Fibroblasts Derived From Marrow and Lung Differ in the Production of Factors that Influence the Growth of Rat Hemopoietic Colonies

Running Title: Function of Fibroblast Subpopulations

Hong Wang

M.D. (Beijing Medical University), 1983

Department of Physiology/McGill Cancer Center

McGill University

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The journey of a thousand miles begins with one step.

Lao-Tse

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ABSTRACT

Normal hemopoiesis is restricted to specific organs and is governed by a network of cellular and humoral factors. The work of this thesis explored some of the functional differences between stromal cells derived from hemopoietic and non-hemopoietic organs that might be relevant to the control of blood cell growth. As determined by *in vitro* assays of hemopoietic precursor cells, colony-stimulating activity predominated in the culture medium of fibroblastoid cells derived from rat bone marrow but not those from the lung. However, the medium in which lung fibroblastoid cells had been grown contained primarily an inhibitory activity. This inhibitory activity decreased the clonogenic growth of both normal and leukemic (BNML cell line) rat myeloid cells, but not that of eight other rat tumor cell lines derived from various organs. Nether murine hemopoietic precursors nor human myeloid cell lines were affected by the inhibitor(s). Fibroblastoid cells cultured in the presence of hydrocortisone, appeared to release less inhibitory activity into the media. Antisera to transforming growth factor-beta, interferon-beta, or ferritin did not neutralize the inhibitory activity, which migrated in the 100-120 kDa range by gel filtration, and bound to immobilized heparin and wheat germ agglutinin. At this time I am not able to attribute this constellation of characteristics to any other identified molecule.

RESUME

L'hematopoiese est controlee par un reseau de facteurs humoraux et cellulaires et se produit normalement uniquement dans certains organes specifiques. Le travail presente dans cette these decrit quelques-unes des differences fonctionnelles retrouvees entre les cellules stromales derivees d'organes hematopoietiques et non-hematopoietiques pouvant etre impliquees dans le controle de la croissance des cellules sanguines. Les techniques in vitro de croissance de cellules precurseurs a l'hematopoiese permettent de demontrer une activite stimulatrice de colonies dans le milieu de culture de cellules fibroblastoides obtenues de la moelle osseuse de rat. On ne retrouve cependant pas cette activite dans le milieu de culture lorsque ces cellules sont obtenues de poumons de rat. Au contraire, le milieu de culture dans lesquelles ces cellules sont incubees contient plutot une activite inhibitrice qui diminue la croissance de clones de cellules normales et de leucemie myeloide de rat (BNML) mais pas celle de huit autres lignees tumorales de rat derivees d'organes divers. Ni les cellules precurseurs hematopoietiques de souris, ni les lignees myeloides humaines ne sont affectees par cet(s) inhibiteur(s). Les cellules fibroblastoides cultivees en presence d'hydrocortisone semblent relacher moins d'activite inhibitrice dans le milieu. Les antrsera contre le facteur de croissance transformont beta (TGF-B), l'interferon beta ainsi que la ferritine ne neutralisent pas cette activite

inhibitrice qui, en filtration sur gel apparait dans un pic migrant aux environs de 100-120 kDa. Cette activite se lie aussi a l'heparine immobilisee et a la lectine de germe de ble (WGA). Pour l'instant, nous sommes incapables d'attribuer ces caracteristiques a toute autre molecule connue.

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Chapter 1 INTRODUCTION[1]

[1]The references cited in this chapter are listed in "References" from Page 157 to Page 195.

Hemopoiesis is a regulated process through which mature blood cells reach the circulation after they have developed within the bone marrow. The earliest multipotential stem cells (CFU-S in Fig.1) may remain quiescent, self-renew, or begin to differentiate further into progenitors that are committed to a given lineage and that can be identified by <u>in vitro</u> clonogenic assays (e.g., BFU-E, CFU-E, CFU-GM, CFU-G, CFU-M, CFU-Eos, and CFU-MK in Fig.1). Through further stages, the committed precursors complete their proliferative phase and finally mature into functioning erythrocytes, lymphocytes, platelets, granulocytes, and macrophages. To maintain a steady state in the circulation, the rate of exit of mature blood cells from the marrow is closely linked to the demands of the organism.

Although stem cells can circulate, the hemopoietic process is not ubiquitous throughout the body. During embryonic and fetal development of mammals the major focus of blood formation shifts from the yolk sac to the liver and spleen, and finally to the bone marrow. Although the marrow remains the major hemopoietic organ throughout adult life, the spleen and liver can support a limited growth of blood cells under certain pathological conditions.

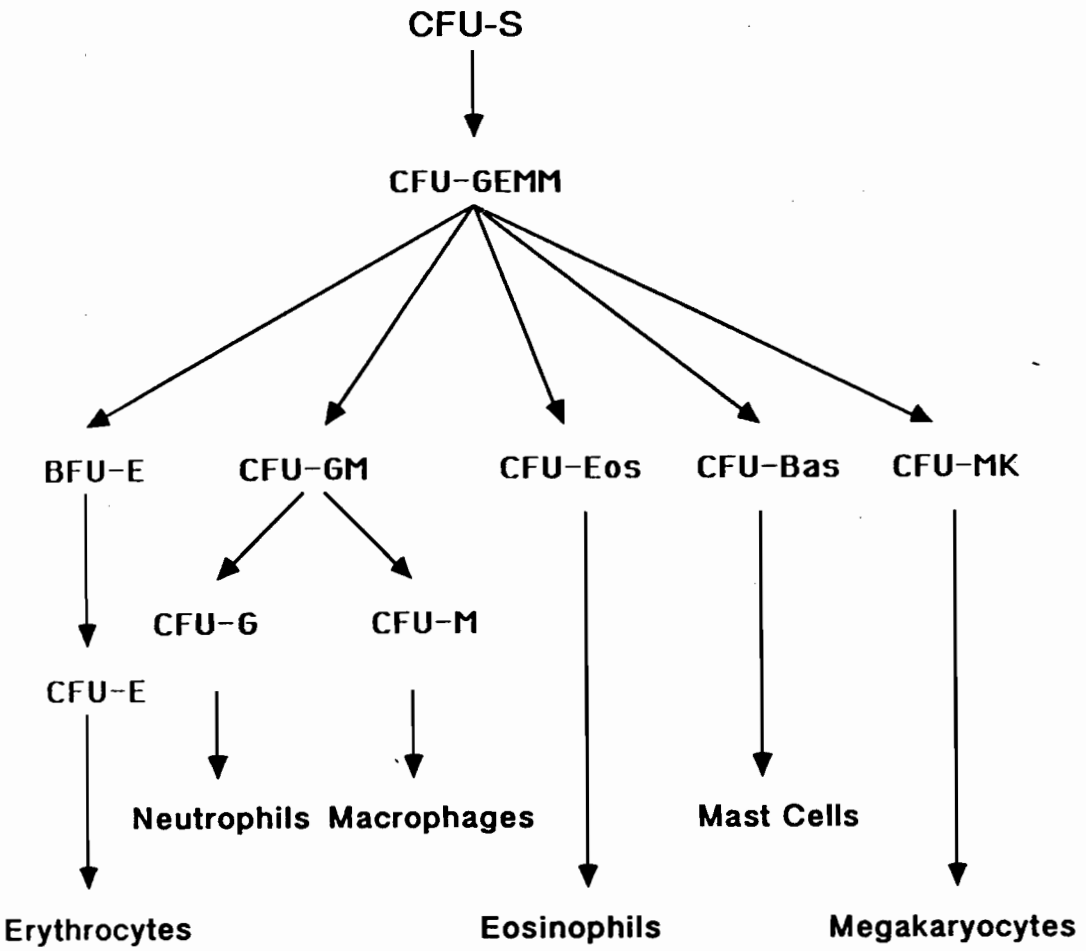

Figure 1 Relationship among precursors of mature blood cells.

The problem that was studied initially in this thesis was the search for a cellular basis to explain why hemopoiesis is restricted to the bone marrow, and for some of the important regulatory mechanisms that contribute to it. The following sections will review the current understanding of how cellular and humoral components interact within the marrow microenvironment to regulate differentiation of blood cells.

1.1 The concept of a hemopoietic inductive microenvironment.

A "hemopoietic microenvironment" can be defined as the cellular and non-cellular components of an organ that support proliferation and differentiation of hemopoietic stem cells. Thus, mechanisms to be explained include the process of stem cell lodgement, induction of lineage commitment, and regulation of terminal maturation [Allen 1990].

Early experimental evidence for restricted sites of hemopoiesis came from the studies of Till & McCulloch [1961] and Wolf & Trentin [1968], who showed that after donor marrow was infused into the veins of an irradiated mouse, hemopoietic colonies developed only in the spleen and bone marrow. Further experiments demonstrated that marrow and spleen differ in the degree to which each one supports myeloid or erythroid proliferation - in the marrow, granulocytic colonies predominated over erythroid in a 2:1 ratio, but in the spleen, erythroid loci outnumbered myeloid 3:1. This preference continued to be expressed in fragments of marrow tissue that had been implanted by trochar into the spleen. The individual colonies that grew across junctions between stromal cells in the spleen or in the marrow maintained their respective tendencies for erythroid and granulocytic development. Subsequent transplantation of spleen colonies into another irradiated mouse gave rise to secondary colonies in the marrow and spleen with the previously-observed lineage

preference [Trentin 1970]. These phenomena were interpreted to indicate that the controlling determinant was not a selection (homing) of precursors committed to a different lineage, but to the influence of the local environment [Trentin 1970].

Even within the marrow the proliferative and maturational components of hemopoietic activity appear not to be distributed uniformly. The concentration of stem cells (CFU-S) is highest near the endosteal surface and decreases toward the bone axis, the more committed CFU-C are maximal at approximately one third of the radius [Lord 1975], and the mature granulocytes and megakaryocytes are most evident in the central region [Shackney 1978]. Although the overall rate of CFU-C proliferation does not vary in these regions, that of CFU-S decreases with the distance from the bone surface [Lord 1978]. The capacity for self-renewal of CFU-S, however, is higher in the central than in the endosteal region, indicating a possible gradient in the controlling elements for stem cell differentiation [Mason 1989]. This may be related in part to the existence of opposing gradients of CFU-S inhibitors and stimulators, the former being greater near the bone surface and the latter near the center [Lord 1988].

In addition to maturational gradients there also may exist microdomains related to lineage, which may not take the same form in different species. For example, in rat marrow erythroid precursors are localized in apposition to the adventitial surface of the venous sinuses, in contrast

to the granulocytic precursors which are found in the center of the cord-like hemopoietic tissue between the sinuses [Weiss 1976]. In embryonic chick bone marrow, however, erythropoietic precursors proliferate and differentiate within sinusoids, while granulopoiesis develops in extravascular regions [Sorrell & Weiss 1980]. The endocloning experiments of Lambertsen & Weiss [1983, 1984] using murine bone marrow corroborate those of Lord described above. They found that undifferentiated colonies resided along the bone surface in the endosteal region, erythrocytes proliferated centrally around small arteries and arterioles, and granulocytes initially developed in all areas of marrow and then concentrated along the inner bone surface. Megakaryocytic colonies, in a reverse pattern, originated along the bone surface but differentiated centrifugally. Similar to the marrow, in the spleen (murine) lineage precursors resided in specific areas. Erythroid colonies usually are observed in the red pulp, in contrast to granulocytic colonies which grow under the capsule [Wolf 1979]. Different from both of them, megakaryocytic colonies appear in a region of about 500 um from the splenic surface [Choudhury 1989a].

