IN VITRO GROWTH CHARACTERISTICS OF HUMAN LYMPHOID MALIGNANCIES IN PRIMARY CELL CULTURE

(In vitro groei-eigenschappen van humane lymfatische maligniteiten
in primaire celkweek)

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ABBREVIATIONS

AET	- 2-aminoethylthiouronium bromide hydrobromide
Ag	- antigen
ALL	- acute lymphoblastic leukemia
AML	- acute myeloblastic leukemia
ATL	- adult T cell lymphoma
B-act	- activated B lymphocyte
BCDF	- B cell differentiation inducing factor
BCGF	- B cell growth inducing factor
BFUe	- burst forming unit-erythroid
BSA	- bovine serum albumin
CALLA	- common acute lymphoblastic leukemia related antigen
CFU-e	- colony forming unit-erythroid
CFU-eo	- colony forming unit-eosinophil
CFU-GM	- colony forming unit-granulocyte/monocyte
CFU-Mast	- colony forming unit-mast cell
CFU-Meg	- colony forming unit-megakaryocyte
CLL	- chronic lymphocytic leukemia
CML	- chronic myelogeneous leukemia
Con A	- concanavalin A
CTLL	- murine cytotoxic interleukin 2 dependent cell line
Cy Ig	- cytoplasmic immunoglobulin
DNA	- deoxyribonucleic acid
E.coli	- Escherichia coli
EGF	- epidermal growth factor
EPO	- erythropoietin
EPA	- erythroid potentiating activity
FAB	- French-American-British cooperative group
FACS	- fluorescence activated cell sorter
FITC	- fluorescein isothiocyanate
GAHu-Ig	- Goat anti Human immunoglobulin antibody
GAM-Ig	- Goat anti mouse immunoglobulin antibody
G-CSF	- granulocyte colony stimulating factor
GM-CSF	- granulocyte/monocyte colony stimulating factor
HBSS	- Hanks balanced salt solution
HLA	- human leukocyte associated antigens
HPCM	- human placenta conditioned medium
HTLV	- human T cell lymphotropic virus
IFN	- interferon
Ig	- immunoglobulin
IL1	- interleukin 1
IL2	- interleukin 2
IL3	- interleukin 3
LAK cell	- lymphokine activated killer cell
L-CFU	- leukemia colony forming unit
LTBMC	- long term bone marrow culture
MAF	- macrophage activating factor
MCA/McAb	- monoclonal antibody
M-CSF	- monocyte/macrophage colony stimulating factor
MHC	- major histocompatibility complex
MoAb	- monoclonal antibody
multi-CFU	- multilineage colony forming unit
NHL	- non Hodgkin's lymphoma

NK cell	- natural killer cell
PBS	- phosphate buffered saline
PDGF	- platelet derived growth factor
PHA	- phytohaemagglutinin
PGE1	- prostaglandin E1
PGE2	- prostaglandin E2
rIL2	- recombinant interleukin 2
RNA	- ribonucleic acid
SAC	- Staphylococcus aureus strain Cowan I
sIg	- surface membrane associated immunoglobulin
SSV	- simian sarcoma virus
T-act	- activated T lymphocyte
TCR	- T cell receptor complex
TdT	- terminal deoxynucleotidyl transferase
TL-CFU	- T lymphocyte colony forming unit
TPA	- 12-O-tetradecanoylphorbol-13-acetate
TRITC	- tetramethylrhodamine isothiocyanate

CHAPTER 1

General Introduction

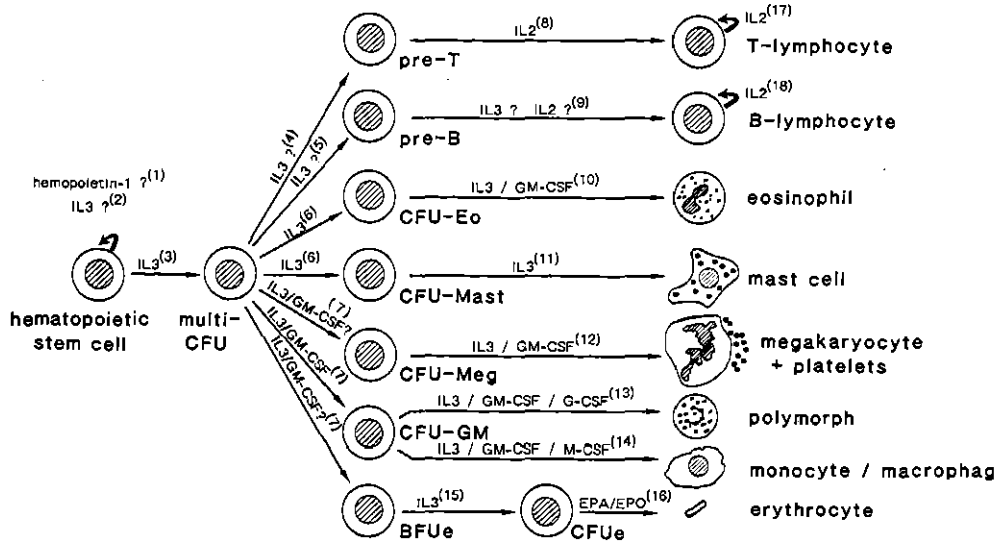

Figure 1: Scheme of blood cell production and the action of hematopoietic growth factors according to the literature (adapted from Metcalf, 1984)

(1) Bartelmez and Stanley, 1985
(2) Garland and Crompton, 1983; Dorssers et al., 1984
(3) Iscove et al., 1982
(4) Ihle et al., 1981
(5) Mc Kearn et al., 1985
(6) Greenberger et al., 1983; Metcalf, 1984
(7) Iscove et al., 1982; Metcalf et al., 1980
(8) Raulet, 1985
(9) Palacios et al., 1983; Paige, 1984; Mc Kearn et al., 1985; this thesis
(10) Greenberger et al., 1983; Metcalf, 1980
(11) Nabel et al., 1981; Ihle et al., 1983
(12) Iscove et al., 1982; Quessenberry et al., 1985
(13) Metcalf, 1984; Metcalf and Nicola, 1983; Ihle et al., 1983
(14) Metcalf, 1984; Byrne et al., 1981; Ihle et al., 1983
(15) Wagemaker et al., 1978; Iscove et al., 1982; Ihle et al., 1983
(16) Gasson et al., 1985; Stephenson et al., 1971
(17) Morgan et al., 1976
(18) Tsudo et al., 1984; this thesis
Note: IL3 is a murine factor of which a human homologue is still elusive

1.1. Hematopoiesis

The peripheral blood contains a number of cell types with highly specialized functions, i.e., red blood cells, platelets, lymphocytes, monocytes and granulocytes. Blood cells have a limited life span and need to be produced continuously. Hematopoietic stem cells, localized in the bone marrow, guarantee the permanent supply of functional blood cells. Hematopoiesis is regulated by a number of polypeptide hormones which act at various stages of differentiation. The hematopoietic compartment can be subdivided in a) pluripotent stem cells with self-renewal capacity, b) progenitor cells committed to differentiation and c) functional end cells. In mice, pluripotent stem cells can be assayed by injecting lethally irradiated animals with bone marrow cells and counting of spleen nodules that develop 8-12 days later (Till and McCulloch, 1961). Human as well as murine progenitor cells (except those of the lymphoid lineages) can be studied conveniently in vitro using semi-solid colony culture systems. A simplified scheme of hematopoietic cell differentiation is shown in Fig. 1. The stimulatory hormone like factors involved in this process are indicated as well.

1.2. Leukemia

Leukemias are neoplastic diseases which are characterized by the accumulation of hematopoietic cells. In several types of leukemia (with the notable exception of chronic myeloid leukemia) there is an excess of a homogeneous morphological cell type that closely resembles that of a normal cell at a given stage of differentiation. Thus, for example, leukemias may either present as well differentiated lymphocytic or monocytic cells, as half-way differentiated cells (e.g., lymphoblastic, promyelocytic, megakaryoblastic, erythroblastic), or, instead, as undifferentiated cells that share properties with the hematopoietic stem and progenitor cells. In contrast, in chronic myeloid leukemia (CML), the neoplastic cells cover a broad range of differentiation stages of myelomonocytic, erythroid, megakaryocytic and B lymphocytic (Fialkow et al., 1977; 1978), and rarely also of T lymphocytic lineages (Griffin et al., 1983). Evidence is multifold to indicate that leukemic cell proliferation is a clonal event, i.e., originating from one transformed cell. It is not certain whether the differen-

tiation stage of the leukemia in general reflects that of the originally transformed cell, or rather represents an accumulation of more differentiated cells descending from precursors (Fig. 2). Clearly, in CML, the latter situation exists.

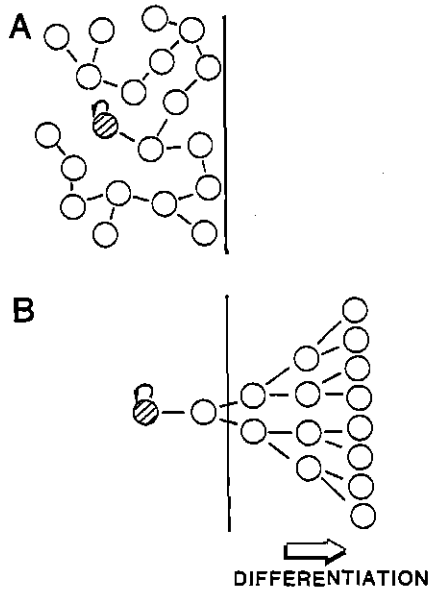

Figure 2: Two models of leukemic cell differentiation. Progeny of the leukemic stem cells (hatched) retains the original grade of differentiation (A) or expresses a more mature phenotype than that of the leukemic stem cells (B).

1.3. Growth factors in neoplasia

An important objective of cancer research is to understand how neoplastic cells escape the normal regulatory mechanisms of growth. One hypothesis is that abnormalities in response to stimulatory (hormone-like) factors result in the unbalanced production of neoplastic cells. Three abnormal patterns of growth stimulation of neoplastic cells have been postulated. Firstly, as a result of neoplastic transformation, tumour cells may have acquired the ability of autostimulation by producing growth factors themselves (i.e., autocrine stimulation) (Sporn and Todaro, 1980). Evidence in favour of an autocrine mechanism of tumour growth was obtained from studying the expression of a viral oncogene (transforming gene of a tumour-inducing virus) derived from simian sarcoma virus (SSV), a virus causing sarcomas and gliomas in experimental animals. The putative trans-

forming protein ($p28^{v-sis}$) of SSV structurally resembles platelet derived growth factor (PDGF) (Waterfield et al., 1983), a polypeptide hormone that stimulates the growth of fibroblasts. Moreover, antibodies against PDGF inhibit the growth of SSV-transformed humen fibroblasts in culture. These findings support the notion that autocrine stimulation, mediated by a PDGF-like factor, contributes to the growth of the SSV-transformed cells (Johnsson et al., 1985).

Secondly, oncogenic tranformation may result in the aberrant expression of membrane receptors for growth factors. This possibility is illustrated by the finding that the oncogene V-erb B is homologous to the gene encoding for epidermal growth factor receptor (Downward et al., 1984).

Finally, it is possible that following oncogenic transformation the cells become completely independent on external growth regulation. This may result from the autonomous activation of intracellular events leading to proliferation. For example, the product of the src oncogene, a protein tyrosine kinase, may directly trigger the intracellular effects of epidermal growth factor (Collett et al., 1978); Levinson et al., 1978; Ushiro et al., 1980). Growth factor independent cellular proliferation occurs also in human tumour cells that contain products of the ras oncogenes (i.e., guanosine diphosphate and guanosine triphosphate binding proteins) (Weinberg, 1984, 1985).

Similar mechanisms may be operational in the neoplastic transformation of hematopoietic cells. In mice, leukemic cell growth has been associated with alterations in the normal response mechanism to external growth stimuli, i.e.:

1) Infection of murine myeloid cells with Abelson - Mu LV renders the cells growth factor (IL3) independent (Pierce et al., 1985; Cook et al., 1985).
2) Recombinant murine retroviruses expressing v-myc oncogenes abrogate factor requirements of IL2 and IL3 dependent cell lines (Rapp et al., 1985);
3) The constitutive synthesis of IL3 by the murine leukemia cell line WEHI-3B is due to the presence of a retroviral insertion close to the promotor region of the IL3 gene (Ymer et al., 1985);
4) The product of the oncogene fms is closely related (perhaps identical) to the cell surface receptor of the murine monocyte/macrophage colony

stimulating factor CSF-1 (Sherr et al., 1985; Sariban et al., 1985).

In man, the HTLV induced T-cell neoplasms provide as yet the only example of altered growth factor response in leukemia. HTLV-I induces the constitutive expression of membrane receptors and renders the cells independent on activation by antigen for proliferation. Eventually, the HTLV-I transformed cells may also lose dependence on IL2 (Wong-Staal and Gallo, 1985).

The examples mentioned above all deal with transformation by viruses. It is not clear how these findings relate to the growth characteristics of leukemias with a non-viral etiology.

1.4. In vitro models for neoplastic cell growth

To assess the in vitro response of malignant cells to growth and differentiation stimuli most investigators have utilized continuous cultures of immortalized tumour cells (cell lines). Rarely, these approaches have dealt with primary tumour material of individual patients. The advantages of cell lines are their relatively easy handling in culture and their supply of cells in unlimited numbers. However, the growth characteristics of a cell line are far from representative of the original neoplasm from which it was derived, as fresh tumours only rarely develop immortalized cells in culture. Moreover, as a result of prolonged in vitro maintenance, initial requirements for external stimuli may have altered in cell lines. Because of the highly "artificial" growth features of cell lines their contribution to our understanding of tumour growth is restricted.

Fresh tumour specimens are often difficult to obtain and provide limited cell numbers. Nonetheless, there is a great interest in the development of convenient culture assays for primary tumour specimens as these directly reflect growth factor responses of cells following their malignant transformation in the host.

1.5. Classification and phenotyping of lymphoid malignancies

Different pathological subtypes of lymphoid malignancy are associated with certain stages of lymphoid maturation. Utilizing immunologic markers (in combination with morphological analysis) a precise definition of human

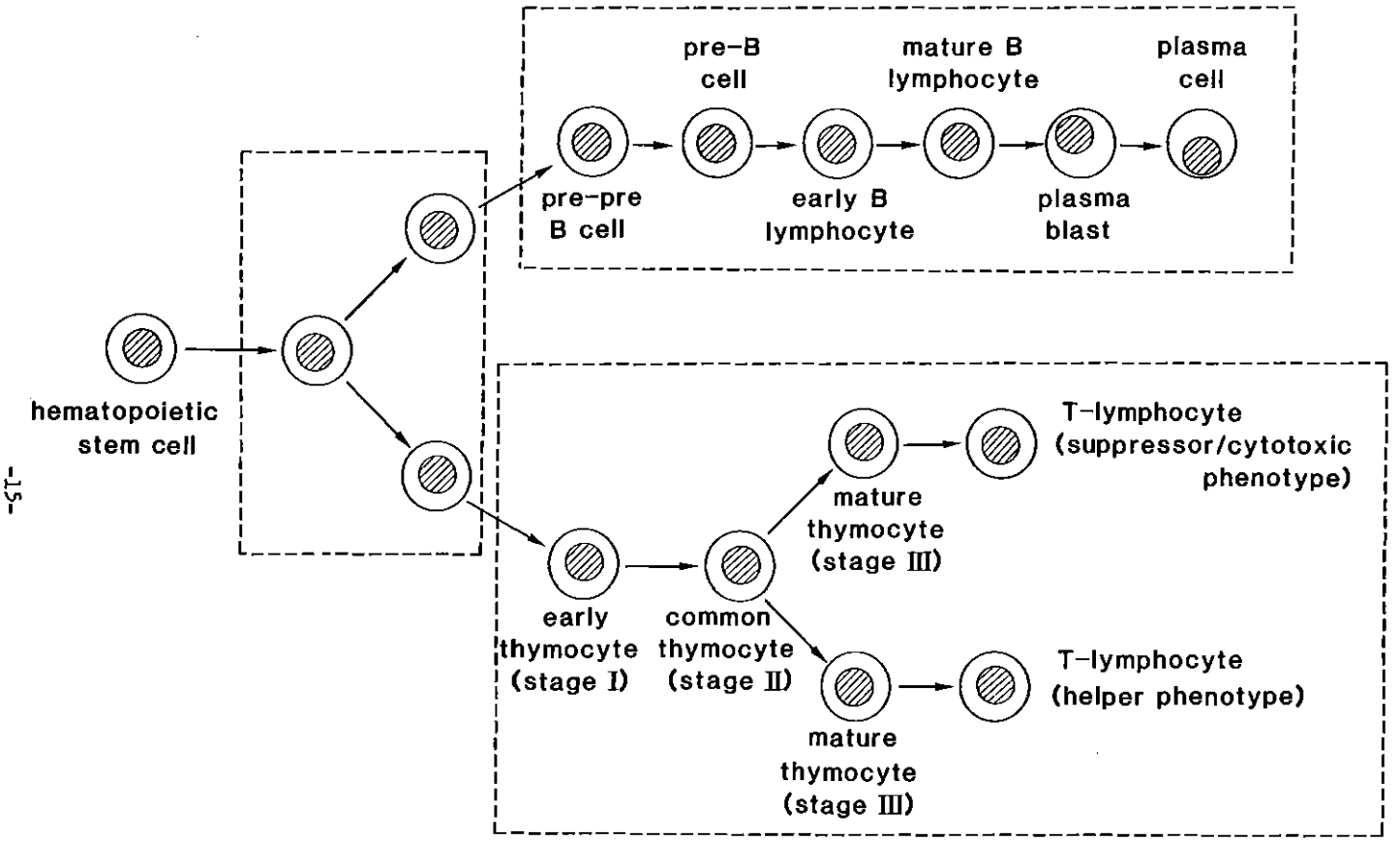

Figure 3: Scheme of lymphoid cell differentiation (adapted from Foon, Schroff and Gale, 1982)

leukocyte differentiation stages and their neoplastic counterparts is possible. This approach has benefited immensely from the development of monoclonal antibody (MCA) technology (Köhler and Milstein, 1975).
A scheme of lymphoid differentiation proposed on the basis of immunologic marker analysis is depicted in Figure 3. In figures 4, 5 and 6 the relationship between immunophenotypes and the lymphoid malignancies relevant to the experimental work described in this thesis is shown in detail.

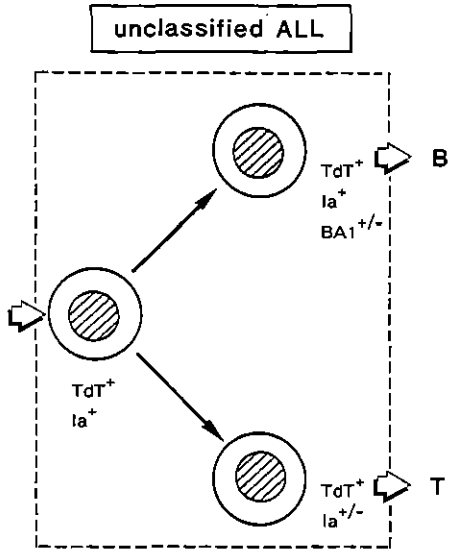

Figure 4: Unclassified early stages of lymphoid differentiation (detail of figure 3). Immunologic markers and type of malignancy associated with these differentiation stages are indicated. Markers are described in chapters 2 and 3.

1.6. Interleukin 2

1.6.1. In vitro culture of T lymphocytes

The existence of a soluble factor involved in the proliferation of T cells emerged from the work of Morgan and colleagues. They noted that T lymphocytes can be cultured in vitro for months under the stimulatory influence of supernatants conditioned by lectin-activated leukocytes (Morgan, Ruscetti and Gallo, 1976). This factor, according to its function, was originally designated as T cell growth factor but later it was named, more neutrally, interleukin 2 (IL2). IL2 is released by activated normal T lymphocytes, in particular by those belonging to the T helper subset (Robb, 1984). In addition, several T lymphocytic leukemia cell lines were found to

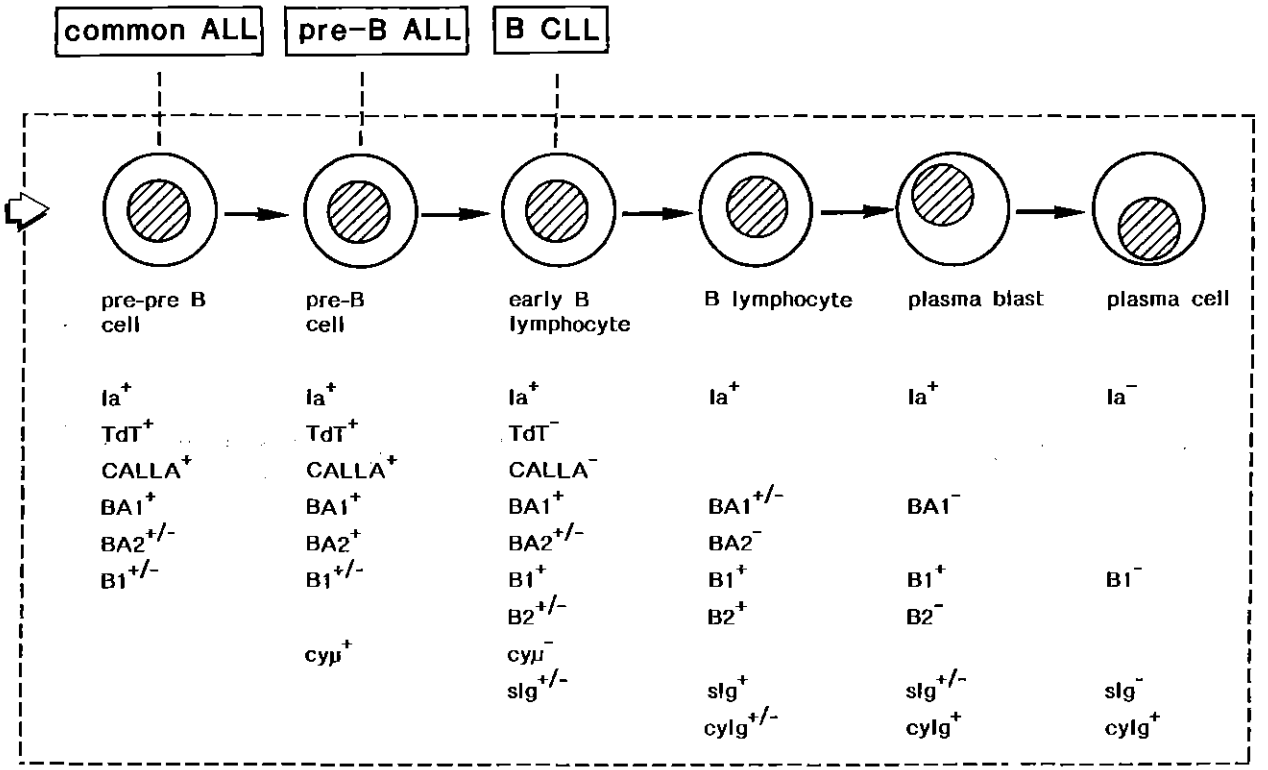

Figure 5: B lymphocytic differentiation (detail of figure 3). Immunologic markers and malignancies associated with the various stages of B-cell development are indicated. Details on the markers are given in chapters 2, 3 and 5.

synthesize IL2, sometimes in excessive amounts (Gillis and Watson, 1980).

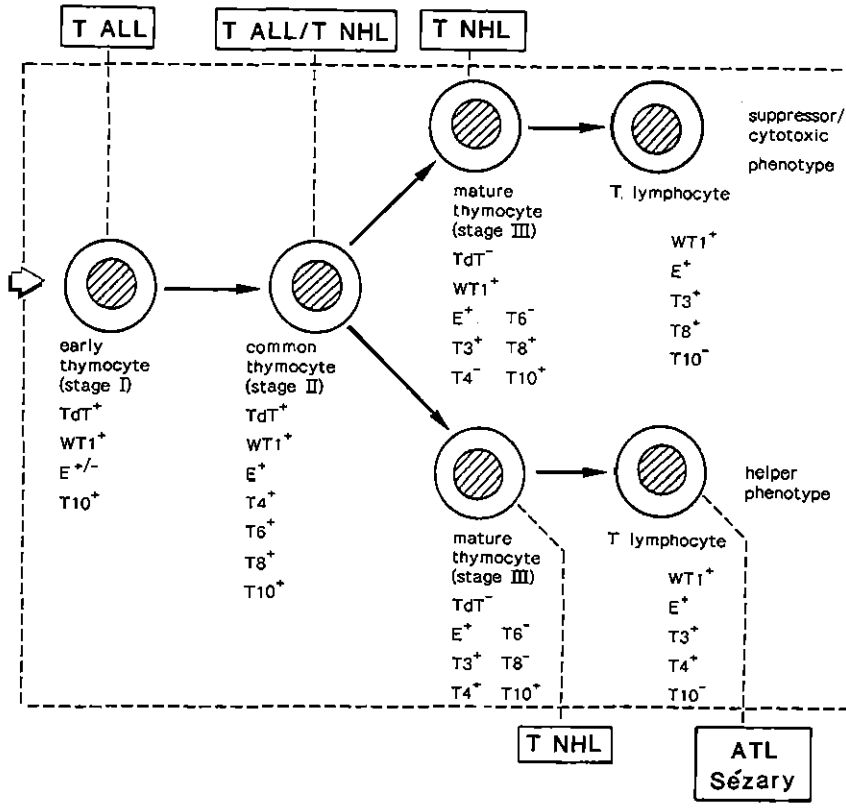

Figure 6: T lymphocytic differentiation (detail of figure 3). Immunologic markers and malignancies associated with the various stages of T-cell development are indicated. Markers are described in chapter 7.

1.6.2. Biochemistry of interleukin 2

Purification of IL2 from crude culture supernatants revealed some of its biochemical characteristics. Human IL2 has been identified as a glycoprotein with a variable molecular weight (ranging from 17,000 to 22,000 D) (Robb, 1984). This variability can be ascribed to differences in glycosylation of the IL2 molecule. Following treatment with neuraminidase and glycosidase to remove carbohydrate components, IL2 has a mere molecular weight of about 15,000 D and an isoelectric point of 8.2 (Robb and Smith,

1981). Removal of the carbohydrates exerts no effect on the in vitro biological activity of IL2 (Robb, 1982).

The gene for IL2 is located on human chromosome 4 q (Seigal et al., 1983). Molecular cloning of human IL2 cDNA revealed that this DNA encodes for a polypeptide of 153 amino acids of which the first 20 starting from the N-terminal region of the polypeptide presumably function as a signal peptide, to be cut off when "mature" secretable IL2 is produced (Taniguchi et al., 1983; Clark et al., 1984). Expression of IL2 cDNA in monkey COS cells or in E. coli bacteria has led to the availability of large amounts of highly purified (recombinant) IL2 (Taniguchi et al., 1983; Devos et al., 1983; Rosenberg et al., 1984).

1.6.3. Characterization of membrane receptors for interleukin 2

At an early stage, it was already recognised that IL2 exerts its effect on T cells by interaction with receptor molecules present on the cell membranes (Bonnard et al., 1979; Ruscetti and Gallo, 1981). Studies by Robb et al. with radiolabeled material then disclosed that IL2 acts as a hormone as regards affinity, specificity and saturability of its binding to T cells (Robb et al., 1981). Robb et al. estimated the number of receptor sites per normal activated T cell at 5,000 - 15,000.

Uchiyama et al. prepared the monoclonal antibody anti-Tac directed against the human IL2 membrane receptor. The antibody blocks the interaction between growth factor and its receptor (Uchiyama et al., 1981; Leonard et al., 1982). Using this monoclonal antibody, the human IL2 receptor was further characterized and identified as a glycoprotein with a molecular weight of 47,000 - 53,000 (Leonard et al., 1982). Later, cloning of an IL2 receptor cDNA and its expression in monkey - COS cells led to the biochemical definition of the receptor structure (Cosman et al., 1984). The human IL2 receptor gene is located on chromosome 10 (Leonard et al., 1985). Studies with the anti-Tac monoclonal antibody in combination with radiolabeled IL2 affinity experiments revealed the existence of two distinct receptor classes, one with high and one with low affinity for IL2, similar to what has been found for the receptors of epidermal growth factor and nerve growth factor (Robb and Greene, 1983; Kawamoto et al., 1983; Buxser et al., 1983). Normal T blasts stimulated with PHA for 72 hrs were found to

express approximately 3,500 high affinity (Kd 3.6 x 10^{-12}M) and 25,000 low affinity (Kd 28,300 x 10^{-12}M) receptors per cell (Robb et al., 1984). Molecular heterogeneity of IL2 receptors is probably caused at the post-translational level, as examination of several T cell types with the cDNA clone does not provide indications for the existence of more than one structural IL2 receptor gene (Leonard et al., 1984).

It is at present unknown whether or how low-affinity IL2 receptors are involved in the physiological response to the growth factor. IL2 concentrations, assumed to be physiological in vivo, would occupy less than 1% of the low-affinity sites, whereas the high-affinity sites would be saturated (Robb et al., 1984). This argues against a direct participation of the low affinity sites in the IL2 response. On the other hand, it cannot be excluded that in close cellular interaction with IL2-producing cells, the local IL2 concentrations would be excessively high, leading to an increased occupation of the low affinity receptors (Robb et al., 1984). It is also possible that the low affinity sites reflect a pool of non-functional "precursor" components awaiting their biochemical modification to become highly affinitive (Robb et al., 1984).

1.6.4. Physiological roles of interleukin 2 in the immune response

Over the years, it has become clear that IL2 is not only a simple proliferation factor for T cells, but that the factor has a central role in the immune response (Fig. 7). IL2 induces the secretion by T cells of other lymphokines, thereby indirectly influencing a variety of immune functions. One of these is gamma interferon (IFNγ), a multifunctional factor that, e.g., induces macrophage activation and antibody secretion by B cells (Pace et al., 1983; Sidman et al., 1984; Leibson et al., 1984).
Other lymphokines produced by T cells under the direct regulatory influence of IL2 include so called B cell growth- and differentiation factors (Leanderson et al., 1982; Howard et al., 1983; Inaba et al., 1983).

