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# FUNCTIONAL STUDIES ON THE *IN VITRO* HEMOPOIETIC MICROENVIRONMENT IN ACUTE MYELOGENOUS LEUKEMIA

BY

HECTOR MAYANI-VIVEROS

A THESIS

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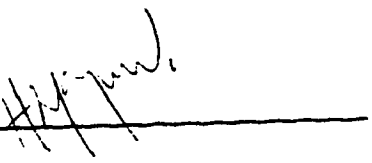
(signed) 

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Functional Studies on the *in vitro* Hemopoietic Microenvironment in Acute Myelogenous Leukemia** submitted by **Hector Mayani-Viveros** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Medical Sciences (Medicine).

Anna Janowska-Wieczorek

Larry J Guilbert

Carol E Cass

Timothy R Mosmann

Andrew R Belch

Armand Keating

June 29, 1992

## ABSTRACT

Blood cell production, or hemopoiesis, takes place within a specialized environment composed of different cell types and their products. This hemopoietic microenvironment is localized in very specific organs, such as bone marrow and spleen. Under normal conditions, there is a continuous and tightly regulated interaction between hemopoietic and microenvironmental cells, that leads to the steady-state production of blood cells. It has been suggested that alterations in the components of the hemopoietic microenvironment may be implicated in the development and/or progression of certain hematological diseases. To date, this hypothesis has been tested by several investigators, however, little is still known about the functional integrity of the hemopoietic microenvironment in pathological conditions.

The present thesis is a study on the functional integrity of the *in vitro*-developed hemopoietic microenvironment from patients with acute myelogenous leukemia (AML), a hematological malignancy characterized by the accumulation of blast cells both in bone marrow and peripheral blood. The experimental model used throughout this study was the long-term marrow culture (LTMC), a system that reproduces many of the conditions present *in vivo*. The experimental approach was based on the well-known ability of microenvironmental cells to (i)

produce macrophage colony-stimulating factor (CSF-1), a hemopoietic regulator that specifically affects the macrophagic lineage, (ii) respond to CSF-1, and (iii) support the growth of hemopoietic progenitor cells.

The results obtained suggest that the hemopoietic microenvironment developed in AML LTMC is functionally defective. This seems to be due to the presence of functionally abnormal macrophages, which (i) constitutively produce hemopoietic inhibitors, such as TNFα, and (ii) abnormally respond to exogenous CSF-1. Fibroblastoid cells developed in AML LTMC seemed to be functionally normal, thus, they may not be involved in the defective hemopoietic supportive capacity of the AML-derived microenvironment.

This study contributes to the understanding of the biology of hemopoiesis *in vitro* and, particularly, to our understanding of the role of the hemopoietic microenvironment in both normal and AML conditions.

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| | |
|---|---|
| ABMT | Autologous bone marrow transplantation |
| AML | Acute myelogenous leukemia |
| AML-ACl | AML-associated cluster |
| BFU-E | Burst-forming unit-erythroid |
| CFU-C | Colony forming unit-culture (indicates myeloid, non-erythroid progenitors) |
| CFU-E | CFU-erythroid |
| CFU-F | CFU-fibroblast |
| CFU-G | CFU-granulocyte |
| CFU-GM | CFU-granulocyte/macrophage |
| CFU-M | CFU-macrophage |
| CFU-MIX | also known as CFU-GEMM (CFU-granulocyte, erythrocyte, macrophage, megakaryocyte) |
| CFU-S | CFU-spleen |
| CSF-1 | Colony-stimulating factor-1, also known as M-CSF (macrophage-CSF) |
| EPO | Erythropoietin |
| FIBR | Fibroblasts or fibroblast culture |
| G-CSF | Granulocyte-CSF |
| GM-CSF | Granulocyte-macrophage CSF |
| HGF | Hemopoietic growth factor |
| IFNγ | Interferon-gamma |
| IL-3 | Interleukin-3 |
| LTMC | Long-term marrow cultures |
| MDS | Myelodysplastic syndrome |
| MIP-1α | Macrophage inhibitory protein-1alpha |
| Mo | Macrophages |
| PGE | Prostaglandin E |
| PHA-LCM | Phytohemagglutinin-stimulated leukocyte conditioned medium |
| TGFβ | Transforming growth factor-beta |
| TNFα | Tumor necrosis factor-alpha |
| SAL | Stromal adherent layer |

# I. INTRODUCTION

Understanding the mechanisms involved in the origin and development of blood cells is of great importance. Firstly, because blood cells represent an excellent model for the study of cell proliferation and differentiation, which are key processes in the development of multicellular organisms. Secondly, because understanding the biology of blood cell formation has a potential application in the diagnosis and treatment of several hematological diseases. During the last three decades, impressive advances in the study of blood cell formation, or hemopoiesis, have been made. Hemopoietic stem/progenitor cells have been identified and purified (Visser & Van Bekkum, 1990), the genes of several hemopoietic regulators have been cloned (Nicola, 1989), and techniques that allow the proliferation and differentiation of hemopoietic cells *in vitro* have been developed (Metcalf, 1984; Dexter et al, 1984). However, we are still far from having a complete and detailed picture at the cellular and molecular levels of the complex process of hemopoiesis, especially in terms of understanding the origin and progression of hematological malignancies such as the different types of leukemias.

## A) RATIONALE FOR THIS STUDY

Acute myelogenous leukemia (AML) is a hematological malignancy characterized by increased levels of blast cells both in bone marrow and in peripheral blood. Patients with such a disease have a rather short survival period (usually less than one year) unless treated with chemotherapy and bone marrow transplantation, in which case survival can be improved (Mayer, 1987). AML is a genetic disorder of somatic cells. It arises from clonal expansion of a single

transformed stem/progenitor cell, which gives rise to a functionally abnormal progeny with growth advantages over normal cells. As a result of this, normal hemopoiesis is dramatically reduced in such patients (Messner & Griffin, 1986).

Leukemogenesis is a multi-step process in which many different factors and mechanisms are involved. Thus, understanding the biology of AML has not been an easy task. One approach to this problem has been the development of *in vitro* models that reproduce, to a certain extent, the *in vivo* situation. This has allowed the identification of AML progenitor cells and their functional characterization in culture (Griffin & Lowenberg, 1986).

Blood cell formation depends to a large extent on the interaction between stem/progenitor cells and the hemopoietic microenvironment, a network of different cell types (fibroblasts, macrophages, endothelial cells, adipocytes, and T lymphocytes) and their products (extracellular matrix and cytokines), localized primarily in the medullary cavity. By different mechanisms, that include cell-to-cell contact and secretion of cytokines, the hemopoietic microenvironment regulates steady-state production of blood cells and also mediates increased blood cell formation in cases of blood demand (Greenberger, 1991). Several investigators have suggested that alterations in the hemopoietic microenvironment may contribute to the abnormal production of blood cells, leading to hematological disorders (Ershler et al, 1980; Juneja & Gardner, 1985; Takahashi et al, 1985). A functionally abnormal hemopoietic microenvironment may, on the one hand, contribute to the manifestation of certain diseases. On the other hand, the growth of normal stem/progenitor cells during treatment of a disease may not be optimal if they are surrounded by a defective microenvironment. Thus, studies on the biology of the hemopoietic

microenvironment may give important insights into understanding and treating blood disorders.

To date, little is known about the functional integrity of the environment in which leukemic (AML) hemopoiesis takes place. Some investigators have suggested that AML is a disease of the hemopoietic system without involving the hemopoietic microenvironment (Sawyers et al, 1991). In contrast, some *in vitro* studies indicate that certain elements of the hemopoietic microenvironment in AML are functionally abnormal (Greenberg et al, 1978; Greenberg et al, 1981).

Using liquid suspension cultures, Langley and colleagues have shown that AML blasts can be induced to mature into macrophages. In fact, those investigators demonstrated that macrophages produced in the cultures do not derive from coexisting normal progenitor cells (Langley et al, 1986). Interestingly, studies by the same group of investigators showed that when the macrophagic regulatory cytokine macrophage colony-stimulating factor (CSF-1) is added to the cultures, maturation of blasts into macrophages is greatly enhanced (Miyauchi et al, 1988b). This seems to be a direct effect, since AML blasts express the CSF-1 receptor on their surface (Ashmun et al, 1989).

## B) HYPOTHESIS AND EXPERIMENTAL APPROACH

The major goal of this thesis was to assess some specific functional parameters of the AML-derived hemopoietic microenvironment developed *in vitro*. Based on the observations described above, the hypothesis for the present study was that the AML marrow-derived hemopoietic microenvironment developed *in vitro* contains macrophages that are of leukemic origin (derived from AML blasts). Therefore, they may be functionally abnormal despite their

apparently normal morphology. To test this hypothesis, I followed an approach based on the interaction between CSF-1 and macrophages. Since macrophages (i) are one of the major components of the hemopoietic microenvironment developed *in vitro* (Allen & Dexter, 1984), (ii) produce and secrete CSF-1 (Clark & Kamen, 1987), and (iii) are regulated by CSF-1 (Stanley et al, 1983), it seemed reasonable to suggest that a defective microenvironment, due to the presence of functionally abnormal, leukemia-derived macrophages, would not be able to produce and/or respond to CSF-1 adequately. This, in turn, would have significant implications on the whole hemopoietic system, since macrophages regulate different hemopoietic lineages via the production of cytokines capable of stimulating and inhibiting stem/progenitor cell growth (Warren & Ralph, 1986).

Based on this, three major parameters were assessed: the ability of the AML-derived *in vitro* hemopoietic microenvironment to (i) produce CSF-1, (ii) respond to exogenous CSF-1, and (iii) sustain normal hemopoiesis. The main experimental system used in this study was the long-term marrow culture (LTMC), which has been shown to reproduce many of the conditions present *in vivo*. Since one of the objectives of this work was to determine the functional integrity of AML-derived macrophages, the above parameters were assessed on cell populations containing and lacking macrophages. Throughout this thesis, parallel experiments were performed using LTMC containing a hemopoietic microenvironment derived from normal bone marrow, in order to establish a comparative analysis.

# II. LITERATURE REVIEW

The purpose of this chapter is to give an overview of the complex process of blood cell formation, or hemopoiesis, in mammals. I will cover general aspects of the structural organization of the hemopoietic system, the role of the cellular components of the hemopoietic microenvironment in the regulation of hemopoiesis, as well as the role of different hemopoietic cytokines. Finally, I will focus on the biology of AML in different *in vitro* systems.

## A) HEMOPOIESIS

### The blood cells and the hemopoietic tissues

The cells circulating in the blood perform a variety of functions that are essential for survival of the organism. Erythrocytes transport oxygen molecules from the lungs to every single tissue in the body, through a process involving the oxygen-binding intracellular protein hemoglobin (Perutz, 1976). Neutrophils, eosinophils and monocytes participate in the killing of microorganisms and other parasites (Klebanoff & Clark, 1978; Cline, 1972; Territo & Cline, 1975). The monocyte, in addition, performs an important role in the presentation of antigens to lymphocytes, which are the cells that control the cellular and humoral arms of the immune response (Greaves et al, 1973). Platelets, on the other hand, are crucial in the mechanisms of coagulation (Weiss, 1975).

All mature blood cells have a rather short life span (from 1 to 120 days, depending on the cell type). This has made necessary their continuous production throughout life at incredibly high rates, i.e. in an adult human, $10^{10}$ erythrocytes and $4 \times 10^8$ leucocytes are produced every hour (Gordon & Barret, 1985). Blood cell formation, or hemopoiesis, takes place within specific sites of

the body known as hemopoietic tissues (Metcalf & Moore, 1971). In adult mammals, the hemopoietic tissues are restricted in location to the bone marrow and spleen. The vast bulk (more than 95 percent) of hemopoietic tissue is located in the bone marrow in the form of scattered deposits in the cavities of bones such as the sternum, ribs, sacrum and long bones (Erslev & Gabuzda, 1979).

Hemopoietic cell populations originate from cells formed during embryogenesis, a discrete event of quite short duration. Studies on the chicken and mouse indicate that ancestral hemopoietic cells are generated in the yolk sac and then migrate into the developing liver of the fetus (Tavassoli, 1991). Later on, a second migration process occurs in which hemopoietic cells leave the liver and colonize the developing spleen and bone marrow. With expansion of the marrow and spleen populations, the hemopoietic population in the liver declines shortly after birth. The liver then becomes a non-hemopoietic organ but retains a latent capacity to support hemopoiesis again in abnormal situations where both the marrow and spleen are damaged or diseased (Erslev and Gabuzda, 1979).

In contrast to other organs such as the skin or gut, where extensive cell production occurs throughout life, hemopoietic tissues are not arranged in stratified layers of progressively more differentiated cells. Indeed, microscopic inspection of sections of bone marrow suggests that the tissue is a random mixture of cells of different lineages and differentiation stages, with little evidence of either structural organization or segregation of cells of different lineages (Islam et al, 1984). In spite of this, hemopoiesis has been shown to be a tightly regulated process, with a complex and well defined hierarchical organization.

**The clonal nature of hemopoiesis: A historical perspective**

In 1961, Till and McCulloch demonstrated the presence, in the bone marrow of mice, of cells capable of forming hemopoietic colonies in the spleen when injected to irradiated, syngeneic mice (Till & McCulloch, 1961). Such cells were termed colony-forming units-spleen (CFU-S). Studies using radiation-induced chromosomal markers demonstrated that all the cells present in a hemopoietic spleen colony derive from a single CFU-S (Becker et al, 1963). Furthermore, since it was found that such colonies contain cells of three different cell lineages (erythrocytic, granulocytic, and megakaryocytic), it was concluded that the CFU-S are, in fact, stem cells (Wu et al, 1967). Subsequent studies indicated that at least some CFU-S are stem cells for lymphoid as well as myeloid differentiation (Nowell et al, 1970; Abramson et al, 1977).

The crucial property of CFU-S to self-generate was identified in experiments in which single colonies were dispersed and retransplanted into secondary recipients; colonies were frequently found in the spleen of the secondary hosts containing a distribution of cell classes similar to those present in the primaries (Lewis & Trobaugh, 1964). However, only a small minority of cells in the primary colonies were able to give rise to secondary colonies (Siminovitch et al, 1964). Presumably, this was because most of the cells in the primary colonies had already differentiated to the point where their capacity for proliferation was insufficient to permit formation of a detectable macroscopic colony (Till et al, 1964).

In the mid 60's, *in vitro* methods were developed that allowed the identification and characterization of a particular type of bone marrow cell, capable of forming hemopoietic colonies in semisolid cultures (Pluznik & Sachs,

1965; Bradley & Metcalf, 1966). These cells, known as colony-forming units in culture, are progenitor cells derived from primitive stem cells and committed to a particular lineage of differentiation. Such progenitors were also found and characterized in human bone marrow (Pike & Robinson, 1970). In contrast to stem cells, progenitor cells are uncapable of self-replication. The progeny of the these cells consists of morphologically-recognizable precursor cells, which, in turn, give rise to the mature circulating blood cells. It became clear, then, that the hemopoietic system is organized in a hierarchical manner, consisting of four compartments: stem cells, progenitor cells, precursor cells, and mature cells. The development of semisolid cultures for bone marrow cells also permitted the identification of soluble factors, known as colony-stimulating factors (CSF), which regulate the proliferation, differentiation, and function of the hemopoietic cells (Metcalf, 1985; Sieff, 1987; Clark & Kamen, 1987).

Although it has been demonstrated that both myeloid and lymphoid cells have a common origin (Abramson et al, 1977; Keller et al, 1985; Spangrude et al, 1988), most of the studies on hemopoietic stem/progenitor cells and their regulation have been focused on the myeloid lineages (granulocytic, monocytic, erythrocytic, and megakaryocytic). Indeed, much less is known about the lymphoid stem cell(s) and their immediate progeny (B and T progenitor cells) (Phillips, 1989). In the present review, and due to its relevance to this thesis, I will focus on the studies regarding the myeloid arm of the hemopoietic system.

**Hemopoietic stem cells**

Until 1982, the spleen colony assay was considered an indication of mouse and rat hemopoietic stem cell numbers and it was used as an endpoint in

purification studies. It then became clear that at least a distinction had to be made between spleen colony formation at 8 and at 12 days after transplantation. In other words, it was shown that day-8 CFU-S are different (more mature) from day-12 CFU-S (Magli et al, 1982; Baines & Visser, 1983; Lord & Spooncer, 1986; Spangrude et al, 1988). Subsequently, it was also observed that the day-12 CFU-S are heterogeneous (Bertoncello et al, 1985; Mulder & Visser, 1987). And recently, it was shown that long-term repopulation of hemopoiesis in irradiated mice can be obtained without any CFU-S, indicating the presence of a more primitive cell or pre-CFU-S (Ploemacher et al, 1989; Jones et al, 1990).

Because of their very low frequency in the bone marrow (only 0.05% of the total number of nucleated cells), the morphology and physiology of murine stem cells have been difficult to characterize. However, during the last decade great advances in their purification have been made (Visser & Van Vekkum, 1990; Spangrude, 1991). It is now known that the murine hemopoietic stem cells are small (7 $\mu$m in diameter) lymphocyte-like cells, with a density of 1.070 g/ml. The majority are in a quiescent state and will go into cycle only when stimulated. They express unique surface antigens Thy-1 (at low levels) and Sca-1, and do not express antigens present on the surface of mature hemopoietic cells.

For many years, detection and eventual isolation of the human equivalent to the murine CFU-S and pre-CFU-S were hindered by the absence of a suitable assay system for its detection. A major break through occured when Nakahata and Ogawa identified a primitive murine hemopoietic progenitor cell capable of producing *in vitro* colonies containing blast cells only (Nakahata & Ogawa, 1982). These progenitors (CFU-Blast) were also identified in normal human bone marrow and it was shown that they share with stem cells two important features:

namely, their potential to self-renew, and their capacity to differentiate along various hemopoietic lineages (Gordon et al, 1987; Rowley et al, 1987).

More recently, a more complex *in vitro* system for the assessment of human stem cells has been developed. The assay is based on the capacity of certain primitive cells to initiate and sustain hemopoiesis for several weeks when cultured on pre-established stromal adherent cell layers in the absence of any other cell type and without adding any hemopoietic stimulator. Such cells have been referred to as long-term culture initiating cells (LTC-IC). By separating bone marrow cells on the basis of cell surface antigen expression, it has been shown that the LTC-IC express the antigens CD34 and CD45RO, but do not express HLA-DR or any other antigen present on lineage-committed blood cells (Sutherland et al, 1989, 1990; Andrews et al, 1990; Verfaillie et al, 1990; Lansdorp et al, 1990; Terstappen et al, 1991). The functional properties of LTC-IC suggest that they can be considered as the human hemopoietic stem cell. They are capable of self-renewal; when cultured in semisolid cultures, they do not produce hemopoietic colonies containing morphologically recognizable blood cells, but give rise to blast colonies (thus, they are CFU-Blast); when cultured on stromal adherent layers, they give rise to progenitors that will produce multipotential, myeloid and erythroid colonies (Verfaillie et al, 1990; Srour et al, 1991). By using the supravital dye rhodamine-123, it has been demonstrated that LTC-IC are in a quiescent state (Udomsakdi et al, 1991). Their number in normal bone marrow is <0.05% of the total number of nucleated cells (Andrews et al, 1990). Morphologically, the LTC-IC are primitive blasts, slightly larger than lymphocytes, with an irregularly shaped nucleus, prominent nucleoli, and scanty cytoplasm (Terstappen et al, 1991).

**Committed progenitor cells**

The immediate progeny of the hemopoietic stem cells are progenitors capable of forming colonies, in agar or methylcellulose, that contain granulocytes, erythrocytes, macrophages and megakaryocytes (CFU-GEMM) (Fauser & Messner, 1979). Although these are multipotential cells, they are not considered stem cells because of their inability to self-renew (Messner, 1984). CFU-GEMM give rise to progenitors committed to a particular lineage of differentiation. Two classes of erythroid progenitors have been identified: immature erythroid progenitors (BFU-E), which form large colonies of hemoglobinized cells, and more mature progenitors (CFU-E), which form smaller colonies (Axelrad et al, 1974). CFU-E are the progeny of BFU-E; these two populations differ in their sensitivity to erythropoietin, the major erythropoietic regulatory factor (Eaves & Eaves, 1984). Within the myeloid lineage, an immature bipotential progenitor, capable of forming colonies of granulocytes and macrophages (CFU-GM), has been identified. This cell gives rise to single lineage committed progenitors, which form colonies of granulocytes (CFU-G) or macrophages (CFU-M) (Metcalf, 1984). The megakaryocytic lineage consists of immature (BFU-Mk) and mature (CFU-Mk) progenitors, which follow a maturation pattern similar to the one observed in the erythroid lineage (Briddell et al, 1989).

The combined frequency of these progenitor cells in bone marrow is very low (0.1-0.3% of the total number of nucleated cells). Like stem cells, hemopoietic progenitors express the surface antigen CD34 (Civin et al, 1984; Baines et al, 1988). However, they also express lineage specific antigens (Janowska-Wieczorek et al, 1984), absent on the surface of stem cells. A significant proportion of progenitor cells are in active cell cycle, which is also in

contrast to what has been observed in stem cells (Lajtha et al, 1979). The progenitor cells cannot be recognized in bone marrow smears, however, by analyzing the morphology of CD34-positive bone marrow cells, it is known that they are blast-like cells (Baines et al, 1988).

**Morphologically-recognizable precursor and mature cells**

The progeny of committed progenitors can be identified by morphological methods (light microscopy). Such precursor cells, which include erythroblasts, myeloblasts and their progeny, monoblasts, and megakaryocytes, comprised the great majority of the cells in the bone marrow. Their progeny are mature cells that leave the bone marrow and enter the circulation, with the exception of erythrocytes, which reach their final stage of maturation in the peripheral blood (Erslev & Gabuzda, 1979). Mature blood cells remain in the circulation for short periods of time (from a few hours, in the case of granulocytes, to 120 days for erythrocytes), after which, they will migrate into the tissues (granulocytes and monocytes) or will be removed from the circulation and destroyed in the liver (erythrocytes) (Erslev & Gabuzda, 1979).

## B) THE HEMOPOIETIC MICROENVIRONMENT

*In vivo* and *in vitro* studies performed during the last three decades have shown that effective hemopoiesis is the result of an interplay between hemopoietic stem/progenitor cells and a supporting stroma localized in very specific organs (Metcalf & Moore, 1971; Wolf, 1979; Wilson, 1984; Greenberger, 1991). As mentioned before, in normal adult mammals the bone marrow is the only site in which myelopoiesis, erythropoiesis, and lymphopoieis proceed

simultaneously. Thus, local tissue influences critical for hemopoiesis appear to operate primarly in the medullary cavity (Tavassoli & Friedenstein, 1983).

**Evidence for a hemopoietic microenvironment**

The first evidence for the presence of specific conditions in the bone marrow and spleen that are necessary for hemopoietic development came from experiments in which mouse marrow cells were injected into syngeneic, irradiated mice. The first capillary bed those cells encounter is in the lungs, but the lungs do not become hemopoietic, nor do the skeletal muscles or other body parts to which those injected cells that escape the lungs may be shunted next. Hemopoiesis is established in the bone marrow and spleen only (Ford et al, 1956; Till & McCulloch, 1961). Later on, it was observed that the hemopoietic colonies developed in the mouse spleen were primarily erythroid, whereas those developed in the marrow were mainly granulocytic (Wolf & Trentin, 1968; Trentin, 1971). Furthermore, it was also shown that among the colonies developed in the spleen, those developed in the red pulp were almost exclusively erythroid, whereas the granulocytic colonies developed under the splenic capsule and the trabeculae (Trentin, 1970).

Microgeographical regions have also been described in the bone marrow. Lord and colleages have shown that the spatial distribution of CFU-S, as well as that of erythroid and myeloid committed progenitors in the mouse bone marrow, is not random (Lord et al, 1975; Frassoni et al, 1982). They suggested that the hemopoietic tissue possess a well-defined structure, although not evident in histological sections, that is relevant to the proliferation and differentiation of hemopoietic cells. Interestingly, in 1978, Schofield proposed the existence of a

stem cell "niche", a functional compartment of marrow stroma that isolates conditions for self replication from conditions conducive to differentiation (Schofield, 1978).

**Definition of the hemopoietic microenvironment**

The marrow stromal tissue is a network of cells (fibroblasts, endothelial cells, macrophages, adipocytes) and extracellular matrix (collagen, laminin, fibronectin, proteoglycans), that physically supports the hemopoietic cells and influences their proliferation and differentiation (Dorshkind, 1990). Stromal cells have been considered to be organized into hemopoietic microenvironments that support blood cell development. The term hemopoietic microenvironment was created to distinguish different microgeographic stromal cell influences within an organ on the pluripotent stem cells, as opposed to whole organ differences, each of which is composed of the sum of its stromal microenvironments (Trentin, 1989). The hemopoietic microenvironment, however, is not easy to define. If it is taken to be a broad term describing those cells that regulate hemopoietic development, other cells in addition to the stromal cells may be components of it. This is, in fact, the case for T lymphocytes, which are known to regulate hemopoiesis by secreting multiple hemopoietic cytokines (Torok-Storb et al, 1982; Nakao et al, 1984; Gillis, 1989). Thus, using this definition, stromal cells contibute to the hemopoietic microenvironment, but not all microenvironmental cells are stromal cells. In the present thesis, I will define the hemopoietic microenvironment as *the local network of stromal cells (fibroblasts, macrophages, endothelial cells, adipocytes), accessory cells (T lymphocytes and monocytes), and their products (extracellular*

*matrix and cytokines), capable of influencing the self-renewal, proliferation and differentiation of hemopoietic stem/progenitor cells*. Due to its relevance to this thesis, I will focus the rest of this review on the structure and physiology of the hemopoietic microenvironment of the bone marrow.

**Composition of the hemopoietic microenvironment *in vivo***

Reticular cells are the major cellular component of the bone marrow stroma (Tavassoli & Yoffey, 1983). They consist of two types, adventitial and fibroblastic. Adventitial reticular cells are located around venous sinuses forming a layer that partially covers the endothelium's abluminal side. Morphological studies indicate that one of their major roles is the regulation of the migration of mature blood cells from the marrow to the circulation (Shaklai, 1989). Fibroblastic reticular cells, on the other hand, are located within the marrow hemopoietic cord; their thin cytoplasmic processes envelop maturing hemopoietic cells in the marrow compartment (Shaklai & Tavassoli, 1979). These fibroblastic cells are usually in close contact with immature granulocytic cells, which suggests that there are functional interactions between both cell types (LaPushin & Trentin, 1977; Westen & Bainton, 1979).

Macrophages are the second major cellular component of the marrow stroma (Weiss, 1976; Tavassoli & Friedenstein, 1983; Shaklai, 1989), and the only one of hemopoietic origin (Allen & Dexter, 1984). Topographically, they are found in three different sites in the marrow: the erythroblastic islands (central macrophages), the abluminal side of the sinus endothelium (perisinal macrophages), and dispersed between the developing hemopoietic cells. The central macrophage is usually surrounded by maturing red cells (erythroblasts),

thus forming the anatomic unit commonly referred to as an erythroblastic island (Bessis & Breton-Gorius, 1962). It has been suggested that the central macrophage plays a key role in erythroid maturation. The possibility exists that macrophages of the erythroblastic island produce or process erythropoietin that is then transferred directly to the erythroblasts (Shaklai & Tavassoli, 1979). Hypoxia, which stimulates red cell production, also leads to an increase in the central macrophage's size and its phagocytic activity (Shaklai, 1989). Perisinal macrophages cover varying proportions of the sinus endothelium's abluminal surface. Their cytoplasmic protrusions penetrate the endothelium, engulf senescent or defective red cells, and remove them from the circulation (Tavassoli, 1974).

Endothelial cells and adipocytes are also major components of the marrow stroma (Shaklai, 1989). Endothelial cells are the primary cell type forming the blood vessels of the marrow. Adipocytes, on the other hand, occupy the majority of the marrow cavity in adult mammals (Erslev & Gabuzda, 1979). Their role in hemopoiesis is not clear, although *in vitro* studies suggest that they are capable of producing hemopoietic growth factors (Lanotte et al, 1982).

Marrow stromal cells produce and secrete macromolecules that form a highly organized structure known as the extracellular matrix (Urist, 1965; Reddi & Huggins, 1972; Zuckerman et al, 1989). The major components of the extracellular matrix are (i) collagens, fibrillar proteins produced by reticular and endothelial cells, (ii) fibronectin, a glycoprotein produced by reticular cells and macrophages, (iii) laminin, another large glycoprotein produced by endothelial cells, and (iv) proteoglycans, composed of a core protein to which one or more glycosaminoglycans are attached (Zuckerman, 1989). *In vitro* studies indicate

that the extracellular matrix of the bone marrow not only acts as a physical support for the hemopoietic elements, but also plays a dynamic role in the regulation of hemopoiesis, i.e. localization of hemopoietic cells within the medullary cavity and presentation of hemopoietic cytokines to progenitor cells (Keating & Gordon, 1988; Zuckerman et al, 1989).

**Composition of the hemopoietic microenvironment *in vitro***

Among the different *in vitro* models that have been developed to study the hemopoietic microenvironment, the long-term marrow culture (LTMC) system seems to be the one that most closely reproduces the conditions observed *in vivo*. Both murine and human hemopoiesis can be sustained for several weeks in LTMC (Dexter et al, 1977; Gartner & Kaplan, 1980). This is due to the development of an adherent cell layer, consisting of different cell types, which provides both physical support and the hemopoietic cytokines (soluble and cell-associated proteins) that are necessary for the growth of hemopoietic progenitors (Greenberger, 1991).

It is noteworthy that the adherent cell layers of the LTMC are very similar in their composition to the bone marrow stroma. The major components of such adherent layers are fibroblasts, capable of producing collagen types I and III as well as fibronectin (Bentley, 1982). These cells are the most numerous, comprising 45 - 55% of the total cell number (Andreoni et al, 1990). Macrophages are also present in significant levels, comprising 25 - 35% of the adherent cells (Andreoni et al, 1990). Endothelial cells, on the other hand, comprise 5 - 20% of the total cell number (Bentley, 1984). Adipocytes are usually observed in both murine and human LTMC, although their presence may

depend on certain culture conditions (Greenberger, 1978). Lymphocytic, as well as myeloid, cells have also been observed in the adherent layers of LTMC, although in reduced levels (Andreoni et al, 1990). Like its *in vivo* counterpart, the extracellular matrix developed in LTMC consists of collagen I, III, and IV, laminin, fibronectin and proteoglycans (Bentley, 1982; Zuckerman et al, 1983; Keating et al, 1984; Wight et al, 1986).

**Interactions between the marrow microenvironment and hemopoietic cells**

Microenvironmental cells can influence hemopoiesis, in a positive or negative way, by at least two general mechanisms: direct cell-to-cell contact, or via the production and secretion of proteins (extracellular matrix or cytokines) (Torok-Storb, 1988). However, due to the cell heterogeneity observed both in the hemopoietic microenvironment and the hemopoietic system, it is difficult to describe all the different types of interactions between them. Thus, in order to provide an overall appreciation for the complexity of those interactions, I will describe only a few examples:

*a) Stromal cells - stem cells.* Stromal cells have been shown to regulate proliferation and differentiation of stem cells by the production of soluble stimulators and inhibitors (Lord & Wright, 1980). Direct cell-to-cell contact has also been implicated; Dexter and colleagues have shown that CFU-S need to interact physically with stromal cells in order to grow in LTMC (Roberts et al, 1987). Similar requirements for cell-to-cell contact have been reported for growth of CFU-blasts (Gordon et al, 1987). More recently, it has been demonstrated that stromal cells produce a factor (stem cell factor or c-kit ligand) which can be secreted or remain as a cell-associated protein, that stimulates stem

cells by interacting with a cell-surface receptor (c-kit) (Ikuta et al, 1991).

*b) Stromal cells - progenitor cells.* Several studies have shown that progenitor cells adhere to stromal cells *in vitro* (Coulombel et al, 1983), and that the extracellular matrix produced by stromal cells is critical for the optimal growth and differentiation of these progenitors (Tsai et al, 1987). On the other hand, stromal cells also produce soluble hemopoietic cytokines (Clark & Kamen, 1987). Interestingly, a recent report indicates that factors like GM-CSF (see section C of this chapter) can be produced by stromal cells and then be selectively retained (bound) by the glycosaminoglycans found in the extracellular matrix (Gordon et al, 1987b; Keating & Gordon, 1988)), suggesting that the extracellular matrix plays a role in the presentation of cytokines to progenitor cells.

*c) Accessory cells - progenitor cells.* Monocytes and T lymphocytes produce soluble factors that positively or negatively regulate the growth of hemopoietic progenitors (Clark & Kamen, 1987). For example, T cells produce and secrete interleukin-3 (IL-3), which acts on myeloid and erythroid progenitors stimulating their growth. On the other hand, monocytes produce tumor necrosis factor (TNF), which inhibits the proliferation of hemopoietic progenitor cells (Broxmeyer et al, 1986).

*d) Accessory cells - stromal cells.* Monocytes produce activities, such as TNF and IL-1, which can, in turn, stimulate fibroblasts and endothelial cells to produce hemopoietic cytokines (Bagby et al, 1983; Sieff et al, 1987). It is attractive to speculate that monocytes or T cells from the peripheral blood could be triggered to infiltrate specific sites in the marrow, and induce the production of localized concentrations of growth factors sufficient to trigger progenitor proliferation.

**Origin of the microenvironmental cells**

The origin of the cells of the hemopoietic microenvironment has been of great controversy during the last decade. As demonstrated by several investigatiors (see section A of this chapter), bone marrow macrophages as well as circulating monocytes and T lymphocytes, derive from the pluripotent hemopoietic stem cell. The origin of the so-called "non-hemopoietic" stromal cells, on the other hand, is not so clear. Among them, fibroblasts are the ones that have been best characterized in terms of their origin; thus, I will focus in this subsection on these cells.