Experiments on extramedullary transplantation of marrow stroma to sites such as under the renal capsule have been instructive [Tavassoli 1968]. When bone matrix was transplanted, hemopoiesis was observed only after stromal cells had regrown [McCarthy 1984]. Although bone formation and proliferation of myeloid cells occurred after transplantation of marrow stroma, lymphopoiesis was

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W/Wv type lesion - "White Sports (Ws) was also identified in the rat [Niwa 1991 and reviewed by witte 1990].) When cells from the spleen or femur of either Sl/Sld or Sl/+ mice were transplanted into individual Sl/Sld mice, the number of CFU-S in the Sl/+ grafts were several times greater than that of the Sl/Sld grafts [Fried 1973]. Direct evidence for a defect in the supporting cells has come from the observation that stromal elements from Sl/Sld marrow grown on cellulose ester membranes provided suboptimal hemopoietic colony formation [Knospe 1985]. In addition, the Sl/Sld stromal cell lines derived from long term bone marrow culture (LTBMC) (details of LTBMC can be found in next section), when engrafted with either hemopoietic progenitors from LTBMC or an interleukin-3-dependent cell line B6SUtA, had less capacity to support the BFU-E and CFU-GEMM compared to the Sl/+ cell lines in the absence of exogenous interleukin-3 [Anklesaria 1987]. This may be related in part to a suppressive effect of Sl/Sld stromal cell lines [Zuckerman 1986, Onoue 1989]. Recently, the product of Sl locus was proven to be the ligand for the receptor encoded by a proto-oncogene, c-kit [Huang 1990, Zsebo 1990, Williams 1990 and reviewed by Witte 1990] that probably is identical to the W locus [Geissler 1988, Chabot 1988]. This ligand appears to be a multipotent growth factor, which is active on erythroid, myeloid and lymphoid precursors [Anderson 1990, Martin 1990, McNiece 1991]. In addition, several mutations thought to originate in the Sl locus appear now to be associated with deletions or

alterations of the ligand's gene [Zsebo 1990, Copeland 1990]. Therefore, the nature of the microenvironmental deficiency in mice bearing the Sl/Sld genetic lesion can be, at least partly, the result of a deficiency or an alteration of this ligand on the stromal cells.

Occasionally, rare states of marrow failure in humans have been attributed to a microenvironmental defect. For example, Ershler [1980] described the case of a woman with chronic anemia whose bone fragments inhibited CFU-E, although her marrow precursors gave rise to an increased number of erythroid colonies. The authors proposed that the etiology of the anemia was due to an "unfavorable microenvironment".

In summary, the evidence discussed above affirms that hemopoiesis is initiated, maintained and regulated within an inductive microenvironment. Deficiency or distortion of this microenvironment can result in hemopoietic failure. Although their importance is well recognized, the components, differentiation, and function of the marrow stroma are not well understood.

1.2 Role of the bone marrow stroma in hemopoietic regulation.

All organs are composed of both supporting (stroma) and functional (parenchyma) elements. In the bone marrow, the parenchyma consists of developing blood cells and their progenitors; the stroma includes the extracellular matrix and a heterogeneous assortment of mesenchymal cells. The long-term bone marrow culture (LTBMC) model developed by Dexter has formed the basis for much of what is known about how the marrow stroma influences blood cell growth [Dexter 1973]. In this system, a marrow-derived adherent cell layer is established to which a fresh sample of marrow is later added ("recharging"). The adherent layer thereby forms a microenvironment permissive to both stem cell proliferation and differentiation, which can be maintained for several months or even longer than a year [Dexter 1977]. Further modifications showed that recharging was not necessary if hydrocortisone was added to the culture during formation of the adherent cell layer [Greenberger 1978].

Ultrastructural studies on the LTBMC system have yielded clues to the functional organization of the stroma, which consists predominantly of fat cells, sinus endothelium, fibroblasts, and in some instances, osteoblasts. These cells, along with a subpopulation of marrow macrophages, supply the necessary components for a hemopoietic microenvironment. It appears that cellular "niches" form into which stem cells migrate, and as

determined by mechanisms yet to be identified, either remain dormant or differentiate [Allen 1981]. Within this complex, histological studies have shown that there are areas of preference for different blood cell lineages. For example, progenitors of all lineages were observed in association with fibroblastoid cells, and undifferentiated types were usually close to osteoblasts [Lambertsen & Weiss 1983].

During the first few days of a "Dexter" culture, macrophages migrate under what appear to be a form of specialized fibroblast called a "blanket cell", forming what has been suggested to be the in vitro equivalent of the "stem cell niche" [Schofield 1978]. Myeloid precursors can "home" to these areas, mature, and then pass out into the liquid phase. The proliferation and maturation of erythroid cells, on the other hand, proceeded on the surface of macrophages in the "erythroblastic islets" (specific areas for erythroid maturation), but were not directly associated with fibroblasts [Allen 1991]. However, fibronectin, a fibroblast product, may be involved in the homing of erythroid precursors to these areas [Coulombel 1991, detail see section 3]. Another candidate EbR (erythroblast receptor), which is specifically expressed on macrophages in the erythroblastic islets, may also participate in the process [Crocker 1991, Morris 1991]. It was hypothesized that in vivo macrophages not only supply a surface for erythroid cell maturation, but also carry the mature erythroid cells toward the sinus and deliver them into the circulation

[Allen 1990, 1991]. Each of the major cell types that contribute to the formation of a functional stroma will now be summarized.

Fibroblastoid cells are an essential element of the LTBMC and can be defined operationally by the fact that they are not phagocytic, produce collagens type I and III, stain for alkaline phosphatases (at least in some species) [Bentley 1980], but lack von Willebrand's factor (vWF) [Song 1984]. Several observations have suggested that different surface antigens (e.g., Thy-1 in mice [Piersma 1985a] and CALLA, common acute lymphoblastic leukemia antigen in humans [Ndumbe 1985]) may be expressed on fibroblastoid cells derived from different organs. The investigation of pure populations of fibroblasts became possible only after applications of modern techniques such as fluorescence-activated cell sorting (FACS) [Lennon 1986], and the ability to grow colony-forming units of fibroblasts (CFU-F) [Castro-Malaspina 1980]. It was shown that the increase of CFU-F precedes the increase of hemopoiesis in the yolk sac, liver, spleen, and bone marrow during fetal development [Van Den Heuvel 1987] and also precedes the increase in blood cellularity and granulocyte/macrophage precursors in the hemolytic anemia induced by phenylhydrazine [Piersma 1985b]. This is indirect evidence that proliferation of fibroblasts is a prerequisite for the development of hemopoietic cells. Furthermore, the proliferation of granulocyte, monocyte, and even early erythroid precursors (BFU-E) was facilitated by fibroblastoid cell lines derived from

either murine [Garnett 1984] or human [Harigaya 1985] LTBMC. However, fibroblastoid cells derived from anemic patients failed to do so [Hotta 1985]. This may be related to the decreased production of CSF by these cells [Gordon 1983].

Another essential component of the marrow stroma is the sinusoidal endothelium which forms the first barrier between the circulation and the extra-vascular sites of hemopoiesis. There is evidence that one of their functions is to act as a sensor and "gate-keeper" to control the exit of mature blood cells into circulation [Weiss 1970]. In LTBMC they can be identified by immunostaining with antibodies to vWF [Keating 1982] and collagen type IV [Zucherman 1983], although vWF may not be detected on sinusoidal endothelium due to the absence of Weibel-Palade bodies in which vWF is stored [Irie 1986]. Apart from their barrier function, endothelial cells possess a lectin-like substance which specifically recognizes galactosyl residues [Kataoka 1985]. This may be related to homing of stem cells from circulation to marrow (detail see section 3). In addition, the endothelium can be stimulated by other cells or cell products to release growth factors active on both hemopoietic (see section 4) and other stromal cells (e.g., fibroblasts [Scarra 1985]).

Although the marrow fat composes a significant portion of the marrow space in adult humans, it is less prominent in rodents, and its role in the function of the hemopoietic microenvironment remains controversial. Furthermore, some reports have claimed that it may differ

from peripheral fat in its metabolic control (e.g., lipogenesis), being less responsive to insulin and more dependent on corticosteroids [Greenberg 1978]. The evidence linking adipocytes with a possible regulatory role in maturation of blood cell precursors is indirect. In conditions of erythroid hypoplasia the fat spaces increase in the adipose cells, whereas in hyperproliferative disorders such as polycythemia or acute leukemia they decrease, suggesting a reciprocal relationship between hemopoietic activity and fat deposition [reviewed by Tavassoli 1984a]. This reciprocal relationship may be associated with retardation of the differentiation of adherent cells [Harigaya 1986] instead of their proliferation [Lanotte 1982]. Furthermore, there may be two subpopulations of adipocytes that constitute red and yellow marrow respectively. When fibroblasts associated with red or yellow marrow were implanted under rabbit kidney capsule, the red gave rise to hemopoietic islands, in contrast to the yellow which produced only fat [Bainton 1986]. In the LTBMC, the quantity of adipocytes appeared to be greater in marrow cultured in the presence of hydrocortisone and to correlate with the steroid-enhancement of hemopoietic activity [Greenberg 1986]. The close contact observed between adipocytes and granulocytic precursors [Allen 1981] in the LTBMC have led to speculations that adipocytes also might contribute to the control of granulocyte migration into the circulation [Tavassoli 1984a].

Being derived from the myeloid lineage, macrophages are not included in the strict definition of the "stroma", but nonetheless appear to be an essential component of the hemopoietic microenvironment. It is possible that some of them may have undergone further differentiation, in that subpopulations expressing the Forssman antigen appear to be involved in cell-cell interactions [Sadahira 1988]. In both in vivo [Weiss 1976] and in vitro [Allen 1981] experiments, they can be identified by their phagocytic properties and high concentration of acid phosphatase. They have been observed in various locations including islets of erythroblastic activity, beneath the sinusoidal endothelium, and in aggregates of granulocyte precursors. The capacity to secrete several products that influence hemopoiesis, including M-, G-, GM-CSF, erythropoietin [reviewed by Rich 1985, Vogt 1989] and hemopoietic inhibitors (details in section 4 & 5) implies a role in regulation of cell proliferation. Also, they may phagocytose imperfect or damaged cells and assist in extrusion of nuclei from mature erythrocytes [Weiss 1976]. In addition to the often close proximity of adipocytes and macrophages to endothelial cells, further interaction in nutrient control is suggested by the presence of lipoprotein lipase on the membranes of all three of them. [Allen 1984].

1.3 Contribution of the extracellular matrix (ECM) to stromal function.

Components of the extracellular matrix (ECM), which include the collagen framework and its associated glycoproteins and proteoglycans [Campbell 1985, 1988], play a major role in the organization of stromal function. Collagen itself is produced by stromal cells [Stuart 1980, Zuckerman 1983]), and as first demonstrated by Reddi [1976], transplantation of a crude collagen-containing matrix into subcutaneous sites is followed by the colonization of mesenchymal cells that can support the growth of hemopoietic islands. Further studies in LTBMC demonstrated that inhibition of collagen biosynthesis by cis-hydroxyproline led to decreased production of CFU-S and CFU-GM. This implies that the production of collagen is a necessary part of the sequence that leads to the formation of a hemopoietic microenvironment [Zuckerman 1985].

Like all other organs, the bone marrow contains the matrix-associated glycoproteins laminin and fibronectin. Early and late erythroid precursors (BFU-E and CFU-E) bind to both fibroblasts [Tsai 1987] and culture dishes coated with ECM or fibronectin [Coulombel 1988]. This adhesion process can be blocked by antibodies to fibronectin [Coulombel 1988, Tsai 1987], or by the active-site tetrapeptide (Arg-Gly-Asp-Ser) of its binding fragment (110 kDa) [Gianocotti 1986]. As the CFU-E mature, binding decreases and the cells are released [Vuillet-Gaugler

1990]. Furthermore, for erythroid precursors to complete their maturation, binding to fibronectin in the stroma might be required. Evidence for this includes the observation that DMSO-induced cells are arrested at the erythroblast stage and fail to enucleate unless they have been in contact with fibronectin [Patel 1987]. The possible physiologic relevance of this process has been suggested by similar observations using cell lines (e.g., hemin-induced human erythroleukemia K562 and DMSO-induced murine erythroleukemia) [Virtanen 1987, Patel 1984, 1986], and reticulocytes from patients with hemolytic anemia [Patel 1985].

A novel 60 kDa protein called hemonectin [Campbell 1985, 1987], which appears not to affect erythroid cells, has been implicated in the maturation of granulocytic and megakaryocytic precursors. It appears to be produced by cells of the marrow, but not those of the spleen, and tends to be distributed in an organ specific manner to preferred sites of granulopoiesis. Within the marrow, it is concentrated at the endosteal surface of the bone, which has been shown to be a locus of stem cell renewal and differentiation [Campbell 1987]. Analogous to what had been observed with erythroid cells and fibronectin, the ability of granulocytic precursors to adhere to hemonectin was lost during maturation of normal CFU-GM or leukemic cell lines (HL-60) [Campbell 1987, 1990].