A number of authors have recently published data indicating that IL2 directly regulates the proliferation of B cells. Functional IL2 membrane receptors were detected on human B lymphocytes activated with Staphylococcus aureus strain Cowan I (SAC) and SAC activated B cells proliferated in response to pure IL2 (Tsudo et al., 1984; Mingari et al., 1984; Prakash

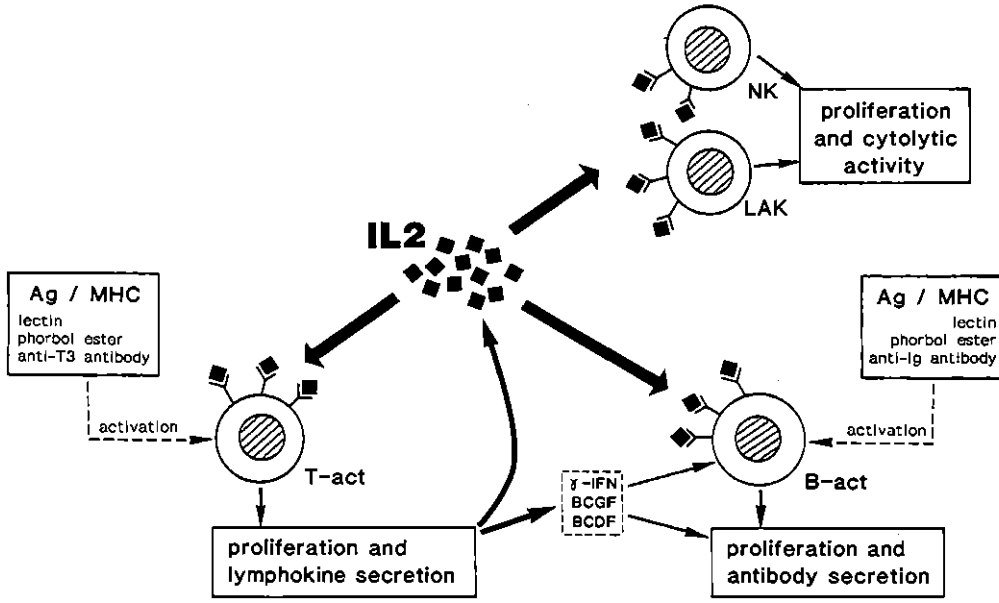

Figure 7: Schematic representation (according to literature data) of the central role of IL2 in the immune response (adapted from Robb, 1984)

et al., 1985; Nakagawa et al., 1985; Zubler et al., 1984; Muraguchi et al., 1985). IL2 receptors on the activated B lymphocytes (B blasts) are structurally identical to those present on activated T cells (Mingari et al., 1985; Lowenthal et al., 1985; Boyd et al., 1985). The total amount of IL2 receptors on B blast cells, including high- and low-affinity types, is approximately half of that on activated T cells (Lowenthal et al., 1985). IL2 receptors on B and T blasts have identical binding-characteristics and show the same, i.e., 1 : 10, ratio of high- versus low-affinity receptor numbers (Lowenthal et al., 1985).

Differentiation of B cells (i.e., stimulation of immunoglobulin secretion) occurs at very high concentrations of IL2, i.e., above 100 units $.ml^{-1}$, with an optimal response at 10^4 units $.ml^{-1}$ (Ralph et al, 1984). This could suggest that a low-affinity interaction between receptor and growth factor is involved in the induction of the differentiation of B

cells by IL2. Blocking of the IL2 receptors on SAC activated human B cells by the monoclonal antibody anti-Tac resulted in a 90 per cent inhibition of Ig secretion by these cells (Mittler et al., 1985).

How the effect of IL2 on B cell proliferation and maturation relates to the activities of other materials such as B cell growth factors (BCGF's) and B cell differentiation factors (BCDF), still aweits clarification. Studies in this direction will greatly benefit from the availability of cytokine preparations obtained by recombinant techniques and of antibodies reactive with the receptors for these cytokines.

1.6.5. Regulation of interleukin 2 production and interleukin 2 response

From limiting dilution culture experiments in which large numbers of T lymphocytes are expanded from a single clonogenic cell, it has been estimated that approximately 60 per cent of all T lymphocytes can produce IL2 and do not require exogenous IL2 for proliferation (Wee and Bach, 1984; Gullberg and Smith, 1986). This has led to the notion that the majority of T lymphocytes are self-perpetuating in their IL2 driven proliferative response (Meuer et al., 1984).

The physiological signal for IL2 production by T lymphocytes is activation through the simultaneous recognition of foreign antigen and major histocompatibility (MHC) antigens on the surface of macrophages which present the foreign antigen (Figure 8). In this interaction, the T-cell receptor complex is directly involved (review; Acuto and Reinherz, 1985). Subsequently, the T lymphocytes release factors (γ IFN; MAF) that activate macrophages. The activated macrophages enhance IL2 production by the T cells via elaboration of the lymphokine interleukin 1 (IL1). Activated macrophages also produce prostaglandins, which can, dependent on concentration, eventually down-regulate IL2 production by the activated T cells.

Efrat and colleagues have obtained evidence for another regulation mechanism for IL2 production. They assume the existence of labile as yet unidentified protein(s) with an inhibitory effect on the formation of biologically active IL2 mRNA. Accordingly, inhibition of synthesis of these putative repressor proteins by blocking of translation (with the drug cycloheximide) leads to superinduction of IL2 mRNA (Efrat et al., 1984; Efrat and Kaempfer, 1984).

The capacity of T and B cells to respond to IL2 is strictly dependent on the expression of membrane receptors with high affinity for the growth factor (1.7.2. and 1.7.3.). Normally, expression of IL2 membrane receptors occurs after activation of the cells. In addition to the mechanism described above for antigen activation, T lymphocytes can be directly activated in vitro by lectins, e.g., PHA or Concanavalin A (Con A), the phorbol ester 12-O-tetra decanoyl phorbol-13-acetate (TPA) or monoclonal antibodies (anti-T3) reactive with the T-cell receptor complex. Likewise, B lymphocytes express IL2 receptor antigens following activation by lectins (e.g., pokeweed mitogen), TPA or anti-Ig antibodies (Fig. 7).

Little is known about the regulation of IL2 receptor expression and function after activation. It has been suggested that IL2 itself is involved in this process and that IL2 augments transcription of the IL2 receptor gene (Wakasugi et al., 1985; Welte et al., 1984, Smith and Cantrell, 1985; Depper et al., 1985). Experiments designed to investigate the intracellular mechanisms by which IL2/IL2 receptor interaction promotes the proliferation of T cells have indicated that protein kinase C is involved in intracellular signal transduction (Farrar and Anderson, 1985).

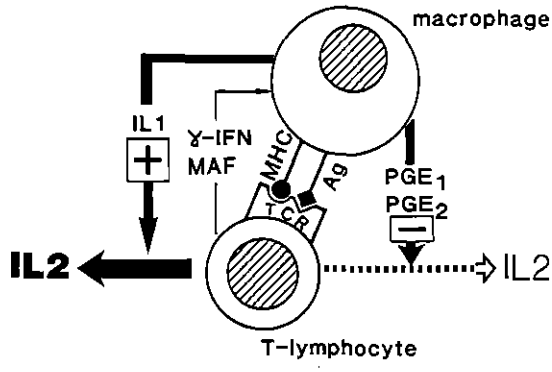

Figure 8: Physiological regulation of IL2 production (adapted from Schreier, 1984)

1.7. Introduction to the experimental work

The experimental work presented in this thesis deals with the analysis of the in vitro growth and differentiation characteristics of acute lymphoblastic leukemia (T and non-T), T cell non Hodgkin's lymphoma and B cell chronic lymphocytic leukemia in primary cell culture. For these studies, reproducible cell culture assays for the growth of these lymphoid tumours first needed to be developed. Considerable attention has been paid to the response of these neoplasms to the polypeptide hormone interleukin 2. The experiments described in chapters 2 and 3 deal with the in vitro colony growth of non-T acute lymphoblastic leukemia (ALL) and with the effect of interleukin 2 on these cells in combination with a factor elaborated by feeder leukocytes. The requirements of the ALL cells for activation with a lectin (PHA) or a phorbol ester (TPA) for colony growth have also been investigated. To assess whether non-T ALL cells differentiate toward more mature cell types during in vitro growth, the morphological and immunological phenotypes of colony cells were determined. To compare the differentiation capacities of ALL with those of acute leukemia of the myeloid differentiation lineage (AML) the abilities of AML to produce more mature progeny under comparable in vitro conditions were studied (chapter 4). Chapter 5 deals with an analysis of growth requirements of B cell type chronic lymphocytic leukemia (CLL) in colony culture and specifically with the role of IL2 in the proliferation of B CLL cells. This analysis is extended in chapter 6, in which the results of binding experiments with radiolabeled IL2 are presented. These experiments were carried out to determine numbers and affinity of IL2 receptors expressed by B CLL cells. In addition, the hypothesis that certain CLL cells might be capable of self-stimulation via the autocrine production of IL2 is approached in this chapter. In chapter 7, culture characteristics of acute lymphoblastic leukemia and non Hodgkin's lymphoma (NHL) of the T cell differentiation lineage are presented. The studies described in chapter 8 are our first attempts toward the characterization of membrane phenotypes and growth requirements of normal T-lymphocytic precursor cells in the human bone marrow. For this purpose, we applied the human long-term bone marrow culture system. Knowledge of the growth and differentiation features of normal lymphoid progenitors is essential for our understanding of whether or how

the leukemic counter parts of these cells reflect a modified response to growth and differentiation stimuli. In chapter 9 a brief overview of our current understanding of the role of IL2 in the proliferation of neoplastic T and B cells is presented. Moreover, the results of this thesis are discussed in this chapter with reference to their implications for our insight into the control of proliferation and differentiation of the different types of lymphoid leukemia/lymphoma.

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CHAPTER 2

Common and Pre-B Acute Lymphoblastic Leukemia Cells Express Interleukin 2 Receptors, and Interleukin 2 Stimulates *In Vitro* Colony Formation

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Common and Pre-B Acute Lymphoblastic Leukemia Cells Express Interleukin 2 Receptors, and Interleukin 2 Stimulates In Vitro Colony Formation

By Ivo Touw, Ruud Delwel, Reinder Bolhuis, George van Zanen, and Bob Löwenberg

The role of interleukin 2 (IL 2) as a possible regulator of in vitro proliferation and differentiation of non-T acute lymphoblastic leukemia (ALL) cells was investigated. For this purpose, leukemic cells from the blood or bone marrow of eight untreated patients with common or pre-B ALL were analyzed using the anti-Tac monoclonal antibody (reactive with the IL 2 receptor) in indirect immunofluorescence. The receptors for IL 2, which were initially absent from the cell surface, were induced on high percentages of the ALL cells after the in vitro exposure to the lectin phytohemagglutinin or the phorbol ester 12-0-tetradecanoylphorbol-13-acetate in six patients, suggesting that the cells had become sensitive to IL 2. In colony cultures to which feeder leukocytes and IL 2 had been added, colony growth was obtained in five of eight cases. Whereas the cells from one patient formed colonies in the absence of exogenous stimuli, the cells from others were dependent on the addition of feeder leukocytes plus IL 2. In the latter cases, feeder leukocytes alone, releasing some IL 2, stimulated growth suboptimally at different cell concentrations. Their stimulative effect was significantly enhanced when leukocyte-derived IL 2 or pure recombinant IL 2 was supplemented. Alone, IL 2 (up to 500 U/mL) did not support colony formation. Apparently, IL 2 and feeder leukocytes are both required for the induction of colonies in these cases of ALL. From cell sorting of fluorescent anti-common ALL antigen (CALLA) stained cells it appeared that colonies descended from cells with high as well as low or negative CALLA expression. Immunophenotyping demonstrated the presence of the original leukemia markers on colony cells, but was not indicative of maturation of ALL toward more differentiated B cells. We suggest that IL 2 can stimulate the in vitro proliferation of certain neoplastic B lymphocyte progenitors.

INTERLEUKIN 2 (IL 2) is the soluble factor responsible for the in vitro proliferation of antigen or lectin-activated T lymphocytes.[1] Prior activation is required to induce receptors for IL 2 on the cell surface membrane. The monoclonal antibody anti-Tac specifically binds to these receptors (also called Tac antigens).[2,3]

Recently, activated human B cells have also been demonstrated to express IL 2 receptors, and results from ^{3}H-thymidine incorporation assays indicate that a subpopulation of these cells proliferates in the presence of immunopurified IL 2 without the addition of other factors.[4] In addition, it has been shown that in vitro colony formation by B chronic lymphocytic leukemia cells is induced by IL 2.[5]

Here we present our first attempts directed toward the characterization of factors required for the in vitro colony formation by neoplastic B cell progenitors, ie, common and pre-B acute lymphoblastic leukemia (ALL) cells. The findings suggest that, in certain cases, IL 2 may be involved in the proliferation of these leukemias. ALL cells initially lack but express IL 2 receptors following exposure to 12-0-tetradecanoylphorbol-13-acetate (TPA) or to phytohemagglutinin (PHA). The addition of IL 2 to colony cultures can stimulate ALL colony formation.

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MATERIALS AND METHODS

Patients and separation of leukemia cells. Eight untreated patients with ALL were studied. The leukemic cells were classified as common or pre-B ALL cells on the basis of immunologic phenotyping[6] (Table 1). Leukemic cells were isolated from peripheral blood or bone marrow following Ficoll-Isopaque (Nygaard, Oslo) separation.[7]

Contaminating E rosette-positive lymphocytes were then removed from the leukemic cell fractions by rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes and Ficoll-Isopaque sedimentation according to Madsen et al.[8]

Immunofluorescence studies. Surface antigens on fresh and cultured cells were assayed by means of indirect immunofluorescence using murine monoclonal antibodies (MoAb) and goat anti-mouse immunoglobulin (Ig) coupled with fluorescein isothiocyanate (GAM/FITC, Nordic, Tilburg, The Netherlands). Details on the labeling procedures have been given elsewhere.[9] Cells were evaluated for specific fluorescence with either a Zeiss fluorescence microscope (200 cells per slide were scored) or a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, Calif). Control incubations with GAM/FITC alone, included in each test to check for nonspecific binding of the conjugate, were negative in all experiments. Cell surface membrane-associated immunoglobulins (sIg) were assayed as described.[10] Cytoplasmic immunoglobulin M heavy chains (Cy μ) were stained in cytocentrifuged cells (Shandon, Cheshire, England) with 1:25 diluted goat anti-human Ig M/FITC (GAHuIgM/FITC, Nordic).[11] Two hundred cells per slide were examined with a fluorescence microscope. Control stainings with goat anti-human IgG/FITC and goat anti-human κ or λ light chain/FITC were negative.

Dual stainings: after the first MoAb (anti-Tac; IgG2a) treatment and labeling with 1:40 diluted goat anti-mouse IgG2a coupled to tetramethylrhodamine isothiocyanate (GAM/IgG2a/TRITC; Nordic), the cells were spun on a microscope slide and fixed for ten minutes at −20 °C in 5% vol/vol acetic acid in methanol. After three washings with phosphate-buffered saline (PBS), they were further treated with either 1:10 diluted MoAb VIL A1 (IgM) and 1:40 diluted goat anti-mouse IgM FITC conjugate (GAM/IgM/FITC)

Table 1. IL 2 Receptor Expression in Eight Cases of ALL

Patient No.	Age (yr)	Source*	Leukemic Phenotype (Percentage of Positive Cells)† CALLA	Cy μ	BA_1	IL 2 Receptor Expression (Percentage of Positive Cells)† Before Incubation	18 h PHA Incubation‡	18 h TPA Incubation‡
1	61	PB	60	50	82	0	53	43
2	2	PB	83	86	87	0	57	62
3	17	BM	76	45	70	0	38	51
4	7	BM	75	75	76	0	24	26
5	3	PB	60	0	95	0	63	64
6	16	BM	60	0	27	0	0	0
7	43	PB	53	0	74	0	62	65
8	38	BM	91	0	99	0	0	0

Blast cell phenotyping also included the T cell markers T3, T11, and WT1. These were negative in all cases. Patients No. 1 through 4, pre-B ALL; patients No. 5 through 8, common ALL.

*Leukemic cells wee isolated from peripheral blood (PB) or bone marrow (BM).

†Determined by fluorescence microscopy.

‡Details are given in Materials and Methods.

or GAHuIgM/FITC for the simultaneous visualization of IL 2 receptors and common ALL antigens or IL 2 receptors and cytoplasmic IgM heavy chains, respectively.

Control stainings (VIL A1 [IgM] + GAM/IgG2a/TRITC, anti-Tac [IgG2a] + GAM/IgM/FITC, GAM conjugates without MoAb pretreatment) were all negative.

Monoclonal antibodies. The following MoAb were used in this study (in parentheses are the dilutions used in indirect immunofluorescence): (1) anti-Tac (1:1,000), anti-IL 2 surface membrane receptor[2] (Dr T. Uchiyama, Kyoto, Japan); (2) VIL A1 (1:100), anti-common ALL antigen (CALLA)[12] (Dr W. Knapp, Vienna); (3) BA2 (1:250), anti-pre-B cell differentiation antigen[13] (Hybritech, San Diego); (4) WT1 (1:100), anti-pan-T cell antigen[14] (Dr W.J.M. Tax, Nijmegen, The Netherlands); (5) OKT11 (1:40), anti-sheep erythrocyte rosette receptor (Ortho Pharmaceutical Corp, Raritan, NJ); (6) OKT3 (1:40), anti-mature thymocyte and T lymphocyte antigen (Ortho Pharmaceutical Corp); and (7) S4-7 (1:500), anti-myeloblast — granulocyte and monocyte antigen[15] (Dr G. Rovera, Philadelphia).

Colony culture. Colony culture experiments were performed according to a previously described culture system with PHA and irradiated (2,500 rad) human peripheral blood leukocytes as stimulators.[15] This system supports the formation of normal T lymphocyte as well as myeloid leukemia colonies[16,17]; 2×10^5 cells were plated in each culture dish. On certain occasions, modifications were made to the system: (1) replacement of PHA (reagent grade; Wellcome, Dartford, England) by 100 ng of the phorbol ester TPA (Sigma Chemical Corp, St Louis); (2) addition of IL 2; and (3) omission of feeder leukocytes. Two sources of IL 2 were used: 15,000 mol wt fractions of gel-filtered (Ultrogel AcA 54; LKB, Bromma, Sweden) culture media from lectin-stimulated human leukocyte cultures and pure IL 2 obtained by recombinant DNA techniques (rIL 2; Biogen SA, Geneva).

Suspension culture. Suspension cultures were performed in 6-mL tubes; 2×10^6 cells were cultured in 1 mL of alpha medium (with 10% vol/vol fetal calf serum) with either 100 ng/mL TPA or 1% vol/vol PHA for 18 hours in a fully humidified atmosphere of 5% CO_2 at 37 °C. They were washed three times with PBS and prepared for indirect immunofluorescence.

Cell sorting. In one series of experiments, cells were inoculated into culture following MoAb VIL A1 (anti-CALLA) and GAM/FITC incubations and fluorescence-activated cell sorting (by FACS 440).

RESULTS

Induction of IL 2 membrane receptors on pre-B ALL cells. Fresh ALL cells did not express IL 2 receptors on their membranes as assessed with the MoAb anti-Tac. On

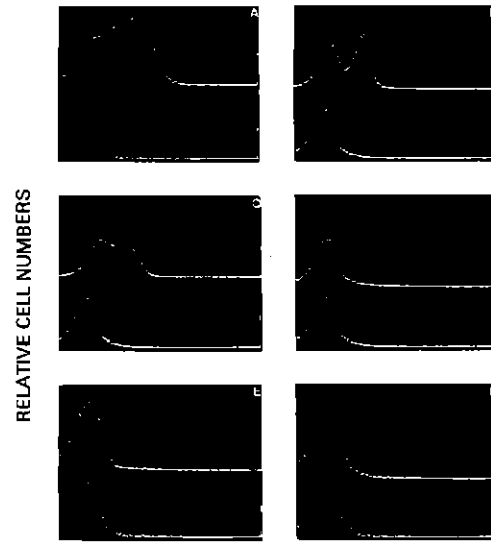

Fig 1. Flow cytofluorometric analysis of membrane antigens expressed by pre-B ALL cells of patient No. 1 following incubation with TPA. In each panel, the histograms of the log fluorescence intensity (horizontal axis) v relative cell numbers (vertical axis) are shown. The upper graphs represent stainings with MoAb and goat anti-mouse Ig coupled to fluorescein isothiocyanate (GAM/FITC); the lower graphs represent control stainings with GAM/FITC alone. (A) anti-Tac, (B) VIL A1, (C) BA2, (D) WT1, (E) OKT11, and (F) OKT3.

the other hand, IL 2 receptors were found in high percentages of cells following incubation in suspension with TPA or PHA in each of four cases of pre-B ALL and in two of three cases of common ALL (Table 1). Less than 1% of the incubated cells formed E rosettes, indicating that the anti-Tac binding was not due to contaminating E-positive T lymphocytes. Evidence for the presence of IL 2 receptors on pre-B cells following TPA exposure was obtained when the cells from patient No. 1 were phenotyped in more detail. Cytofluorometric analysis (Fig 1) demonstrated the binding of anti-Tac (57% positive), anti-CALLA (54% positive), and anti–pre-B cell antigen (BA2) (66% positive) antibodies on the incubated cells, whereas the T cell markers T3, T11, and WT1 all remained negative. A similar binding pattern (not shown here) was found after incubation with PHA. In addition, two-color fluorescence labeling methods revealed the simultaneous expression of the IL 2 receptors with CALLA and with Cy μ on individual cells; 80% of the CALLA-positive cells expressed the IL 2 receptors (200 CALLA-positive cells counted) (Fig 2A1 and A2), and such receptors were found on 47% of the Cy μ-positive cells (Fig 2B1 and B2). As expected, IL 2 receptors could be induced on normal peripheral blood T lymphocytes using these methods (24% to 55% anti-Tac–positive cells after PHA and TPA incubation, respectively).

ALL colony formation. Colony data from PHA-supplemented cultures are summarized in Table 2. In four cases (patients No. 1, 4, 7, and 8) colony formation was obtained (in a range of 47 to 63 colonies per 10^5 plated cells) in the presence of irradiated feeder leukocytes (in the culture underlayer) and IL 2.

The stimulative effect of IL 2 is evident from the comparison with cultures with leukocytes but without the addition of IL 2. Colony formation by cells from patients No. 1, 4, and 7 was enhanced by the extra IL 2. We hypothesized that suboptimal concentrations of IL 2 had been produced by the feeder leukocytes.

In these cases, PHA and IL 2 alone, ie, without leukocytes, were not active (Table 2) even when IL 2 was present in concentrations up to 100 U/mL (not shown). Apparently feeder leukocyte-derived factor(s) other than IL 2 were also essential for these ALL cells to form colonies.

The additional role of leukocyte stimulation is illustrated in cultures reconstituted with increasing numbers of feeder cells in the presence of 25 units of IL 2 (Fig 3). Colony numbers increased progressively when titrated numbers of feeder leukocytes were added to the cultures. In addition, the stimulative effect of IL 2 is evident from these experiments: colony numbers were significantly higher in the cultures supplied with exogenous IL 2 than in those without extra IL 2. These results suggest that IL 2, in combination with leukocyte-derived factors, was required for in vitro colony formation by the pre-B ALL cells.

To exclude the possibility that contaminating molecules in the IL 2 preparation had been responsible for the stimulation of colony growth, the cells of patient No. 1 were cultured with increasing concentrations (25 to 500 U/mL) of pure rIL 2 in the presence of PHA and 2×10^6 feeder leukocytes. The addition of rIL 2 enhanced colony growth in a dose-dependent fashion. Colony numbers in cultures with 500 rIL 2 were about three times higher than those in cultures without rIL 2. Similarly to IL 2, rIL 2 (500 U/mL) could not induce colony formation in the absence of additional feeder leukocyte stimulation.

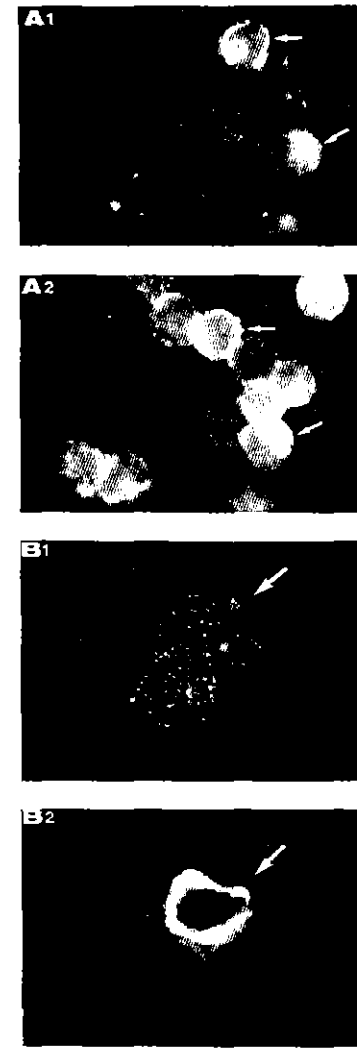

Fig 2. Two-color immunofluorescence microscopy of pre-B ALL cells of patient No. 1 after incubation with TPA for 18 hours. The arrows indicate double fluorescent cells. (A) anti-Tac (A1) and VIL A1 (anti-CALLA) (A2); (B) anti-Tac (B1) and anti-Cy μ (B2).

Cultures from the cells of patients No. 2, 3, and 5 did not give rise to colonies under any of the conditions tested. On the other hand, cells from patient No. 6 produced colonies in PHA containing culture medium without further additives,

Table 2. ALL Colony Formation in PHA-Supplemented Cultures

Patient No.	Growth Stimulation	Colonies per 10^5 Plated Cells: 2×10^6 Irradiated Leukocytes + 25 U IL 2	Colonies per 10^5 Plated Cells: 2×10^6 Irradiated Leukocytes	Colonies per 10^5 Plated Cells: 25 U IL 2	Colonies per 10^5 Plated Cells: None	Immunologic Markers on Colony Cells* (Percentage Positive): CALLA	BA2	Cy μ	sIg	E	WT1
1		49	31	0	0	53	69	29	0	0	0
2		0	0	0	0	—	—	—	—	—	—
3		0	0	0	0	—	—	—	—	—	—
4		63	0	0	0	10	58	ND	0	12	8
5		0	0	0	0	—	—	—	—	—	—
6		25	22	80	80	49	76	0	0	22	ND
7		47	36	5	0	57	77	0	0	0	10
8		54	49	2	0	22	67	0	0	0	11

All experiments included control cultures without the addition of PHA; no colonies were formed in these cultures. ND, not determined.

*Pooled colonies were harvested from the plates with a Pasteur pipette, suspended to a single-cell suspension, and prepared for immunofluorescence microscopy as described in Materials and Methods.

indicating that proliferation occurred independent of exogenous growth stimulators.

Phenotyping of ALL colony cells. Colony cells were examined morphologically and immunologically. Their microscopic appearance was consistent with that of lymphoblasts. Immunologic phenotyping (Table 2) of the colony cells confirmed the presence of the preculture ALL markers (CALLA, BA2, Cy μ), although the percent expressions before and after culture differed in some instances. Indications for the in vitro maturation of ALL cells were not obtained. A loss of CALLA expression was noted following culture in two of eight cases, but this was not accompanied by the acquisition of cytoplasmic IgM heavy chains or the appearance of sIg (Table 2).

The cultured cells were checked for the presence of T lymphocytes with markers E and WT1 inasmuch as the applied culture conditions are known to be highly permissive for T cell colony formation (Table 2). Significant contaminating proliferation of T lymphocyte colony-forming cells did not occur in each of these patients. The presence of myelomonocytic cells was excluded using marker MoAb S4-7.

Expression of CALLA on ALL colony-forming cells. Using a FACS 440 cell sorter, VIL Al- and GAM/FITC- stained cells from patients No. 1 and 8 were sorted in two fractions: (1) cells with a high CALLA density and (2) cells without CALLA or with a low CALLA density. The results listed in Table 3 show that colony-forming cells were recovered from both fractions.

DISCUSSION

IL 2, previously referred to as T cell growth factor,[1] has been identified as the essential stimulatory component for in vitro T lymphocyte proliferation. However, recent studies have suggested that IL 2 can also stimulate the proliferation of human B lymphocytes.[4] Very little is known about factors involved in the growth of precursor cells of the human B lymphocyte lineage. The present experiments provide indications that IL 2 can act on leukemic B cell progenitors, ie, common and pre-B ALL colony-forming cells. Cells obtained from eight patients at diagnosis were studied. In six cases, the ALL cells could be induced to express membrane receptors for IL 2 (which they initially lacked) by the in vitro exposure to a lectin (PHA) or a phorbol ester (TPA), similar to normal peripheral blood T lymphocytes. These findings raised the question of whether ALL cells may respond to IL 2 and proliferate in culture. To investigate this possibility, we plated the cells in a colony culture system containing PHA or TPA and leukocyte feeder cells either with or without the addition of human IL 2. Colony growth was obtained with the cells of five patients.

The addition of leukocyte-derived IL 2 to the culture medium significantly enhanced colony formation by the leukemic cells of patient No. 1, whereas those from patient

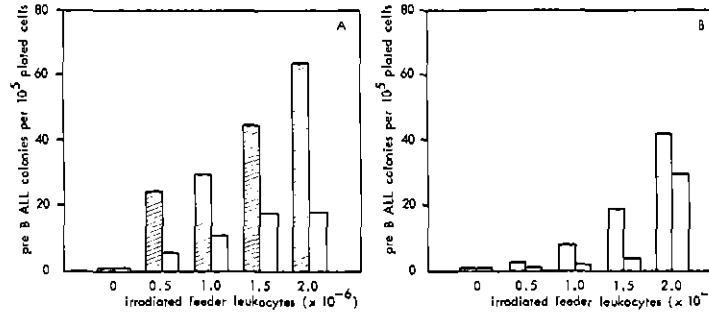

Fig 3. Pre-B ALL colony formation in patient No. 1 with (▨) or without (□) 25 units of IL 2 added to the upper layers of the cultures. Colonies (50 cells or more) were counted on day 7 of the culture. The effect of IL 2 on colony growth was assessed in relationship with varying numbers of irradiated peripheral blood leukocytes in the underlayer. Sections A and B indicate the experiments in which cultures were supplemented with TPA (100 ng per dish) and PHA (0.01 mL per dish), respectively.