Since the early work of Friedenstein and colleagues (Friedenstein et al, 1970), and years later by Castro-Malaspina and collaborators (Castro-Malaspina et al, 1980), it became clear that fibroblasts derive from adherent, nonphagocytic progenitors capable of forming fibroblastic colonies *in vitro* (colony-forming unit-fibroblast, or CFU-F). By using a complement-mediated cytotoxicity assay, it was shown that CFU-F are different from CFU-C, which may also imply a different origin (Castro-Malaspina et al, 1980). Very recently, Simmons and Torok-Storb demonstrated that CFU-F express a cell-surface antigen, absent on any other hemopoietic progenitor, recognized by the monoclonal antibody STRO-1 (Simmons & Torok-Storb, 1991a). Interestingly, cells giving rise to adipocytes and endothelial cells are also positive for STRO-1 (Simmons & Torok-Storb, 1991a). The same investigators have performed extensive phenotypical studies on CFU-F and found that, although these cells share some characteristics with the hemopoietic progenitors, such as the expression of the CD34 antigen, they are distinct cell types (Simmons & Torok-Storb, 1991b).

*In vitro* studies in human LTMC have suggested the existence of a

pluripotent cell in the bone marrow that gives rise to hematopoietic cells and to their stromal microenvironment (Singer et al, 1984). Such an idea has been supported by *in vitro* studies in rats showing that the marrow stroma grown in culture contains cells with hemopoietic potential (Islam et al, 1988). These reports seem to give support to the study of Keating and colleagues, in which they found that the human hemopoietic microenvironment developed *in vitro* after bone marrow transplantation is of donor origin (Keating et al, 1982). However, more recent studies have indicated that the human *in vitro* microenvironment after bone marrow transplantation is, with the exception of macrophages, of host origin (Simmons et al, 1987; Laver et al, 1987; Agematsu & Nakahori, 1991), suggesting independent origins for hemopoietic and stromal cells. The apparent discrepancies between the above studies seem to be due to differences in the techniques used to assess cultured cells and the type of cells analyzed. For instance, Laver and colleagues used chromosomal analysis, which determines the origin of stromal cells capable of undergoing mitosis, whereas Keating and colleagues, by determining the presence of the Y-body chromosome, analyzed non-dividing cells. Thus, the question of whether hemopoietic and stromal (fibroblastic) cells derive from a common stem cell or from independent stem cells, is still open.

## C) HEMOPOIETIC CYTOKINES

The participation of soluble factors in the regulation of blood cell production was first suggested in 1906, when Carnot and Deflandre postulated the presence, in serum, of a soluble molecule capable of stimulating red cell production. However, it was not until the early 50's that conclusive evidence for

the humoral regulation of erythropoiesis was provided by Reissmann (Reissmann, 1950) and Erslev (Erslev, 1953).

In the 1960's, the development of semisolid cultures for bone marrow cells allowed the discovery and characterization of a group of factors with the capacity to stimulate the growth of hemopoietic colonies (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966). Such colony-stimulating factors (CSFs) were biologically and biochemically characterized during the seventies and early eighties (Metcalf, 1985), and their genes have been cloned during the last five years (Golde, 1990).

In recent years, it has become evident that hemopoiesis is regulated by more than twenty different factors, also known as hemopoietic cytokines. Some of them, such as erythropoietin (Epo; the major erythropoietic regulator) and CSFs, act mainly as positive regulators (Krantz, 1991; Clark & Kamen, 1987). Others, such as the tumor necrosis factors (TNF), interferon-$\gamma$ and transforming growth factor-$\beta$ (TGF-$\beta$) act mainly as inhibitors (Axelrad, 1990). Some cytokines, such as interleukin-1 (IL-1) and IL-6, are characterized by having little or no intrinsic capacity to stimulate or inhibit hemopoietic colony formation by themselves but enhance the effects of other cytokines (Stanley et al, 1986; Mochizuki et al, 1987; Ikebuchi et al, 1987; Quesenberry, 1986). The physiology of the hemopoietic cytokines, however, is very complex: most of them are produced by different cell types, they all have more than one function, act on different target cells, and may influence the production and function of other cytokines (Clark & Kamen, 1987; Sieff, 1987; Wheeton, 1990). In this section, I will present an overview of the biochemistry and biology of some of the cytokines involved in hemopoiesis. Among such hemopoietic cytokines, macrophage colony-stimulating factor (CSF-1) is the one that, in the context of the present

thesis, has more relevance. Thus, in this section I will devote particular attention to this factor.

Macrophage colony-stimulating factor (M-CSF or CSF-1)

CSF-1 is a lineage specific hemopoietic cytokine that stimulates proliferation and supports differentiation and survival of cells of the mononuclear phagocyte series (Stanely, 1979; Stanley et al, 1983). CSF-1 induces formation of pure macrophage colonies from individual bone marrow progenitors plated in semisolid medium and can stimulate proliferation of mature macrophages (Stanley et al, 1983). It also potentiates the ability of mature mononuclear phagocytes to perform their functions by enhancing their ability to kill infectious microorganisms and tumor cells, and by regulating release of macrophage cytokines, such as interferon (IFN), TNF, IL-1, and granulocyte-CSF (G-CSF) (Sherr, 1990).

CSF-1 is a 70 - 90 kD homodimeric glycoprotein produced by several cell types, including fibroblasts, endothelial cells and macrophages (Clark & Kamen, 1987). Biologically active secreted and membrane-bound forms of CSF-1 are encoded by alternatively spliced mRNAs transcribed from a single gene (Ladner et al, 1987) that was originally assigned to the long arm of human chromosome 5 at band q33.1 (Pettenati et al, 1987). Recently, the human CSF-1 gene has been reassigned to chromosome 1 (Morris et al, 1991). In the hemopoietic system of adult mammals, CSF-1 exerts its pleiotropic effects by binding to a single class of high affinity receptors (Guilbert & Stanley, 1986) expressed predominantly on monocytes, macrophages, and their progenitors (Byrne et al, 1981). The CSF-1 receptor, which is encoded by the *c-fms* protooncogene (Sherr et al, 1985), is also

expressed on the surface of blast cells from patients with acute myelogenous leukemia (Ashmun et al, 1989). Apart from its direct action on macrophagic progenitors (Sato et al, 1991), CSF-1 also influences hemopoiesis by modulating the production of several positive and negative hemopoietic regulators (Warren & Ralph, 1986).

Granulocyte colony-stimulating factor (G-CSF)

G-CSF is the primary regulator of the production of neutrophilic granulocytes (Lotem et al, 1980; Demetri & Griffin, 1991). It is a hydrophobic glycoprotein with a molecular weight of 24 kD (Nicola et al, 1983). G-CSF is encoded by a single gene localized on the long arm of chromosome 17 (Le Beau et al, 1987). Macrophages, fibroblasts and endothelial cells are the major sources of this factor. Its production is highly regulated and not constitutive. In normal humans, the circulating levels of G-CSF can be increased up to 100-fold under stress conditions.

*In vitro*, G-CSF stimulates proliferation and differentiation of neutrophil colony-forming cells and alters several functions of mature neutrophils (Metcalf & Nicola, 1983). Like each of the other hemopoietic cytokines, G-CSF exerts its biologic activities through binding to specific receptors (Nicola & Metcalf, 1985). Although G-CSF acts primarily on a relatively mature cell population of progenitors committed to neutrophilic differentiation, it also synergises with cytokines, such as IL-3 and IL-6, that act on more primitive cells (Ikebuchi et al, 1988a; 1988b).

Granulocyte-Macrophage colony stimulating factor (GM-CSF)

As its name suggests, GM-CSF's primary action is to enhance the survival as well as to stimulate the proliferation and differentiation of progenitor cells committed to the neutrophilic granulocyte and macrophage cell lineages (Tomonaga et al, 1986; Gough & Nicola, 1990). GM-CSF is a glycoprotein with a molecular wight of 23 kD, produced by T lymphocytes, macrophages, fibroblasts and endothelial cells (Nicola et al, 1979). The GM-CSF gene has been localized on the long arm of chromosome 5 (Huebner et al, 1985). The most surprising revelation arising from the gene localization of GM-CSF is its close proximity to the IL-3 gene (as little as 10 kbp in humans). The most obvious explanation for this tight linkage is that the two genes are ancestrally related, and indeed, IL-3, as we shall see later on, is a functionally related cytokine.

As mentioned before, the major target cells for GM-CSF are the myeloid (granulocytic and macrophagic) progenitors, on which this factor can act directly or indirectly, via accessory cells (Baines et al, 1988). However, several investigators have also demonstrated a direct effect of GM-CSF on multipotential as well as on erythroid progenitors (Migliaccio et al, 1987, Mayani et al, 1989).

Interleukin-3 (IL-3)

IL-3 is a glycoprotein with a molecular weight near 20 kD (Ihle et al, 1981). It is encoded by a single gene located on the long arm of chromosome 5, very close to the GM-CSF gene. Due to its ability to induce the growth of hemopoietic colonies in semisolid cultures, IL-3 has been classified as a colony-

stimulating factor (Leary et al, 1987; Clark & Kamen, 1987). In such cultures, IL-3 supports the development of mixed myeloid/erythroid, macrophage, neutrophil, eosinophil, and basophil colonies (Hapel et al, 1985). By using single-cell cultures, it has been shown that IL-3 acts directly on myeloid progenitors (Clark-Lewis & Schrader, 1988). The primary source of IL-3 appears to be the antigen-reactive T lymphocyte (Kelso & Metcalf, 1985; Yang et al, 1987); many investigators therefore consider IL-3 to be solely a mediator of the inflammatory events that follow T-cell stimulation. However, others have demonstrated constitutive production of IL-3 by endothelial cells (Luger et al, 1985) and the yolk sac (Labastie et al, 1984), suggesting that this factor is actually involved in steady-state hemopoiesis.

Erythropoietin (Epo).

Epo is a glycoprotein produced primarily by the kidney, and is the principal factor regulating red blood cell production (Erslev, 1953; Krantz, 1991). Its production is part of a classic feedback control system. Hypoxia results in a significant increase in Epo production, which enhances the manufacture of new red cells, whereas hyperoxia reduces the amount of Epo and, consequently, decreases red cell production (Krantz & Jacobson, 1970).

The molecule has a molecular weight of 34 kD and contains approximately 30% carbohydrate (Miyake et al, 1977). The human Epo gene has been localized on chromosome 7 (Law et al, 1986). Although the kidney is the major site of Epo production, the liver has also been shown to produce it (Erslev et al, 1980). The major target cell for Epo is the CFU-E, which is able to respond to low concentrations of the factor (Sawada et al, 1987). BFU-E and early

erythroblasts also respond to Epo, although they require higher concentrations. Megakaryocytes and their progenitors have also been shown to respond to Epo (Dessypris et al, 1987).

c-kit ligand or stem cell factor (SCF)

SCF was discovered and characterized only two years ago (Zsebo et al, 1990; Williams et al, 1990; Martin et al, 1990). By itself, SCF shows modest effects on early myeloid and lymphoid cells (Witte, 1990), however, when added to hemopoietic cultures in combination with other cytokines, such as GM-CSF, IL-3, G-CSF, and Epo, it induces significant increases in the growth of myeloid and erythroid progenitor cells (Bernstein et al, 1991; McNiece et al, 1991). Administration of SCF into $Sl/Sl^d$ mice results in the reversal of the anemia and mast cell deficiency, characteristic of such animals (Zsebo et al, 1990).

Tumor necrosis factor-α (TNF-α)

TNF-α is a multifunctional cytokine produced mainly by monocytes and macrophages (Nathan, 1987). It is a nonglycosylated protein with a molecular weight of 17,350 Daltons that can be present as a soluble or cell-associated molecule (Aggarwal et al, 1985). TNF-α plays several roles within the hemopoietic system, by acting directly on hemopoietic progenitors (Roodman et al, 1987), and by inducing the production and release of several other cytokines by different cells (Munker et al, 1986; Koeffler et al, 1987; Oster et al, 1987). TNF-α is a potent inhibitor of myeloid (CFU-C) and erythroid (BFU-E and CFU-E) progenitors both *in vivo* and *in vitro* (Roodman et al, 1987; Broxmeyer et al, 1986; Akahane et al, 1987; Johnson et al, 1990). Its inhibitory effects have

also been documented in LTMC (Eliason & Vassalli, 1988). Recent reports indicate that blast cells from patients with acute myelogenous leukemia (AML) produce and secrete TNFα (Oster et al, 1989; Kobari et al, 1990).

Transforming growth factor-β (TGF-β)

TGF-β is synthesized by many normal and neoplastic cells (Assoian et al, 1983; Hooper, 1991). TGFβ is secreted as an inactive precursor and later activated by certain conditions; this means that this factor is often not active at the site of synthesis (Hooper et al, 1991). Within the hemopoietic microenvironment, macrophages are important producers of this factor (Assoian et al, 1987). TGF-β is a disulfide linked dimer of 25 kD, and can be found in at least three distinct forms as either homo- or hetero-dimer (Chiefetz et al, 1988). Several studies have demonstrated inhibitory effects of TGF-β on both myeloid and erythroid cells (Hino et al, 1988; Sing et al, 1988; Cashman et al, 1990).

Interferon-γ (IFN-γ)

IFN-γ is a cytokine produced by several cell types in response to viral infections and other stimuli (Broxmeyer, 1986). Cells of the hemopoietic microenvironment, such as T lymphocytes, produce it in significant levels upon appropiate stimulation (Mamus et al, 1985). Apart from its anti-viral effects, IFN-γ is a potent inhibitor of hemopoietic progenitors as well as of stromal cells (Zoumbos et al, 1984; Coutinho et al, 1986).

Macrophage inflammatory protein-1α (MIP-1α)

Also referred to as stem cell inhibitor (SCI), MIP-1α was discovered a few

years ago. It is a protein with a low molecular weight (< 10 kD), secreted by bone marrow macrophages (Graham et al, 1990). *In vitro* studies have shown that MIP-1α is a primary negative regulator of stem cell proliferation with no significant effects on more mature progenitors (Graham et al, 1990).

## D) ACUTE MYELOGENOUS LEUKEMIA

Alterations in the mechanisms regulating hemopoiesis may result in the development of hematological disorders, in which production of blood cells may be abnormally reduced (i.e. pure red cell aplasia or aplastic anemia) or increased (i.e. polycythemia vera or leukemia). Most of these diseases arise from alterations in the genome of primitive stem/progenitor cells, which give rise to abnormal progenies with growth advantages over their normal counterparts (Jacobs, 1991; Sawyers et al, 1991).

Acute myelogenous leukemia (AML) can be defined as the uncontrolled proliferation or expansion of hemopoietic (myeloid) cells that do not retain the capacity to differentiate normally to mature blood cells (Sawyers et al, 1991). As a result of this dysfunction, 30 - 95% of the cells in the bone marrow of AML patients are blasts, and such cells are also increased in the peripheral blood (Messner & Griffin, 1986). While the cause of AML remains unknown in most cases, it is clear that this disease has a strong environmental component (i.e. exposure to toxic agents and radiation). AML incidence rates are greater in industralized cities within North-America and Europe and in developed countries (Sandler, 1987).

Chromosome abnormalities are detected in 50 - 65% of adult AML patients (Bloomfield & de la Capelle, 1987). Such abnormalities are detected in

almost all the cases of AML arising after chemotherapy or radiotherapy for a prior cancer (Kantarjian & Keating, 1987). In *de novo* AML, a history of occupational exposure to environmental toxins has been significantly associated with higher incidence of chromosome abnormalities (Mitelman et al, 1981). Some of the most common abnormalities include: t(15;17), t(8;21), del(5q), del (7q), trisomy 8, and monosomy 7 (Fenaux et al, 1989).

In some cases, AML is preceded by a state known as myelodysplastic syndrome or preleukemia, characterized by refractory anemia and by increased levels of blast cells (up to 30%) in bone marrow (Jacobs, 1991). Although reduced to a great extent, normal hemopoiesis is not totally suppressed in AML. Based on this observation, different treatments have been developed, which try to eliminate the leukemic cells and to stimulate the growth of the normal ones (Burke, 1991).

By using genetic methods based either on chromosomal markers or on the distribution of the molecular variants of glucose-6-phosphate-dehydrogenase, it has been demonstrated that AML arises from the clonal expansion of a single transformed myeloid stem/progenitor cell with proliferative advantages over its normal counterpart (McCulloch, 1983; Fialkow et al, 1987). The resulting leukemic cell population is biologically heterogeneous despite morphological homogeneity (Griffin & Lowenberg, 1986). *In vivo* studies using $^{3}$H-thymidine labeling have shown that the entire leukemic cell population is sustained by a small fraction of cells that are actively synthesizing DNA. Such cells have been identified *in vitro* and referred to as clonogenic blasts (Griffin & Lowenberg, 1986).

## AML *in vitro*

During the past two decades, significant advances in understanding the biology of AML have been achieved, mainly through *in vitro* studies. By using semisolid and liquid cultures, a subpopulation of AML cells, known as AML colony-forming units (AML-CFU), has been recently characterized. Several observations suggest that AML-CFU function as leukemic progenitor cells. Firstly, AML-CFU are capable of forming colonies consisting of blast cells carrying cytogenetic AML markers (Lowenberg et al, 1980). Secondly, a very high proportion (> 60%) of AML-CFU are in the S phase of the cell cycle; interestingly, thymidine labeling index of blasts correlates with the incidence of leukemic colony formation (Marie et al, 1982). Finally, liquid culture studies have shown that AML-CFU are capable of self-renewal (Nara & McCulloch, 1985).

### AML in liquid cultures

AML-CFU can be induced to self-renew when cultured in liquid cultures in the presence of stimulating factors (Nara & McCulloch, 1985). IL-3, GM-CSF and G-CSF, alone or in combination, are potent stimulators of AML-CFU self-renewal (Vellenga et al, 1987; Miyauchi et al, 1988a). These effects seem to be direct, since receptors for such factors have been demonstrated on the AML cell surface (Onetto-Pothier et al, 1990). Under these conditions, AML-CFU can be sustained for up to 3 months. Although to a lesser extent, stimulation of AML-CFU self-renewal has also been observed in cultures supplemented with IL-1 (Murohashi et al, 1990).

Constitutive expression of hemopoietic stimulator genes has been observed by different investigators, which has lead to the hypothesis that autocrine mechanisms play key roles in the malignant growth of AML cells (Young et al, 1987). Interestingly, Murohashi and colleagues have suggested that there are at least two blast cell subpopulations in AML: one is a proliferating subpopulation with self-renewal capacity and the other is a supporting subpopulation with the capacity to produce hemopoietic stimulators (Murohashi et al, 1989).

In contrast to other stimulatory cytokines, CSF-1 has practically no effect on the self-renewal of AML-CFU (Suzuki et al, 1988). Its major effect is the induction of such cells to differentiate into adherent, macrophage-like cells (Miyauchi et al, 1988b). AML-CFU self-renewal is significantly inhibited by TGF$\beta$ and also by TNF$\alpha$ and IFN$\gamma$ (Nara et al, 1989; Kerangueven et al, 1990).

AML in semisolid cultures

When bone marrow cells from AML patients are cultured in semisolid cultures, AML-CFU give rise to colonies consisting of blast cells expressing cytogenetic AML markers (Lowenberg et al, 1980). In these culture conditions, leukemic cell growth also depends on the presence of hemopoietic stimulators, such as GM-CSF and G-CSF (Murohashi et al, 1988).

The growth of normal progenitor cells (i.e. CFU-C, BFU-E, and CFU-MIX) in semisolid cultures of AML bone marrow cells is dramatically reduced (Metcalf, 1984). In fact, BFU-E and CFU-MIX are usually absent in such cultures. Some investigators have observed the production of soluble inhibitory factors by the leukemic cells (Broxmeyer et al, 1981; Kobari et al,1990), which

may explain, at least in part, the inhibition of normal progenitors in AML semisolid cultures. On the other hand, this may simply reflect the *in vivo* situation, in which normal hemopoiesis is drastically reduced.

## E) LONG-TERM MARROW CULTURES

The long-term marrow culture (LTMC) system was first described fifteen years ago by Dexter and colleagues (Dexter et al, 1977). As I have mentioned before in this work, both normal and murine hemopoiesis can be maintained in LTMC for several months due to the development of an adherent cell layer, consisting of marrow stromal cells (fibroblasts, macrophages, endothelial cells and adipocytes), which, together with marrow accessory cells (lymphocytes and monocytes), provide the microenvironment that stem/progenitor cells need in order to proliferate and differentiate (Greenberger, 1991). In this section, I will focus on the biology of human LTMC initiated with normal and AML cells.

### Normal bone marrow in LTMC

Hemopoietic progenitor cells from human normal bone marrow can be observed in LTMC for an average period of 10 weeks (Gartner & Kaplan, 1980) and up to 20 weeks (Preiksaitis & Janowska-Wieczorek, 1991). Different studies have demonstrated that LTMC allow the actual growth, i.e. self-replication and differentiation of stem cells as well as differentiation of progenitor cells (Cashman et al, 1990; Sutherland et al, 1990; Verfaillie et al, 1990), and not only act as a passive environment that delays the death of the progenitors. The fact that human LTMC cannot be sustained for longer than 4 - 5 months seems to be due to the continuous depletion of stem/progenitor cells from the culture

(Varma et al, 1992).

After 3 weeks of culture, the majority of the progenitor cells are localized in the adherent fraction (Coulombel et al, 1983), which supports the idea that hemopoiesis proceeds in close association with the marrow stroma. Myeloid progenitors (CFU-C) are the ones that are sustained the longest (> 10 weeks); erythroid (BFU-E and CFU-E) as well as multipotential (CFU-MIX) progenitors disappear from the culture significantly faster (Eaves & Eaves, 1988).

Recently, it has been demonstrated that the progenitor cells developed in human LTMC are derived from a primitive cell known as long-term culture initiating-cell (LTC-IC; Sutherland et al, 1989; 1990). The physiological characteristics of these cells have been described before (see section A of this chapter), and it is noteworthy that the great majority of these cells have been localized in the adherent fraction, interacting with stromal cells (Verfaillie et al, 1990).

It has been suggested that stromal cells in human LTMC play key roles in regulating the proliferation of immature progenitor cells. However, the exact mechanisms operating in this process are not fully understood. Cashman and colleagues have found that immature erythroid progenitors present in the nonadherent fraction are actively cycling at all times, whereas the same cell population is in a quiescent state when localized in the adherent layer (Cashman et al, 1985). Addition of fresh growth medium to the culture stimulates these cells to go transiently into cycle (Cashman et al, 1985). These authors have suggested that stromal cells keep closely located hemopoietic progenitors out of cycle ($G_0$) by secreting TGF$\beta$. On the other hand, factors present in horse serum (essential component of the growth medium), as well as cytokines such as IL-1 or

platelet-derived growth factor, stimulate stromal cells to produce stimulatory cytokines, such as GM-CSF, and these, in turn, stimulate the hemopoietic progenitors to proliferate (Eaves & Eaves, 1988; Eaves et al, 1991).

Addition of recombinant growth factors into LTMC has significant effects on the levels of progenitor cells. IL-3 and GM-CSF have potent stimulatory effects on the growth of CFU-C, whereas G-CSF has been shown to be less effective (Coutinho et al, 1990). Interestingly, IL-3, GM-CSF and G-CSF constitutively produced by genetically engineered fibroblasts, showed more significant effects on progenitor cells than the recombinant proteins added to the culture (Hogge et al, 1991; Otsuka et al, 1991). This observation suggests that the way in which the hemopoietic stimulators are presented to the progenitor cells may determine the magnitude of the biological response.

**AML bone marrow in LTMC**

In 1985, Coulombel and colleagues reported a very interesting finding. When bone marrow cells from patients with AML are cultured in LTMC, AML blasts and AML-CFU disappear, in most cases, during the first 3 - 4 weeks. Concommitantly, morphologically normal blood cells and CFU-C become detectable and reach significant levels, although usually lower than those observed in normal LTMC, after 2 - 4 weeks (Coulombel et al, 1985; Janowska-Wieczorek et al, 1991)). This observation raises two interesting points. Firstly, the physiological advantage that AML cells show *in vivo* over their normal counterparts is not reproduced in LTMC, suggesting that LTMC may not be the ideal model to study the development and progression of AML. However, it is interesting to speculate that the behavior of AML cells in LTMC may reproduce

the biology of AML during remission. Secondly, that leukemic progenitors in LTMC are at a physiological disadvantage that leads to their disappearance after a few weeks in culture suggests the possibility that LTMC could be used as an *in vitro* method for purging leukemic cells for autologous bone marrow transplantation (ABMT). Indeed, groups in Manchester (Chang et al, 1989) and Vancouver (Barnett et al, 1989) have already used LTMC for such purposes.

The origin of the hemopoietic progenitors (CFU-C) developed in AML LTMC has been a controversial issue during the past five years. On the one hand, some studies using chromosomal analysis have shown that morphologically normal CFU-C observed in AML LTMC have a normal karyotype, in spite of the fact that the cells in the initial innoculum exhibited chromosome abnormalities (Coulombel et al, 1985; Shiro et al, 1990), suggesting that such progenitors are of normal origin. On the other hand, by using the G6PD assay, Singer and colleagues have shown that morphologically normal CFU-C may be of leukemic origin (Singer et al, 1988). In keeping with this observation are the results of Iland and collaborators, who found that mature myeloid cells, including polymorphonuclear neutrophils, developed in AML LTMC contain Auer rods, which are a morphological feature of AML cells (Iland et al, 1987).

Despite these apparently contradictory results, the fact that progenitor cells grown in LTMC initiated with AML bone marrow are being used for ABMT indeed suggests that LTMC favors the growth of normal progenitors. In this regard, it is noteworthy that the Vancouver group has used LTMC for purging bone marrow cells for ABMT in seven AML patients, and this method has been used by the Manchester group in more than 15 cases (AC Eaves; personal communication).

# III. MATERIALS AND METHODS

## A) SOURCE OF CELLS

### Normal bone marrow cells

With the donors' fully informed consent, normal bone marrow samples were obtained from the sternums of hematologically normal patients undergoing cardiac surgery. The marrows were aspirated into sterile syringes containing 1000 U preservative-free heparin. This protocol has been approved by the Ethical Committe of the University of Alberta Hospitals.

### AML bone marrow/peripheral blood cells

Bone marrow and/or peripheral blood cells were obtained during diagnostic sampling from twelve AML patients, either at time of presentation (ten cases) or relapse (two cases). The marrows were aspirated into sterile syringes containing 1000 U preservative-free heparin. Blood samples were collected in sterile tubes also containing 1000 U preservative-free heparin. This protocol has been approved by the Ethical Committe of the Alberta Cancer Board. The samples were provided by Dr. Andrew R. Belch (Cross Cancer Institute, Edmonton, Alberta). All the patients fulfilled the diagnostic criteria defined by the French-American-British (FAB) classification (Bennett et al, 1976). According to the FAB criteria, seven categories of AML have been identified: M1 (myeloblastic leukemia without maturation), M2 (myeloblastic leukemia with maturation), M3 (hypergranular promyelocytic leukemia), M4 (myelomonocytic leukemia), M5 (pure monocytic leukemia), M6 (erythroleukemia), M7 (megakarioblastic leukemia). Table 1 shows the patients' clinical information and hematological parameters.

**Table 1**

**Clinical data on the AML patients**

| Patient | FAB | Sample[a] | Hb[b] (g/dL) | WBC[c] ($x10^{-9}$/L) | Blasts (%) |
|---|---|---|---|---|---|
| 1 | M1 | BM | 11.7 | 1.4 | 79 |
| 2 | M1 | BM | 11.8 | 2.3 | 43 |
| 3 | M2 | BM/PB | 11.4 | 155.7 | 83/87 |
| 4 | M2 | BM | 8.3 | 134.0 | 88 |
| 5 | M2 | PB | 6.9 | 51.6 | 89 |
| 6 | M4 | BM | 5.5 | 2.0 | 49 |
| 7 | M4 | PB | 11.5 | 26.3 | 60 |
| 8 | M4 | BM | 7.8 | 1.2 | 57 |
| 9 | M4 | BM | 10.7 | 16.9 | 48 |
| 10 | M5 | BM/PB | 6.2 | 537.0 | 93/89 |
| 11 | M5 | BM | 8.5 | 69.6 | 64 |
| 12 | UC* | BM | 7.6 | 84.3 | 95 |

a BM = bone marrow
PB = peripheral blood

b Hb = hemoglobin

c WBC = white blood cells

* UC = unclassified

**Cryopreservation of cells**

When not cultured immediately, normal or AML cells were resuspended, at a concentration of $1.5 \times 10^7$ cells/ml, in Iscove's medium supplemented with 20% fetal calf serum. An equal volume of Iscove's medium supplemented with 20% dimethyl sulfoxide (DMSO) was added dropwise to the cell suspension (final concentration 10% DMSO). Then, 1.5-ml aliquots were dispensed into NUNC freezing vials. The vials were sealed and immediately placed at -70° C. After 1 - 10 days, vials were transferred to liquid nitrogen (-196° C).

For thawing of the cells, vials were rapidly removed from liquid nitrogen and placed in a 37° C waterbath until the contents were thawed. The contents were then gently placed in a 15-ml plastic tube. Immediately afterwards, 12 ml of Iscove's medium supplemented with 20% fetal calf serum were added slowly and the suspension was gently mixed. The cells were collected by centrifugation (400 x g for seven minutes), the supernatant was discarded and the cells were resuspended in fresh medium .

## B) CELL CULTURE METHODS

**Long-term marrow cultures (LTMC)**

LTMC were performed based on the method described by Coulombel and colleagues (Coulombel et al, 1983). Bone marrow buffy coat cells, from either normal or AML subjects, were obtained by centrifugation (400 x g for 7 minutes) and then resuspended in growth medium consisting of $\alpha$-medium (GIBCO, Ontario) supplemented with extra glutamine (400 mg/L; GIBCO), inositol (40 mg/L; GIBCO), folic acid (10 mg/L; Lederle, Montreal), horse serum (12.5%; GIBCO), fetal calf serum (12.5%; GIBCO), 2-mercaptoethanol ($10^{-4}$ mol/L;

Eastman Kodak, Rochester, NY), hydrocortisone ($10^{-6}$ mol/L; Upjohn, Ontario), and a mixture of the antibiotics penicillin and streptomycin (1%; GIBCO). Between $20 \times 10^6$ - $30 \times 10^6$ cells were loaded into a 25-$cm^3$ tissue culture flask and incubated at 37° C in an atmosphere of 5% $CO_2$ in air. After three days, flasks were transferred to a different incubator and maintained at 33° C thereafter. Four days later (seven days after initiation of the cultures), half of the supernatant (including nonadherent cells) was removed and replaced with fresh growth medium. The cultures were fed in this manner at weekly intervals. The nonadherent cells, obtained weekly during the medium change, were counted (using a Coulter Counter, Coulter Electronics Inc. Florida), analyzed morphologically (see section D), and assayed for hemopoietic progenitors (see section C). On certain days, some of the cultures were sacrificed for evaluation of the adherent cells, which were detached by trypsinization, resuspended in growth medium, and processed in the same way as nonadherent cells.

**Stromal adherent layers**

In order to establish stromal adherent layers both from normal and AML bone marrow, LTMC were initiated as described above. At week three (day 21), all the nonadherent cells were removed and the adherent cells were irradiated with 1000 Rads of gamma-irradiation ($^{137}Cs$) to inhibit the proliferative capacity of the hemopoietic progenitors residing in the cultures. At this point, adherent layers were considered to be confluent when >80% of the flask surface area was covered by adherent cells. Previous studies have shown that stromal cells (i.e. fibroblasts and macrophages) are capable of proliferating and performing a variety of functions, such as production and secretion of hemopoietic cytokines,

after being exposed to 1000 Rads X rays or gamma-irradiation (Tavassoli, 1982; Song & Quesenberry, 1984). In contrast, hemopoietic progenitors are unable to proliferate after being exposed to a similar dose of radiation (Tavassoli & Friedenstein, 1983). In the present study, one hour or five weeks after irradiation, some of the adherent layers were tested for the presence of hemopoietic progenitors (see section C). In every case, the yield of progenitors was zero.

By morphological criteria (see section D), the two major cell populations detected in the stromal adherent layers were cells with fibroblast morphology and macrophages, which is in keeping with the studies by Andreoni et al (Andreoni et al, 1990). Since we did not have a direct evidence (i.e. cell surface marker) that the former were actual fibroblasts, they were referred to as fibroblastoid cells. Fat cells were also observed (Figure 4). However, no specific analysis for endothelial cells was performed, thus, although we assume that they were present in these cultures (Allen & Dexter, 1984), no quantitative data is presented.

**Fibroblast cultures**

Cultures containing practically pure populations of fibroblastoid cells (>95%, as determined by morphology) were established based on the method described by Friedenstein and colleagues (Friedenstein et al, 1970). Bone marrow buffy coat cells were resuspended in Iscove's medium supplemented with 15% fetal calf serum, at a concentration of $1 \times 10^5$ - $2 \times 10^5$ cells/ml. Eight ml of the cell suspension were loaded into a 25 $cm^3$ tissue culture flask and incubated at 37° C and 5% $CO_2$ in air. After three days, all the supernatant and nonadherent cells were removed and fresh culture medium was added. This

procedure was repeated every four days. Confluency was reached three weeks after initiation of the culture. At this time, these adherent layers were also irradiated (1000 Rads gamma-irradiation). Friedenstein et al (1970), as well as Castro-Malaspina et al (1980) have previously demonstrated that adherent layers established in this manner consist of a practically pure population of fibroblasts.

**Chimeric LTMC**

Chimeric LTMC were established by co-culturing hemopoietic progenitors derived from normal bone marrow with stromal adherent layers from either normal or AML bone marrow. This procedure was based on the one described by Coutinho et al (1990). Normal bone marrow buffy coat cells, resuspended in LTMC-growth medium at 2 x $10^6$ cells/ml, were depleted of adherent cells by two consecutive incubations (overnight and two hours long, respectively) in tissue culture plastic flasks. Then, the medium containing nonadherent cells was recovered and placed on top of the pre-established stromal or fibroblast adherent layers, from either normal or AML bone marrow, at a concentration of 0.5 x $10^6$ nonadherent cells/ml (4 x $10^6$ cells /flask). In every experiment, control flasks were established in which nonadherent cells from normal bone marrow were cultured in the absence of a pre-established adherent layer. All the chimeric and control (lacking a pre-established adherent layer) cultures were kept for 4 - 5 weeks. At weekly intervals, hemopoietic progenitors in the non-adherent fraction were assessed in colony assays (see section C of this chapter). On certain days, some of the cultures were sacrificed for evaluation of progenitors residing in the adherent layer.