Oligosaccharide moieties of plasma membrane glycoproteins of sinusoidal endothelial cells have been implicated in the process of "homing" by circulating stem

cells. Using lectin histochemistry in both in situ and in vitro models, it has been shown that the lumenal and ablumenal sides of the endothelium present different structures [Pino 1984, Tavassoli 1985]. Further experiments demonstrated that artificial glycoproteins bearing defined oligosaccharides bind preferentially to lectin-like proteins on the endothelial cell surface [reviewed by Tavassoli 1990]. Stem cells and other precursors also may possess lectin-like proteins that could be specifically recognized by oligosaccharides. Both CFU-S and CFU-GM can be agglutinated by galactose and mannose-containing oligomers [Aizawa 1987a, 1987b], BFU-E by mannose, and CFU-E by fucose [Konno 1990]). Furthermore, a 110 kDa protein with specificity for mannose and galactose-containing chains was purified from the membrane of precursor cell lines (B6SUtA and FDC-P1) [Hardy 1986, Matsuoka 1989]. Based on experiments with mice demonstrating that, after intravenous injection in the presence of galactose and mannose, fewer CFU-S and CFU-GM appeared in the marrow [reviewed by Tavassoli 1990], it was suggested that this lectin-like protein might play a role in the homing of circulating stem cells.

Several subgroups of sulfated proteoglycans, including chondroitin, dermatan, heparan and hyaluronic acid, have been detected in LTBMC [Wight 1986], and may be produced by marrow fibroblastoid cells [Heremans 1989]. By perturbing their relative concentrations, such as by adding B-D-xylosides to cultures to augment chondroitin sulfate, the production of CFU-S and CFU-GM was increased

[Spooncer 1983]. Selective digestion of heparan sulfate from the surface of marrow-derived fibroblasts diminished the adhesion of granulocytes [Del Rosso 1981]. Further insight into the interaction between the ECM and hemopoiesis has been provided by observations that growth factors can be selectively concentrated by specific proteoglycans [reviewed by Keating & Gordon 1988; Gordon 1987; Roberts 1988]. Furthermore, the concentration of certain proteoglycans may vary in hemopoietic disorders. For example, marrow and spleen chondroitin sulfate A, B, and C were found to be decreased in experimental polycythemia [Noordegraaf 1979], whereas sulfated acid mucopolysaccharides accumulated in mice bearing the Sl/Sl^d genetic lesion [McCuskey 1973] and in those with starvation-induced anemia [Pearson 1967].

In summary, through its ability to concentrate hemopoietic growth factors and to induce progenitors to differentiate <u>in situ</u>, the extracellular matrix actively participates in the stromal control of hemopoiesis. Its capacity to selectively bind hemopoietic progenitors may be part of the homing mechanism used by circulating stem cells to find its appropriate niche, both naturally and after bone marrow transplantation.

1.4 Hemopoietic growth factors.

Hemopoiesis appears to be controlled in part by soluble growth factors that act on precursors of each of the different blood cells. Of those that have been identified, the genes have been isolated as genomic clones and the protein products obtained in pure form by recombinant techniques [reviewed by Metcalf 1989a]. At pharmacological concentrations *in vivo* they can stimulate an increase in peripheral blood cellularity, but with the exception of erythropoietin, their physiologic role is not well understood [reviewed by Morstyn 1988]. In *in vitro* culture systems they must be present for the precursors to survive, proliferate, and differentiate into recognizable colonies (CFU-C), and it is this ability to support clonogenic growth that enabled the proteins to be identified and gave them the name of colony-stimulating factors (CSFs). Thus, multi-CSF (also known as IL-3) is a multilineage factor promoting the growth of both myeloid (CFU-GM) and erythroid (BFU-E) colonies. Granulocyte/macrophage-CSF (GM-CSF) induces the formation of colonies containing both of granulocytes and macrophages (CFU-GM). Macrophage-CSF (M-CSF), however, induces only macrophage colonies (CFU-M). Erythropoietin (Epo), which is essential for the growth of CFU-E, also can be considered as a CSF. However, for any individual CSFs, by themselves, they are very poor stimulators of colony growth, which is more evident in serum-deprived conditions. The development of any lineage of blood cells

involves the interplay of at least several growth factors. The CSF requirements change during the process of precursor differentiation. For example, the requirement of IL-3 decreased with precursor differentiation, while Epo, G-CSF and M-CSF increased with precursor differentiation [reviewed by Migliaccio 1990]. The characteristics of each of CSF listed above will be summarized.

Multi-CSF.

Multi-CSF, also called IL-3, enhances the survival, proliferation, and differentiation of pluripotent marrow cells. In *in vitro* clonogenic assays it stimulates the growth of BFU-E, the earliest detectable erythroid precursor, and CFU-GEMM, which contains granulocytes, erythrocytes, macrophages, and megakaryocytes [reviewed by Schrader 1986]. However, for the full development of any these lineages, multi-CSF must act in synergy with other growth factors or interleukins (details discussed in later sections) [Morris 1990]. Because it can bind to marrow ECM [reviewed by Keating & Gordon 1988], it may act *in situ* in the bone marrow. In addition, the production of multi-CSF by the leukemic cell line WEHI-3B, and its action to stimulate the proliferation of other leukemic cell lines (e.g., FDC-P1) [Hapel 1985] suggest that in certain situations it might play a role in leukemogenesis.

Multi-CSF is a glycoprotein [Ihle 1982] with a mol wt of 41 kDa which reduces to 23 kDa under dissociating conditions. It was identified originally from mitogen-stimulated spleen cell conditioned medium [Nicola 1982], and is produced predominantly by T lymphocytes [Kelso 1985]. Although controversial, it appears to be secreted also by other cell types including keratinocytes [Luger 1985], yolk sac [Labastie 1984], and astrocytes [Frei 1986, Farrar 1989]). The activity of multi-CSF is restricted to a given species or at higher concentrations to others closely-related to the source [reviewed by Sieff 1987].

Granulocyte/macrophage CSF.

Granulocyte/macrophage-CSF (GM-CSF) acts on the precursors of granulocytes and macrophages inducing the formation of CFU-GM, and in synergy with other CSFs to promote the growth of CFU-GEMM, BFU-E, CFU-Eos, and CFU-MK [Dexter 1987; Metcalf 1982b]. In addition to its effects on immature cells, GM-CSF acts also on mature blood cells. It extends the time in the circulation of granulocytes and macrophages [Begley 1986], and enhances their metabolic (e.g., synthesis of membrane and nuclear proteins) and phagocytic activities [reviewed by Gough 1990; Lopez 1983]). In inflammatory sites, GM-CSF is chemotactic to neutrophils and macrophages and inhibits their migration, contributing to the efficient elimination of pathogens [reviewed by Morstyn 1988]. Under other conditions it can

either induce terminal maturation of myeloid leukemic cell lines or stimulate their proliferation [Metcalf 1979; Begley 1987].

The action of GM-CSF also is restricted to the same or closely-related species. It is a glycoprotein with 56% amino acid sequence homology between mice and humans. The murine form consists of 124 amino acids and its mol wt in reduced form is about 14 kDa [Sparrow 1985], whereas the human form is three residues longer [Motoyoshi 1982]. Although both of them are highly glycosylated, the oligosaccharides are not necessary for the biological activity [Metcalf 1985]. The protein originally was purified from human urine [Motoyoshi 1982] and murine lung conditioned medium after the cells had been stimulated in vivo by endotoxin [Burgess 1977]. In contrast to multi-CSF, GM-CSF is produced by many cell types in almost all organs [Nicola 1979].

Granulocyte CSF.

Granulocyte-CSF (G-CSF) initially was discovered as a differentiation-inducer of the WEHI-3B/D+ cell line, but later was shown to be a CSF. At lower concentrations it acts almost exclusively on granulocytic precursors in clonogenic assays [Nicola 1983], but in higher concentration it may stimulate other precursors such as CFU-E. In synergy with multi-CSF [Ikebuchi 1988] and M-CSF [McNiece 1988b], it stimulates the formation of CFU-GEMM, BFU-E and CFU-M. Similar to GM-CSF, G-CSF also enhances

the survival of human mature neutrophils [Begley 1986], stimulates their function [Lopez 1983], and can induce the M1 and WEHI-3B/D+ murine myelomonocytic leukemic cell lines and human HL-60 cells to mature into macrophage-like cells [reviewed by Nicola 1990] or granulocytes [Hara 1985].

In contrast to that of the previous factors, the activity of G-CSF is less restricted to species. Although originally purified from murine lung conditioned medium [Nicola 1983] and the human bladder carcinoma cell line 5637 [Welte 1985], it can be produced by almost all tissues. There is about 75% amino acid homology between the murine (24 kDa) and human (18 kDa) forms [Morstyn 1988; Welte 1985]

Macrophage CSF.

Macrophage-CSF (M-CSF) acts primarily on the mononuclear phagocytic lineage and promotes the growth and maturation of precursors [Dexter 1987], although some synergistic effects with multi-CSF [Bartelmz 1985] and GM-CSF [McNiece 1988a] have been reported. It also enhances various functions of monocytes and macrophages, including phagocytosis and migration [Khwaja 1991]. Because there is a 10,000-fold increase in M-CSF production by the murine uterus during pregnancy [Pollard 1986], which appears to be regulated by chorionic gonadotrophin [Bartocci 1986],

M-CSF might be involved in placental and fetal development.

This glycoprotein is produced by a broad spectrum of tissues and cells (monocytes, endothelial cells, fibroblasts, liver, placenta, lung, brain, heart, spleen and uterus [Rambaldi 1987; reviewed by Kawasaki 1990], and its activity is not restricted to species. The variability of its carbohydrate content accounts for the wide range of reported molecular weights (47-76 kD) [Das 1982, Csejtey 1986, Ralph 1986]. Overall, it is a highly conserved molecule, maintaining about 75% homology between the human and murine forms of M-CSF [reviewed by Kawasaki 1990].

Erythropoietin.

Erythropoietin also is a CSF. It is produced mainly by the kidney and can be purified from the urine of anemic patients. It selectively induces the terminal maturation of erythroid precursors, and in in vitro clonogenic assays it stimulates BFU-E in the presence of multi-CSF. In addition, it has been reported to potentiate human CFU-MK stimulated by multi-CSF [Reviewed by Krantz 1991].

Interleukins and hemopoiesis.

Almost all of the interleukins (ILs) have been shown to stimulate hemopoiesis either directly or indirectly, although the presence of other CSFs usually is required [reviewed by Mizel 1989]. IL-1 has been demonstrated to

act synergistically with multi-CSF, GM-CSF, and G-CSF to stimulate CFU-S [Zsebo 1988a], CFU-GEMM, BFU-E [Moore 1987], CFU-Eos [Warren 1988] and acute myeloblastic leukemic cells [Hoang 1988]. It also amplifies the effects of these CSFs by inducing the production of GM-CSF, G-CSF, M-CSF and IL-6 (detail will be discussed later) [Nicola 1990, Rodriguez-Cimadevilla 1990, Beauchemin 1991]. IL-4, also known as B-cell stimulatory factor-1, enhances basophil colony formation [Favre 1990] and mast cells growth [reviewed by Paul 1991] in addition to its action on B-cells. In the presence of erythropoietin, G-CSF, and M-CSF, IL-4 further enhances CFU-E, CFU-G and CFU-M respectively; in the presence of multi-CSF it suppresses CFU-GM formation [Rennick 1987b]. In addition, it also may augment production of M-CSF by fibroblasts [Henschler 1990]. On the other hand, IL-5 has a more lineage-restricted action. It selectively induces eosinophil proliferation and differentiation [Saito 1988]. Also known as B-cell stimulatory factor-2, IL-6 acts synergistically with multi-CSF to stimulate CFU-GEMM [Ikebuchi 1987], CFU-GM, and CFU-MK [Lotem 1989]. Furthermore, its <u>in vivo</u> administration results in increased BFU-E and CFU-GM in mice [Pojda 1990]. However, it may inhibit CFU-G formation in the presence of G-CSF [Katayama 1990].

The effect of CSFs on LTBMC.

The effect of growth factors on long-term bone marrow cultures is complex. For example, M-CSF is an inhibitor when added in recombinant form to human LTBMC. Although it is a stimulator of macrophage colonies *in vitro* and macrophage proliferation *in vivo*, it blocks hemopoietic precursors in LTBMC [Mayani 1991]. Similar observations were noted with combinations of M-CSF and GM-CSF or multi-CSF [Dexter 1992a, 1992b]. They strongly decreased CFU-M in LTBMC, in association with increased macrophage differentiation.

The extent to which CSFs support hemopoiesis in LTBMC is controversial. Initial studies showed that addition of pure CSFs or antibodies against them had little effect on myelopoiesis [Dexter 1980]. Subsequently, with other techniques such as the double layer agar system, rapid medium exchange, and serum deprived cultures, it has been possible to demonstrate that CSFs promote the production of blood cell precursors [Gualtieri 1984]. Rapid medium exchange (50% daily), together with a combination of added multi-CSF, GM-CSF and Epo. also increased CFU-GM production [Schwartz 1991]. In human LTBMC, addition of GM-CSF and multi-CSF affects the cells in both the adherent and non-adherent layers. In the non-adherent layer they increased the output of hemopoietic cells and CFU-F, and in the adherent layer they decreased the number of fat cells. This effect was not noted in irradiated cultures [Wang 1990].