Table 3. CALLA on ALL Colony-Forming Cells

Sorted Fractions*	Cell Recovery (%)	Colony-Forming Cells per 10^5
Patient No. 1		
Unfractionated	100	47
CALLA-negative or weakly positive‡	35.4	40†
CALLA-positive	64.6	46
Patient No. 8		
Unfractionated	100	24
CALLA-negative or weakly positive‡	25.4	6
CALLA-positive	74.6	27

Colonies were grown in the PHA leukocyte feeder system supplemented with 25 units of IL 2. It was not possible in these cases to separate the CALLA-negative cells from the cells weakly expressing the antigen since histogram analysis of the CALLA fluorescence distribution did not reveal distinct CALLA-positive and CALLA-negative populations.

*Cells were stained with MoAb VIL A1 and GAM/FITC and sorted on the basis of fluorescence intensity.

†Colony cells were harvested and analyzed for CALLA expression. Forty-three percent of the cells showed bright fluorescence after VIL A1 and GAM/FITC staining.

‡Fluorescence intensity did not exceed the maximal fluorescence of cells stained with GAM/FITC alone.

No. 4 were absolutely dependent on the supply of IL 2. Pure (recombinant) IL 2 exerted a similar effect. These results support the idea that the in vitro proliferation of ALL cells can be stimulated by IL 2. The findings also suggest that IL 2 was not the only growth factor involved in these cases: omission of feeder leukocytes from IL 2-containing cultures abrogated colony growth, and a positive relationship between feeder cell numbers and ALL colony response was established (Fig 3). In this respect, the stimulation of the ALL colony-forming cells clearly differs from that of clonogenic T lymphocytes. Normal E rosette-positive peripheral blood T cells produce colonies in PHA and IL 2-containing cultures and do not need additional feeder cell stimulation (data not shown).

A second difference relates to the optimal IL 2 concentration needed for colony growth: T colony-forming cells are stimulated maximally by 10 to 25 U/mL of IL 2, whereas ALL colony numbers (patient No. 1) do not reach plateau values in the presence of 500 U/mL of IL 2 (data not shown).

In view of the IL 2 receptor expression on high numbers of the cells following stimulation with PHA or TPA, a role for IL 2 in ALL colony formation through a direct interaction between IL 2 and IL 2 receptors on the clonogenic ALL cells is strongly suggested. Nevertheless, the alternative explanation for these findings, ie, that the effect of IL 2 had been indirect (on the feeder cells), has to be considered. Unfortunately, we have not yet been able to produce a cell-free source of stimulation that can efficiently replace the leukocytes, which is a prerequisite to address this problem.

Although we have, so far, only studied a small group of patients, a marked diversity in growth requirements was noted. In discrepancy with the essential culture conditions described above for patients No. 1 and 4, the cells from patient No. 6 produced colonies independently of exogenous growth factors. In addition, the leukemic cells of patients No. 2, 3, and 5 did not give rise to colonies, although they were capable of IL 2 receptor expression. This inability to proliferate may reflect different requirements for growth factors other than IL 2. For example, this could indicate that in certain cases the applied leukocyte feeder does not provide sufficient concentrations of these factors for proliferation.

Recently, the murine growth factor, IL 3, has been shown to support the growth and maturation of mouse pre-B cell clones.[17] It is possible that an analogous regulator produced by the feeder leukocytes is involved in pre-B ALL cell proliferation in humans.

ALL colony-forming cells were analyzed for CALLA expression by fluorescence-activated cell sorting (Table 3). In the two cases investigated, colony-forming cells were recovered from cell fractions with bright as well as dull or negative CALLA fluorescence. These findings are consistent with the CALLA positivity of ALL colony-forming cells, but they do not exclude the possibility of a coexistent CALLA-negative precursor subset. Further investigations along this line appear to be of extreme importance in view of the immunotherapeutic use of anti-CALLA monoclonal antibodies.

We have not obtained indications for the in vitro differentiation of ALL cells to a more mature cell type, neither in PHA- nor in TPA-supplemented colony cultures. The very limited capacity of ALL to differentiate in vitro has been noted by others,[19,20] and so far, only one case of ALL has been reported in which the leukemic cells matured to sIg-bearing B cells.[21] Recently, Ralph and co-workers demonstrated that high concentrations (ie, 10^3 to 10^4 U/mL) of IL 2 can induce the maturation of normal human B lymphocytes.[22] The question of whether high IL 2 concentrations are also effective in inducing differentiation of ALL cells cannot yet be answered.

We conclude that the induction of IL 2 receptors and the subsequent proliferative response in the presence of IL 2 in vitro found in ALL cells raises the possibility of a direct regulatory role of IL 2 in early stages of human B cell differentiation.

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CHAPTER 3

In Vitro Colony Forming Cells of Acute Lymphoblastic Leukemia: Analysis of 24 Cases with Recombinant Interleukin 2 as Growth Stimulus

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IN VITRO COLONY FORMING CELLS OF ACUTE LYMPHOBLASTIC LEUKEMIA: ANALYSIS OF 24 CASES WITH RECOMBINANT INTERLEUKIN 2 AS GROWTH STIMULUS

Ivo Touw, Willem Hofhuis, George van Zanen, Ruud Delwel and Bob Löwenberg

1. Introduction

In vitro colony assays are considered promising techniques for the detection of clonogenic leukemia cells. It is thought that leukemia cells, giving rise to colony growth in vitro (L-CFU), contribute to the perpetual growth of the tumor in vivo. Therefore, L-CFU are regarded as relevant in vitro end points for evaluating the remission status of leukemias, and for assessing the efficacy of purging procedures applied to autologous marrow transplants.

Efficient colony systems for acute myeloid leukemia (AML) exist since the mid-seventies (1-3). On the other hand, attempts at developing colony assays for acute lymphoblastic leukemia (ALL) have met less success.

Positive results on the growth of ALL colonies were initially reported by Izaguirre and colleagues, who used a colony system with both irradiated T cells and culture media conditioned by activated T cells added as growth stimuli (4). Although this system was later applied with good results to the colony growth of B cell chronic lymphocytic leukemia (5), its reproducibility for ALL appeared unsatisfactory (6). Using a modification of the colony system developed for AML in our laboratory (3), we have recently reported on the successful culturing of ALL colonies in 5 patients (7).

We established that both the lymphokine interleukin 2 (IL2) and an additional factor produced by peripheral blood leukocytes are essential stimuli for colony formation. Activation of the ALL cells with the lectin phytohaemagglutinin (PHA) or a phorbol ester is also required for colony growth. From suspension culture experiments, it became evident that in vitro activation induces membrane receptors for IL2, which had been lacking from the fresh ALL cells (7).

Here, we present the data of studies on IL2 receptor expression and in vitro colony formation in 24 cases of non-T ALL, including the immunological subtypes pre-B ALL, common ALL, and unclassified ALL.

2. Materials and Methods

2.1. Leukemia cells

ALL cells were isolated from the peripheral blood or bone marrow of newly diagnosed patients not receiving cytoreductive therapy. They were collected from light density fractions after Ficoll Isopaque centrifugation and sedimentation of E rosette forming lymphocytes (7). On the basis of immunologic phenotyping three subtypes of ALL were distinguished, i.e., pre-B ALL, common ALL and unclassified ALL (for definition see under table 2).

2.2. Immunofluorescence

Surface antigens on fresh and cultured cells were detected in indirect immunofluorescence. Cells were first treated with monoclonal antibody (30 min. at 0° C, followed by washing with phosphate buffered saline to remove excess antibody), and then with goat anti mouse immunoglobulin conjugated with fluorescein isothiocyanate (GAM/FITC) (30 min. at 0° C, followed by washing to remove excess GAM/FITC conjugate). Subsequently, the cells were prepared for fluorescence microscopy (Zeiss microscope with epiluminescence) or flow cytometry (FACS 440, Becton-Dickinson).
Surface and cytoplasmic immunoglobulins were assayed using goat anti human immunoglobulins coupled to FITC as described (7). The presence of terminal deoxynucleotidyl transferase (TdT) in the nuclei of cytocentrifuged cells was assessed using an indirect immunofluorescence kit (Gibco).

2.3. Monoclonal antibodies (MCA)

MCA used in this study with their specifications are listed in Table 1.

TABLE 1. Monoclonal antibodies

Antibody	Specificity	Source
VIL A1	common ALL antigen	Dr. W. Knapp, Vienna Austria
VIM 2	myelo monocytic antigen	Dr. W. Knapp, Vienna, Austria
BA 1	B cell associated antigen	Hybri tech, San Diego, USA
BA 2	B cell associated antigen (p 24)	Hybri tech, San Diego, USA
B 1	B cell specific antigen (p 30)	Coulter Corp., Hialeah, USA
B 2	B cell specific antigen (p 140)	Coulter Corp., Hialeah, USA
anti-IL2 receptor	membrane receptor for IL2	Becton - Dickinson, Mountain View, USA
WT1	T cell specific antigen	Dr. W. Tax, Nijmegen, The Netherlands

2.4. Colony culture

ALL colonies were grown in a double layer culture system in 35 millimeter diameter culture dishes (1.4 milliliter culture volume) supplemented with 1% vol./vol. PHA, recombinant IL2 (rIL2) (25 units per culture dish), and 2 x 10^6 irradiated (2500 rad) feeder leukocytes. Colonies containing 50 or more cells were scored on day 7 of culture. The colony system has been described in more detail elsewhere (7). After counting, colony cells were mass harvested and their phenotypes were assessed in indirect immunofluorescence.

2.5. Suspension culture

To evaluate ALL cells for their capacity to express membrane receptors for IL2 after in vitro activation, 2 x 10^6 cells were incubated for 18 hrs at 37° C/5% CO_2 in culture medium supplemented with 1% vol./vol. PHA (7). The cells were then prepared for indirect immunofluorescence with the MCA aIL2r (anti-IL2 receptor).

3. Results

3.1. Induction of membrane receptors for IL2 on ALL cells

Data on the presence of receptors for IL2 on the surface membrane of ALL cells are summarized in Table 2. In all cases, non-activated cells were IL2 receptor negative. On the other hand, significant proportions of the ALL cells of most patients expressed the IL2 membrane receptors, following incubation in vitro in the presence of PHA.

Table 2. Expression of IL2 membrane receptors in cases of non-T ALL

ALL immunologic subtype*	non stimulated cells expressing IL2 receptors	in vitro activated (PHA) cells expressing IL2 receptors		
	incidence of cases	incidence of cases	% of IL2 receptor expressing cells	
			mean	range
pre-B ALL (n = 8)	0/8	7/8	31	5-57
Common ALL (n = 12)	0/12	9/12	41	3-78
Unclassified ALL (n = 4)	0/4	4/4	21	6-30

pre-B ALL: TdT, CyIgM, CALLA positive

Common ALL: TdT, CALLA positive; CyIgM negative

Unclassified ALL: TdT positive; CALLA, CyIgM negative; no T cell or myeloid markers

3.2. Induction of ALL colony formation

Under optimal culture conditions, a linear colony response with titrated cell numbers plated in culture was apparent (Fig. 1).

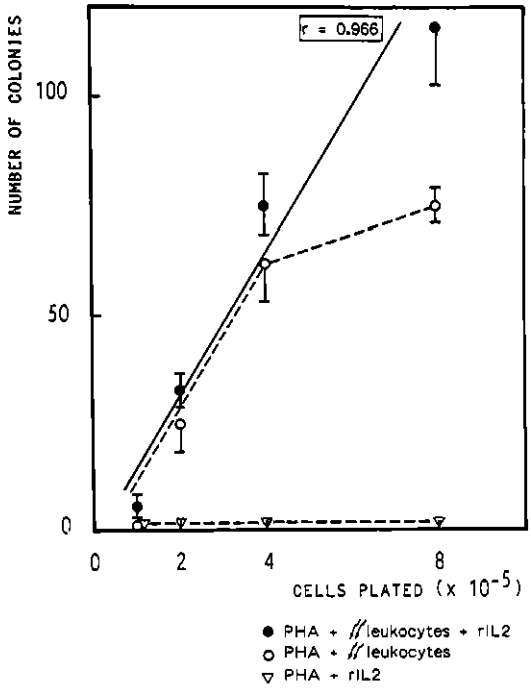

FIGURE 1. ALL colony formation with titrated cell numbers inoculated in culture under various stimulatory conditions. Optimal stimulation was obtained with PHA, rIL2 plus irradiated feeder leukocytes (●-●), and resulted in a linear colony response (r=0.966). O-O: stimulation with PHA plus feeder leukocytes but no IL2; ▽ - ▽: stimulation with PHA plus rIL2 but no feeder leukocytes.

Omission of IL2 from the cultures generally reduced colony formation, whereas the absence of feeder leukocytes resulted in a complete lack of colony growth in most cases (Fig. 1). Plating efficiencies among the different immunological categories of ALL are shown in Fig. 2. From the series of 24 cases, the cells of 18 patients gave rise to ALL colonies, thus indicating a positive colony response in 75% of non-selected cases. Plating efficiencies ranged from 15 to 127 per 10^5 plated cells (mean: 44 colony forming cells per 10^5). No significant differences were evident between the pre-B ALL, common ALL and unclassified ALL with respect to colony forming abilities.

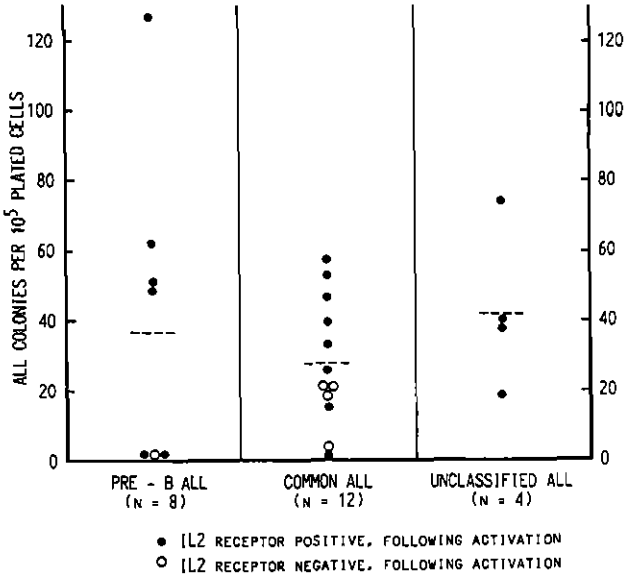

FIGURE 2. Plating efficiencies in different immunological subtypes of ALL Closed symbols: ALL cells expressed membrane receptors for IL2 after 18 hrs suspension culture (Table 2). Open symbols: ALL cells were IL2 receptor negative after suspension culture (Table 2), but in the colony forming cases more than 15% of the colony cells expressed IL2 receptors.

A comparison between childhood ALL (age younger than 12 yrs) and adult ALL (age 12 yrs and older) revealed that the culture system is equally permissive for colony growth of childhood and adult ALL (Fig. 3).

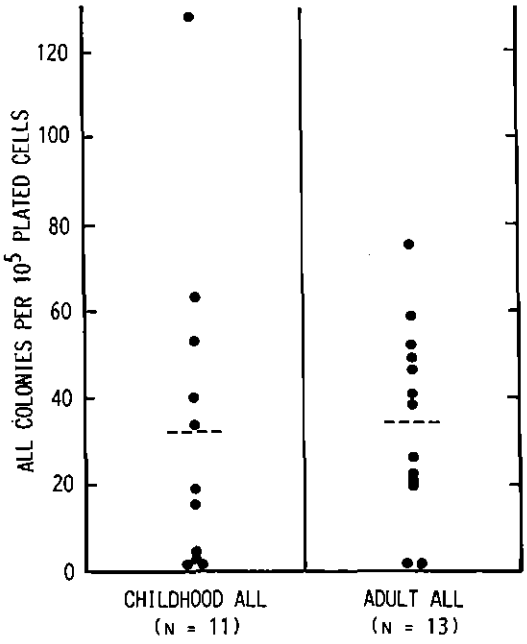

FIGURE 3. Plating efficiencies in childhood ALL and adult ALL

TABLE 3. Immunological markers expressed on fresh cells and on colony cells: Three examples of different immunological subtype.

Marker	CASE A (unclassified ALL)		CASE B (common ALL)		CASE C (pre B-ALL)	
	% positive fresh cells	% positive colony cells	% positive fresh cells	% positive colony cells	% positive fresh cells	% positive colony cells
VIL A1	0	0	91	22	60	53
BA 1	2	0	84	94	n.d.	n.d.
BA 2	0	0	67	64	82	69
B1	0	0	0	31	0	0
B2	0	0	0	0	0	0
Cy μ	0	0	0	0	50	29
sIg	0	0	0	0	0	0
E	0	0	0	0	0	0

3.3. Phenotyping of ALL colony cells

Morphological analysis of colony cells revealed that these had a lymphoblastic appearance. Immunofluorescence studies indicated that the immunophenotype of the colony cells closely resembled that of the fresh cells. Convincing indications for in vitro differentiation of ALL cells, as evidenced by the acquisition of new maturation antigens were not obtained. Representative colony phenotypes as compared with the preculture blasts of each immunological subtype of ALL are given in Table 3. In these analyses, colony cells always contained less than 10 per cent contaminant normal cells as assessed by E rosetting (T cells), immunofluorescence analysis with MCA WT 1 (T cells), VIM 2 (myelomonocytic cells) and staining of surface Ig (B cells).

4. Conclusion

The data presented in this paper confirm and extend our initial experiences on the in vitro growth of ALL colonies (7). The culture system, with IL2 and feeder leukocytes as growth stimuli, and PHA for the activation of the ALL cells, permits colony formation in 18/24, i.e., 75% of the cases. The system is efficient for the different immunological subtypes (i.e., pre-B, common, unclassified) of ALL and can be applied to childhood as well as adult leukemia. The linearity observed between colony response and cell numbers plated allows quantitative analysis. These features open perspectives for the general applicability of this colony assay in monitoring residual clonogenic ALL cells in bone marrow aspirates during the remission phase of the disease. For this purpose, it will be first necessary to investigate the behaviour of normal marrow progenitors in the colony system. It can be anticipated that the feeder leukocytes added to the culture will not only elaborate the factors into the medium required for ALL colony formation but also factors that stimulate the outgrowth of normal hematopoietic colony forming cells (e.g., CFU-GM). To avoid contaminant growth of non-leukemic cells it may be necessary to remove the normal progenitors by monoclonal antibody labeling followed by cell sorting or complement lysis procedures before plating the remission bone marrow sample in culture.

A second matter of importance, which deserves extensive investigation, is the question whether the cells forming colonies in the culture system represent the total ALL clonogenic cell population or only a subset of proliferative ALL cells. Testing the stimulating capacities of purified lymphokines, separately or in combination with other stimuli, will be necessary to address this problem.

Colony cultures are of great value for studying the cell biology of leukemia. For example, comparisons of the colony forming responses of leukemic cells and their normal counterparts to proliferation and differentiation stimuli will add to our understanding how growth and differentiation is altered in leukemia. At the same time, the analysis of factors required for leukemic cell growth may provide insights into the regulation of normal cell proliferation and differentiation. Recent studies have shown that IL2 stimulates the growth of normal as well as leukemic B lymphocytes, thus establishing that the activity of IL2 (previously termed T cell growth factor) is not limited to T cells (8-10). Very little is known about factors involved in early stages of B cell development. Our data on the induction of IL2 receptors on ALL blasts, and the stimulative effect of IL2 on ALL colony forming cells raise the possibility that IL2 acts on cells of the B cell lineage from the earliest recognisable differentiation stage, i.e., that of unclassified (non-T) ALL.

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CHAPTER 4

Variable Differentiation of Human Acute Myeloid Leukemia during Colony Formation *in Vitro:* a Membrane Marker Analysis with Monoclonal Antibodies

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Variable differentiation of human acute myeloid leukaemia during colony formation *in vitro*: a membrane marker analysis with monoclonal antibodies

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SUMMARY. The capacities of human acute myeloid leukaemia (AML) cells for differentiation were evaluated by the degree of maturation of the cells following *in vitro* colony formation. For this purpose highly purified blast cells were seeded into culture. Exogenous differentiation inducers, other than the colony stimulating materials, were not added to the cultures. Phenotypes of the colony cells were determined by use of monoclonal antibodies directed against a number of maturation antigens. In all 10 cases marked changes in the surface phenotypes of the cells post culture were seen. Differentiation was incomplete and quite variable as compared with normal myeloid colonies. Following colony formation myeloid maturation antigens became apparent on the cells of the one patient with morphologically undifferentiated leukaemia. The residual differentiation capacities among individual cases of human AML, as revealed *in vitro*, are diverse and do not parallel the morphological maturation features of the blasts prior to culture according to the FAB nomenclature. This approach may be utilized to improve the classification of human AML and to disclose the lineage relationship of certain cytologically unclassifiable leukaemias.

The induction of maturation in human myeloid leukaemias in culture has been studied with a number of differentiation inducing agents (i.e. phorbolesters, retinoic acid, dimethylsulfoxide). Thus, human leukaemic cell lines can be stimulated to mature (Koeffler, 1983). Although these AML-derived cell lines serve as useful model systems, they do not reflect the variability of the disease in individual patients. Attempts at differentiation induction of AML cells from individual patients have been carried out in suspension cultures. These studies

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demonstrated that AML cells, following exposure to phorbolesters or retinoic acid, did develop to macrophage-like cells in a variable proportion of cases (Breitman *et al.* 1981; Fibach & Rachmilewitz, 1981). Pure AML blasts were not used in the experiments, so that one cannot quantify the contribution of normal cell admixture to the different degrees of maturation.

In this report we describe the differentiation capacities of AML during proliferation *in vitro*. These studies were carried out using a colony culture technique to which leucocyte feeder cells and phytohaemagglutinin (PHA), but no specific differentiation inductive agents, were added. This system has been demonstrated specifically to grow acute myeloid leukaemia colonies as evidenced by cytogenetic analysis (Swart *et al.* 1982).

Purified blast cells from 10 patients with untreated AML were cultured. Murine monoclonal antibody (McAbs), directed against granulocyte and monocyte differentiation antigens as well as those specific for lymphocytic, erythroid and megakaryocyte lineages, were employed for the detection of surface antigens present on the blasts before and after culture.

MATERIALS AND METHODS

Human subjects. Ten patients with AML were studied at the time of diagnosis before chemotherapy was started. Either bone marrow aspirates (patients 1, 2, 4, 6 and 10), peripheral blood cells (patients 3, 5, 7 and 9) or a mixture of marrow and blood cells (patient 8) were used. Leukaemia subtypes were classified according to the FAB nomenclature (French–American–British Cooperative Group, 1976). The marrow cells of three normal donors were used to obtain reference data.

Cell separation. 97–100% pure leukaemic blasts were prepared from bone marrow aspirates and blood by means of discontinuous albumin gradient centrifugation and E rosette sedimentation as described previously (Swart *et al.* 1982). Normal marrow mononuclear cells were collected after Ficoll–Isopaque separation (Böyum, 1968).

Colony assays. Leukaemic colonies were grown in the PHA leucocyte feeder system as reported in detail (Swart *et al.* 1982). On day 7 of culture, colonies of 50 cells or more were harvested with a Pasteur pipette and a monocellular suspension was established by vigorous pipetting. Cells were washed twice with Hanks Balanced Salt Solution (HBSS) and prepared for immunofluorescence studies. It has previously been reported that contamination of the purified blast suspensions with T lymphocyte colony forming cells (TL-CFC) which interfere with leukaemic growth in these cultures does not occur under these conditions (Swart *et al.* 1982). Nevertheless, by means of E rosette tests, we always excluded that T cell colony growth was involved. Normal myeloid colonies were grown from bone marrow mononuclear cells in the Pike & Robinson double agar layer system, with minor modifications (Swart & Löwenberg, 1980), and in a colony method containing human placenta conditioned medium (HPCM), according to Burgess *et al* (1977). On day 12 of culture, dishes were flushed with HBSS, as a result of which the upper layer containing the colony cells is detached from the underlayer. The collected colony cells were washed twice with HBSS to remove residues of agar prior to immunofluorescence staining procedures.

Indirect immunofluorescence studies. The presence of surface antigens on uncultured and

cultured cells was assessed by indirect immunofluorescence. Following fixation with paraformaldehyde (Schuit *et al.* 1980), cells were incubated with murine monoclonal antibody and afterwards with goat anti-mouse Ig immunoglobulin coupled to fluorescein isothiocyanate (GAM/FITC) at 4°C according to standard procedures. Incubations were carried out in duplicate at two plateau concentrations of McAb to verify maximal labelling. GAM/FITC (Nordic Immunological Reagents. The Netherlands) was used in a 1:40 dilution, providing optimal brightness of the fluorescence. Two hundred cells per slide were scored for specific membrane fluorescence with a Zeiss fluorescence microscope using Plan-neofluar 25× and 63× lenses. Control incubations with GAM/FITC only, included in each test to check for nonspecific binding of the reagent to the cell surface, were negative in all experiments.

Monoclonal antibodies. The binding patterns of the monoclonal antibodies used in this study are summarized in Table I.

Table I. Specifications of monoclonal antibodies (McAb)

McAb	Titres used in indirect immuno-fluorescence assays	Reactivity	Reference or source
B2.12	1:500–1:1000	Granulocyte and monocytes	Van der Reijden *et al.* 1983
B4.3	1:500–1:1000	Granulocytes	Van der Reijden *et al.* 1983
B13.9	1:500–1:1000	Granulocytes	Van der Reijden *et al.* 1983
S4-7	1:500	Myeloblasts→granu-locytes, monocytes	Ferrero *et al.* 1983
IV B5	1:500–1:1000	Erythrocytes (glycophorin A)	Dr P. M. Lansdorp*
C2	1:500–1:1000	Platelets (glycoprotein IIa)	Dr P. M. Lansdorp
C15	1:500–1:1000	Platelets (glycoprotein IIIa)	Dr P. M. Lansdorp
WT1	1:100	All thymocytes and T lymphocytes	Tax *et al.* 1981
OKIa	1:40	HLA-Dr bearing cells	Ortho Pharmaceutical Corporation
OKT3	1:40	Mature thymocytes and T lymphocytes	Ortho Pharmaceutical Corporation
OKT6	1:40	Common thymocytes and epidermal Langerhans cells	Ortho Pharmaceutical Corporation

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RESULTS

The results from immunofluorescence studies on AML blasts before and after colony culture in the PHA-leucocyte feeder assay are summarized in Table II.

Myeloid and monocytic differentiation antigens

The colonies contained higher percentages of cells reactive with the marker B4.3 (granulocyte) and S4-7 (myelo-monocytic) as compared with the pre-culture cells in all instances tested. In five out of 10 cases a higher expression of granulocytic antigens reactive with the McAb B13.9 was evident after colony culture.

On the other hand, no significant shifts in reactivity with the myelomonocytic marker McAb B2.12 were apparent.

HLA-Dr ('Ia-like') antigens

Reactivity with the anti Ia McAb was not significantly altered after colony formation with the exception of patient 1. In this case, initially 5% of the leukaemic blasts were Ia positive, which increased to 72% in the colonies.

Erythroid and platelet associated antigens

In none of the cases the expression of the erythroid specific marker glycophorin A (reactive with McAb IVB5) or the platelet associated glycoproteins IIa and IIIa (reactive with McAb C2 and C15 respectively) was seen on the AML blasts prior to or after colony growth.

Induction of the T6 antigen during colony formation

In one patient (no. 4) 48% of the colony cells expressed the T6 antigen, normally present on a subpopulation of thymocytes. This antigen was not found on the cells before culture.

On the other hand, 39% of the pre-culture cells expressed another T cell marker, i.e. WT1, which could not be detected after culture. The cells did not react with anti-T3 McAb or with sheep red blood cells (E rosette formation) before or after colony formation.

Normal granulocyte-macrophage colonies

Reference immunofluorescence data from normal granulocyte-macrophage colonies were collected (Table III). Colonies were grown from three different bone marrow samples, in Pike & Robinson and HPCM assays. All three samples showed high reactivities with B2.12 (52–79%), B4.3 (44–75%) and with S4–7 (40–89%) after culture in both systems; the colony cells showed variable staining with B13.9 (18–65%), while reactivity with OKT6 was negative (less than 1%).

Table II. Membrane immunofluorescence with monoclonal antibodies of purified AML blasts before and after colony culture

		Percentage of cells reactive with monoclonal antibody*											
		B4.3		B13.9		B2.12		S4–7		Ia		T6	
Patient	Cytologic subtype (FAB)†	Before culture	Colonies	Before culture	Colonies	Before culture	Colonies	Before culture	Colonies	Before culture	Colonies	Before culture	Colonies
1	M1	0	64	0	6	0	1	24	93	5	72	0	2
2	M1	3	49	<1	16	28	30	0	47	83	50	1	4
3	M1	1	59	0	11	10	6	11	68	66	63	0	0
4‡	M2	10	31	<1	2	23	20	23	86	64	72	0	48
5	M2	3	45	0	40	11	19	n.d.	n.d.	62	59	0	2
6	M2	0	8	0	0	3	1	43	48	41	42	0	2
7	M4	<1	18	<1	0	7	1	17	79	64	58	0	0
8	M4	4	72	<1	<1	3	1	3	87	54	58	0	0
9	M4	21	46	8	8	17	8	53	68	58	68	6	5
10	U§	3	71	<1	64	42	56	2	21	26	12	0	0

Monoclonal antibodies are described in Table I. Purified AML blasts were plated in the PHA-leucocyte feeder assay for colony formation. The numbers of colonies per 10^5 plated blasts from these patients ranged from 127 to 1075 (mean $334/10^5$).

* Reactivity with McAb C2, C15 (antiplatelet) and IVB5 (antiglycophorin) was negative (less than 2% staining) in all patients before and after culture. Data from patients 3, 7 and 10 represent means of two separate experiments. Positive staining in individual experiments differed less than 6% from these mean values.

† Morphologic subtypes based on the classification of the French–American–British Cooperative Group.