**AML blast cultures**

AML blast cells were purified by loading 4 - 6 ml AML peripheral blood or bone marrow buffy coat cells (40 - 90% blasts; at a concentration of $5 \times 10^6$ cells/ml) on top of 4 ml 60% Percoll (density = 1.077 g/ml; Pharmacia LKB, Uppsala) in 15-ml plastic tubes. The samples were centrifuged at 1800 rpm for 30 min. Red cells as well as mature and immature granulocytic cells formed a pellet on the bottom of the tube. Mononuclear cells (>95% blasts) formed a clear band between the Percoll solution and the plasma. These cells were recovered with a Pasteur pipette, washed twice in Iscove's medium and resuspended in Iscove's medium supplemented with 20% fetal calf serum. The cells were cultured for 2 - 21 days, at 37° C in an atmosphere of 5% $CO_2$ in air, with weekly medium changes.

**Cell culture supernatants**

In every single experiment, cell culture supernatants were obtained weekly during medium change. After centrifugation of cells (400 x g for 7 min), the supernatants were recovered using a Pasteur pipette, placed in 3-ml plastic tubes, and stored at -20° C. Few minutes prior to being used, supernatants were thawed by placing the tubes in warm water.

## C) CLONOGENIC ASSAYS

**Hemopoietic colony assays**

These were performed based on the method described by Fauser & Messner (Fauser & Messner, 1979). Hemopoietic progenitor cells were assayed in Iscove's medium (GIBCO) containing 0.9% methylcellulose (Fluka AG,

Chemische Fabrischen, Basel) and supplemented with 30% human plasma (collected in our lab), 10% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM; source of hemopoietic stimulatory cytokines), 2-mercaptoethanol (final concentration $10^{-4}$ M), and 1 U/ml erythropoietin (EPO, Terry Fox Laboratory, Vancouver). When assaying hemopoietic progenitors from LTMC, cells were plated in 35-mm dishes at a final concentration of 0.5 - 2 x $10^5$/ml (1 ml/dish), depending on the number of cells recovered from the flasks. When assaying hemopoietic progenitors from freshly isolated bone marrow, cells were always plated at 2 x $10^5$ cells/ml. Each assay was set up in duplicate. All hemopoietic colonies were scored in the same dish, after 8 - 17 days of incubation at 37° C at 5% $CO_2$ in air, using an inverted microscope. In order to obtain the total number of hemopoietic progenitors per LTMC flask, the number of progenitors observed per semisolid culture was multiplied by the total number of nucleated cells per LTMC flask, and the resulting number divided by the number of cells plated in semisolid culture.

**Morphology of the hemopoietic colonies**

Hemopoietic colonies were scored according to previously reported standard criteria (Metcalf, 1984). Colonies derived from mature erythroid progenitors (CFU-E) consisted of small colonies containing 20 - 50 hemoglobinized cells and were scored after 8 - 10 days of incubation. Colonies derived from immature erythroid progenitors (BFU-E) consisted of >50 hemoglobinized cells grouped in one or several clusters and were scored after 14 - 17 days of incubation. Colonies derived from myeloid progenitors (CFU-C) consisted of >50 granulocytes and/or macrophages. CFU-C were classified as

granulocytic (CFU-G), macrophagic (CFU-M), and granulocyte/macrophage (CFU-GM) colonies. They were also scored after 14 - 17 days of incubation. Colonies derived from immature multipotential progenitors (CFU-GEMM or CFU-MIX, scored on day 14 - 17 of incubation) consisted of >50 erythroid and myeloid cells. AML-associated clusters (AML-ACl), which were present exclusively in cultures derived from AML bone marrow, were scored on day 14. They consisted of clusters (10 - 40 cells) containing blast cells only. This type of colony seems to correspond to the one described by Coulombel et al (1985) and that has been referred to as AML-CFU. The accuracy of colony identification by direct visualization was confirmed by random staining of cytospin-prepared individual colonies with Wright-Giemsa stain. Typical examples of the colonies mentioned above are shown in Figure 1.

**Assay for fibroblastic progenitors**

Fibroblast colony-forming cells (CFU-F) present in bone marrow, were assayed according to the method described by Castro-Malaspina et al (1980). Cells obtained from fresh bone marrow samples or by trypsinization of stromal adherent layers were resuspended at a concentration of $1 \times 10^5$ cells/ml in Iscove's medium containing 20% fetal calf serum. Cell suspensions were incubated in 35-mm petri dishes (1 ml/dish), at 37° C in an atmosphere of 5% $CO_2$ in air. After three days, the medium and all the nonadherent cells were removed and fresh medium was added. The cultures were returned to the incubator for a further seven days. At the end of this period, the medium was discarded and the adherent cells were stained with Wright-Giemsa. Colonies of more than 50 fibroblastoid cells were scored as fibroblastic (Figure 2).

**Figure 1.** Morphology of human hemopoietic colonies developed in semisolid cultures. (a) CFU-MIX (the dark area is actually red due to the presence of erythroid cells); (b) BFU-E (the whole colony is red); (c) CFU-G; (d) CFU-M; (e) CFU-GM; (f) AML-ACl.

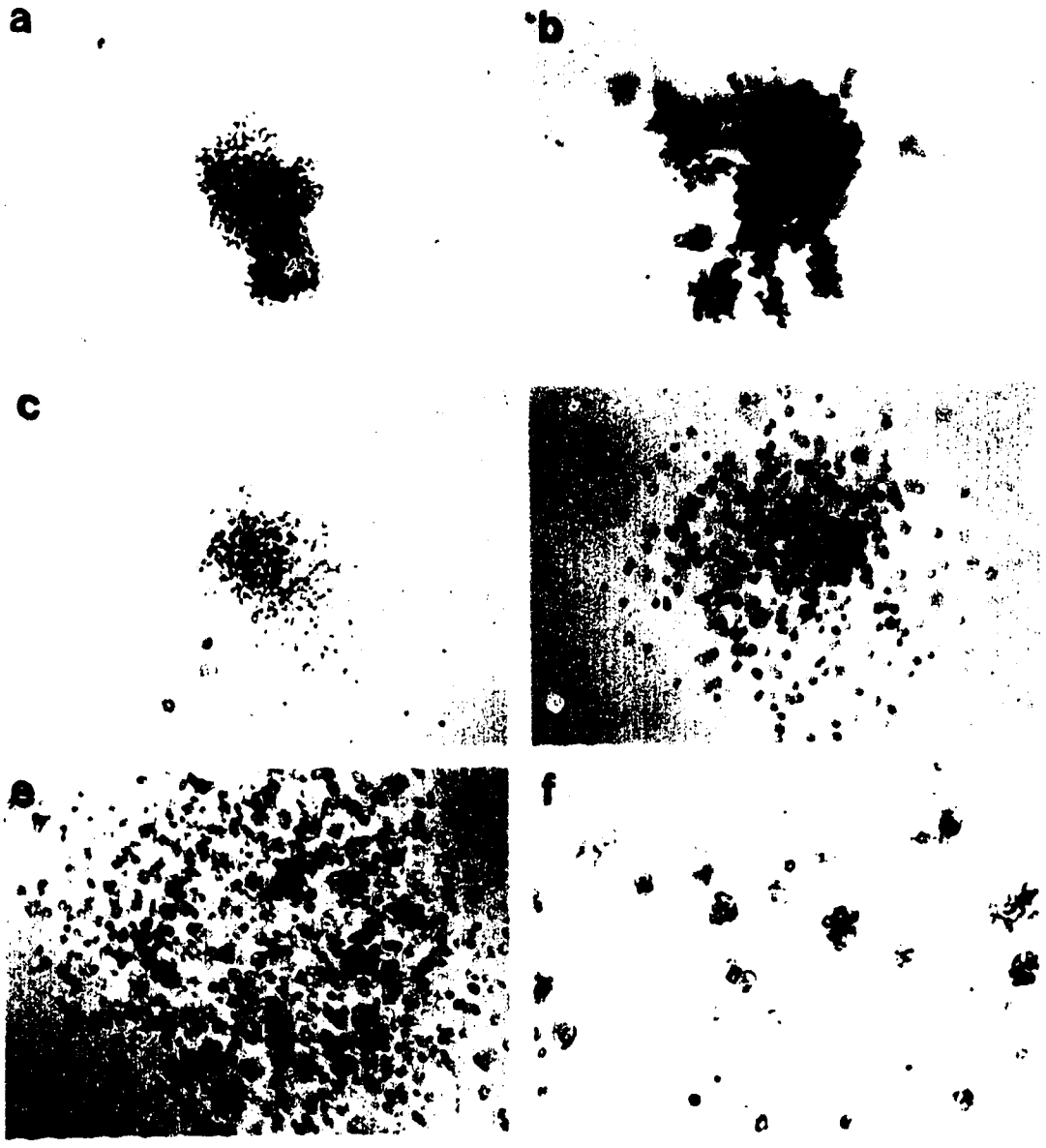
a
b
c
e
f

**Figure 2.** Morphology of a fibroblastic (CFU-F) colony

## D) MORPHOLOGICAL ANALYSIS OF NUCLEATED CELLS

Cells obtained from fresh bone marrow samples or from the cell cultures were examined on cytocentrifuge preparations stained with Wright-Giemsa. By using standard morphological criteria (Zucker-Franklin et al, 1981), nucleated cells were classified as: granulocytes (comprising the whole granulocytic series from myeloblasts to polymorphonuclear cells), erythroblasts (from proerythroblasts to orthochromatic erythroblasts), lymphoid cells, monocytes/macrophages, and blasts (immature cells with no features corresponding to any specific lineage). In order to identify macrophages and fibroblastoid cells present in the adherent layer of the cultures, cell were removed by trypsinization, resuspended in Iscove's medium containing 20% fetal calf serum, at 1 x $10^5$ cells/ml, and incubated in 35-mm petri dishes at 37° C, 5% $CO_2$ in air. After 24 hours, the supernatant and nonadherent cells were removed and the adherent cells were fixed and stained with Wright-Giemsa. The cells were then analyzed under an inverted microscope. This procedure permitted a clear morphological identification of macrophages (large, usually round, cells with a small round nucleous) and fibroblastoid cells (spindle cells) according to previously reported criteria (Friedenstein et al, 1970; Castro-Malaspina et al, 1980). In some cases, the cells were stained with double esterase (α-napthyl butyrate esterase and naphtol AS-D chloroacetate esterase) for macrophagic identification.

## E) CYTOKINES AND CYTOKINE ASSAYS

### Recombinant human CSF-1 (rhCSF-1)

Chinese hamster ovary (CHO)-cell-derived purified recombinant human

CSF-1 (kindly provided by Dr. Steven C Clark; Genetics Institute, Cambridge MA) was used throughout this study. This rhCSF-1 corresponds to the long form of the CSF-1 molecule reported by Wong and colleagues (554 aminoacids; Wong et al, 1987). CSF-1 activity was 1 x $10^6$ U/mg, where 1 U/ml yields half the maximum colony formation in the murine bone marrow assay, as determined by Dr. Clark. In one particular experiment, the effects of *E coli*-derived purified recombinant human CSF-1 (Cetus Corporation, California) were compared with those of the CHO-cell-derived CSF-1. This bacteria-derived rhCSF-1 corresponds to a truncated form of CSF-1 (218 amino acids; Halenbeck et al, 1989). These CSF-1 forms share a common N-terminus of 149 amino acids and a common C-terminus of 69 amino acids; the *E coli*-derived rhCSF-1, however, lacks the rest of the internal aminoacid sequence (Halenbeck et al, 1989).

**Anti-CSF-1 monoclonal antibody**

Highly purified mouse-derived anti-human CSF-1 monoclonal antibody ($IgG_1$; Cetus Corporation, California), with a specific activity of 1.2 x $10^5$ neutralizing untis (NU)/ml, was used to neutralize the effects of pure rhCSF-1 in LTMC. This antibody has been described elsewhere (Halenbeck et al, 1989). Two hundred and fifty NU anti-CSF-1 were needed to completely neutralize 3 ng/ml rhCSF, as measured by radioreceptor assay (RRA) as described below.

**CSF-1 assay**

Supernatants from normal, AML and chimeric cultures were thawed and tested serially in RRA for CSF-1. Human CSF-1 (G.I.) was radioiodinated to approximately $10^{18}$ cpm/mol as described previously (Stanley & Guilbert, 1981)

and used within three weeks. The RRA was performed as described previously (Das et al, 1981) with some modifications. Cells from the CSF-1-receptor-bearing murine macrophage line P388D were collected from log-phase cultures by centrifugation, washed twice in ice-cold buffered saline (PBS), resuspended at $2 \times 10^6$ cells/ml in IMB10 culture medium and kept on ice. Cell viability was always greater than 95% as determined by trypan blue exclusion.

All procedures were carried out at 2° C. Samples and standards (50 $\mu$l/well) were added in triplicate to 96-well microtiter plates. A total of $2 \times 10^5$ cells in 100 $\mu$l was added to each well and incubated for 2 hours on ice before the addition of $^{125}$I-CSF-1 ($5 \times 10^4$ cpm/well in 25 $\mu$l). After a further 1 hour incubation, 100 $\mu$l (from 175 $\mu$l) aliquots from each well were layered on top of 200 $\mu$l of ice-cold fetal calf serum in 500 $\mu$l polyethylene microfuge tubes. The tubes were centrifuged (9000 rpm for 2 min) and rapidly frozen in liquid nitrogen. The tips of the tubes, containing the cell pellets, were cut off, and amounts of both cell-bound (pellet) and free (supernatant) radioactivity were determined in a gamma counter. The extent of competition of a given sample was expressed as the ratio of bound/free radioactivity. Purified rhCSF-1 (Genetics Institute, Cambridge MA) was used to obtain a standard curve for each assay. Final values were calculated from the standard curve and expressed as ng/ml. The limit of detection of this assay is 0.9 ng/ml.

**TNF assay**

TNF was quantified using a cytolytic bioassay as described by Branch and Guilbert (Branch et al, 1991). Cells of the murine fibroblastoid L929-8 cell line were added to microtiter wells to a final concentration of $5 \times 10^4$ cells per well in

50 $\mu$l of Iscove's medium containing 5% (v/v) fetal bovine serum plus the sensitizing agent actinomycin D (2 $\mu$g/ml). The microtiter plates were incubated at 37° C in 5% $CO_2$ in air for 2 hours. After this period, the samples (culture supernatants) and standards (different dilutions of rhTNF$\alpha$) were added (50 $\mu$l/well) and the plates incubated at 40° C for 16 - 18 hours. After this incubation, 50 $\mu$l of a filtered solution of the vital stain neutral red (Sigma, 0.05% [w/v] in normal saline) was added to each well. Plates were incubated for 2 hr at 37° C in an atmosphere of 5% $CO_2$, emptied by inversion, and rinsed once with 0.2 ml PBS. One hundred $\mu$l of a sodium phosphate-alcohol solution (0,05 M $NaH_2PO_4$ in 50% [v/v] ethanol) was added to each well and the plates gently mixed on a shaker for at least 20 min. Absorbence (optical density) at 570 nm was determined using an automated microplate spectrophotometer with a reference wavelength of 630 nm. This assay detects levels as low as 1 pg/ml of human TNF. To quantitate cell-associated TNF cells were lyzed by three consecutive cycles of freezing and thawing, after which, the suspension was assayed as described above. It is important to mention that this assay detects both TNF$\alpha$ and TNF$\beta$.

**Anti-TNFα monoclonal antibody**

Anti-TNFα monoclonal antibody (Boehringer Mannheim, Laval, Canada) was used to neutralize the effects of TNF both in semisolid and LTMC. One ng of anti-TNFα neutralized 1 pg of TNFα, as determined by the TNF bioassay described above. This 1000-fold difference may be due to a low purity of the antibody preparation or to a low affinity of the antibody. It is also possible that most of the material in the antibody preparation is inactive.

**Prostaglandin E assay**

The levels of prostaglandin E (PGE) in LTMC supernatants were measured by a specific [$^3$H] radioimmunoassay. The PGE RIA kit was a gift from Dr. Paul Slowey (INCSTAR Corporation, Stillwater, MN). Briefly, culture supernatants (1 ml) were treated with 0.1 ml of 1 N NaOH in order to convert PGE to PGB. Then, 0.6 ml of gel tris buffer were added to the standards and culture supernatants. Tubes (duplicates) were prepared by adding 50 $\mu$l of each PGB standard or 400 $\mu$l of each sample, and then 50 $\mu$l of [$^3$H] PGB tracer. Afterwards, 50 $\mu$l of rabbit anti-PGB serum were added to all the tubes. After mixing thoroughly, the tubes were incubated for 60 min in a 37$^o$ water bath and 100 $\mu$l of normal rabbit serum were added to all the tubes followed by 100 $\mu$l of goat anti-rabbit serum. The tubes were then incubated for 20 hr at 4$^o$ C. The tubes were centrifuged for 30 min at 1600 x g. Each tube was decanted until all the supernatant was removed. One ml of 0.1 N NaOH was added and the tubes were mixed thoroughly. Each solution was placed into scintillation vials and 10 ml scintillation fluid were added to each vial. Each vial was then counted for 5 minutes. A standard curve was obtained based on the percent bound for each standard. Final values were calculated from the standard curve and expressed in pg/ml. The limit of detection of the assay was 8.2 pg/ml.

**Assay for undefined hemopoietic stimulators and inhibitors**

To test for possible hemopoietic stimulators and/or inhibitors produced in the cell cultures, the effects of supernatants from normal, AML, and chimeric LTMC on colony formation by normal bone marrow cells in semisolid cultures were assessed. Semisolid cultures were performed as described above (section

C). Stimulation by 10% (v/v) supernatant was defined as colony formation in the absence of any other cytokine source but EPO. Inhibition was defined as a reduction in the number of colonies by 10% (v/v) supernatant in the presence of EPO plus PHA-LCM. This procedure was based on the one described by Gualtieri et al (1984). These investigators have demonstrated that conditioned medium from normal marrow-derived LTMC, tested in colony assay at 10% v/v, does not have an inhibitory effect on hemopoietic cell growth. Thus, this procedure seems to be adequate for assessing the presence of hemopoietic inhibitors in supernatants from AML LTMC.

**F) STATISTICS**

The Student's *t*-test was used to determine the significance of differences among groups of paired observations or means. Except when indicated, results presented in figures 3, 5 - 18 and in tables 2 - 32 represent mean $\pm$ SD. *n* indicates number of experiments performed, and each experiment corresponds to the bone marrow from a different normal subject or AML patient.

# IV. RESULTS

## CHARACTERIZATION OF HUMAN LTMC

As stated in chapter I, LTMC is the main experimental system that was used throughout this study to investigate the functional integrity of the *in vitro* hemopoietic microenvironment in AML. Thus, the first specific objective for this work was to characterize in detail the biology of human LTMC established with bone marrow cells from both normal and AML subjects.

### A) LTMC FROM NORMAL BONE MARROW

#### Kinetics and morphology of nucleated cells

The cultures were initiated with $20 \times 10^6$ - $30 \times 10^6$ bone marrow cells in 8 ml of culture medium (see Materials and Methods). After one week of culture, a dramatic decrease (90%) in the total number of nonadherent cells was observed (Figure 3). During the following weeks, the levels of nonadherent cells decreased gradually, reaching 1% of the initial innoculum by week 10. As shown in Table 2, most of the nucleated cells present in the initial innoculum were granulocytic cells. However, after 5 weeks, macrophages were the most numerous cell population. Interestingly, erythroblasts disappeared from the cultures by week 3 - 4, which is in keeping with previous reports indicating that LTMC sustain erythroblast growth for only few weeks (Dexter et al, 1984)

From week 3, the majority of the nucleated cells were localized in the adherent layer (Figure 3). Such an adherent layer, which reached confluency usually by week 3, consisted mainly of cells with fibroblast morphology and macrophages (Table 2). Fat cells were also observed (Figure 4a), however, it was

not possible to quantitate their number due to the difficulty to distinguish individual cells. At week 3, some cultures (n = 12) were sacrificed to determine the number of fibroblastic progenitors present in the adherent layer. The values obtained were 180 - 260 CFU-F per flask, which is within the range reported by Coutinho et al (1990) for normal bone marrow. As stated previously in this thesis (Chapter III, section D), the criteria for the distinction between macrophages and fibroblastoid cells was based on morphological analysis using Wright-Giemsa stain and, in some cases, double-esterase stain (which stains macrophages). A more accurate characterization can be made by determining the presence of specific markers. Fibroblasts react with antibodies against collagen types I and III, are positive for the antibody STRO-1, and stain positive for alkaline phosphatase. On the other hand, macrophages are positive for anti-CD45. In this study, no specific assays to determine the presence of endothelial cells (the third most numerous cell type present in bone marrow stroma) were performed. These could include looking at the expression of collagen type IV, laminin or factor VIII. Thus, although it can be assumed that such a cell population was present in our cultures (Allen & Dexter, 1984), no particular comments can be made on this issue.

**Kinetics of hemopoietic progenitors**

Table 3 shows the numbers of hemopoietic progenitors present in the nonadherent and adherent fractions of the LTMC throughout 10 weeks of culture. CFU-MIX were the least numerous at day 0 and their levels were always low until they became undetectable by week 7. From week 3, the majority of CFU-MIX were localized in the adherent layer, which is in keeping with the

results of Coulombel and colleagues (Coulombel et al, 1983). These authors have suggested that the presence of most of the hemopoietic progenitors in the adherent layers of LTMC reflects the close association between hemopoietic and stromal cells *in vivo*.

Although considerably abundant at the beginning of the culture, erythroid progenitors decreased very rapidly in LTMC. This reduction was particularly dramatic for CFU-E, which reached undetectable levels after only 4 weeks, in spite of the fact that their immediate progenitors (BFU-E) persisted for up to 8 - 9 weeks. The reason for this is not clear. Some investigators (Eliason et al, 1979), however, have suggested that the maturation step from BFU-E to CFU-E is blocked, either because of a lack of a promoting agent or the presence of an inhibitory factor. Most of the erythroid progenitors were present in the adherent layer. Interestingly, the BFU-E observed in the adherent fraction were usually larger (3 - 5 clusters) than their nonadherent counterparts (1 - 3 clusters). It has been suggested that colony size can be taken as an indication of maturity, in other words, large colonies derive from primitive progenitors whereas smaller colonies derive from mature progenitors (Eaves & Eaves, 1984). Thus, it seems that those BFU-E present in the adherent fraction of the LTMC were more immature than those observed in the nonadherent fraction.

At all time points, CFU-C were the most numerous progenitors, persisting for up to 16 weeks. At weeks 14 - 16, the CFU-C levels were 34 $\pm$ 19 per flask. Most of them were also localized in the adherent layer, in fact, from week 14 - 16 practically all of them were observed in the adherent layer. Among these myeloid progenitors, those giving rise to pure macrophagic colonies (CFU-M) were usually the most numerous (Table 4), followed by CFU-G and CFU-GM,

respectively. This proportion was observed both in the nonadherent and adherent fractions of the culture. Similar to what was observed for erythroid progenitors, most of the adherent CFU-C were larger than those present in the nonadherent fraction, suggesting that more immature myeloid progenitors are physically associated with stromal cells.

**Effects of exogenous erythropoietin**

The fact that erythroid progenitors in human LTMC were sustained for significantly shorter periods than myeloid progenitors made it difficult to study hemopoiesis, as a whole, for periods longer than 4 weeks. Erythropoietin (Epo) is known to play a central role in both *in vivo* and *in vitro* erythropoiesis (Krantz, 1991). Since the major site of Epo production is not the bone marrow but the kidney (Erslev & Caro, 1983), it was possible that the levels of Epo in LTMC were lower than those occuring *in vivo*, resulting in the deficient erythroid growth in this experimental system. Thus, in an attempt to sustain erythropoiesis for longer periods of time, LTMC were supplemented with human Epo, either natural (purchased from the Terry Fox Laboratory, Vancouver, Canada) or recombinant (a gift from Dr. Steven Clark, Genetics Institute, Cambridge MA). Epo was added weekly at a concentration of 1 U/ml, which (i) is significantly higher than the physiological concentration observed in human plasma under normal conditions (10 - 40 mU/ml; Erslev & Caro, 1983), and (ii) has been found to be adequate for the growth of erythroid progenitors in human semisolid cultures and in murine LTMC (Eliason et al, 1979).

Addition of Epo to LTMC did not affect the levels of CFU-MIX, either nonadherent or adherent, indicating that this factor does not act on such

primitive cells (Figure 5). As a whole, erythropoiesis was, as expected, stimulated by Epo, however, such a stimulation was only transient (Figure 6). Epo had no effect on adherent BFU-E (Figure 6a), suggesting that either (i) Epo was not accessible to those cells, or (ii) immature BFU-E do not respond to Epo. In contrast, the levels of nonadherent BFU-E were significantly reduced by Epo. Concommitantly, a significant increase in the levels of CFU-E (Figure 6b) and erythroblasts (Figure 6c) was observed. These results suggested that Epo acts at the level of mature (nonadherent) BFU-E, inducing their maturation into CFU-E, which, in turn, mature into erythroblasts. Interestingly, addition of Epo into LTMC resulted in the inhibition of myelopoiesis, as assessed by the reduction in the total numbers of CFU-C (Figure 7) and mature myeloid cells, i.e. macrophages and granulocytes (Figure 8). It is noteworthy that the effects of Epo on erythropoiesis and myelopoiesis in LTMC were observed using either natural or recombinant Epo.

**Discussion**

Confirming previously reported results (Eaves & Eaves, 1988), we have shown that human myeloid progenitors can be sustained in LTMC for up to 16 weeks, whereas erythroid progenitors are supported for less than 8 weeks. These results suggest that the conditions of LTMC particularly favor the growth of myeloid cells. Because this preference correlates with the presence of myeloid growth factors, such as GM-CSF, G-CSF and IL-3 (Gualtieri et al, 1987; Fibbe et al, 1988; Janowska-Wieczorek et al, 1988) and the possible absence of erythroid factors in LTMC, we have studied the effects of the erythroid growth and differentiation factor Epo.

As expected, addition of Epo resulted in the stimulation of erythropoiesis, however, two interesting observations were made: Firstly, erythropoiesis was stimulated only transiently and never to the *in vivo* proportions. Secondly, myelopoiesis was inhibited in cultures treated with Epo. The deficient stimulation of erythropoiesis by Epo was evident when we look at the ratios of committed progenitors and erythroblasts in Epo-treated LTMC. At day 0, when cultures were initiated, the ratio BFU-E:CFU-E:erythroblasts was 1:1.6:1300, whereas after 5 weeks of culture the ratio was 1:0.1:340. This indicates that Epo alone is not sufficient to restore erythropoiesis in LTMC and that some other factor(s) or condition(s) necessary for erythropoiesis are absent in LTMC. Throughout this study we added Epo at 1 U/ml, and since no other concentration was tested, we cannot rule out the possibility that this concentration was not optimal. However, because this concentration (i) is significantly higher than the physiological one in plasma under normal conditions (10-40 mU/ml; Erslev & Caro, 1983), and (ii) has been found to be adequate for the growth of erythroid cells in semisolid cultures (Eaves & Eaves, 1984) and in murine LTMC (Eliason et al, 1979), we believe that the concentration of Epo used in this study was not a limiting factor. To rule out the possibility of the loss of activity throughout the culture period, in three of the cultures Epo was measured weekly using a specific RIA (assayed by Dr. Jaime Caro, Cardeza Foundation, Philadelphia) in both control and treated flasks, before addition of Epo. Although great variation of such levels was observed in treated cultures (250 $\pm$ 151 mU/ml), they were significantly higher than those in control cultures (19 $\pm$ 12 mU/ml), indicating that at all time points the effective levels of Epo in treated LTMC were significantly higher than in their untreated counterparts.

The reason for the negative effects of Epo on myelopoiesis is not clear. Since Epo had no effect on immature progenitors (CFU-MIX, adherent BFU-E and adherent CFU-C), it is difficult to suggest that Epo acts on pluripotent cells inducing their differentiation into the erythroid lineage, which would result in the down-modulation of myelopoiesis, as suggested by others (Van Zant & Goldwasser, 1979; Salvado et al, 1988). On the other hand, it is possible that the Epo-induced inhibition of the myeloid compartment in LTMC occurs only at the level of mature nonadherent progenitors, although the mechanism remains to be elucidated. In any case, it is important to note that these findings agree with previous reports indicating that accelerated differentiation into either the erythroid or myeloid lineage may be accompanied by diminished differentiation into the other pathway (Bradley et al, 1967; Van Zant & Goldwasser, 1979; Salvado et al, 1988).

Due to the fact that addition of Epo to human LTMC resulted only in a transient, although significant, stimulation of erythropoiesis, and also in a dramatic inhibition of myelopoiesis, this cytokine was not added to LTMC in subsequent experiments. Furthermore, analysis of hemopoiesis in normal LTMC was restricted to 7 weeks in order to analyze both arms of hemopoiesis simultaneously. During this culture period, analysis of erythropoiesis was focused to immature erythroid progenitors (BFU-E).

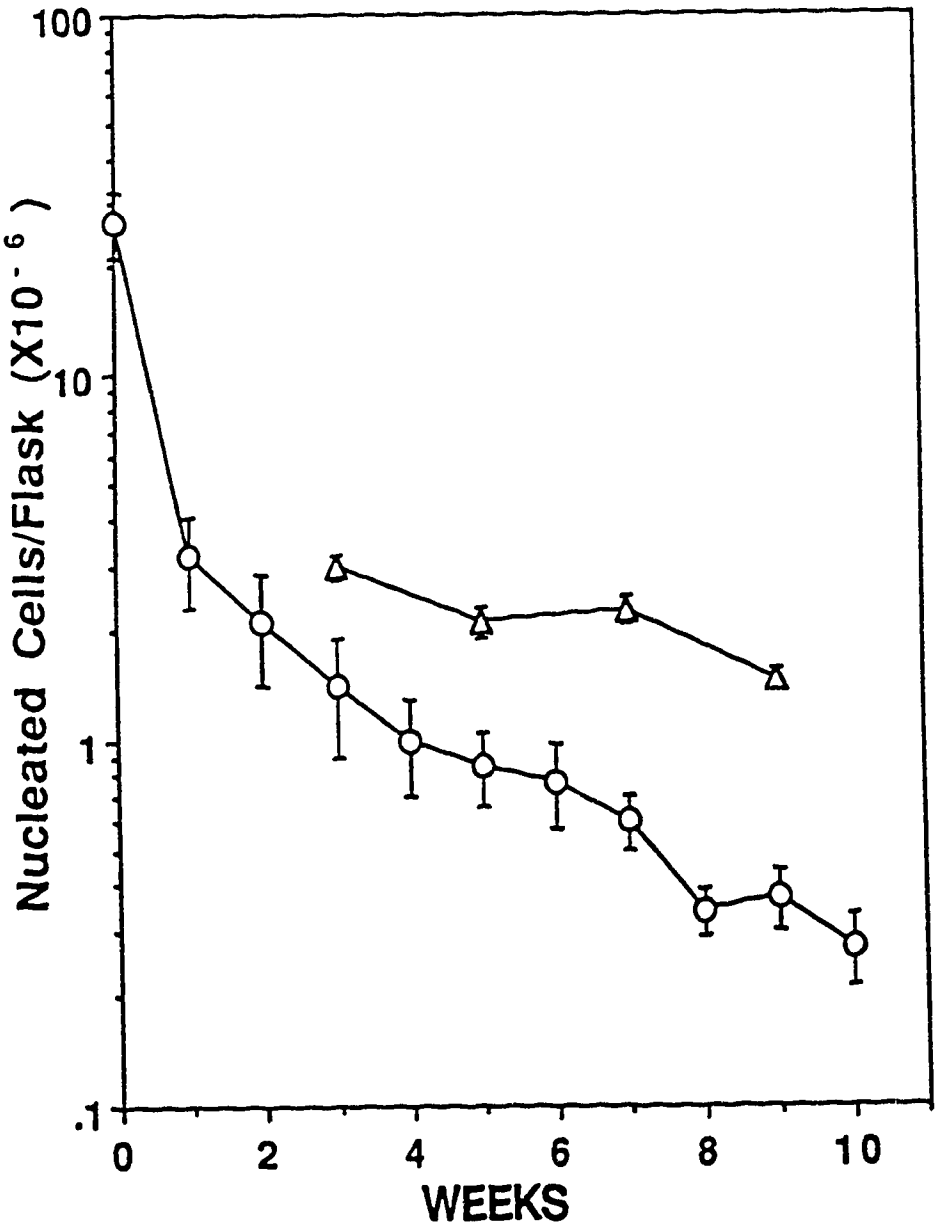


Figure 3. Total number of nucleated cells in the nonadherent (circles; n = 12) and adherent (triangles; n = 6) fraction of normal LTMC. Results represent mean + SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different normal subject.

**Figure 4.** Morphology of stromal adherent layers from untreated (a) and rhCSF-1-treated (b) normal LTMC at week 3. Note the absence of fat cells in treated cultures.