CSFs produced by stromal cells.

Although colony-stimulating activity (CSA) was observed in conditioned medium in which marrow cells had been cultured [Chan 1972], initial attempts to detect it in LTBMC was not successful [Dexter 1977]. However, with the use of improved techniques, such as radioimmunoassay, it subsequently was demonstrated [Shadduck 1983]. The failure to detect CSFs in LTBMC may be related to the presence of inhibitors [Toksoz 1980], adsorption by ECM components [Gordon 1987], or consumption by progenitors [Heard 1982]. It appears that the level of production of CSA in LTBMC depends on the stage of myelopoiesis. In patients who had received chemotherapy, the concentration of CSA is elevated during regenerative hemopoiesis but declines with stabilization [Takeichi 1987]. An increase in the production of CSA is usually followed by increased proliferation of myeloid cells in LTBMC [Lipshitz 1987].

All types of stromal cells in LTBMC appear to produce at least one of the CSFs. The CSA production by fibroblasts was demonstrated in CFU-F [McCarthy 1984], fibroblastoid cell lines [Piersma 1984] and purified primary marrow fibroblasts [Brockbank 1986], although for some factors direct contact with the progenitors may be essential [Greenberg 1981]. In addition to fibroblasts, macrophages [Oblon 1983, Takeichi 1987], pre-adipocytes [Lanotte 1982], and vascular endothelial cells [Quesenberry 1980] also produce CSA.

The specific type of CSF produced by stromal cells has been demonstrated by antibody neutralization for GM-CSF [Godard 1983, Gualtieri 1984] and M-CSF [Hunt 1987] in LTBMC and in derived cell lines such as murine MBA-1 [Zipori 1985], H-1 [Harigaya 1981, Garnett 1982a, 1982b], and corresponding human lines [Harigaya 1985]. Analysis of specific mRNA by Northen blotting has confirmed that the factors were produced by the cells under study [Eliason 1988; Woodward 1990]. With assays based on the growth of factor-dependent cell lines [Brockbank 1986; Woodward 1990], G-CSF production also was demonstrated. However, multi-CSF has not been detected either by antibody neutralization [Gualtieri 1987] or mRNA expression in either media or cells derived from LTBMC [Eliason 1988], although material that potentiates the formation of CFU-S [Li 1987, Leung 1987] and BFU-E [Li 1985] usually was observed.

CSFs produced by stimulated stromal cells.

The production of CSFs by stromal cells can be augmented as part of the response to infection. Bacterial endotoxin is a potent inducer [Quesenberry 1980, Gualtieri 1987], as are IL-1, TNF [reviewed by Bagby 1987], lipopolysaccharide (LPS) [Wang 1991], viruses (e.g., retroviruses) [Koury 1982], synthetic peptides (e.g., N-acetylmuramyl-L-alanyl-D-isoglutamine; MDP) [Galelli 1985], and the synthetic double-stranded RNA poly(rI):poly(rC) [Fibbe 1988a].

The inducing effect of IL-1 and TNF originally was identified in macrophage conditioned medium [Broudy 1986b]. These products can induce or increase production of M-CSF, G-CSF, GM-CSF [Fibbe 1988a, 1988b] and other CSA to stimulate BFU-E and CFU-GEMM [Zucali 1987, Fibbe 1988c]. Between the two forms of TNF, the effect of TNF-alpha is stronger than TNF-beta [Broudy 1987, Koeffler 1987]. The in vivo administration of TNF results in an increase in production of GM-CSF and M-CSF, which was demonstrated both by expression of mRNA and release into the serum [Kaushansky 1988].

When LTBMC are cultured in the presence of IL-1 and TNF, monocytes, endothelial cells, and fibroblasts release CSA. Monocytes also release activity in the presence of IFN-gamma [Lu 1988]. Endothelial cells increase the production of both GM-CSF [Zsebo 1988b; Broudy 1986a; Kaushansky 1989] and G-CSF [Zsebo 1988b, Segal 1988]. Similarly, human fibroblasts derived from either lung [Munker 1986, Koeffler 1987] or marrow [Rennick 1987a, Lee 1987] augment their production of GM-CSF and G-CSF in response to TNF [Munker 1986, Koeffler 1987] and IL-1 [Yang 1988] at both the transcriptional and post-transcriptional levels.

In summary, the increase in production of CSFs under conditions of infection is an example of how cell-cell interactions among stromal cells can control hemopoiesis. Monocytes stimulated by bacterial products (such as endotoxin) release IL-1 and TNF, which induce or further increase production of CSFs by stromal cells (fibroblasts

and endothelial cells) [Fig. 2]. This increased production of CSFs results in increased production of neutrophils and monocytes in the marrow, and potentiation of their activities in the circulation, all contributing to the elimination of invading microbes.

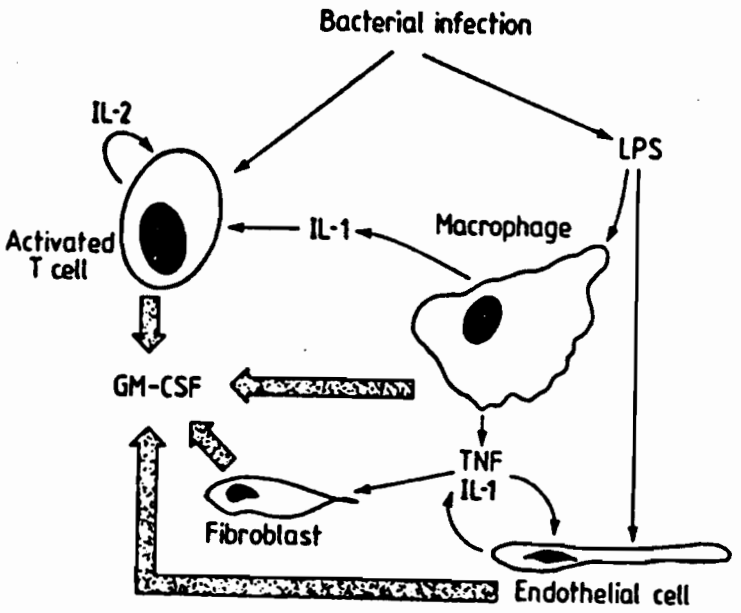

Figure 2 Potential signals stimulating GM-CSF production after bacterial infection [Gough 1990].

1.5 Stem cell factor/ c-kit ligand

Stem cell factor (SCF) [Zesbo 1990a, 1990b], identified also as "mast cell growth factor" (MGF) [Boswell], was purified from a rat liver-derived cell line and from marrow-derived fibroblasts. It is deficient in the stromal cells of Sl/Sl^{d} mice. Its mRNA also is expressed in human endothelial cells and the gene was cloned from marrow stromal cells [Aye 1992]. Subsequently, it was identified as the ligand for the product of the c-kit proto-oncogene, a member of the tyrosine kinase family and deficient in W/W^{V} mice [Copeland 1990, Williams 1990, Huang 1990, Zsebo 1990, and Bernstein 1989, 1990; also see Introduction 1.1]. Because of the multiple terms used to refer to the ligand, SCF will be used here for convenience.

SCF as multipotent growth factor.

SCF is necessary for the development of hemopoietic progenitors. Unless it is present in the nutrient medium, BFU-E will not grow in serum free culture even in the presence of IL-3 [Dai 1992]. Furthermore, as shown in experiments measuring human, murine, and rat myeloid and erythroid colonies, SCF alone has little stimulatory effect on hemopoietic precursors, but usually acts in synergy with other CSFs such as IL-3, IL-6, GM-CSF, G-CSF, and erythropoietin [McNiece 1991, Bodine 1992, Metcalf 1991]. Likewise, its effect on a purified subpopulation of

high proliferative potential $CD34^+$ cells [Brandt 1992] Carow 1991] and CD34+ cells lacking lineage-related markers (i.e., early progenitors) [Bernstein 1991] was evident only when it was added in the presence of other factors such as IL-3 or GM-CSF. In synergy with IL-3, GM-CSF and G-CSF, SCF markedly increases BFU-E, CFU-E and CFU-GM in LTBMC from a subpopulation of aplastic anemia patients [Wodnar-Filipowicz 1992].

The observed <u>in vivo</u> effects of SCF also implies that it has a biological significance. Injection of SCF into rats was followed by neutrophilia, lymphocytosis, and increased mast cells in the peripheral circulation [Ulich 1991]. In combination with G-CSF, SCF increased the CFU-GM proliferative rate and neutrophil (storage and proliferative) pools in new born rat bone marrow [Cairo 1992]. For Sl/Sl^d mice, SCF increased the CFU-S in bone marrow and spleen [Bodine 1992]. Experiments with a c-<u>kit</u> specific antibody (ACK2), which functionally disrupts the SCF receptor, further support the notion that SCF is relevant to the development of hemopoietic progenitors. Injection of ACK2 into mice resulted in depletion of hemopoietic progenitors, and eventual loss of granulocytes and erythroid cells from the marrow [reviewed by Broxmeyer 1991].

SCF and LTBMC.

SCF appears to be involved in supporting hemopoiesis in LTBMC, although strong evidence for a similar role for other single growth factors is lacking. The deficiency of SCF in Sl/Sl^d mice was proven to be a key factor in Dexter's observation that stromal cells derived from Sl/Sl^d mice failed to support hemopoiesis *in vitro* [Dexter 1977]. Later experiments with pure SCF showed that addition to cultures with IL-6 resulted in an increase in CFU-S. The increased CFU-S, however, were found only in the CFU-S-enriched but not the depleted fractions, indicating that the existing CFU-S pool had been expanded and probably had not differentiation from more primitive cells [Bodine 1992].

SCF can be expressed in two biologically active forms, either bound to the plasma membrane or released as a soluble factor [Anderson 1990]. The SCF/c-*kit* interaction is of special interest in the study of bone marrow stroma because the membrane-bound form of the ligand was found to be reduced on the surface of fibroblasts of the Sl mutant mouse, although the soluble form was normal [Flanagan 1990]. This argues that such interactions between stromal cells and early precursors is relevant to normal hemopoietic function *in vivo* [reviewed by Broxmeyer 1991], and to the initiation a long-term marrow cultures [Carow 1991].

Interaction between c-kit and its ligand also may be involved in the attachment of hemopoietic precursors to fibroblasts. Also, deficiency of either c-kit or SCF resulted in poor attachment of mast cells to fibroblasts. Because the c-kit protein has both intracellular and extracellular domains, cells from W/W^V strains carrying a mutation in the internal tyrosine kinase domain (W^V and W^{42}) were able to adhere to fibroblasts normally. Monoclonal antibodies directed to the extracellular domain inhibited binding of mast cells to fibroblasts, indicating this part of the c-kit molecule is necessary for attachment [Adachi 1992].

SCF and mast cell development.

Mast cells are heterogenous. In the mouse, both "mucosal" (MMC) and "connective tissue" types (CTMC) have been identified. They are distinguished from each other by their distribution; MMC are prominent along the gastrointestinal and respiratory tracts, whereas CTMC are in the skin, lung, pretoneal cavity, and elsewhere. The MMC are larger and contain more granules and more chondroitin-E, while CTMC contain mostly heparan proteoglycan. In cell culture, MMC are IL-3 dependent, whereas CTMC are stroma dependent.

The two types of mast cells appear to develop from precursors that have migrated to peripheral sites and have differentiated in response to local microenvironmental factors. Bone marrow mast cells, when cultured in the

presence of IL-3, become phenotypically MMC (more chondroitin-E), and when cultured on a layer of 3T3 fibroblasts, become CTMC (more heparan) [Tsuji 1990]. The human analogs of these two types can revert to one or the other in culture, depending on the microenvironment [Kanakura 1988]. Human bone marrow mast cells expanded in response to IL-3 shift the pattern of proteoglycan synthesis from chondroitin-E to heparan when cocultured with murine embryo or human adult skin fibroblasts. However, the question remains whether this reflects true subpopulations, or simply, plasticity of their phenotypes [Gilead 1990].