‡ Blast cells of this patient were tested for T lymphoid markers, i.e. E rosetting and McAb OKT3 and WT1. Prior to culture 39% of the blasts stained with WT1, whereas E and T3 fluorescence were negative. 11% of the blast cells were positive in Sudan black B staining; non specific-esterase, acid phosphatase and Periodic acid Schiff stainings were all negative. Colony cells had lost WT1 reactivity (0% staining) while E and T3 fluorescence remained negative.

§ Undifferentiated leukaemia, negative for Sudan black B, non specific-esterase, acid-phosphatase and Periodic acid Schiff stainings.

Table III. Membrane immunofluorescence of normal GM-colony cells with a series of monoclonal antibodies (McAb)

	Percentage positive colony cells					
	A		B		C	
McAb	Leucocyte feeder	HPCM	Leucocyte feeder	HPCM	Leucocyte feeder	HPCM
B2.12	73	53	70	52	—*	79
B4.3	56	44	65	75	—	74
B13.9	19	18	52	40	—	65
S4–7	46	46	40	72	—	89
T6	<1	<1	<1	<1	—	—

Bone marrow samples from three different donors designated A, B and C. Two colony methods were applied: one with a leucocyte feeder according to Pike & Robinson (1970) and one with human placenta conditioned medium as stimulator.

* Not determined.

DISCUSSION

Acute myeloid leukaemias display a morphological and functional block in differentiation. The above-described experiments were designed to determine the capacities of AML cells from individual patients to differentiate during proliferation in culture. Maturation stages of AML colonies were compared with those of the noncultured blast cells. After colony growth, the progeny of AML cells exhibited a more mature myeloid phenotype than did the preculture blast population (Table II). This was evident in all cases and thus appears to be a common feature in human AML. Recently, it has been shown in our laboratory that the B4.3 antigen was not expressed on the leukaemic colony forming cells from patients 1 and 3, which supports the suggestion that this marker was acquired during proliferation and differentiation in culture (Wouters & Löwenberg, 1984).

Marie *et al* (1981) have previously demonstrated that the number of cells carrying a granulocyte maturation antigen (My-1) was higher following AML colony formation as compared with preculture values. These authors did not use purified blast suspensions in their studies to avoid interference of normal cells with AML phenotyping and therefore the question as to whether AML cell differentiation occurred during *in vitro* colony growth was not answered conclusively. In another series of experiments (Ozawa *et al*, 1983) differentiation in colony culture of progenitor cells of individual AML patients was suggested on the basis of cytochemical staining of the colonies. Limited data of the preculture blast phenotypes were given, so that a strict comparison between pre and post culture data cannot be derived from their studies. The experiments reported herein with pure blast cells and an extended number of monoclonal antibodies add evidence that human AML cells undergo further differentiation into granulocytic-monocytic directions upon proliferation *in vitro*.

We used mass harvested cells for the analysis after culture. One could raise the question as to whether the non-colony cells on the plates contributed to the results. We estimated that the numbers of single cells in the culture dishes represented less than 1% of the cells present in colonies. This makes it unlikely that individual cells persisting in culture are a significant factor in the observed phenomena of differentiation.

As compared with the progeny of normal granulocyte-macrophage colony formers (Table II), the expression of myeloid surface markers on the AML colony cells was incomplete. Morphologically, the AML cells found in the PHA-leucocyte feeder colony cultures did not mature to granulocytes or monocytes but remained of blast appearance (Swart *et al.* 1982). Apparently, AML cell differentiation during colony formation was partial, also from a morphological point of view. It appears unlikely that differences between normal and leukaemic colonies were due to the variations in the culture techniques. In patients from which we were able to grow colonies in the Pike & Robinson assay (patients 3, 4, 8 and 10) the colony cells showed phenotypes comparable to those obtained from the PHA-leucocyte feeder system (data not shown).

The finding that colony cells of patient 4 expressed the common thymocyte related antigen T6 was unexpected. Earlier, the expression of myeloid markers on acute lymphoblastic leukaemia cells has been reported (Bettelheim *et al.* 1982). It was suggested that this phenomenon may reflect derepression of gene activity. Our observation that the T6 marker was acquired in culture while it had been completely absent from the preculture AML blasts (also confirmed by Fluorescence Activated Cell Sorter analysis; data not shown) raises the possibility that leukaemic cells showed aberrant gene expression as a result of proliferation in culture. Alternatively, a certain relationship between the AML cells of this case and the T6 positive epidermal Langerhans cells, which belong to the haematopoietic system (Volc-Platzer *et al.* 1984), may be suggested by this observation.

Although we investigated only a limited number of patients, it appears that the capacity of the AML blasts to acquire myeloid differentiation antigens during *in vitro* colony formation is not related to the morphological maturation stage of the preculture cells. Analysis of the maturation of AML blasts cells in colony culture provided information on the differentiation capacities of the leukaemic cells that was at variance with the differentiation stages as defined by means of FAB criteria. We suggest that the application of colony culture systems in combination with cell surface marker analysis for determining the differentiation capacities of proliferating leukaemic blasts in individual patients, may eventually be of use for refining the current classification of leukaemias based on morphological and cytochemical parameters.

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CHAPTER 5

Interleukin 2 Stimulates Chronic Lymphocytic Leukemia Colony Formation *In Vitro*

Blood 66:237-240, 1985

CONCISE REPORT

Interleukin 2 Stimulates Chronic Lymphocytic Leukemia Colony Formation In Vitro

By Ivo Touw and Bob Löwenberg

The requirements of clonogenic cells of B cell-type chronic lymphocytic leukemia (B CLL) for interleukin 2 (IL 2) were analyzed. Using the cells of five patients, we measured IL 2 receptor expression on the cell surface and the colony-forming abilities of the cells in response to IL 2. In four of the cases, significant percentages of the CLL cells expressed IL 2 membrane receptors (as assessed with the monoclonal antibody anti-Tac), indicative of their potential sensitivity to IL 2. Pure recombinant interleukin 2 (r-IL 2) was added to colony cultures that also contained the lectin phytohemagglutinin (PHA) or the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) to activate the CLL cells. Colony formation completely depended on the presence of r-IL 2 and PHA or TPA in culture, with the exception of one case, in which the addition of IL 2 was not required for colony growth in TPA-supplemented cultures. Twenty-five to fifty units of r-IL 2 per milliliter of culture medium provided optimal stimulation. Under these conditions, a linear relationship was observed between plated cell numbers and colony numbers formed. Morphological and immunologic analysis of colony cells indicated that these were monoclonal CLL cells that had matured toward plasmacellular lymphocytes and plasma cells.

INTERLEUKIN 2 (IL 2) is the soluble factor required for the in vitro proliferation of activated T lymphocytes.[1] With the availability of pure IL 2 preparations (obtained by recombinant techniques)[2] and the development of monoclonal antibodies reacting with IL 2 receptor molecules on the cell surface membrane,[3,4] it now becomes clear that IL 2 can also directly act on normal human B lymphocytes as well as on neoplastic B cell progenitors, and induce a proliferative response of those cells.[5-9]

Earlier, we reported that the proliferation of common and pre-B acute lymphoblastic leukemia (ALL) cells depends on IL 2 plus additional leukocyte factor(s). On this basis, a colony culture technique for ALL has been developed.[8,9] Using a modification of this culture assay, it is shown here that B cell-type chronic lymphocytic leukemia (B CLL) cells, when activated by the lectin phytohemagglutinin (PHA) or the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA), form colonies in response to recombinant IL 2 (r-IL 2). In contrast to clonogenic ALL cells, CLL colony formers do not require extra leukocyte stimulation for proliferation.

MATERIALS AND METHODS

Patients and separation of leukemia cells. Nucleated cells of five patients with CLL were isolated from the peripheral blood by Ficoll-Isopaque separation. None of the patients had received cytoreductive therapy for at least 12 weeks prior to examination. Residual T lymphocytes were removed from the Ficoll interface cells by rosetting with 2-aminoethylthiouronium bromide (AET)-treated sheep erythrocytes and sedimentation of the rosetted cells through Ficoll-Isopaque.[9] In the non-rosetting (less than 0.5% positive) leukemic cell fractions, 97% to 100% of the cells were characterized morphologically as small lymphocytes.

Colony culture. Colony cultures were performed in 35-mm diameter culture dishes (1.4 mL culture volume), essentially as described for non-T ALL.[8] However, one major modification was introduced, ie, that feeder leukocytes were not included in the culture system. Instead, pure r-IL 2 (Biogen SA, Geneva) added to the culture upper layer served as stimulator in combination with either 0.75% vol/vol PHA (reagent grade, Wellcome, Dartford, England) or 70 ng/mL TPA (Sigma Chemicals, St Louis). Colonies (50 cells or more) were counted on day 7 of culture. Colony cells were then mass-harvested with a Pasteur pipette, washed three times with phosphate-buffered saline, and prepared for immunofluorescence, morphological and cytochemical analysis (May-Grünwald-Giemsa, nonspecific esterase and Sudan black B staining of cytocentrifuged cells), and E rosette formation.

Immunofluorescence studies. The presence of immunoglobulin (Ig) chains on the cell surface and in the cytoplasm was assessed by immunofluorescence microscopy as described,[8] using goat anti-human (GaHu) immunoglobulin antisera coupled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). GaHu IgG/TRITC, GaHu κ/FITC, GaHu λ/TRITC (Kallestad, Austin, Tex), GaHu IgD/FITC, GaHu IgM/TRITC, and GaHu IgA/FITC (Nordic, Tilburg, The Netherlands) were all used in titers of 1:40.

In dual staining procedures, incubations with TRITC-coupled antisera always preceded those with the FITC-coupled antibodies. Cell surface membrane receptors for IL 2 were assayed in indirect immunofluorescence using the monoclonal antibody anti-Tac[3] and a goat anti-mouse Ig FITC conjugate (GAM/FITC; Nordic).

RESULTS

Colony stimulation. In four of the five cases, a significant proportion of the fresh CLL cells was found to express IL 2 receptors on the cell surface (Table 1). Culture experiments then disclosed that B CLL colony formation was induced by IL 2 when PHA or TPA was added as well. No colonies were formed in cultures containing IL 2 alone, PHA or TPA alone, or without any of these substances (Table 1). As an exception, TPA alone induced colony growth from the cells of patient No. 4. In all instances, colonies consisted of monoclonal B cells (see below); E rosette tests, and Sudan black B and nonspecific esterase stainings of cells harvested from the cultures were negative, indicating that contaminating T lymphocyte and myelomonocytic growth did not occur. To assess the optimal dose of IL 2 required for colony

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Table 1. IL 2-Dependent Colony Formation in Five Cases of B CLL

Patient No.	Peripheral Blood Leukocyte Count ($\times 10^{-9}$/L)	Percentage of Tac-positive CLL Cells	Colony No. per Culture Dish*					
			PHA + IL 2	TPA + IL 2	PHA	TPA	IL 2	None†
1	379	36	38	24	0	0	0	0
2	244	61	26	8	0	0	0	0
3	44	80	16	36	0	0	0	0
4	123	85	44	70	0	66	0	0
5	15	0	19	31	3	0	0	0

*CLL cells (2×10^5) were cultured per dish; values are means of triplicate cultures.

†Colony stimulation: PHA, 0.7% vol/vol; TPA, 100 ng per culture dish; IL 2, 25 units per culture dish (culture volume, 1.4 mL).

formation, IL 2 was added in increasing amounts (0.05 to 1,000 units) to the culture dishes. In PHA-supplemented cultures, colony numbers (patient No. 1) already reached plateau values at 10 units of IL 2 per dish (Fig 1A). In contrast, a maximal size of colonies was obtained at a higher dose, ie, 50 units per dish. Further increases of IL 2 concentrations in culture (100 to 1,000 units per dish) reduced the colony size (Fig 1A). Similar results were obtained with the cells of patients No. 3 and 5 in TPA plus IL 2-supplemented cultures. A cell titration experiment performed in the presence of 50 units of IL 2 (optimal growth) revealed a linear relationship between cell numbers plated and colony numbers formed (Fig 1B). This feature makes the colony system suitable for quantitative studies.

The polyclonal B cell mitogen *Staphylococcus aureus* strain Cowan I was ineffective in the induction of IL 2-stimulated B CLL colony growth either alone or in combination with PHA or TPA.

Immunologic and morphological analysis. Immunofluorescence analysis was performed on the cells after colony formation and the immunophenotypes of the colony cells were compared with those of the preculture cells. These studies revealed that high percentages of colony cells expressed the same immunoglobulins with single light chains in the cytoplasm or on the cell surface as the CLL cells prior to culture (Table 2). Bright cytoplasmic immunofluorescent staining suggested plasmacellular maturation within the CLL colonies. Substantial morphological changes were noted during colony growth in the TPA- and IL 2-stimulated cultures, indicative of the differentiation of CLL colony cells into plasma cells (Fig 2, a2 and b2). In the PHA- and IL 2-supplemented cultures, the majority of colony cells resembled plasmacellular lymphocytes (Fig 2, a1 and b1). In all cases, 15% to 20% of the colony cells expressed IL 2 receptors on their cell surface.

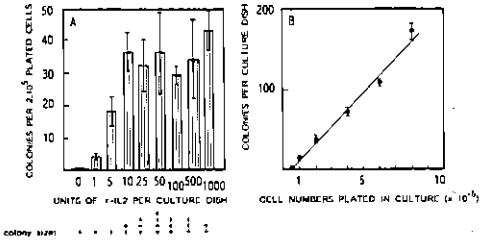

Fig 1. B CLL colony formation (patient No. 1) induced by r-IL 2 and PHA. Colony numbers are expressed as mean values of triplicate cultures ± SD. (A) Colony numbers and size following stimulation with titrated concentrations of r-IL 2 (culture volume, 1.4 mL). Estimated cell numbers present in the predominant type colonies serve as a measure of colony size. +, 50 to 100 cells per colony; ++, 100 to 200 cells per colony; +++, 200 to 500 cells per colony; ++++, 500 to 1,000 cells per colony. (B) Linear dose–response relationship ($r = .9935$) between cell numbers plated and the numbers of colonies formed in the presence of 50 units of r-IL 2 per culture dish.

DISCUSSION

It has been shown previously that B CLL cells produce colonies in vitro following activation by the plant lectin PHA.[11,12] Successful culturing of the CLL colonies was dependent on the presence of (irradiated) T lymphocytes. The nature of the stimulatory components produced by the T lymphocytes has remained unknown. Moreover, the question of whether the lectins exerted a direct effect on the clonogenic CLL cells or an indirect influence through the T cells (or both) has not been answered. The data presented in this report show that lectin (PHA)- or phorbol ester (TPA)-activated B CLL colony formation occurs in the absence of T lymphocytes (or other stimulator cells) when pure IL 2 is added to the cultures as the single source of stimulation (Table 1). Membrane receptors for IL 2 were found on high percentages of the fresh CLL cells, with the exception of patient No. 5 (Table 1). In addition, such receptors were also detectable on the colony cells, including those of patient No. 5. These results provide evidence that IL 2 in the presence of PHA or TPA directly stimulates CLL proliferation.

In common and pre-B ALL, PHA and TPA were found to induce IL 2 receptors on the cell membrane initially not expressed on the untreated cells.[8,9] The role of these factors in CLL proliferation is not immediately clear, as high percentages of untreated CLL cells may already express IL 2 receptors (Table 1). Recent results from ^{3}H-thymidine incor-

Table 2. Immunoglobulin Chains in CLL Colony Cells

Patient No.	Cytoplasmic Ig		Cell Surface Ig	
	Positive	Negative	Positive	Negative
1*	M (95) λ (95)	A, D, G, κ	Mλ (14)	A, D, G, κ
2*	M (28) κ (89)	A, D, G, λ	M + Dκ (95)	A, D, G, λ
3†	M (45) κ (64)	A, D, G, λ	M + Dκ (84)	A, D, G, λ
4*	M (87) κ (84)	A, D, G, λ	Mκ (50)	A, D, G, λ
5†	M (64) κ (65)	A, D, G, λ	M + Dκ (84)	A, D, G, λ

Numbers in parentheses are the percentages of positive colony cells (200 cells counted).

*Assessed in PHA + IL 2-induced colonies.

†Assessed in TPA + IL 2-induced colonies.

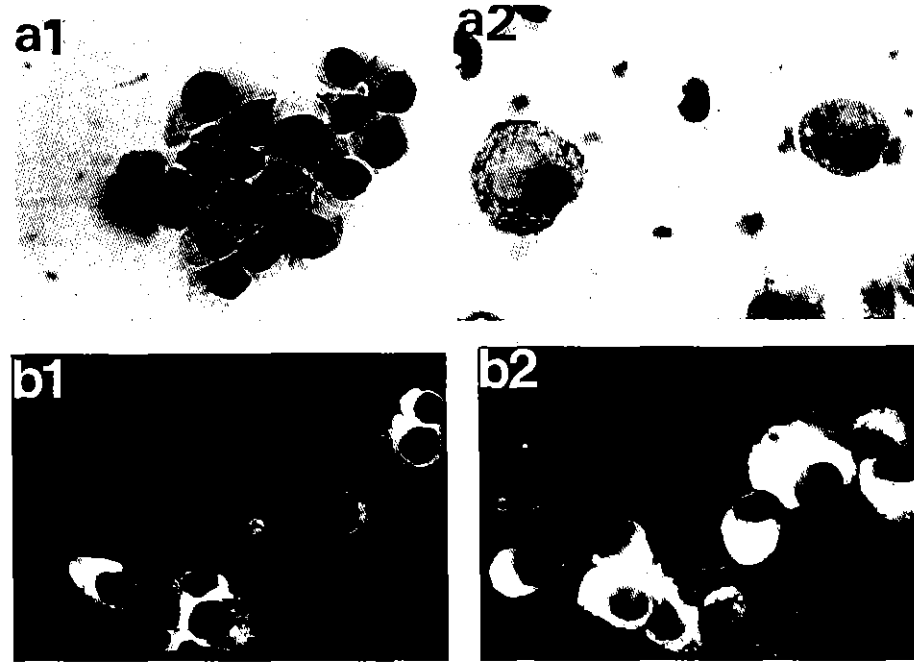

Fig 2. May-Grünwald-Giemsa morphological (a) and anti-κ Ig light chain/FITC immunologic staining of cytocentrifuged colony cells. a1 and b1, PHA plus IL 2-induced colonies (patient No. 2); a2 and b2, TPA plus IL 2-induced colonies (patient No. 5). Original magnification ×630; current magnification ×410.

poration experiments suggest that PHA (or TPA) activation of the CLL cells is required for the continuous expression of IL 2 receptors on the proliferating cells in culture from day 3 on (Ivo Touw and Bob Löwenberg, unpublished data).

Morphological analysis revealed maturation of the CLL colony cells. In the TPA-supplemented cultures, colony cells expressed the morphology of plasma cells (30% to 75%) or intermediate plasmacellular differentiation stages (Fig 2a2 and b2). In vitro differentiation of CLL cells induced by TPA has been noted by others.[13,14] In the colonies formed in the cultures stimulated with PHA, plasma cell development was less prominent (2% to 5%). Nevertheless, the abundant presence of M and κ or λ immunoglobulin chains in the cytoplasm (Table 2) and the plasmacytoid appearance of the colony cells (Fig 2a1 and b1) indicated that maturation occurred to some extent. High concentrations of IL 2 have recently been reported to induce plasmacellular maturations of normal B lymphocytes.[15] We wondered whether higher IL 2 concentrations in PHA cultures would drive CLL colony cells toward further differentiation stages, but morphological analysis of the colony cells grown in the presence of 500 to 1,000 units of IL 2 did not provide evidence for this.

The cells of patient No. 4 were exceptional in that they did not require addition of IL 2 to culture for TPA-activated colony formation (Table 1). This raised the question of whether minimal numbers of residual (E-positive) T lymphocytes had been responsible for the effect through the elaboration of IL 2 into the culture medium. This possibility appears unlikely, since a second E rosette depletion of the CLL cells did not eliminate colony growth in cultures with TPA alone (data not shown). More plausible explanations are that the CLL cells of this patient produced stimulatory components themselves or that their colony-forming abilities do not depend on growth factors. These alternatives are presently under investigation.

To this end, it appears that IL 2 has become established as a growth and differentiation inducer of normal and neoplastic B cells,[5-9,15] including CLL colony-forming cells. Notably, CLL cells proliferate in vitro without the need of other growth factors, similar to normal B lymphocytes. The existence of other lymphokines with the capacity to stimulate B cell proliferation, referred to as B cell growth factors (BCGFs), has been postulated in a large series of publications, as recently reviewed.[16] In order to understand the putative role of BCGFs in normal and neoplastic B cell regulation additional to IL 2, purification to homogeneity of these activities is now of crucial importance.

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CHAPTER 6

The Proliferative Response of B Cell Chronic Lymphocytic Leukemia to Interleukin 2: Functional Characterization of the Interleukin 2 Membrane Receptors

Submitted for publication

THE PROLIFERATIVE RESPONSE OF B CELL CHRONIC LYMPHOCYTIC LEUKEMIA TO INTERLEUKIN 2: FUNCTIONAL CHARACTERIZATION OF THE INTERLEUKIN 2 MEMBRANE RECEPTORS

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In order to determine the growth properties of B cell chronic lymphocytic leukemia (B CLL) and to identify possible abnormalities thereof, we examined the in vitro action of interleukin 2 (IL2) in 4 patients. Using radiolabeled IL2 and monoclonal antibodies reactive with IL2 membrane receptors we show that CLL cells, following their activation in vitro, express IL2 receptors of high as well as low affinity type, exactly as has been reported for normal T and B blasts. In three of the four reported cases, CLL proliferation (measured with ^{3}H-thymidine incorporation) depended on the addition of phytohaemagglutinin (PHA) to activate the cells and IL2 (optimal concentration 10-100 units of IL2 per ml). In contrast, the cells of the fourth case of CLL (CLL-4) proliferated in an autonomous fashion, i.e., without a need for PHA and IL2 in culture. Specific blocking of the IL2 binding sites with anti IL2 receptor monoclonal antibodies almost completely inhibited the proliferation of these cells, indicating that functional IL2 receptors were required for the autonomous proliferation. The demonstration of low concentrations of IL2 activity in the culture medium conditioned by the cells suggests that endogenous IL2 had been responsible for the spontaneous ^{3}H-thymidine uptake by the CLL cells of patient 4. However, we were unable to extract IL2 mRNA from the cells (neither fresh nor after various in vitro incubations) in quantities detectable by Northern blot analysis which would prove that the CLL cells of patient 4 were actively synthesizing IL2 during culture. Thus, individual cases of B CLL are subject to variable growth regulation involving functional IL2 receptors on the cell surface: a) following activation with PHA the cells respond to exogenous IL2 in a fashion similar to normal B lymphocytes, or b) the cells are stimulated by endogenous IL2 (or an IL2-like activity) and do not require activation with PHA.

INTRODUCTION

Interleukin 2 (IL2) is one of the polypeptide hormones controlling the proliferation and differentiation of B cells via surface membrane receptors (1-10). Studies in our laboratory have focused on the role of IL2 in the proliferation of leukemic B cells, one of the purposes being to analyze whether the leukemic cells express abnormal responsiveness to the hormone (11). Using a colony culture technique, we have shown that in acute lymphoblastic leukemia (ALL) of B cell origin, i.e., common ALL and pre-B ALL, IL2 stimulates proliferation, although only when additional factors derived from leukocyte feeder cells are present in culture as well (12,13). The nature of the latter factors has not been established yet.

In B cell chronic lymphocytic leukemia (B CLL), IL2 as a single stimulus induces in vitro colony formation, provided that the CLL colony forming cells are activated with the phorbol ester 12-O-tetradecanoyl phorbol 13 acetate (TPA) or the lectin phytohaemagglutinin (PHA) (14). Proliferation of CLL cells stimulated by IL2 and TPA has also been reported in ^{3}H Thymidine incorporation assays (15).

In the present study we characterize the function of the IL2 membrane receptors of B CLL cells by using a radiolabeled IL2 preparation and further analyze IL2 responsiveness in micro culture. We demonstrate that in vitro activated CLL cells express two classes of IL2 receptors, one with a high affinity and one with a low affinity for IL2, a property which these cells share with normal activated T and B blasts (5). Results from the micro culture experiments show that CLL cells of three patients require IL2 and activation by PHA for proliferation. On the other hand in a fourth case, CLL cells proliferate without supplementation of IL2 and PHA to the cultures. The results of additional experiments suggest that the "spontaneous" proliferation of these CLL cells is in fact not IL2 independent, but controlled by endogenous IL2.

MATERIALS AND METHODS

Leukemic cells

CLL cells were isolated by Ficoll-Isopaque separation from the peripheral blood of patients which had not received cytoreductive therapy for at least 12 weeks (16). T lymphocytes were removed by rosetting with 2-aminoethyl-thiouronium bromide (AET) - treated sheep erythrocytes followed by a second Ficoll-Isopaque separation (17). The T cell depleted leukemic cell fractions contained 97-100% small lymphocytes and always less than 0.5% E-rosette forming cells. Peripheral blood leukocyte counts and immunotypes are listed in Table 1.

TABLE 1. HEMATOLOGICAL DATA OF CLL PATIENTS

Patient	Peripheral blood leukocyte count ($\times 10^{-9}$/l)	Immunotype	
		Ig heavy chain	Ig light chain
CLL ≠ 1	379	M	λ
CLL ≠ 2	161	M+D	κ
CLL ≠ 3	268	M+D,A,G	κ
CLL ≠ 4	152	M+D	λ

Cell cultures

Proliferation of CLL cells was measured by ^{3}H thymidine (^{3}H TdR) uptake. Cells (1×10^{5}) were cultured in triplicate in round bottom microtiter wells (Greiner, Alphen a/d Rijn, The Netherlands) in 100 µl culture medium. This culture medium consisted of Dulbecco's modified Eagle's minimal essential medium, heat inactivated fetal calf serum (6.7% v/v), heat inactivated horse serum (6.7% v/v), trypticase soy broth (6.7% v/v) supplemented with dialysed bovine serum albumin (0.75% w/v), egg lecithin (3×10^{-5} M), $Na_2 Se O_3$ (10^{-7} M), iron saturated human transferrin (7.7×10^{-6} M)), and beta-mercaptoethanol (10^{-4} M) as modified from Guilbert and Iscove (18). Additions to the cultures included 0.1% v/v phytohaemagglutinin (PHA, reagent grade, Wellcome, Dartford, England), pure recombinant IL2 with a specific activity of 1.9×10^{6} units per milligram protein or approximately 3×10^{13} units per Mol (Biogen SA, Geneva, Switzerland), and monoclonal antibodies reactive with IL2 membrane receptors (see below). Cultures were performed in triplicate. Sixteen hours before harvesting of the cells (Titertek cell harvester 550), 0.1 µCi of ^{3}H-TdR (Amersham, U.K.) specific activity 2 Ci/mmol) was added to each microwell. Radioactivity was measured by liquid scintillation counting (Beckmann LS 3800).

Suspension cultures of CLL cells and normal peripheral blood mononuclear cells were performed in test tubes a) to assess IL2 activities elaborated into the culture medium, b) to extract RNA from the in vitro stimulated cells, and c) to determine binding of radiolabeled IL2 to in vitro stimulated cells. Cells (2×10^{6} per ml culture medium) were incubated either with PHA (0.1% v/v) plus TPA (10 ng/ml), PHA alone, or without additions. After various incubation periods (ranging from 4 hrs to 7 days) followed by centrifugation, the culture supernatants were collected, filter sterilized (0.22 um filter, Millipore, Bedford, Mass.) and stored at -20° C. For the purpose of RNA extraction, the cells were washed twice with Hanks Balanced Salt solution (HBSS), collected in Eppendorf micro centrifugation tubes, quickly frozen (30 sec.) in liquid nitrogen and stored at -80°C. For radiolabeled IL2 binding experiments, the cells were incubated for one hour in fresh culture medium and washed five times with ice cold alpha-medium supplemented with 1% w/v BSA.

IL2 producing Jurkat cells (a gift from Dr. L.A. Aarden, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) were cultured in RPMI medium supplemented with 10% heat inactivated FCS in T75 culture flasks (Greiner). For induction of IL2 mRNA the cells were cultured in the presence of PHA (1% v/v) and TPA (10 ng/ml) for 6 hrs. After two washings with HBSS, the cells were collected for RNA extraction, frozen in liquid nitrogen and stored at -80°C as described above.
Murine IL2 dependent CTLL cells (a gift from Mrs. S. Knaan-Shanzer, Radiobiological Institute TNO, Rijswijk, The Netherlands) were cultured as described (19). To estimate IL2 concentrations in CLL culture supernatants, 10, 20 and 40% v/v of these supernatants were tested for their stimulatory effects on the proliferation of the CTLL cells. These estimations were compared with the proliferative response of the CTLL cells to titrated rIL2 concentrations.

Cytogenetic analysis

Spread metaphases of cultured CLL cells were checked for cytogenetic abnormalities using Q-, R-, and G Banding Techniques (20). These analyses were performed by Dr. A. Hagemeijer and Mrs. E.M.E. Smit (Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands).

Monoclonal antibodies reactive with IL2 receptors

Four MoAbs reactive with membrane receptors for IL2 were used: three of these, i.e., mouse MoAbs anti-IL2 receptor (Becton Dickinson, Mountain View, California), anti-TAC (21) and rat MoAb 18 E 64 (22) compete with IL2 for binding to the receptor.
The fourth, rat MoAb 36 A 1.2., has been shown not to interfere with IL2 binding (22).

Indirect immunofluorescence

For immunologic detection of IL2 membrane receptors, cells were treated with anti IL2 receptor MoAb and with goat anti mouse immunoglobulin coupled with fluorescein isothiocyanate (GAM/FITC, Nordic, Tilburg, The Nether-

lands) and analyzed on a fluorescence activated cell sorter (FACS 440, Becton-Dickinson, Sunnyvale, CA) as described (12).