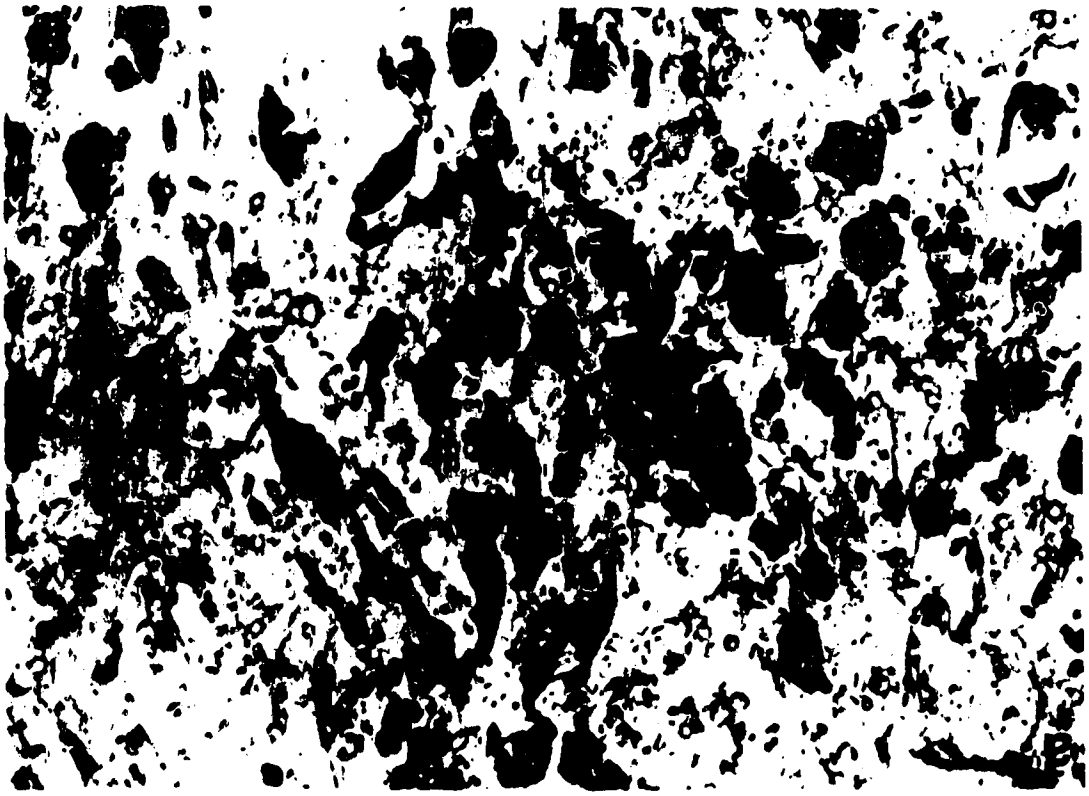

**Table 2**

**Morphology of nucleated cells in normal LTMC**

| | | Differential counts (%) | | | | | |
|---|---|---|---|---|---|---|---|
| Week | Fraction | Blasts | Granulocytes | Lymphoid | Erythroid | Macrophages | Fibroblastoid |
| 0 | Total | 1 ± 1 | 59 ± 6 | 16 ± 3 | 24 ± 5 | <1 | <1 |
| 3 | NA | 6 ± 3 | 42 ± 8 | 22 ± 6 | 2 ± 1 | 27 ± 5 | <1 |
| | A | 1 ± 1 | 5 ± 1 | <1 | <1 | 43 ± 4 | 52 ± 5 |
| 5 | NA | 12 ± 4 | 24 ± 5 | 19 ± 4 | <1 | 45 ± 6 | <1 |
| | A | <1 | 1 ± 1 | <1 | <1 | 39 ± 4 | 59 ± 4 |
| 7 | NA | 13 ± 2 | 21 ± 4 | 17 ± 2 | <1 | 49 ± 3 | <1 |
| | A | <1 | <1 | <1 | <1 | 41 ± 6 | 60 ± 3 |

Results represent mean ± SD of 6 experiments. Each experiment corresponds to the bone marrow from a different normal subject. 300 cells per experiment were analyzed.

NA = nonadherent, A = adherent

Table 3

**Numbers of multipotential, erythroid and myeloid progenitors in normal LTMC**

| | | No. progenitors/flask | | | |
|---|---|---|---|---|---|
| Week | Fraction[a] | CFU-Mix | CFU-E | BFU-E | CFU-C |
| 0 | Total | 871 ± 273 | 6123 ± 887 | 4031 ± 1387 | 13380 ± 4729 |
| 1 | NA | 91 ± 26 | 267 ± 146 | 232 ± 59 | 2975 ± 716 |
| 2 | NA | 48 ± 16 | 41 ± 21 | 180 ± 56 | 892 ± 123 |
| 3 | NA | 26 ± 10 | 29 ± 13 | 113 ± 20 | 414 ± 84 |
| | A | 84 ± 21 | 61 ± 9 | 195 ± 9 | 1053 ± 247 |
| 4 | NA | 12 ± 6 | <32 | 134 ± 57 | 325 ± 43 |
| 5 | NA | 14 ± 8 | <12 | 77 ± 15 | 265 ± 80 |
| | A | 56 ± 11 | <44 | 140 ± 14 | 617 ± 56 |
| 6 | NA | 3 ± 4 | <9 | 40 ± 16 | 183 ± 53 |
| 7 | NA | <8 | <8 | 32 ± 10 | 94 ± 17 |
| | A | 19 ± 2 | <19 | 86 ± 12 | 384 ± 38 |
| 8 | NA | <6 | <6 | 19 ± 7 | 64 ± 12 |
| 9 | NA | <8 | <8 | <8 | 49 ± 9 |
| | A | <9 | <9 | 16 ± 8 | 206 ± 17 |
| 10 | NA | <6 | <6 | <6 | 52 ± 20 |

Results represent mean ± SD of 12 experiments. Each experiment corresponds to the bone marrow from a different normal subject.

a NA = nonadherent, A = adherent

**Table 4**

**Morphology of myeloid (CFU-C) colonies in normal LTMC**

| Week | Fraction[b] | Percent of total CFU-C | | |
|---|---|---|---|---|
| | | CFU-G | CFU-M | CFU-GM |
| 0 | Total | 37 ± 3 | 48 ± 3 | 15 ± 2 |
| 3 - 9[a] | NA | 27 ± 3 | 68 ± 4 | 5 ± 2 |
| | A | 26 ± 5 | 65 ± 6 | 9 ± 3 |

Results represent mean ± SD of 9 experiments for NA cells and 7 experiments for A cells. Each experiment corresponds to the bone marrow from a different normal subject. See Table 3 for absolute numbers of CFU-C (CFU-G + CFU-M + CFU-GM) as a function of time.

a Cultures were analyzed on weeks 3, 5, 7 and 9.

b NA = nonadherent A = adherent

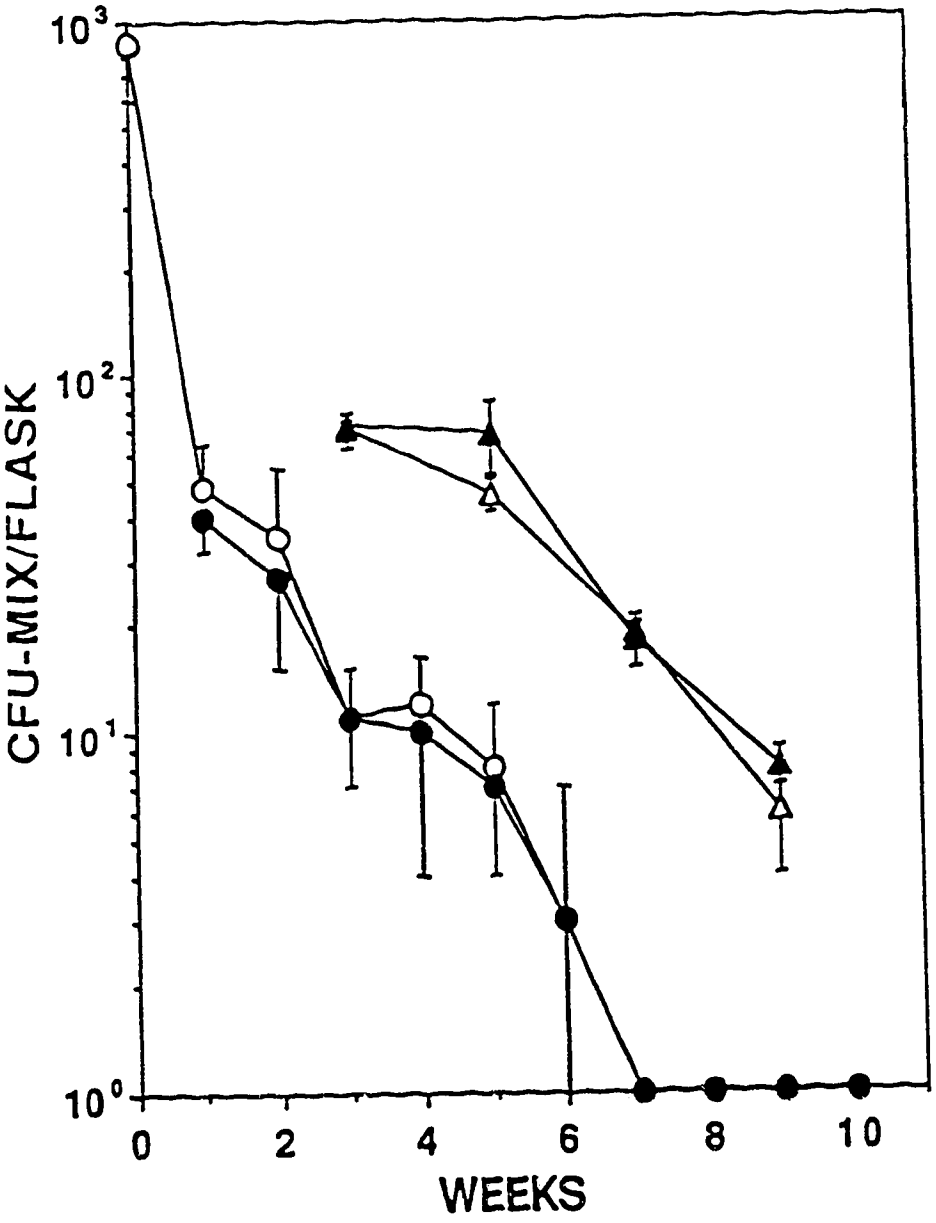


**Figure 5.** Total number of nonadherent (circles; n = 12) and adherent (triangles n = 6) CFU-MIX in untreated (open symbols) or EPO-treated (filled symbols) human LTMC. Results represent mean ± SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different normal subject.

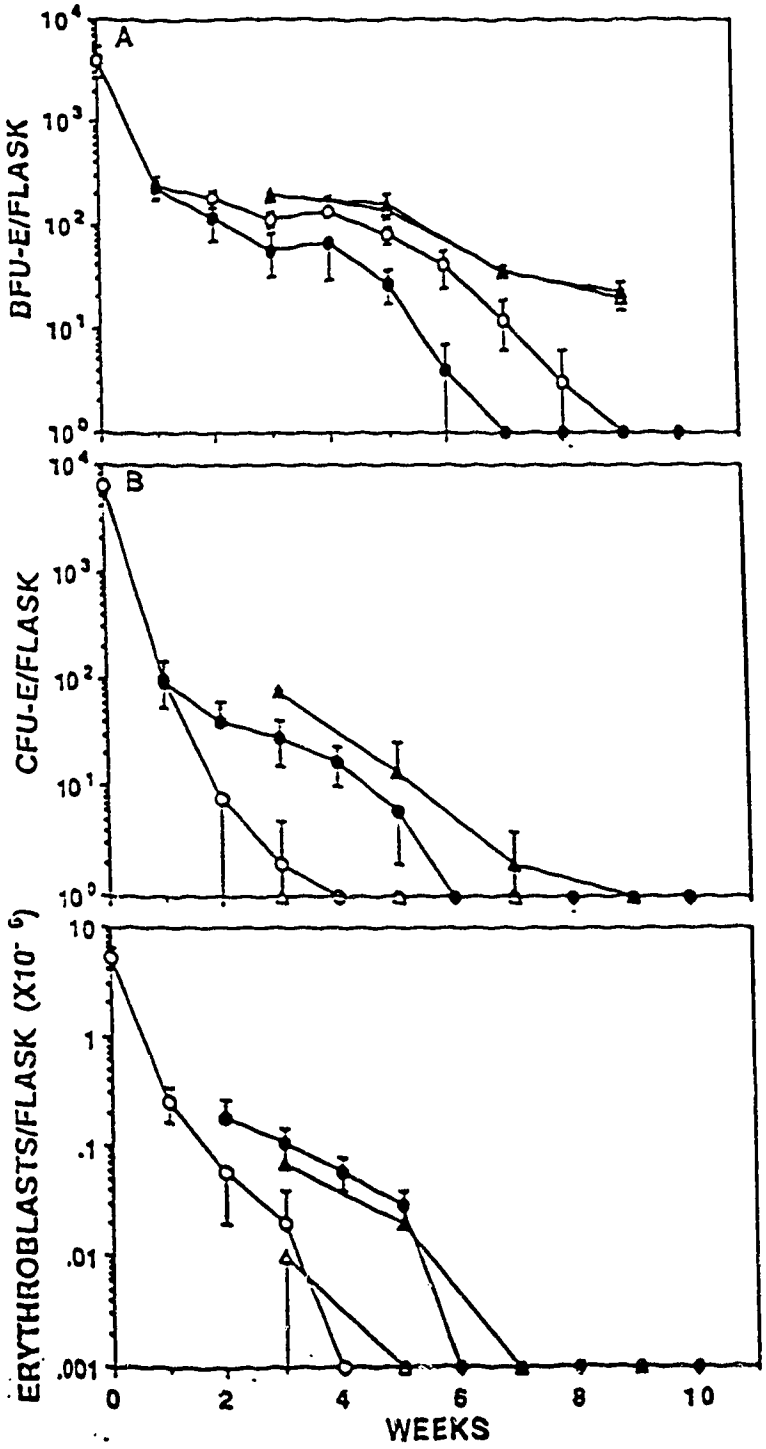


**Figure 6.** Total number of BFU-E, CFU-E and erythroblasts in the nonadherent (circles; n = 12) and adherent (triangles; n = 6) fraction of untreated (open symbols) or EPO-treated (filled symbols) human LTMC. Results represent mean + SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different normal subject.

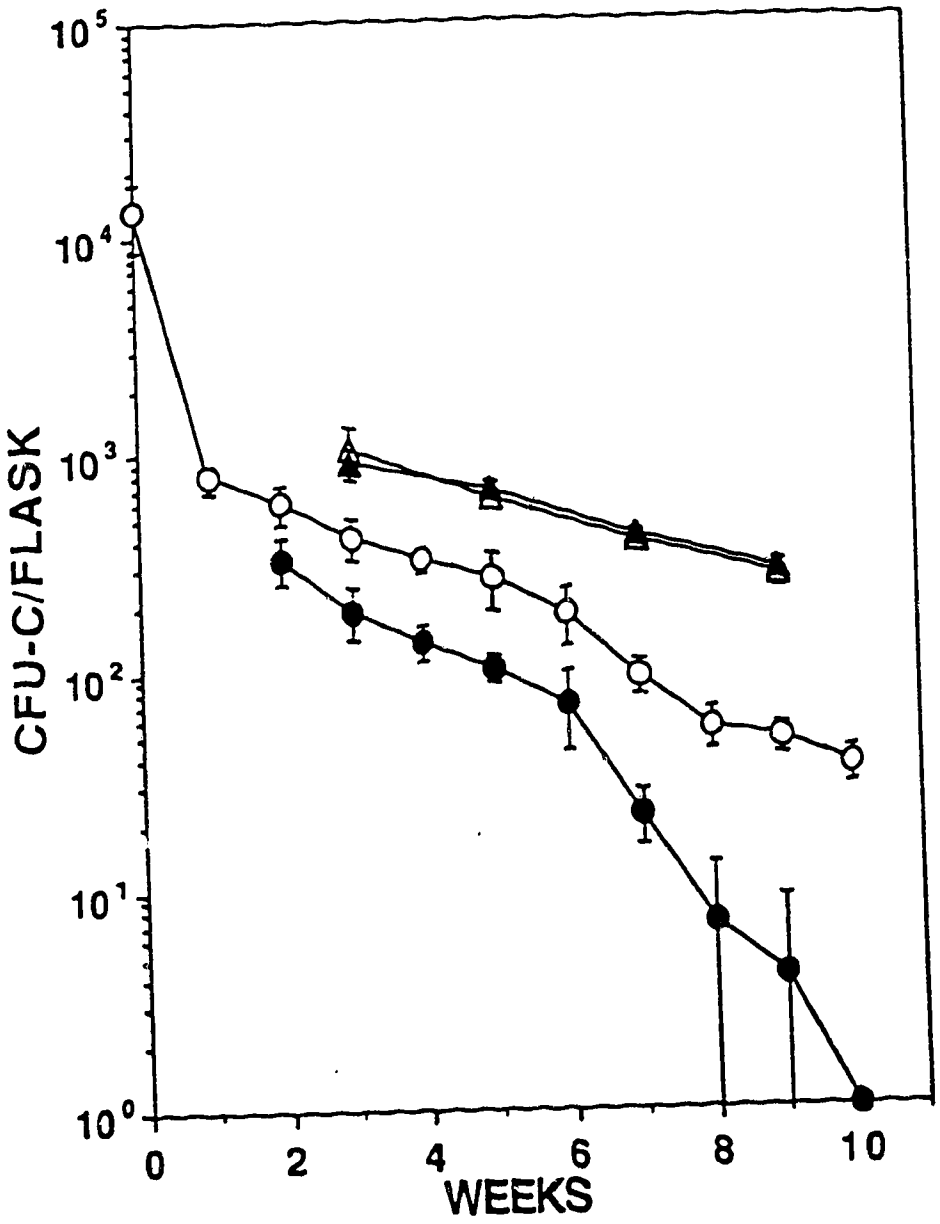


**Figure 7.** Total number of CFU-C in the nonadherent (circles; n = 12) and adherent (triangles; n = 6) fraction of untreated (open symbols) and EPO-treated (filled symbols) human LTMC. Results represent mean ± SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different normal subject.

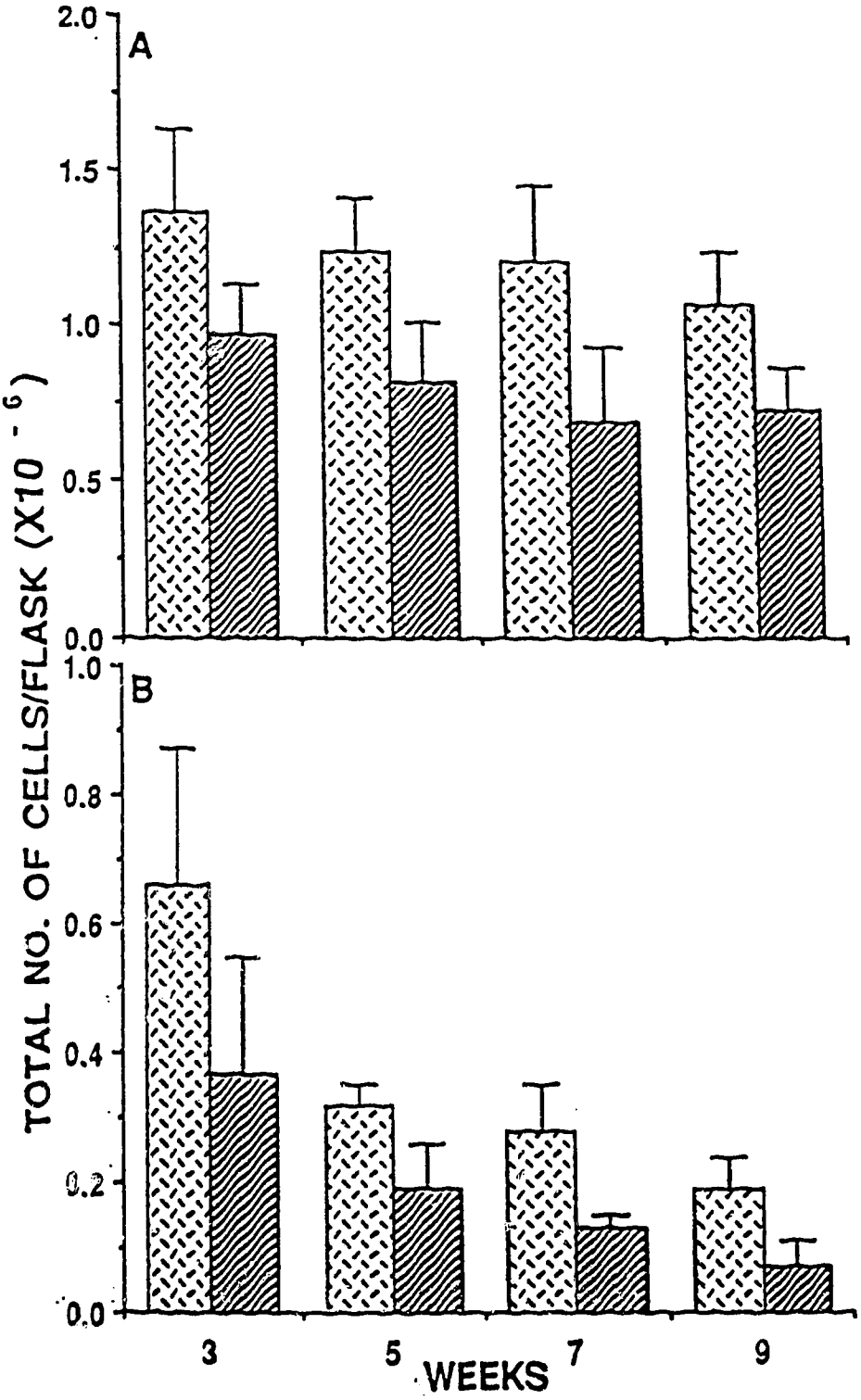


**Figure 8.** Total number of macrophages (A) and granulocytes (B) present in untreated (hatched; n = 6) or EPO-treated (diagonal; n = 6) human LTMC. Results represent mean + SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different normal subject.

## B) LTMC FROM AML BONE MARROW

### Kinetics and morphology of nucleated cells

The kinetics of nonadherent cells in AML LTMC (Figure 9) were similar to those observed in normal LTMC, although more patient-to-patient variability was observed. Although the great majority of the cells (75%) present in the initial inocula were blasts (Table 5), they were dramatically reduced during the following weeks and comprised less than 10% of the total number of cells after 5 weeks of culture. Macrophages and granulocytes became the predominant cell populations in the nonadherent fraction. As expected, erythroid cells were practically absent throughout the culture period.

From week 3, most of the nucleated cells were present in the adherent fraction (Figure 9). In contrast to normal LTMC, significant variability in terms of adherent cell numbers was observed. Bone marrow from AML patients is typified by a large number of blast cells (Islam et al, 1984). This could "dilute", in a relative sense, stromal elements or their progenitors. In order to determine whether the fibroblast progenitors (CFU-F) were present in reduced numbers in the AML marrow samples used in this study, their relative numbers were compared to those in normal marrows. As shown in Table 6, CFU-F levels in bone marrow from AML patients showed great variabililty, ranging from 12 - 122/$10^6$ nucleated cells. In three AML patients (no. 6, 10, 11) CFU-F levels were below the normal range (66 - 138 CFU-F/$10^6$ BMC, defined as the mean $\pm$ 2SD). It is not clear whether the reduced numbers of CFU-F in such patients reflect a decreased absolute number of CFU-F in the marrow or their relative dilution by marrow blasts. However, the degree of reduction in CFU-F numbers in such patients did not correlate with the percent of blasts (Table 1). These

results are in keeping with previous studies showing reduced levels of fibroblastic progenitors in the bone marrow of some AML patients (Kaneko et al, 1982; Nagao et al, 1983; Wlodarski & Janowska-Wieczorek, 1984; Hirata et al, 1986). It is noteworthy that the reduced levels of marrow CFU-F reported in the present study were observed in marrows from patients classified as M4 (myelomonocytic leukemia, patient 6) and M5 (pure monocytic leukemia, pateints 10 and 11), which are leukemias that predominantly involve the monocytic lineage. The significance of this observation, however, is not clear.

As shown in Fig. 9, a significant variability was observed in terms of the total number of adherent cells developed in AML LTMC. In some cultures (patients 1,2,3,4,8 nd 12), the numbers of adherent cells were similar to those in normal LTMC, whereas in others (patients 6, 10 and 11), the numbers of adherent cells were significantly reduced. Interestingly, a direct correlation was observed between the numbers of CFU-F in the patients' marrows and the numbers of adherent cells in LTMC (Fig. 10). Also in contrast to normal LTMC, a significant variability was observed in the proportion of the different cell types in AML adherent layers (Table 7). At week 3 of culture, CFU-F numbers in AML LTMC were between 6 and 235 per flask, and these numbers also correlated with the CFU-F levels in the initial innoculum (Fig. 11).

**Kinetics of hemopoietic progenitors**

AML LTMC showed significant differences, as compared to normal LTMC, in terms of the levels and types of progenitor cells present in the nonadherent and adherent fractions. CFU-MIX were practically absent in AML LTMC (not shown). On the other hand, BFU-E were detected only in two

cultures between weeks 3 and 4, and at very low levels (4 - 56 colonies per flask). At day 0, morphologically abnormal colonies (Figure 1f) were present in 6 out of 9 AML LTMC (patients 1,3,4,6,11,12). They consisted of 10 - 40 cells showing blast morphology, and it is important to mention that these colonies were not present in normal LTMC. According to previously reported criteria (Coulombel et al, 1985) these colonies seem to be derived from leukemic progenitor cells. In this study they have been referred to as AML-associated clusters (AML-ACl). Although they were numerous at the beginning of the culture (Table 8), their numbers dropped very fast during the following weeks, reaching undetectable levels by weeks 4 - 6. AML-ACl were not detected in the other 3 AML LTMC analyzed (patients 2,8,10). It is important to note that there was no correlation between the presence of AML-ACl and the development of the adherent layer. The above results indicate that the conditions present in LTMC are not adequate for the growth of leukemic progenitors, which suggests that this experimental system does not reproduce the *in vivo* situation, where AML progenitors have a growth advantage over the normal progenitor cells.

Morphologically normal colonies (identical to the CFU-C observed in normal LTMC) were present in 8 out of 9 AML LTMC. Interestingly, these colonies, referred to as CFU-C, showed two different growth patterns. In cultures from patients 6,10, and 11, significant levels of CFU-C (297 - 3241 CFU-C per flask) were present in the nonadherent fraction at day 0 (Figure 12). Then, they gradually decreased, becoming undetectable by week 8. In cultures from patients 1,2,3,4 and 8, CFU-C were not detected at the beginning of the culture, they were detected from week 1, reaching a peak between weeks 2 - 4, and reaching undetectable levels also by week 8. Regardless of the growth pattern,

the levels of non-adherent CFU-C in AML LTMC were usually lower (30-60%) than the levels of CFU-C observed in normal LTMC and they always reached undetectable levels by week 8. The distinction between the two growth patterns was based only on the levels of CFU-C at day 0: Undetectable in 5 out of 8 cultures *vs* large numbers in 3 out of 8 cultures. From week 2, practically a single growth pattern was observed. These two growth patterns have been previously described by Coulombel et al (1985), who found that in 8 out of 13 AML LTMC no CFU-C were detected on day 0, whereas in the other 5 cultures. large numbers of CFU-C were observed from the initiation of the cultures. Adherent CFU-C levels in AML cultures were also usually lower than in normal cultures (Table 9). Interestingly, a positive correlation was observed between the relative numbers of CFU-F in the patient's bone marrow and the numbers of CFU-C in the adherent layer of AML LTMC (Fig. 13).

**Discussion**

In contrast to normal bone marrow, AML-derived marrow samples showed significant variations in terms of fibroblast progenitor numbers. This, in turn, resulted in a significant variability in several LTMC parameters. LTMC established from AML bone marrow containing CFU-F levels similar to those observed in normal marrow showed high levels of adherent cells, fibroblastoid cells, adherent fibroblast progenitors, and adherent hemopoietic progenitors (CFU-C). In contrast, all the above parameters were significantly reduced in LTMC from patients whose marrow contained low levels of CFU-F. These results suggest that the presence of CFU-F in adequate levels (70 - 140 CFU-F/$10^6$ BMC) in bone marrow is important in the development of the stromal

adherent layer in LTMC, and the development of such a layer, in turn, is necessary for the growth of hemopoietic progenitors (adherent CFU-C).

The results presented in this section confirm previous observations indicating that LTMC are unable to sustain the growth of leukemic progenitors (Coulombel et al, 1985; Singer et al, 1988; Shiro et al, 1990). A possible explanation for this is that some cell population(s) and/or cytokine(s) necessary for the development of leukemic cells is(are) absent in the LTMC system. Our results also indicate that the growth of putative normal hemopoietic progenitor cells in AML LTMC is clearly deficient, i.e. reduced numbers and faster disappearance, as compared to the CFU-C growth in normal LTMC. The reason for this is not so clear, however, different explanations can be suggested. Firstly, it is possible that although those progenitors give rise to morphologically normal colonies, they are derived from the leukemic clone, thus, they are not sustained in LTMC. Secondly, it is possible that the hemopoietic microenvironment developed in AML LTMC is functionally defective, thus, it is not capable of sustaining the growth of normal progenitor cells. A third possibility would be a combination of the first two.

The first possibility has already been explored by different investigators. Coulombel and co-workers (Coulombel et al, 1985) demonstrated that morphologically normal CFU-C present in LTMC from some AML patients possess normal karyotype, even though the cultures were established from bone marrow cells that were of abnormal karyotype. The authors, then, suggested that at least some of the CFU-C developed in AML LTMC derive from normal progenitors. This idea has been recently supported by Shiro and colleagues (Shiro et al, 1990). It is important to mention that the development of non-clonal

hemopoietic progenitors in LTMC from patients with hematological malignancies has also been observed in patients with chronic myelogenous leukemia (Coulombel et al, 1983; Dube et al, 1984; Hogge et al, 1987) and acute lymphoblastic leukemia (Shiro et al, 1990). On the other hand, by using the G6PD enzyme-variants assay, Singer and collaborators have presented evidence that neither phenotypic nor cytogenetic normalcy can be taken as rigorous evidence that the CFU-C observed in AML LTMC are not neoplastic (Singer et al, 1988). In keeping with this idea are the results of Iland and colleagues (Iland et al, 1987),who observed that mature granulocytes developed in AML LTMC possess Auer rods, a morphological feature of AML cells. These latter results are in keeping with those by Fearon and colleagues (Fearon et al, 1986), showing that blast cells from AML patients can mature *in vivo* into polymorphonuclear leukocytes. Interestingly, these authors observed that in 3 of 13 patients in complete clinical remission polymorphonuclear cells were from clonal origin.

In the present study, I was unable to perform any type of analysis to determine the origin of the colonies morphologically classified as AML-ACl or CFU-C. Thus, although it is highly likely that the so-called AML-ACl are of leukemic origin, the possibility that this is not so cannot be ruled out; neither can be the possibility that the CFU-C observed in AML-LTMC are of leukemic origin. It is noteworthy, however, that the LTMC system has already been used as a method for purging leukemic cells for autologous bone marrow transplantation (ABMT). Based on the observation that leukemic cells are not supported in LTMC, and that morphologically and cytogenetically normal cells are sustained for several weeks in this system, cells from leukemic (CML or AML) patients have been cultured in these conditions for 10 days, and then,

reinfused into the patient, who has been treated with chemo and radiotherapy (Chang et al, 1989; Barnett et al, 1989). To date, the Vancouver group has used LTMC in this manner in 20 CML and 7 AML cases and the results have been encouraging. In fact, one of the CML patients is in his fifth relapse-free year after transplantation (A Eaves; personal communication). These clinical studies would support the idea that the progenitors developed in AML LTMC are of normal origin.

The second possibility mentioned above, that is, that the CFU-C developed in AML LTMC are actually normal, but their growth is deficient due to the development of a functionally defective hemopoietic microenvironment has not been explored to date.

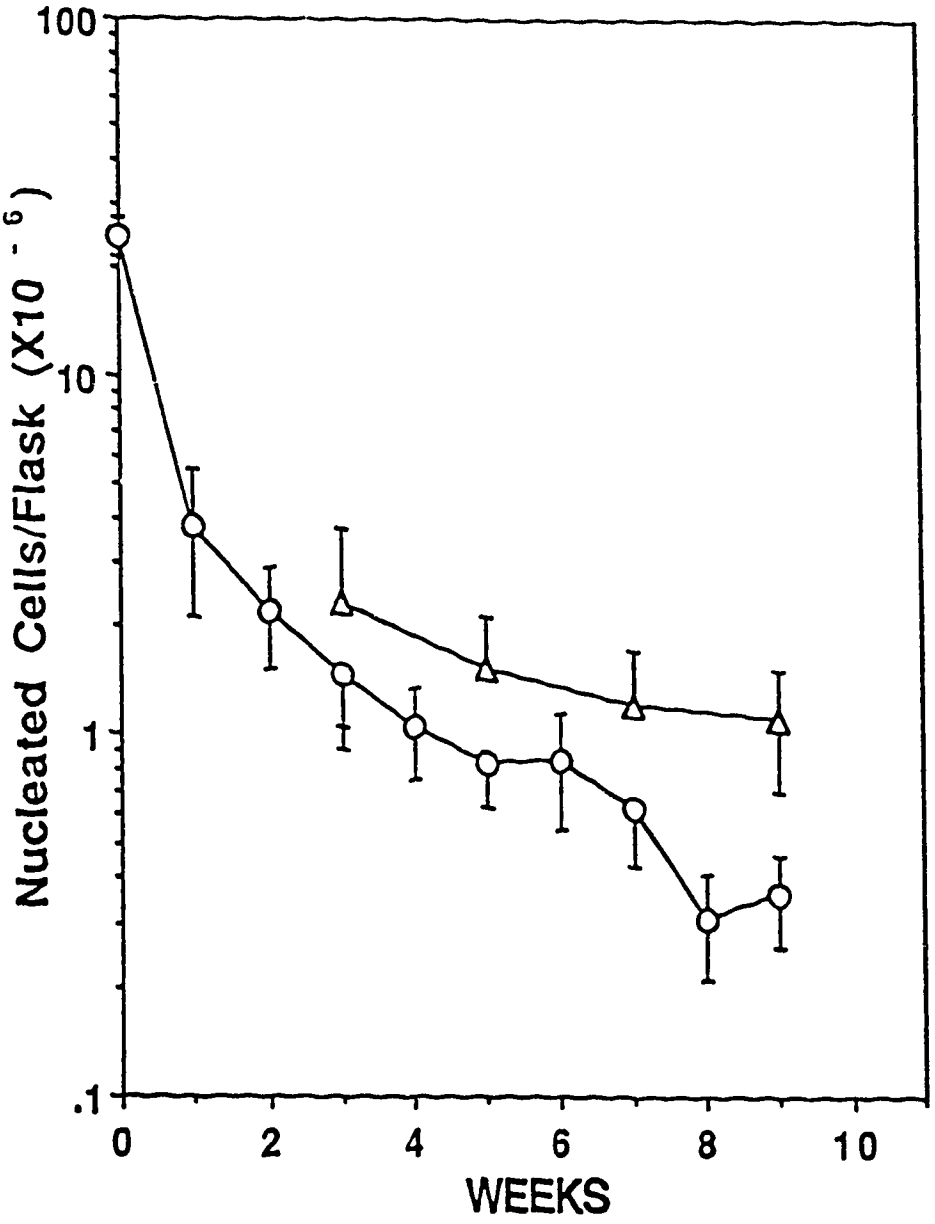


**Figure 9.** Total number of nucleated cells in the nonadherent (circles; n = 9) and adherent (triangles; n = 9) fraction of AML LTMC. Results represent mean ± SD from a given number of experiments (n). Each experiment corresponds to the bone marrow from a different AML patient.

