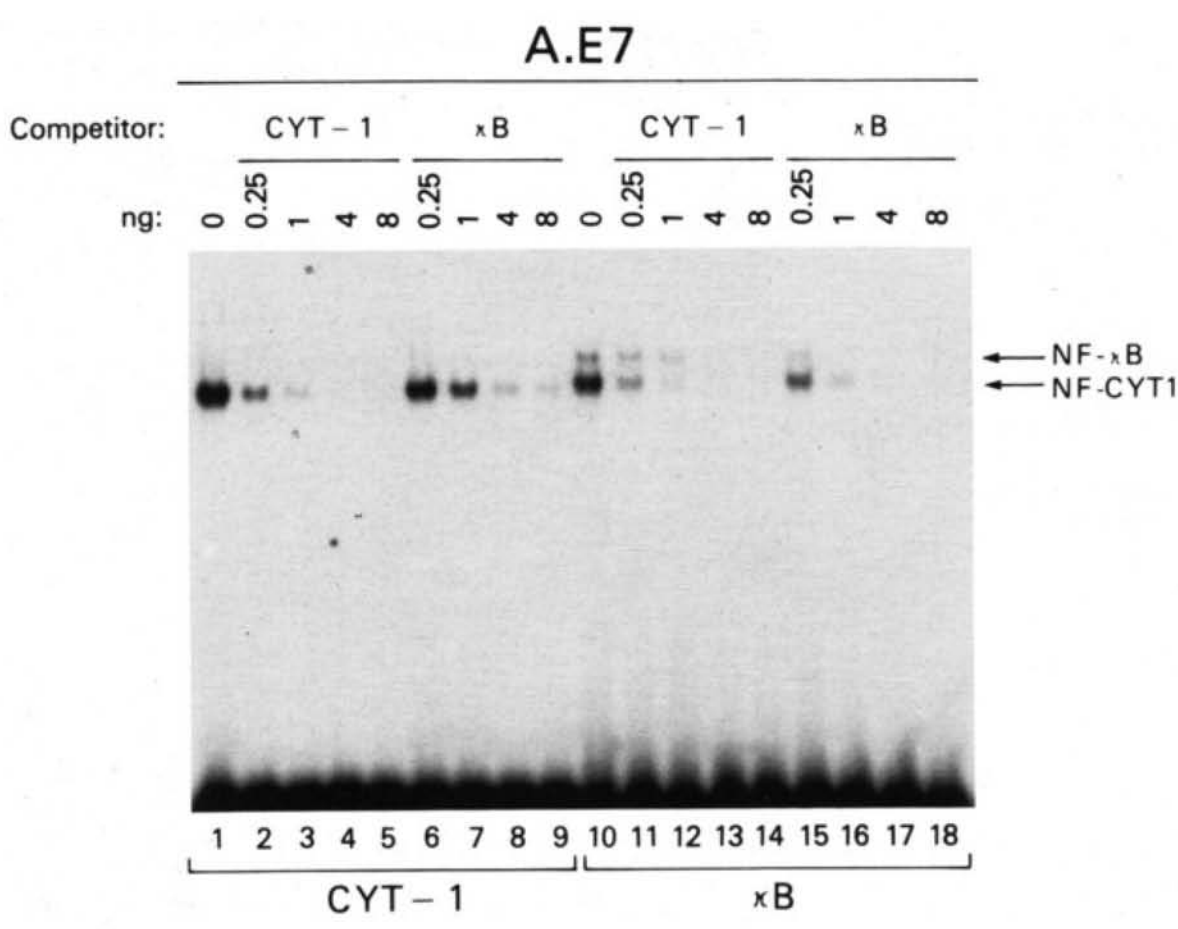

Fig. 2. EMSA: extract from resting A.E7 T-cell clones. *Lanes 1–9*, IL-2 site used as labelled probe; *lanes 10–18*, κB site from the κ-light chain enhancer used as labelled probe. Competitions as noted above the lanes, representing approximately 2.5-, 10-, 40-, and 80-fold molar excess. Sequences of synthetic oligos (with Sal 5′ overhangs): IL-2 site TCGACCAAGAGGGATTTCACCTAAAT CC and κ-light chain site TCGACAGAG GGGACTTTCCGAGAGGG

CYT1 are non-specific, as assayed by competition studies (not shown).

In summary, we have found the decrease in NF-CYT1 activity only in a T-cell clone which produces IL-2. We have also found significant binding activity in non-T cells.

NF-CYT1 is Regulated Differently in Transformed T cells

We examined the regulation of NF-CYT1 activity in the well-studied T-cell tumor lines EL-4 (a mouse thymoma line), and Jurkat (a human T-cell line) (Fig. 4). Both lines produce IL-2 upon stimulation with PMA, and phytohemagglutinin (PHA) + PMA, respectively. In EL-4 cells, a significant amount of binding activity was seen in unstimulated extracts (lane 8). In contrast to A.E7, however, the binding activity was increased with stimulation at 3 h (lane 9). A similar result was obtained in the Jurkat line (lanes 10, 11). Thus, the regulation of NF-CYT1 in the transformed T cells appears to differ from that found in normal T-cell clones. The answer may lie in the observation that these tumor lines have different kinetics of IL-2 induction than in the T-cell clone A.E7: for example, IL-2 message peaks at 4 h in A.E7 and at 8 h in EL-4. Thus, transcriptional events may be taking place at a later time point. Studies are underway to determine whether the difference in regulation of NF-CYT1 between normal and transformed T cells is simply a kinetic one.

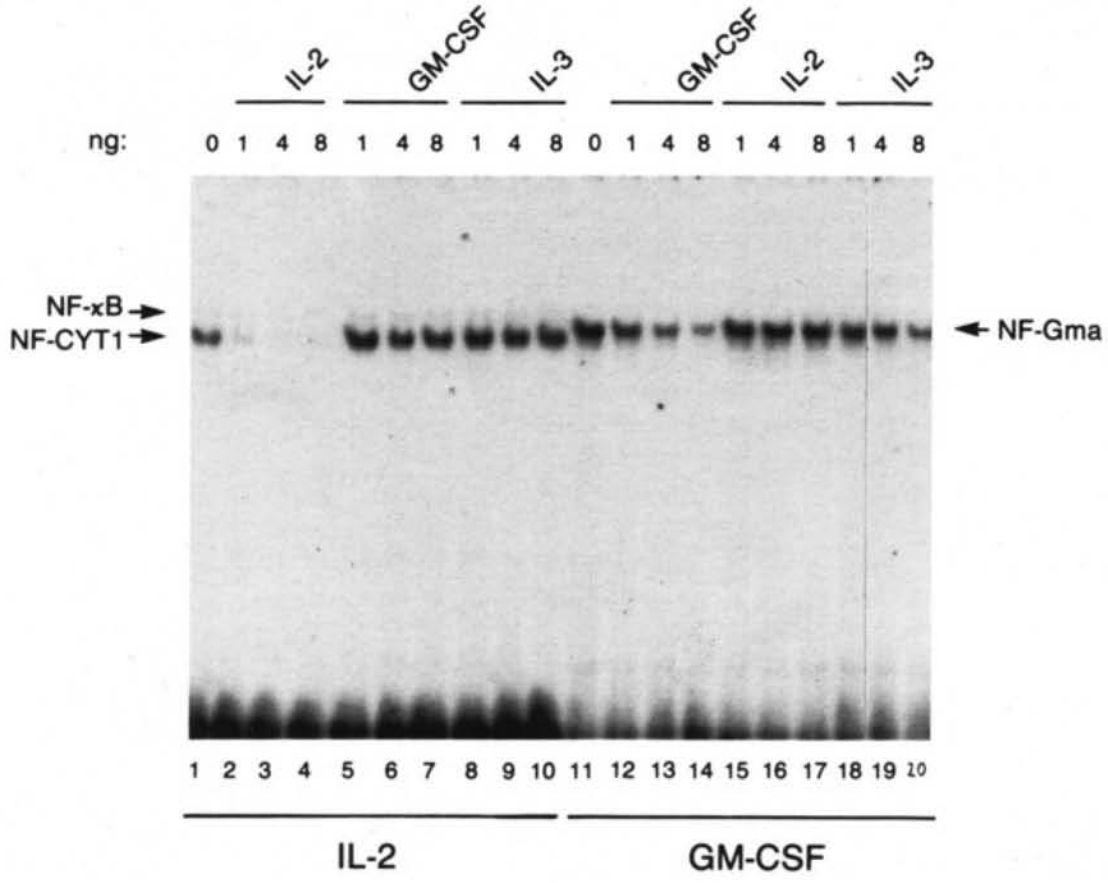

Fig. 3. EMSA: PMA stimulated EL4 extract used in all lanes. *Lanes 1–10*, IL-2 site used as labelled probe; *lanes 11–20*, GM-CSF site used as labelled probe. Competitors are noted above the lanes, representing approximately 10-, 40-, and 80-fold molar excess. GM-CSF oligo: TCGAAGGGCCAGGAGATTCCA CAACT. IL-3 oligo: TCGAAGGATGAGA TTCCACTGCATA

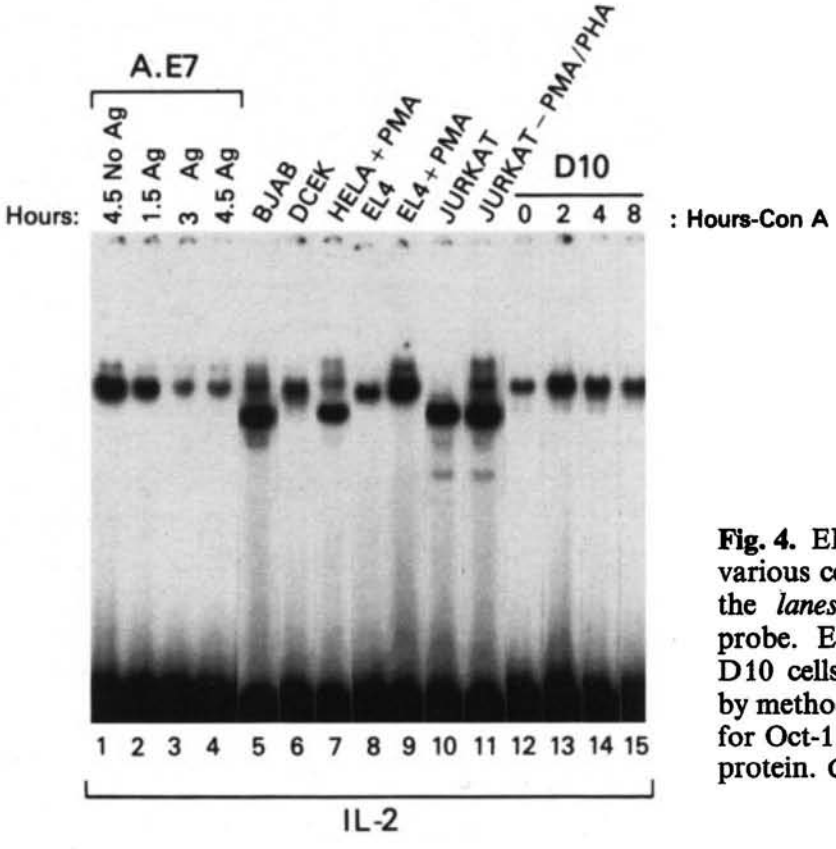

Fig. 4. EMSA: comparison of CYT1 in various cell types. Extracts noted above the *lanes*.. IL-2 site used as labelled probe. Extracts from A.E7 cells and D10 cells were normalized for protein by method of Bradford, and by assaying for Oct-1, a ubiquitous and constitutive protein. *Con A:* concanavalin A

Table 1. DNA binding proteins of the κB site family

Factor	Site	Genes regulated	Distribution	Inducers	Mode
NF-κB	GGGACTTTCC (most frequent)	Igκ light chain β-interferon, TNF-α IL-2 receptor-α, *HIV* IL-6, lymphotoxin serum amyloid A, angiotensinogen	Precursor is ubiquitous; constitutive – B and some T cells	Phorbols TNF-α viruses ds RNA IL-1, $p40^{tax}$	Post-translational
H2- TF1	GGGGATTCCCC	MHC H2-K^b	ubiquitous	LPS	N.D.
KBF-1	GGGACTTTCC	β_2-microglobulin MHC H2-K^b	?ubiquitous	N.D.	N.D.
HIVEN86A	GGGACTTTCC	IL-2 receptor-α, HIV	?T lymphocytes	PMA/PHA	N.D.
EBP-1	GGGACTTTCC	HIV, SV-40	?ubiquitous	N.D.	
PRDII-BF1 MBP-1	GGGAAATTCC	?β-interferon	almost ubiquitous	serum viruses	transcriptional
NF-CYT1	GGGATTTCACC	IL-2	almost ubiquitous	Ag + APC (decrease levels)	N.D.

Summary

We have identified a novel nuclear factor, NF-CYT1, which is present in both T and non-T cells and binds with highest affinity to the κB motif in the IL-2 gene. NF-CYT1 appears to bind with approximately 6-fold lower apparent affinity to the NF-κB sites in the κ-light chain enhancer, the HIV LTR, and the IL-2R alpha chain enhancer. Preliminary studies appear to implicate the purine residue in the second half-site as the major contributor to the binding specificity (Kang et al., unpublished results).

We believe that NF-CYT1 is a new member of the "κB site binding" family (Table 1). We show here that NF-CYT1 is distinct from NF-κB and NF-GMA, based on affinity and regulatory differences. We believe that NF-CYT1 is not related to H2-TF1, KBF-1 or EBP-1 because these have been found to be constitutive factors. The relationship to HIVEN86A and PRDII-BF-1 is unclear because these factors have not been demonstrated in mobility shift assays. It will be interesting to compare the structural differences between NF-CYT1 and NF-κB once they are cloned, to help understand how two factors can bind to similar sequences, but with reciprocal affinities.

The mechanism of the decrease in binding activity of NF-CYT1 and how it is restricted to activated TH1 cells is unknown. The rapid kinetics of the decrease make a posttranslational mechanism more likely. Studies may provide insights into the signal transduction pathways involved in T-cell activation.

NF-CYT1 is the first κB-motif binding factor shown to decrease in binding activity with cellular activation. In fact, it may be the first transcription factor that apparently decreases during lymphocyte activation. The functional implications of this decrease are unclear at present. However, the regulation of NF-CYT1 in various cell types is consistent with that of a repressor molecule. The observation that NF-CYT1 binds with highest affinity to the IL-2 site and only decreases with activation in T cells which make IL-2 suggests that NF-CYT1 may be important in keeping IL-2 gene transcription off in situations inappropriate for IL-2 production. However, NF-CYT1 may also play a modulatory role in the transcription of genes to which it can bind with lower affinity. Functional studies will serve to define the interplay of NF-CYT1 and NF-κB in the transcriptional regulation of both T- and non-T-cell restricted genes.

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Soluble Cytokine Receptors as Immunomodulators

D. Cosman [1]

Introduction

During the past few years it has become clear that many membrane proteins can be found in soluble forms in body fluids. Examples include histocompatibility antigens [1] and Fc receptors [2]. The extracellular portion of the membrane protein may be released by proteolysis, or by phospholipase action in the case of those molecules that are linked to the membrane by a phosphoinositol linkage. Alternatively, the soluble protein may be encoded by an alternatively spliced messenger ribonucleic acid (mRNA) species. Frequently the soluble, extracellular portion of the protein retains the same ligand-binding properties as the membrane-bound form.