RNA extractions, Northern blotting and hybridization with IL2 cDNA probe

RNA was extracted by two different methods yielding comparable amounts and quality of total RNA, i.e., hot phenol extraction (in the presence of Vanadyl-ribonucleoside complexes to inhibit RNase activity), and the Guanidinium/Cesium chloride method (23). Poly $(A)^+$ RNA was isolated by paper affinity chromatography using poly (U) coated paper (Medac, Hamburg, Germany) (24). RNA was electrophoresed on formaldehyde-agarose gels (21) and blotted onto nylon hybridization membrane (Gene Screen Plus, New England Nuclear, Boston, Massachusetts). The human IL2 cDNA used for hybridization was obtained from TPA stimulated Jurkat cells and cloned in a G-tailed pBR322 vector (prepared and kindly supplied by Dr. H. Pannekoek, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) before use in hybridization. The gel purified IL2 cDNA fragment containing nucleotides 377-837 (24) was isolated from the vector by digestion with Pst I (Boehringer, Mannheim, FRG) followed by polyacrylamide gel electrophoresis of the digest. The IL2 cDNA fragment was radioactively (^{32}P) labeled by random priming using oligodeoxynucleotides, essentially according to Feinberg and Vogelstein (26). Hybridizations were carried out as described (23). Autoradiography using Kodak Xomat AR X-ray film occurred at -70°C in the presence of an intensifying screen.

Binding of radiolabeled IL2

Before treatment with radiolabeled IL2, the cells were incubated (1 h at 37° C) in fresh culture medium and washings (5 times with alpha medium containing 1% w/v BSA) to remove endogeneous IL2 (24). Then, 10^6 cells in 50 µl alpha-BSA medium were placed in wells of medium pre-wetted (alpha-BSA) millititer 96 well 5.0 µm filter bottom plates (Millipore). To determine the amount of specific binding, serial dilutions of pure, recombinant ^{125}I IL2 (Amersham, U.K.) ranging from 6.25 - 800 pM were added to the cells. The cells were incubated with the radiolabeled IL2 for one hour at 37° C. These conditions allow for optimal saturation of high and low affin-

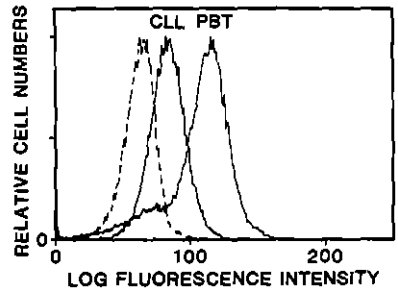

Figure 1

Flow cytometric fluorescence (histogram) analysis of IL2 receptors on activated CLL cells (CLL ≠ 3) and activated normal peripheral blood T lymphocytes (PBT)

Following 36 hrs of in vitro incubation in the presence of 0.1% v/v PHA and washings, cells were stained with MoAb aIL2r and GAM/FITC. The dotted curve represents the fluorescence profile of the cells stained with GAM/FITC alone. Staining with a nonrelevant MoAb of the same immunoglobulin subclass as MoAb aIL2r (IgG1) revealed a fluorescence profile identical to that of the GAM/FITC control.

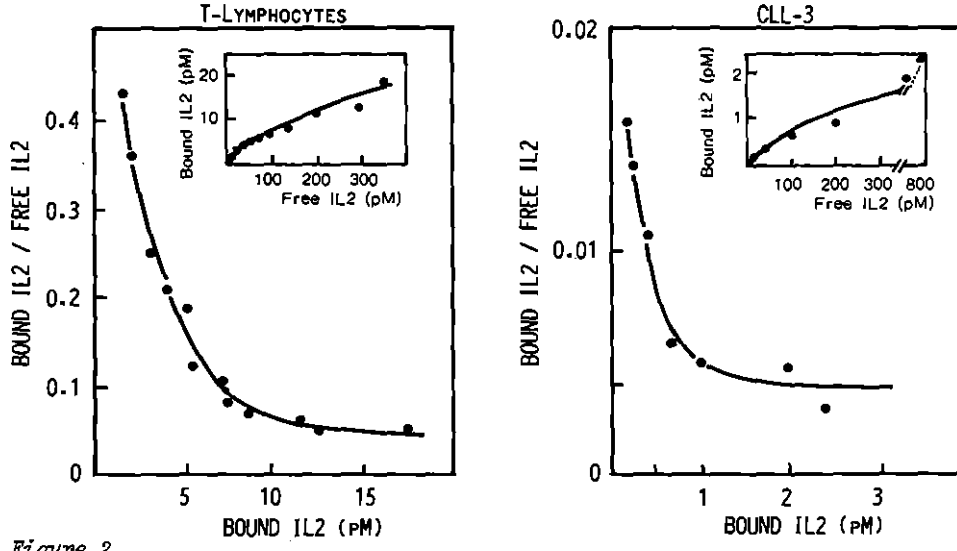

Figure 2

Specific binding of ^{125}I-IL2 to activated normal peripheral T lymphocytes and activated CLL cells (patient ≠ 3)

T cells and CLL cells had been incubated in the presence of 0.1% v/v PHA for 36 hrs. The data are expressed as Scatchard plots; the inserts show the relationships between the concentrations of free and specifically bound ^{125}I-IL2.

ity binding sites (27). To correct for nonspecific binding of the ^{125}I IL2, incubations were also performed in the presence of excess (i.e., thousand fold concentrations) of non-labeled recombinant IL2. All incubations were carried out in duplicate. At the end of the incubations, the medium containing the unbound radiolabeled IL2 was sucked away by placing the filtration plates on a vacuum filtration holder (Millipore Filter System). Duplicate samples from the incubation medium were taken to determine unbound radioactivity. The cells retained by the filter bottoms were then washed twice with 100 µl icecold alpha-BSA medium. After drying (30 min. at room temperature) the filter bottoms were punched out and collected in gamma counter vials. Radioactivity was counted in a 4/200 automatic gamma counter (Micromedic System, Horsham, Pa). The data were analyzed using the "ligand" computer program of Munson and Rodbard (28).

RESULTS

Expression of IL2 receptors on the CLL cells and binding of radiolabeled IL2

IL2 receptors, as assessed in indirect fluorescence with MoAb aIL2r and flow cytometry, were absent or weakly expressed on the fresh CLL cells. In all cases IL2 receptors were maximally expressed on the cells after 18 hrs of suspension culture in the presence of 0.1% PHA. Flow cytometric histogram analysis indicated that IL2 receptor densities on the in vitro activated CLL cells were lower than on PHA activated normal T lymphocytes (Fig. 1).

A binding assay using ^{125}I-IL2 was performed to determine affinity and numbers of IL2 receptors on the activated CLL cells. For comparison, IL2 receptors on activated T lymphocytes were also studied. Specific binding of the radiolabeled IL2 was measured at concentrations ranging from 6.25 to 800 pM. Scatchard plot analysis (Fig.2) indicated that CLL cells expressed two classes of receptors similar to the T cells, i.e., one with an affinity for IL2 in the picomolar range and one with an affinity in the nanomolar range. The data of these experiments are listed in Table 2.

TABLE 2. BINDING OF RADIOLABELED IL2

Cell type[$]	High affinity binding[#]		Low affinity binding[#]	
	Kd(pM)	Mean number of receptors per cell	Kd(nM)	Mean number of receptors per cell
CLL ≠ 1	34	90	n.d.	n.d.
CLL ≠ 2	7.5	64	n.d.	n.d.
CLL ≠ 3	28	13	25	13.0×10^3
CLL ≠ 4	1.2	11	62.5	20.5×10^3
normal T lymphocytes[+]				
donor ≠ 1	10.4	171	28.6	41.0×10^3
donor ≠ 2	19	154	n.d.	n.d.

$ Cells had been cultured for 72 h in the presence of 0.1% v/v PHA

Estimated using the "ligand" computer program of Munson and Rodbard (27)

n.d. Not determined

+ Isolated from normal peripheral blood

Proliferative response of CLL cells to IL2

DNA synthesis by CLL cells in culture was estimated by ^{3}H-TdR incorporation at various time points during 11 days. Cells were cultured under different stimulatory conditions, i.e., in the presence of PHA (0.1% v/v) and IL2 (50 units per ml), PHA alone, IL2 alone, and without these additions (Fig.3). In CLL cases ≠ 1, 2 and 3, PHA and IL2 were required for induction of proliferation. Omission of one or both of these components resulted in significantly less or no incorporation of ^{3}H-TdR. ^{3}H-TdR uptake

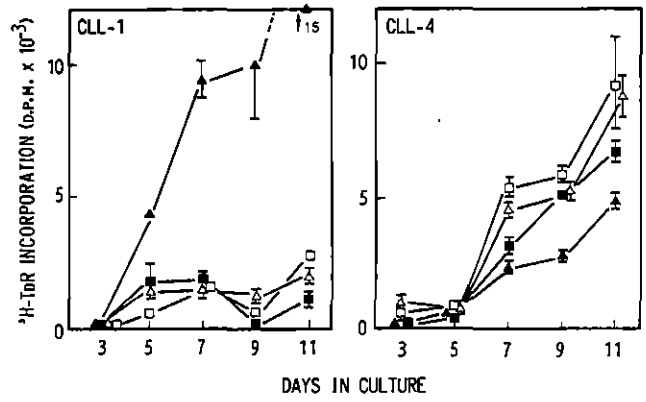

Figure 3

^{3}H-TdR incorporation by CLL cells under different stimulatory conditions in micro culture during 11 days of culture

(▲ - ▲): PHA (0.1% v/v) + IL2 (50 u ml^{-1}); (■ - ■) PHA alone; (△ - △): rIL2 alone; and (□ - □): no additions. Left panel shows the data of the experiments with CLL ≠ 1. Comparable results were obtained with the cells of CLL ≠ 2 and CLL ≠ 3. In the right panel, the data of ^{3}H-TdR uptake of CLL ≠ 4 are plotted.

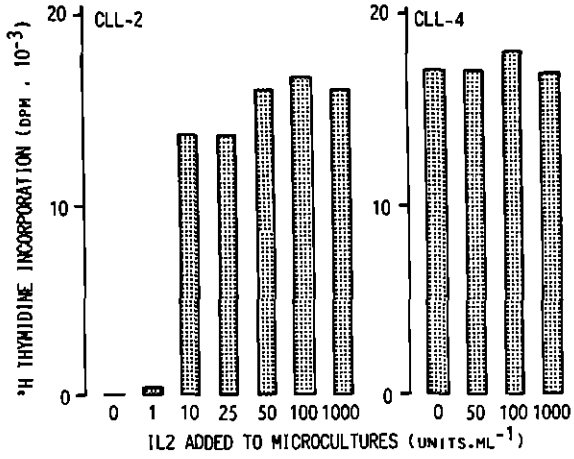

Figure 4

^{3}H-TdR incorporation by CLL cells in relation to the concentration of IL2 added to the cultures

The cells were incubated with or without IL2 for 10 days. Left section: CLL ≠ 2 (cells were cultured in the presence of 0.1% v/v PHA). Right section: CLL ≠ 4 (cells were cultured in the absence of PHA).

was significantly reduced, i.e. by 45-77 per cent when anti IL2 receptor MoAb interfering with the IL2 binding had been added to the cultures (at their initiation). This confirmed that stimulation occurred through IL2 - IL2 receptor interaction.

In contrast, ^{3}H-TdR incorporation by the cells of CLL ≠ 4 was completely independent on the addition of PHA and IL2 to the micro cultures. To assure that the proliferating cells of the four patients were indeed the leukemic cells (and not, e.g., residual normal T lymphocytes) immunologic analysis was performed. From this it appeared that on day 7 of culture the cells expressed the clonal phenotypes characteristic of the neoplasm (Table 1). The cultured cells contained less than 1% T cells, as assessed by E rosetting and MoAb anti-T3 labeling in indirect immunofluorescence. Thus, T cells did not significantly contaminate the in vitro growth of the CLL cells. Leukemic cell growth was also confirmed by cytogenetic analysis. On day 7 of culture of the cells of CLL ≠ 1, 13 of 15 of the spread metaphases expressed abnormal karyotypes, including inv. (X), 22p+ and +22. In CLL ≠ 4, 30 of 31 analyzed metaphases appeared abnormal (i.e., expressing 14 q+) after 7 days of culture.

IL2 dependence was investigated by adding IL2 in increasing concentrations to the microcultures (Fig.4). In CLL ≠ 1 through 3, optimal proliferation occurred in the presence of 50-100 units of IL2 per ml. On the other hand, in CLL ≠ 4, maximal incorporation of ^{3}H-TdR was obtained in cultures without any addition of IL2. This could suggest that the cells of CLL ≠ 4 lacked IL2 dependence, or that the cells produced IL2 and that proliferation was stimulated by endogenous IL2 (see below).

Analysis of the "spontaneous" proliferation of CLL cells (case # 4)

In CLL ≠ 1-3, proliferation depended on the presence of PHA and IL2. In contrast, in CLL ≠ 4 the rate of proliferation reached a maximum although no IL2 (and PHA) had been supplemented to the cultures (Figs.3 and 4). Epstein Barr Virus nuclear antigen (EBNA) was absent in these cells, indicating that these cells had not been transformed by EBV. We addressed the question as to whether "spontaneous" proliferation in CLL was truly IL2 independent or possibly mediated through endogenous IL2.

First, we examined the effect of four different MoAbs, reactive with IL2 receptor determinants, on the proliferation of the cells of CLL ≠ 4 (Table 3). The addition of MoAbs, aIL2r, anti-Tac and 18 E6.4, which compete with IL2 for receptor binding, completely inhibited ^{3}H-TdR incorporation. In addition, incubation of the cells with MoAb 36 A1.2, an anti IL2 receptor antibody that does not block IL2 binding, did not exert inhibitory effect on proliferation. These results indicated that the IL2 receptors (and more specifically the IL2 binding sites of these receptors) had a role in the proliferation although the cells had not been exposed to exogenous IL2. We hypothesized that these receptors were activated by endogenously produced IL2. To test this possibility, IL2 activities elaborated by the CLL ≠ 4 cells into the culture medium, were estimated. Media conditioned by CLL cells in suspension cultures were collected at various times (1-7 days) and assayed on a murine IL2 dependent (CTLL) cell line. IL2 activities (i.e., at concentrations varying from 0.15 to 1.5 units per ml) were indeed detectable in these CLL cell conditioned media. Finally, we applied Northern analysis using an IL2 cDNA probe to test whether IL2 mRNA was present in the cells of CLL ≠ 4 (fresh and after various in vitro incubations), which would indicate that the cells were capable of synthesizing IL2. We were repeatedly unable to detect IL2 mRNA in the cells of CLL ≠ 4, even when relatively large quantities (i.e., estimated at 2 - 5 μg) of purified, poly(A)$^{+}$, mRNA preparations had been loaded onto the gels (Fig.5). At the same time, IL2 mRNAs from control cells, i.e., PHA/TPA-activated Jurkat cells, as well as from PHA/TPA-activated normal peripheral blood T cells were readily demonstrated (Fig.5). The inability to show IL2 mRNA in cell samples of CLL ≠ 4 could suggest that the endogenous IL2 in the culture media conditioned by these cells was not due to active production but represented an activity previously absorbed by the CLL cells from an exogenous source. On the other hand it remains possible that the CLL cells did synthesize small quantities of IL2, but that the amount of IL2 mRNA remained below the detection level of the Northern Blot analysis.

TABLE 3. EFFECT OF FOUR DIFFERENT ANTI IL 2 RECEPTOR MoAbs ON THE "SPONTANEOUS" PROLIFERATION OF CLL CELLS (CLL ≠ 4)[#]

MoAb[$]	Blocking of IL2 binding	^{3}H-TdR incorporation (dpm x 10^{-3})*
-		4.30 ± 0.29 (100)
aIL 2 r	+	0.04 ± 0.01 (0.8)
anti-Tac	+	0.02 ± 0.01 (0.4)
18 E 6.4	+	0.12 ± 0.02 (2.9)
36 A 1.2	-	4.31 ± 0.67 (100.3)

[#] IL 2 (or PHA) were not added to the cultures.

[$] Each of the MoAbs was added at saturating concentrations (as assessed in indirect immunofluorescence) to microcultures at the initiation of the cultures.

* Assessed on day 8 of culture. Data are expressed as mean ± standard deviation of triplicate cultures. Figures in parentheses represent the percentages of control values.

DISCUSSION

Recently, it was shown that IL2 is a growth stimulator of B lymphocytes, and detailed information with respect to the responsiveness of B cells to IL2, their requirements of activation for the expression of membrane receptors for IL2, and the binding properties of the IL2 receptors

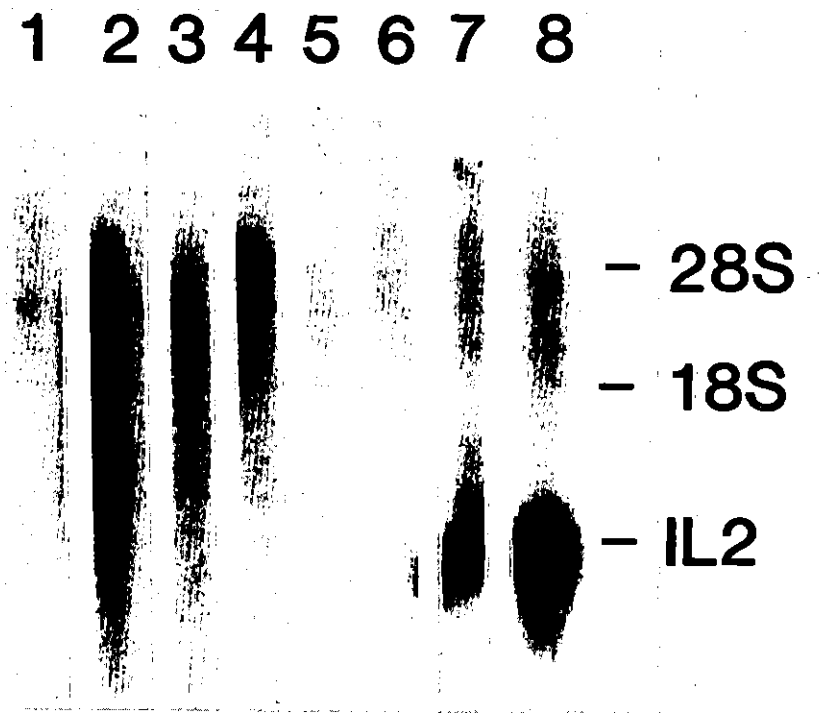

Figure 5

Autoradiograph of a northern blot of poly(A)$^+$ RNA samples from activated and nonactivated CLL cells, Jurkat cells and peripheral blood T lymphocytes

The blot was hybridized with a radioactive cDNA IL2 probe. Ribosomal RNA (28S and 18S) was run in a separate lane (stained with ethidium bromide) as a size marker.
Lane 1: CLL-4, nonincubated cells; Lane 2: CLL-4, after incubation (4 hrs) in the presence of PHA and TPA; Lane 3: CLL-4, 6 hrs PHA/TPA; Lane 4: CLL-4, 20 hrs PHA/TPA; Lane 5: CLL-4, incubation (20 hrs) without additions; Lane 6: CLL-1, 20 hrs PHA/TPA; Lane 7: peripheral blood T lymphocytes, 20 hrs PHA/TPA; Lane 8: Jurkat, 6 hrs PHA/TPA.

has become available (1-10). Similar studies carried out with leukemic cells should reveal whether the response of the cells to growth and differentiation stimuli has been altered as a result of neoplastic transformation. The present experiments were undertaken to determine the properties of IL2 receptors and the IL2 responsiveness of leukemic B lymphocytes, i.e., in four cases B CLL, and to establish whether these features were different from those reported for normal B lymphocytes.

From radiolabeled IL2 binding assays it became apparent that activated CLL cells express two classes of IL2 receptors, i.e., with a high (picomolar range) affinity and with a low (nanomolar range) affinity (Table 2). Similar data have been reported for murine B cell blasts (5). Mean densities of IL2 receptors per cell on the mouse blast cells appeared to be considerably (about 200 times) higher than those on human B CLL cells. On the other hand, Muraguchi et al., without discriminating between high and low affinity binding sites, estimated the mean numbers of IL2 receptors per normal activated human B cell at 320 (with a mean Kd of 457 pM) (4). The low mean numbers of high affinity IL2 binding sites per cell that we observed in CLL (i.e., ranging from 11 to 90) could indicate that these functional receptors are not equally distributed over the total CLL cell population, but concentrated on the IL2 responsive clonogenic subfraction which comprises only a small minority (less than 0.1 per cent) of the total CLL cell population (14). It should be emphasized that the cell suspensions had not been enriched for blast cells as was described by Robb et al. (25). This may also explain why the mean numbers per cell of high affinity IL2 receptors on normal activated T lymphocytes that we observed were 10-20 times less than those reported by Robb et al. for density gradient purified T blasts (25).

In three cases (CLL ≠ 1, 2 and 3) proliferation as measured by ^{3}H-TdR incorporation was stimulated by IL2. The optimal IL2 concentration in these cases was 50-100 units per ml, i.e., in the same order as reported for proliferation of normal B lymphocytes (Fig.2) (4,10). For an optimal response to IL2 the cells of CLL ≠ 1, 2 and 3 also required activation by PHA. This lectin has been previously shown to activate CLL cells (14,29, 30). In separate experiments not reported here, the phorbol ester TPA was used as the activation signal with comparable results.

In contrast to the above described response pattern of CLL ≠ 1, 2 and 3, the cells of CLL ≠ 4 proliferated independently on exogenous IL2 and did not require PHA as an activating factor (Figs. 1 and 2). Nonetheless, after in vitro incubation the CLL ≠ 4 cells expressed functional IL2 receptors with a high affinity for IL2 (Table 2). Upon further analysis it became evident that spontaneous proliferation of the CLL ≠ 4 cells was most likely induced by endogenous IL2: Specific blocking of IL2 receptors with monoclonal antibodies almost completely abrogated cell proliferation (Table 3). Moreover, concentrations of IL2 activity within the picomolar range were detected in the growth medium in which the cells from CLL ≠ 4 had been cultured. One could argue that this endogenous IL2 activity had been produced by activated contaminant T lymphocytes. For two reasons we consider this possibility unlikely:

1) In the fresh (T cell depleted) material no T lymphocytes were detectable, and immunological and cytogenetic analysis indicated that T lymphocytic cells had not expanded during culture either.
2) T lymphocytes require activation (e.g., by the lectin PHA) or a phorbol ester for IL2 production, whereas "spontaneous" proliferation of the CLL ≠ 4 cells and elaboration of IL2 into the culture medium occurred in the absence of PHA.

Therefore it is most plausible to assume that indeed the CLL cells (CLL ≠ 4) elaborated IL2 into the culture medium. The suggestion that normal and transformed B cells can produce cytokines, including IL2, was recently raised by several investigators (31-33). To investigate whether the cells of CLL ≠ 4 were able to synthesize IL2 we attempted to detect IL2 mRNA in the cells, however, with negative results (Fig.5). Thus, if the cells really produced IL2, they formed IL2 mRNA in a quantity below the detection level of the Northern analysis. Typically, this could be the case when only a small portion of the CLL cells, e.g., the prolifereting subpopulation under the applied in vitro conditions, synthesized IL2. More sensitive detection methods, e.g., S1 nuclease mapping or in situ hybridization techniques, will be needed to clarify this issue. Other explanations for the failure to detect IL2 mRNA are that the cells of CLL 4 produced an IL2-like activity with a protein structure discrepant from T cell derived IL2, or that the CLL cells released IL2 that had previously been absorbed by the cells from external sources.

Taken together, the present experiments have established that the proliferation of CLL cells in vitro is regulated by IL2 through direct IL2-IL2 receptor interaction, similarly to that of normal B lymphoblasts. In addition, the experimental data could suggest that in some cases the CLL cells are capable of autocrine IL2 mediated stimulation. Autocrine growth may be the result of neoplastic transformation and it has been suggested as a mechanism of maintaining tumor cell proliferation (34). On the other hand, it has become clear that normal cells may be controlled by autocrine mechanisms of growth as well. For example, T lymphocytes proliferate in response to autocrine IL2 (35). It is unknown whether (subpopulations of) normal B cells may also be capable of autostimulation. Therefore, at the present time, it remains uncertain as to whether the growth characteristics of the cells of CLL $\neq$ 4 represent those of a normal B-lymphocytic subset, or whether they are typical of the transformed status of the neoplastic cells. Irrespective of this question, it is intriguing that primary tumor cells from individual patients, that apparently descended from the same type of cell (i.e., early B lymphocyte), may show markedly different in vitro growth characteristics.

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CHAPTER 7

Acute Lymphoblastic Leukemia and non Hodgkin's Lymphoma of T Lineage: Colony Forming Cells Retain Growth Factor (Interleukin 2) Dependence

Blood, in press, 1986

ACUTE LYMPHOBLASTIC LEUKEMIA AND NON HODGKIN'S LYMPHOMA OF T LINEAGE: COLONY FORMING CELLS RETAIN GROWTH FACTOR (INTERLEUKIN 2) DEPENDENCE

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The regulatory role of interleukin 2 (IL2) in the proliferation of T acute lymphoblastic leukemia (T ALL) and T non Hodgkin's lymphoma (T NHL) cells from six individual patients was analyzed in a colony culture system to which pure recombinant IL2 (r-IL2), and the lectin phytohaemagglutinin (PHA) or the phorbolester 12-O-tetradecanoyl phorbol-13-acetate (TPA), had been added. The proliferative response was correlated with the inducibility of receptors for IL2 on the surface membrane of T ALL and T NHL cells by incubation with TPA or PHA for 18 hours. Leukemic T cell colonies, identified by immunophenotyping or cytogenetic analysis, appeared in vitro following TPA and IL2 stimulation in all six cases. Accordingly, receptors for IL2, initially absent from the cell surface, were found on high proportions of the T ALL and T NHL cells after in vitro exposure to TPA. In contrast, colony formation stimulated by PHA, and the induction of IL2 receptors by PHA, were limited to the one case of T NHL with the mature thymocyte immunophenotype. The cells from the other patients, expressing common or prothymocyte phenotypes, did not respond to PHA. No colonies were formed in any of these cases when PHA or TPA were withheld from the IL2 containing cultures. While colony growth depended absolutely on exogenous IL2 in three cases (ALL), in the three other cases (1 ALL, 2 NHL) some colonies grew also when no IL2 had been added to the cultures. Upon further analysis of the cells of one of the latter patients, it was found that the cells produced IL2 and proliferated in response to this endogenous IL2. The results from this study indicate that the requirements of endogenous versus exogenous IL2 for cell proliferation in T ALL and T NHL, and IL2 receptor activation by PHA and TPA vary from patient to patient. In addition, they support the notion that T ALL and T NHL cells have not lost dependence on IL2 and IL2 receptor activation for in vitro growth.

INTRODUCTION

Attempts at understanding the underlying mechanisms of tumor growth are concerned with the possibility that neoplasms are disturbed in their response to growth factors. Their uncontrolled outgrowth may be caused by an altered sensitivity to growth regulators (e.g., as a result of aberrant expression or function of membrane receptors) or to the acquired ability to self-produce factors (1). These phenomena have been described for a variety of tumors and in a number of cases they were found to correlate with the expression of oncogenes (2-4). Alternatively, proliferation of tumor cells may become completely independent on normal growth regulators (4).

The availability of interleukin 2 (IL2) gene probes, pure recombinant IL2 preparations and monoclonal antibodies reactive with cell surface membrane receptors for IL2 (5,6) allow for the analysis of growth requirements of human T cell leukemias. These approaches have been pursued mainly in leukemias of mature T cell type, in particular those associated with the human T cell lymphotropic virus I (HTLV I) (7). Until now, however, scarce information is available on the control of proliferation by IL2 in immature type T cell neoplasms i.e., T acute lymphoblastic leukemia (T ALL) and certain T cell non Hodgkin's lymphomas (T NHL).

In the present study, we have examined T ALL and T NHL cells from six individual patients for their proliferative response to IL2. We applied a colony culture technique in which pure recombinant IL2 (r-IL2) was added as the single growth factor. The lectin phytohemagglutinin (PHA) or the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was supplemented simultaneously for activation of the cells. Our findings indicate that distinct patterns of IL2 and activation requirements can be recognized in individual cases of T ALL and T NHL. However, like normal T cells, the leukemia cells remain dependent on TPA or PHA activation and IL2 for proliferation. The proliferating neoplastic cells do not acquire new surface antigens indicative of maturation to later cell stages, suggesting that their capacity to differentiate in vitro is very limited. We conclude that proliferation of T ALL and T NHL cells cannot be simply explained by the loss of control by IL2 or by the aberrant (i.e., independent on activation) expression of membrane receptors for IL2.

Table 1 SELECTED DATA OF PATIENTS WITH T ALL AND T NHL

patient	age	sex	diagnosis	blood leukocyte count ($x10^{-9}/l$)	leukemic immunophenotype[x] (% positive cells)					
					WT1	T3	T4	T6	T8	E
1	8	F	T ALL	180	98	0	0	0	0	0
2	4	F	T ALL	100	45	0	0	0	0	75
3	10	M	T ALL	200	100	W	7	3	13	93
4	30	M	T ALL	53	98	0	34	98	96	92
5	19	M	T NHL	40	n.d.	0	34	37	64	80
6●	63	M	T NHL	166	96	98	94	0	0	95

Patients no. 1-5 TdT positive; patient no. 6 TdT negative. In none of the cases the leukemic cells expressed Ia antigens.

x : assessed by indirect immunofluorescence and E rosetting (200 cells counted)
W : weak expression (see Fig.1)
● : 88% of the cells expressed T10 antigens
n.d. : not determined

patient 5: diffuse, lymphoblastic lymphoma
patient 6: diffuse, well differentiated lymphocytic lymphoma.

MATERIALS AND METHODS

Patient data and immunologic phenotypes of leukemic T cells

Selected clinical and immunological data of the patients entered into this study are summarized in Table 1. Patients with prothymocyte-like (stage I) leukemic cells (TdT^+, $WT1^+$, $E^{+/-}$, $T3^-$, $T4^-$, $T6^-$, $T8^-$) had been diagnosed cyto-histopathologically as cases of T ALL (patients no 1 to 3), patients carrying thymocyte-like (stage II, stage III) malignant T cells ($T3^-$, $T6^+$, $T4/T8^+$ or $T3^+$, $T4^+$, $T10^+$, $T6^-$, $T8^-$) as T ALL (patient 4) or leukemic T NHL (patients no 5 and 6) (8,9).