It has been noted that the mast cells of both the Sl and W mutants were defective. When skin of W/W or Sl mice were grafted onto the backs of normals, mast cells colonized the engrafted skin of W but not that of Sl, suggesting that there may be a stromal cell defect in the Sl mutant. Investigating the nature of defect, the authors suggested that fibroblasts may be involved. Fibroblast lines derived from normal embyros supported normal mast cell growth, but the cell lines derived from Sl/Sl^d embyros failed to do so [reviewed by Bernstein 1991]. Subsequently, it was shown that the progenitors from W/W^v mice lacked the ability to respond to fibroblast conditioned medium (due to the lack of c-kit). However, fibroblasts from Sl/Sl^d did not induce the differentiation of mast cells [Jarboe 1990]. W mast cells grow in response to soluble factors (IL-3 and IL-4), but not after contact with fibroblasts (mouse embryo 3T3). 3T3 cells from Sl do

not support mast cell growth, and may even suppress their proliferation in response to soluble factors (PWM-SCM) [Zuckerman 1986; Onoue 1989].

SCF stimulates mast cells growth as well as the attachment of mast cells to stromal cells. Injection of SCF also causes a profound increase in mast cells in the circulation of both of normal and Sl mice. It also acts as a chemo-attractant to mast cells [Meininger 1992]. Based on the discussion above, it appears that SCF may have a profound effect on the development of CTMC but not MMC.

Unanswered questions regarding the Sl and W mutants.

The discovery of SCF ushered in a new understanding of the hemopoietic disorder seen in Sl mutant mice. However, other factors (such as NK cells or hemonectin) also may be involved. NK cells appear to inhibit the proliferation of murine and human hemopoietic cells. Another mutant mouse strain (mi/mi) seems to have a mast cell defect similar to W/W, but they also have osteopetrosis, microphthalmia, and deficient NK cells, which in the W/W are normal [Ebi 1990]. Furthermore, injection of antibodies against NK cells into Sl/Sl^d mice increased the number of CFU-S in cycle, as well as BFU-E, CFU-E and CFU-GM [Pantel 1991].

Hemonectin (see Introduction 1.3), a protein involved in interaction of granulocytic precursors and stromal cells, also may contribute. Normal bone marrow progenitors bind poorly to Sl/Sl^d stromal lines. Hemonectin is

decreased in bone marrow of Sl/Sl^d, and is not detected at all in some Sl/Sl^d derived stromal cell lines. Incubation of Sl/Sl^d stromal cells with hemonectin increases precursor cell binding and CFU-S production in LTBMC. Hemonectin binds to BFU-E and CFU-GM but not to CFU-MK, of interest in view of the fact that megakaryopoiesis is only minimally affected in Sl/Sl^d mice [Anklesaria 1991].

1.6 Hemopoietic growth inhibitors.

Hemopoietic growth inhibitors are a group of molecules of natural origin that block or retard the proliferation and/or differentiation of blood cells. They are produced by different tissues and cells (stromal and non-stromal), and usually are recognized by their ability to prevent the growth of hemopoietic CFU in vitro. Most of them were discovered in the context of actions other than their ability to inhibit hemopoiesis. Their properties and biological effects will be summarized below, concentrating on how they alter the growth and maturation of bone marrow cells in culture.

Transforming growth factor-beta (TGF-beta).

The effect of TGF-beta on hemopoietic precursors is mainly inhibitory, although in some instances it can be stimulatory [Roberts 1985a]. It suppresses the growth of human BFU-E, CFU-E and CFU-GM, and murine BFU-E, CFU-GM, CFU-G [Hino 1988], and CFU-MK [Ishibashi 1987], as well as the M-CSF dependent cell line BMM-8 [Strassmann 1988] and the human leukemic lines K-562 and HL-60. Overall, it has greater inhibitory effect on immature (such as CFU-GEMM, BFU-E, and CFU-GM) than more mature (such as CFU-G, CFU-M and CFU-E) precursors. The counteract effects of CSF by TGF-beta is different between human and murine. It inhibits response to GM-CSF in human but does not in murine. In both human and murine, TGF-beta has no effect

TGF-beta dose not have any effect on the response to G-CSF [reviewed by Hooper 1991]. The inhibitory effect on proliferation can occur either with or without induction of terminal maturation [Chen 1989, Tessier 1988]. Another level of TGF-beta inhibition of hemopoiesis is mediated by its modulation of the expression of CSF receptors (such as multi-CSF, GM-CSF and G-CSF) on progenitor cell lines [Jacobsen 1991]. As well as hemopoietic precursors, normal epithelial cells and certain tumor cells are also inhibited, whereas anchorage-independent clonal growth of certain fibroblasts (e.g., as in the NRK assay) is augmented [Roberts 1985b].

The predominant focus for the biological effects of TGF-beta appears to be directed at the genesis and repair of tissues [Massague 1987]. Elevated concentrations occur in active areas such as the bone marrow, fetal liver, Hassall's corpuscles, and osteocytes, but the highest has been found in platelets, indicating that it may be a major controller of collagen synthesis and wound healing [Massague 1987; Roberts 1985b].

The molecule itself is a disulfide-linked homodimer of a 112 amino acid protein (mol wt = 25 kDa) [Roberts 1985b], and is resistant to heat and acid [Roberts 1981]. It is highly conserved, the human and murine forms differing from each other by only one amino acid, and thus it has maintained a broad spectrum of reactivity across species boundaries. Other normal adult tissues (e.g., kidney, liver, muscle, heart and brain) and cells (e.g., osteocytes, chondrocytes, megakaryocytes, and thymocytes)

also produce an inactive form; many types of neoplastic cells release either the active or inactive form of the molecule [Roberts 1981, 1985a, 1985b; Ellingsworth 1986].

Tumor necrosis factor (TNF).

This cytokine originally was identified by its in vitro (cytotoxic) and in vivo (hemorrhagic necrosis) antitumor effects [Old 1988], but now it is recognized to be a regulatory molecule of more general significance. Of the alpha and beta forms, which share 30% sequence homology and differ in glycosylation (157 and 171 amino acids; mol wt 17 vs 25 kDa), the former ("cachectin") is derived predominantly from macrophages, and the latter from lymphocytes ("lymphotoxin") [Pfizenmaier 1987; Aggarwal 1985]. Both bind to the same cellular receptor and have overlapping, but not identical, biological activities [Keski-oja 1987].

Cachectin has diverse effects on hemopoietic precursors. It inhibits the growth of human CFU-GM, BFU-E, and CFU-GEMM [Zucali 1988], either alone or in synergy with interferon [Broxmeyer 1986, Peetre 1986]. On mature blood cells, TNF enhances phagocytosis by neutrophils and stimulates their adherence to the endothelium. It also can induce stromal cells to produce CSFs (further discussed in section 4). Like TGF-beta, it stimulates the growth of fibroblasts, but inhibits K562, HL-60 [Pfizenmaier 1987],

and U937 [Schutze 1988] cell lines. In vivo, chronic administration of TNF to mice, at least in some studies, result in anemia [Johnson 1990].

Until recently TNF (see above) and IFN (see below) were viewed as inhibitors of hemopoiesis. However, more recent experiments indicate that their effects depend on the target cells, other growth factors, and the time at which one observes. With enriched CD34+ cells (i.e., stem cells), TNF potentiates their response to IL-3 and GM-CSF [Hoang 1991; Piacibello 1990 and Caux 1990]. Similar observations also were obtained with acute myeloblastic leukemia blasts that remain partially dependent on IL-3 and GM-CSF [Hoang 1989; Salem 1990]. Conversely, it antagonizes the effect of G-CSF on CD34+ and acute myeloblastic leukemia blasts [Elbaz 1991]. The potentiating effect of TNF on the CD34+ cells is transient and ceases after 10 to 12 days; whereupon TNF displays an inhibitory effect. [Caux 1991].

TNF appears to potentiate the maturation of monocytic cells, whereas it inhibits granulocyte development [Caux 1991]. The inhibition of granulopoiesis may result from inhibiting differentiation of CD34 blasts and inhibiting proliferation of granulopoietic precursors. While, it potentiates differentiation in the macrophage lineage and then later inhibits further proliferation. The dual effect of TNF appears not to be mediated by induction of the release of other CSF's [Backx 1991], but by regulation of

the CSF receptors (e.g., upregulation of receptors for IL-3 and GM-CSF and down regulation of G-CSF receptors [Elbaz 1991]).

A similar stimulatory effect of IFN also was observed [Hoang 1990]. The ability of IFN to induce accumulation of TNF transcripts and the fact that an antibody against TNF can abrogate the stimulatory effect of IFN all support the notion that this action of IFN is mediated by TNF [Murohashi 1991].

Interferon (IF).

The IFs were discovered as a result of their antiviral action. They are produced by a wide variety of cells (both normal and neoplastic) in response to viruses, bacterial products, foreign cells and macromolecules, and numerous other agents. There are three major classes, alpha, beta and gamma, produced by leukocytes, fibroblasts, and lymphocytes respectively.

In addition to its antiviral activity, the biological effects of IF include immunomodulation, antitumor activity, macrophage activation, and inhibition of cell growth [Pestka 1981]. After incubation of human bone marrow cells in the presence of IF, CFU-GEMM, BFU-E, CFU-MK, and CFU-GM, but not CFU-E, are inhibited [Carlo-Stella 1988; Parker 1982]; murine CFU-GM and CFU-E also are inhibited [McNeill 1973, Greenberg 1977, Hull 1978]. These effects may be related to the retarded

engraftment seen in some patients who have received a bone marrow transplant, as demonstrated by the fact that incubation of marrow cells from these patients with antibodies to IF resulted in an increase in hemopoietic colonies [Raghavachar 1986]. Among different types of IF, the inhibitory effect by type gamma is more potent than alpha [Rigby 1985]; type alpha is more potent than beta [Hull 1978]. Similar to the other interacting cytokines, the effects of IF on hemopoietic cells can be amplified by its ability to induce the production of TNF [Cannistra 1988].

Lactoferrin.

The hemopoietic inhibitory effect of the iron-binding glycoprotein lactoferrin is mediated in part by its ability to repress the production of GM-CSF by monocytes and macrophages. Colony formation by marrow progenitors that were stimulated by exogenous CSA was not affected [Broxmeyer 1978b]. The effect on CSF production may be related to the inhibition of the synthesis and release of IL-1 by mononuclear phagocytes [Zucali 1989].

Acidic isoferritin.

Ferritin, a major iron storage protein of mammalian cells, consists of multiple subunits and has a mol wt of 550 kDa. It can be separated into isoforms composed of two types of subunit (H and L with mol wt of about 21 and 19

kDa). The H form is the predominant component of ferritin isolated from heart, whereas L is the major form from liver. Acidic isoferritin is composed mainly of H subunits; the basic is mainly L [Munro 1978].

The inhibitory activity of acidic isoferritin on hemopoietic progenitors was originally described as a product of marrow and blood cells from patients with acute leukemia. The H form, which is the most active in this context, suppresses the formation of CFU-GM by S-phase cells [Broxmeyer 1978a, 1981; Dezza 1988]. Analysis of a panel of over 20 hemopoietic cell lines showed that only those derived from monocytes and macrophages released acidic isoferritin. Therefore, a negative feedback regulatory role has been suggested [Broxmeyer 1982].

Prostaglandin E (PGE).

PGE, a 20-carbon and hydroxylated fatty acid, inhibits CFU-GM formation [Kurland 1977], and its effect is more pronounced on precursors of macrophages than on granulocytes or megakaryocytes [Pelus 1979, 1981, Williams 1979].

Hemopoietic inhibitors derived from bone marrow-derived cells.

The molecules to be discussed in the next two sections include a heterogeneous group of more recently-described factors which inhibit hemopoietic cell

growth in at least one type of assay. Some have been well characterized molecularly, whereas others remain to be purified.

An activity that appeared to be directed specifically toward CFU-S was identified in an extract of murine marrow, and later was shown to be released by macrophages from other sites such as the spleen and peritoneal fluid [Pojda 1988]. It was sensitive to trypsin, had a mol wt of 50-100 kDa, and prevented CFU-S from entering into DNA synthesis, but it did not retard the growth of cells already in S-phase [Lord 1976]. The fact that resting (a low proportion of CFU-S in S-phase) had a higher concentration than regenerating marrow cells (more in S-phase) [Tokoz 1980], implies that it might have a role in maintaining stem cells in a steady state. It also inhibited the proliferation of IL-3 dependent cell lines [Lord 1987]. Recently, it has been demonstrated that this activity might be identical to the macrophage inflammatory protein-1a (MIP-1a) [Graham 1990], isolated from both rat [Cork 1981] and murine cells [Riches 1987].