Cytokine receptors also show the same behavior. Soluble receptors that retain ligand-binding properties have been found in urine and serum for the interleukin-2 (IL-2) receptor-alpha [3], tumor necrosis factor (TNF) receptor (two forms) [4], IL-6 receptor [5], gamma-interferon (γ-IFN) receptor [5], growth hormone receptor [6], and nerve growth factor receptor [7]. Recently we have identified alternatively spliced mRNA species that encode soluble forms of the IL-4 and IL-7 receptors [8, 9]. Here we discuss these results and describe how soluble cytokine receptors, either naturally occurring forms or generated by recombinant deoxyribonucleic acid (DNA) manipulation, can be used as immunomodulatory agents, both in vitro or in vivo.

Cytokine Receptor Families

Elucidation of the primary amino acid sequences of many cytokine receptors as a result of cDNA cloning has allowed the grouping of these receptors into families, based on similarities in their extracellular, ligand-binding domains. For those receptors whose ligands regulate hematopoiesis and immunity, three families have emerged. The first family is the well-known and very large immunoglobulin superfamily, most of whose members are not cytokine receptors. However, the receptors for IL-1, colony stimulating factor-1 (CSF-1), and PDGF belong to this group, with three, five, and five immunoglobulin-like domains respectively in their extracellular portions [10, 11]. CSF-1 and PDGF receptors have intracellular tyrosine kinase domains, the IL-1 receptor does not.

The second, more recently recognized, family consists solely of cytokine receptors. We have designated this as the hematopoietin receptor family as almost all of these receptors mediate effects on hematopoietic cells [12]. The members of this family currently consist of the receptors for IL-2 (β subunit) [13, 14], IL-3 [15], IL-4 [8, 12], IL-6 [16, 17], IL-7 [9], GM-CSF [18, 19], G-CSF [20], erythropoietin [21], prolactin (two forms of receptor) [22, 23], and growth hormone [6]. The common sequence element in the extracellular domains of these receptors is a stretch of about 200 amino acids that

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

shows considerable sequence conservation between the different members of the family [8, 18, 24, 25]. When these sequences are compared using the ALIGN program to generate pairwise scores that measure the degree of amino acid sequence relatedness, scores are mostly in the range of 3–12 [24]. Any score greater than 3 is considered to indicate significant sequence relatedness [10].

Within these 200 amino acids there are certain features that show particular conservation (see Fig. 1). These include the positions of four N-terminal cysteines (although many family members have additional nonconserved cysteines) and a WSXWS motif located at the C-terminus of the conserved region, usually just outside the transmembrane domain. The C-terminal 90–100 amino acids of the conserved region show significant homology to type III fibronectin domains [26], and the G-CSF receptor is so far unique in having three additional fibronectin-like domains between the conserved region and the transmembrane domain [19, 20]. It can be speculated that the fibronectin-like domains play a role in interaction of the growth factor receptors with extracellular matrix components or other cell surface proteins.

The IL-3 receptor has a duplication of the 200 amino acid conserved region [15], and the IL-6 and G-CSF receptors have N-terminal immunoglobulin-like domains [16, 19, 20], showing that receptors can belong to more than one family.

In contrast to the striking degree of sequence relatedness between the extracellular domains of the receptors, the

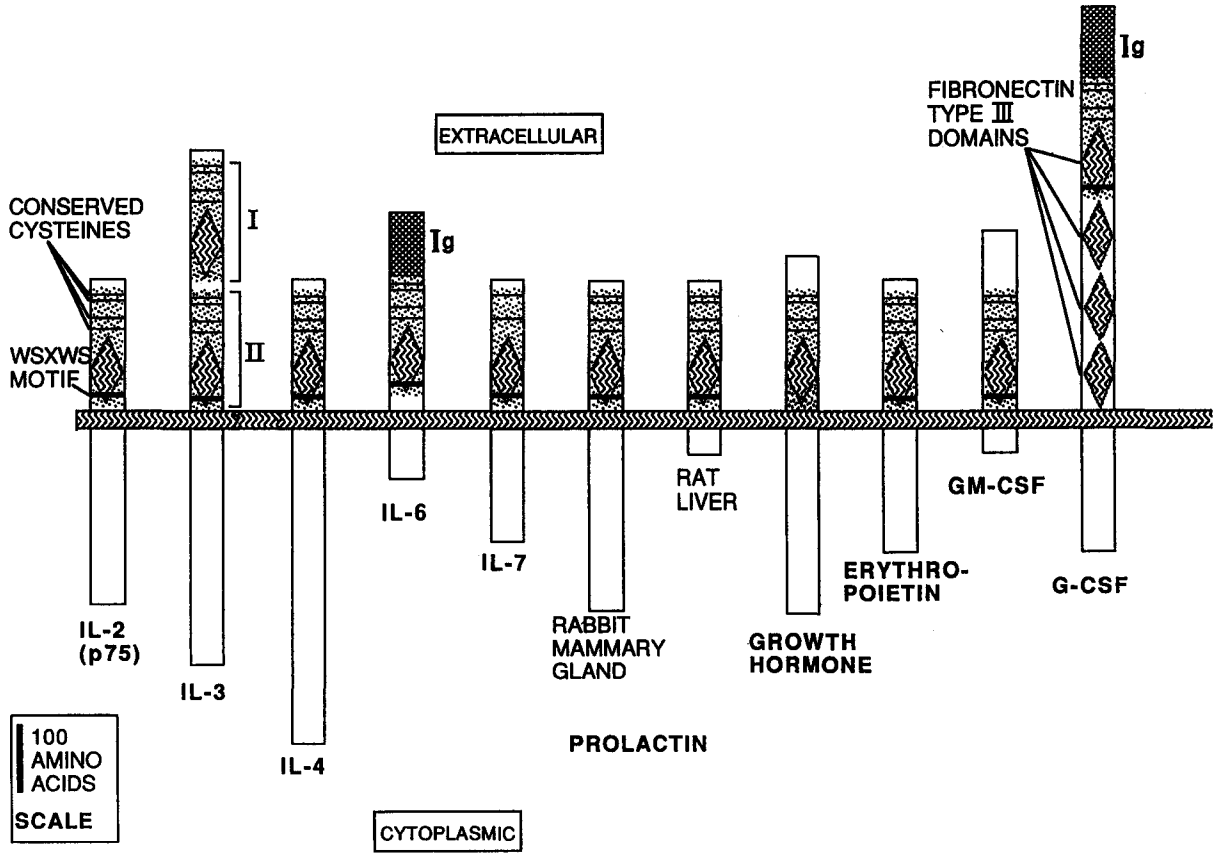

Fig. 1. The hematopoietin receptor superfamily. Schematic representations of the structures of all known members of the family are shown. *Thin horizontal bars* represent conserved cysteine residues. *Thick horizontal bars* represent the conserved Trp-Ser-X-Trp-Ser motif (WSXWS). Fibronectin type III domains are shown as *diamond shapes*, and the stretch of 200 amino acids characteristic of these receptors is *shaded*. The immunoglobulin-like domains at the N-termini of the IL-6 and G-CSF receptors are also indicated

cytoplasmic sequences show little if any similarity apart from a general tendency towards a high content of serine, proline, and acidic amino acids. This reflects our current ignorance as to the mechanisms of signal transduction by these receptors.

The third family centers around the newly cloned TNF receptors p80 and p60 [27–29]. Both these molecules have a cysteine-rich, extracellular, ligand-binding domain that can be subdivided into four internally homologous subdomains. Other members of the family sharing this structure are the nerve growth factor receptor [30]. CD40, a cell surface protein involved in B cell activation [31], 4-1BB, characterized as a mRNA species induced upon T cell activation [32], and OX40, a membrane protein present on rat $CD4^+$ T cells that can contribute to T cell proliferation [33]. The last three proteins may well be cytokine receptors with unknown ligands.

An additional member of this family, with particularly strong homology to TNF receptor p80, is the T2 open reading frame from Shope fibroma virus, a rabbit pox virus [34]. The predicted protein sequence has characteristics of a secreted TNF receptor, and we have shown that the T2 ORF can be expressed in mammalian cells. The protein is secreted and binds TNF [35]. It seems likely that the virus has acquired a rabbit TNF receptor during evolution and that it expresses a soluble TNF receptor as a defense against the portion of the host's immune response mediated by TNF.

Once again, the members of the TNF receptor family show little or no sequence relatedness in their cytoplasmic domains, nor do the IL-1 and TNF receptors, despite the fact that IL-1 and TNF share many biological activities.

Soluble Cytokine Receptors

The existence of soluble extracellular domains of cytokine receptors that retain their ligand-binding capabilities suggested that such molecules might be able to block interaction of their cognate ligands with cell surface receptors. This might have a normal immunoregulatory role in vivo, or could be exploited pharmacologically to down-modulate undesirable immune reactions, such as allergy, autoimmunity, or graft rejection.

In order to test this hypothesis, we have expressed soluble murine IL-1 and IL-4 receptors in mammalian cells and purified the recombinant proteins by affinity chromatography. The soluble IL-1 receptor was generated by inserting a translation termination codon immediately 5′ to the transmembrane domain [36], and the soluble IL-4 receptor used a cDNA from a naturally occurring, alternatively spliced mRNA species [8]. The purified receptors were tested for their ability to block specifically the biological activities of their respective ligands. IL-1 and IL-4 can each stimulate B cell proliferation when anti-immunoglobulin is used as a co-mitogen. IL-1 mediated B cell proliferation was completely inhibited by soluble IL-1 receptor, whereas soluble IL-4 receptor had no effect. Conversely, IL-4 mediated B cell proliferation was inhibited by soluble IL-4 receptor but not by soluble IL-1 receptor [37]. These results demonstrate not only that soluble IL-1 and IL-4 receptors have highly specific neutralizing capacity, but also that IL-1 and IL-4 mediate B cell proliferation by independent pathways.

Following the demonstration of in vitro biological activity, the soluble receptors were tested in vivo in two models that involve lymphocyte activation in response to alloantigenic challenge [38, 39]. In the first, Balb/c mice were injected in the footpad with irradiated allogeneic spleen cells from C57BL/6 mice. Over the course of 7 days there was a host-versus-graft response leading to lymphoproliferation and consequent swelling of the draining popliteal lymph nodes. The strength of this reaction could be quantitated by excision and weighing of the lymph nodes. As a control, each mouse was injected in the contralateral footpad with an equal number of syngeneic,

irradiated spleen cells, so that the specific response could be measured as the weight of the lymph nodes draining the site of allogeneic cell injection minus the weight of the lymph nodes draining the site of syngeneic spleen cell injection. Daily injections of soluble IL-1 receptor or soluble IL-4 receptor could completely block the lymphoproliferative response. Injections were given intraperitoneally or subcutaneously for 4 days, using mouse serum albumin as a negative control. As little as 100 ng–1 μg per dose of receptor showed significant inhibition, and the optimum time to commence treatment was 1 day prior to challenge with the allogeneic spleen cells [38, 39]. In each case, the inhibitory effect of the soluble receptor could be reversed by its cognate ligand.

In a second model system, hearts from newborn C57BL/6 mice were grafted into ear pinnae of Balb/c mice. The hearts continued to beat until rejected by the hosts at around 12 days after transplantation. Daily administration of soluble IL-1 receptor or soluble IL-4 receptor for 4–6 days, starting on the day of transplantation, significantly prolonged graft survival [38, 39].

These data implicate both IL-1 and IL-4 as being important in the initiation of an immune response to alloantigenic challenge in vivo, and suggest that both soluble receptors may be clinically useful in preventing graft rejection. Based on the known biological activities of IL-1 and IL-4, it might be predicted that the soluble receptors would be of therapeutic value in other disease states. IL-1 has many pro-inflammatory properties; examples include induction of prostaglandin release, stimulation of cartilage breakdown, and induction of cytokines with chemotactic activity for neutrophils and monocytes. Soluble IL-1 receptor might be a useful anti-inflammatory agent in diseases such as rheumatoid arthritis. IL-4 promotes synthesis of IgE by an isotype class-switching mechanism in B cells and is a growth factor for mast cells in conjunction with IL-3. It is thought to be a central mediator of allergic responses and consequently soluble IL-4 receptor may have therapeutic value in controlling allergy. The demonstrated efficacy of soluble cytokine receptors as immunomodulators opens up possibilities for clinical intervention in many disease states, and this promises to be an area of active investigation.

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Early Life and Evolution

Immunological Self-Nonself Discrimination and Numerous Peptide Fragments Shared by Unrelated Proteins

S. Ohno [1]

Introduction

Ever since the X-ray crystallographic analysis of a class I major histocompatibility complex (MHC) antigen revealed the presence of an alien peptide fragment sandwiched between its two parallel α-helices [1], the immunological self became a multitude of such peptide fragments, usually 15–20 residues long, derived from host proteins after intracellular processing. For the mainly intrathymic education of self to cytotoxic T cells, these fragments are presented in association with class I MHC antigens, while for the education of helper T cells, they are presented with class II MHC antigens.

For those who believe that proteins represent random assemblages of 20 amino acid residues, the above manner of presentation of self poses no problem, for 15–20 residues long peptide fragments represent an astronomical variety of 20^{15}–20^{20}. With this much variety, homologous peptide fragments are to be found only among proteins related by the propinquity of their descents. Thus, viral and other pathogenic peptide fragments would be distinct from most of the host peptide fragments.

The purpose of this paper is to show that the above is far from the truth. Many peptide fragments are syntactical in construction, and are therefore to be found in many totally unrelated proteins.

The average amino acid composition deduced from 18383 entries in Database is as follows: (1) The top four residues, Leu, Ala, Gly, and Ser, in this order, comprise 32% of the total, and (2) the bottom four residues, His, Met, Cys, and Trp, in this order, comprise only 7% of the total. All 20 homodipeptides occurred at above their expected rates, thus, homodipeptides in the average protein acounted for 14% of its length. While the Leu-Leu homodipeptide was the most numerous of the 400 dipeptides, the second in rank was Leu-Val, occurring at nearly twice the expected rate, while its reciprocal Val-Leu was only one-third as numerous [2]. The above can be viewed as a rudimentary indication of syntatic structures in amino acid sequences. In order to expand on this theme, I have chosen four totally unrelated proteins as representatives of the warm-blooded verterate host. They are: (1) human ET.REC (estrogen receptor), 595 residues long [3]; (2) chicken C-SRC (tyrosine kinase), 533 residue long [4]; (3) human S.ALB (serum albumin), 585 residue long [5]; and (4) human PGK (phospholglycerate kinase) 415 residue long [6].

Lys-Leu- and Leu-Lys-Containing Oligopeptides in Four Host Proteins

We shall now start our inquiry by choosing a pair of Leciprocal dipeptides, Lys-Leu and Leu-Lys. According to the aforementioned extensive survey of 18383 entries in Database, Lys-Leu occurred at about the expected rate, while the incidence of its reciprocal Leu-Lys was slightly less [2]. In the case of four host proteins totalling 2128 residues, there were only 12 Lys-Leu and 13 Leu-

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Lys. Yet, three of the 12 Lys-Leu dipeptides appeared as Val-Lys-Leu and two of them as Ser-Lys-Leu tripeptides. These are indisputable cases of preferential associations, for the most abundant tripeptide ending in Lys-Leu should have been the palindromic Leu-Lys-Leu which, on a random basis, had the expected incidence of 1.08. The fact is that there was not a single Leu-Lys-Leu tripeptide among the four proteins. As to its carboxyl end partners, the Lys-Leu dipeptide showed a distinct preference for Val and the next Gly, for there were four Lys-Leu-Val and two Lys-Leu-Gly. Accordingly, it was no surprise that two totally unrelated proteins, C-SRC and S.ALB, shared a pair of homologous tetrapeptides. Lys-Leu-Val-Gln and Lys-Leu-Val-Asn, as shown in Fig. 1a. As to the 13 Leu-Lys found in four host proteins, this dipeptide showed a definite preference to associate with Phe as its amino terminal partner (four Phe-Leu-Lys in C-SCR, S.ALB, and PGK) and a preference for Ser as its carboxyl terminal partner (three Leu-Lys-Ser in ET.REC and PGK). Accordingly, a pair of homologous pentapeptides containing Leu-Lys was shared between ET.REC and PGK and a pair of identical tetrapeptides, Thr-Phe-Leu-Lys, between S.ALB and PGK. As to two pairs of homologous tetrapeptides containing Leu-Lys or Ile-Lys, the first was shared by S.ALB and PGK and the second by ET.REC and C-SRC, as also shown in Fig. 1a.