**Table 5**

**Morphology of nonadherent cells in AML LTMC**

| | Differential counts (%) | | | | |
|---|---|---|---|---|---|
| Week | Blasts | Granulocytes | Lymphoid | Erythroid | Macrophages |
| 0 | 77 ± 14 | 14 ± 8 | 7 ± 4 | 2 ± 4 | <1 |
| 3 | 28 ± 11 | 39 ± 13 | 11 ± 6 | <1 | 21 ± 7 |
| 5 | 9 ± 4 | 48 ± 11 | 6 ± 3 | <1 | 37 ± 10 |
| 7 | 4 ± 3 | 32 ± 8 | 10 ± 7 | <1 | 54 ± 16 |

Results represent mean ± SD of 9 experiments. Each experiment corresponds to the bone marrow from a different AML patient.
300 cells were analyzed per experiment.

**Table 6**

**Total number of CFU-F in bone marrow samples from AML patients and normal subjects**

| Patient | CFU-F/$10^6$ bone marrow cells |
|---|---|
| 1 | 86 |
| 2 | 122 |
| 3 | 79 |
| 4 | 93 |
| 6 | 12 |
| 8 | 109 |
| 10 | 31 |
| 11 | 19 |
| 12 | 96 |
| normal range (n=9)* | 66 - 138 |

* Normal range represents mean $\pm$ 2 SD from 9 experiments, each of them corresponding to the bone marrow from a different normal subject.

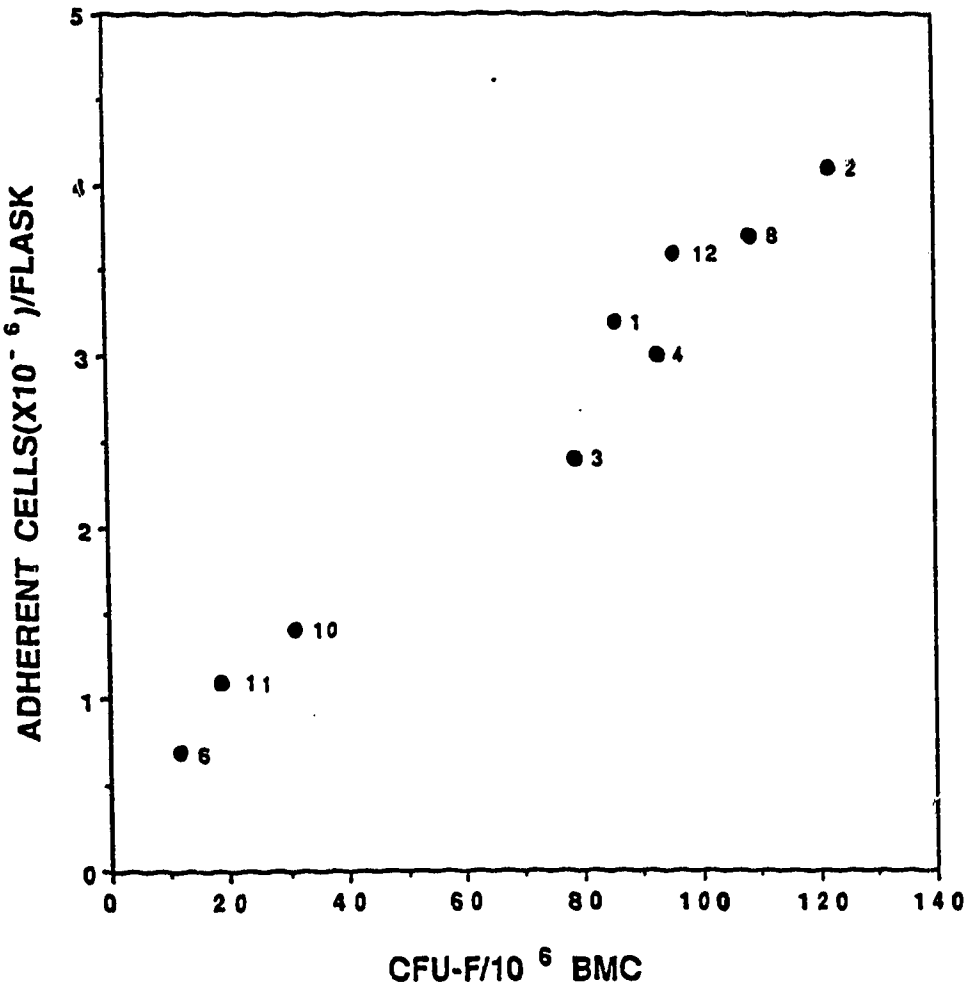


Figure 10. Correlation between CFU-F relative numbers in the patients' marrow and the numbers of adherent cells in LTMC at week 3. Each point represents a particular patient.

**Table 7**

**Morphology of adherent cells in AML LTMC**

| | Cell Type (%) | | | |
|---|---|---|---|---|
| Week | Blasts | Macrophages | Fibr[a] | Other[b] |
| 3 | 5 ± 4 | 52 ± 16 | 33 ± 21 | 9 ± 3 |
| 5 | <1 | 58 ± 18 | 41 ± 19 | 1 ± 2 |

Results correspond to differential counts, and represent mean ± SD of 9 experiments. Each experiment corresponds to a particular AML patient.

a Cells with fibroblast morphology

b granulocytes and lymphoid cells

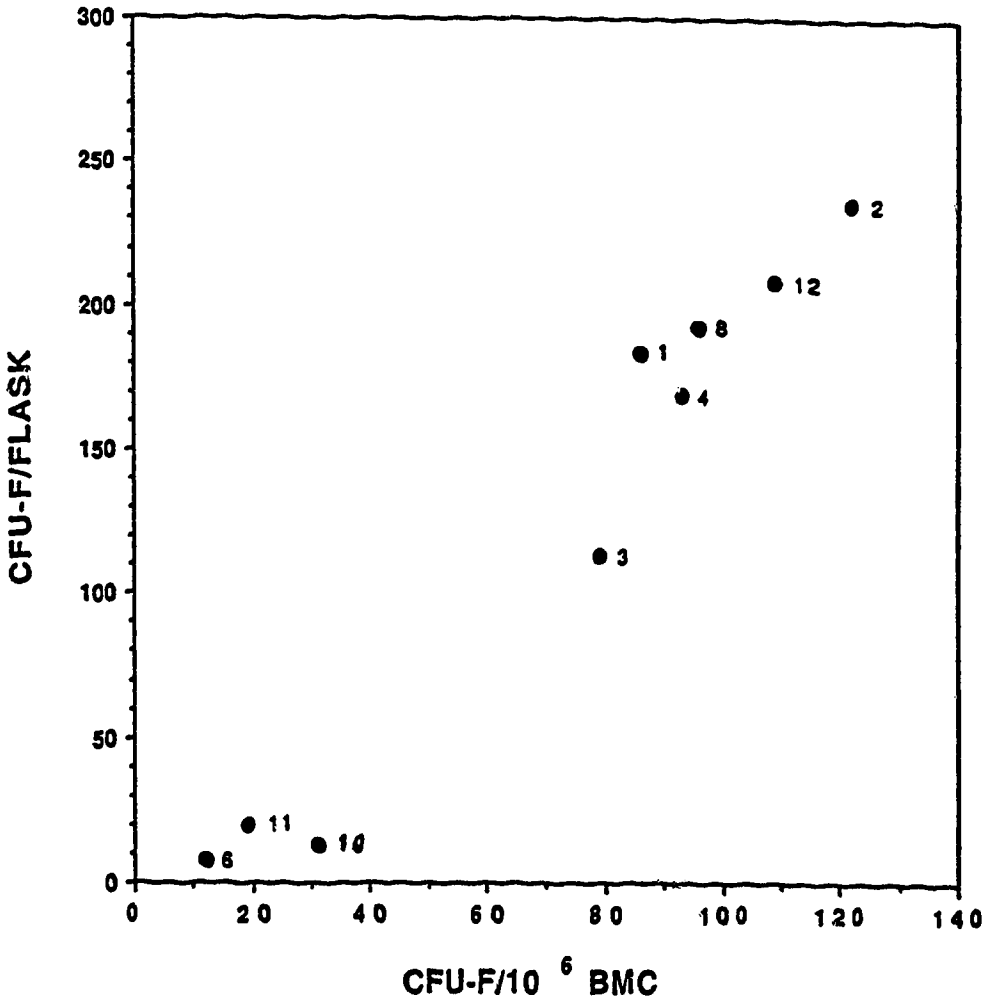


Figure 11. Correlation between CFU-F relative numbers in the patients' marrow and the numbers of CFU-F present in LTMC at week 3. Each point represents a particular patient.

**Table 8**

**Numbers of AML-ACl in AML LTMC**

| Week | Fraction | AML-ACl/flask |
|---|---|---|
| 0 | Total | (6200 - 165400) |
| 1 | NA | 1134 ± 962 |
| 2 | NA | 228 ± 86 |
| 3 | NA | 56 ± 33 |
| | A | 122 ± 36 |
| 4 | NA | 21 ± 28 |
| 5 | NA | 3 ± 5 |
| | A | 11 ± 8 |
| 6 | NA | <2 |
| 7 | NA | <2 |
| | A | <6 |

Results represent mean ± SD of 6 experiments. Each experiment corresponds to the bone marrow from a different AML patient (patients 1, 3, 4, 6, 11, 12).

NA = nonadherent

A = adherent

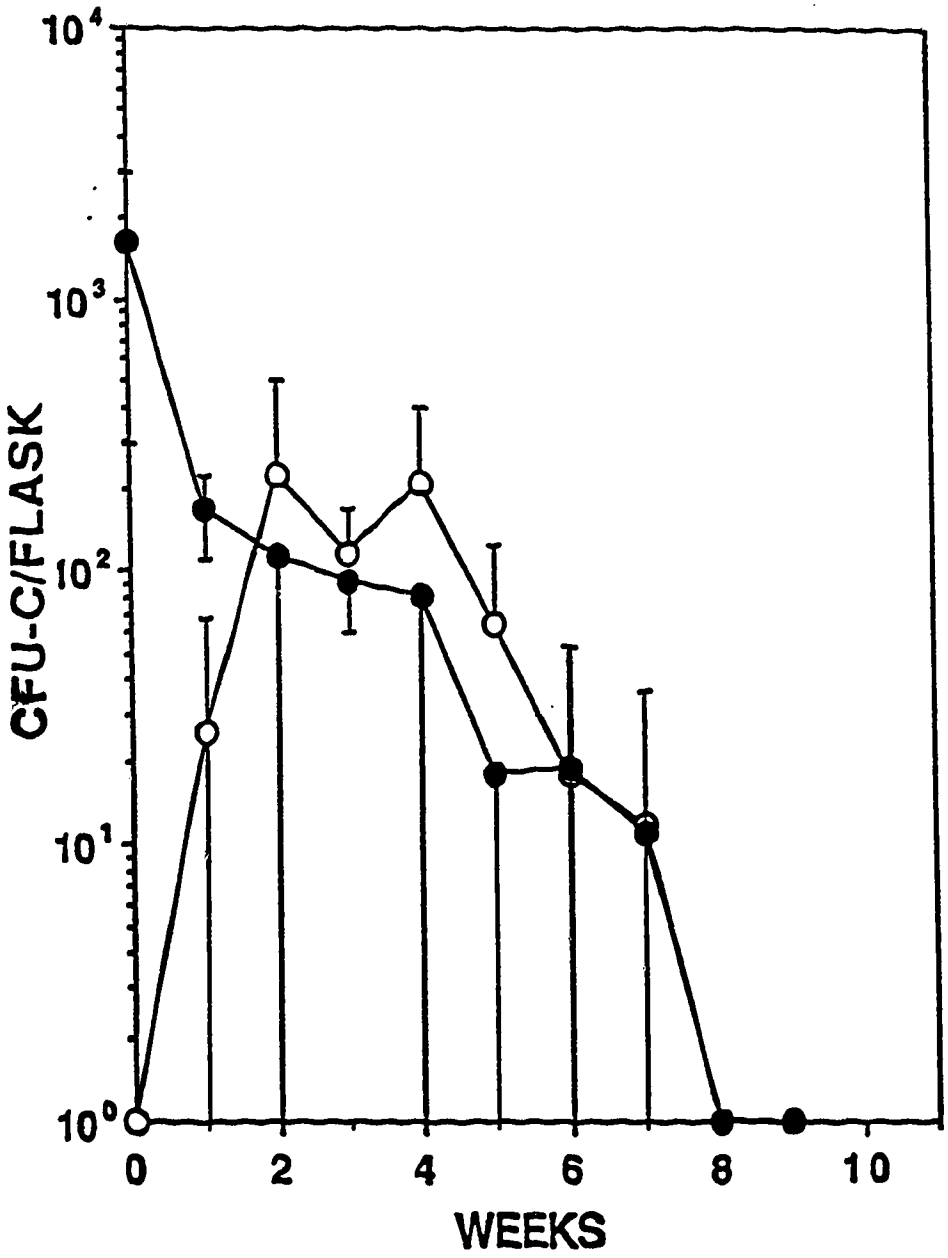


**Figure 12.** Total number of nonadherent CFU-C in AML LTMC. Growth pattern I (open symbols; patients 1,2,3,4,8,12) and growth pattern II (filled circles; patients 6,10,11). Results represent mean + SD of a given number of experiments. Each experiment corresponds to the bone marrow from a different AML patient. The distinction of two different growth patterns is based on the number of CFU-C on day 0, and is in agreement with the CFU-C growth patterns in AML LTMC described by Coulombel et al (1985).

**Table 9**

**CFU-C in the adherent layer of AML LTMC**

| Week | CFU-C/flask |
|---|---|
| 3 | 257 ± 140 |
| 5 | 136 ± 95 |
| 7 | 58 ± 57 |

Results represent mean ± SD of 8 experiments. Each experiment corresponds to the bone marrow from a different AML patient.

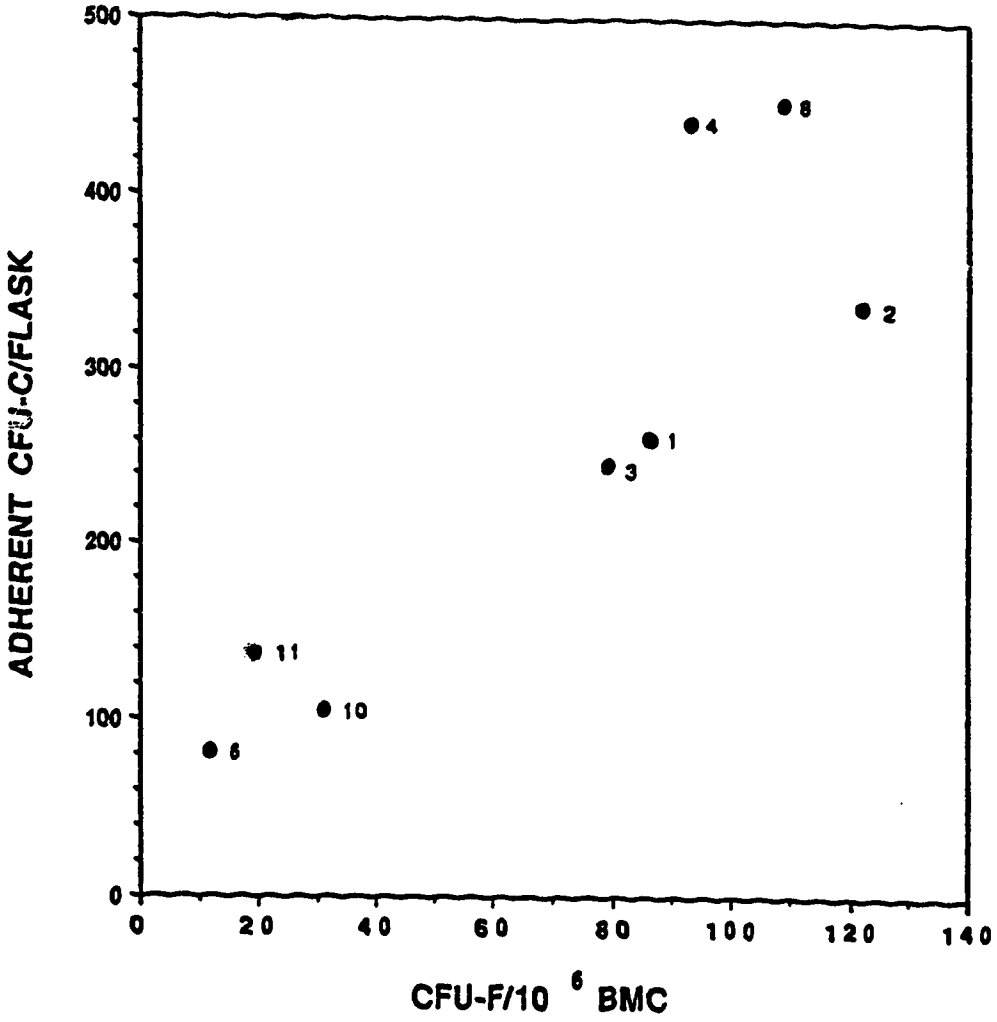


Figure 13. Correlation between CFU-F relative numbers in the patients' marrow and the levels of adherent CFU-C in LTMC at week 3. Each point represents a particular patient.

# V. RESULTS

## CSF-1 IN HUMAN LTMC

From the results presented in the previous chapter, it is clear that a possible explanation for the deficient growth of hemopoietic progenitors in AML LTMC is the development of a functionally abnormal hemopoietic microenvironment in such cultures. In order to test this possibility, three different parameters were tested: The ability of the AML-derived hemopoietic microenvironment to (i) produce CSF-1, (ii) respond to exogenous CSF-1, and (iii) sustain the growth of progenitor cells derived from normal marrow. These parameters were also tested in normal marrow-derived hemopoietic microenvironments, as controls. In this chapter, the results regarding the first two parameters are presented.

### A) CSF-1 IN NORMAL LTMC

#### CSF-1 production in LTMC

As shown in Figure 14, CSF-1 was constitutively produced in normal LTMC. The steady-state levels observed were between 2 - 8 ng/ml. In an attempt to identify the CSF-1-producing cell(s), LTMC, as well as cultures of fibroblasts were established. At week 3, the nonadherent cells in LTMC were completely removed, resuspended in fresh growth medium, and cultured for 2 days. Fresh growth medium was also added to the adherent cells from LTMC and fibroblast cultures. Two days later, the supernatant from each culture was collected and CSF-1 was measured. Nonadherent cells produced small amounts of CSF-1 (Table 10), whereas the CSF-1 levels in cultures of stromal adherent

cells were similar to those in LTMC. Significant levels were also observed in fibroblast cultures. These results indicate that the great majority of the CSF-1 present in LTMC was produced by adherent cells (macrophages and fibroblasts), and suggested that fibroblasts accounted for up to 50% of the total CSF-1 production. These results, however, need to be interpreted with caution. Establishment of stromal and fibroblast adherent layers was performed by using different technical protocols, which involved different culture media (see Chapter III, section B). Thus, we are not certain that the fibroblasts developed in stromal adherent layers are the same population from the one developed in fibroblast cultures. Furthermore, in stromal adherent layers, fibroblasts interact with different cell populations, such as macrophages, which may influence some of their functions (i.e. CSF-1 production); in contrast, fibroblast cultures seem to consist of a homogeneous cell population, thus, their physiology may be quite different.

**Effects of exogenous CSF-1**

When human bone marrow cells were cultured in semisolid cultures supplemented with recombinant human CSF-1 (rhCSF-1) as the only exogenous hemopoietic cytokine, only macrophagic progenitors developed (Table 11). This is in keeping with the fact that CSF-1 is a macrophagic stimulator (Stanley et al, 1983). However, two interesting observations were made. Firstly, the optimal concentration of CSF-1 for the growth of CFU-M was 10 - 40 ng/ml, which is higher than the endogenous CSF-1 levels observed in normal LTMC (Fig. 14). Secondly, the fact that CFU-M levels were slightly increased when the cultures were supplemented with PHA-LCM suggested the presence of a subset of CFU-

M which requires other factors, in addition to CSF-1, for optimal growth.

Based on the first observation, rhCSF-1 was added to LTMC at 15 ng/ml. In a preliminary set of experiments, it was found that the half-life of CSF-1 in LTMC is about 1 day: CSF-1 levels in LTMC (week 2) before addition of rhCSF-1 = 3.5 $\pm$ 2.9 ng/ml; 1 hour after rhCSF-1 addition = > 19.5 ng/ml; 1 day after rhCSF-1 addition = 9.8 $\pm$ 4.1 ng/ml; 2 days after rhCSF-1 addition = 4.9 $\pm$ 2.4 ng/ml. Thus, in the subsequent experiments rhCSF-1 was added to LTMC every second day at 15 ng/ml.

Addition of rhCSF-1 to normal LTMC had no significant effects on the development of the stromal adherent layer, except for the inhibition of fat cell formation (Fig 4b). Macrophage numbers were slightly increased (from 1.16 $\pm$ 0.19 x $10^6$/per flask to 1.42 $\pm$ 0.27 x $10^6$/per flask), although the difference was not significant. Similarly, CFU-F levels were practically unaffected (227 $\pm$ 42/flask in untreated cultures *vs* 206 $\pm$ 31/flask in rhCSF-1-treated LTMC).

When rhCSF-1 was added from the beginning of the culture (day 0 - 20), there was a significant inhibition of hemopoiesis (Table 12). The levels of all the progenitors analyzed (CFU-MIX, BFU-E, CFU-C) were significantly reduced from week 1, reaching the highest level of inhibition, both in the adherent and nonadherent fractions, by week 3. When the addition of rhCSF-1 was stopped at week 3, there was an increase in the levels of CFU-C and BFU-E; however, during the 4 following weeks the numbers of these progenitors never reached the levels observed in control cultures.

In order to confirm that the hemopoietic inhibition observed in LTMC was actually due to the presence of rhCSF-1 (Genetics Institute; GI) and not to a contaminant in the rhCSF-1 preparation, the effects of a bacteria-derived rhCSF-

1 (Cetus Corp.) were tested in a similar experimental protocol. These two types of rhCSF-1 differ in several aspects. GI-rhCSF-1 (derived from CHO cells) corresponds to the long form of CSF-1 (554 amino acids; Wong et al, 1987) and the protein is glycosilated. Cetus-rhCSF-1 (derived from *E coli*) corresponds to a truncated form (218 amino acids; Halenbeck et al, 1989) and the protein is not glycosilated. Both molecules, however, contain the same N-terminus region (149 amino acids) and C-terminus region (69 amino acids), and have similar biological activities (Halenbeck et al, 1989). In a preliminary experiment using the 5.10.14-S1 cell proliferation assay (Branch & Guilbert, 1986), we observed that in order to have the same biological activity, Cetus-rhCSF-1 had to be added at a concentration twice as high as GI-rhCSF-1, thus, Cetus-rhCSF-1 was added to LTMC at 30 ng/ml. The cultures were studied for only two weeks, however, during this period there were no significant differences between the effects of both types of rhCSF-1: At week 2, the levels of BFU-E and CFU-C in LTMC treated with GI-rhCSF-1 were 26% and 42% of the levels in untreated cultures, respectively. BFU-E and CFU-C levels in LTMC treated with Cetus-rhCSF-1 were 19% and 31% of control, respectively.

To further confirm the inhibitory effect of rhCSF-1 in human LTMC, one of the cultures was treated with GI-rhCSF-1 incubated with anti-rhCSF-1 ($3 \times 10^3$ NU/ml) for 1 hr before its addition to LTMC every second day. At week 2, the levels of BFU-E and CFU-C were 86% and 93% of those in untreated cultures, respectively.

When rhCSF-1 addition was delayed 3 weeks to allow the establishment of the adherent layer, fat cell formation was unaffected, and its inhibitory effects on hemopoietic cells were significantly reduced (Table 13). In fact, at week 5 (i.e.

after 2 weeks of continuous addition of rhCSF-1) the levels of nonadherent CFU-C and BFU-E were about 85% and 80% of those in control cultures, respectively. This result is significantly different from what was observed in cultures treated with rhCSF-1 from day 0, in which the levels of CFU-C and BFU-E, at week 2, were about 30% and 10% of control, respectively. Although the levels of adherent CFU-C and BFU-E were reduced to 66% and 60% of control, respectively, they were significantly higher than those observed at week 3 in cultures treated with rhCSF-1 from week 0 (25% and 2% of control, respectively).

One possible explanation for the diminished inhibitory effects of rhCSF-1 when added after the formation of the stromal layer is that rhCSF-1 utilization diminished once the adherent layer was developed. In order to test this possibility, CSF-1 levels were measured in control and rhCSF-1-treated LTMC. The endogenous levels of CSF-1 in control LTMC during the first 5 weeks of culture were between 1 - 8 ng/ml, which is within the normal range reported before for normal LTMC (Figure 14). Although the levels in rhCSF-1-treated cultures were usually higher, at week 1 and 2 (days 7 and 14) the values were not significantly different from those in control flasks (Table 14). Since rhCSF-1 (15 ng/ml) was added to these cultures on days 6 (one day before the CSF-1 determination at day 7) and 12 (two days before the CSF-1 determination at day 14), there appeared to be a very rapid reduction of CSF-1 levels during the first two weeks of culture. In contrast, a significant difference was observed at week 3 (day 21, i.e. one day after rhCSF-1 addition on day 20) and in cultures treated with rhCSF-1 from day 21 to 33. Thus, addition of rhCSF-1 had the largest biologic effect precisely when such an addition had the least effect on total CSF-1

concentration.

The inhibitory effects of rhCSF-1 in LTMC could be due to (i) a direct inhibitory effect on hemopoietic progenitors, (ii) the suppression of production of hemopoietic stimulators, or (iii) the production of hemopoietic inhibitors. The first possibility seemed unlikely, since addition of rhCSF-1 to semisolid cultures of marrow cells resulted in the stimulation of macrophagic progenitors and did not affect the growth of progenitors stimulated by PHA-LCM (Table 12). Furthermore, a recent report by Sato and colleagues (Sato et al, 1991) demonstrated a direct stimulatory effect of CSF-1 on macrophagic cells. To distinguish between the other two possibilities, the effects of LTMC supernatants on colony formation in semisolid cultures of normal bone marrow cells were examined. As shown in Table 15, supernatants from neither control nor rhCSF-1-treated LTMC stimulated colony formation in combination with Epo. Because we could not detect hemopoietic stimulators in control supernatants by this assay, it is not possible for us to comment on whether rhCSF-1 suppressed the production of stimulators in LTMC.

Supernatants from control and rhCSF-1-treated LTMC were next tested for activity able to inhibit colony formation. Although control supernatants did not inhibit PHA-LCM-stimulated colony formation, supernatants from LTMC treated with rhCSF-1 from day 0 strongly inhibited the formation of all types of colonies (Table 15). In contrast, supernatants from LTMC supplemented with rhCSF-1 after a 3-week delay were much less inhibitory. These results clearly demonstrated the presence of a CSF-1-induced soluble activity in LTMC that strongly suppressed the development of hemopoietic progenitors.

**Discussion**

The results presented above indicate that CSF-1 is constitutively produced in normal human LTMC. CSF-1 production seems to involve both fibroblasts and macrophages. The average steady state level observed between weeks 2 - 7 was 3.9 ng/ml. Previous studies by Guilbert and Stanley have demonstrated that murine bone marrow macrophages express a single class of high affinity receptor for CSF-1 with an apparent Kd = $4 \times 10^{-10}$ M (Guilbert & Stanley, 1986). Assuming that the CSF-1 receptor expressed on human marrow macrophages has a similar Kd to the one of murine macrophages, and considering (i) the concentration observed in untreated cultures (3.9 ng/ml = $4.3 \times 10^{-11}$ M) and (ii) the equation for the interaction receptor(R)-ligand(L) Kd=[R][L]/[RL], the CSF-1 receptor occupancy in these conditions corresponds to approximately 9%. When rhCSF-1 was added to LTMC every second day at 15 ng/ml, a significant decrease in the levels of hemopoietic progenitors was observed. The net CSF-1 concentration in these treated cultures was > 18.9 ng/ml ($2.1 \times 10^{-10}$ M), at least during the first few hours. In these conditions, CSF-1 receptor occupancy corresponds to > 35%. Thus, there is a transient 4-fold increase in the numbers of CSF-1 receptors occupied in LTMC treated with rhCSF-1 as compared to untreated cultures. Our results, then, could be explained by the fact that in untreated cultures, CSF-1 receptor occupancy is low (9%), whereas in the presence of rhCSF-1 receptor occupancy rises close to saturation levels.

Two major observations suggest that the inhibitory effects of rhCSF-1 on hemopoietic progenitors were not direct but mediated by microenvironmental cells. (i) When rhCSF-1 was added, at 15 ng/ml, to semisolid cultures of normal bone marrow cells in the absence of any other cytokine, but Epo, it stimulated

the growth of macrophagic progenitors in a dose-dependent manner. If this factor was added in the presence of Epo and PHA-LCM (a source of hemopoietic cytokines), it did not inhibit the growth of progenitor cells. (ii) Addition of rhCSF-1 to LTMC induced the production of a soluble activity capable of inhibiting the growth of hemopoietic progenitors.

Among the different cell populations present in the normal hemopoietic microenvironment, macrophages seem to be the only ones capable of directly responding to CSF-1, since only macrophages bear CSF-1 receptors (Byrne et al, 1983). Thus, it seems reasonable to suggest that the cells producing the soluble inhibitory activity in response to CSF-1 were macrophages. The above results also suggest that the presence or absence of the adherent layer determined the magnitude of the inhibitory effect of rhCSF-1. Although the nature of this is not clear, several possibilities can be suggested: (i) nonadherent-inhibitory activity-producing cells are more responsive to rhCSF-1 than their adherent counterparts, (ii) due to the different interactions occuring in the adherent layer between hemopoietic cells, stromal cells and extracellular matrix elements, rhCSF-1 is less accessible to CSF-1-responsive cells located in the adherent fraction, and (iii) once the adherent layer is formed, the inhibitory activity is either absorbed to extracellular matrix elements or actually removed by cells that bind it. In keeping with the first two suggestions are the observations that there are significantly more nonadherent monocytic cells at week 1 ($1.12 \pm 0.18 \times 10^6$/flask) than at week 4 ($0.42 \pm 0.15 \times 10^6$/flask), and that CSF-1 consumption (a measure of CSF-1 binding and therefore an index of potential CSF-1 response; Guilbert & Stanley, 1986) is higher during the first two weeks of culture than later. The studies on the identification of the CSF-1-induced soluble inhibitory activity and

the cells that produce it will be presented in chapter VI.

It is noteworthy that among the CSFs, CSF-1 is the only one that has negative effects on hemopoiesis when added to human LTMC from normal marrow. GM-CSF, G-CSF and IL-3 have been shown to stimulate the growth of hemopoietic progenitors in this experimental system (Coutinho et al, 1990). Interestingly, Dexter and colleagues reported that when murine LTMC are treated with L-cell supernatant (which contains mostly CSF-1), hemopoiesis is inhibited (Dexter et al, 1977). Recently, Mergenthaler and colleagues have reported that when cytokines such as GM-CSF, IL-3, or G-CSF were added to human micro-LTMC hemopoiesis was stimulated. In contrast, addition of TNFα, IFNγ or rhCSF-1 resulted in a significant inhibition of hemopoieis (Mergenthaler et al, 1991), which confirms the observations presented in this study.