A molecule acting specifically on BFU-E called "negative regulatory protein" (NRP) was isolated and purified from the supernatant of murine marrow cells [Axelrad 1987] and identified as cytosolic superoxide dismutase [Pluthero 1990]. In a manner similar to the CFU-S inhibitor, it blocks the entry of BFU-E into the DNA synthetic phase, but does not alter the growth of CFU-GM and CFU-E [Del Rizzo 1988; Axelard 1981]. Other small molecules have been identified that can inhibit CFU-S. A

tetrapeptide (AcSDKP) (mol wt = 487) was purified from fetal calf and murine bone marrow [Lenfant 1989], and appeared not to be produced elsewhere [Frindel 1977]. It was active on CFU-S both in vivo and in vitro, preventing their entry into S-phase [Guigon 1987]. Recent experiments indicated that thymosin B4 may be the precursor of AcSDKP [Lenfant 1991]. Another completely different pentapeptide (pEEDCK) with similar activity was purified from conditioned medium of rat bone marrow [Paukovits 1987], but also was found at sites that contained the greatest quantity of mature granulocytes. In addition to CFU-S, both the natural and synthetic peptides inhibited the growth of CFU-GM, and the HL-60 and THP-1 (monocytic) cell lines [Paukovits 1986]. Mice injected with the peptide showed a prolonged neutropenia. Interestingly, the peptide can form disulfide bridges through cysteines and become a potent stimulator of myelopoiesis [Laerum 1987].

A plasmacytoma inhibitor that does not act on normal hemopoietic progenitors is produced by an endothelial-like cell line [Zipori 1985, 1986]. Subsequently, this and other related activities have been termed "restrictins" [Zipori 1990]. Another class of natural hemopoietic inhibitors that act as a receptor antagonist is exemplified by a molecule of 18-22 kDa released from human monocytes. It has a structure similar to IL-1, and binds to the receptor. However, it does not lead to the subsequent events that are associated with IL-1 action [Hannum 1990].

Hemopoietic inhibitors derived from leukemic cells.

Normal hemopoiesis is suppressed in patients with acute leukemia, and a search for a cause led to the recognition of other types of inhibitors. Some of these will be discussed below.

Bone marrow cells from some patients with acute myeloid leukemia release what has been called the "leukemia-associated inhibitor" (LAI) which blocks CFU-GM, but not BFU-E from entering S-phase [Olofsson 1980b]. LAI, subsequently was demonstrated also in normal marrow [Olofsson 1984]. The molecule is a glycoprotein with mol wt 150-170 kDa [Olofsson 1980a].

Leukemia inhibitory factor (LIF), produced by a variety of cell types including T lymphocytes [Moreau 1988] and certain tumor cells such as the Krebs II ascites line [Hilton 1988a], has been purified and its gene cloned [Gearing 1987, Gough 1988, Hilton 1988a, Godard 1988]. It suppresses the proliferation and induces the monocytic differentiation of a murine leukemic cell line (M1), and appears to bind specifically to normal murine hemopoietic tissues including marrow and spleen [Hilton 1988b]. Although LIF is classified as a "hemopoietic inhibitor" here, its biological effects are not restricted to hemopoietic elements or inhibitory action. In synergy with CSFs, it supports human CFU-GEMM, BFU-E, CFU-Eos [Verfaillie 1991] and CFU-MK [Metcalf 1991] formation, and IL-3 dependent cell lines [Leary,1990]. On mature eosinophils it can act as a chemoattractant and activator

[Moreau 1987]. For non-hemopoietic cells, it can inhibit the differentiation of embryonic stem cells [Smith 1988, Williams 1988], induce the differentiation of cholinergic neurons [Yamamori 1989], inhibit lipoprotein lipase [reviewed by Gearing 1991], and induce calcium release from murine calvaria in vitro [Abe 1986]. Demonstrating its wide range of biologic activities, in vivo experiments in which LIF-producing cells had been implanted into mice showed that the animals became cachectic, and underwent excess new bone formation, ectopic calcifications (heart, liver and skeletal muscle), pancreatitis, thymic atrophy, and deficiency of the ovarian corpora lutea [Metcalf 1989b].

An inhibitory substance active on normal BFU-E, CFU-GM, and CFU-MK has been derived from leukemic hairy cells (a type of B lymphocyte) [Lauria 1987], although the BFU-E appears to be the most sensitive [Gaggioli 1987]. Another activity (mol wt 5-6 kDa) derived from the same type of cells suppressed only CFU-GM and CFU-E, but not BFU-E and CFU-MK. These observations suggest that the leukemic cells themselves may be responsible for the marked neutropenia and anemia seen in this disease [Taniguchi 1989].

Several other leukemic cell-derived hemopoietic inhibitors have been reported but have not been well characterized. A CFU-S and CFU-GM inhibitor derived from a murine leukemic cell line (C1498) was demonstrated by Quesenberry [1978]. Another substance dependent on the presence of adherent cells for its activity was released

from acute non-lymphoblastic leukemia cells; it could suppress BFU-E and appeared to be different from the other inhibitors discussed previously [Najman 1987]. Still another activity which suppressed BFU-E, CFU-E, and CFU-G in vivo was shown to be produced by both rat acute myeloid leukemia and human leukemic cell lines, prior to but not after induction of their maturation [Steinberg 1987].

1.7 Relevant results from Dr. Sullivan's Lab.

As discussed above, the results of experiments observing the effects of ectopic transplantation, the behavior of cells in the long-term bone marrow culture system, and histologic studies all support the notion that stromal cells are a significant regulator of hemopoiesis. The interactions are complex, mediated by diffusible factors and direct cell-cell contacts. However, many of the underlying mechanisms for how stromal cells control hemopoiesis are not well understood.

To further investigate this problem it became necessary to objectively identify different types of stromal cells. For this purpose, a series of murine monoclonal antibodies to rat marrow cells were developed in Dr. Sullivan's laboratory (Details can be found in the Appendix). The antibodies used in the work to be presented here include the following: BN35 that recognizes mature macrophages and myeloid precursors (including CFU-GM and CFU-M), ST3 and ST4 that recognize subpopulations of fibroblastoid cells with a distinct tissue distribution. The predominant fibroblastoid cell grown from rat bone marrow is ST3+/ST4-, but that from several non-hemopoietic organs (e.g., lung, diaphragm, and epididymal adipose stroma) is ST3-/ST4+. Fibroblasts derived from the spleen, an organ that can support hemopoiesis under certain conditions, are an equal mixture of both types. Thus,

histological evidence indicated that organs could contain at least two distinct subpopulations of fibroblastoid cells, as defined by the ST3 and ST4 antibodies.

1.8 Rationale for the development of this project.

The long-range goal of this project, of which this thesis forms a part, is to explain why hemopoiesis occurs in the bone marrow and not in other tissues of a normal adult mammal.

The purpose of these experiments, begun in 1987, was to explore what was then and still remains a novel phenomenon. As has been discussed in the preceding pages, activities inhibitory to *in vitro* assays of hemopoietic growth had been identified from a wide variety of sources, but few of them had been characterized. Based upon the observation by Sullivan *et al* that fibroblastoid cells derived from bone marrow and lung differed in their expression of certain membrane antigens, the work of this thesis was designed to explore some of the possible functional differences between similar-appearing cells from these two organs. The experiments progressed logically in five phases.

The first was to determine whether secretion products from antigen-defined fibroblastoid cells of the bone marrow differed from those of the lung, as indicated by how they modified the growth of committed hemopoietic precursors. From the perspective of physiologic regulation, the goal was to examine the effect of the *total pool of synergistic and antagonistic* activities present in conditioned media from the two types of cells.

This led to the second phase focusing on the action of the inhibitory component on normal and leukemic hemopoietic precursors. Much of this effort was directed to eliminating the possibility that certain other known molecules, acting alone, might be responsible for it.

To confirm that these patterns were consistent, the third phase was to establish fibroblastoid cell lines from lung and marrow and show that the different patterns were similar to what had been observed with primary cells.

In the forth, we began to address the question of physiologic significance. Of the multitude of approaches that one might take to this end, we began to explore it in the context of inflammatory mediators and response to infection, and their control by corticosteroid hormones. Evidence is beginning to accumulate that glucocorticoids regulate a set of genes that encode many of the cytokines of the response to stress and inflammation [Tobler, 1992].

Finally, to set the stage for work by others who will follow in the future, an initial strategy for purification was established.

This work has led to four peer-reviewed publications.

The results that form the basis of this thesis are organized into chapters using duplicated original papers (Published, Chapter 2 & 5; accepted, chapter 3 & 4) according to Guidelines Concerning Thesis Preparation: "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper,

or papers. ..... It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis". In Chapter 2 is demonstrates differences in the release of CSA and inhibitory activity from two antibody-defined cell types. In Chapter 3 is discussed the characterization of a possibly novel inhibitory factor produced by lung-derived fibroblastoid cells (ST3-/ST4+). In Chapter 4 is presented the properties of the cell lines derived from these two antibody-defined cells and the modulation of the inhibitory activity by corticosteroids. Finally, in Chapter 5 is described initial steps in the purification of the molecule(s) that mediates the inhibitory activity.

Chapter 2 FIBROBLASTOID CELLS DERIVED FROM RAT BONE MARROW AND LUNG DIFFER IN THEIR PATTERN OF PRODUCTION OF HEMOPOIETIC STIMULATORS AND INHIBITORS[2]

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[2]This chapter is a duplication of the paper by H. Wang and A.K. Sullivan, which has been published in Leukemia Research 16:385-393 (1992).

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FIBROBLASTOID CELLS DERIVED FROM RAT BONE MARROW AND LUNG DIFFER IN THEIR PATTERN OF PRODUCTION OF HEMOPOIETIC STIMULATORS AND INHIBITORS

HONG WANG and ARTHUR K. SULLIVAN

Department of Medicine, Division of Hematology, Royal Victoria Hospital, 687 Pine Avenue, West Montreal, Canada H3A 1A1

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Abstract—Blood cells develop in the bone marrow, controlled by a complex network of regulatory factors, some of which originate in the non-hemopoietic stroma. Previously we found that the predominant fibroblastoid (FB) cell growing in primary cultures of rat marrow bears surface antigens different from similar cells derived from certain other tissues. As determined by two monoclonal antibodies, the 'marrow type' is $ST3^+/ST4^-$, and the 'peripheral type' is $ST3^-/ST4^+$. Here we describe some of the functional differences between these two cell types. After primary cultures were depleted of hemopoietic elements and 'other' type FB, colony-stimulating activity was detected in the conditioned media (CM) of cells derived from marrow ($ST3^+$), but not from lung ($ST4^+$). Conversely, lung FB produced a CM that inhibited the clonogenic growth of normal marrow precursors. This substance (or substances) was produced by lung cells in the absence of exogenous stimulation, but by marrow cells only after they had been cultured in the presence of macrophage-derived products. The inhibitory activity did not have any measurable effect on murine blood cell precursors. We conclude that these isolated antibody-defined fibroblast subpopulations from different organs differ in their capacity to stimulate or inhibit normal hemopoietic precursors.

Key words: Bone marrow, stromal cells, fibroblasts, lung, colony-stimulating factors, hemopoiesis, inhibitors.

INTRODUCTION

BLOOD cell precursors proliferate and mature in the bone marrow under the influence of a complex network of regulatory molecules that include 'colony-stimulating factors' (e.g. M-, G-, and GM-CSF) and certain interleukins (e.g. IL-3 and IL-6) [1, 2]. Other molecules such as prostaglandins [3], acidic isoferritin [4], lactoferrin [5], tumor necrosis factor (TNF) [6], beta-transforming growth factor [7, 8], and interferons [9, 10], appear to be inhibitory. Although isolated marrow fibroblasts and peripheral vascular endothelial cells [11, 12] can produce some of these regulators, *in vivo* they may require signals from other cells or cytokines (e.g. IL-1 and TNF) to perform their physiologic role [13, 14, and reviewed in 15]. At present, it is not well understood how the stimulatory and inhibitory factors might interact within the architectural constraints of the bone marrow organ, or how stromal cells from different organs might vary in their ability to produce them.

Using two monoclonal antibody probes called ST3 and ST4, we demonstrated previously that the predominant population of fibroblastoid cells cultured from rat bone marrow appears to be different from the major type derived from other non-hemopoietic organs [16]. The phenotype of the marrow species is $ST3^+/ST4^-$, and that from representative peripheral organs (i.e. lung, diaphragm, and adipose tissue stroma) is $ST3^-/ST4^+$; those from spleen are an equal mix of both types. Because lung-derived cul-

Abbreviations: *CFU*, colony-forming unit; *rCFU or mCFU*, rat or mouse CFU; *CM*, conditioned medium; *LF*, lung fibroblasts, treated as per 'Methods'; *LFmcm*, LF cultured with MCM; LF_{NT}, LF 'not treated' to remove other cells; *MCM*, macrophage-conditioned medium; *Med*, medium alone; *MF*, marrow fibroblasts, treated as per 'Methods'; *MFmac*, MF cultured with macrophages; *MFmcm or MFm*, MF cultured with MCM; MF_{NT}, MF 'not treated' to remove other cells; *NRS*, normal (pre-immune) rabbit serum; *SCCM*, spleen cell-conditioned medium.