Lys-Leu- and Leu-Lys-Containing Oligopeptides in Two Influenza A Virus Hemagglutinins

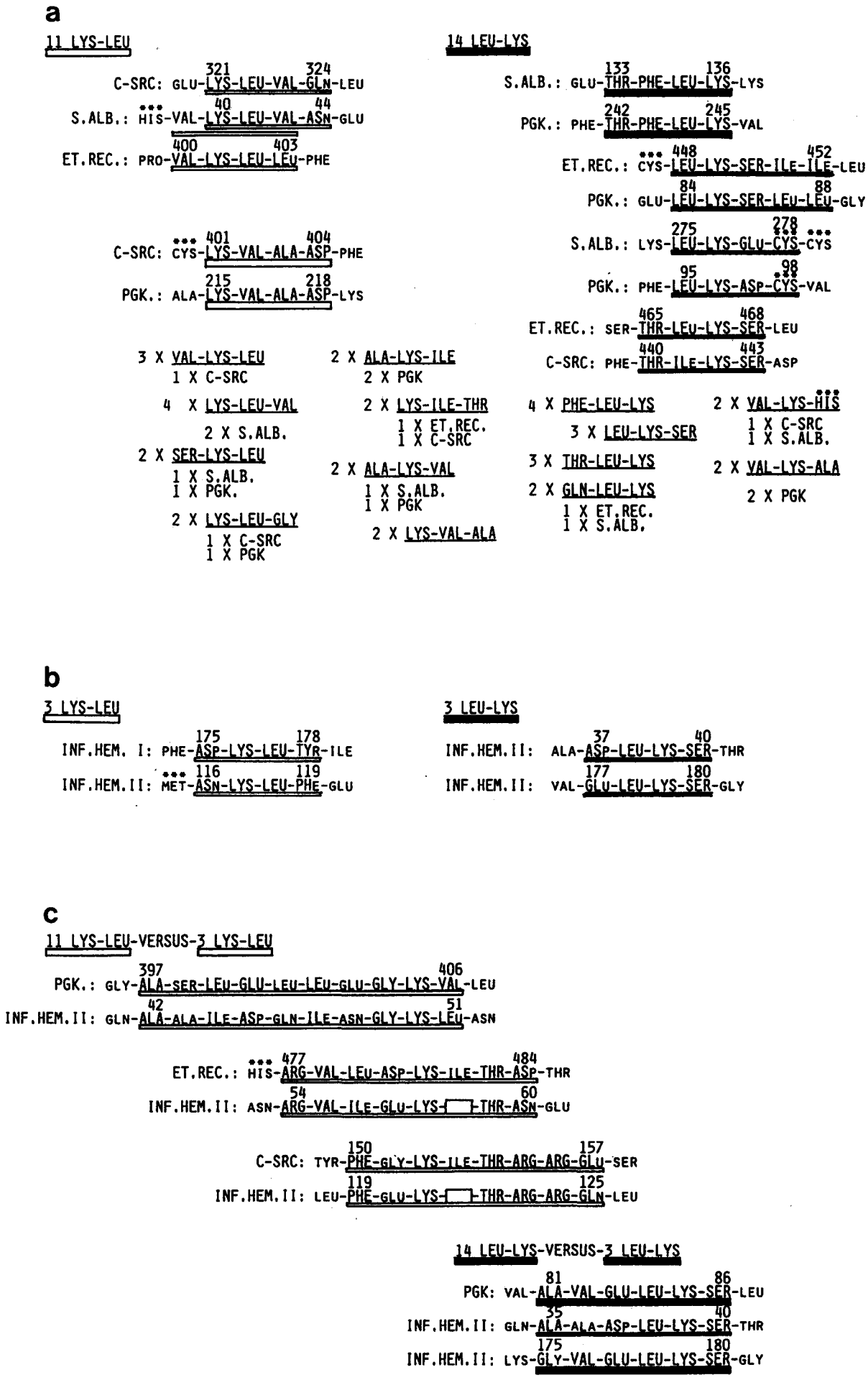
As it has now become clear that totally unrelated host proteins commonly share homologous and identical penta- and tetrapeptides between them, comparison between vertebrate host proteins and viral proteins becomes quite interesting. For this comparison, I have chosen two hemagglutinins of influenza A virus: INF.HEM I and INF.HEM II [7]. Together, these two hemagglutinins comprise only 550 residues, and so, there were only three each of Lys-Leu and Leu-Lys. Nevertheless, it should be noted that within these two hemagglutinins, they were parts of two pairs of homologous tetrapeptides, as shown in Fig. 1b. It would also be noted that two of the three Leu-Lys appeared as Leu-Lys-Ser in INF.HEM II. Thus, the preference of Leu-Lys for Ser as its carboxyl end partner is truly catholic.

The above aroused interest on the long-standing question of self versus nonself. Confining ourselves only to Lys-Leu- and Leu-Lys-containing oligopeptides, how long a fragment of influenza virus hemagglutinins was homologous with that contained in one or the other of the four vertebrate host proteins?

Lys-Leu- and Leu-Lys-Containing Oligopeptides in Host Versus Virus

Although there were only three Lys-Leu in two hemagglutinins of influenza A

Fig. 1. a Lys-Leu- and Leu-Lys-containing oligopeptides in four host proteins. On the *left* are the number of Lys-Leu dipeptides, two pairs of Lys-Leu-containing homologous tetrapeptides, and a pair of Lys-Val-containing identical tetrapeptides found in four unrelated proteins of the vertebrate host. They are underlined by open bars; *thick bars* are for identical tetrapeptides and *thinner bars* for homologous ones. As to the identity of protein sources of these oligopeptides, see the text. Below these three pairs of homologous and identical tetrapeptides, eight Lys-Leu-containing tripeptides that were found more than once are identified and each's source is also indicated, if not already shown. Identical residues are shown in *all capital letters*, while the third letters of homologous residues are shown in *small capitals*. On the *right*, the same with regard to Leu-Lys dipeptides and Leu-Lys-containing oligopeptides are shown. They are underlined by *solid bars*. **b** Lys-Leu to the *left* and Leu-Lys to the *right* of homologous tetrapeptides found within INF.HEM I and II. **c** Three Lys-Leu- and one Leu-Lys-containing oligopeptide of host proteins that were homologous and identical with those of INF.HEM II


a
11 LYS-LEU
C-SRC: GLU-LYS-LEU-VAL-GLN-LEU
S.ALB.: HIS-VAL-LYS-LEU-VAL-ASN-GLU
ET.REC.: PRO-VAL-LYS-LEU-LEU-PHE
C-SRC: CYS-LYS-VAL-ALA-ASP-PHE
PGK.: ALA-LYS-VAL-ALA-ASP-LYS
3 X VAL-LYS-LEU
1 X C-SRC
4 X LYS-LEU-VAL
2 X S.ALB.
2 X SER-LYS-LEU
1 X S.ALB.
1 X PGK.
2 X LYS-LEU-GLY
1 X C-SRC
1 X PGK
2 X ALA-LYS-ILE
2 X PGK
2 X LYS-ILE-THR
1 X ET.REC.
1 X C-SRC
2 X ALA-LYS-VAL
1 X S.ALB.
1 X PGK
2 X LYS-VAL-ALA
14 LEU-LYS
S.ALB.: GLU-THR-PHE-LEU-LYS-LYS
PGK.: PHE-THR-PHE-LEU-LYS-VAL
ET.REC.: CYS-LEU-LYS-SER-ILE-ILE-LEU
PGK.: GLU-LEU-LYS-SER-LEU-LEU-GLY
S.ALB.: LYS-LEU-LYS-GLU-CYS-CYS
PGK.: PHE-LEU-LYS-ASP-CYS-VAL
ET.REC.: SER-THR-LEU-LYS-SER-LEU
C-SRC: PHE-THR-ILE-LYS-SER-ASP
4 X PHE-LEU-LYS
3 X LEU-LYS-SER
3 X THR-LEU-LYS
2 X GLN-LEU-LYS
1 X ET.REC.
1 X S.ALB.
2 X VAL-LYS-HIS
1 X C-SRC
1 X S.ALB.
2 X VAL-LYS-ALA
2 X PGK
b
3 LYS-LEU
INF.HEM. I: PHE-ASP-LYS-LEU-TYR-ILE
INF.HEM.II: MET-ASN-LYS-LEU-PHE-GLU
3 LEU-LYS
INF.HEM.II: ALA-ASP-LEU-LYS-SER-THR
INF.HEM.II: VAL-GLU-LEU-LYS-SER-GLY
c
11 LYS-LEU-VERSUS-3 LYS-LEU
PGK.: GLY-ALA-SER-LEU-GLU-LEU-LEU-GLU-GLY-LYS-VAL-LEU
INF.HEM.II: GLN-ALA-ALA-ILE-ASP-GLN-ILE-ASN-GLY-LYS-LEU-ASN
ET.REC.: HIS-ARG-VAL-LEU-ASP-LYS-ILE-THR-ASP-THR
INF.HEM.II: ASN-ARG-VAL-ILE-GLU-LYS-[ ]-THR-ASN-GLU
C-SRC: TYR-PHE-GLY-LYS-ILE-THR-ARG-ARG-GLU-SER
INF.HEM.II: LEU-PHE-GLU-LYS-[ ]-THR-ARG-ARG-GLN-LEU
14 LEU-LYS-VERSUS-3 LEU-LYS
PGK: VAL-ALA-VAL-GLU-LEU-LYS-SER-LEU
INF.HEM.II: GLN-ALA-ALA-ASP-LEU-LYS-SER-THR
INF.HEM.II: LYS-GLY-VAL-GLU-LEU-LYS-SER-GLY

virus, compared to 11 Lys-Leu among the four host proteins, these three Lys-Leu of the virus can also be considered as homologous to six Lys-Val and six Lys-Ile of the host. As shown in Fig. 1c, the decapeptide ending in Lys-Val of host PGK occupying the 397th–406th positions was seven-tenths homologous with the decapeptide ending in Lys-Leu of INF.HEM II occupying the 42nd–51st positions. In view of the fact that the total number of proteins possessed by the vertebrate host is of the order of 10^4, it would be no surprise if the decapeptide identical to the above of INF.HEM II were found in at least one unknown host protein. If such is the case, this viral decapeptide is an indisputable self. On the other hand, if the homology of seven-tenths or thereabouts is the maximal obtainable between this viral peptide fragment and a multitude of host peptide fragments, can it be universally recognized as a nonself?

Most instructive concerning this question is the finding reported on human cytotoxic T cell responses to the nuclear matrix protein of influenza A virus [8]. It has been shown that only internal viral proteins, such as the matrix and nucleoproteins of influenza A virus, can invoke a cytotoxic T cell response in infected human and mouse hosts. As far as the matrix protein was concerned, however, it proved incapable of eliciting cytotoxic T cell responses from those human individuals whose class I MHC haplotypes contained HLA-C7 [8]. For those individuals, all peptide fragments of the influenza matrix protein must have appeared as self. Although cytotoxic T cells of HLA-A2 individuals infected with influenza A virus readily responded to the matrix proteins, the test of various peptide fragments revealed that even HLA-A2 cytotoxic T cells recognized only one 19-residue-long peptide fragment representing positions 55–73 of the matrix protein as nonself [8].

It is probable that positions 42–51 of INF.HEM II shown in Fig. 1c are the type of peptide fragments that are recognized as nonself only by helper T cells of particular class II MHC haplotypes, thus creating classical responders and nonresponders among individuals.

Figure 1c also shows that two Lys-Ile-containing octapeptides of the host (one derived from ET.REC and the other from C-SRC) enjoyed seven-eighths and six-eighths homology with two heptapeptides of INF.HEM II, if Ile or Lys-Ile of each was deleted.

As to Leu-Lys-containing oligopeptides, I shall be content to show only the identical pentapeptide, Val-Glu-Leu-Lys-Ser, shared by PGK of the host and INF.HEM II. Actually, positions 81–86 are entirely homologous with positions 175–180 of INF.HEM II. In addition, this PGK hexapeptide was also five-sixths homologous with positions 35–40 of INF.HEM II.

All Proteins as Divergent Essays Written in One Language

During the past several years, we have advanced the notion that all coding sequences in this world are scriptures written in one and the same DNA language [9]. Here, it was shown that the same applies to amino acid sequences of proteins as well. As long as they are written in the same language, two essays on entirely different subjects may have surprisingly many identical and similar components. Witness the following:

"The term *high ceiling* has been used to denote a group of diuretics that have a distinctive action on renal tubular function."

"The term *high ceiling* has been used to denote a group of stocks that show a distinctive pattern of price fluctuations."

The first was derived from an essay on diuretic drugs, while the second was from one on stocks and stock markets, yet 15 of the 22 words are identical. Is it a surprise, then, if totally unrelated proteins derived from vertebrates and from a virus share a multitude of identical and homologous oligopeptides?

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Multigene Families: The Problem of Molecular Recapitulation

S. N. Rodin, A. Y. Rzhetsky, and A. A. Zharkikh [1]

Introduction

The multigene families (MF) are known to have been formed in the course of evolution mainly by sequential duplication of ancestor genes. Almost all MFs are characterized by some specified order of homologous gene expression in the course of ontogenesis. The question arises: are the genes expressed in early ontogenetic stages more "ancient" than their ontogenetically later expressed homologues? Zuckerkandl [1] was the first to formulate and study this question with respect to the MFs. Taking into account that divergence of α- and β-subfamilies of globins occurred much earlier than those of β-like genes, he compared human β-globins – namely, γ (fetal) and β (adult) protein sequences – with α-globin. The latter protein sequence was taken as a marker close to the "ancestor". Zuckerkandl supposed that if the fetal β-like globin (γ) was closer to the α-globin that the adult one (β), the former protein could be assumed to be more ancient than the latter one, and thus evidence in favour of molecular recapitulation would be found. Nevertheless, he discovered that both γ- and β-sequences showed the same number of amino acid dissimilarities (55) with the α-globin [1]. This result compromised the idea of molecular recapitulation for a rather long period.

It is a priori evident that if the phenomenon of molecular recapitulation really takes place, it must be caused by the stabilizing natural selection: the earlier a gene is expressed in ontogeny, the wider is the range of possible undesirable consequences of any mutation in the gene. Selection of this kind must preserve the structure of "functional" domains of the gene much more carefully than those which are "subneutral". Thus, it is not unlikely that a large number of subneutral substitutions is masking a smaller number of substitutions located in the functional sites. Therefore, we decided to verify this suggestion using more representative samples of globin nucleotide sequences and more adequate and rigorous methods than Zuckerkandl of phylogenetic analysis and of differentiating the mutations in the globin functional sites from all the others.

Results

All of the sequences employed were taken from the GenBank data base. Trees were constructed by means of the maximum parisimony method of Zharkikh [2] (program UNISUB). A number of other programs from the VOSTORG package were also used [3].