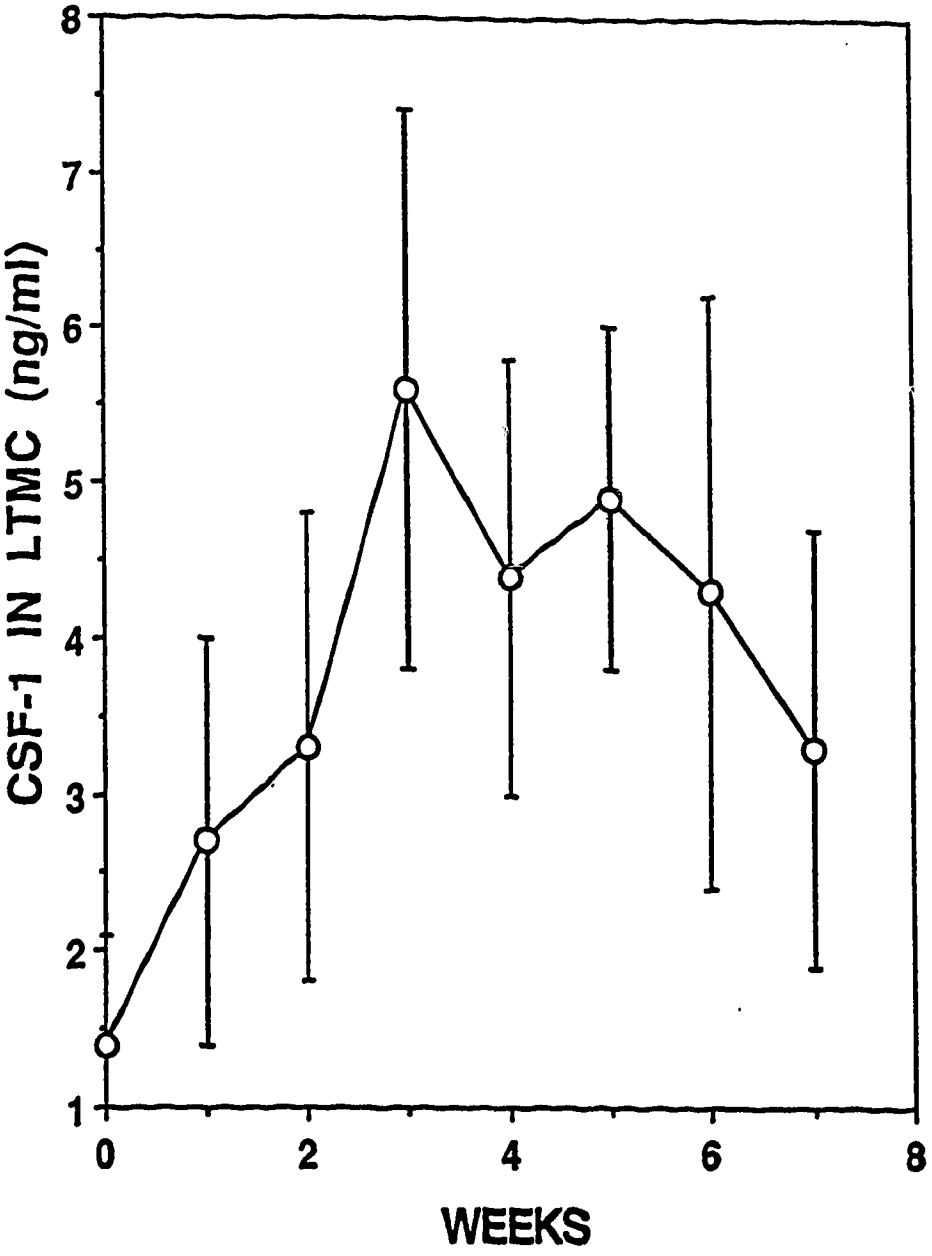


**Figure 14.** CSF-1 levels in supernatants from normal human LTMC. Results represent mean + SD of 8 experiments. Each experiment corresponds to the bone marrow from a different normal subject.

**Table 10**

**CSF-1 production in different cell cultures derived from normal bone marrow**

| Culture | Cells in culture[a] | | | | |
|---|---|---|---|---|---|
| | No./flask ($\times 10^{-6}$) | Mϕ | G,L,B | Fibr | CSF-1 (ng/mL)[b] |
| Nonadherent cells | 1.67 ± 0.3 | 26 ± 4 | 74 ± 5 | <1 | < 0.9 |
| Stromal adherent layer | 2.87 ± 0.1 | 39 ± 4 | 5 ± 3 | 56 ± 2* | 4.7 ± 1.1 |
| Fibroblast culture | 2.06 ± 0.1 | <1 | <1 | 100 | 2.9 ± 0.9 |

Results represent mean ± SD of three experiments. Each experiment corresponds to the bone marrow from a different normal subject.

a Total number of cells per flask and relative proportions of macrophages (Mϕ), granulocytes, lymphocytes and blasts (G,L,B), and fibroblasts (Fibr) at week 3 + 2 days.

b CSF-1 was quantitated in supernatants obtained at week 3 + 2 days.

* Absolute fibroblast number = $1.66 \pm 0.07 \times 10^6$.

**Table 11**

**Hemopoietic colony growth in semisolid cultures supplemented with rhCSF-1***

| Supplement | | Colonies/ $2 \times 10^5$ cells | | | | |
|---|---|---|---|---|---|---|
| PHA-LCM (10% v/v) | rhCSF-1 (ng/mL) | CFU-Mix | BFU-E | CFU-G | CFU-M | CFU-GM |
| -- | 0 | <1 | 2 ± 1 | 2 ± 2 | 1 ± 1 | <1 |
| -- | 5 | <1 | <1 | <1 | 9 ± 2 | <1 |
| -- | 10 | <1 | 1 ± 1 | 2 ± 2 | 26 ± 2 | <1 |
| -- | 15 | <1 | <1 | <1 | 29 ± 3 | <1 |
| -- | 20 | <1 | <1 | <1 | 30 ± 3 | 1 ± 1 |
| -- | 40 | <1 | <1 | <1 | 27 ± 2 | 3 ± 1 |
| + | 0 | 6 ± 2 | 38 ± 3 | 26 ± 2 | 37 ± 4 | 7 ± 2 |
| + | 15 | 5 ± 1 | 31 ± 2 | 25 ± 4 | 34 ± 3 | 7 ± 1 |

* Bone marrow cells from a normal subject were used as target cells.

Results represent mean ± SD of duplicate cultures from a representative experiment. Three other experiments were performed in exactly the same way and the results were highly reproducible.

**Table 12**

**Nucleated cells and hemopoietic progenitors in normal LTMC treated with rhCSF-1 from day 0 through day 20 (% of control)[1]**

| Week | Fraction | Nucleated Cells | CFU-MIX | CFU-C | BFU-E |
|---|---|---|---|---|---|
| 0 | | 100 | 100 | 100 | 100 |
| 1 | NA | 88 ± 26 | 41 ± 33 | 34 ± 18 | 24 ± 16 |
| 2 | NA | 73 ± 12 | 34 ± 17 | 32 ± 21 | 11 ± 22 |
| 3 | NA | 65 ± 10 | 0* | 18 ± 18 | 13 ± 24 |
| | A | 83 ± 19 | 0 | 25 ± 15 | 2 ± 8 |
| 4 | NA | 72 ± 6 | 0 | 47 ± 17 | 40 ± 12 |
| 5 | NA | 78 ± 26 | ** | 56 ± 29 | 64 ± 28 |
| | A | 79 ± 24 | 0 | 46 ± 20 | 43 ± 12 |
| 6 | NA | 85 ± 13 | ** | 68 ± 15 | 59 ± 31 |
| 7 | NA | 73 ± 22 | ** | 65 ± 21 | ** |
| | A | 82 ± 18 | ** | 64 ± 10 | 41 ± 16 |

Results represent mean ± SD of 9 experiments (week 0-5) and 3 experiments (week 6 & 7), and are expressed as % of the number of nucleated cells or colony growth in control cultures, which corresponds to 100% at all time points. Each experiment corresponds to the bone marrow from a different normal subject.

1 rhCSF-1 was added every second day from 0 - 20 at 15 ng/ml.

* Colonies were not detected in rhCSF-1-treated LTMC

** Colonies were not detected in control or in rhCSF-1-treated LTMC

**Table 13**

**Nucleated cells and hemopoietic progenitors in normal LTMC treated with rhCSF-1 from day 21 through day 33 (% of control)**

| Exp | Week | Fraction | Nucleated cells | CFU-MIX | CFU-C | BFU-E |
|---|---|---|---|---|---|---|
| 1* | 3 | NA | 102 | 100 | 100 | 99 |
| | | A | na | na | na | na |
| | 4 | NA | 74 | 51 | 86 | 76 |
| | 5 | NA | 75 | ** | 83 | 75 |
| | | A | 110 | 48 | 67 | 62 |
| 2* | 3 | NA | 100 | 100 | 101 | 100 |
| | | A | na | na | na | na |
| | 4 | NA | 93 | 72 | 95 | 92 |
| | 5 | NA | 86 | ** | 89 | 82 |
| | | A | 100 | ** | 66 | 58 |

Results are expressed as % of the number of nucleated cells or colony growth in control cultures, which corresponds to 100% at all time points.

1* & 2* refer to cultures that were not treated with rhCSF-1 during the first 3 weeks and then treated with rhCSF-1 which was added every second day from day 21-33 at 15 ng/ml.

na = not assessed

** Colonies were not present in control or in rhCSF-1-treated cultures

**Table 14**

**CSF-1 Levels (ng/ml) in normal LTMC**

| Exp | Week | Control | rhCSF-1 | (significance) |
|---|---|---|---|---|
| 1, 2, 3 | 0 | 1.6±0.2 | | |
| | 1 | 3.5 ± 2.9 | 9.8 ± 5.0 | (NS) |
| | 2 | 5.6 ± 2.8 | 11.7 ± 6.1 | (NS) |
| | 3 | 3.3 ± 2.1 | 16.0 ± 5.7 | ($p < 0.05$) |
| 1* | 4 | 3.1 | > 19.5 | |
| | 5 | 5.6 | > 19.5 | |
| 2* | 4 | 5.4 | > 19.5 | |
| | 5 | 4.9 | > 19.5 | |

(NS) Not significant

1* & 2* refer to cultures that were not treated with rhCSF-1 during the first three weeks and then treated with rhCSF-1 which was added every second day from day 21-33 at 15 ng/ml.

**Table 15**

**Hemopoietic colony growth in the presence of different supplements (% of growth)**

| Supplement* | CFU-MIX | CFU-C | BFU-E |
|---|---|---|---|
| Epo | <6 | ± 3 | 9 ± 4 |
| Epo + rhCSF-1 | <6 | ± 3 | <2 |
| Epo + C-LTMC[a] | <6 | 6 ± 2 | 7 ± 3 |
| Epo + CSF-1-LTMC/0-20[b] | <63 | ± 3 | <2 |
| Epo + PHA-LCM | 100 | 100 | 100 |
| Epo + PHA-LCM + rhCSF-1 | 97 ± 7 | 96 ± 6 | 97 ± 2 |
| Epo + PHA-LCM + C-LTMC[a] | 95 ± 9 | 102 ± 5 | 99 ± 6 |
| Epo + PHA-LCM + CSF-1-LTMC/0-20[b] | 29 ±9@ | 59 ±4@ | 38 ±9@ |
| Epo + PHA-LCM + C-LTMC[c] | 96 ± 6 | 94 ± 2 | 101 ± 3 |
| Epo + PHA-LCM + CSF-1-LTMC/21-33[d] | 71 ± 7 | 86 ± 3 | 82 ± 4 |

[a] Supernatants from control LTMC from 8 experiments, at week 2, tested separately.

[b] Supernatants from rhCSF-1-treated LTMC from 8 experiments, at week 2, tested separately.

[c] Supernatants from control LTMC from experiments 1*, 2* & 3*, at week 5, tested separately.

[d] Supernatants from rhCSF-1-treated LTMC from experiments 1*, 2* & 3*, at week 5 tested separately.

* Epo added at 1 U/ml; rhCSF-1 at 15 ng/ml; PHA-LCM at 10% (v/v) and LTMC supernatants at 10% (v/v)
Results represent mean ± SD. Each experiment corresponds to the bone marrow from a normal subject. Experiment 3* was performed in the same way as experiments 1* and 2* (see legends in Tables 14 & 15).

@ Significantly lower ($P<0.025$) than 100%;

## B) CSF-1 IN AML LTMC

### CSF-1 production in AML LTMC

As for their normal counterparts, AML LTMC constitutively produced significant levels of CSF-1, although a marked patient-to-patient variation was evident (Table 16). Interestingly, a direct correlation was observed between the levels of CSF-1 in the culture supernatant and CFU-F numbers in the initial innoculum (Fig. 16). This suggested that the presence of fibroblastoid cells is important for CSF-1 production in LTMC. This, of course, is in keeping with the fact that fibroblasts are one of the major sources of CSF-1 (Stanley et al, 1983). As shown in Table 17, most of the CSF-1 produced in AML LTMC that developed a counfluent adherent layer was produced by the stromal adherent cells (macrophages and fibroblastoid cells). CSF-1 levels in fibroblast cultures derived from the same patients corresponded to 54% of the levels observed in LTMC. These results seem to suggest that adherent macrophages and fibroblasts contribute to CSF-1 production in equal proportions. However, as stated in the previous section, we are not certain that the fibroblastoid population developed in stromal adherent layers is the same population present in fibroblast cultures. Thus, it is not clear whether CSF-1 production by fibroblasts in fibroblast cultures is equivalent to CSF-1 production by these cells in LTMC. In AML LTMC that developed a poor adherent layer, nonadherent and adherent cells produced similar levels of CSF-1 (Table 18), whereas the levels of CSF-1 in fibroblast cultures were undetectable. This observation suggests that in those LTMC macrophages account for most of the CSF-1 production.

**Effects of exogenous CSF-1**

Different concentrations of rhCSF-1 were tested in semisolid cultures of AML bone marrow cells. As shown in table 19, rhCSF-1 had practically no effect on AML-ACl, whereas CFU-C numbers were stimulated by this factor (10 - 40 ng/ml) to approximately 40% of the growth stimulated by PHA-LCM. These results are in keeping with previous reports showing that CSF-1 does not stimulate AML-CFU growth as compared to other CSFs (Miyauchi et al, 1988a; Suzuki et al, 1988).

In order to study its effect in AML LTMC, rhCSF-1 was added every second day at 15 ng/ml. This is the same schedule as the one used in normal LTMC. In contrast to its effects in normal LTMC, where it had little, if any, effect, rhCSF-1 significantly increased (3-fold) the numbers of adherent macrophages in AML LTMC (Table 20). Studies by Miyauchi and colleagues (Miyauchi et al, 1988b) have shown that CSF-1 induces the maturation of blast cells into adherent macrophagic cells. This seems to be a direct effect since AML blasts express CSF-1 receptors on their surface (Ashmun et al, 1989). Thus, it is possible that similar CSF-1 effects occur in AML LTMC. Indeed, blast cell numbers in rhCSF-1-treated AML LTMC were 70 $\pm$ 4%, at week 3, and 12 $\pm$ 6%, at week 5, of the levels observed in untreated AML LTMC. As expected, AML-derived CFU-F were not affected by rhCSF-1, whereas fat cell formation was inhibited.

Hemopoietic progenitors were significantly affected by addition of rhCSF-1 to AML LTMC. AML-ACl growth was dramatically reduced from the first week of culture (Table 21). Thus, although these progenitors disappeared from the culture rather fast, their disappearance was even faster in rhCSF-1-treated

cultures. In contrast, CFU-C growth in most AML cultures was stimulated by this factor. Only cultures from patient 8 did not respond at all (no changes in AML-ACl or CFU-C numbers) to rhCSF-1. Interestingly, the endogenous levels of CSF-1 in such cultures were already higher (10 - 14 ng/ml) than normal. A possible explanation for the lack of effects of rhCSF-1 in this particular patient could be the absence of CSF-1 receptors on the AML blast cell surface.

The rhCSF-1 effects on CFU-C observed in AML LTMC (stimulation) were opposite to those observed in normal LTMC (inhibition; Table 13). At least two different possibilities can explain these apparently contradictory results: (i) despite giving rise to morphologically normal colonies, CFU-C developed in AML LTMC are of leukemic origin and respond differently to rhCSF-1. Although this is a possibility we cannot rule out, it seems unlikely because rhCSF-1 inhibited the growth of both normal marrow-derived CFU-C and AML-ACl, thus, even if the CFU-C observed in AML LTMC were leukemic, we would expect them to be reduced by rhCSF-1. (ii) the rhCSF-1 effects are mediated by microenvironmental cells, which in AML LTMC may be functionally defective. Thus, they would mediate differently the effects of rhCSF-1. An obvious first approach to explore this latter possibility was to test for the presence of the soluble inhibitory activity produced in normal LTMC treated with rhCSF-1. As shown in Table 22, two interesting observations were made from those experiments. Firstly, in contrast to what was observed in normal LTMC, a soluble activity capable of inhibiting the growth of hemopoietic progenitors was constitutively produced in untreated AML LTMC. Secondly, no further inhibition was observed in the presence of supernatants from rhCSF-1-treated AML LTMC, which suggested that rhCSF-1 did not induce microenvironmental

cells to produce more inhibitory activity, as it did in normal LTMC.

**Discussion**

As in normal LTMC, CSF-1 was constitutively produced in AML LTMC in a process that seems to involve both macrophages and fibroblastoid cells. A direct correlation was observed between CSF-1 levels and the development of the adherent layer. In other words, CSF-1 levels were usually higher in AML LTMC containing high levels (within the normal range) of CFU-F and adherent cells. These results are not surprising, since it is well known that the major sources of CSF-1 are fibroblasts and macrophages (Stanley et al, 1983; Clark & Kamen, 1987).

Contrary to its effects in normal LTMC, rhCSF-1 stimulated the growth of putative normal myeloid progenitors (CFU-C) in AML LTMC. Since rhCSF-1 effects seem to be mediated by microenvironmental cells, the above results suggested that the hemopoietic microenvironment in AML LTMC is functionally abnormal. This idea is supported by two findings: Firstly, microenvironmental cells in AML LTMC constitutively produced a soluble inhibitory activity that is not produced in normal LTMC. This is in keeping with previous studies showing that leukemic cells produce inhibitory molecules *in vitro* (Broxmeyer et al, 1981; Kobari et al, 1990). Secondly, rhCSF-1 did not induce microenvironmental cells to produce a soluble inhibitory activity, as it did in normal LTMC. Results on the identification of the cells responsible for the production of the inhibitory activities in human LTMC and the nature of such activities will be presented in Chapter VI.

In contrast to the effects of other CSFs, such as GM-CSF, G-CSF, and

IL3, which stimulate the growth of leukemic progenitors (Miyauchi et al, 1988a), CSF-1 accelerated the disappearance of AML-ACl in AML LTMC. Although the mechanism for this effects is not fully understood, it seems that CSF-1 acts directly on AML blasts, which express the CSF-1 receptor (Ashmun et al, 1989) and induces their maturation into macrophagic adherent cells (Miyauchi et al, 1988b). Since macrophages developed in AML LTMC seem to be derived from leukemic blasts, it is possible that they are functionally distinct from normal macrophages. If this idea is correct, it would explain the abnormal response to rhCSF-1 observed in AML LTMC.

**Table 16**

**CSF-1 levels in AML LTMC**

| Week | CSF-1 (ng/mL) |
|---|---|
| 0 | 1.4 ± 0.3 |
| 1 | 4.1 ± 2.8 |
| 2 | 4.3 ± 3.5 |
| 3 | 3.7 ± 3.1 |
| 4 | 4.5 ± 3.0 |
| 5 | 4.7 ± 3.4 |
| 6 | 3.9 ± 2.8 |
| 7 | 4.2 ± 1.1 |

Results represent mean ± SD of 9 experiments. Each experiment corresponds to a different AML patient.

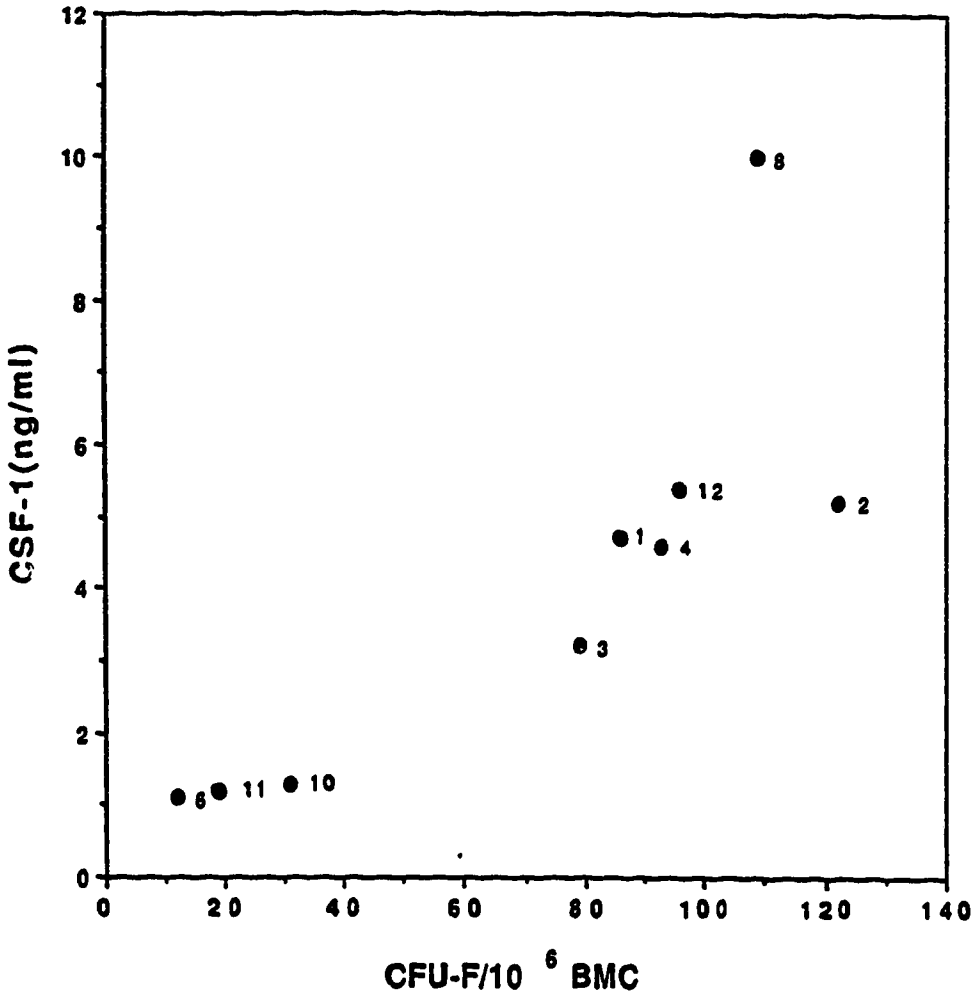


Figure 15. Correlation between relative numbers of CFU-F in the patients' marrow and CSF-1 levels in LTMC at week 3. Each point represents a particular patient.

**Table 17**

**CSF-1 production in different cell cultures derived from AML-I bone marrow**

| Culture | Cells in culture[a] | | | | |
|---|---|---|---|---|---|
| | No./flask ($\times 10^{-6}$) | Mϕ | G,L,B | Fibr | CSF-1 (ng/mL)[b] |
| Nonadherent cells | 1.46 ± 0.7 | 30 ± 4 | 70 ± 3 | <1 | 1.2 ± 0.2 |
| Stromal adherent layer | 2.61 ± 0.3 | 39 ± 4 | 6 ± 3 | 54 ± 3 | 4.2 ± 0.8 |
| Fibroblast culture | 1.91 ± 0.2 | 2 ± 1 | <1 | 98 ± 1 | 2.9 ± 0.9 |

Results represent mean ± SD of three experiments. Each experiment corresponds to the bone marrow from a different AML patient (patients 2,4,8).

a Total cell number per flask and relative proportions of macrophages (Mϕ), granulocytes, lymphocytes and blasts (G,L,B), and fibroblastoid cells (Fibr) at week 3 + 2 days.

b CSF-1 was quantitated in supernatants obtained at week 3 + 2 days.

**Table 18**

**CSF-1 production in different cell cultures derived from AML-II bone marrow**

| Culture | Cells in culture[a] | | | | |
|---|---|---|---|---|---|
| | No./flask ($\times 10^{-6}$) | Mϕ | G,L,B | Fibr | CSF-1 (ng/mL)[b] |
| Nonadherent cells | 1.79 ± 0.6 | 27 ± 4 | 74 ± 2 | <1 | 1.3 ± 0.3 |
| Stromal adherent layer | 0.97 ± 0.3 | 70 ± 5 | 22 ± 4 | 7 ± 3 | 1.1 ± 0.1 |
| Fibroblast culture | 0.08 ± 0.01 | 7 ± 2 | 1 ± 1 | 92 ± 1 | <0.9 |

Results represent mean ± SD of three experiments. Each experiment corresponds to the bone marrow from a different AML patient (patients 6,10,11).

a Total cell number per flask and relative proportions of macrophages (Mϕ), granulocytes, lymphocytes and blasts (G,L,B), and fibroblastoid cells (Fibr) at week 3 + 2 days.

b CSF-1 was quantitated in supernatants obtained at week 3 + 2 days.

**Table 19**

**AML-derived colony growth in semisolid cultures supplemented with rhCSF-1**

| Supplement | | Colonies / $2 \times 10^5$ | |
|---|---|---|---|
| PHA-LCM (10% v\v) | rhCSF-1 (ng/mL) | AML-ACl | CFU-C |
| -- | 0 | 6 ± 2 | <1 |
| -- | 10 | 9 ± 3 | 8 ± 2 |
| -- | 20 | 9 ± 1 | 6 ± 1 |
| -- | 40 | 7 ± 2 | 7 ± 2 |
| + | 0 | 64 ± 10 | 21 ± 3 |
| + | 20 | 61 ± 7 | 19 ± 1 |

Bone marrow cells from patient 4 were used as target cells.

Results represent mean ± SD of duplicate cultures from a representative experiment. Two other experiments were performed using bone marrow cells from patients 2 and 6, respectively. Although significant variation in colony numbers was observed from patient to patient, the same trend was observed in every experiment, i.e. no effect of rhCSF-1 on AML-ACl numbers. CFU-C numbers were always significantly lower than those in normal bone marrow (see Table 12).

**Table 20**

**Effect of rhCSF-1 on the composition of the adherent layers established from normal and AML bone marrow cells**

| | Normal (n = 11) | | AML (n=6) | |
|---|---|---|---|---|
| Parameter | C | CSF-1[a] | C | CSF-1[a] |
| Adherent Cells (% of control) | 100 | 119 ± 24 | 100 | 234 ± 46 |
| Macrophages (% of control) | 100 | 122 ± 27 | 100 | 292 ± 39 |
| CFU-F (% of control) | 100 | 94 ± 13 | 100 | 109 ± 17 |
| Fat Cells[b] | 11 | 1 | 3 | 0 |

Results correspond to week 3 of culture.

a rhCSF-1 (15 ng/mL) was added to the cultures every second day, from day 0 to day 20.

b number of cultures containing fat cells

**Table 21**

**Hemopoietic colony growth in rhCSF-1-treated AML LTMC**

| Week | Fraction[a] | AML-ACl | (significance) | CFU-C | (significance) |
|---|---|---|---|---|---|
| 1 | NA | 27 ± 24 | ($p < 0.025$) | 132 ± 34 | (NS) |
| 2 | NA | 34 ± 29 | ($p < 0.025$) | 88 ± 38 | (NS) |
| 3 | NA | 11 ± 15 | ($p < 0.025$) | 195 ± 108 | ($p < 0.05$) |
| | A | 45 ± 10 | ($p < 0.025$) | 332 ± 155 | ($p < 0.05$) |
| 4 | NA | b | | 239 ± 162 | (NS) |
| 5 | NA | b | | 230 ± 77 | ($p < 0.05$) |
| | A | b | | 314 ± 154 | ($p < 0.05$) |
| 6 | NA | b | | c | |
| 7 | NA | b | | c | |
| | A | b | | 367 ± 238 | ($p < 0.05$) |

Results represent mean ± SD of 6 (AML-ACl) and 7 (CFU-C) experiments and are expressed as the percentage of the growth in untreated AML LTMC, which corresponds to 100% at all time points. Each experiment corresponds to the bone marrow from a different patient.

a NA = nonadherent, A = adherent

b AML-ACl were not present in control or rhCSF-1-treated cultures.

c CFU-C were present only in rhCSF-1-treated cultures.

**Table 22**

**Hemopoietic colony growth in the presence of different supplements**

| Supplement** | Colony growth (%) CFU-Mix | CFU-C | BFU-E |
|---|---|---|---|
| Epo | <7 | <2 | 17 ± 4 |
| Epo + rhCSF-1 | <7 | 34 ± 6 | 14 ± 3 |
| Epo + AML-LTMC$_c$[a] | <7 | <2 | 4 ± 3 |
| Epo + AML-LTMC$_{CSF-1}$[b] | <7 | <2 | <3 |
| Epo + PHA-LCM | 100 | 100 | 100 |
| Epo + PHA-LCM + rhCSF-1 | 96 ± 3 | 96 ± 4 | 102 ± 6 |
| Epo + PHA-LCM + AML-LTMC$_c$[a] | 26 ± 4@ | 72 ± 3* | 56 ±4@ |
| Epo + PHA-LCM + AML-LTMC$_{CSF-1}$[b] | 33 ± 3@ | 90 ± 4* | 74 ±5@ |

Normal bone marrow cells were used as target cells.

** Epo added at 1 U/mL; rhCSF-1 at 15 ng/mL; PHA-LCM at 1% (v/v) and LTMC supernatants at 10 % (v/v).

a Supernatants from control flasks of AML LTMC (patients 1,2,3,4,6,10,11), at week 2, tested separately.

b Supernatants from rhCSF-1-treated AML LTMC (patients 1,2,3,4,6,10,11), at week 2, tested separately.

* 90 ± 4 significantly different ($p < 0.05$) from 72 ± 3.

@ Significantly lower ($p<0.025$) than 100%

# VI. RESULTS

## *IN VITRO* HEMOPOIETIC SUPPORTIVE CAPACITY OF THE AML-DERIVED HEMOPOIETIC MICROENVIRONMENT

In the previous chapters, a series of observations were presented that indicated that normal and AML LTMC differ in several aspects: (i) progenitors giving rise to morphologically normal colonies (CFU-C) in AML LTMC were sustained for significantly shorter periods of time (5 - 7 weeks) than in normal LTMC (12 - 16 weeks); (ii) the CFU-C levels in AML LTMC were usually lower (30-60%) than in normal LTMC; (iii) when added to normal LTMC, rhCSF-1 induced production of a soluble activity capable of inhibiting the growth of hemopoietic progenitors, whereas it did not when added to AML LTMC; (iv) a soluble inhibitory activity was consitutively produced in AML LTMC, and its levels seem to be slightly reduced when the cultures were supplemented with rhCSF-1.

The above results, particularly those regarding the effects of rhCSF-1 in both types of LTMC, suggested that the hemopoietic microenvironment developed in AML LTMC might be functionally defective. To test this hypothesis, some points need to be analyzed: (i) As mentioned before, previous reports (Singer et al, 1988) suggest that morphologically normal colonies developed in AML LTMC may still be derived from the leukemic clone, thus, in order to prove the presence of a deficient hemopoietic microenvironment in AML LTMC, it is necessary to asses its capacity to sustain the growth of hemopoietic progenitors derived from normal bone marrow. (ii) Although macrophages are the most likely candidates, it is not known which cell

population(s) produce(s) the soluble inhibitory activity in response to rhCSF-1 in normal LTMC. (iii) It is also unknown which cells constitutively produce the soluble inhibitory activity in AML LTMC. (iv) Finally, it is unclear which factor(s) is(are) present in the above activities.

### A) CHIMERIC LTMC

In order to assess the ability of the hemopoietic microenvironment developed in normal and AML LTMC to sustain the growth of normal hemopoietic progenitors, chimeric LTMC were developed in which progenitor cells from normal bone marrow were cultured on stromal adherent layers established from either normal or AML bone marrow. To further characterize the response of the microenvironmental cells to CSF-1, the stromal adherent layers were established in the absence or presence of rhCSF-1. As described in chapter III (Materials and Methods), normal marrow-derived nonadherent cells were seeded on pre-irradiated stromal layers from normal or AML bone marrow. The levels of CFU-MIX, BFU-E, and CFU-C present in the nonadherent fraction of the chimeric cultures, were determined weekly. On week 5, adherent progenitors were also evaluated. For each experiment, control flasks were established in which normal nonadherent bone marrow cells were cultured in the absence of a pre-established adherent layer.

#### Chimeric LTMC containing a normal stromal adherent layer

As shown in Figure 16, CFU-C, BFU-E, and CFU-MIX were better supported by normal adherent layers established in the absence of rhCSF-1. Stromal adherent layers established in the presence of rhCSF-1 showed a

reduced hemopoietic supportive capacity. After five weeks of culture, most of the progenitors were located in the adherent layer (Table 23). At this point, the adherent layers established with rhCSF-1 contained fewer numbers of progenitors in comparison to the numbers found in adherent layers established without rhCSF-1. The above results are in keeping with the observations described before showing that even 3 - 5 weeks after rhCSF-1 addition has been stopped, the levels of hemopoietic progenitors are lower than in untreated cultures (Table 12). Cultures of nonadherent cells lacking a pre-established adherent layer showed high levels of progenitors, particularly CFU-C, during the first two weeks. However, the numbers of progenitors decreased rapidly thereafter (Figure 16), indicating the importance of the stromal cells in the long-term maintenance of hemopoiesis *in vitro*.

**Chimeric LTMC containing AML stromal adherent layers**

The hemopoietic supportive capacity of stromal adherent layers established from AML bone marrow was tested in the same way as their normal counterparts. When normal nonadherent marrow cells were seeded on AML adherent layers established in the absence of rhCSF-1, the levels of nonadherent CFU-C were reduced by 30% and BFU-E by more than 75% in comparison to the levels in cultures of normal adherent layers (Figure 17). Establishing AML adherent layers with rhCSF-1 increased their ability to support both CFU-C (almost to normal levels) and BFU-E. CFU-MIX were practically absent in cultures of untreated AML adherent layers. In contrast, when cultured on rhCSF-1-treated AML adherent layers, they showed detectable levels during the first three weeks of culture. Significantly higher levels of adherent progenitors

were observed at week 5 in all the adherent layers established with rhCSF-1 (Table 23).

**Presence of a soluble inhibitory activity**

Considering the results obtained in normal LTMC treated with rhCSF-1 and in untreated AML LTMC, a possible explanation for the reduced hemopoietic supportive capacity of (i) normal stromal adherent layers established with rhCSF-1 and (ii) AML adherent layers established without rhCSF-1 is that they produce hemopoietic inhibitors. In order to address this possibility, supernatants from the chimeric LTMC were tested in colony assays of normal bone marrow cells. As shown in Table 24, we were unable to detect any colony stimulating activity in any of the supernatants. In contrast, a soluble inhibitory activity was detected in the supernatants from cultures containing rhCSF-1-treated normal adherent layers and AML adherent layers. Interestingly, the levels of the inhibitory activity were reduced in supernatant from AML adherent layers established in the presence of rhCSF-1.