Correspondence to: Dr A. K. Sullivan, Rm 714 McGill Cancer Center, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6.

tures usually contained very few ST3$^+$ cells, they were selected for futher study. In the work presented here, we compare the capacity of these two antigen-defined fibroblastoid cell subpopulations to elaborate stimulatory and inhibitory factors.

MATERIALS AND METHODS

Animals and cell culture

Bone marrow and other tissues were obtained from 200–300 g Brown Norway rats sacrificed by overdose of inhaled ethyl ether. Unless otherwise stated, cells were cultured by standard techniques in RPMI 1640 medium supplemented with 10% fetal bovine serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in tissue culture flasks (T75-No. 25110-75 or T25-No. 25100; Corning, supplied through Fisher Scientific, Montreal, Quebec) in a 37°C humidified incubator with an atmosphere of 5% CO_2/95% air.

Clonogenic assays: marrow colony-forming units (CFU-C)

These were enumerated by an adaptation of established techniques [17]. Briefly, 3–4.5 × 10^6/ml of rat marrow cells in 10% FCS were mixed with 0.8% methyl cellulose to give a final concentration of 1–1.5 × 10^5 cells/ml, 30% FCS, 5 × 10^{-5} M 2-mercaptoethanol, 5% spleen cell-conditioned medium (or other stimulators as indicated) in Iscove's medium. They were then distributed into 35 mm Petri dishes, and after seven days culture in 5% CO_2 at 37°C the colonies were enumerated using an inverted microscope (colony defined as >50 cells). Spleen cell-conditioned medium (SCCM) was prepared as described by Johnson and Metcalf [18], except that phytohemagglutinin (PHA-L Pure, E.Y. Labs, San Mateo, CA) was used as the stimulator.

For determination of CFU-E, bone marrow cells were separated by flotation on a barrier of Ficoll–Paque before being suspended in media containing 20% FCS/1% BSA with a final concentration of 2 U/ml erythropoietin (step III, purified from sheep; Connaught Laboratories, Willowdale, Ontario; No. 1501-5-7); the colonies were counted on day two. Murine colonies were measured in an identical manner, except that media conditioned by WEHI-3B cells were used at 5% concentration, instead of SCCM, for experiments requiring maximal stimulation.

Unless otherwise stated, all CM from fibroblast cultures (see below) were added at a final concentration of 5%; when two factors were assessed for competitive effects, each was at a final concentration of 5%. In experiments testing the effect of recombinant M-CSF (human rhM-CSF [MAC-H], Genzyme through Intermedico, Markham, ON, Canada), the stated concentration of the pure factor was added instead of the stimulating CM.

Preparation of macrophage-conditioned medium (MCM)

Rat spleens were removed and minced, and the pieces were pushed through a stainless steel screen; clumps and recovered cells were resuspended by vigorous pipetting with RPMI 1640 medium, and seeded into a single T75 flask. On day 4, the non-adherent cells were decanted, and those remaining were incubated for 30 min with antibodies ST3 and ST4, at pretested optimal dilution for cytotoxicity, followed by 45 min with mouse complement (Low-Tox M No. CL3015, Cedarlane, Hornby, Ontario), washed, and finally replenished with 40 ml of medium. By day 6 this resulted in a rich growth of macrophage-like cells staining with BN35 and ST2 anti-macrophage antibodies [described in 16], and only a rare fibroblastoid cell identified by immunostaining with ST3 and ST4 antibodies. On day 6 the medium was changed, and on day 8 the supernatant (designated as 'MCM') was collected; before being used, it was depleted of any suspended cells by centrifugation. If stored at 4°C, MCM retained its ability to stimulate fibroblasts (see Results) for at least 3 months. For experiments using macrophages instead of MCM, the cells were harvested by trypsinization of the adherent layer, and then were added to the designated mixed cultures.

Preparation of fibroblast conditioned medium (MFCM and LFCM)

Both femora and tibiae were removed from rats, the marrow cavities were vigorously flushed with 10 ml of 1640 medium, and the resulting cell suspension was transferred to a T75 flask in a final volume of 50 ml, after which the cultures received the following manipulations:

Day 3: Non-adherent cells were decanted, and fresh media were added to those remaining.

Day 7: The adherent layer, consisting of macrophages and emerging fibroblast colonies [16], was passaged into a new T75 flask using a rubber scraper.

Day 14: The cells were distributed into 8–10 T25 flasks containing 10 ml of medium.

Day 17: To each T25 flask was added mycophenolic acid (MPA, Sigma No. M5255) at a final concentration of 5 μg/ml to inhibit growth of hemopoietic precursors [19].

Day 21: The supernatant was decanted, and fresh 1640 medium without MPA was added.

Day 24: Cells were exposed to a second cycle of MPA-containing medium, which was replaced with fresh medium on day 27.

Days 31 and 33: The remaining cells were treated with ST4 and BN35 antibodies in the presence of complement (as for preparation of MCM described above) to deplete remaining macrophages and the minor subpopulation of 'non-marrow' type fibroblasts bearing the ST4 antigen. The conditioned media from these cultures were designated as 'MF CM' (i.e. 'marrow fibroblast-conditioned medium').

It is relevant to note that even when not treated in this manner, the predominant population (>90%) of marrow FB was ST3$^+$; and macrophages comprised no more, and usually less, than 10% of the total adherent cell population. Likewise, for lung cells, the ST4$^+$ population was >90% ST4$^+$. Thus, the major selective pressure on the cells in the cultures was that of their ability to replicate under the conditions described. There was not any attempt made to assess the total number of fibroblastoid colonies in the starting lung or marrow tissue that might enable an estimate of how representative the resulting cultures cells were of the entire population.

Day 35: Individual cultures were exposed to either MCM (40% final volume) or added macrophages (6.0 × 10^4/ml) for 24 h; the resulting supernatants were collected, processed identically to that described for MCM, and designated as 'MFmcm CM' or 'MFmac CM' respectively. At the time supernatants were collected, the cultures were >75% confluent by visual inspection.

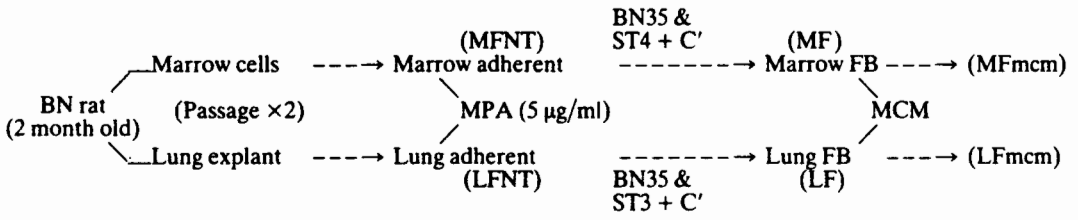
Control cultures, not exposed to either mycophenolic

acid or antibodies, were grown in parallel to those described, and the resulting supernatant was designated 'MF_{NT} CM' (i.e. 'non-treated marrow fibroblast-conditioned medium').

Fibroblasts were prepared from the lung of the same rat by placing pieces of minced tissue in RPMI 1640 in a T75 flask for 14 days, changing the media twice weekly, but retaining the fragments. On day 14 the lung pieces were removed, the adherent cells were harvested and distributed into 8–10 T25 flasks in RPMI 1640 containing 5 μg/ml MPA. The same scheme was followed as described for marrow cells, except that on days 31 and 33, the cultures were treated with antibodies BN35 and ST3 to deplete residual macrophages and 'marrow type' fibroblasts. Corresponding supernatants were harvested and called 'LF_{NT} CM', 'LF CM', and 'LFmcm CM'.

At this juncture, samples of the cultures used to obtain the CM from MF and LF were greater than 99.9% homogeneous by immunostaining (i.e. we did not see any $BN35^+$ or $ST3^+$ cells in 1000 lung cells, or any $BN35^+$ or $ST4^+$ cells in marrow-derived adherent cells). By immunohistochemical staining, both $ST3^+$ and $ST4^+$ cells contained collagens type I and III in a perinuclear granular pattern (Chemicon International, Inc., Temecula, CA, cat. No. 755 and No. 757), but did not react with rabbit antisera to human Factor VIII-related antigen (Dakopatts code A082, obtained through Dimension Laboratories, Mississauga, Ontario). The diluted serum was absorbed twice each with rat erythrocytes and spleen cells, after which it retained strong reactivity toward both human umbilical vein endothelial cells and rat megakaryocytes. Thus, MF and LF will be referred to operationally as fibroblastoid cells (FB).

The sequence of manipulations is summarized below.



For some experiments, to follow the kinetics of factor production, supernatants from MCM-stimulated fibroblast cultures were harvested on days 36–41, and each day fresh medium was replaced and collected after 24 h for use in the CFU-C assay.

In other experiments, as stated in the text, the CM was obtained from an $ST4^+$ cell line that had developed spontaneously from a culture of lung FB. The production of CSA and inhibitory activity, both constitutive and in response to MCM, was as described for primary LF.

Further treatment of the conditioned media (CM): antibody neutralization and acid stability

To determine which of the known factors might be the predominant hemopoietic stimulator in the CM from MF stimulated by MCM (i.e. MFmcm), an aliquot of the appropriate supernatant was incubated for 1 h at room temperature with an equal volume of either rabbit anti-mouse M-CSF, or pre-immune serum from the same animal, (diluted 1/10 in RPMI 1640; a generous gift from Dr Richard Shadduck, University of Pittsburgh School of Medicine, Pittsburgh, PA). Dilutions were adjusted so that there was a final concentration of 5% CM in the methyl cellulose of the CFU-C assay. To test for acid stability, aliquots of the CM of LF were acidified to pH 1.5–2.0 with 1 M HCl at 4°C for 60 min, brought to pH 8.0 with 1 M NH_4Cl, and then immediately frozen and lyophilized. Before further use, the sample was reconstituted in the original volume of distilled water.

RESULTS

Hemopoietic stimulatory activity in media conditioned by FB subpopulations

The activities in the conditioned media (CM) from several different fibroblast cultures that had been manipulated in different ways were assessed by their effects on the evolution of bone marrow colonies in the CFU-C assay. To assist with interpretation of the figures and tables, the reader is referred to the list of abbreviations and to the flow diagram of the 'sequence of manipulations' in the 'Methods' section.

The homogeneous populations of fibroblastoid cells derived from either lung or marrow were cultured, and the resulting 'conditioned media' were harvested, as described in the 'Methods'. From each of these organs, in three different states of culture (i.e. non-treated fibroblasts [e.g. MFNT], after treatment with mycophenolic acid and antibodies [e.g. MF], and after co-culture of treated fibroblasts in the presence of macrophage products [e.g. MFmcm]; as per flow diagram), the CM were evaluated for their ability to stimulate the growth of hemopoietic colonies in methyl cellulose. The results summarized in Fig. 1A show that before the minor population of macrophages and $ST3^+$ cells had been immunodepleted from lung cultures or the minor population of macrophages and $ST4^+$ cells from marrow cultures, both of the CM (bars MFNT and LFNT of the figure) contained CSA, although the activity of the CM of MFNT appeared to be stronger than that of LFNT. After macrophages, the minor population of 'other' FB, and hemopoietic precursors had been depleted, neither of the resulting CM (bars MF and LF of the figure) stimulated myeloid colonies above the background level. When these same FB were exposed to medium conditioned by macrophages

(MCM), those from marrow but not from lung produced CM (bars MFmcm and LFmcm of the figure) containing measurable CSA. At a concentration equivalent to that used in other colony assays, neither MCM alone nor a mixture of MCM and CM of MF had significant CSA, indicating that the effect of the CM from stimulated MF (i.e. MFmcm) was not one of simple synergy between pre-existing factors at suboptimal concentrations. The time course of stimulation of CSA by MCM showed a rise to a maximum level by 24 h and a return to baseline by 3–4 days (Fig. 1B). Even though the media had been changed daily, the MF continued to produce CSA between days 1 and 2 under the influence of the MCM to which they had been exposed on day 0.