Using the data of Perutz [4], we have divided the amino acid sites of the globins into two groups: "functional" and "nonfunctional" (or "subneutral"). All amino acid sites that participate in some important functional contacts were assigned to the former group. This group includes sites involved in: the α- and β-contacts with haem, the Bohr effect, the α–β bonds between the haemoglobin sub-

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

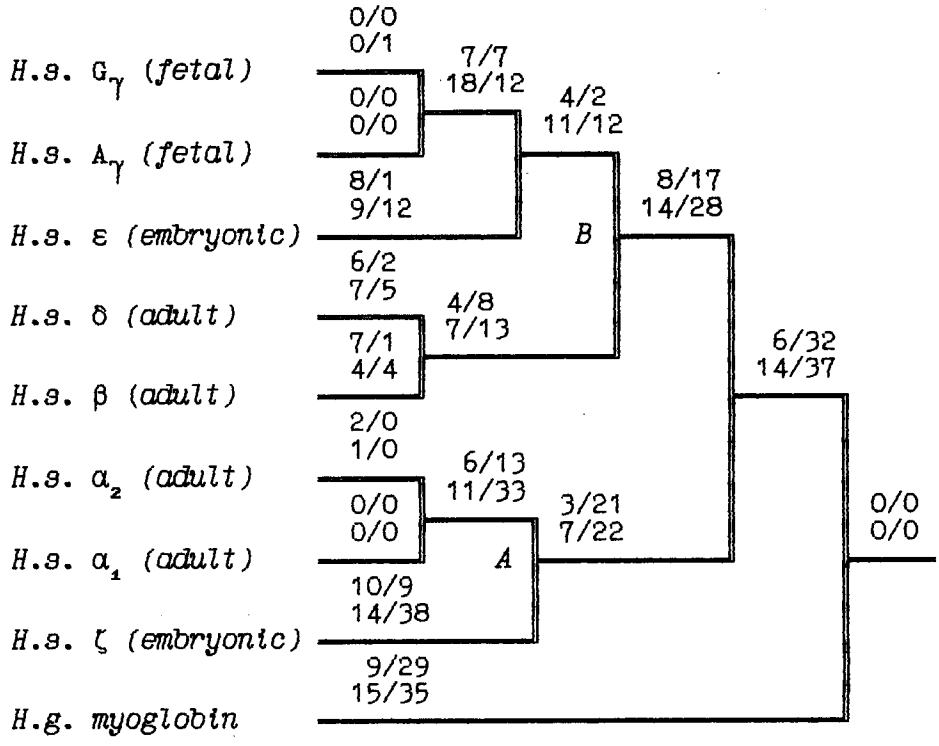

Fig. 1. Phylogenetic tree inferred by maximum parsimony method for eight human globin genes. The *Halichoerus grypus* myoglobin gene was used as a homologous but relatively distant gene to determine the position of the tree root. The total number of substitutions is 593. The ancestral sequences were reconstructed for tree nodes *A* and *B* (for α-like and β-like sequences, respectively).

Abbreviations: *H.s.*: Homo sapiens; *H.g.*: Halichoerus grypus.

Upper line of numbers above each branch, the numbers of synonymous/nonsynonymous reconstructed nucleotide substitutions in the "functional" sites. *Lower line of figures* above each branch, the same for "nonfunctional" sites

units, the binding of 2,3-diphosphoglycerate (for β-like chains) and the salt bridges. The nonfunctional group includes all the other sites.

On the base of the primary DNA sequence alignment, phylogenetic trees for the globin genes of *Homo sapiens* (see Fig. 1), *Capra hircus* and *Xenopus laevis* (not presented) were inferred. In order to determine the position of the tree root, we used the *Halichoerus grypus* myoglobin gene as a homologous but relatively distant gene.

When estimating branch lengths of the trees we sorted the reconstructed nucleotide substitutions in a special way. Each nucleotide substitution was characterized from two points of view: on the one hand, as affecting a functional or nonfunctional site of the protein, and on the other hand, as synonymous or nonsynonymous. Using the estimated branch lengths we computed the distances between the present day sequences and the corresponding ancestor ones reconstructed for each α- and β-gene cluster. The results are presented in Table 1.

Studying both α- and β-like human sequences revealed the same regularity: the number of reconstructed nonsynonymous substitutions fixed in the functional sites of the embryonic genes (ζ and ε) is threefold less than in adult genes (α_1, α_2 in α-cluster and β, δ in β-cluster). The analogous values for the fetal and adult β-like genes are almost equal (about nine substitutions) (see Table 1).

In fact, the same could be said about the *C. hircus* genes. The goat β-cluster consists of three groups of genes [5]: the β^c, β^A and β^F genes (the last one is also often designated γ); they are orthologous to the human β-globin, $\psi\beta^X$, $\psi\beta^Y$ and $\psi\beta^Z$ pseudogenes and the δ-globin gene.

Table 1. Evolutionary distances between the human present-day globin genes and the reconstructed ancestral sequences

Gene	"Functional" sites			"Nonfunctional" sites			Total
	Synonymous	Nonsynonymous	Total	Synonymous	Nonsynonymous	Total	
ε	12	3	15	20	24	44	59
G_γ	11	9	20	29	25	54	74
A_γ	11	9	20	29	24	53	73
δ	10	10	20	14	18	32	52
β	11	9	20	11	17	28	48
α_1	6	13	19	11	33	44	63
α_2	8	13	21	12	33	45	66
ζ	10	9	19	14	38	52	71

In the individual development of a goat, besides embryonic (ε^I and ε^{II} genes), fetal (β^F/γ) and adult stages (β^A) of globin gene expression, an additional "preadult" or "juvenile" stage is found which is characterized by the expression of β^C gene [5].

Thus, the ε^I (goat β-like embryonic) gene appears to be the closest of the β-like genes to the "ancestor" gene (if only the nonsynonymous substitutions in functional sites are considered). Almost negligible regularity $\beta^A > \beta^C > \gamma$ is observed for the other three genes (see Table 2).

As for the goat ε^{II} gene, it was noticed that it exceeds all other β-like genes both in the total number of substitutions and in almost any particular group of distances (see Table 2). Taking into account that this gene

1) significantly differs from the goat ε^I and human ε genes,
2) has accumulated large numbers of nonsynonymous substitutions in the "functional" sites (Table 2), and
3) is orthologous to the primate gene ($\psi\beta_1$) that was proved to be a pseudogene, it is reasonable to suggest that the goat ε^{II} gene is not an active one, but could be involved in some other processes, e.g. regulation of ontogenetic expression of the globins, as proposed by Goodman et al. [6] for the primate $\psi\beta_1$ gene.

Finally, the most significant regularity was found for the *X. laevis* globin genes [7]: the tadpole genes from both α- and β-clusters are approximately twice as close to the corresponding ancestors than the adult ones and it was the class of nonsynonymous substitutions in the functional sites that revealed this difference (see Table 3).

Table 2. Evolutionary distances between the globin genes of the goat and the reconstructed ancestral sequence

Gene	"Functional" sites			"Nonfunctional" sites			Total
	Synonymous	Nonsynonymous	Total	Synonymous	Nonsynonymous	Total	
ε^I	5	4	9	26	18	44	53
ε^{II}	13	14	27	32	23	55	82
γ	7	9	16	10	27	37	53
β^C	8	10	18	14	29	43	61
β^A	7	11	18	11	27	38	56

Table 3. Evolutionary distances globin genes of the clawed frog and the reconstructed ancestral sequences. The tadpole genes are designated as (*t*), and the adult ones as (*a*)

Gene	"Functional" sites			"Nonfunctional" sites			Total
	Synonymous	Nonsynonymous	Total	Synonymous	Nonsynonymous	Total	
$\alpha(t)$	8	8	16	20	34	54	70
$\alpha_1(a)$	21	19	40	26	42	68	108
$\alpha_2(a)$	23	19	42	29	43	72	113
$\beta_1(t)$	15	21	36	30	49	79	115
$\beta_2(t)$	17	20	37	32	49	81	118
$\beta_1(a)$	17	27	44	21	39	60	104
$\beta_2(a)$	17	28	45	21	39	60	105

Summing up, let us note that the effect expected by Zuckerkandl can be clearly seen when embryonic/"ancestor" and adult/"ancestor" distances are compared. It does not hold true when comparing fetal/"ancestor" and adult/"ancestor" distances. The latter conclusion is obviously in agreement with Zuckerkandl's idea: there were no embryonic-stage globins in his sample of amino acid sequences. There are good reasons to consider the fetal-stage globins (and the goat "preadult" globin) as the product of relatively recent gene duplications. Thus, the time span after the last duplication might have been insufficient to accumulate the differences in the degree of evolutionary conservatism of the fetal- and adult-stage globin genes.

It should be emphasized that when analysing phylogenetic relations in some other MFs [immunoglobulin genes of mammals [8], insect chorion protein genes [9], and even homeoboxes of some regulatory genes of *Drosophila melanogaster* responsibel for embryonic morphogenetic gradients, segmentation and differentiation of the segments (S. N. Rodin, unpublished)] we found a tendency resembling that described here for globin genes. For example, the order of duplication of immunoreceptor progenitor genes in the evolutionary past was in good agreement with the order of gene rearrangements and their expression in the course of B- and T-lymphocyte differentiation [8].

Discussion

"Relay-Race" Regime of Molecular Evolution

Any significant increase in the rate of substitution fixation in a particular gene from a multigene family could be explained in two ways. The first explanation implies that the pressure of stabilizing (negative) natural selection is lessened. The second possible cause of the same phenomenon might be the improvement in the gene function that is provided by positive natural selection. In the second case, the higher the rate of adaptive evolution, the larger the substitution load, i.e. Haldane's dilemma must be playing an important role in evolutionary periods of just this kind. These two possible reasons might appear to be combined in the case of globin gene family evolution [10–13]. Although gene multiplications seem to be quite an ordinary event in genome evolution, they far more often give rise to silent pseudogenes than to novel functional genes.

The above may imply that multigene family evolution occurs in this "relay-race" mode, i.e. at any moment, most probably only one gene within the same family is allowed to evolve in an adaptive manner [11].

In fact, the relay-race mode of molecular evolution may be considered as a general theoretical substantiation of a cascade-like pattern of switches in ex-

pression from one structural gene to another in the course of ontogenesis.

Regulation of Development and Anaboly

The majority of authors (see [14]) are unanimous in assuming that ontogenesis is regulated by a number of genes that are organized as a "Bickford fuse" or a "relay-race with a specified time of last participant arrival". This means that the expression of "the right gene in the right time and in the right cell" requires a chain of intermediate regulatory gene activations. The last participant of this relay-race must activate the target gene. This chain of activations must be characterized by strict adherence to the expression timetable. Each regulatory gene might be responsible for multiple gene activations. In turn, a group of regulatory genes is often controlled by a higher order regulatory gene. Thus, the scheme of gene interactions in ontogeny is undoubtedly a hierarchic one.

The mode of terminal addition of new stages (called anaboly by Severtsov [15]) appears to be the least dangerous mode of gaining ontogenetic complexity. The latter does not mean that "nonanabolic" evolutionary rearrangements of individual development are forbidden, but in reality they are likely to occur far more rarely than the anabolic ones.

There are well-studied examples where the prolonged activity of an earlier expressed gene compensated for a malfunction in its later expressed homologues (see [16]), i.e. the earlier expressed gene could be said to recapitulate the ancestral mode of expression. Notably, among all the reported cases of human globin gene malfunctions (thalassaemias) there are no examples of compensating embryonic gene damage by expression of fetal or adult globin genes. Thus, one can conclude that, for example, a normal activation of fetal globins takes place only provided that the embryonic gene was expressed normally etc. Thus, the structural globin genes are also organized into some analogue of the regulatory hierarchy and the later expressed genes are more open to evolutionary changes.

Recapitulation and Selective Strategies

The so-called "biogenetic law" of Haekkel was proved to hold true only in some cases and not in others (see [14]). However, one can explain (and maybe even predict) whether recapitulation will be found in any particular case if the following speculations are valid.

There are two main "poles" of natural selection that are recognized by ecologists [17]. The complexity of any ecological system is thought to be determined, on the one hand, by the quantity of free energy available and, on the other hand, by the stability of the environment.

An environment which is characterized by low probability of intensive disastrous fluctuations is usually most densely populated. Plant and animal communities in these conditions are known to form complex trophic chains that utilize free energy in the most efficient way. The intensive intra- and inter-specific competition that is observed in these cases favours the increase of organism complexity. Selection of this kind is called "K-selection" [17].

When the environment is unstable (large parts of populations are randomly eliminated) the individuals which have more offspring are most successful. This kind of selection is known as "r-selection". A prolonged period of r-selection may cause a drastic reduction in the morphologic and ontogenetic complexity.

It is quite reasonable to suggest that the anabolic complication of ontogenesis must be demonstrated by species evolving under pronounced K-type natural selection. On the other hand, it is unlikely that traces of a recent terminal addition of new stages will be found when typical r-strategy species are considered.

Of course, when real organisms are being dealt with, the picture might appear to be much more complex. First of all,

ancestors of almost any present-day animal surely underwent multiple successions of r- and K-selection. This means that what could be observed a posteriori is a complicated tangle of tendencies. Apart from that, there are a great number of species which could not be definitely classified according to the r/K scheme. Thus, the hypothesis suggested may be applied only to relatively "recent" spans of evolutionary time when the species observed are known to evolve under one kind of selection.

Summary and Conclusions

Multigene families (MF) represent the most promising level of genome organization when studying the molecular basis of both developmental and evolutionary processes. Haldane's cost of selection "allows" almost all MFs to increase their complexity in evolution in a relay-race manner. Each MF is in turn characterized by a strict ontogenetic order of expression of homologous structural genes. According to Zuckerkandl, if any earlier expressed gene resembles in structure the ancestor gene more than its later expressed homologue, this could be considered as a case of molecular recapitulation. We showed here that this phenomenon does occur invarious MFs when comparison is performed only for sites that are known to be involved in selectively important functional bonds. For all other sites, conditionally denoted nonfunctional or subneutral, this regularity is not valid. The dichotomic mode of switches in gene expression, unreciprocity of ontogenetic compensation of human globin gene malfunctions (adult by fetal but not reverse), allelic and isotypic exclusions in expression of immunoglobulin genes clusters are certainly associated with the molecular recapitulation phenomenon.

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Repeated Intragenome "Parasites" as a Factor in Molecular Coevolution

S. N. Rodin, Y. G. Matushkin, and J. S. Krushkal[1]

Introduction

Any genome, except for its presently neutral DNA (i.e. without coding sequence), comprises a perfect ensemble of functional genetic units, the range of these units having its roots in individual exons and being crowned by the most complicated supergene complexes. The whole ensemble is undoubtably a product of mutually adaptive molecular coevolution. Any ecosystem, in turn, is the result of concerted molecular evolution of the species making up the system. At present, when the sequencing of entire genomes is running at a phenomena rate, to construct a theory of molecular coevolution would be of utmost importance both for theoretical molecular biology and genetics and for evolutionary theory itself.

All specific forms of adaptive molecular coevolution may be subdivided into intra- and intergenome and into directed and nondirected processes [1].

It is typical of nondirected molecular coevolution that, whatever its mode, mutations occur and are fixed at a rate which, despite their apparent adaptive value, remains on average constant. This fact, however, is at variance with one of the keystone postulates of Kimura's neutralistic theory. Up until now, only nondirected processes of molecular coevolution have been proposed and studied in details: intergenomically, concerted fixations of mutant reception and absorption genes in bacteria and phages, respectively [2], different variants of coevolving antigens and antibodies [3], original interactions between natural selection and molecular drive in the coevolution of multiple promoter and enhancer regions in rDNA loci, on the one hand, and the RNA *PolI* gene, on the other [4], specific pairs of base substitutions compensating for each other to maintain the rRNA secondary structure [5], etc. All these cases of molecular co-evolution, both intra- and intergenome (in different parasite–host pairs), are in fact variations on a theme, i.e. coevolution. The question of whether coevolution could provide development of multigene systems ab simplecioribus ad complexiora is of especially profound interest.