Isolation of leukemic T cells

Leukemic cells were isolated from peripheral blood or bone marrow using Ficoll Isopaque separation (10). A further separation was applied to remove residual normal (E^+, $T3^+$) T lymphocytes as, in most cases, these could interfere with leukemic growth in the IL2 supplemented cultures. In one case of E rosette negative T ALL (i.e., patient no. 1) normal T cells were eliminated by E rosette depletion using 2-aminoethylthiouronium bromide (AET) treated sheep erythrocytes as described (1). In the other cases (i.e., patients no. 2, 3, 4 and 5) residual normal T lymphocytes were removed from the leukemic cell fractions on the basis of differential T3 antigen expression on normal and neoplastic T cells by fluorescent staining of the T3 antigen (see below) followed by fluorescence activated cell sorting (FACS 440, Becton- Dickinson, Sunnyvale, Ca) (Figure 1). The cells of patient no. 6 were studied without further separation as both cytogenetic and immunologic analysis of fresh and cultured cells indicated that contaminating growth of normal T cells did not occur.

Isolation of E rosette forming lymphocytes from normal peripheral blood samples

E rosette forming lymphocytes were recovered from the sediment after centrifugation of AET-E rosetted cells through Ficoll-Isopaque as described (1). Sheep erythrocytes were removed from these fractions by hypotonic

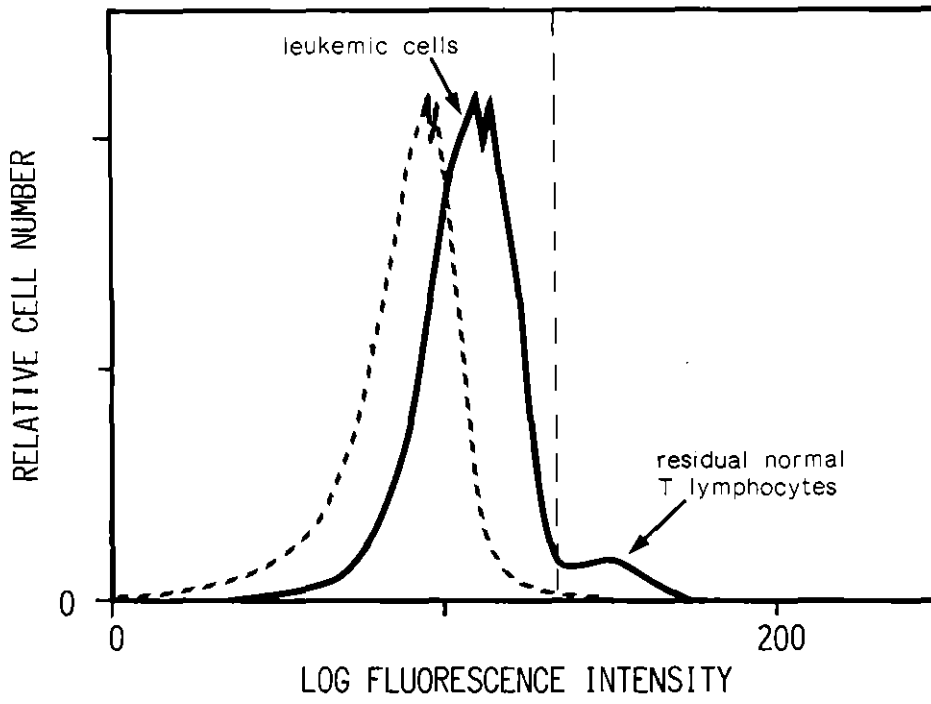

Figure 1 : Fluorescence activated cell sorter histograms of Ficoll-Isopaque isolated cells of patient no. 3 (T ALL) after OKT3 + GAM/FITC (——) and GAM/FITC control (---) staining.
The ALL cells of this patient showed a weak T3-antigen expression, whereas residual normal T lymphocytes (interfering strongly with leukemic colony growth) express the T3-antigens in a high density on their cell surface. To eliminate the normal T cells from the ALL cell sample of this patient (and of patients no. 2, 4 and 5) cell sorting was performed on the basis of T3 fluorescence intensity. The vertically drawn interrupted line indicates the level at which the cells were separated; ALL cells were recovered left from the fraction with low fluorescence intensity.

lysis (incubation for 5 min at 37°C in 0.75% w/v NH_4Cl in 3mM Tris-HCl pH = 7.2).

Immunofluorescence

Surface membrane antigens on fresh and cultured cells were assayed by indirect immunofluorescence microscopy using murine monoclonal antibodies (MoAb) and goat anti-mouse Ig immunoglobulins coupled to fluorescein isothiocyanate (GAM/FITC; Nordic, Tilburg, The Netherlands) (12). The presence of terminal deoxynucleotidyl transferase (TdT) in the cell nuclei was assessed on cytocentrifuged cells using an indirect immunofluorescence kit (Gibco, Ghent, Belgium).

Monoclonal antibodies (MoAb)

MoAb reactive with the T3, T4, T6, T8 and T10 antigens and with the Ia-antigen were obtained from Ortho Pharmaceutical Corp. (Raritan, N.J.) and all used in a 1:40 dilution. MoAb WT1, reactive with a pan-T cell antigen (3) was supplied by Dr. W.J.M. Tax (Nijmegen, The Netherlands) and used in a titer of 1:100. MoAb anti-IL2-receptor (aIL2r) obtained from Becton-Dickinson (Mountain View, CA) was used in a 1:20 dilution. The reactivity of this antibody is identical to that of anti-Tac (14). MoAb B13.9, detecting a myelocytic antigen, was a gift from Dr. P. Lansdorp (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Colony culture

Colony cultures were performed basically as described for normal T cell colony formation (15) with the following modifications:

1) Feeder leukocytes were not included in the system as a source of growth stimuli. Instead, pure recombinant IL2 (Biogen SA, Geneva, Switzerland) was added to the culture upper layer.
2) Either PHA (reagent grade, Wellcome, Dartford, England) at a concentration of 0.7% v/v, or the phorbol ester TPA at a concentration of 7 ng/ml (Sigma Chemicals, St. Louis) were used as mitogens in culture.

Colony cells were mass-harvested with a Pasteur pipette, washed three times with phosphate buffered saline (PBS) and prepared for indirect immunofluorescence and E rosette formation. In one case (patient no. 6), the colony cells were analyzed cytogenetically (Dr. A. Hagemeyer, Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam).

Induction of IL2 receptors

Short-term (18 hrs) suspension cultures supplemented with PHA or TPA to induce membrane receptors for IL2 were performed as described (16). After culture, the cells were washed three times with PBS and prepared for indirect immunofluorescence with the anti IL2 surface receptor (aIL2r)-antibody.

Micro culture of NHL cells and in vitro blocking of IL2 receptors

Proliferation of NHL cells in culture as measured by ^{3}H-Thymidine uptake was examined with and without blocking of IL2 receptors. Parallel cultures were established to which MoAb aIL2r (IgG1, non cytotoxic), control MoAb (OKT3, B13.9) or no MoAb had been added at their initiation. $1x10^{5}$ cells were cultured in triplicate in round bottom microtiter wells (Greiner, Alphen aan den Rijn, The Netherlands) for 3 days in 100 μl RPMI 1640 medium (Gibco) with 10% heat-inactivated fetal calf serum supplemented with $5x10^{-5}$M b-mercaptoethanol and l-glutamine (referred to as complete RPMI medium) supplemented with 0.1% PHA. Sixteen hours before harvesting of the cells (Titertek cell harvester 550) 0.1 μCi of (^{3}H)-Thymidine (Amersham, U.K., specific activity 2 Ci/mmol) was added to each microwell. Radioactivity was measured by liquid scintillation counting (Beckman LS 3800). All cultures were performed in triplicate. Data are expressed as mean d.p.m. $\pm$ standard deviation of triplicate cultures.

IL2 assay

Murine IL2 dependent CTLL cells (a gift from Mrs. S. Knaan-Shanzer, Radiobiological Institute TNO, Rijswijk, The Netherlands) were cultured in triplicate after three washings with Hanks Balanced Salt Solution (HBSS) in

Table 2 INDUCTION OF IL2 RECEPTORS AND COLONY FORMATION

PATIENT*	IL2 RECEPTOR EXPRESSION (% positive cells)			COLONY FORMATION^x (different stimulatory conditions)					
	Untreated	TPA≠	PHA≠	TPA + IL2 (25 u)	TPA	PHA + IL2 (25 u)	PHA	IL2 (25 u)	None
1	0	97	0	76	0	0	0	0	0
2	0	47	0	30	0	0	0	0	0
3	3	61	5	47	36	0	0	0	0
4	0	20	0	14	0	0	0	0	0
5	5	18	2	35	23	8	0	0	0
6	0	22	95	41	0	166	106	0	0
normal T$	0	55	24	n.d.	n.d.	539	88	0	0
normal T$	0	26	55	410	266	872	72	0	0

≠: Determination of IL2 receptors following 18 hours of TPA/PHA supplemented suspension culture (see Materials and Methods)

x: Colony numbers per 2.10^5 plated cells

*: See Table 1 for description of patients

$: E positive lymphocytes purified from normal donor peripheral blood

complete RPMI medium at $1x10^5$ cells/100 µl in microtiter wells. At 20 hours, cultures were supplemented with 0.1 µCi ^{3}H-TdR and 4 hours later the cells were harvested and assayed for ^{3}H-TdR incorporation by liquid scintillation counting. To estimate IL2 concentrations in T NHL culture-supernatant, the proliferation of CTLL cells in the presence of these supernatants was compared with proliferation in response to titrated r-IL2 concentrations (ranging from 0.01 to 100 units per ml).

Preparation of T NHL culture-supernatant

$2x10^6$ cells T NHL cells per ml complete RPMI medium with or without 1% v/v PHA in 6 ml tubes (Greiner) were placed in the incubator for 3 days. Supernatants were then recovered, filter sterilized through a 0.22 µm filter (Millipore, Bedford, Mass) and stored at -20°C.

RESULTS

Induction of IL2 receptors

Untreated T ALL and T NHL cells, like normal peripheral blood T lymphocytes, did not express membrane receptors for IL2 as measured in indirect immunofluorescence using MoAb aIL2r (Table 2). Studies on cells from short-term (18 hrs) suspension-cultures then disclosed that IL2 receptors appeared on significant proportions of the cells when TPA or PHA had been added to these cultures (Table 2). In the absence of TPA/PHA, IL2 receptor induction did not occur. A striking discrepancy with respect to PHA sensitivity was evident between the prothymocyte or immature thymocyte-like leukemia cells (i.e., patients no. 1-5) and the normal T lymphocytes. These leukemias, in contrast to the normal T cells, did not respond to PHA. In contrast, the cells of patient 6 carrying a mature thymocyte phenotype were sensitive to activation by both TPA and PHA, thus sharing this property with normal peripheral T cells.

Colony stimulation

To assess the proliferative response of the normal T and the T NHL/ T

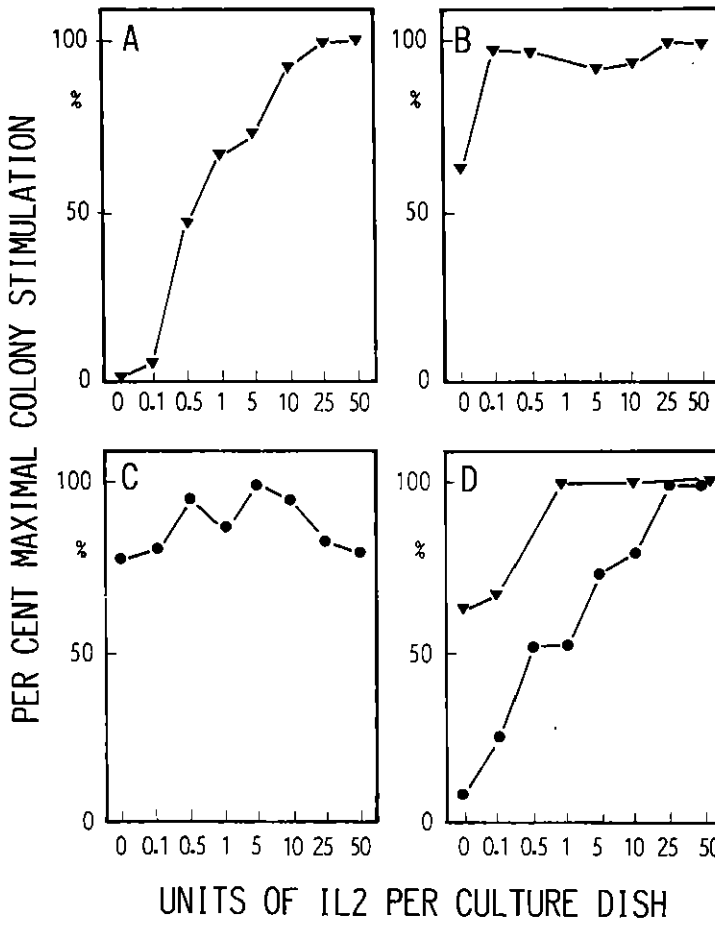

Figure 2 : Induction of ALL/NHL (A,B,C) and normal (D) T colony formation with titrated doses of rIL2. The data are expressed as percentages of maximal colony stimulation.
A) T ALL patient no. 1
B) T ALL patient no. 3
C) T NHL patient no. 6
D) E^+ lymphocytes purified from normal peripheral blood ▼—▼ *: TPA costimulation;* ●—● *: PHA costimulation.*

ALL cells to IL2, the cells were plated in colony culture under selected stimulatory conditions (Table 2). Colony formation by the normal T lymphocytes depended on the presence of both IL2 and mitogen (either PHA or TPA). When the addition of mitogen (i.e., PHA or TPA) was omitted from IL2 containing cultures, no colonies were formed, which is in agreement with the finding that non-activated T cells lack membrane receptors for IL2. On the other hand, suboptimal colony formation was noted in the presence of TPA or PHA when no IL2 had been added to the cultures. The fact that some colonies develop in non IL2 supplemented cultures is most likely to be explained by endogenous production of IL2 by the T cells, stimulated by the "interleukin 1 like" activity of TPA (17) and/or some residual monocytes present among the cells.

The colony forming abilities of the cells from patients no. 1-5 were clearly different from those of normal blood T lymphocytes. The cells did not form colonies in response to PHA, even when IL2 had been added to culture. In contrast, when combined with TPA, IL2 stimulated the formation of considerable numbers of colonies in all of these cases. The capacity of the cells to form colonies in response to TPA but not to PHA, was entirely in accordance with, respectively, the ability and inability of these compounds to induce IL2 receptors in the individual cases. The cells of patient 6 showed a divergent response pattern: Colony formation and IL2 receptor induction occurred with either TPA or PHA.

To define the IL2 dependence of T ALL and T NHL colony forming cells, IL2 dose-titration experiments were performed (Fig. 2). These experiments show that the T ALL cells of patients 1 and 3, activated by TPA, like normal T lymphocytes require the addition of IL2 to the cultures for optimal colony formation (panels A, B and D). Patient no. 1 is representative of the pattern in which the cells are typically absolutely dependent on the exogenous supply of IL2 to culture. This type of response also applies to that in patients no. 2 and 4. Plateau IL2 requirements for leukemic colony formation were variable, i.e., 25 units per culture dish for patient 1 and 0.1 units per dish for patient 3. Further increases in IL2 concentrations (up to 500 units per culture dish) did not significantly alter the numbers of (normal and leukemic) colonies. The TPA activated T ALL cells of patient 3 produced colonies (although in suboptimal numbers) without the addition of IL2. A similar phenomenon was evident with the T NHL cells of patient 6,

cultured with PHA (panel C). These findings raise the possibility that certain T ALL and T NHL cells are capable of IL2 release and autostimulation (see below).

Colony typing

Membrane marker analysis was performed after colony culture (Table 3) and the immunophenotypes of colony cells were compared with those of the fresh cells. Immunologic phenotypes of the leukemic colony cells in general reflected similar stages of maturation as the cells before culture. For example, colony cells from patient 1, 2 and 3 still expressed the surface antigens, characteristic of their preculture immaturity (Table 1).

Table 3 SURFACE MEMBRANE ANTIGENS ON COLONY CELLS

Patient≠	Percentage of positive colony cells*					
	WT1	T3	T4	T6	T8	E
1	96	0	0	5	1	0
2	77	1	13	0	0	95
3	88	3	0	3	0	85
4	86	0	0	0	98	90
5	65	4	70	16	9	73
6	100	58	85	0	0	96
normal T	65	90	60	0	26	86
normal T	88	87	67	0	27	90

≠ Preculture phenotypes of the leukemic cells are given in Table 1

* Assessed by indirect immunofluorescence (200 cells counted)

Colony cells of patients no. 4 and 5 appeared slightly more mature than the cells before culture, since they had largely lost the T6 antigen. In neither of these two cases, however, maturation had progressed toward the T3 positive mature-thymocyte differentiation stage. Normal T-cell

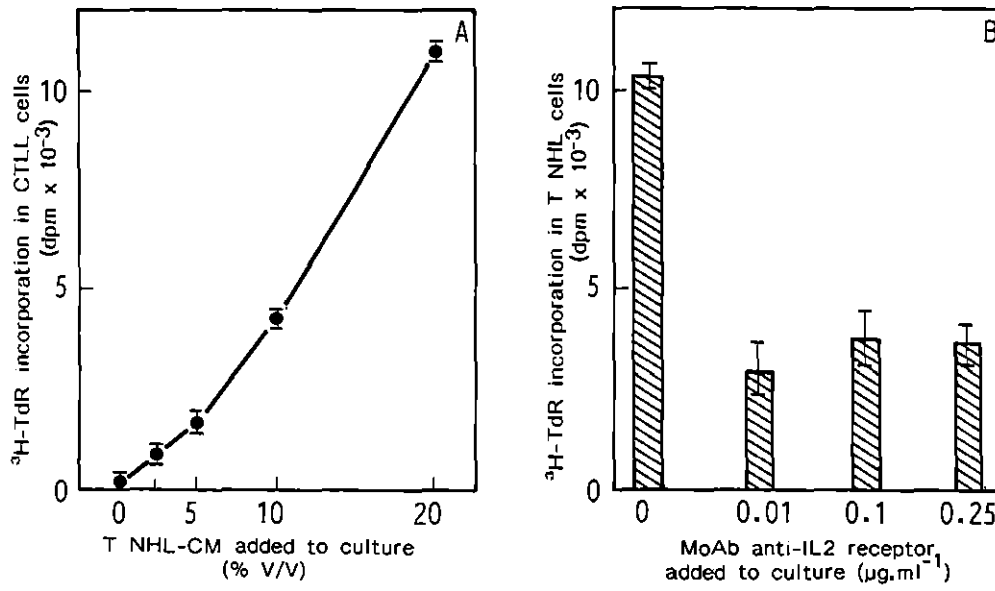

Figure 3 : Microculture analysis of IL2 production (section A), and of the effect of IL2 receptor blocking on proliferation (section B) (T NHL patient no. 6).

A) Dose dependent proliferation induction of fully IL2 dependent murine CTLL cells by increasing concentrations of culture medium conditioned by the leukemic T cells (T NHL-CM).

B) Inhibition of proliferation of T NHL cells in cultures with PHA and without exogenous IL2 following the addition of MoAb anti-IL2-receptor at three different concentrations.

colonies (Table 3) contained mature activated T lymphocytes with blastic appearance and a mature T-cell immunophenotype, i.e., $T3^+$, T4 or $T8^+$, $T6^-$, $WT1^+$ and E^+. Colony cells of patient 6 expressed the normal phenotype. It was identical to that of the pre-culture cells i.e., resembling that of mature activated normal helper T cells ($T3^+$, $T4^+$, $T6^-$, $T8^-$, $T10^+$). The fact that these colonies were indeed leukemic was apparent from cytogenetic analysis. It was found that 91% of the colony cells in metaphase had the complex cytogenetic abnormality 46, XY, 6q-, 7p+, t(8p+; 11p-q?), 12 q+, -14, 15p+, + Mar (14q-?) which had been demonstrated in 76% of the fresh NHL cells in metaphasis.

Analysis of growth requirements of leukemic T cells that do not depend on the addition of IL2 for proliferation

Colony formation in three cases occurred in spite of the absence of exogenous IL2 (i.e., in patients no. 3 and 5 with TPA alone and in patient no. 6 with PHA alone (Table 2, Fig. 2). Possible explanations for these findings are that these leukemias either proliferated independently on IL2 or that IL2 was needed but provided by the neoplastic cells themselves. To clarify this question, the cells of patient no. 6 (NHL) were studied in ^3H-Thymidine incorporation microculture assays (Fig. 3).

First, it was investigated whether the cells were capable of IL2 production. Culture supernatants conditioned by PHA activated T NHL cells (T NHL-CM) were tested for IL2-activity by inducing proliferation of CTLL cells (murine IL2 dependent cell line). From figure 3A it can be seen that increasing concentrations of T NHL-CM stimulated CTLL proliferation progressively. IL2 activity of the T NHL-CM was estimated at approximately 5 u/ml by comparison with dose response relationships of pure rIL2. Without PHA activation, the culture media conditioned by the NHL cells contained only 1 u/ml IL2 activity.

The elaboration of IL2 in the culture medium by the NHL cells of patient no. 6 and the appearance of IL2 receptors on these cells following PHA stimulation suggested that their proliferation depended on the release of endogenous IL2. Therefore, we investigated the effect of blocking of IL2 membrane receptors (with the anti IL2 receptor MoAb aIL2r) on the proliferation of the T NHL cells in the absence of exogenous IL2 (Fig. 3B). Cul-

turing of the cells in the presence of MoAb aIL2r resulted in a 60-70% inhibition of ^{3}H-TdR incorporation. On the other hand, a) the incubation of T NHL cells with control MoAb OKT3 (IgG2a) or B13.9 (IgG1), or b) the incubation of control (AML) cells with MoAb aIL2r did not result in a significant suppression of ^{3}H-TdR incorporation.

Taken together, these data confirm that the T NHL cells of patient 6 indeed require IL2 for proliferation. Notably, however, exogenous IL2 is not required as the cells elaborate their own IL2 at effective concentrations.

DISCUSSION

The induction of proliferation of normal T lymphocytes in vitro involves a dual step mechanism in which an activator (e.g., antigen, lectin, phorbol ester) and the lymphokine IL2 (previously designated as T cell growth factor) are both essential components (18). Activation is required to induce receptors for IL2 on the T cell-membrane, thereby rendering the cells sensitive to the growth factor. The discovery that certain (HTLV-infected) leukemias of mature T cell type continuously express IL2 receptors without prior activation (8) and the finding that some cutaneous T cell leukemia/lymphoma cell lines produce and respond to IL2 (19,20) supported the idea that the growth of leukemic T cells depends on a permanent responsiveness to IL2 in association with the ability to self-produce this growth factor. Such a model of autostimulation, driven by IL2, was initially thought to be crucial in the development of HTLV associated neoplasms (19). However, this suggestion was later considered less likely, as it was found that not all HTLV induced leukemias are capable of IL2 production. In addition, the cells of the latter neoplasms did not always require (endogenous or exogenous) IL2 for proliferation (21).

In another class of mature T cell leukemia, i.e., malignant cutaneous T cell lymphoma (Sézary's syndrome) the cells were found to produce and respond to IL2 in vitro following stimulation with PHA and TPA (22). Sézary cells, with few exceptions, are not infected with HTLV (23). They generally lack spontaneous IL2 receptor expression and are therefore dependent on mitogenic stimulation. The autocrine secretion of IL2 by Sézary cells, however, is not a tumor associated characteristic, as normal T-helper

lymphocytes express the same ability (24,25).

The role of IL2 in the regulation of the growth of T ALL and T NHL has as yet remained a rather unexplored area. The present study was undertaken to establish the in vitro culture requirements of these T cell neoplasms. The results from colony culture and ^{3}H-Thymidine incorporation experiments indicate that T ALL and T NHL cells from 6 individual patients had remained dependent on IL2 (either added to the cultures or endogenously produced) for proliferation (Table 2, Figs. 2 and 3). Besides IL2, T ALL and T NHL cells require activation by TPA for colony growth. In all cases, colonies were formed in TPA plus IL2 supplemented cultures (Table 2). TPA is needed for the induction of IL2 surface membrane receptors on the T ALL and T NHL cells. Fresh leukemic T cells lacked these receptors, whereas exposure to the phorbol ester resulted in their appearance on a high proportion of the cells (Table 2). The induction of receptors for IL2 by TPA on certain leukemic T cell lines initially lacking such receptors has also been reported (26). Thus, T ALL and T NHL cells share with normal T lymphocytes the requirement of activation before they are capable of responding to IL2.

Whereas it is clear that the interaction of foreign antigen with antigen receptors on the cell surface is the in vivo activation signal for T lymphocytes and stage III thymocytes, no in vivo mechanism underlying activation of immature T cell types (i.e., not expressing the T3 antigen receptor complex on the cell membrane) is known. Therefore, no conclusions can as yet be drawn from our in vitro experiments as regards the role of IL2 in the proliferation of stage I and stage II thymocyte like T ALL and T NHL cells in vivo. However, it was recently shown that IL2 receptors are present on a population of freshly isolated murine thymocytes with an immature ($Ly2^{-}$, $L3T4^{-}$) immunophenotype (27,28), and on this basis it was suggested that thymocytes can be activated by a stimulus intrinsic to the thymus (27). If this hypothesis is correct it raises the possibility that, in vivo, the IL2 responsiveness of leukemic T cells with stage I or stage II thymocyte immunophenotypes can be induced, in situ, in the thymus. Notably, thymic involvement is frequently observed in T ALL and T NHL.

Unlike TPA, PHA exerted an effect only in the one case of T NHL expressing a mature thymocyte (stage III) immunophenotype (Table 2, patient no. 6). The expression of IL2 receptors and colony formation were induced

by PHA (as well as by TPA) in this mature (stage III thymocyte) positive NHL. The other, pro- or immature (stage I, stage II) thymocytelike tumors lacked PHA responsiveness. They share this inability with their putative normal thymic counterparts (29). Using Jurkat cells and a variety of mutants derived from this cell line it was recently shown that PHA responsiveness of T cells is directly related to the expression of the $T3^-$ antigen receptor complex on the cell surface (30).

In three cases (i.e., patients no. 1, 2 and 4), colony formation was strictly dependent on the addition of IL2 to culture. The fact that IL2 had to be added suggests that the neoplastic cells were not capable of IL2 production. However, on the basis of our experiments we cannot exclude the possibility that some IL2, insufficient for autostimulation, was actually released by the cells of these patients. On the other hand, in the three other cases (i.e., patients 3, 5 and 6) colony growth was apparent with TPA or PHA as single additive to culture and did not require exogenous IL2. It was documented in one of these cases (patient no. 6) that the neoplastic cells produced considerable amounts of IL2. Culture media conditioned by the T NHL cells of patient 6 contained IL2 activity in the order of 5 units per ml. Thus, we propose that T ALL and T NHL may be subdivided into categories on the basis of in vitro proliferation characteristics, i.e., (1) responsive to both TPA and PHA, (2) responsive to TPA but not to PHA, (3) capable of IL2 production in sufficient amounts for self-stimulation in colony culture and (4) not capable of significant IL2 production. Normal T cells at different stages of maturation may express identical features. Therefore, the results of our in vitro studies as yet do not provide indications for a disordered response to IL2 underlying the uncontrolled proliferation in T ALL and T NHL. However, it is at present not known whether critical abnormalities exist in these T cell leukemias as far as the detailed requirements of activation of IL2 synthesis, activation and down regulation of IL2 receptors, and function of these receptors are concerned. Such abnormalities have been described for adult T cell leukemia and certain leukemic T cell-lines (6,31).

Results from immunophenotyping provided no positive evidence for differentiation of the T ALL and T NHL cells during colony formation in cultures supplemented with TPA. No acquisition of new differentiation antigens (e.g., E, T3, T6) indicative of in vitro maturation of the leu-

kemic T cells was apparent (Table 1, Table 3). These data contrast with those of experiments with certain leukemic T cell lines (Jurkat, CEM and Molt-3) in which TPA was found to induce the expression of E rosette receptors and T3 antigens (32,33,34).

Cloning efficiencies of T ALL/T NHL cells in IL2 plus mitogen containing cultures were significantly lower than of normal peripheral T cells (Table 2). This raises the question as to whether culture conditions were suboptimal for T ALL and T NHL, for example, because T ALL and T NHL cells require other lymphokines for proliferation besides IL2. The finding that the afore mentioned subpopulation of mouse thymocytes (i.e., early $Ly2^-/L3T4^-$ thymocytes) do not respond in vitro to IL2, although the cells express functional (i.e., high affinity) IL2 membrane receptors (27,28) suggests that other growth factors, in combination with IL2, act on early stages of (murine) T cell maturation and possibly on the leukemic counterparts of the analogous cell types. Results from previous work on non-T ALL indicated that in vitro colony formation by common and pre-B ALL cells depends on the combined exposure to IL2 and a second factor elaborated by feeder leukocytes (16). However, we could not demonstrate a significant effect of the same crude source of stimulation on colony forming cells of ALL/NHL of T lineage (results not shown). To further elucidate the critical requirements additional to IL2 for in vitro colony growth of T ALL and T NHL cells, the availability of pure (recombinant) growth factor preparations will be essential.

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CHAPTER 8

Production of T Lymphocyte Colony-Forming Units from Precursors in Human Long-Term Bone Marrow Cultures

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Production of T Lymphocyte Colony-Forming Units From Precursors in Human Long-Term Bone Marrow Cultures

By Ivo Touw and Bob Löwenberg

T cell differentiation in human marrow was studied in Dexter type long-term bone marrow cultures. In these cultures, T lymphocyte colony-forming units (TL-CFU), E rosette-forming cells (E^+), and $T3^+$, $T4^+$, and $T8^+$ cells (assayed by indirect immunofluorescence) were found to be present for at least 7 weeks. It was investigated whether the existence of T cells in long-term culture resulted from the persistence of inoculated T lymphocytes or from the production by immature progenitors. No significant numbers of E^+, $T3^+$, $T4^+$, or $T8^+$ cells were detected in cultures that were established from E^+ lymphocyte-depleted bone marrow, indicating little or no production of T lymphocytes from E-negative precursors. On the other hand, bone marrow cells purged of E^+ lymphocytes did not contain TL-CFU, but appeared to regain high numbers of TL-CFU during Dexter culture; this suggested that an earlier step in T cell differentiation may take place in this culture system. The generation of TL-CFU in the E-negative long-term marrow cultures only occurred when an adherent stroma layer had been established in the culture flask; it did not require added mitogens or detectable interleukin 2 in the culture medium. TL-CFU in fresh marrow (TL-CFU II) are mature (E^+, $T3^+$) T cells and are capable of producing helper ($T4^+$) and suppressor/cytotoxic ($T8^+$) phenotype cells in colonies. The TL-CFU newly formed in E-depleted Dexter cultures (TL-CFU I) are distinct from this population, as they are E-negative and give rise to colonies of the helper type only. T3 cell depletion of the marrow inoculum prior to culture did not prevent the appearance of TL-CFU I in long-term culture; this suggests that TL-CFU I are derived from an E^- and $T3^-$ precursor (pre-TL-CFU).