**Discussion**

In comparison to their normal counterparts, AML adherent layers showed an impaired hemopoietic supportive capacity. This functional deficiency observed in AML adherent layers, particularly in terms of sustaining the growth of BFU-E and CFU-MIX, cannot be explained by deficiencies in the numbers of macrophages, fibroblasts or fat cells, since in the cultures used for chimerid LTMC these numbers were similar to those observed in normal adherent layers. Rather, our results suggest that there is a functional alteration in some of the

components of the AML adherent layers. Previous studies by Bernard Greenberg et al (1981) indicated that fibroblast layers derived from AML patients were unable to sustain the growth of normal progenitor cells, whereas normal fibroblasts were able to do so. Peter Greenberg et al (1978), on the other hand, demonstrated a reduced production of stimulatory cytokines by AML-derived macrophages. In this study, we were unable to detect, by colony assays, any stimulatory activity in the culture supernatants. Thus, we cannot comment on the possibility that AML-derived stromal layers produced reduced levels of stimulators. However, a soluble inhibitory activity was detected in supernatants from AML adherent layers. These results indicate that the reduced hemopoietic supportive capacity of the AML-derived hemopoietic microenvironment may be due to the constitutive production of hemopoietic inhibitors.

Hemopoietic support by AML adherent layers was improved when they were established in the presence of rhCSF-1, which is opposite to the effects of rhCSF-1 on normal adherent layers. Addition of rhCSF-1 during the establishment of the adherent layers resulted in a slight reduction in the levels of the inhibitory activity, as detected in colony assays, thus, it is possible that rhCSF-1 down-regulates the production of inhibitors by AML adherent cells.

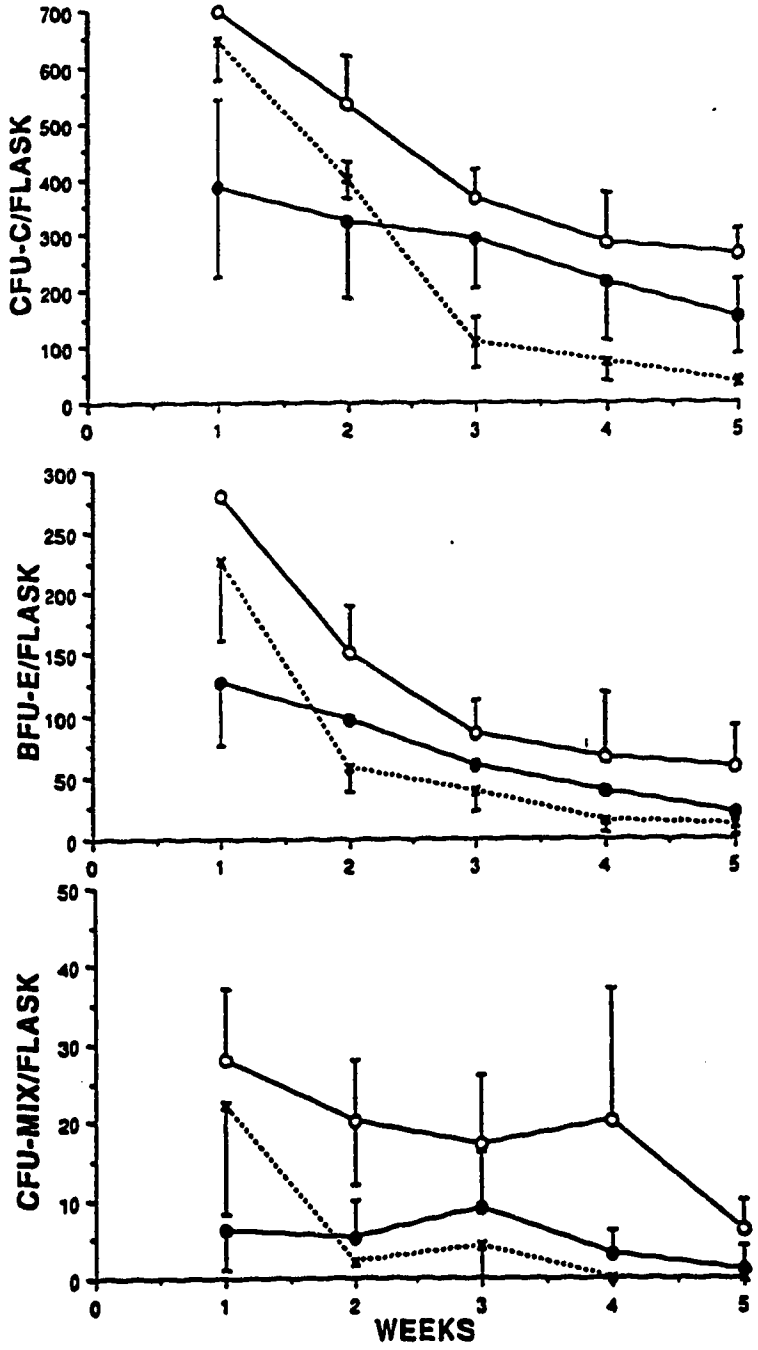


**Figure 16.** Total number of nonadherent CFU-C, BFU-E and CFU-MIX in chimeric LTMC (n = 6) containing normal stromal adherent layers established without (open circles) or with (filled circles) rhCSF-1. X = levels of hemopoietic progenitors in cultures lacking a pre-established adherent layer. Each experiment corresponds to the stromal layer derived from the bone marrow of a normal subject.

**Table 23**

**Number of progenitor cells in the adherent layer of chimeric LTMC at week 5**

| | | No. Progenitors/flask | | |
|---|---|---|---|---|
| Stromal layer | rhCSF-1 | CFU-MIX | BFU-E | CFU-C |
| Normal[a] (n = 6) | -- | 24 ± 10 | 96 ± 21 | 371 ± 54 |
| | + | 6 ± 3 | 43 ± 12 | 243 ± 39 |
| AML[b] (n = 4) | -- | <2 | 41 ± 17 | 260 ± 36 |
| | + | 3 ± 4 | 74 ± 12 | 324 ± 41 |
| No SAL (n = 6) | | <1 | 16 ± 7 | 76 ± 46 |

a In every single experiment, progenitor numbers were significantly higher ($p<0.05$) in cultures containing normal adherent layers established without rhCSF-1.

b In every single experiment, progenitor numbers (except for CFU-MIX) were significantly lower ($p<0.05$) in cultures containing AML adherent layers established without rhCSF-1.

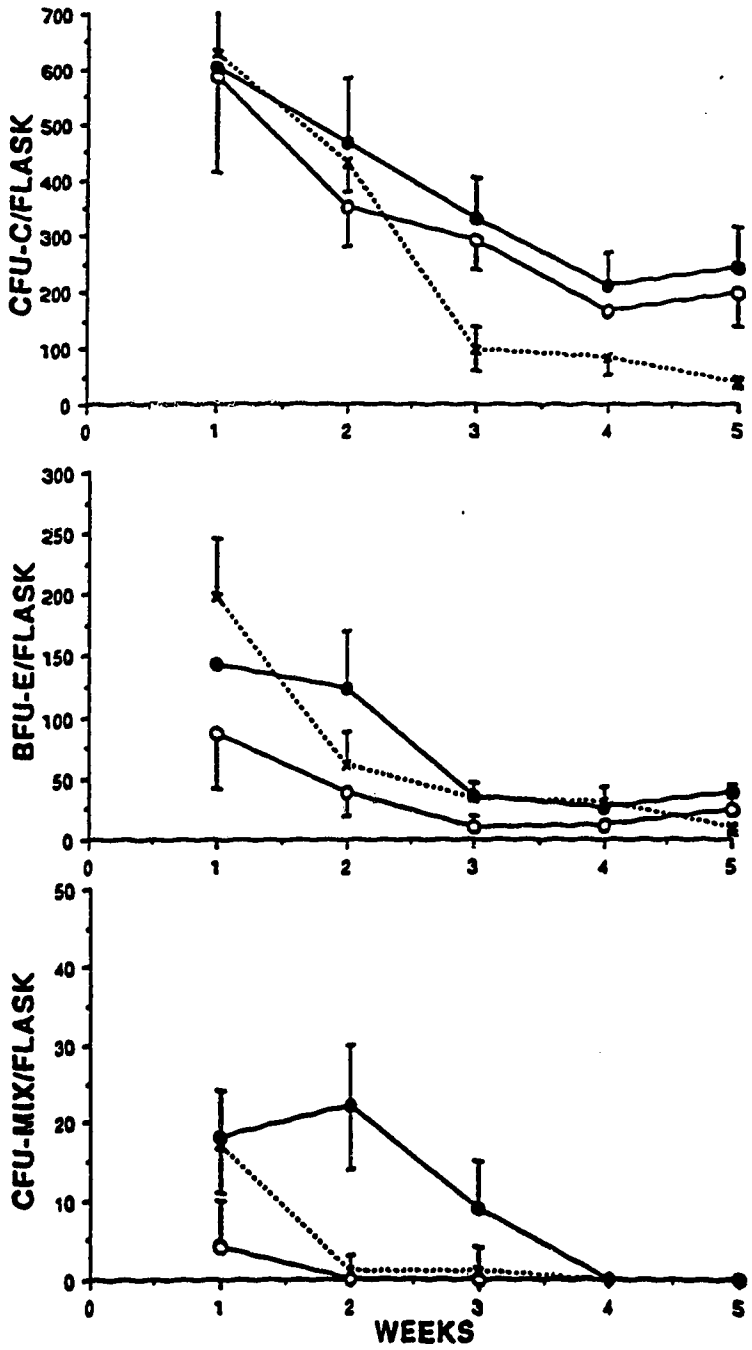


**Figure 17.** Total number of nonadherent CFU-C, BFU-E and CFU-MIX in chimeric LTMC (n = 4) containing AML stromal adherent layers established without (open circles) or with (filled circles) rhCSF-1. X = levels of hemopoietic progenitors in cultures lacking a pre-established adherent layer. Each experiment corrsponds to the stromal layer derived from the bone marrow of a particular AML patient (patients 1,2,3,4).

**Table 24**

**Hemopoietic colony growth in the presence of different supplements**

| Supplement** | Colony growth (%) CFU-Mix | BFU-E | CFU-C |
|---|---|---|---|
| Epo | <7 | 12 ± 4 | 6 ± 3 |
| Epo + rhCSF-1 | <7 | 8 ± 3 | 46 ± 4 |
| Epo + N-$SAL_C$[a] | <7 | 9 ± 3 | 5 ± 2 |
| Epo + N-$SAL_{CSF-1}$[b] | <7 | 2 ± 2 | <2 |
| Epo + AML-$SAL_C$[c] | <7 | <3 | 3 ± 2 |
| Epo + AML-$SAL_{CSF-1}$[d] | <7 | 6 ± 1 | 5 ± 3 |
| Epo + PHA-LCM | 100 | 100 | 100 |
| Epo + PHA-LCM + rhCSF-1 | 96 ± 4 | 96 ± 5 | 97 ± 4 |
| Epo + PHA-LCM + N-$SAL_C$[a] | 102 ± 2 | 104 ± 3 | 99 ± 3 |
| Epo + PHA-LCM + N-$SAL_{CSF-1}$[b] | <7* | 46 ± 5* | 62 ± 5* |
| Epo + PHA-LCM + AML-$SAL_C$[c] | 26 ± 4* | 57 ± 3* | 79 ± 4* |
| Epo + PHA-LCM + AML-$SAL_{CSF-1}$[d] | 44 ± 7* | 75 ± 4* | 91 ± 3@ |

**Table 24 - Legend**

** Epo added at 1 U/mL; rhCSF-1 at 15 ng/mL; PHA-LCM at 10% (vol/vol) and LTMC supernatants at 10% (vol/vol). All supernatants from the chimeric LTMC were collected at week 2 and tested separately in colony assays. Normal bone marrow cells were used as target cells.

a Supernatants from cultures containing normal stromal adherent layers (n=6) established in the absence of rhCSF-1, tested separately.

b Supernatants from cultures containing normal stromal adherent layers (n=6) established in the presence of rhCSF-1, tested separately.

c Supernatants from cultures containing AML stromal adherent layers (from patients 1,2,3,4,6,10) established in the absence of rhCSF-1, tested separately.

d Supernatants from cultures containing AML stromal adherent layers (from patients 1,2,3,4,6,10) established in the presence of rhCSF-1, tested separately.

* significantly lower ($p<0.05$) than the control (100%)

@ not significantly different from the control

## B) CELLS PRODUCING THE INHIBITORY ACTIVITIES

The results presented above clearly indicated the possibility that functional abnormalities are present in the hemopoietic miroenvironment developed in AML LTMC. On the one hand, it was shown that its hemopoietic supportive capacity was significantly reduced as compared to the hemopoietic microenvironment developed in normal LTMC. This correlated with the constitutive production of a soluble activity capable of inhibiting the growth of hemopoietic progenitors. On the other hand, rhCSF-1 did not stimulate microenvironmental cells in AML LTMC to produce a soluble inhibitory activity as did in normal LTMC. We, then, tried to identify the cells producing the inhibitory activities in rhCSF-1-treated normal LTMC and in AML LTMC.

### Detection of the inhibitory activity in different cell cultures

Stromal adherent layers (SAL) and fibroblast cultures (FIBR) from normal bone marrow were established in the presence of rhCSF-1. Whereas SAL contained a significant proportion of macrophages (up to 44%), FIBR cultures consisted almost entirely of fibroblastoid cells (Table 25). As shown in Table 26, only supernatants from SAL showed detectable levels of the inhibitory activity, which suggests that an adherent, non-fibroblastic cell population produces such an activity in response to rhCSF-1. In a similar approach, SAL and FIBR from AML bone marrow were established in the absence of rhCSF-1, since this factor seems to decrease the levels of the inhibitory activity. In order to determine whether AML blasts were capable of producing the inhibitory activity, AML blast cell cultures were also established. The cellular composition of the cultures is presented in Table 25. As for their normal counterparts, supernatants from AML

FIBR did not show detectable levels of the inhibitory activity, in contrast, the activity was detected in supernatants from SAL and blast cultures (Table 26). These results suggest that AML blasts, as well as adherent, non-fibroblastic cells produce the inhibitory activity.

**Hemopoietic supportive capacity of normal and AML fibroblastoid cells**

The above results indicate that the cells involved in the production of the inhibitory activities in normal rhCSF-1-treated LTMC and in AML LTMC are adherent, non-fibroblastic cells. If, as suggested by these results, (i) fibroblasts are not involved in the production of such activities, and (ii) the reduced hemopoietic supportive capacity observed in those cultures is due, at least in part, to the production of the inhibitory activities, we would expect to see no effect of rhCSF-1 on the hemopoietic supportive capacity of fibroblastoid cells. We also wanted to compare the hemopoietic supportive capacity of fibroblast layers from both normal and AML bone marrow.

As shown in Table 27, rhCSF-1 had no effect on the ability of normal marrow-derived fibroblast layers to sustain the growth of CFU-C or BFU-E. During the first two weeks, the capacity of fibroblast layers to sustain CFU-C was slightly lower. However, it is difficult to make conclusions since the results presented correspond to a single experiment and no statistical analysis was made. On weeks 3 and 4, practically no difference was observed. At all time points, BFU-E numbers were lower in cultures containing fibroblast layers, however, as for CFU-C, no definite conclusions can be made from a single experiment.

As expected, rhCSF-1 had no effect on the hemopoietic supportive capacity of AML-derived fibroblast layers (Table 28). CFU-C levels in cultures

of fibroblasts layers were usually higher than in untreated SAL, and similar to those in rhCSF-1-treated SAL. Interestingly, the levels of CFU-C in cultures of normal and AML fibroblast layers were similar, suggesting equivalent myelopoietic supportive capacities. BFU-E, on the other hand, were better supported in fibroblast cultures than in SAL, which suggests that AML-derived macrophages had a negative influence on erythroid progenitors. As for CFU-C, normal and AML fibroblast layers supported similar levels of BFU-E.

**Discussion**

The experiments presented above were conducted to identify the cell population(s) producing the soluble inhibitory activities in normal LTMC treated with rhCSF-1 and in AML LTMC. Our results suggest that in normal LTMC, an adherent, non-fibroblastic cell population is responsible for the production of the inhibitory activity. Considering that (i) among the microenvironmental cells, macrophages are the only cells that have been clearly shown to bear CSF-1 receptors on their surface, and (ii) macrophages comprise up to 44% of the adherent cells in normal rhCSF-1-treated LTMC, it seems reasonable to suggest that the inhibitory activity present in such cultures is produced by macrophages. However, we cannot completely rule out the possibility that an adherent, non-macrophagic, non-fibroblastic cell population, which would account for 5 - 10% of the total cell number, is the producer of the inhibitory activity

Supernatants from both AML blast cultures and AML-derived SAL were shown to contain the inhibitory activity. Since blast cultures contained 95 - 99% blasts, it seems highly likely that such cells produced the inhibitory activity. As for normal cultures, our results suggest that AML-derived macrophages were the

adherent cells producing the soluble inhibitory activity in AML LTMC.

The hemopoietic supportive capacity of fibroblastoid cells from either normal or AML marrow was not affected by rhCSF-1, which indicates that CSF-1 does not directly act on such cells. This is in keeping with previous works demonstrating that within the hemopoietic microenvironment, only monocytes/macrophages are directly responsive to CSF-1 (Byrne et al, 1983; Stanley et al, 1983). On the other hand, it is important to note that the ability of normal and AML fibroblastoid cells to sustain CFU-C and BFU-E in chimeric LTMC was very similar. This observation, however, needs to be taken with caution since only one experiment of this type was performed. Furthermore, this result is not a proof that fibroblastoid cells do not derive from the leukemic clone, although it does suggest that no functional differences exist between normal and AML fibroblastoid cells.

Previously in this thesis, I have pointed out the possibility that, due to differences in culture conditions, fibroblastoid cells developed in fibroblasts cultures may be functionally different from those present in stromal layers. Thus, although the results presented in this section suggest that (i) there were no functional differences between normal and AML marrow-derived fibroblastoid cells in fibroblast cultures, and (ii) fibroblastoid cells present in fibroblast cultures did not produce the inhibitory activities, we are not certain that these two observations apply to fibroblastoid cells in stromal adherent layers. On the other hand, the fact that fibroblastoid cells in SAL interact with macrophages and this does not seem to occur in FIBR cultures makes it difficult to compare the results observed in both types of cultures. Indeed, the possibility exists that fibroblastoid cells are capable of producing the inhibitory activity but they

require a signal from macrophages, thus, in FIBR cultures no production of the inhibitory activity was observed.

**Table 25**

**Cellular composition of the normal and AML cultures**

| Culture | | rhCSF-1 | Fraction[a] | Cell Type(%) Mφ | Fibr | Blasts | Other[b] |
|---|---|---|---|---|---|---|---|
| normal | LTMC | + | NA | 29 ± 6 | <1 | 3 ± 2 | 67 ± 3 |
| | LTMC | + | A | 43 ± 4 | 52 ± 5 | 1 ± 1 | 5 ± 2 |
| | SAL | + | A | 39 ± 5 | 49 ± 4 | 2 ± 1 | 11 ± 3 |
| | FIBR | + | A | 1 ± 1 | 99 ± 2 | <1 | <1 |
| AML | LTMC | - | NA | 30 ± 3 | <1 | 17 ± 4 | 53 ± 7 |
| | LTMC | - | A | 34 ± 5 | 50 ± 2 | 6 ± 3 | 9 ± 2 |
| | SAL | - | A | 32 ± 4 | 46 ± 5 | 11 ± 3 | 12 ± 3 |
| | FIBR | - | A | 2 ± 3 | 97 ± 2 | <1 | 1 ± 1 |
| | BLAST | - | NA | <1 | <1 | 97 ± 2 | 3 ± 3[c] |

Results represent mean ± SD of 3 - 6 experiments for each type of culture. Each experiment corresponds to the bone marrow from a different normal subject or AML patient. 300 cells per experiment were analyzed. Cultures were established as described in Chapter III.

a NA = Nonadherent A = adherent

b immature myeloid cells, mature myeloid cells and some lymphoid cells

c lymphoid cells

**Table 26**

**Growth of hemopoietic progenitors from normal bone marrow in semisolid cultures containing different supplements**

| Supernatant | % of growth | | |
|---|---|---|---|
| | CFU-Mix | BFU-E | CFU-C |
| -- | 100 | 100 | 100 |
| N-LTMC$_c$ (n=10) | 98 ± 7 | 103 ± 7 | 97 ± 4 |
| N-LTMC$_{CSF-1}$ (n=10) | 26 ± 8[a] | 46 ± 8[a] | 62 ± 7[a] |
| N-SAL$_{CSF-1}$ (n=8) | 22 ± 5[a] | 43 ± 10[a] | 64 ± 9[a] |
| N-FIBR$_{CSF-1}$ (n=7) | 101 ± 6 | 97 ± 4 | 99 ± 3 |
| AML-LTMC$_c$ (n=8) | 34 ± 8[a] | 71 ± 6[b] | 79 ± 6[b] |
| AML-LTMC$_{CSF-1}$ (n=8) | 46 ± 9[a] | 84 ± 7[b] | 96 ± 3 |
| AML-SAL$_c$ (n=8) | 30 ± 5[a] | 66 ± 4[a] | 74 ± 3[b] |
| AML-FIBR$_c$ (n=7) | 94 ± 6 | 98 ± 2 | 97 ± 4 |
| AML-BLAST$_c$ (n=6) | 44 ± 7[a] | 76 ± 5[b] | 75 ± 4[b] |

Results represent mean ± SD of a given number (n) of supernatants tested separately. Each supernatant was collected from a culture established with bone marrow cells from a different normal subject or AML patient. Supernatants were collected at week 3 + 2 days, except for AML BLAST cultures, in which they were collected at day 2.

All the semisolid cultures contained Epo (1 U/mL) and PHA-LCM (10% v/v). Supernatants were added at 10% (v/v).

a $p<0.025$ as compared to 100%

b $p<0.05$ as compared to 100%

**Table 27**

**Progenitor cell numbers in the nonadherent fractions of chimeric LTMC containing normal marrow-derived adherent layers (No. colonies/flask)**

| | CFU-C[a] | | | | BFU-E[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | SAL[b] | | Fibr[c] | | SAL[b] | | Fibr[c] | |
| Week | C | CSF-1 | C | CSF-1 | C | CSF-1 | C | CSF-1 |
| 1 | 614 | 407 | 539 | 517 | 286 | 193 | 239 | 261 |
| 2 | 496 | 352 | 400 | 421 | 192 | 101 | 142 | 172 |
| 3 | 337 | 227 | 309 | 297 | 101 | 73 | 90 | 109 |
| 4 | 216 | 132 | 220 | 231 | 74 | 32 | 37 | 42 |

Results presented correspond to a single experiment. Fibroblast numbers were $1.81 \pm 0.16 \times 10^6$/flask in SAL, and $2.11 \pm 0.23 \times 10^6$/flask in FIBR.

a Input values were: CFU-C = 2438/flask; BFU-E = 1247/flask

b SAL = stromal adherent layer

c Fibr = fibroblast layer

**Table 28**

**Progenitor cell numbers in the nonadherent fractions of chimeric LTMC containing AML marrow-derived adherent layers (No. colonies/flask).**

| | CFU-C[a] | | | | BFU-E[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | SAL[b] | | Fibr[c] | | SAL[b] | | Fibr[c] | |
| Week | C | CSF-1 | C | CSF-1 | C | CSF-1 | C | CSF-1 |
| 1 | 513 | 584 | 508 | 491 | 68 | 127 | 190 | 184 |
| 2 | 321 | 387 | 417 | 402 | 13 | 39 | 115 | 101 |
| 3 | 238 | 306 | 289 | 306 | 19 | 46 | 91 | 84 |
| 4 | 102 | 222 | 239 | 233 | 22 | 39 | 43 | 37 |

Results presented correspond to a single experiment. Fibroblast numbers were $1.72 \pm 0.09 \times 10^6$/flask in SAL, and $1.95 \pm 0.23 \times 10^6$/flask in FIBR.

a Input values were: CFU-C = 2438/flask; BFU-E = 1247/flask

b SAL = stromal adherent layer

c Fibr = fibroblast layer

## C) TNFα IS PRESENT IN THE INHIBITORY ACTIVITIES

TNFα is a cytokine produced by activated macrophages, which can be found as a soluble or as a cell-associated molecule localized on the macrophage cell surface (Nathan, 1987). *In vivo* and *in vitro* studies have demonstrated that TNFα is a potent hemopoietic inhibitor that significantly affects the erythroid and, to a lesser extent, the myeloid arms of hemopoiesis (Peetre et al, 1986; Johnson et al, 1989; Ulich et al, 1990; Means et al, 1990). Its production is a tightly regulated process in which several factors are involved. One of the major inducers of TNFα production is CSF-1 (Warren & Ralph, 1986).

Recently, different reports have shown that blast cells from patients with AML are capable to constitutively produce significant amounts of TNFα in liquid cultures (Oster et al, 1989; Kobari et al, 1990). In fact, it has been suggested that such a cytokine may be implicated in the inhibition of normal hemopoiesis in AML. Based on the above observations, it seemed reasonable to suggest that the presence of the inhibitory activities in normal rhCSF-1-treated LTMC and in AML LTMC may have been due to the production of TNFα by normal macrophages and AML blasts and macrophages.

### Presence of TNFα in the culture supernatants

Our first approach in order to test this hypothesis was to measure the levels of TNF in the supernatants from normal and AML cultures. As shown in Figure 18, significant levels of TNF were detected in supernatants from cultures that contained the soluble inhibitory activities, suggesting that TNF is one of the factors present in such activities. Interestingly, whereas addition of rhCSF-1 to normal LTMC stimulated TNF production, rhCSF-1 down-modulated the

production of TNF by the leukemic cells. Cell-associated TNF was also quantitated, however, the levels were below the detection limit (not shown), which suggested that practically all the TNF produced is released into the supernatant.

To demonstrate that TNFα is part of the soluble inhibitory activities, anti-TNFα monoclonal antibody (125 ng/ml) was added to the culture supernatants for two hours before they were added to the semisolid cultures. Incubation with anti-TNFα resulted in a significant reduction of the inhibition of CFU-MIX, BFU-E, and CFU-C (Table 29). However, anti-TNFα was never able to completely neutralize the inhibitory activity present in rhCSF-1 treated normal LTMC, which suggested that TNFα was present in such an activity but was not the only factor involved. On the other hand, anti-TNFα completely neutralized the inhibitory activity produced in AML LTMC, which indicated that TNFα was practically the only cytokine responsible for the soluble activity produced in such cultures.

**Effect of anti-TNFα in LTMC**

In order to assess to what extent production of TNFα contributes to the inhibition of hemopoiesis in rhCSF-1-treated normal LTMC and in AML LTMC, anti-TNFα monoclonal antibody was added to LTMC. When normal LTMC were treated with rhCSF-1, a dramatic reduction in CFU-C and BFU-E was observed (Figure 19), confirming our previous results. Addition of anti-TNFα to LTMC resulted in a slight (not significant) increase in CFU-C levels. In contrast, a larger increase was observed in the levels of BFU-E. However, they never reached the values in untreated cultures, which is in keeping with the results

observed in semisolid cultures (Table 29).

Addition of anti-TNFα to AML LTMC resulted only in a slight increase in the levels of CFU-C (Figure 20). Addition of both anti-TNFα and rhCSF-1 to AML LTMC resulted in higher levels of CFU-C, however, the effect observed was a little less than additive. This may be due to the fact that part of the effect of rhCSF-1 in AML LTMC is, actually, down-regulation of TNFα production. It is important to note that TNFα was undetectable in LTMC treated with anti-TNFα monoclonal antibody, indicating that the activity of the cytokine was completely blocked.

Similar results to the ones mentioned above were obtained when anti-TNFα was added to chimeric LTMC containing AML-derived stromal adherent layers. A slight increase (not significant) was observed in the levels of CFU-C (Table 30), and higher increments were observed in BFU-E levels. In neither case, anti-TNFα restored the levels of hemopoietic progenitors to those observed in untreated cultures containing normal adherent layers. Although these results suggest that TNFα production is not the only mechanism by which AML cells inhibit normal hemopoiesis in chimeric LTMC, no definite conslusions can be made since only one experiment of this type was performed.

**Prostaglandin E levels in LTMC**

Different reports have shown that prostaglandin E (PGE) has negative effects on hemopoiesis both *in vivo* and *in vitro* (Kurland & Moore, 1977; Pelus & Gentile, 1988). It has also been shown that PGE production by human monocytic cells is induced by CSF-1 (Raghavachar, 1988). Thus, we measured the levels of PGE in two normal and two AML LTMC. PGE levels in normal untreated

LTMC were 43 and 51 pg/ml, respectively, whereas the levels in CSF-1-treated cultures were 700 and 740 pg/ml, respectively. Leukemic untreated LTMC showed extremely high levels of PGE (1650 and 1500 pg/ml, respectively) and the levels were not different in CSF-1-treated LTMC (1470 pg/ml).

**Discussion**

The results described above demonstrate that TNFα is present in the soluble inhibitory activities produced in rhCSF-1-treated normal LTMC and in AML LTMC. The presence of the inhibitory activity in the supernatants from rhCSF-1-treated normal LTMC correlated with increased levels of TNF. However, addition of anti-TNFα monoclonal antibody to colony assays supplemented with supernatants from rhCSF-1-treated normal LTMC or to rhCSF-1-treated normal LTMC resulted only in partial neutralization of the inhibitory activity. It seems, then, that TNFα is not the only factor present in the soluble inhibitory activity. In addition to TNFα, other hemopoietic inhibitors, such as interferon-$\tau$ and prostaglandin-$E_2$ have been shown to be produced by monocytic cells in response to CSF-1 (Warren & Ralph, 1986; Raghavachar et al, 1988), thus, it is possible that those factors may be involved in the CSF-1-induced inhibition of human hemopoiesis in normal LTMC. In keeping with this idea, we observed that PGE levels were significantly increased (15-fold) in CSF-1-treated LTMC.

TNF levels in AML LTMC also correlated with the presence of the soluble inhibitory activity. The TNF levels observed in AML LTMC (3 - 45 pg/ml) were rather low when compared to the levels reported in the sera of patients with AML (218 - 4672 pg/ml, Cimino et al, 1991), myelodysplasia (297

± 65 pg/ml, Zoumbos et al, 1991), or in cultures of AML blasts (<20 - 8100 pg/ml, Oster et al, 1989; <50 - 1200 pg/ml; Kobari et al, 1990). This difference may be due, at least to some extent, to the fact that in most of those studies TNFα was quantitated by radioimmunoassay, which can detect biologically inactive material, whereas in this study we used a bioassay (Branch et al, 1991). On the other hand, the culture conditions reported in those studies were different from the ones used in our study and this may influence the production of TNFα.

Despite the fact that TNFα seemed to account for 100% of the soluble inhibitory activity detected in AML LTMC, addition of anti-TNFα into AML LTMC or into chimeric LTMC containing AML SAL caused only a slight (not significant) effect. This indicated that other mechanisms may also participate in the inhibition of normal hemopoiesis in AML LTMC. These may involve other factors produced by AML cells that are located in the extracellular matrix developed in the cultures, and thus, would not be detected in the culture supernatant. Another related possibility is that the AML cells produced inhibitors that remained on their surfaces and were presented to the normal hemopoietic cells during direct cell-to-cell contact between normal and AML cells. A third possibility is that some inhibitor(s) present in the culture acted via stromal cells, and thus, although present in the culture supernatant, it had no direct inhibitory effect on hemopoietic cells in semisolid cultures. This may be, in fact, the case for PGE, an inhibitor of hemopoiesis, which was present at very high levels both in untreated and CSF-1-treated AML LTMC. Interestingly, Marley and colleagues have recently demonstrated the production of a PGE-mediated hemopoietic inhibitory activity in IL-1-treated LTMC (Marley et al, 1992). Such an activity seemed to be produced by macrophages and appeared to

be confined to the adherent layer, since supernatants from those cultures were not inhibitory to myeloid progenitors. Thus, similar mechanisms may be operating in our experimental system.

In this study, TNFα activity was blocked by using an anti-TNFα monoclonal antibody. An alternative way, not explored in this thesis, for blocking TNFα may invlove the use of the hemorrheologic agent pentoxifylline (PTX), which has been shown to modulate TNFα production via inhibition of TNFα mRNA transcription (Han et al, 1990). Thus, it would be of interest to test the effects of PTX in AML LTMC.