Regarding the genes of the immune system, we have suggested that HIV-like viruses could be involved in coevolution of this sort [6].

Coevolutionarily Motivated Complication of Immune Multigene Families

Let us consider a hypothetical ancestral organism with a primitive, poorly differentiated immune system. Suppose the corresponding ancestral immune cells (prelymphocytes) change their state from L to T in the course of ontogenesis, where L and T are the immature and mature prelymphocytes, respectively. Suppose also that viruses (V) can only strike the L-cells, i.e. immature prelymphocytes. We then admit that the molecular-genetic system of immunity is simple enough for

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the virus to make use, by adsorption, of the very receptor of the L-cell that T-cells, in turn, use to identify and inactivate the virus. The adsorption of V on L leads to the formation of infected cells (denoted Z), from which, via lysis, the daughter viral particles come. The T/V binding, by contrast, leads to the elimination of the viruses. Accordingly, we can derive the following system of differential equations describing the dynamics:

$$\begin{aligned} \dot{L} &= [F(L) - \alpha L] - G(L,V) \\ \dot{V} &= [\omega\beta Z - G(L,V)] - Q(T,V) \\ \dot{Z} &= G(L,V) - \beta Z \\ \dot{T} &= \alpha L - kT \end{aligned} \qquad (1)$$

where α, ω, β and k are constants of the respective process rates.

The state of equilibrium $(\dot{L},0,0,\dot{T})$ in Eq. 1, where $\dot{T} = (a/k)\dot{L}$ and $\dot{L}$ is the root of equation $F(L) = aL$, is assumed to be health. This state is locally stable (which implies that the prelymphoid tissue is resistant to minor infections) if $Q'_V(\dot{T},0) > (W-1)G'_V(\dot{L},0)$ and unstable if $Q'_V(\dot{T},0) < (W-1)G'_V(\dot{L},0)$. In our model, an increase in the stability of the "healthy" state can be obtained by increasing the value of the term $Q'_V(\dot{T},0)$ and/or decreasing $G'_V(\dot{L},0)$. However, since $\dot{T} = (a/k)\dot{L}$, then a drop in $\dot{L}$ causes a drop in $\dot{T}$. To increase $\dot{T}$, it is necessary to increase $\dot{L}$; note that $\dot{T} < (a/k)\dot{L}$.

Therefore, there are two ways of increasing the resistance of the prelymphoid system to minor infections (in terms of a simplified model): first, by increasing the number of clones of those prelymphocytes that are specific to various antigen determinants; and secondly, by changing in the course of the prelymphocytes' maturation the avidity of the antigen specific receptor. Both ways are found in the immune systems of contemporary vertebrates.

The second way actually implies that no entirely identical receptor molecules can participate in either the absorption of viruses upon the target immunocytes or in the recognition and destroying of viruses (or their antigens), since to acquire homologous but not identical receptors, progressive divergence of the molecular genetic system of immunity is required.

However, what factor(s) could direct the evolutionary complication of all the other multigene families (MFs)? What if the role of intragenome "parasites", such as human *Alu* repeated sequences, retroviruses, and mobile genes of *Drosophila*, in the evolution of MFs is similar to that of HIV-like pathogens in the evolution of immune supergenes?

Intragenome Parasites and Genome: a Coevolutionary Aspect

We have studied [7] the processes of concerted variability which actually result from cooperation of such entities as, on the one hand, various mobile elements (a kind of "intragenome parasite", GP) and, on the other, the genome itself ("host").

Several systems of differential equations similar to Eq. 1 have been built in order to analyse the following situations:

1) the GP is insertable in the vacant sites only, its free state (not in the "host" but still in the cell) not being durable;
2) the GP is insertable in the vacant sites only, its free state being durable;
3) the GP is insertable in both vacant and occupied sites („molecular memory"), its free state not being durable (mammalian *Alu*-like repeats taken as a prototype);
4) the GP is insertable in both vacant and occupied sites and is able to exist "on its own" (retroviruses taken as a prototype).

We then admitted that the genome is tolerant to the "selfish" proliferation of GP until the share of the occupied sites exceeds the limit $1/K$. Our analysis revealed that the coevolutionary complication of GP – from the simplest, which is only able to insert in vacant sites, through the ongoing acquirement of terminal re-

peats ("molecular memory"), to perfectly integrated complexes with an extragenomial life style – is accompanied by change in the selective coevolutionary restrictions on genome size: upper limit–no limit–lower limit. Thus, mobile elements may be regarded as an inner factor inducing progressive, coevolutionarily motivated complication of genomes, including multiplication of coding regions.

Our models are based on the assumption that there is always a superior selective force (from the "host" side) that restricts the number of GPs and influences the pattern of GP distribution in the host genome. However, the following question arises here: Are there any inferior restrictions directly related to the GP structure as such? We go on to show below that *Alu*-like repeated sequences, even with extremely simple structures, could have such restrictions.

CpG-Rich Promoters as an Inner Constraint on Amplification of *Alu*-Like Sequences

With the aid of the package of applied programs VOSTORG [8], designed in our laboratory, 83 *Alu* repeats (60 human included) from seven species of primates and 13 *Alu*-like *B*1 repeats from three rodent species were subjected to phylogenetic analysis, in particular, for mutations fixed in RNA polymerase III promoters (Fig. 1).

Using the method of diagnostic positions [9] enabled us to divide all 60 human *Alu* sequences into three different classes (Fig. 2) corresponding to *J*, *Sa* and *Sb* (identification of the *Sc* class was certain) according to Britten et al. [9]. The topologies of the phylogenetic tree constructed on the complete sample of *Alu* sequences and of the tree derived from the comparison of the consensus for all classes revealed a good agreement with the order of appearance of these classes in the course of evolution (Fig. 3): progenitor (*7SL* RNA gene) $\rightarrow J \rightarrow Sa \rightarrow Sc(?) \rightarrow Sb$.

As is known, the CpG positions evolve on average 10.5 times as much as other positions of *Alu* repeats, which is due to methylation of the cytosines. In particular, the A (enhancing) and B (initiating) boxes of promoters contribute considerably to the concentration of CpGs (Fig. 1). We tried to build a dichotomic dendrogram from CpG positions of the promoter alone but failed. This could be an argument in favour of the "burst"-like formation of the *Alu* classes.