DURING THE PAST FEW YEARS, factors regulating the proliferation and activation of mature T lymphocytes have been identified, and it has now become possible to culture these cells under well-defined in vitro conditions.[1] Still, very little is known about the regulation of earlier steps of T cell differentiation. The thymus plays a major role in this process during ontogeny. However, recent studies in congenitally athymic (nude) mice have shown that functionally active T lymphocytes may also be produced in the absence of this organ.[2,3] Dexter type long-term bone marrow cultures (LTBMC) have been employed to study in vitro T cell differentiation in murine bone marrow.

Schrader and Schrader[4] were the first to demonstrate the potential of LTBMC to induce T cell commitment in marrow-derived hematopoietic precursors. They showed that LTBMC gave rise to cells capable of differentiation into mature T lymphocytes when injected into irradiated mice. This finding was further substantiated by Jones-Villeneuve et al,[5] who reported the maintenance in LTBMC of immature, Thy-1–negative, progenitor cells in LTBMC, which could give rise to colonies of Thy-1–positive cells. These cells, developing in vitro in the absence of a thymic microenvironment, showed helper activity for cytolytic T lymphocyte precursors, a characteristic of a mature T lymphocyte subpopulation.[6] To what extent T cell differentiation can occur in murine LTBMC has not been established. In one series of experiments,[7] no Thy-1–positive cells were detected in the cultures. This suggested that terminal differentiation to T lymphocytes was prohibited. By contrast, in investigations by Dorshkind and Phillips,[8] under appropriate culture conditions, Thy-1–positive functional lymphocytes were maintained in LTBMC, although it was suggested that this was due to contamination of already mature T cells rather than the result of differentiation of immature (Thy-1–negative) precursors.

The LTBMC system has been recently adapted for cells from other than the murine species.[9-11] In this article, we describe experiments directed towards T cell kinetics and differentiation in long-term cultures of human bone marrow. Our data indicate that terminal differentiation from precursor cells does not take place, but that E-negative progenitors are produced in culture. These progenitors (TL-CFU I) give rise to mature helper phenotype progeny (ie, $T3^+$, $T4^+$, $T8^-$) upon transfer to a T cell colony assay system. In this respect, they differ from the E^+, $T3^+$ TL-CFU subset (TL-CFU II) in fresh marrow, which is capable of the production of colonies of both the helper ($T4^+$) and the suppressor/cytotoxic ($T8^+$) phenotypes.

MATERIALS AND METHODS

Bone Marrow

Bone marrow cells obtained from posterior iliac spine aspirates from hematologically normal donors were collected in Hanks' bal-

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anced salt solution (HBSS) containing preservative-free heparin. Excess red blood cells were removed by sedimentation (20 minutes) at unit gravity in 0.1% methylcellulose. Light density (≤1.077 g/mL) marrow fractions were obtained by Ficoll-Isopaque separation.[12]

E Rosetting and E Depletion

E rosetting of lymphocytes was achieved by using the method of Madsen and Johnsen,[13] with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (AET-SRBC). E rosette-negative (E^-) cell fractions were obtained after centrifugation of the rosetted cells through Ficoll-Isopaque. According to evaluations following a second rosetting procedure, the E^- fractions contained less than 2% E-positive (E^+) lymphocytes.

Antibody- and Complement-Mediated Cytotoxicity

Some of the marrow specimens were treated with cytotoxic monoclonal antibody (MoAb) OKT3 and rabbit complement (C') to remove T3-positive ($T3^+$) lymphocytes. First, 2×10^6 marrow cells in 100 μL were incubated on ice with 5 μL OKT3 for 30 minutes. When more than 2×10^6 cells were treated, the incubation volume and the amount of OKT3 were increased proportionally. Subsequently, cells were washed with HBSS (1×), resuspended in 1 mL C' (30% vol/vol in HBSS), and incubated for 30 minutes at 37 °C. After adding a few grains of deoxyribonuclease (bovine pancreas DNA-se I, B-grade; Calbiochem, San Diego) to avoid cell clumping and washing with HBSS (2×), cells were prepared for both viability (trypan blue exclusion) and indirect immunofluorescence (after a second OKT3 labeling) assays to evaluate the efficiency of the lysis and for tissue culture experiments. Control incubations with OKT3 alone and with C' alone were included in each experiment. These incubations did not show a cytoreductive effect. In one experiment, a T3 depletion was applied to an E-depleted marrow suspension that contained less than 2% $T3^+$ cells. In this case, the efficiency of the in vitro treatment was assessed in a parallel incubation of peripheral blood mononuclear cells with OKT3 and C'.

Indirect Immunofluorescence Studies

Cells positive for T3, T4, T6, and T8 cell surface antigens were assessed with indirect immunofluorescence assays using MoAbs of the OKT series (Ortho Pharmaceutical Corporation, Raritan, NJ) as the first layer and purified goat anti-mouse Ig immunoglobulin G coupled to fluorescein isothiocyanate (GAM/FITC) as the second layer. After fixation with paraformaldehyde,[14] cells were treated on ice with MoAb (in titers of 1:20) and subsequently with 1:40 diluted GAM/FITC (Nordic Immunological Reagents, Tilburg, The Netherlands), according to standard procedures.[15] Using a Zeiss fluorescence microscope with plan-neofluar 25× and 63× lenses, 200 cells per slide were studied. Control incubations with GAM/FITC alone, included in each test to check for nonspecific binding of the reagent to the cell surface, were negative in all experiments. Normal granulocyte macrophage (CFU-GM) and acute and chronic myeloid leukemia (AML and CML) colony cells were found to lack reactivity with the OKT MoAbs used, indicating that these reagents were valid for studying T cell differentiation in colony culture.

T Cell Colony Assay

T lymphocyte colonies were grown in the PHA–leukocyte feeder system, as previously described.[16] In brief, 0.5×10^5 cells were plated in a 0.4-mL liquid upper layer, supplemented with 0.01 mL PHA (Wellcome Diagnostics, Dartford, England) upon a 1-mL 0.5% agar layer containing 2×10^6 irradiated (2,500 rad) peripheral blood nucleated cells in 35-mm Petri dishes (Costar, Cambridge, Mass). After seven days of incubation (37 °C, 5% CO_2 in air) in a humidified atmosphere, colonies of more than 50 cells were counted with a Zeiss inverted microscope. Colony cells were harvested with a Pasteur pipette, washed twice with HBSS, and prepared for E rosette and indirect immunofluorescence assays.

Long-Term Bone Marrow Culture (LTBMC)

Dexter-type cultures for human bone marrow were set up in two phases. In the first two to four weeks of culture, an adherent stroma cell layer, consisting of fibroblast-like cells, adipocytes, and macrophages, was allowed to form. A second portion of autologous marrow cells, cryopreserved[17] on the day of aspiration, was then added. The time of addition of this second inoculation (or recharge) was considered as the initiation or time zero of LTBMC. Culture conditions have been described in detail elsewhere.[18] However, slight modifications were introduced to adapt the culture system for the studies reported here. Second inoculates contained 3 to 10×10^6 Ficoll-Isopaque isolated marrow cells (either nondepleted, E depleted, or E depleted plus T3 depleted). Prior to the reinoculation, all the spent medium, including the remaining nonadherent cells of the first inoculate, was discarded, the adherent layer washed twice with 5 mL HBSS, and fresh medium added. LTBMC were then monitored weekly, ie, the nonadherent cells in the cultures were counted and used for E rosetting, immunofluorescence, and T cell colony formation.

Detection of Interleukin 2 (IL-2) Activity

IL-2 activities of LTBMC conditioned media were determined as described by Gillis et al[19] for murine IL-2. The media were kept at −20 °C until use. After thawing, they were added in a 10% dilution in culture medium (Iscove's with 10% fetal calf serum and 10^{-4} mol/L beta-mercaptoethanol) in a vol of 0.2 mL to microtiter wells containing 5×10^3 cells of a murine IL-2-dependent cytotoxic lymphocyte line (Dr L. Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Significant proliferation of these cells was obtained with samples containing 0.1 U/mL of partially purified (mitogen-free) human IL-2.

RESULTS

Kinetics of T Lymphocytes in LTBMC

Marrow cells in LTBMC were checked for the presence of E rosette (nine experiments) and T3, T4, T6, and T8 positive cells (three experiments). Cultures inoculated with nondepleted and with E rosette-depleted marrow cells were compared. In the nondepleted LTBMC, cells showing mature T cell characteristics (ie, E^+, $T3^+$, and $T6^-$) were maintained in relatively constant concentrations (20% to 40% of the cells in suspension). Both T lymphocytes with the helper ($T4^+$) and with the suppressor/cytotoxic ($T8^+$) phenotype were present. On the other hand, in the cultures established from E rosette-depleted marrow, numbers of cells expressing these T cell markers remained low, indicating that production of mature T cells from precursors did not occur. Results of the weekly determinations of T cell markers on LTBMC suspension cells in a complete experiment are given in Fig 1.

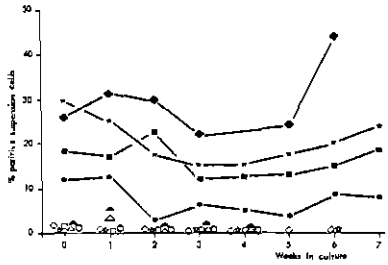

Fig 1. Kinetics of suspension cells carrying T cell surface antigens in LTBMC derived from nondepleted (closed symbols) and from E rosette-depleted marrow (open symbols). (◆) E; (☆) T3; (■) T4; (▲) T6; (●) T8.

TL-CFU in the Marrow at the Time of Inoculation in LTBMC (TL-CFU II)

Some characteristics of the TL-CFU present in the bone marrow prior to inoculation in long-term culture are presented in Table 1. In all cases, TL-CFU were removed from the marrow following E rosette depletion. Most likely, this was the result of separation of the TL-CFU and not of removal of factors essential for colony growth, as colony formation was not restored in E-negative marrow samples by the reconstitution of the plated marrow suspension with irradiated E lymphocytes or by the addition of human leukocyte-derived IL-2 to the colony culture. These results indicate that the TL-CFU in the marrow are all E-positive cells. In separate experiments, it was found that TL-CFU were completely eliminated from the bone marrow following in vitro treatment with MoAb OKT3 and complement, but not with complement or MoAb alone. Thus, marrow TL-CFU are also positive for the T3 antigen and apparently represent a population of mature T lymphocytes. We have designated this colony former as TL-CFU II.

Kinetics of TL-CFU in LTBMC

At first, TL-CFU numbers in nondepleted LTBMC were estimated in six experiments. In five experiments, it was found that TL-CFU numbers had increased during culture. Data corrected for the weekly culture medium replacements[20] reveal a mean production of 323% (range, 158% to 760%) as compared with TL-CFU numbers in culture at time zero. An example of the TL-CFU kinetics in a nondepleted LTBMC (Fig 2) indicates a constant increase of TL-CFU during six weeks of incubation. It was subsequently investigated as to whether E-negative cells were responsible for this TL-CFU production. LTBMC were established from E-depleted bone marrow, so that no TL-CFU II were inoculated in culture. The results of three separate experiments are given in Fig 3. It appeared that new TL-CFU were produced in these LTBMC and remained detectable in culture during five to six weeks (upper panel of the figure).

Meanwhile, the consistent E-negative nature of these LTBMC throughout this period was evident in each experiment (lower panel). These results suggest that the new TL-CFU were generated in LTBMC from immature (E^-) cells. To exclude the possibility of production of TL-CFU due to the incomplete elimination of mature T lymphocytes from the marrow suspension at the onset, LTBMC were set up with marrow cells that had been subjected to a double procedure of T lymphocyte elimination. After the E rosette separation, the cells underwent antibody- and complement-mediated lysis with MoAb OKT3 (E^- cells treated with C' alone serving as a control). Table 2 shows that the numbers of TL-CFU generated in LTBMC after inoculation with one step (E) and double step (E as well as T3) depleted bone marrow were comparable. Thus, the additional T3 depletion did not significantly affect the TL-CFU production in culture; this makes it unlikely that the newly formed TL-CFU originated

Table 1. T Lymphocyte Colony Growth From Human Bone Marrow (TL-CFU II)

	Percent E^+ Lymphocytes Before Colony Culture	T Lymphocyte Colonies†
Nondepleted marrow (n = 9)		
(low-density Ficoll-Isopaque fraction)	33.8 ± 8.5*	301 ± 183*
E rosette-depleted marrow (n = 9)	1.2 ± 0.8*	11 ± 14*
+ 25 U IL-2‡ (n = 2)	0.25	2
+ 50 U IL-2‡ (n = 1)	1.5	28
+ 100 U IL-2‡ (n = 1)	1.5	8
+ irradiated (2,500 rad) E^+ lymphocytes‡§ (n = 2)		0
OKT3 + complement-treated marrow (n = 2)		0

*Mean ± standard deviation.

†Numbers per 10^5 plated cells; E positivity of the colony cells was always verified.

‡Added to the upper layer of the cultures.

§Added numbers reconstituted the concentrations present in nondepleted marrow.

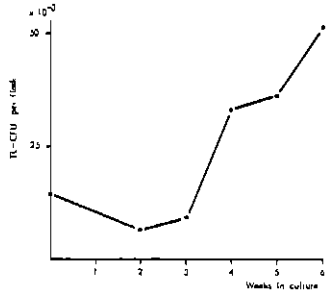

Fig 2. Kinetics of TL-CFU in a nondepleted LTBMC. Total TL-CFU numbers per culture flask are expressed as values corrected for weekly medium replacements, according to Gregory and Eaves.[20]

from a residual population of mature phenotype T cells insufficiently removed from the marrow inoculates. In addition, it was excluded that TL-CFU production was the result of detachment of TL-CFU present in the preformed adherent stroma layer. In stroma preestablished control cultures to which an irradiated (2,500 rad) marrow portion had been added or in cultures without a second marrow charge, no TL-CFU were detected in suspension (data not shown). This provided evidence to indicate that E^-, $T3^-$ precursor cells (without colony-forming capacities) gave rise to TL-CFU in LTBMC. We considered the possibility that these newly formed TL-CFU in consistently T cell-devoid cultures (Fig 1) lacked T cell surface characteristics and were different in this respect from the common TL-CFU (TL-CFU II) in fresh bone marrow. E depletion experiments, performed on LTBMC suspension cells after one week of culture, were carried out to test this possibility. It was found that two LTBMC suspensions still showed high concentrations

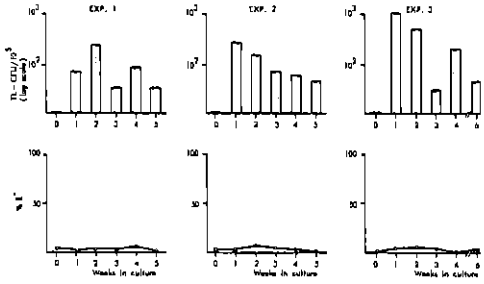

Fig 3. T lymphocyte colony-forming cells (TL-CFU I) per 10^5 suspension cells measured at weekly intervals in LTBMC (upper panel) in comparison with the kinetics of E^+ lymphocytes (lower panel). LTBMC were established from E rosette-depleted bone marrow. Results are from three separate experiments.

Table 2. Comparison of TL-CFU Production (TL-CFU I) in One-Step (E) and Two-Step (E as well as T3) T Lymphocyte-Depleted LTBMC

	Numbers of TL-CFU per Culture Flask†				
Marrow*	Week 0	Week 1	Week 2	Week 3	Week 4
E deplotion‡	0	4,108	6,044	1,240	4,935
E as well as T3 depletion§	0	3,267	4,290	1,862	3,115

*In vitro treatment of the bone marrow was done at the time of the inoculation onto the preestablished stroma layer (time zero). TL-CFU were then assessed at weekly intervals.

†Data are not corrected for the weekly removal of nonadherent cells due to medium replacements.

‡E rosette-depleted bone marrow was incubated with C' only.

§E rosette-depleted bone marrow was incubated with OKT3 and C'. Since E-negative bone marrow contained less than 2% OKT3-positive cells (Fig 1), the efficacy of the procedure was assessed in a parallel incubation on peripheral blood mononuclear cells using the same OKT3 and C' batches in identical concentrations. The blood cells contained 54% OKT3-positive cells and were all viable (on the basis of trypan blue exclusion); after the MoAb and C' treatment, OKT3 staining was reduced to 5%, and 54% of the cells had ingested trypan blue.

of TL-CFU (ie, 543 and 207/10^5 cells) after E depletion. This indicated that the TL-CFU generated in LTBMC, in contrast to TL-CFU II, do not express the E rosette receptor. We have designated this E-negative TL-CFU population as TL-CFU I.

Phenotyping of Colony Cells

The immunologic phenotypes of the colony cells grown from (E^-) TL-CFU I harvested at week 2 and week 4 of E^- LTBMC and from unfractionated fresh marrow-derived (E^+, $T3^+$) TL-CFU II are given in Table 3. The data indicate that both TL-CFU I and TL-CFU II produced mature T lymphocyte, ie, E^+, $T3^+$, and $T6^-$ progeny. A marked difference, however, was evident with respect to the helper and suppressor/cytotoxic phenotype of the colony cells. Colonies derived from the TL-CFU I were almost entirely of the

Table 3. T Lymphocyte Surface Markers on Marrow Cells Before and After T Cell Colony Formation (Percent Positive Cells)

	Normal Marrow (TL-CFU II)		E^- LTBMC Suspension† (TL-CFU I)			
			Week 2		Week 4	
Markers	Before Culture*	Colonies	Before Culture	Colonies	Before Culture	Colonies
T3	46	90	<1	72	<1	72
T4	30	60	<1	56	<1	75
T6	<1	<1	<1	<1	<1	<1
T8	16	26	<1	1	<1	8
E	39	86	1	91	0	97

*Ficoll-Isopaque–separated light density (≤ 1.077 g/mL) marrow cells.

†Nonadherent cells from E rosette-depleted marrow inoculated cultures, assayed at week 2 and at week 4 of culture.

helper ($T4^+$, $T8^-$) phenotype, whereas those arising from the TL-CFU II contained $T4^+$ and $T8^+$ cells in the usual peripheral blood ratio. It appeared from morphological analysis, that both the TL-CFU I and the TL-CFU II colony cells resembled lymphoblasts.

Determination of IL-2 Activity in LTBMC

Supernatants of two LTBMC were assayed for IL-2 at different times of culture. We detected no IL-2 activities in the media at weeks 2, 3, and 4 of these cultures.

DISCUSSION

In this article, we have described experiments on T lymphocyte kinetics in human Dexter-type long-term bone marrow cultures (LTBMC) and have distinguished between three stages of T cell differentiation (ie, a putative pre-TL-CFU, TL-CFU I, and TL-CFU II) on the basis of PHA-induced in vitro colony formation. Our results demonstrate that T lymphocytes are maintained in LTBMC when an unfractionated light density marrow sample is inoculated (Fig 1). However, when an E-depleted marrow fraction is used, cells with E, T3, T4, T6, or T8 markers are not detected in significant numbers in these cultures. Thus, differentiation from immature precursors to T cells with a more mature immunologic phenotype does not occur. Similar findings have been described in the mouse system.[8]

T lymphocyte colony-forming cells are produced in LTBMC. This was evident in nondepleted cultures (Fig 2) as well as in cultures set up with marrow from which T lymphocytes had been removed prior to inoculation in LTBMC (Fig 3, Table 2). The T cell-depleted marrow had been rendered devoid of TL-CFU (Table 1). The TL-CFU newly formed in LTBMC in the absence of T lymphocytes stemmed from immature (E^-, $T3^-$) cells without colony-forming capacities. This step did not require the addition of mitogens to the system and occurred in the absence of assayable amounts of interleukin 2 in the culture medium.

On the other hand, the generation of TL-CFU in E^- LTBMC was dependent on the presence of stroma cells. Typically, cultures set up in the absence of a preestablished adherent cell layer failed to produce significant numbers of E^- TL-CFU (data not shown). Thus, the production of TL-CFU was stimulated by the marrow stroma analogue in these cultures, similar to the production of the granulocyte/macrophage colony-forming cells.[18] We have no data to indicate the identity of the stroma-derived factors needed for this production. A recently discovered interleukin, IL-3, is a possibility, as Hapel and coworkers[21] have demonstrated that, in mice, IL-3 promotes the commitment of Thy-1^- precursors to T cell differentiation in the absence of IL-2. It should be emphasized that in concordance with the IL-3-induced progenitor cells, the LTBMC-derived immature TL-CFU in mice[5] and in man (Table 2) produced no significant amounts of cytotoxic/suppressor phenotype T lymphocytes, but mainly, if not only, helper phenotype progeny. We designated the E^- TL-CFU, which is generated in the human Dexter culture and is capable of the production of helper ($T4^+$) colonies, TL-CFU I.

It was found that TL-CFU detectable in uncultured marrow samples carry a mature T cell (ie, E^+ and $T3^+$) phenotype, which is in agreement with a previous report.[22] We have now designated these as TL-CFU II. Similar results have been published for peripheral blood-derived TL-CFU.[22-24] By contrast, other investigators have described the presence of E^-, $T3^-$ TL-CFU in the peripheral blood[25] and marrow,[26] using somewhat different colony culture techniques. The relationship between these and the Dexter culture-derived TL-CFU I is not yet clear, but the results of colony phenotyping by Triebel et al[26] reveal progeny of both the helper ($T4^+$) and the suppressor/cytotoxic ($T8^+$) phenotypes and, therefore, suggest that they are different cell types.

At present, we propose that the production of E^- TL-CFU (TL-CFU I) in LTBMC reflects an early step in bone marrow T lymphocyte differentiation. This hypothesis is also of interest in view of the observations of Messner et al[27] that multilineage in vitro colony-forming cells from normal bone marrow are capable of T lymphocyte differentiation, notably only towards the helper ($T4^+$, $T8^-$) subset.

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CHAPTER 9

General Discussion

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9.1. Interleukin 2: its role in the proliferation of neoplastic T and B cells

Interleukin 2, previously termed T cell growth factor, stimulates the proliferation of normal peripheral blood or bone marrow T lymphocytes through its interaction with specific receptors. These receptors appear on the cell membrane following activation by antigens or lectins (Morgan, Ruscetti and Gallo, 1976). Recently, it has become clear that IL2 exerts a similar effect on activated B lymphocytes (Tsudo, Uchiyama and Uchino, 1984; Mingari et al., 1984; Nakagawa et al., 1985). Investigations were then initiated to clarify the role of IL2 in the proliferation of different types of B and T cell neoplasia. Studies along this line have made use of the availability of pure IL2 preparations (obtained by recombinant techniques) (Devos et al., 1983), IL2 gene probes (Taniguchi et al., 1983) and monoclonal antibodies reacting with the IL2 membrane receptors (Uchiyama, Broder and Waldmann, 1981). The lymphoid malignancy most intensively studied so far has been adult T cell leukemia/lymphoma (ATL), the etiology of which is associated with the human T cell lymphotropic virus I (HTLV I) (see Wong-Staal and Gallo, 1985, for review).

The fact that HTLV transformed cells can be grown relatively easily in vitro has contributed to the progress made in the analysis of proliferation of this malignancy (Wong-Staal and Gallo, 1985). Attempts to unravel the growth abnormalities of other lymphoid neoplasms, e.g., acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non Hodgkin's lymphoma (NHL) have been hampered by the lack of adequate in vitro proliferation assays.

The experiments of this thesis represent the first efforts to characterize the factors involved in the growth of certain human lymphoid malignancies (i.e., non-T ALL, B CLL, T ALL/T NHL) and have in particular been concerned with the role of IL2.

9.1.1. Non-T acute lymphoblastic leukemia (non-T ALL)

Fresh non-T ALL cells of the undifferentiated (CALLA negative), the common (CALLA positive) or the pre-B (CALLA positive + cytoplasmic M heavy chain positive) immunophenotype did not express receptors for IL2 on their

surface as assessed with monoclonal antibody (MCA) anti Tac in indirect immunofluorescence (Chapters 2 and 3). Within 18 hrs following incubation of the cells with either PHA or the phorbol ester TPA significant proportions (25-100%) of the cells exhibited receptors for IL2 on their membrane. It became clear that the induction of these receptors on the cells directly relates to their proliferation ability. IL2, when added to cultures of ALL cells, stimulated colony growth, although only when PHA or TPA and a leukocyte feeder layer were incorporated in the culture as well (Table 1). IL2 supplemented in sufficient concentrations was essential for colony formation by non-T ALL cells, and a dose effect relationship between IL2 concentration and colony response was apparent. The frequency of colony forming cells under optimal IL2 concentrations in the presence of PHA (or TPA) and a leukocyte feeder was estimated at 25-70 CFU per 10^5 cells.

In a minority of patients, the ALL cells, though displaying IL2 receptors, did not respond to IL2 in colony culture even when very high concentrations of IL2 (1000 u/ml) were supplied.
The reason for this failure of response is as yet not understood but suggests variation of regulation of growth. We speculate that these differences relate to functional abnormalities of the IL2 receptor or to additional growth factor requirements.

Non-T ALL cells appeared to be firmly arrested in their maturation. The cells did not demonstrate alterations to later stages of differentiation upon proliferation in culture. This was evident from morphological examination, analysis of B cell differentiation antigens using a panel of monoclonal antibodies and immunoglobulin expression. Notably, impaired differentiation in vitro was also evident in the cultures supplemented with TPA, a well established differentiation inducer of normal and neoplastic B lymphocytes.

9.1.2. B cell chronic lymphocytic leukemia (CLL)

B CLL cells spontaneously expressed IL2 receptors in 4 out of 5 cases (Chapter 5). The cells proliferated as measured in colony techniques and thymidine incorporation assays in response to PHA + IL2 or TPA + IL2 (Table 1, Chapters 5 and 6). Proliferation in response to IL2 could be inhibited by simultaneous blocking of the IL2 receptor with the anti-IL2 receptor

MCA. Because of the spontaneously activated IL2 receptors on B CLL cells, the addition of PHA (or TPA) to culture was not immediately required and could be delayed for 24 hrs without reducing the efficiency of stimulation of proliferation of the cells by IL2. When B CLL cells were kept devoid of PHA (or TPA) for longer periods, however, the cells lost their responsiveness to IL2. As a consequence, when PHA was withheld from these cultures for more than one day, the rate of proliferation of B CLL cells in vitro declined.

In one case of CLL, a spontaneous ^{3}H-TdR uptake by the CLL cells, i.e., independent on the addition of PHA and IL2 to the cultures, was observed (Chapter 6). This autonomous proliferation was most likely induced by endogenous IL2, since IL2 receptor blocking with MCA shut off ^{3}H-TdR incorporation. Moreover, IL2 activity was detected in culture media conditioned by the CLL cells of this patient. Similar to activated normal T and B cells, activated CLL cells express two types of IL2 receptors, one with a high affinity and one with a low affinity. This was shown in binding studies with radiolabeled IL2 (Chapter 6). B CLL cells differ from pre-B ALL in two aspects: a) they do not need feeder cells as an additional stimulating factor and b) B CLL cells differentiate further in vitro. B CLL colonies produced in culture consisted of plasmacellular lymphocytes or fully developed plasma cells expressing the same heavy chain and light chain type in the cytoplasm, as was characteristic of the original B CLL lymphocytes (Chapter 5).

9.1.3. T non Hodgkin's lymphoma (T NHL) and T acute lymphoblastic leukemia (T ALL)

We have studied the leukemic blood cells from 3 patients with T NHL and 3 patients with T ALL (Chapter 7). This series included one patient of a relatively mature (i.e. stage III) and 5 patients of a more immature (i.e. stages I or II) immunophenotype (Nadler et al., 1982). IL2 receptor expression on the cells was initially negative but such receptors appeared on significant percentages of the cells (20-97%) following exposure to TPA. Consistent with these findings was the observation that in cultures containing TPA + IL2, colony growth was induced (at an average cloning efficiency of 30-76 per 10^{5} cells). On the other hand, PHA was only capable

of activating IL2 receptor expression and inducing colony growth in the single case of NHL which showed the mature thymocyte phenotype (i.e., TdT^-, $WT1^+$, E^+, $T3^+$, $T4^+$, $T6^-$, $T8^-$, $T10^+$) among this limited series of patients. In the other five cell samples from patients with the stage I or II thymocyte phenotype (i.e., TdT^+, $WT1^+$, E^+ or E^-, $T3^-$, $T4^-$, $T6^-$, $T8^-$, $T10^+$; or TdT^+, $WT1^+$, E^+, $T3^-$, $T4^+$, $T6^+$, $T8^+$, $T10^+$) only TPA was effective in activating IL2 receptor expression (Table 1). Furthermore, it is noteworthy that in three of these cases exogenous IL2 was not required for stimulating proliferation. TPA alone in two cases, and PHA alone in one case induced colony growth. Nevertheless, also in the latter instances, it has become clear that proliferation depended on IL2. It appeared that IL2 was provided endogenously by the neoplastic cells in culture: blocking of the IL2 receptor by incubating the cells of these patients with the anti-Tac monoclonal antibody abrogated proliferation. It then also appeared that these cells were self-producers of IL2, since conditioned medium derived from the cells sustained the proliferation of an IL2 dependent cell line. No clear indications for in vitro maturation during colony culture of T ALL and T NHL cells were apparent. Surface marker analysis of colony cells did not provide evidence that the cells had proceeded to later stages of differentiation.