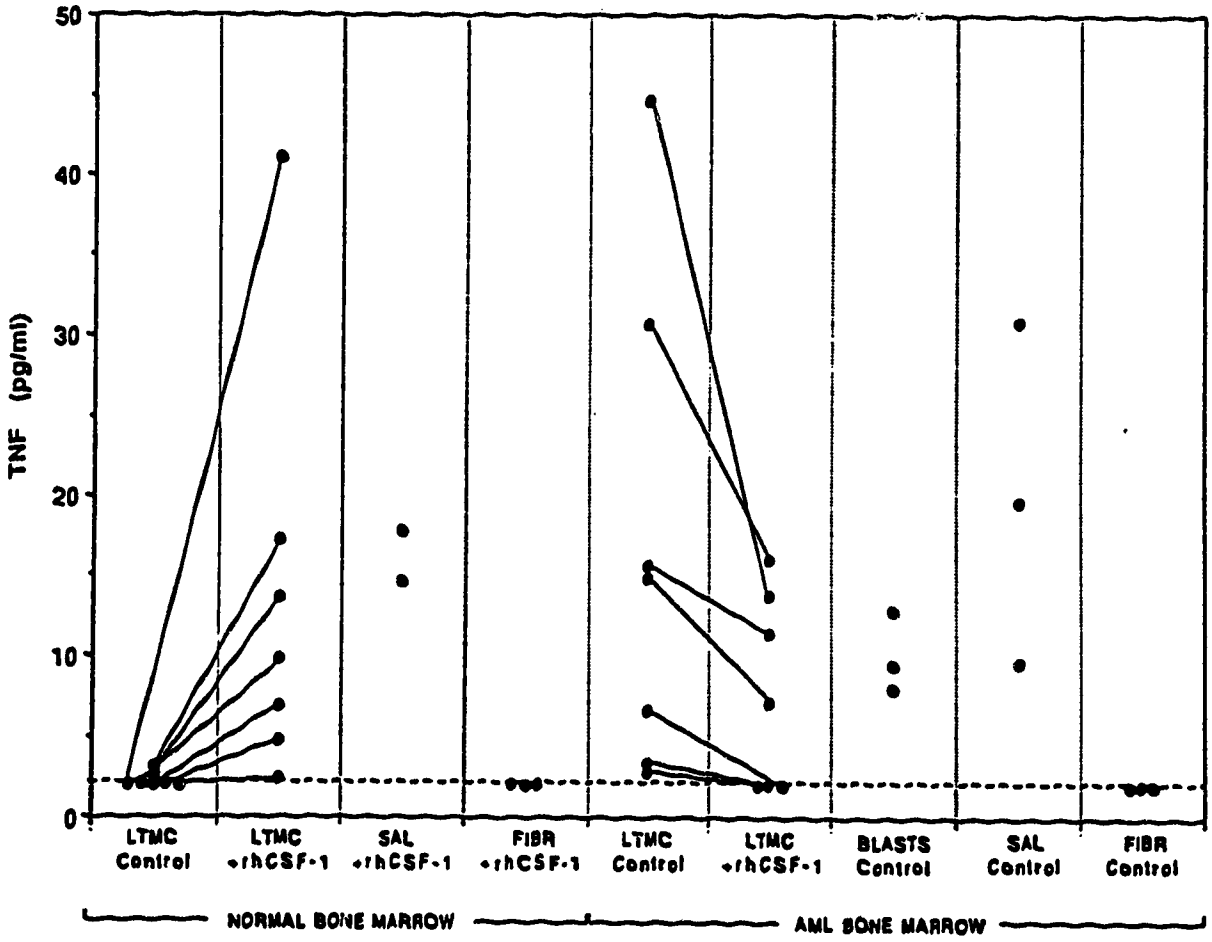


**Figure 18.** TNF levels in supernatants from normal or AML cultures. Supernatants were collected at week 3 + 2 days, except for BLAST cultures, in which they were collected at day 2. Each point represents the culture from a different normal subject or AML patient.

**Table 29**

**Growth of hemopoietic progenitors from normal bone marrow in semisolid cultures containing different supplements.**

| Supernatant | anti-TNFα | % of growth CFU-Mix | BFU-E | CFU-C |
|---|---|---|---|---|
| -- | -- | 100 | 100 | 100 |
| $N\text{-}LTMC_c$ | -- | 104 ± 4 | 94 ± 6 | 103 ± 5 |
| $N\text{-}LTMC_c$ | + | 96 ± 7 | 95 ± 6 | 97 ± 5 |
| $N\text{-}LTMC_{CSF\text{-}1}$ | -- | 16 ± 9[a] | 47 ± 7[a] | 61 ± 5[a] |
| $N\text{-}LTMC_{CSF\text{-}1}$ | + | 51 ± 5[a] | 80 ± 4[b] | 83 ± 4[b] |
| $AML\text{-}LTMC_c$ | -- | 42 ± 6[a] | 66 ± 6[a] | 73 ± 4[a] |
| $AML\text{-}LTMC_c$ | + | 97 ± 7 | 98 ± 5 | 99 ± 6 |
| $AML\text{-}LTMC_{CSF\text{-}1}$ | -- | 50 ± 7[a] | 71 ± 3[a] | 84 ± 5[b] |
| $AML\text{-}LTMC_{CSF\text{-}1}$ | + | 101 ± 6 | 97 ± 3 | 101 ± 4 |

Results represent mean ± SD of three experiments. Supernatants from 2 normal and 2 AML LTMC (each one from a different normal subject or AML patient) were tested separately in each experiment.

All the semisolid cultures contained Epo (1 U/mL) and PHA-LCM (10% v/v) Supernatants were added at 10% (v/v). Anti-TNFα added at 125 ng/mL

a $p < 0.025$ as compared to 100%

b $p < 0.05$ as compared to 100%

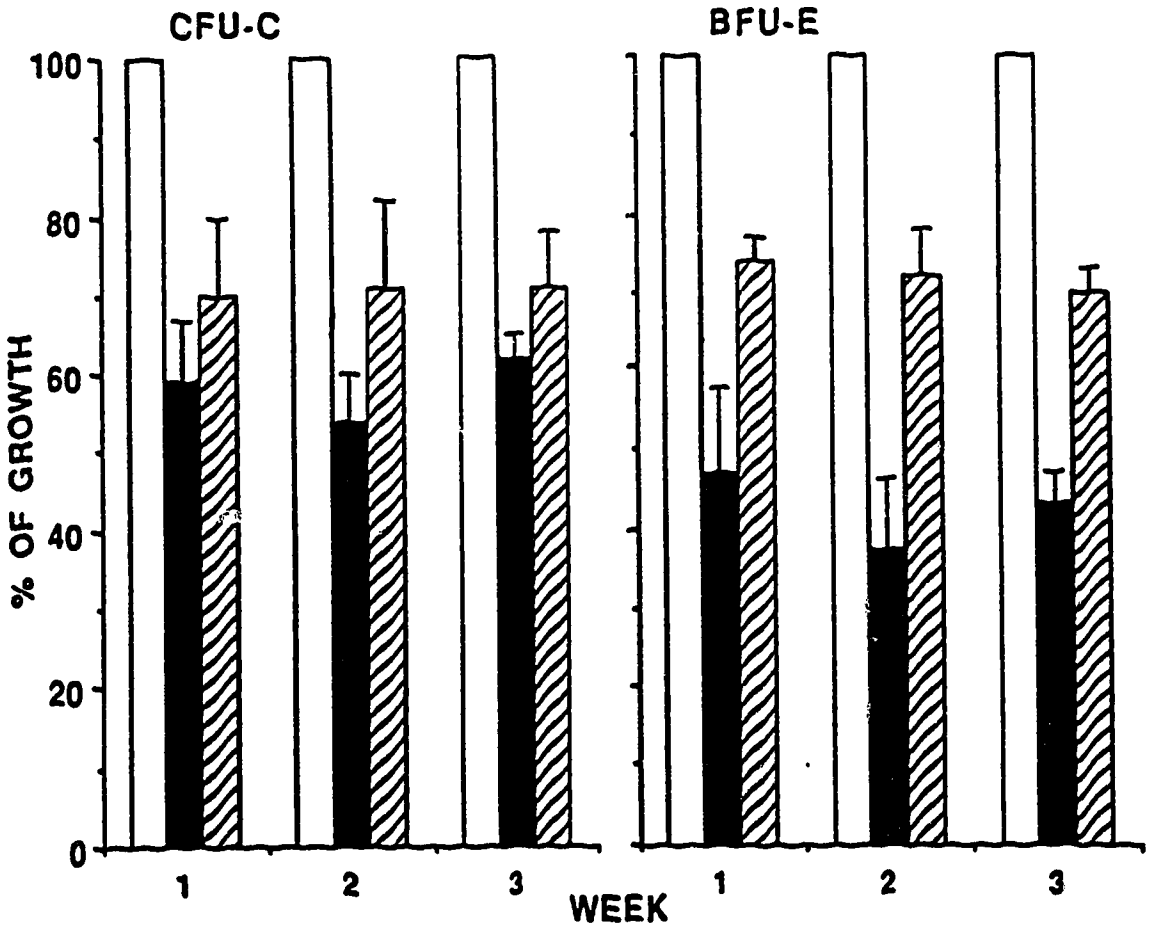


**Figure 19.** Relative growth of nonadherent CFU-C and BFU-E in untreated (open bars) LTMC, and in LTMC treated with rhCSF-1 (filled bars) or rhCSF-1 plus anti-TNFα monoclonal antibody (diagonal bars). Results represent mean ± SD of two experiments, each one established with the bone marrow from a different normal subject.

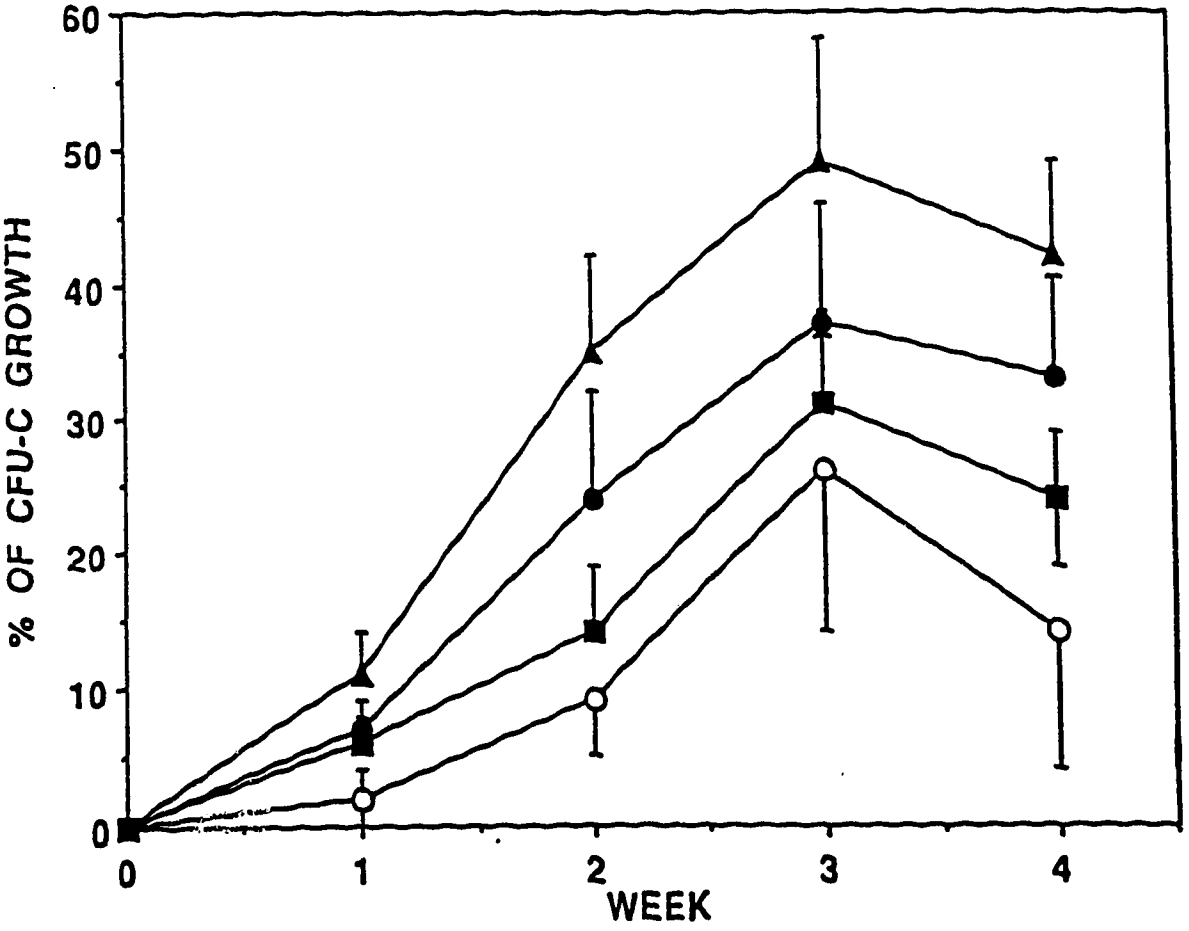


**Figure 20.** Relative growth of CFU-C in AML LTMC established in the absence (open circles) or presence of anti-TNFα (filled squares), rhCSF-1 (filled circles), or anti-TNFα plus rhCSF-1 (filled triangles). Results represent mean + SD of two experiments and are expressed as the percent of the growth in chimeric LTMC containing normal stromal adherent layers. Each experiment was established with the bone marrow from a different AML patient (patients 2 & 4).

**Table 30**

**CFU-C numbers (colonies/flask) in the nonadherent fraction of chimeric LTMC containing AML-derived SAL**

| | AML SAL | | | | |
|---|---|---|---|---|---|
| Week | Control | rhCSF-1[a] | anti-TNFα[b] | rhCSF-1 + anti-TNFα[c] | normal SAL |
| 1 | 536 | 607 | 590 | 649 | 697 |
| 2 | 361 | 421 | 429 | 492 | 526 |
| 3 | 271 | 359 | 342 | 377 | 439 |
| 4 | 139 | 209 | 187 | 226 | 292 |

Results from a single experiment.

a SAL established in the presence of rhCSF-1.

b anti-TNFα was added every 72 hrs, at 125 ng/mL, from day 0 - 21.

c anti-TNFα was added as described above, to AML SAL established in the presence of rhCSF-1.

**Table 31**

**BFU-E numbers (colonies/flask) in the nonadherent fraction of chimeric LTMC containing AML-derived SAL**

| | AML SAL | | | | |
|---|---|---|---|---|---|
| Week | Control | rhCSF-1[a] | anti-TNFα[b] | rhCSF-1 + anti-TNFα[c] | normal SAL |
| 1 | 72 | 131 | 197 | 268 | 307 |
| 2 | 29 | 82 | 131 | 181 | 216 |
| 3 | 12 | 40 | 79 | 92 | 127 |
| 4 | 27 | 44 | 57 | 77 | 96 |

Results from a single experiment.

a SAL established in the presence of rhCSF-1.

b anti-TNFα was added every 72 hrs, at 125 ng/mL, from day 0 - 21.

c anti-TNFα was added as described above, to AML SAL established in the presence of rhCSF-1.

## VII. SUMMARY AND DISCUSSION

The major goal of the present study was to determine whether the AML-derived hemopoietic microenvironment developed *in vitro* is functionally normal. In order to do so, LTMC from both AML and normal (as control) bone marrow were established and specific functional parameters of the microenvironmental cells were analyzed. The experimental approach followed in this study was based on the previously reported observation that CSF-1, a macrophagic stimulating factor, is produced by and acts on microenvironmental cells. Thus, we compared the ability of AML and normal microenvironmental cells to (i) produce CSF-1, (ii) respond to exogenous CSF-1, and (iii) sustain normal hemopoiesis in LTMC.

*CSF-1 production.* CSF-1 was constitutively produced in normal and AML LTMC. Both fibroblastoid cells and macrophages seemed to be involved in this process. A significant patient-to-patient variation was observed in AML LTMC and CSF-1 levels both above and below the normal range were observed. The low levels detected in some AML cultures seemed to be due to the poor fibroblast growth occuring in such LTMC. On the other hand, the increased levels of CSF-1 observed in LTMC from one of the AML patients may be due to an increased production and/or reduced utilization of CSF-1.

When CSF-1 levels in normal and AML cultures containing similar numbers of macrophages and fibroblasts were compared, no significant difference was observed, indicating that the balance between production and utilization of CSF-1 in such cultures was very similar. Thus, this observation suggests that in terms of CSF-1 production, both normal and AML microenvironmental cells behave similarly.

*Response to CSF-1.* Both AML and normal microenvironmental cells responded to rhCSF-1. Since fibroblastoid cells were not affected by this factor (no changes in CFU-F or fibroblast numbers, no induction of inhibitory activty production, no effect on the hemopoietic supportive capacity) our results strongly suggest that the major CSF-1-responsive cell in LTMC is the macrophage. Assuming that this is the case, it is interesting that rhCSF-1 had opposite effects on normal and AML-derived macrophages. On the one hand, CSF-1 induced normal marrow-derived macrophages to produce a soluble activity (containing TNFα and possibly PGE), capable of inhibiting the growth of hemopoietic progenitors. As a result of this, hemopoiesis was significantly reduced in normal LTMC treated with rhCSF-1. In contrast, AML-derived macrophages did not produce the inhibitory activity in the presence of rhCSF-1. In fact, rhCSF-1 down-modulated TNFα production and had no effect on PGE levels. These observations suggested significant differences in the physiology of normal and AML marrow-derived macrophages.

*Hemopoietic supportive capacity.* AML-derived stromal adherent layers (up to 45% macrophages) showed a reduced ability to sustain normal hemopoietic progenitors in chimeric LTMC, as compared to normal marrow-derived adherent layers. Such a reduced hemopoietic supportive capacity seemed to be due to (i) the constitutive production of inhibitory molecules such as TNFα and PGE, and (ii) other mechanisms that may involve direct cell-to-cell interactions between normal and AML cells. AML-derived adherent layers consisting exclusively of fibroblastoid cells showed a similar hemopoietic supportive capacity to the one observed in normal marrow-derived fibroblast layers, suggesting that AML-derived fibroblastoid cells are functionally normal.

Thus, it seemed that the defective hemopoietic supportive capacity of the AML hemopoietic microenvironment was due to the presence of abnormal macrophages, which is in keeping with the results observed in terms of CSF-1 responsiveness.

The fact that macrophages developed in AML LTMC seemed to be functionally abnormal raised the question of whether these cells are of normal or leukemic origin. Langley et al (1986) have presented evidence that macrophages developed in AML liquid cultures derive from AML blasts rather than from normal progenitor cells. I am not certain that in our system this is the case, however, this question may be approached in the future by cytogenetic studies usin purified cell populations from AML patients that have chromosomal alterations or by *in situ* hybridization analysis using specific probes.

Throughout this study, a consistent correlation was observed between the levels of CFU-F in the AML patients' marrow and several LTMC parameters, such as (i) total numbers of adherent cells, (ii) levels of adherent hemopoietic progenitors, and (iii) CSF-1 production. These results suggest that CFU-F play a key role in the development of human LTMC and give support to the idea that effective hemopoiesis is the result of the interplay between hemopoietic progenitor cells and the hemopoietic microenvironment.

The present study has also given an important insight into the biology of CSF-1. For many years, CSF-1 has been recognized as a positive modulator of hemopoiesis and as the primary regulator of the monocytic lineage (Stanley et al, 1983). In this study, however, it has been demonstrated that, depending on the culture conditions, CSF-1 can act as a negative regulator of hemopoiesis by inducing the production by macrophages of hemopoietic inhibitors.

In order to place this study into context, it is interesting to compare our results in normal and AML human LTMC with the *in vivo* situation. Although most of the studies on the *in vivo* effects of hemopoietic growth factors in humans involve the use of Epo, GM-CSF or G-CSF, some phase I/II clinical trials using rhCSF-1 have already been reported. In a preliminary study, melanoma patients showed a dose-dependent rise in peripheral blood monocytes when treated with rhCSF-1 (10 - 80 $\mu$g/kg/day; Bajorin et al, 1989). However, no data was presented regarding the effects of rhCSF-1 on marrow progenitors. Thus, it was not clear whether such an increase in circulating monocytes was due to an actual stimulation of monocytic progenitors or to the release of monocytes from a non-circulating pool.

In a bone marrow transplantation study, patients treated with rhCSF-1 had a decreased number of days with white blood cell counts < 100/ml, compared to historical controls (Peters et al, 1989). However, the authors observed no changes in bone marrow cellularity or marrow progenitor cells. Thus, this study suggests that the positive effects of rhCSF-1 on the patients' recovery may not be due to a direct effect of this factor on the growth of monocytic progenitors in the bone marrow. In a more recent study, Nemunaitis and colleagues assessed the effects of rhCSF-1 in patients with invasive fungal infection before and after BMT and observed no significant changes in monocyte, neutrophil or lymphocyte counts (Nemunaitis et al, 1991).

Since in two out of the three studies mentioned above no data was presented on the effect of rhCSF-1 on hemopoietic progenitors, it is not possible to compare such studies with our results. However, it is interesting that in the report by Peters and colleagues (Peters et al, 1989), rhCSF-1 had no effect on

marrow progenitors. Previous *in vivo* studies in mice have demonstrated the presence of a unidirectional blood-bone marrow barrier to CSF-1, that is to say, CSF-1 can easily be transfered from bone marrow to blood, but the reverse process will take place only if CSF-1 is continuosly present in circulation at high concentrations (Shadduck et al, 1989; Chen, 1991). If a similar mechanism exist in humans, it may explain, at least in part, the results observed in the clinical trials described above.

Recently, Janowska-Wieczorek and colleagues reported increased levels of CSF-1 in the sera of patients with preleukemia (350 - 1700 U/ml), AML (800 - 1600 U/ml), and CML (650 - 2300 U/ml), as compared to the levels observed in normal subjects (250 - 500 U/ml). Interestingly, the CSF-1 levels in sera of patients in remission from AML were significantly lower than during active AML (Janowska-Wieczorek et al, 1991). Thus, it is interesting to speculate that the increased levels of CSF-1 observed in those patients have negative effects on the growth of normal hemopoietic cells. Increased levels of TNFα have also been reported in the sera of patients with preleukemia and AML (Zoumbos et al, 1991; Cimino et al, 1991), which correlates with the fact that CSF-1 induces the production of TNFα by monocytic cells. Based on the above observations and considering the results of the present study, it seems reasonable to suggest that CSF-1 plays a dual role in hemopoiesis *in vivo*. When present at relatively low concentrations, i.e. within the normal range, this factor has positive effects on hemopoietic (macrophagic) progenitors and mature macrophages. On the other hand, when CSF-1 levels are significantly above normal (due to increased production and/or decreased utilization), the production of hemopoietic inhibitors (such as TNFα and PGE) by macrophagic cells increases, which, in

turn, down-regulates normal hemopoietic growth. Supporting this idea are the results described by Shadduck and colleagues, indicating that a single or double injection of CSF-1 into mice causes a marked increase in the number of CFU-C in cycle one day after the injection. However, if a third injection of CSF-1 is administered during the 24-hr cycle, there is a near complete inhibition of cycling of the CFU-C (Shadduck et al, 1989).

Considering previous studies demonstrating that, in normal conditions, monocytes/macrophages are the only marrow microenvironmental cells that bear CSF-1 receptors on their surface (Byrne et al, 1983; Ashmun et al, 1989), and our own data demonstrating that adherent layers (containing up to 45% macrophages) were responsive to rhCSF-1 whereas fibroblast layers were not, it seems reasonable to suggest that macrophages are the cells mediating the CSF-1 response both *in vitro* and *in vivo*.

The correlation of our results in AML LTMC with the *in vivo* situation is not so clear. We observed that at high concentrations, rhCSF-1 inhibited leukemic hemopoiesis (decreased levels of blast cells and AML-CFU) and stimulates normal hemopoiesis (increased levels of macrophages and CFU-C). As mentioned before, CSF-1 levels in sera of AML patients are significantly above normal (Janowska-Wieczorek et al, 1991). If this were also true in the marrow hemopoietic microenvironment, and considering the results observed in AML LTMC, we would expect to find reduced levels of AML blasts and leukemic progenitors, and increased levels of macrophages and normal progenitors. However, it is obvious that this is not the case.

The above results, then, lead to the question of whether or not AML LTMC reproduce the *in vivo* situation during active AML. The answer seems to

be no, and at least two observations support this answer: (i) Whereas AML cells have a growth advantage *in vivo* over their normal counterparts, they are not sustained in LTMC for periods longer than 6 weeks. The reason for this is not clear, however, a possible explanation is that a particular cell type, critical for the growth of AML cells, does not develop in LTMC. Related possibilities involving specific cytokines can be suggested. (ii) High levels of CSF-1 in AML patients correlate with a decreased normal hemopoiesis, whereas high levels of CSF-1 in AML LTMC result in the stimulation of putative normal hemopoiesis. One might speculate that *in vivo*, AML blasts do not mature into macrophages, and that the macrophages present in the marrow microenvironment derive from normal progenitors, which, although reduced in number, are present in bone marrow. Thus, such macrophages respond to high CSF-1 levels by producing inhibitory molecules.

In spite of the fact that AML LTMC do not seem to reflect the *in vivo* situation during active AML, the characterization of this experimental system is important since LTMC have already been used as a method for purging leukemic cells for ABMT (Barnett et al, 1989; Chang et al, 1989). Thus, the manipulation of AML LTMC, by using different cytokine combinations, may improve the capacity of this system to eliminate leukemic cells and stimulate the growth of their normal counterpart. On the other hand, it is noteworthy that AML LTMC reflect a situation similar to the *in vivo* condition during remission from AML, that is to say, down-modulation of leukemic hemopoiesis and stimulation of normal hemopoietic growth. Thus, the characterization of AML LTMC may be relevant to the understanding of the biology of hemopoiesis during remission from AML, and this, in turn, may help in the development of clinical treatments

for certain leukemic patients.

**Future directions**

In this section, I would like to briefly discuss two specific lines of investigation that seem to me of particular importance. One of them refers to the use of LTMC as a method for purging leukemic cells for ABMT, the second one refers to an *in vivo* model for the study of the interactions between hemopoietic and microenvironmental cells.

(i) As I have mentioned before, recent reports indicate that LTMC seem to be an effective system for purging leukemic (AML and CML) cells for ABMT (Barnett et al, 1989; Chang et al, 1989). As demonstrated in the present study, AML hemopoiesis in LTMC can be further manipulated by the addition of recombinant cytokines. This suggests that LTMC may be improved as a leukemia-purging system. Previous studies have demonstrated that leukemic (AML) cells can be induced to mature by different cytokine combinations (including CSF-1, G-CSF and IFN-$\gamma$; Salem et al, 1989), other biologic inducers of differentiation, such as dihydroxylated vitamin $D_3$ and retinoic acid (Ferrero et al, 1992), as well as by low doses of cytotoxic drugs (Koeffler et al, 1985) Thus, I think that it would be of significant interest to explore the use of some of these agents in AML and CML LTMC.

(ii) Among the *in vitro* models that have been developed for the study of human hemopoiesis, LTMC is the one that best resembles the *in vivo* situation (Dexter et al, 1984). However, it is clear that this system is still far from reproducing the structural and physiological complexity found in bone marrow. This is particularly evident when AML or CML hemopoiesis are studied in

LTMC (Coulombel et al, 1983, 1985; Janowska-Wieczorek et al, 1991).

During the last few years, there has been a concensus regarding the need for an animal model in which human hemopoiesis could be studied. Recently, several groups have reported the successful transfer of normal and leukemic human hemopoietic cells into severe combined immunodeficiency (SCID) mice (McCune et al, 1988; Kamel-Reid et al, 1989; Sawyers et al, 1992). Indeed, human cells from patients with AML, CML, and AL(lymphocytic)L have been shown to grow in the bone marrow and peripheral blood of SCID mice in a pattern closely resembling human leukemias. Successful transfer of a human lymphoid microenvironment into SCID mice has also been reported (Waller et al, 1991).

Previous *in vitro* studies by Keating et al (1982), Singer et al (1984), and Islam et al (1988) have indicated the existence of a primitive progenitor cell common to hemopoiesis and its microenvironment, suggesting that the hemopoietic microenvironment is transplantable. Using the SCID-human mouse model this hypothesis can be explored in greater detail. Recently, fibroblastic progenitors have been shown to express the cell surface antigen CD34 (Simmons & Torok-Storb, 1991b) as well as a unique antigen recognized by the antibody STRO-1 (Simmons & Torok-Storb, 1991a). Based on these observations, the purification of such cells seems to be out of question. On the other hand, macrophagic progenitors (CFU-M) have also been recently purified (Sato et al, 1991). Thus, it would be of interest to transfer human fibroblastc and macrophagic progenitors from normal and AML subjects into SCID mice and assess their effects on the growth of hemopoietic progenitors and the production of hemopoietic cytokines.

I certainly believe that these two lines of investigation, among others, would give important insights in understanding the biology of human hemopoiesis under both normal and pathological conditions, and would also be helpful in the improvement of treatments for certain hematological disorders.

## VIII. CONCLUDING REMARKS

The study of blood cell formation is a field that has expanded in an impressive manner during the last three decades. Before the classic works of Till and McCulloch, Pluznik and Sachs, and Bradley and Metcalf in the 60's, most of the studies regarding the organization of the hemopoietic system were based on morphological approaches, the existence of a pluripotent stem cell was only a hypothesis, and erythopoietin was the only factor known to play a key role in the regulation of hemopoiesis. Today, different models have been developed that allow the study of hemopoiesis either *in vivo* or *in vitro*, murine and human hemopoietic stem and progenitor cells have been isolated, and more than twenty regulatory molecules have been identified, purified, functionally characterized, and their genes have been cloned. Furthermore, some of those regulators, such as EPO, GM-CSF, and G-CSF are already being used in the clinic.

Hemopoiesis research, however, is a continuously expanding field. More hemopoietic cytokines are being identified; new roles for already known molecules are being discovered; new techniques for the purification of human stem cells are being developed, and new strategies for the treatment of hematological disorders are being designed.

The present study contributes to the understanding of the *in vitro* biology of human hemopoiesis, under both normal and leukemic (AML) conditions. Throughout this project, I have demonstrated that:

(i) CSF-1 is constitutively produced in normal and AML LTMC, in a process that involves both fibroblasts and macrophages [**Mayani H et al(1992) Exp Hematol 20 (in press)**].

(ii) When added exogenously to human LTMC, rhCSF-1 has the capacity to modulate, in a negative or in a positive manner, the myeloid and erythroid arms of hemopoiesis [**Mayani H et al (1991) Blood 78:651-657; Mayani H et al (1991) Leukemia 5:8-13**].

(iii) CSF-1 effects are mediated by microenvironmental cells, which may respond to this factor by secreting hemopoietic regulators, such as TNFα or PGE, which in turn, affect the growth of progenitor cells [**Mayani H et al (1992) Leukemia (in press)**].

(iv) The AML-derived hemopoietic microenvironment developed in LTMC has an impaired hemopoietic supportive capacity, which is due to the presence of functionally defective macrophages derived from the leukemic clone [**Mayani H et al (1992) Exp Hematol 20 (in press); Mayani H et al (1992) Leukemia (in press)**].

(v) Human erythropoietin has a dual role when added to human LTMC. On the one hand, it stimulates erythropoiesis, although in a transient way; on the other hand, it inhibits myelopoiesis. The actual mechanisms of the latter effect need to be elucidated [**Mayani H et al (1990) Exp Hematol 18:174-179**].

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## APPENDIX I

### Effect of anti-TNFα and two other antibodies in human hemopoietic colony assays

The results presented in Chapter VI, section C (Table 29) suggested that TNFα is (i) one of the factors present in the inhibitory activity produced in normal LTMC treated with rhCSF-1 and (ii) probably the only factor responsible for the inhibitory activity detected in AML LTMC. In those experiments, however, no control antibodies (Ab) were used to confirm that the effects observed in cultures containing anti-TNFα were actually due to such an Ab. Thus, an exriment was performed to address this problem.

Normal bone marrow cells were cultured in semisolid colony assays in the presence of EPO + PHA-LCM and supernatants from normal and AML LTMC. Two control Ab were used to compare their effects with those of anti-TNFα.

(a) mouse γ-globulins (IgG1=46%, IgG2a=24%, IgG2b=27%, IgG3=2%).

(b) P1D6 supernatant mouse anti-human α-5 integrin (IgG3).

Both Ab were kindly provided by Dr. Linda Pilarski (University of Alberta). The Ab were incubated with LTMC supernatants for 2 hr before the supernatants were added to the culture. Mouse IgG were added to the culture at a final concentration of 42.5 μg/ml, which gives a final IgG3 concentration of approximately 850 ng/ml. Anti-α-5 integrin (P1D6 supernatant) was added at 30 μl/ml of culture. The Ab concentration in the supernatant was 2-5 μg/ml, thus, the Ab concentration in colony assay was 60-150 ng/ml. Mouse anti-human TNFα (IgG3) was added at 125 ng/ml.

As shown in Table II, the two control Ab had no significant effect, either in the absence or in the presence of LTMC supernatants, on the growth of hemopoietic colony-forming cells. On the other hand, anti-TNFα effects were similar to those observed in the previous experiments (Table 29). These results suggest that the anti-TNFα effects in semisolid cultures supplemented with LTMC supernatants are specific and confirm the presence of TNFα in the inhibitory activities detected in LTMC supernatants.

**Table I**

**Hemopoietic colony growth in the presence of different supplements**

| Supplement* | Colony growth (%) CFU-Mix | BFU-E | CFU-C |
|---|---|---|---|
| -- | 100 | 100 | 100 |
| anti-TNFα | 88 ± 12 | 93 ± 2 | 96 ± 3 |
| IgG | 100 ± 12 | 103 ± 3 | 89 ± 3 |
| anti-α-5 | 88 ± 24 | 89 ± 2 | 90 ± 4 |
| N-LTMC$_c$ | 112 ± 0 | 100 ± 3 | 98 ± 4 |
| N-LTMC$_{CSF-1}$ | 24 ± 12 | 42 ± 6 | 63 ± 4 |
| N-LTMC$_{CSF-1}$ + anti-TNFα | 48 ± 12 | 68 ± 5 | 80 ± 2 |
| N-LTMC$_{CSF-1}$ + IgG | 24 ± 12 | 35 ± 7 | 65 ± 4 |
| N-LTMC$_{CSF-1}$ + anti-α-5 | 12 ± 12 | 38 ± 2 | 60 ± 3 |
| AML-LTMC$_c$ | 36 ± 0 | 53 ± 3 | 64 ± 5 |
| AML-LTMC$_c$ + anti-TNFα | 88 ± 12 | 96 ± 3 | 97 ± 4 |
| AML-LTMC$_c$ + IgG | 36 ± 24 | 51 ± 5 | 66 ± 5 |
| AML-LTMC$_c$ + anti-α-5 | 12 ± 0 | 43 ± 2 | 61 ± 3 |

* All the cultures contained Epo (1U/mL) and PHA-LCM (10% v/v). Anti-TNFα was added at 125 ng/mL, IgG at 42.5 μg/mL, anti-α-5 at 60-150 ng/mL, and LTMC supernatants at 10% v/v.

Results represent mean ± SD of duplicate cultures.