The most intriguing feature of the *Alu* evolutionary tree (Fig. 3c) is the almost absolute lack of mutations in CpG dinucleotides of the promoter region at the

```
          134     139          150
Right   AATTAGCcgGGcgTGGTGGcgcgcCCTGTAATCCCAGCTACTcgGGAGGCTGAGGCAGGA
Left    ----GGCcgGGcgcgGTGGCTCAcgCCTGTAATCCCAGCACTTTGGGAGGCcgAGGcgGGc
              1  4            15

                                210                                 251
Right   GAATcgCTTGAACCcgGGAGGcgGAGGTTGCAGTGAGCcgAGATcgcgCCACTGCACTCCA
Left    gGATCACCTGAGGTCAGGAGTTcgAGA----------------------------------CCA
                                75                                  86

                                                    284
Right   GCCTGGGcgACAGAGcgAGACTCcgTCTCAAAAAAAA
Left    GCCTGGCCAACATGGTGAAACCCcgTCTCTACTAAAA
                                                    120
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Fig. 1. Consensus of human *Alu* repeats [9] with the left and right halves of the sequences aligned. A and B boxes of the promoter region are *underlined*. The CpG dinucleotides are in *lower case letters*. The right promoter is likely to be inactive due to the relatively long inserted sequence

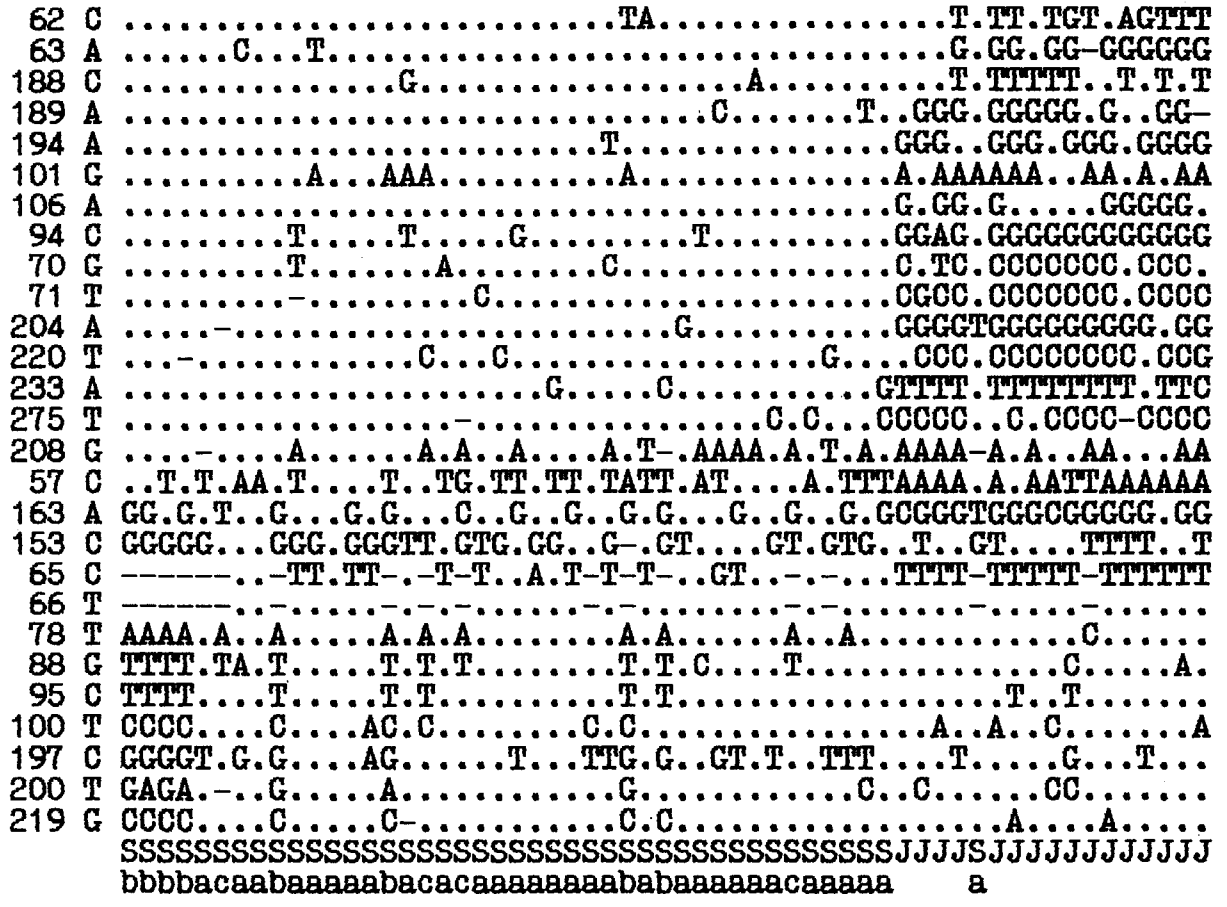

Fig. 2. Variability in the diagnostic positions of 60 human *Alu* repeats. On the *left*, the consensus nucleotides are shown. All the *Alu* repeats rearranged in accordance with a divergence from the consensus. *Letters* at the *bottom* indicate the class to which each *Alu* repeat

upper branches of the tree (when the *Alu* subfamilies were being newly formed), in spite of their extreme mutability. Non-CpG positions show the same regularity (Fig. 3e). It should be noted that mutations in the CpG sites of the "quasi-neutral" part of the *Alu* repeats appear not to be in deficit at that period (Fig. 3d). Thus, a lion's share of mutations in the promoter CpG sites are concentrated in the lower branches of the divergency tree just after the class formation process is over (not shown).

This means that the promoter region of *Alu* repeat progenitors were under very strong negative natural selection pressure until the amplification process started. Moreover, the topology of the dichotomic branching within each class appears to be unstable. Thus, during evolution, first some changes in diagnostic positions (CpG sites not belonging to them) had to be accumulated; secondly, a current class of *Alu* sequences branched off the main stem of the tree; and, finally, mutations at CpG positions predominantly within the promoter occurred most rapidly. The superfamily of *B*1 repeats of rodents, closely related to *Alu*, shows similar regularities.

The results obtained allow us to propose a model where promoter sites play a role of profound importance both in intragenome amplification of the progenitor *Alu* sequence and in the divergence of individual members of the corresponding subfamilies. The model is supposed to explain the limited sizes of a subfamily with the subsequent acquisition of mutational defects in CpG positions of promoters and hence the inevitable slowdown of amplification. As a result, only those of *7SL* RNA-like sequences which have retained the promoters could

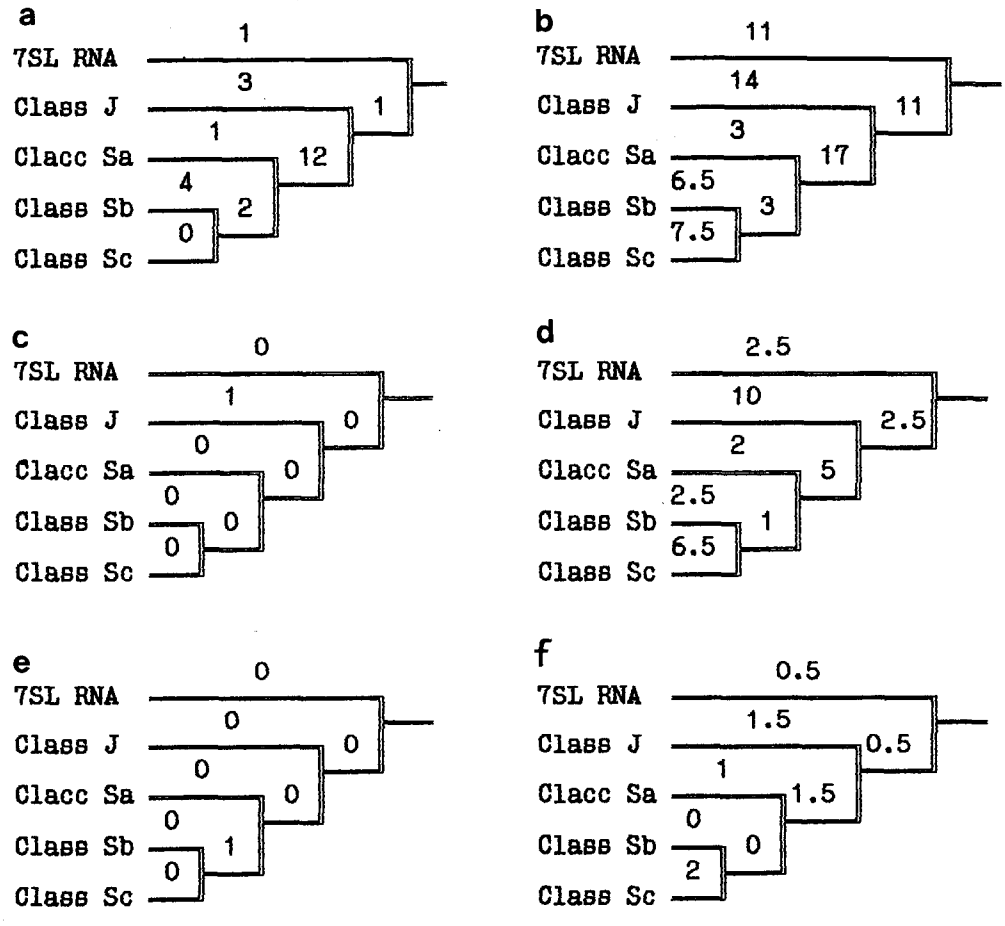

Fig. 3a–f. Phylogeny of the consensus sequences reconstructed for the main *Alu* classes with a human *7SL* RNA sequence as a repeat. Numbers of mutations fixed in various types of positions are shown: **a** in the 23 diagnostic non-CpG positions; **b** in all positions without central and terminal oligo-A parts; **c** in 8 CpG positions in A and B boxes of the left (active) promoter for the host RNA polymerase II; **d** in 38 non-promoter CpG positions; **e** in 16 non-CpG positions in the left promoter; **f** in 6 CpG positions from sites in the right (inactive) domain homologous to A and B boxes

become the progenitor for the following subfamily of *Alu* repeats to amplify and evolve in an active mode.

Each *Alu* repeat is well known to consist of two homologous halves (Fig. 1). Usually, only the leftmost domain is active for amplification by reverse transcription [10]. Figure 3f shows that, in contrast to the single CpG mutation in the leftmost promoter, the rightmost one accepted seven such mutations in CpG dinucleotides at the top part of the tree just when the *Alu* subfamilies were in the making. This is an additional, rather convincing, argument in favour of the importance of the promoter CpG sites, in particular those located in A and B boxes.

Thus, the "selfish" intragenome propagation of any progenitor "pregnant" with a recurrent *Alu* subfamily is destined to slow down and, eventually, to come to a standstill because as any individual *Alu* promoter rapidly accumulates more and more defects, predominantly due to the increased mutability of CpG sites, the host reverse transcriptase becomes less able to recognize the promoter.

This is not so with HIV-like retroviruses. They show unusually high variability, generated by viral reverse transcriptase, the most error-prone of the various RNA and DNA polymerases [11]. In contrast to the short *Alu* repeating unit, the HIV reverse transcriptase is encoded by its own *Pol* gene. It produces

extremely frequent mutations in all regions of the viral genome, including in its own gene. Therefore, there is a good chance for promoter sites and reverse transcriptase to be involved in prolonged steady coevolution, based on the selection of pairs of substitutions compensating for each other. It is an original case of a strikingly rapid intragenome coevolution which should be adaptive but is apparently not directed.

Summary and Conclusions

"Parasitic" DNA may be regarded as a rather active partner in different coevolutionary processes. The basic stages of the processes are likely in most cases to be as follows: parasitism → tolerance → → symbiosis. There are interior and exterior coevolutionary factors complicating molecular-genetic systems within a supersystem "mobile elements-genome". For example, the data presented above indicate clearly that the relatively high concentration of CpG sites in the *Alu* promoter looks prudent as regards the needs of the "parasite" as well as those of the host genome. We consider "prudence" of this kind to be most likely a product of large-scale molecular coevolution.

As to HIV-like retroviruses, they could be simultaneously involved in three different regimes of molecular coevolution:

1) at a level of the parasitic genome as such;
2) as a typical intragenome parasite inserted in the host genome inducing complication in multigenic system (like *Alu*);
3) as a typical intracellular parasite in an "active", infectious state stimulating complication in the immune multigene families.

Evidently, it is only the steadiness of the first coevolutionary process (with "no wheels, no sails") provides for a possible role of HIV-like parasites as a selective factor provoking coevolutionary complication of host genomes.

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Frozen Temporal Pattern in Growing Systems

J. Schwietering [1] and P. J. Plath [2]

Introduction

The coloured patterns of seashells demonstrate great variety. Fascinated by the complexity of the patterns, we were astonished at the similarities between then and the patterns we obtained when trying to model the heterogeneously catalysed oxidation of carbon monoxide.

H. Meinhardt has modelled many shell patterns using a set of coupled one-dimensional differential equations [1–3], whereas our cellular automaton model for the catalysis is based on a discrete description of the process of pattern formation. We have coupled linearly a large set of reactors, characterized by their state $x(t)$ and their phase $p(t)$. The coincidence of our results with the shell patterns encouraged us to apply our model to the growth patterns of seashells as well.

Shells grow at their borders [2, 4], and so Meinhardt worked with one-dimensional differential equations. Instead of using a one-dimensional continuum as he does, we take a one-dimensional cellular automation [5–8], the cells of which now represent biological cells in the border of the shells. The crucial point is that we assume the creation of a cell in the new front at time $(t+1)$ to depend on the current situation of its mother cell in the actual front at time t and the situation of the cells neighbouring the mother cell at time t. The temporal sequence of states of the one-dimensional automaton thus represents the development of the moving front in the growing shells. The pattern of the shell is nothing other than the frozen set of all temporal states of the front which have been created during the growth of the system.

The Model

Let us assume that the incorporation of pigments in a new cell i (daughter cell) at time $(t+1)$ which is born from its mother cell by cell division depends upon the concentration $x(i,t)$ of the reactants (prepigments) in its mother and in both of her neighbouring cells $(i-1)$ and $(i+1)$, which are "aunt" cells to the daughter cells.

Moreover, it is reasonable to assume that each cell i can exist in at least two different states of activity $p(i,t) \in \{0,1\}$ at time t. The activity of the mother cell influences the amount of prepigment $x(i,t+1)$ which the daughter cell inherits from her mother. On the other hand, the daughter's activity $p(i,t+1)$ at time $(t+1)$ is determined by the concentration of the prepigments of her mother $x(i,t)$ and her aunts $x(i-1,t)$ and $x(i+1,t)$ as well as by the activity $p(i,t)$ of her mother.

Therefore, the situation $z(i,t)$ of a cell i at time t is characterized by a vector

$$z(i,t) = (i,t,p,x) = \begin{pmatrix} x(i,t) \\ p(i,t) \end{pmatrix} \qquad (1)$$

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

We can formulate a transformation rule T, which determines the situation $z(i,t+1)$ of the daughter cell i at time $(t+1)$:

$$T: z(i,t) \rightarrow z(i,t+1)$$

$$z(i,t+1) = \begin{pmatrix} x(i,t+1) \\ p(i,t+1) \end{pmatrix} = \begin{pmatrix} f\{p(i,t),\ x(i,t)+x(i-1,t)+x(i+1,t)\} \\ g\{p(i,t),\ x(i,t)+x(i-1.t)+x(i+1,t)\} \end{pmatrix} \quad (2)$$

This transformation T (Eq. 2) is performed at the same time t for all cells of the automaton.

f and g are discrete functions which can be represented by $2 \times k$ matrices:

$$\begin{pmatrix} f(0,0) & f(0,1) & f(0,2) \ldots . f(0,k) \\ f(1,0) & f(1,1) & f(1,2) \ldots f(1,k) \end{pmatrix} \quad (3)$$