9.1.4. Different pathological subtypes of lymphoid malignancy express characteristic response patterns to IL2

The patterns of IL2 dependence of the various lymphoid neoplasms are summarized in Table 1. Some characteristic features of the IL2 response of the different lymphoid malignancies could be recognized: a) B CLL: the spontaneous expression of IL2 receptors in certain cases; b) non-T ALL: the need for additional stimulation by feeder leukocytes for colony formation; c) T ALL/T NHL: immature and intermediate (i.e., stage I and stage II) thymocytes can be induced to express IL2 receptors and to form colonies in the presence of IL2 with TPA but not with PHA, whereas mature (stage III) thymocytes respond to both mitogens. These systematic differences between different disease categories are potentially useful in clinical practice. At the present time, it remains to be established as to whether a classification of the different cytopathological types of lymphoid malignancy on

Table 1 PATTERNS OF IL2 DEPENDENCE OF LYMPHOID NEOPLASIA IN MAN

	Normal T lympho-cytes (blood)	T NHL (mature stage)	T NHL/T ALL (immature or intermediate stage)	B-CLL	Non-T ALL (unclass.; common; pre-B)
IL2 receptor expression					
spontaneously	-	-	-	+/-	-
following TPA induction	+	+	+	+	+
following PHA induction	+	+	-	+	+
Proliferation upon stimulation with IL2					
IL2	-	-	-	-	-
TPA + IL2	+	+	+	+	-
PHA + IL2	+	+	-	+	-
TPA + IL2 + leuko-cyte feeder	+	+	+	+	+
PHA + IL2 + leuko-cyte feeder	+	+	-	+	+
Autocrine IL2 production	+/-	+	+/-	?	?

the basis of in vitro growth criteria will be of diagnostic value.

9.2. Growth control of malignant lymphoid cells by external stimuli: Do abnormalities exist?

To date, no experimental data have been produced that allow explanations for the uncontrolled accumulation of cells in lymphoid malignancies with a nonviral etiology. One could argue that the growth of the malignant lymphoproliferative disorders results from an imbalance of growth regulation, or from a complete loss of control by the complex of regulatory factors. In AML, clonogenic cells proliferate in reaction to granulocyte-macrophage colony stimulating factor (GM-CSF), an established growth factor of normal myeloid progenitor cells. The proliferating cells at the same time are induced to further maturation, which is associated with a decrease in proliferative potential (Chapter 4, and reviewed by Griffin and Löwenberg, 1986). Clearly, the available evidence indicates that clonogenic AML cells have preserved the linkage of differentiation and loss of proliferation, which is a fundamental property of normal myeloid progenitor cells.

One may therefore assume that the expansive growth of AML cells results from an imbalance, rather than a loss, of the regulation of growth and differentiation by external factors. For example, alterations in the sensitivities to growth stimulatory and inhibitory factors may account for this imbalance. One may also hypothesize that leukemia cells respond normally to the physiological growth regulators. In that situation, expansive growth could be due to certain intrinsic defects, which for instance make the cells less susceptible to mechanisms leading to cell death.

Mechanisms by which certain oncogenic viruses have been found to alter the growth of their host cells (i.e., a) induction of constitutive growth factor receptors and b) direct activation of cytoplasmic or nuclear signal pathways resulting in the loss of growth factor dependence) have been mentioned in Chapter 1. Evidence that similar mechanisms are involved in the growth of the investigated types of lymphoid malignancy was not obtained. Although a variety of in vitro growth patterns was observed, the cells nonetheless retained dependence on specific growth stimuli and activation signals. Autocrine stimulation, a third mechanism by which tumour cells have been proposed to escape growth factor control (see Chapter 1), was observed in certain cases of T cell malignancy (Chapter 7) and has been suggested also to occur in a case of B CLL (Chapter 6). However, IL2 driven autostimulation is also observed in a subpopulation of normal T lymphocytes. Therefore, there is no proof that this feature is specific of the neoplastic status of the cells. As little is still known of the growth regulation of normal B cells at various stages of differentiation, we are not sure as to whether self stimulation by IL2 in B CLL is to be considered as typical of the transformed nature of the cells either.

Taken together, the data reported in this thesis raise the possibility that mechanisms, other than those proposed for virally induced leukemias, are responsible for the accumulation of neoplastic cells in non-viral lymphoid malignancies. Possible alternative mechanisms could be based on the loss of differentiation capacity (e.g., observed in ALL) or on a decreased sensitivity to growth inhibitory regulators.

9.3. Analysis of growth requirements of normal immature cell types

To understand how leukemic cells are disturbed in their proliferation

and differentiation characteristics, a detailed knowledge of normal cells at comparable stages of maturation is necessary. This knowledge is relatively easily obtained when the normal cells can be isolated directly from the hematopoietic tissues in large numbers and tested for their response to fully characterized growth stimuli. For instance, T lymphocytes can be grown in clonal cell culture stimulated by pure (recombinant) IL2. These studies are more difficult to conduct when the normal cells represent a minority population amongst the heterogeneous bone marrow. This situation typically applies to the scarcity of precursor cells in normal tissue from which leukemias often descend. As mentioned in chapter 1, some of these cell types, e.g., progenitor cells of myelomonocytic and erythroid differentiation lineages, can be stimulated with pure growth factor preparations and form colonies in vitro (Metcalf, 1984). Nonetheless, the exact factor requirements of these cell types have not been fully elucidated yet, since the admixture of nonclonogenic marrow cells may drastically influence the outgrowth of the colony forming cells. This influence may be achieved by those cells through the elaboration of endogenous stimulatory or inhibitory components. Thus, a conclusive evaluation of factor responses is only possible when not only pure growth factors, but also highly purified cell populations are used. Purification methods for murine and human bone marrow stem cells and progenitor cells on the basis of monoclonal antibody and cell sorting technology are currently in development (Visser et al., 1984; Bodger et al., 1983; Civin et al., 1984). One may expect that the application of flow cytometry of purified fractions in combination with direct cell deposition in microculture (Terasaki) trays will permit studies on the growth requirements of single clonogenic cells in the near future (Lansdorp et al., 1986).

9.4. Comparison of growth requirements of neoplastic lymphoid cells with those of their non transformed analogues

At this stage, it is not possible to compare the growth features of malignant lymphoid cells with those of their normal counterparts.
Surprisingly little is known about the physiological regulation of the early stages of lymphoid proliferation and differentiation. This limited understanding of the regulation of lymphopoiesis is the immediate conse-

quence of the lack of in vitro culture systems and of purified growth factor preparations for normal lymphoid precursor cells. The problem of scarcity of cells, already outlined in the foregoing paragraph (9.3.), is also encountered: the numbers of lymphoid progenitors in the various lymphopoietic tissues are often so small that their identification is difficult. These difficulties apply to the analysis of normal pre-B cells, prothymocytes and early B lymphocytes.

9.5. Application of the long-term bone marrow culture system for the analysis of lymphoid precursor cell stages

Several investigators have recognised the possibility that the long-term bone marrow culture system (LTBMC), initially developed by Dexter and colleagues, could prove useful for the characterization of rare normal lymphocytic precursor cells and the isolation of these cell types. In this in vitro system, hematopoiesis is stimulated by an adherent cell layer of bone marrow stroma components (i.e., fibroblasts, macrophages, adipocytes, endothelial cells). LTBMC have been shown to generate very immature hematopoietic elements, including lineage restricted progenitor cells (CFU-GM, BFU-e, CFU-Meg) and pluripotent stem cells, over periods of more than one year (Dexter et al., 1977). Due to the lack of sufficient concentrations of growth factors that act at the terminal stages of hematopoietic differentiation (i.e., CSFs, IL2, Epo), the production of mature cells is limited. As a result, immature cell populations are enriched in LTBMC suspensions as compared with fresh bone marrow samples.

The mechanism by which the composite adherent cell layer stimulates hematopoiesis in LTBMC has remained unknown. Although it was initially believed that adipocytes (fat cells) played an important role, evidence against this suggestion was later obtained (Touw and Löwenberg, 1983). The establishment of cloned stroma derived cell lines may be of help to elucidate the nature of stromal stimulation of hematopoiesis in the near future (Zipori et al., 1985).

The generation of lymphoid precursors in murine LTBMC was first demonstrated by injecting LTBMC-suspension cells in irradiated mice, which then gave rise to high numbers of concanavalin A (Con A) and lipopolysaccharide (LPS) stimulable lymphoblasts (Phillips et al., 1978; Schrader and

Schrader, 1978; Jones-Villeneuve and Phillips, 1980). It was subsequently documented that murine LTBMC of certain (but not all) strains of mice are capable of generating and maintaining T cell progenitors, including prothymocytes (Boersma and Eliason, 1982; van Bekkum et al., 1984) and T colony forming cells (Jones-Villeneuve et al., 1980).
B cell precursors at various stages of differentiation are also produced in LTBMC (Whitlock and Witte, 1982; Dorshkind and Phillips, 1983; Nagasawa et al., 1985; Denis and Witte, 1986; Dorshkind, 1986). Recently, Muller-Sieburg et al. (1986) reported a hundred fold enrichment of murine pre-B cells in LTBMC suspensions as compared with fresh bone marrow.

In our laboratory we have used LTBMC to study early steps of T lymphocytic differentiation in humans. We have proposed the existence of two precursor cell stages of the T lymphoid differentiation lineage in the bone marrow, which were designated pre-TL-CFU and TL-CFU I (Chapter 8).
Pre-TL-CFU is a postulated entity. It indicates the precursor of the TL-CFU I. Pre-TL-CFU are present in fresh bone marrow which is depleted of T lymphocytes and are devoid of T colony forming capacities in response to stimulation by PHA, IL2 and irradiated leukocytes. Under the stimulatory influence of the adherent stroma layer, pre-TL-CFU give rise to colony forming T cell progenitors (TL-CFU I) in LTBMC. Following PHA/IL2/irradiated leukocyte stimulation, these TL-CFU I produce colonies typically consisting of mature T lymphocytes of the T4 phenotype. TL-CFU I express an immature T cell phenotype, i.e., E^- and $T3^-$. Preliminary results of additional phenotyping of TL-CFU I suggest that these precursors also lack reactivity with other T cell markers (i.e., T4, T8, WT1) but express antigens characteristically found on hematopoietic progenitor cells (Ia, S16-14.4) (Moberts, Touw and Löwenberg, unpublished results). Pre-TL-CFU, which were also E and T3 negative, have not yet been typed with additional membrane markers. The immunophenotype of TL-CFU I is different from that of the cases of T ALL/T NHL described in Chapter 7, and presumably represents a more immature stage of differentiation. Thus, TL-CFU I cannot be regarded as normal counterparts expressing differentiation features of T ALL/T NHL cells. A detailed analysis of the response of pre-TL-CFU and TL-CFU I to various proliferation and differentiation stimuli may nonetheless be of great value to elucidate specific growth abnormalities of immature T cell type malignancies.

9.6. Prospects

Although this thesis has especially dealt with the regulatory role of IL2 in the growth of lymphoid neoplasms, obviously, we have to keep in mind the large body of evidence, mainly derived from experiments on mice, indicating that factors other than IL2 control the growth of lymphoid cells, in particular of the B differentiation lineage. The cDNA of one of these factors, termed IgG1 induction factor, was recently cloned and expressed in COS cells, and the protein purified to homogeneity (Noma et al., 1986). In the near future it will be of importance to test the response of malignant lymphoid cells to these and other stimuli, so that finally the probably highly complex system of growth factor regulation can be analysed to its full extent. Unfortunately, none of these activities have as yet been defined in man. For the study of the in vitro growth requirements of human lymphopoietic malignancies this remains a major limitation. Increasing numbers of research groups in the field of hematopoiesis are currently involved in the molecular cloning and expression of human genes encoding for a variety of growth factors and their receptors, as well as in the generation of monoclonal antibodies directed against these proteins.
This development justifies the expectation that more factors controlling human lymphopoiesis will become available in purified form and their physiological actions evaluated. Such pure and highly specific materials in combination with primary in vitro cell culture are the necessary experimental ingredients to obtain definite insights into the regulation of lymphoid cell growth. Investigations based upon these principles will settle the issue as to whether (and how) abnormal responses to growth and differentiation stimuli result in the uncontrolled cell growth of lymphoid neoplasia.

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Summary - Samenvatting

Summary

Hematopoiesis is regulated by polypeptide hormones acting at the hematopoietic stem cells and at the proliferative cells of the lymphoid, myelomonocytic, erythroid and megakaryocytic differentiation lineages. These hormones (or growth factors) control the properly balanced supply of blood cells. In certain neoplastic disorders of the blood forming system, e.g., leukemia and malignant lymphoma, the homeostasis is disturbed, which results in an accumulation of neoplastic cells. The mechanisms leading to the expansive growth of leukemia/lymphoma cells have not been elucidated. An attractive hypothesis is that these malignant cells escape the hormonal control of proliferation, for example through an acquired ability to produce hormone(s) themselves, through alterations in the function of hormone receptors expressed on the cell membrane, or through a complete loss of hormone dependence. Each of these abnormalities has been reported for experimental tumours. In vitro culture of primary cell specimens appears particularly useful in a search for possible abnormalities of the hormonal responses in human leukemia/lymphoma.

The experiments described in this thesis deal with the development of primary cell cultures for acute lymphoblastic leukemia of B as well as T cell lineage (non-T and T ALL), B cell chronic lymphocytic leukemia (B CLL) and leukemic T cell non Hodgkin's lymphoma (T NHL). These in vitro techniques were then applied to analyse growth and differentiation characteristics of the lymphoid malignancies, and their responsiveness to the hormone interleukin 2 (IL2).

In CHAPTER ONE basic principles of hematopoiesis, leukemia, the physiological action of interleukin 2 and the use of in vitro culture techniques are introduced. In addition, the concept that tumor cells may escape normal regulatory mechanisms of growth, and its experimental support, are briefly reviewed. A scheme of lymphoid differentiation proposed on the basis of immunological phenotyping is also included in this chapter. The cell types, from which the different lymphoid malignancies investigated in this thesis are believed to descend, are indicated in this scheme.

In CHAPTER TWO studies are presented dealing with the definition of culture conditions that permit the in vitro colony growth of common and

pre-B ALL cells. By applying monoclonal antibodies reactive with membrane receptors for IL2 we demonstrated that the ALL cells express IL2 receptors following in vitro incubation in the presence of a lectin (PHA) or a phorbol ester (TPA). IL2 receptors were not detectable on the fresh ALL cells. In a culture system to which IL2, PHA or TPA and irradiated feeder leukocytes had been incorporated ALL colony growth was observed. When one of these components was omitted from the culture system, colony growth was reduced or absent. Apparently, ALL colony growth depended on a complex of growth and activation stimuli including IL2, an unknown leukocyte component and a lectin or phorbol ester. Comparisons of the immunologic phenotypes of colony cells and fresh cells were not indicative of maturation of the ALL cells toward more mature B cell stages during colony culture.

In CHAPTER THREE the studies of the in vitro characteristics of ALL cells are extended to 24 patients. This series also included four cases of unclassified ALL. Inducibility of IL2 membrane receptors and in vitro colony growth were systematically analysed for the three immunological types (i.e., unclassified, common and pre-B) of ALL. Colony formation was observed in 18 of the 24 cases. The analyses revealed no significant differences between the immunological subgroups as regards their colony forming abilities. In addition, no differences in in vitro characteristics between childhood and adult ALL were apparent. Again, in this larger series of patients, no indications were obtained that the ALL cells were capable of in vitro differentiation beyond the stage of the cells in vivo.

In a parallel study presented in CHAPTER FOUR, the in vitro maturation capacities of acute myeloblastic leukemia (AML) under comparable culture conditions were investigated. Using a panel of monoclonal antibodies reactive with myelomonocytic differentiation antigens, we observed in 10 cases of AML that, although the AML colony forming cells produced progeny revealing progressive differentiation, this was clearly incomplete. Thus, ALL and AML colony forming cells share the inability to produce fully mature progeny in vitro.

CHAPTER FIVE comprises studies which settled the conditions permitting in vitro colony growth of B CLL. As compared with the in vitro characteristics of ALL three marked differences became evident: 1) fresh CLL cells frequently expressed IL2 receptors on their cell surface, 2) CLL colony growth depended on the presence of IL2 and PHA or TPA in culture but

did not require additional stimulation by irradiated leukocyte feeder cells and 3) morphological and immunologic analysis of the CLL colony cells revealed that CLL clonogenic cells had retained the capacity to produce more mature progeny (i.e., plasma blasts and plasma cells) in vitro.

Studies to determine the functional properties of IL2 receptors of B CLL cells and additional analyses of the proliferative response of the cells to IL2 in ^{3}H-thymidine incorporation assays are reported in CHAPTER SIX. Binding studies with radiolabeled IL2 revealed that CLL cells following their activation in vitro express two classes of IL2 receptors, one with a high affinity and one with a low affinity for IL2. In this respect, CLL cells resemble normal activated B (and T) cells.
The ^{3}H-thymidine uptake experiments in three cases showed that DNA synthesis by CLL cells is stimulated by IL2 and PHA. Interestingly, however, in one case we observed an apparently spontaneous proliferation, i.e., completely independent on the addition of IL2 and PHA to the microcultures. Experiments involving the blocking of IL2 receptors during culture and determinations of IL2 activity in the growth medium revealed that the proliferation of these cells was stimulated by endogeneous IL2. These findings suggest that the CLL cells themselves were capable of synthesizing IL2. However, we have not been able to prove this at the transcriptional level, i.e., by demonstrating the presence of IL2 mRNA in the cells. As yet an autocrine mechanism of growth stimulation through IL2 had not been reported for human B cell malignancies.

In CHAPTER SEVEN the in vitro growth features of T ALL and leukemic T NHL are presented. These T cell malignancies retained dependence on activation and on IL2 for in vitro proliferation. In this respect they differ from the T cell leukemias/lymphomas with a viral (i.e., HTLV-I induced) etiology, in which dependence on IL2 and activation is abrogated. Among the limited number of cases of T ALL and T NHL that we investigated variations in the in vitro response patterns were evident:
1) activation (and IL2 receptor induction) of the immature T cell type leukemias/lymphomas was achieved with TPA, but the cells did not respond to PHA, whereas mature T cell type malignant cells could be activated by TPA as well as by PHA;
2) in some cases of T ALL/T NHL the cells depended on exogeneous IL2 for proliferation, whereas in others, the cells were capable of producing IL2

themselves. Like in non-T ALL (chapters 2 and 3) the malignant cells in T ALL/T NHL appeared to be firmly arrested in their maturation: No indications of progressive differentiation during colony culture were obtained.

In CHAPTER EIGHT studies are presented that represent our first efforts to analyse the immunophenotypes and growth requirements of normal T-lymphocytic precursor cells. For this purpose we applied the long-term bone marrow culture system (LTBMC). It is shown that under the influence of the bone marrow stroma components, bone marrow devoid of mature T cells, gave rise to clonable T-lymphocytic progenitors. These progenitors, designated TL-CFU I, themselves lacked characteristics of T lymphocytes (i.e., E rosette forming ability and T3 antigen expression), but formed colonies in response to PHA and IL2 consisting of T lymphocytes of predominantly the $T3^{+}T4^{+}$ phenotype. Further experiments along this line (which should also deal with the B-cell differentiation lineage) have to set a basis for comparisons of the growth features of lymphoid leukemia cells with their normal analogues.

Finally, in CHAPTER NINE a brief overview is given of our current understanding of the role of IL2 in the growth of neoplastic T and B cells of the different histological categories. In addition, the results of this thesis are discussed in view of the hypothesis that in leukemia the control of proliferation and differentiation by external stimuli has been altered due to the neoplastic transformation.

Samenvatting

De vorming van bloedcellen wordt gereguleerd door polypeptide hormonen die werken op de hematopoietische stamcellen en op de proliferatieve cellen van de lymfoïde, myelomonocytaire, erythroïde en megakaryocytaire differentiatiereeksen. Deze hormonen (of groeifactoren) zorgen voor een gebalanceerde toevoer van bloedcellen. In leukemieën en maligne lymfomen, neoplastische ziekten van het bloedvormend systeem, is de homeostase verstoord, hetgeen resulteert in een accumulatie van de neoplastische cellen. De mechanismen die ten grondslag liggen aan de expansieve groei van leukemie- en lymfoomcellen zijn onbekend. Een aantrekkelijke hypothese is dat deze kwaadaardige cellen ontsnappen aan de hormonale contrôle van de groei, bijvoorbeeld door de eigenschap te verwerven zelf groeifactoren te gaan maken, door veranderingen in de werking van hormoonreceptoren op de celmembraan, of door een volledig verlies van hormoon-afhankelijkheid. Dergelijke afwijkingen zijn beschreven voor experimentele tumoren.

In vitro kweek van primair celmateriaal is van groot belang voor het onderzoek naar mogelijke afwijkingen in de hormonale respons van leukemie- en lymfoomcellen. De in dit proefschrift beschreven experimenten zijn gericht op de ontwikkeling van primaire celkweken van acute lymfatische leukemieën van zowel de B- als de T-cel differentiatie-reeks (resp. non T ALL en T ALL), chronische lymfatische leukemie van het B type (B CLL), en leukemische T-cel Non Hodgkin lymfomen (T NHL). Deze in vitro technieken zijn vervolgens toegepast om de groei- en differentiatie-eigenschappen van de lymfoïde maligniteiten, alsmede om hun gevoeligheid voor het hormoon interleukine 2 (IL2) nader te analyseren.

In HOOFDSTUK EEN worden enkele grondbeginselen van de bloedcelvorming, leukemie, de fysiologische werking van interleukine 2 (IL2) en het gebruik van in vitro kweektechnieken ingeleid. Daarnaast wordt het concept dat tumorcellen kunnen ontsnappen aan de normale groeiregulatie geïntroduceerd en wordt een kort overzicht gegeven van de experimentele ondersteuning hiervoor. Tevens is in dit hoofdstuk een schema opgenomen van de lymfoïde differentiatie op basis van immunologische typering. In dit schema zijn celtypen, waaruit de verschillende maligniteiten geacht worden te zijn ontstaan, aangegeven.

In HOOFDSTUK TWEE worden studies gepresenteerd die betrekking hebben op de definiëring van de kweekcondities voor de koloniegroei van "common" en pre-B ALL cellen. Met behulp van monoclonale antistoffen gericht tegen de membraanreceptoren voor IL2 werd aangetoond dat ALL cellen deze receptoren tot expressie brengen na incubatie, in aanwezigheid van een lectine (PHA) of een phorbol ester (TPA). IL2 receptoren werden niet gevonden op onbehandelde ALL cellen. In een kweeksysteem, waaraan IL2, PHA of TPA en bestraalde leukocyten werd toegevoegd, werd ALL koloniegroei waargenomen. Wanneer één van deze componenten uit het kweeksysteem werd weggelaten, was de koloniegroei verlaagd of afwezig. Kennelijk is ALL koloniegroei afhankelijk van een complex van groeistimulerende- en activerende factoren, waaronder IL2, een onbekende leukocytenfactor en een lectine of phorbol ester. Vergelijking van het immunologische fenotype van koloniecellen met dat van ongekweekte cellen wees er niet op dat ALL cellen tijdens koloniekweek differentiëren naar meer rijpe stadia van de B lymfocytaire reeks.

In HOOFDSTUK DRIE is de bestudering van de in vitro eigenschappen van ALL cellen uitgebreid naar 24 patiënten. Deze groep omvatte ook 4 gevallen van niet-classificeerbare ALL.
De induceerbaarheid van IL2 membraanreceptoren en in vitro koloniegroei werd systematisch geanalyseerd in de drie immunologische subtypen (niet-classificeerbaar, "common" en pre-B) van ALL. Koloniegroei werd waargenomen bij 18 van de 24 patiënten. Er waren geen verschillen in kolonievormend vermogen waarneembaar tussen de immunologische subtypen en evenmin tussen ALL cellen van kinderen en van volwassenen. In deze uitgebreide groep van patiënten werden opnieuw geen aanwijzingen verkregen dat ALL cellen in staat zijn in vitro verder te differentiëren.

In een vergelijkbare studie beschreven in HOOFDSTUK VIER werd het vermogen van acute myeloblasten leukemie (AML) cellen om in vitro verder uit te rijpen onderzocht. Met behulp van monoclonale antistoffen gericht tegen myelomonocytaire differentiatie-antigenen werd in 10 gevallen van AML waargenomen dat AML kolonievormende cellen nakomelingen produceren die kenmerken van verdere uitrijping vertonen. Deze uitrijping bleek echter incompleet. Dus, kolonievormende cellen in zowel ALL als AML missen het vermogen om in vitro volledig uitgerijpte dochtercellen te vormen.

HOOFDSTUK VIJF omvat de studies waarin de condities voor de in vitro koloniegroei van B CLL werden vastgesteld. In vergelijking tot de in vitro eigenschappen van ALL werden drie duidelijke verschillen zichtbaar:

1) Ongekweekte CLL cellen brengen vaak IL2 receptoren tot expressie op hun celoppervlak;
2) CLL koloniegroei is afhankelijk van IL2 en PHA of TPA, maar behoeft geen aanvullende stimulatie door bestraalde leukocyten en
3) Uit morfologische en immunologische analyse van de CLL koloniecellen bleek dat clonogene CLL cellen het vermogen hebben behouden in vitro meer uitgerijpte nakomelingen (plasmablasten en plasmacellen) voort te brengen.

Studies gericht op de functionele eigenschappen van IL2 receptoren van B CLL cellen, alsmede analyses van de proliferatieve respons van de cellen op IL2 in ^{3}H-thymidine incorporatietests worden beschreven in HOOFDSTUK ZES. Uit bindingsexperimenten met radioactief IL2 bleek dat CLL cellen, na activering in vitro, twee typen IL2 receptoren tot expressie brengen, een met een hoge en een met een lage affiniteit voor IL2. In dit opzicht lijken CLL cellen op normale geactiveerde B- (en T-)cellen. In drie gevallen werd de DNA-synthese door CLL cellen gestimuleerd door IL2 en PHA. Interessant was echter dat in een vierde geval spontane proliferatie, d.w.z. volledig onafhankelijk van de toevoeging van IL2 en PHA, optrad. Experimenten waarin IL2 receptoren tijdens de kweek werden geblokkeerd en bepalingen van de IL2 activiteit in het kweekmedium toonden aan dat de proliferatie van deze cellen gestimuleerd werd door endogeen IL2. Deze waarnemingen suggereren dat de CLL cellen van deze patiënt zelf in staat waren IL2 te maken. Deze hypothese kon echter niet worden bewezen op het niveau van de transcriptie, d.w.z. door het aantonen van IL2 mRNA in de cellen. Een autocrien groeimechanisme, gestuurd door IL2, is nog niet eerder beschreven voor humane B-cel maligniteiten.

In HOOFDSTUK ZEVEN worden de in vitro groei-eigenschappen van T ALL en leukemisch T NHL gepresenteerd. Deze T-cel maligniteiten behielden hun afhankelijkheid van activering en IL2 voor in vitro proliferatie. In dit opzicht verschillen zij van de T-cel leukemieën en lymfomen met een virale etiologie, d.w.z. geïnduceerd door HTLV I, waarin de afhankelijkheid van IL2 en activering verloren is gegaan. In de beperkte groep T ALL en T NHL patiënten die we onderzochten, kwamen variaties in de in vitro respons naar voren:

1) activering (en de inductie van IL2 receptoren) van de T-cel maligniteiten met een onrijp immunologisch fenotype was mogelijk met TPA, maar de cellen reageerden niet op PHA, terwijl maligne cellen met een rijp T-cel fenotype door zowel TPA als PHA konden worden geactiveerd;
2) In enkele gevallen van T ALL en T NHL waren de cellen voor proliferatie afhankelijk van exogeen IL2, terwijl in andere gevallen de cellen zèlf IL2 konden maken. Evenals in non T ALL (hoofdstukken 2 en 3), bleken de maligne cellen in T ALL en T NHL te zijn geblokkeerd in de uitrijping: er werden geen aanwijzingen verkregen dat verdere differentiatie tijdens koloniekweek optrad.

In HOOFDSTUK ACHT wordt een onderzoek gepresenteerd dat de eerste aanzet vormt de immunofenotypen en de groeieigenschappen van normale T-lymfoïde voorloper cellen te analyseren. Ten behoeve hiervan werd het lange termijn beenmergkweeksysteem toegepast. Beenmerg waaruit tevoren T lymfocyten waren verwijderd bleek in het lange termijn systeem onder invloed van stromale componenten kloneerbare voorloper cellen van de T reeks te genereren. Deze voorlopers, aangeduid als TL-CFU I, missen kenmerken van T lymfocyten (te weten het vermogen E rozetten te vormen en de expressie van T3 antigenen), maar vormden onder invloed van PHA en IL2 kolonies van T lymfocyten van voornamelijk het $T3^+$ $T4^+$ immunofenotype. Aanvullende experimenten in deze richting (ook met betrekking tot de B cel differentiatie reeks) dienen een basis te vormen voor een vergelijking van de groeieigenschappen van lymfatische leukemiecellen en analoge normale celtypen.

In HOOFDSTUK NEGEN, tenslotte, wordt een kort overzicht gegeven van onze huidige inzichten inzake de rol van IL2 bij de groei van neoplastische T- en B-cellen. Tevens worden in dit hoofdstuk de resultaten van dit proefschrift besproken in het licht van de hypothese dat in leukemie de regulering van de proliferatie en differentiatie door externe stimuli gestoord kan zijn.

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CURRICULUM VITAE

Ivo Paul Touw was born in Heemstede (The Netherlands) on November 2, 1954. He completed his secondary education (HBS-B) in 1972 and in the same year he started a study in biology at the University of Amsterdam.
His professional training included research work on the biosynthesis of ferredoxin, a chloroplast protein involved in the electron transport in photosynthesis and on the physiology of a putative defense mechanism of tomato fruits to pathogenic fungi.
In addition, he was active as a teaching assistant. The author became interested in biomedical research at the Immunology Department of The Netherlands Cancer Institute in Amsterdam, whilst working on the development of physical separation methods of human peripheral blood leukocytes. He received his degree (cum laude) in April 1981.
The author then became a member of the Institute of Hematology of the Erasmus University, Rotterdam (head: Prof Dr J. Abels). Under the supervision of Dr B. Löwenberg he studied some aspects of the influence of bone marrow stroma components on human hematopoiesis. In August 1982 he joined the cell culture laboratory of the Dr Daniel den Hoed Cancer Center/Rotterdam Radio-Therapeutic Institute in Rotterdam. The investigations described in this thesis were carried out at that institute.