with $f(p,m) \in X$; $m = 0, 1, 2, \ldots, k$; $p \in \{0,1\}$, where X is the set of the possible numbers (concentration) of the prepigments $X = \{0, 1, 2, 3, ..\}$ and k is the largest number of prepigments which can be reached via the addition of the number of prepigments of the cell i and its neighbouring cells $(i-1)$ and $(i+1)$ at time t. The function g is given by:

$$\begin{pmatrix} g(0,0) & g(0,1) & g(0,2) \ldots g(0,k) \\ g(1,0) & g(1,1) & g(1,2) \ldots g(1,k) \end{pmatrix} \quad (4)$$

with $g(p,m) \in P$, where P is the set of possible activities of a cell: $P = \{0,1\}$. Table 1 shows an example for a rule which will be used later on.

To obtain a temporal pattern one has to introduce some special cells into the starting automaton at time $t = 0$ whose situation differs from that of all others. At least one cell j should have a small number of prepigments $x(j,0) > 0$, while the number of prepigments should be zero in all the other cells. However, all cells i of the automaton may have the same high activity $p(i,0) = 0$.

For colouring, the prepigments have to be transformed into the pigments. This process may depend upon the activity and the number of prepigments in the cells. Even the same patterns can be coloured differently, accentuating amounts of special prepigments or the activity of the cells, or only a different way of transforming the situation of a cell into its pigment colour.

In this way, a large variety of coloured patterns can be produced, some of which may resemble the observed seashell patterns.

Table 1. Vector automaton: transformation rule (Eg. 2), 1/6–8/5 rule

The function $f(p(i,t), x_s(i,t))$														
	$x_s(i,t)$	0	1	2	3	4	5	6	7	8	9	10	11	12	13 ...
$p(i,t)=0$	$x(i,t+1)$	0	1	1	2	2	2	3	3	3	4	4	4	5	5 ...
$p(i,t)=1$	$x(i,t+1)$	0	0	0	0	0	0	1	1	1	2	2	2	3	3 ...
The function $g(p(i,t), x_s(i,t)$														
	$x_s(i,t)$	0	1	2	3	4	5	6	7	8	9	10	11	12	13 ...
$p(i,t)=0$	$p(i,t+1)$	0	0	0	0	0	0	0	0	1	1	1	1	1	1 ...
$p(i,t)=1$	$p(i,t+1)$	0	0	0	0	0	1	1	1	1	1	1	1	1	1 ...

$x_s(i,t) = x(i-1,t) + x(i,t) + x(i+1,t)$; this special rule is called the 1/6–8/5 rule, where 1 and 6 are the number of zero values in the first and second row of the f matrix respectively, and 8 and 5 mean the corresponding number of zero values in the g matrix.

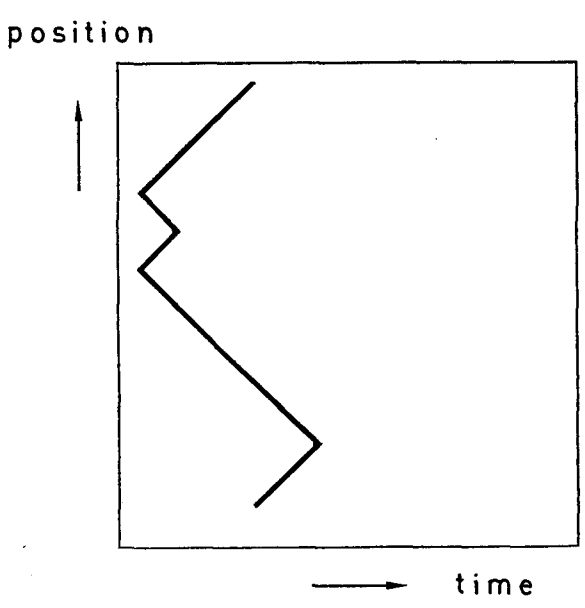

Fig. 1. The activity waves which travel along the one-dimensional automaton (1/6–8/4 rule) like a chemical wave. The automaton is circularly closed. Two special points in the starting automaton (*left side*) have been used

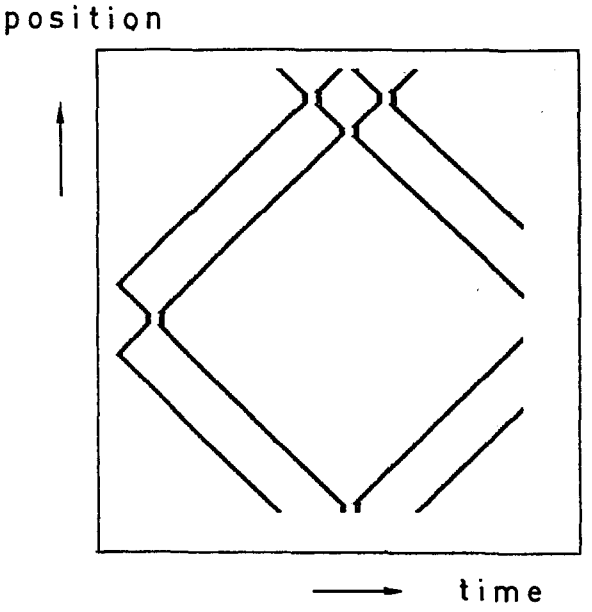

Fig. 2. The activity waves which travel along the one-dimensional automaton (1/6–6/5 rule) like solitons. The automaton is circularily closed. Two starting points (*left side*) have been used

Resulting Patterns

The possible patterns of the simple one-dimensional automata, the states of the cells of which are scalars, have been classified by Wolfram [5]. Apart from his well-known fractal patterns, the one-dimensional vector automaton model presented here exhibits new patterns like chemical waves (also named autowaves) [9, 10] and solitons [11, 12].

Such waves travel along the one-dimensional automaton and, if they behave like chemical waves, may annihilate each other (Fig. 1). They also may cross each other with a phase shift if they are travelling like solitons (Fig. 2). But there are also various waves which exhibit quite different behaviour. For instance, one can observe oscillators periodically creating waves which travel along the

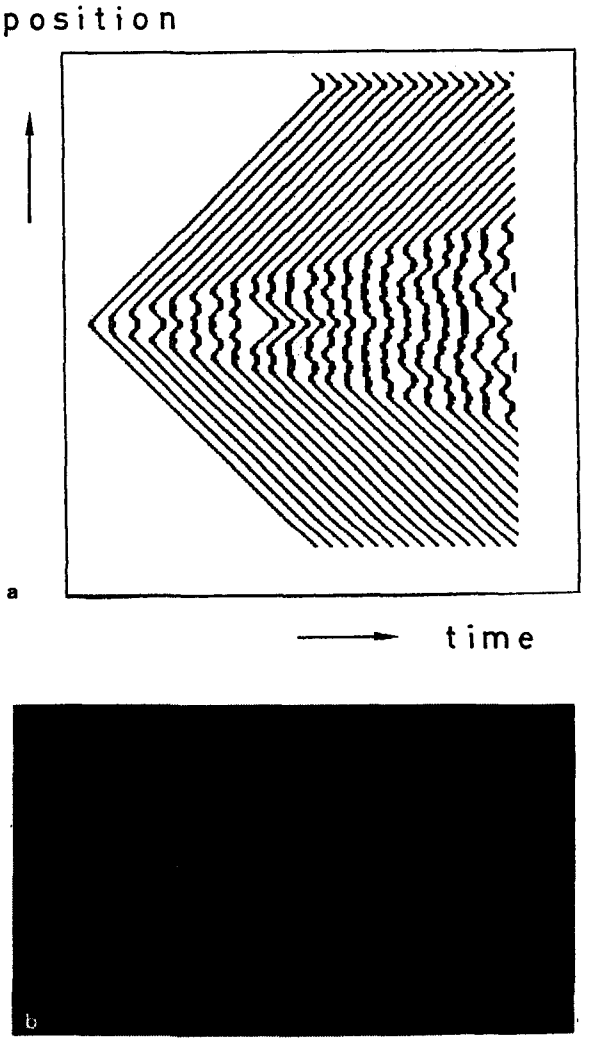

Fig. 3. **a** An automaton with an oscillator (1/6–8/5 rule) which produces a sequence of wave fronts, between which a fractal core is created. **b** A feather of a bird exhibiting a pattern which resembles the pattern of the fractal core in **a**

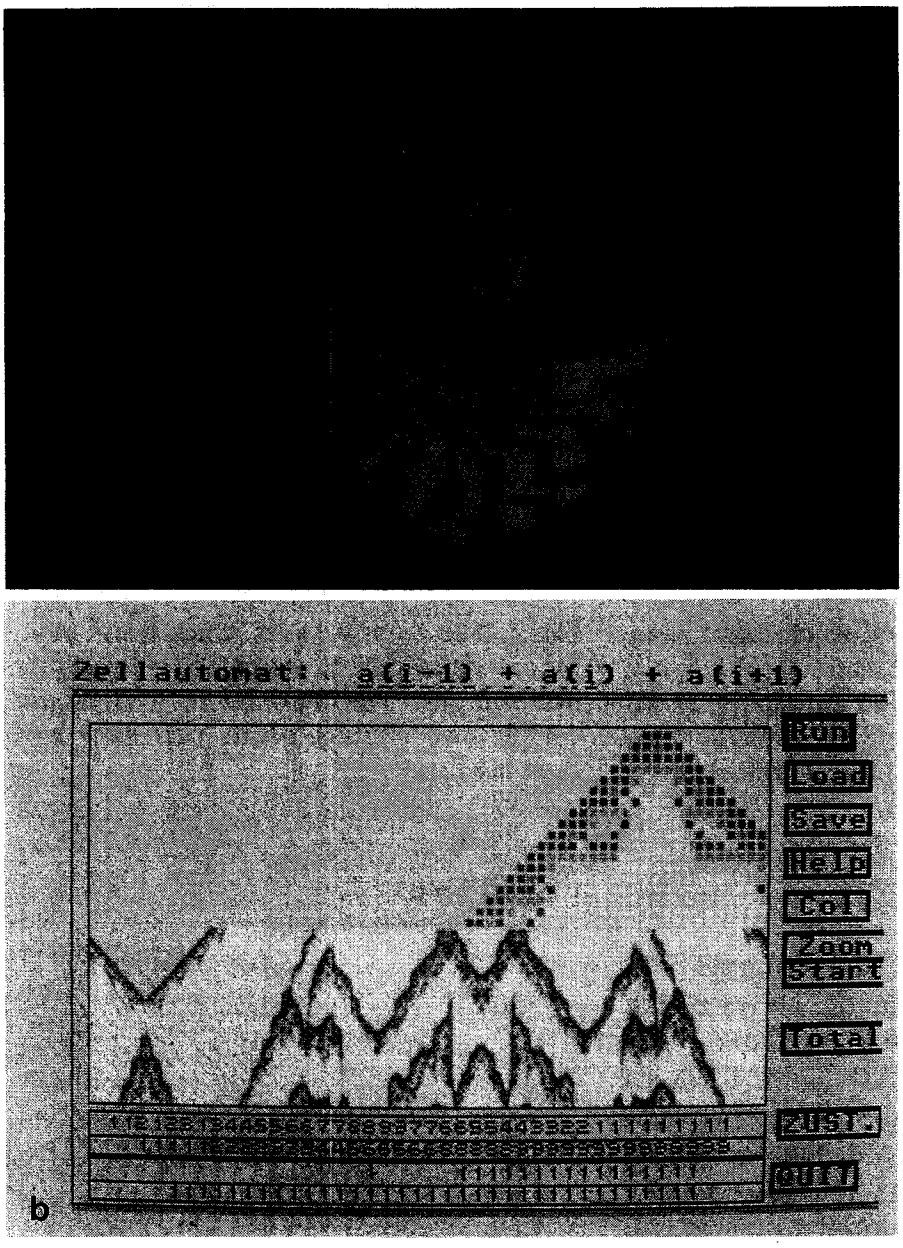

Fig. 4. **a** The pigmentation of a top shell of the family Trochidae. **b** Coloured pattern of the number of prepigments produced by the one-dimensional vector automaton. The rule is shown at the *bottom*

automaton showing interference with each other.

There is another very interesting behaviour (see Fig. 3 a): a wave consisting of two dispersing one-dimensional excited states periodically creates an excited block in between. The end points of the blocks become the starting points of two new waves, which will behave like the original wave. By this means, the wave is creating a fractal core inside itself. Figure 3 b shows the pattern of a feather which greatly resembles this fractal core wave.

Starting not just with one special cell but with a few of them, which may be distributed randomly, a universe of different and complex patterns can be produced. Among these patterns there are classes which strongly resemble the frozen pattern of the seashells (see Fig. 4).

What is really astonishing is the major role of the fractals among the pattern in the seashells (see Fig. 5). For example, the *Cymbiolacca* shell exhibits a pattern of brown triangles of different sizes. The way the triangles are interlocked is typical for patterns of penetrating fractal Sier-

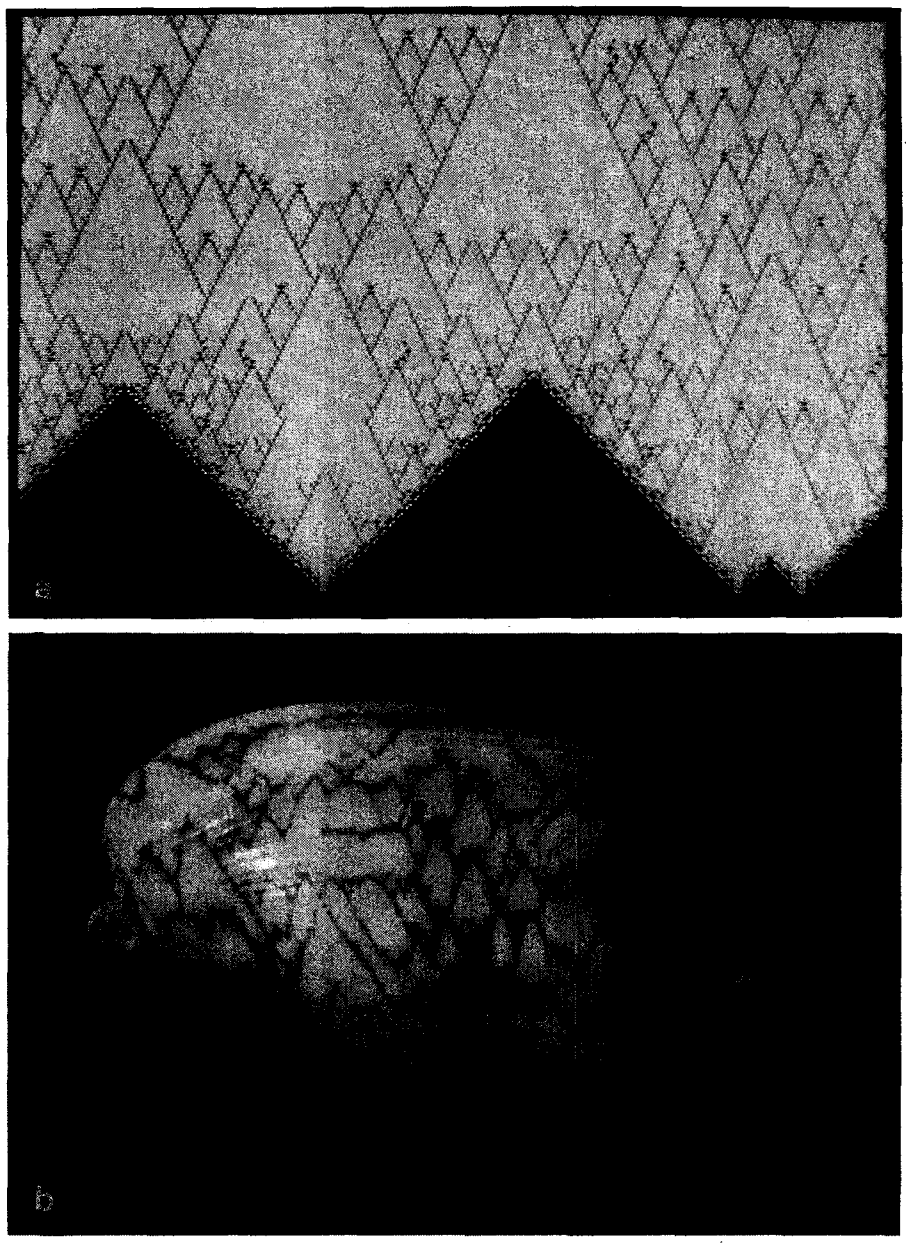

Fig. 5. **a** A *Cymbiolacca* shell (family Volutidae) with a fractal pigment pattern. **b** An automaton, the pattern of which resembles the main elements of *Cymbiolacca* pigmentation

pinsky gaskets [13]. Sometimes one can observe showers of small triangles, while on other positions the sides of large triangles run through pale yellow-grey parts of the shell. A book of seashell patterns look like a zoo of fractal patterns and their combinations, which can be observed especially on cone shells [14, 15]. Another typical fractal pattern can be seen in the shell of *Conus princeps* (Fig. 6). Simulating this pattern with our automaton machine, it can be classified by the type of interpenetrating core waves which have been mentioned above.

Concluding Remarks

The regularities and the irregularities in the pattern of the shells can be reproduced by the automaton model if fractal patterns interact starting from different positions at time $t = 0$. What is so fascinating about the fractal patterns? Fractals are strongly related to the occurrence of deterministic chaos [16], which does not mean that one loses all regularities but only the simple symmetries such as translational or rotational symmetry. If all these symmetries break down in a

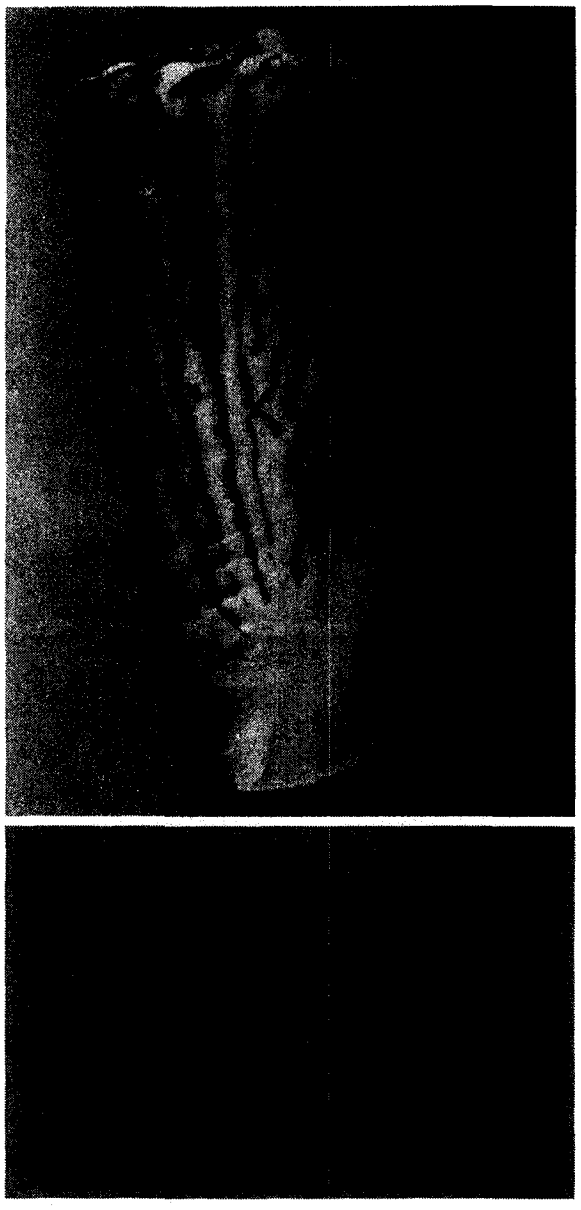

Fig. 6. a *Conus princeps* (Linnaeus 1758), Sinaloa, Mexico. **b** The pattern of an automaton which resembles the main elements of the pigmentation of the *Conus princeps*

state of chaos, one very characteristic symmetry survives: the dilatation symmetry, which is mostly disregarded. In the pattern of the seashells it is precisely this dilatation symmetry which plays the major role, since all the other symmetries vanished.

The growth mechanism of the pattern proposed above is based on a strictly ordered one-dimensional arrangement of cells. This is sensible in the case of the seashells but one can also develop the method for two- or three-dimensional processes to explain other phenomena in pure chemical or living systems. Even reaction in fluids can be modelled in such a way, if the cells stay together for a suitable period of time [17–19]. It would be of great interest to look for the spreading of the patterns of excited cells, even in flowing systems.

Acknowledgement. The photograph in Fig. 7a is reproduced with kind permission of T.F.H. Publication Inc. Ltd., The British Crown Colony Hong Kong, to whom we are indebted.

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On Some Concepts in Modeling Nonlinear Phenomena

U. Krause [1]

Introduction

Applying mathematics to problems in biology and medicine has a long history dating, at least, to the work of Fibonacci in the thirteenth century. In the twentieth century mathematical ideas have had a profound impact on population biology, genetics, epidemiology, and neurophysiology. However, in many ways, mathematical biology is still in its infancy. In such fields as molecular biology, cell biology, immunology, and certain branches of physiology, mathematics is just beginning to be recognized as a useful tool [4].

Many exciting applications of mathematics to the life sciences are today concerned with nonlinear phenomena. Very often the relationship between variables is not linear by its very nature but it has to be treated linearly or by linear approximation because of the lack of mathematical tools handling the nonlinearity directly. Consider, for example, the, in the life sciences ubiquitous, phenomenon of growing (or shrinking) populations (human beings, animals, plants, bacteria, cells, etc.). For carrying out the necessary mathematical analysis, a common idealization then is to assume a constant rate of growth. This is in contrast to the real dependence of the growth rate on the level of population, due to population pressure like crowding, a bounded environment, food restrictions, etc. (see later sections for a more detailed discussion).

During the last two decades there has been great success in developing new mathematical concepts and tools to cope with nonlinear phenomena (see the references given at the end of this paper; for applications in the life sciences, see especially [2, 4, 9, 11, 14, 17]). In particular, techniques were developed dealing with the by now famous concepts of "chaos", "fractals", and "cellular automaton" which have already entered many disciplines. However, these new developments have also shown that it may be extremely difficult to treat even the simplest kinds of nonlinearity analytically in a rigorous manner. Hence, another idea may be useful, which also arose in recent years and by which nonlinearities can be treated analytically if they come with certain properties of positivity, like positive and increasing levels of population. In contrast to what is possible for discrete nonlinear dynamic systems in general, the concept of positive dynamic systems also allows nonlinearities to be treated analytically in higher dimensions, i.e., for many variables.

The aim of this contribution is only to offer a first impression for those who are not yet acquainted with the concepts mentioned, but who want to know roughly what they are about. I do not proceed in a mathematical manner by giving definitions and theorems, but discuss simple examples and illustrate the underlying ideas. The interested reader who wants to know more is referred to the references given at the end of the paper.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

Can the Flap of a Butterfly's Wings Stir Up a Tornado in Texas?

Consider a population in a fixed environment, e.g., bacteria in a Petri dish. Let P_n be the number of (female) individuals of the population in time period $n = 0, 1, ..,$ and let P be the maximum number of individuals which can be carried by the given environment. The *growth factor* (= growth rate + 1) in period n is by definition $w_n = P_{n+1}/P_n$. If the growth factor equals some constant w, then only the following three dynamic modes are possible: Population increases exponentially (for $w > 1$) or decreases exponentially (for $w < 1$) or stays constant (for $w = 1$). Because of population pressure, a more realistic case, however, is a growth factor which diminishes if the population approaches its maximum level. Hence a more realistic manner of modeling the growth of a population would be given by the reproduction curve

$$P_{n+1} = w_n P_n = a\left(1 - \frac{P_n}{P}\right) P_n$$

or letting $x_n = P_n/P$ the relative population level, by

$$x_{n+1} = a x_n (1 - x_n)$$

where a is some constant with $0 \leqq a \leqq 4$ (another possibility of modeling the growth will be discussed in "The Method of Modeling Does Matter"). The last relation may be written also $x_{n+1} = f(x_n)$ with $f(x) = ax(1 - x)$ being the *logistic curve*. The dynamics of this extremely simple model, i.e., the time evolution x_1, $x_2, x_3, \ldots$ of the relative population level starting from x_0, turns out to be extremely difficult. The dynamics depends highly on the value of the parameter a. For some values (e.g., $a = 1$) the dynamics is simple, but for others ($a > 3.57...$) the dynamics looks rather chaotic [see 5, 11, 12, 16]. A first impression of the dynamic complexity can be obtained by applying the technique of *graphic iteration* as shown in Fig. 1.

Although there exists a unique equilibrium (relative) population level x^* different from 0, it may happen that the

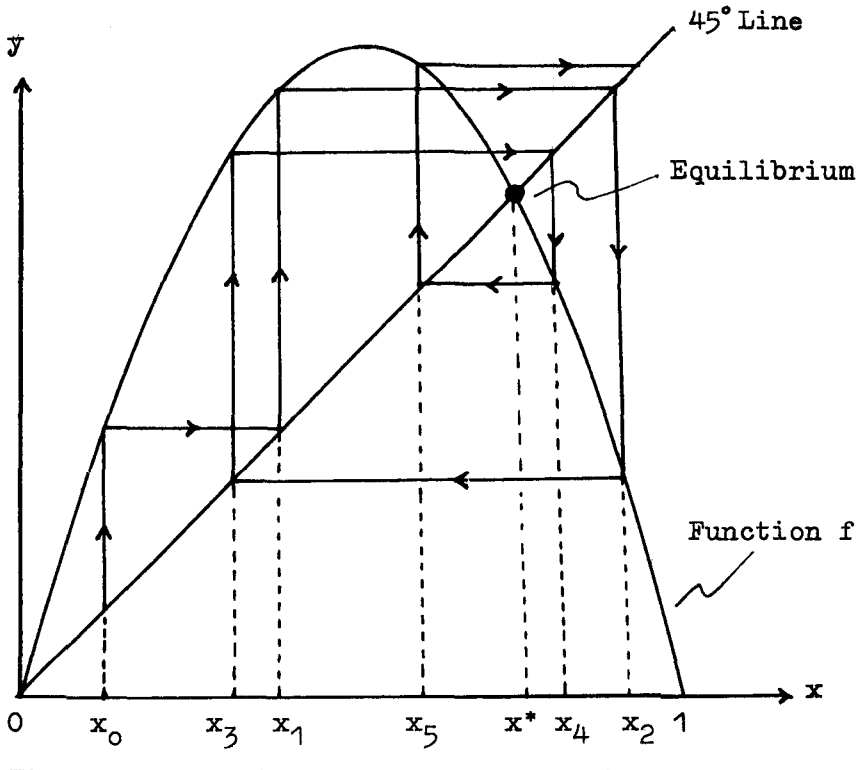

Fig. 1. Complex behavior shown by graphic iteration

successive levels x_1, x_2, x_3,... do not approach this equilibrium, but show a very irregular behavior with respect to it. Beside the parameter a the dynamic behavior depends very sensitively on the initial (relative) population x_0. This is a characteristic feature (but not the only one) of *chaotic dynamics* or (deterministic) *chaos*. More precisely, one speaks of *sensitive dependence on initial conditions* with respect to a dynamic system if a small change in the initial condition x_0 may cause large differences in the course of time. This is the issue addressed by the famous quote of the meteorologist Edward Lorenz given in this sections title. The bad implications for predicting the behavior of a chaotic dynamic system are at hand. What is surprising is that such a simple and completely determined mechanism as the logistic curve may create a behavior which looks very much random. A long time ago, the logistic curve for $a = 4$ was used to generate random numbers [19].

The Fractal Point Of View

Nature presents itself rather differently from what mathematicians usually depict in geometry. Leaves, clouds, coasts, lungs, mountains, radiolaria, etc. are forms too complicated and too fantastic for describing them in terms of Euclidean geometry [3, 10]. Nevertheless, coping with these natural objects the scientist has to find some way to measure those objects. The difficulties of measuring natural forms in common terms have been pointed out by Benoit Mandelbrot (1967) in an article entitled "How long is the coast of Britain?" [cf. 10]. For measuring the true length one has to make the unit of measurement E smaller and smaller and then to take the limit case of all the lengths obtained. This does work even for curved objects when they are smooth enough but it does not work for "fractional" objects or *fractals* as are they named by Mandelbrot (for fractals see [1, 10, 12, 13, 15, 17]). Measuring the fractional coast of Britain by making the unit of measurement smaller does increase the length obtained to infinity, as is indicated in Fig. 2.

In the article mentioned, Mandelbrot refers to the British scientist Lewis Fry Richardson who had already, in 1961, analyzed measurements made empirically for various coasts. Thereby he detected a magnitude not for the length of the coast but for the "roughness" of the coast, which was then called *fractal dimension* by Mandelbrot. More precisely, the fractal dimension measures (in the above example) how much the logarithm of the length L increases compared to an increase in the logarithm of the inverse unit of measurement $1/E$. To put it in a formula, the dependence of L on E is given by the *power law:*

$$L = c\,E^{1-d}$$

where d is the fractal dimension and c is some constant. The fractal dimensions calculated for various coasts are strictly greater than 1 (which is the dimension of a straight line) and strictly smaller than 2 (which is the dimension of a plane). Today, the concepts of a fractal and of a fractal dimension are used to measure surfaces like those of lungs, blood vessel systems, or of materials in chemical reactions. There are several possibilities in giving a precise definition of a fractal. A fractal may be defined by the fact that the fractal dimension does not coincide with the dimension in the usual sense or it may also be defined by its self-similarity. (In fact, several types of fractals have to be distinguished.) Another possibility which, moreover, is very practical in constructing fractals using the computer is the *chaos game* invented by Barnsley [1] which uses a random process as in Fig. 3 a, b.

Draw a (equilateral) triangle in the plane with vertices 1, 2, 3 as shown in Fig. 3 a and mark an arbitrary starting point in the plane. Throw a dice which is assumed to show the numbers 1, 2, 3 only because any two opposite faces of the dice are labeled with the same number. If the

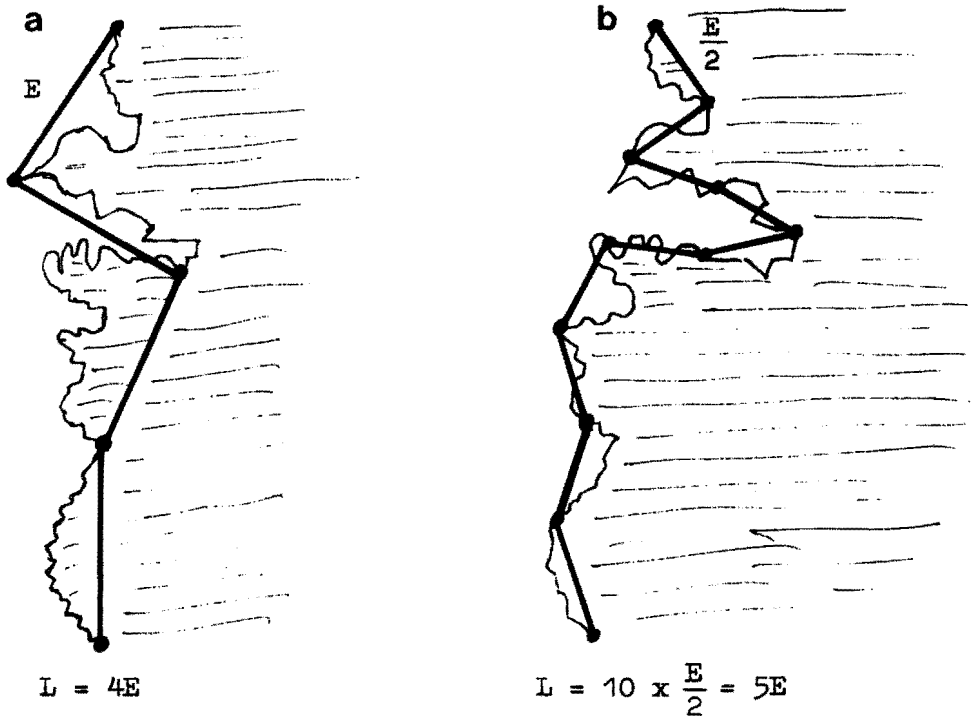

Fig. 2a,b. Length of a coast

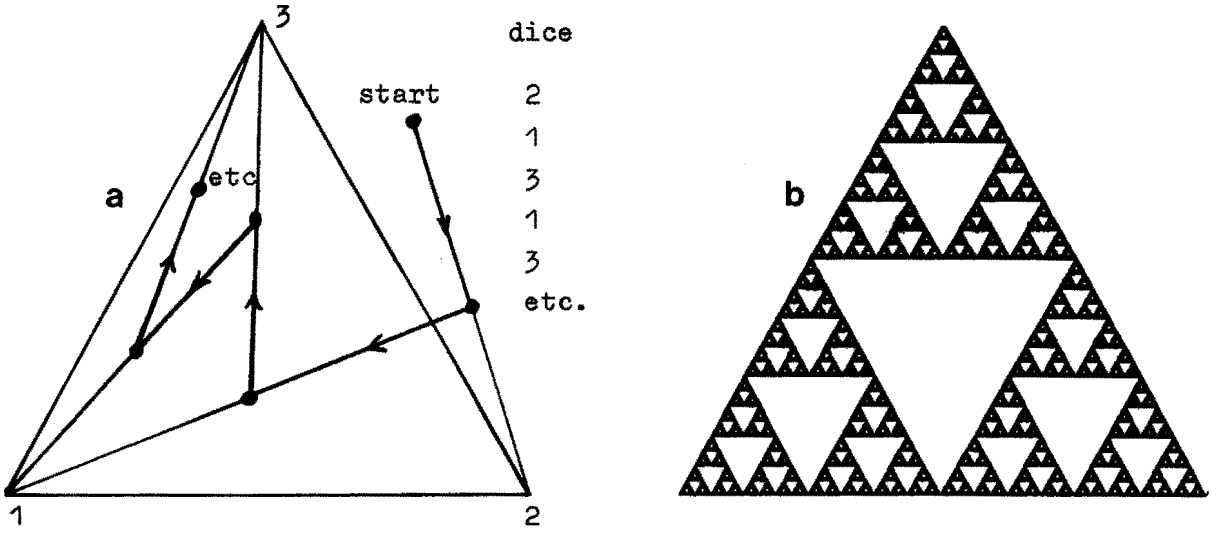

Fig. 3. a The chaos game. **b** The Sierpinski triangle

dice shows number n, then draw a line connecting the starting point with vertex n and obtain a new point by marking the middle point of it. Throw the dice again. If the dice shows number m, connect the new point obtained with vertex m by a line and mark on it the middle point as the new point. This process can be continued. What will be the figure formed by all the marked (middle) points? As it turns out, all these points arrange after a while on a classical fractal known for a long time as the Sierpinski triangle, shown in Fig. 3 b. The process described may also be considered as a dynamic system with the Sierpinski triangle as attractor, that is, the motion of the system is finally attracted to the Sierpinski triangle. Fractals appear as attractors of nonlinear dynamic systems. (For the connection between fractals and dynamic systems as well as for an astonishing gallery of

beautiful fractals, see [12, 13]. The difficult question of how fractal attractors may be found from a statistical analysis of the behavior of a nonlinear system is nicely dealt with in [15].) There is still another possibility, to construct fractals by using cellular automata. For cellular automata, which are interesting in themselves and bear beautiful connections with the life sciences (e.g., Conway's game of life), see [2, 9, 17] and the contribution by Peter Plath in this volume.

The Method of Modeling Does Matter

As seen, even simple nonlinearities may lead to chaos. But it would be wrong to say that nonlinearities necessarily imply chaos and fractals. There exist many nonlinear dynamic systems exhibiting rather regular behavior. It has been recognized that nonlinearities become tame if the system under consideration possesses, in addition, certain properties of positivity [cf. 6–8, 18]. Consider again the example of population growth underlying pressure discussed in "Can the Flap of a Butterfly's Wings Stir Up a Tornado in Texas?". The realistic picture of a growth factor which diminishes if population approaches the maximum level P need not necessarily be modeled by the logistic curve as it was in that section. Another possibility of modeling this phenomenon is given by

$$P_{n+1} = w_n P_n = \frac{P}{b + P_n} P_n$$

or, in terms of the relative population level $x_n = P_n/P$, by

$$x_{n+1} = \frac{x_n}{c + x_n},$$

with some constants $b > 0$ and $c = b/P$, respectively. Here, too, the growth factor decreases if the population increases, and $x_{n+1} = f(x_n)$ with a nonlinear function $f(x) = x/(c + x)$.

Graphic iteration in Fig. 4 shows, in contrast to Fig. 1, that now the relative population levels $x_1, x_2, x_3, \ldots$, do finally approach the equilibrium level x^*. Furthermore, this statement holds for every initial position x_0 different from 0. (If $x_0 = 0$, then the population stays at 0 all the time.) Moreover, the dependence of the dynamics on the parameter c is also simple. For $0 < c < 1$ the (relative) population approaches the positive equilibrium level x^* and for $1 \leqq c$ the population dies out. This stability in behavior is due to an additional property of positivity in the model, viz. the deriva-

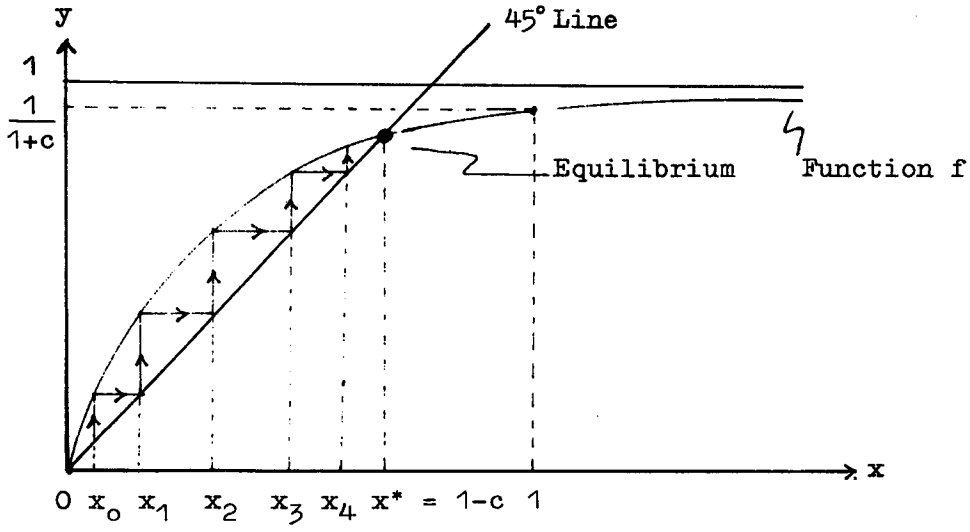

Fig. 4. Stable behavior

tive of $f(x) = x/(c + x)$ remains positive throughout. (Related to this is the fact that overshooting $P_n > P$ is admitted in this model but not in the first model given in this chapter.) The discussion shows that the method of modeling matters. The same qualitative description may be modeled differently in quantitative terms and then result in extremely different conclusions concerning the dynamic behavior, like chaotic behavior versus stable behavior.

Now, the example in Fig. 4 is only a very simple one in the realm of positive discrete dynamic systems. The point in considering positive systems is that there are methods available which work also in higher dimensions, i.e., if many variables are involved [cf. 6, 8, 18]. Also, there are biological processes which are in a natural way positive systems [for examples, see 6, 18]. For positive systems, too, the attractor may be a set, not just a single point, although this set will be not very complicated, in particular not a chaotic or strange attractor, because of the properties of positivity [7].

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Subject Index