Because most of the colonies stimulated by CM from MFmcm were of the macrophage type, it was suspected that the predominant effect was due to M-CSF. As shown in Fig. 1C, most of the activity was neutralized by a polyclonal antiserum to murine M-CSF. The predominance of macrophages reflects more the composition of the CM than an inability of the assay to detect granulocyte-containing colonies. This was shown by subsequent experiments in which the marrow was stimulated by spleen cell conditioned medium (SCCM), a complex mixture known to contain most of the essential hemopoietic growth factors. In these cultures the colonies were increased both in number and in the proportion containing granulocytes (CFU-G and CFU-GM) (Fig. 2A and 2B). Furthermore, increasing the concentration of the CM from control or stimulated marrow or lung fibroblasts (MF, MFmcm, or LFmcm) did not result in a greater number of total colonies or a greater proportion of CFU-GM (Fig. 2A and 2B).

Taken together, these data confirm that primary

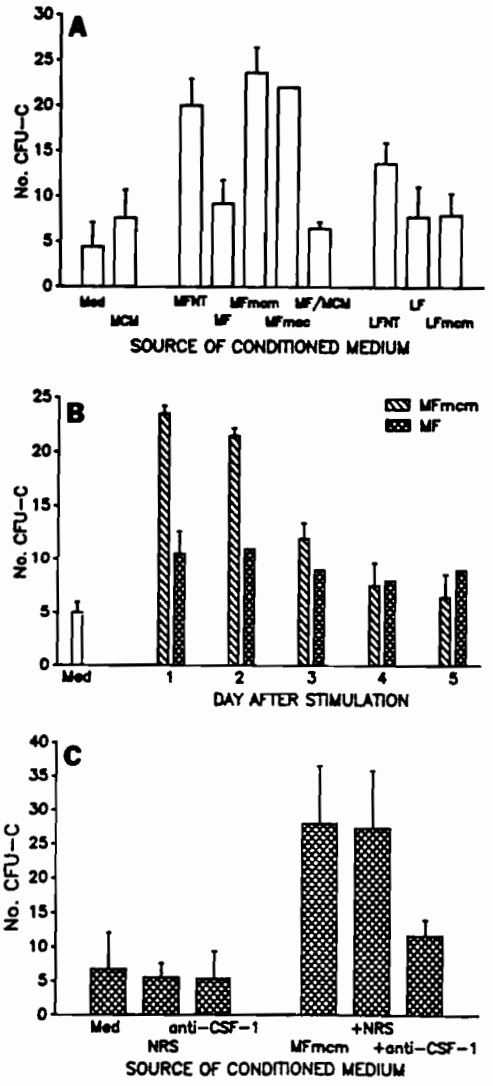


FIG. 1. (A) Effect of different conditioned media on marrow CFU-C. The number of myeloid colonies are expressed in relation to the source of conditioned medium added in the methyl cellulose assay, in the concentrations stated in the 'Materials and Methods'. *Med*, 1640 + 10% FCS alone. *MCM*, macrophage-conditioned medium (CM); *MFNT*, CM from adherent marrow cells not treated to remove hemopoietic precursors, macrophages, or other type fibroblasts; *MF* (marrow fibroblasts), CM from MFNT that had been treated to deplete those elements, as described in the 'Methods'; *MFmac*, CM from MF incubated with added macrophages; *MFmcm*, CM from MF incubated with MCM; *MF/MCM*, mixture of MCM, and CM from MF that had not been exposed to macrophage products; *LFNT*, *LF*, and *LFmcm*, lung cells treated identically to those from marrow of analogous designation. In this and subsequent experiments (unless stated otherwise) error bars indicate the mean ± 1 SD of at least three independent experiments, in each of which the colonies for each data point were enumerated in duplicate culture dishes. (B) Time course of appearance of CSA in CM of marrow fibroblasts (MF) after they had been cultured in the presence of MCM. MCM was added on day 0; medium was harvested at the noted time and used in the CFU-C assay (single hatched bars). Media were harvested also from replicate cultures of MF that had not been cultured with MCM (double-hatched bars). Control cultures not incubated with MCM (not shown) did not produce activity above the background level of the medium (bar 'Med'). (C) Effect of antiserum to murine M-CSF on the colony-stimulating activity present in the CM of stimulated MF (MFmcm). Colony assay as in (A). Either control or conditioned media from MF that had been stimulated by MCM was pre-incubated with antisera to M-CSF or control pre-immune rabbit sera. Additions in sequence noted under the bars of the graph: *Med*, medium alone; *NRS*, medium + pre-immune rabbit serum; *anti-CSF-1*, medium + antiserum at neutralizing dilution; *MFmcm*, CM control; *+NRS*, MFmcm + pre-immune serum; *+anti-CSF-1+*, MFmcm + antiserum.

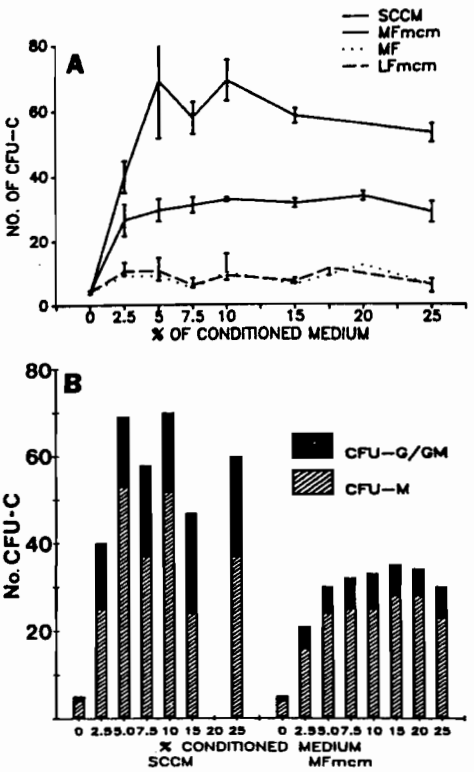


FIG. 2. (A) Dose–response curve of the effect of CM derived from different cultures on the subsequent development of CFU-C. Designation of CM added to marrow cultures for the CFU-C assay is as described in the 'Methods', in the text, and in Fig. 1. The final concentration of CM in the methyl cellulose is noted on the abscissa. (B) The relative effects of SCCM and CM of MFmcm on the subsequent proportion of CFU-M and CFU-G + CFU-GM. Data from (A) expressed to show the relative proportion of the types of colonies developed.

cultures of adherent cells from both marrow and lung can produce CSA, and that the activity elaborated by both of them decreased after the macrophages and heterologous FB had been removed. The results also show that the total net stimulatory activity in the CM of marrow FB, but not that of lung-derived FB, was augmented after they had been cultured in the presence of factors contained in macrophage CM.

Inhibitory activity produced by FB subpopulations

Failure to detect hemopoietic stimulatory activity might be due to either the absence of a given factor, or the presence of inhibitors. To assess these possibilities, the various CM were tested for their ability to decrease the number of colonies formed in the presence of SCCM. Although slight inhibition was observed with MCM alone (Fig. 3A), FB from the lung produced an activity that almost completely reversed the stimulatory effect of SCCM on both myeloid and erythroid rat bone marrow colonies. When CM from primary cultures or cell lines established from them were tested on murine marrow, however, there was not any observed inhibition of either myeloid or erythroid colonies (Fig. 3A and Table 1). The sensitivity of the inhibitory activity to acid was also tested, and it was shown that exposure of the CM to pH 1.5–2.0 for 60 min completely inactivated it (Fig. 3A).

TABLE 1. EFFECT OF RAT-DERIVED CM ON MURINE CFU-C

Cell source of CM added to cultures*	Exp. No. 1		Exp. No. 2	
	CFU-C (% of Max)†	CFU-E	CFU-C (% of Max)†	CFU-E
MF/MFmcm	94/88	ND	105/111	106/94
ST3/ ST3mcm	91/94	95/100	100/95	100/111
LF/LFmcm	98/95	ND	99/109	106/89
ST4/ ST4mcm	95/102	100/111	106/102	83/89

* Source of CM produced from the stated cells were added to test for inhibitory activity, as described for Figs. 1 and 3. 'ST3' is a ST3$^+$ cell line derived from marrow FB; 'ST4' is a ST4$^+$ cell line derived from lung FB.

† For CFU-C (CFU-M + -GM), values expressed relative to that obtained using WEHI-3B cell supernatant (100%); for CFU-E 100% is that obtained in the presence of erythropoietin, as described for Fig. 3A.

ND, not done.

TABLE 2. EFFECT OF CM FROM RAT LUNG FIBROBLASTS ON MARROW CFU-C STIMULATED BY RECOMBINANT M-CSF

Experiment:	Addition to culture:	CFU-M	CFU-G
1	—	5	1
	M-CSF (100 U)	25	1
	M-CSF+LFCM	8	1
2	—	4	1
	M-CSF (200 U)	22	0
	M-CSF+LFCM	8	0
3	—	11	0
	M-CSF (200 U)	20	0
	M-CSF+LFCM	11	0

Conditions identical to those of Fig. 3C, except that rhM-CSF replaced CM of MFmcm. and LFCM was obtained from cultures grown in 10% Nu-Serum. The differences between the paired samples comparing the colonies in the absence and presence of LFCM are significant at the $p < 0.05$ level ($t = 5.71$).

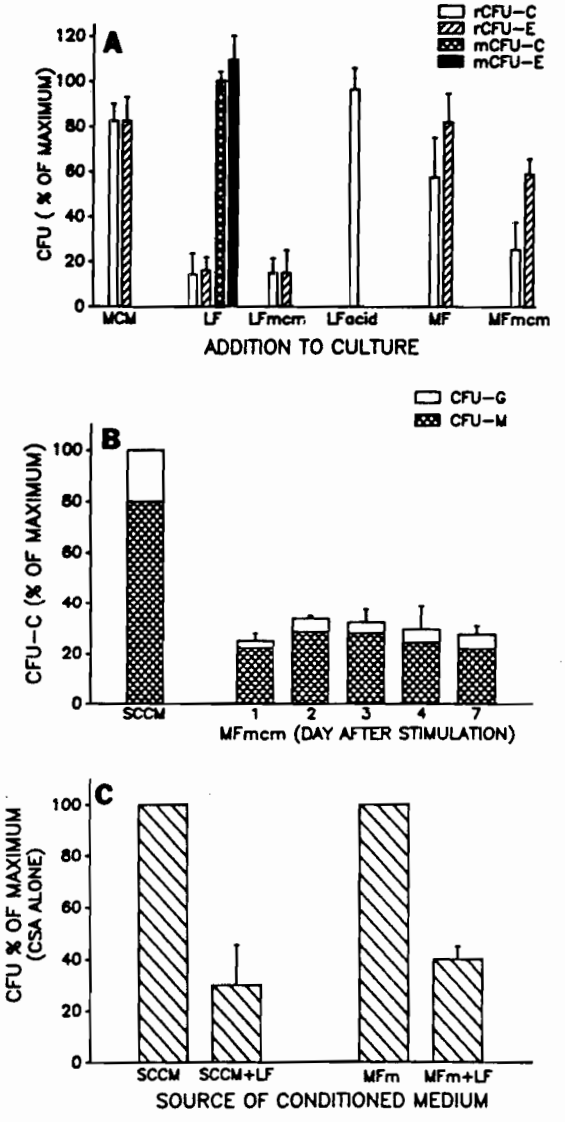
TABLE 3. EFFECT OF CM FROM STIMULATED MARROW FB (MFmcm) ON COLONIES INDUCED BY M-CSF AND SCCM

Addition to culture:	Exp. No. 1* CFU-M	Exp. No. 1* CFU-GM	Exp. No. 2 CFU-M	Exp. No. 2 CFU-GM
Medium alone	5	0	6	0
rhM-CSF (200 U)	16	0	26	0
rhM-CSF + anti-M-CSF	5	0	7	0
rhM-CSF + MFmcm	41	4	40	0
SCCM	61	23	58	19
SCCM + MFmcm	55	10	52	0
SCCM + anti-M-CSF	20	18	32	14

* Values expressed as the absolute number of each CFU-C measured in the assay.

Pre-immune serum from the rabbit yielding the antiserum to M-CSF did not stimulate colonies or inhibit those resulting from SCCM.

Experiments No. 1 and No. 2 were performed with marrow from different rats on separate days, with two different preparations of MFmcm.



Unstimulated marrow FB (MF) produced much less inhibitory activity (Fig. 3A), which was more pronounced on CFU-M and -GM than on CFU-E. After being stimulated by MCM, however, MF produced a CM that decreased myeloid colonies down to the level obtained in the presence of CM of MFmcm alone (i.e. in the absence of SCCM, as in Fig. 1A). In contrast to the time course observed for the elaboration of CSA by marrow fibroblasts after they had been stimulated by MCM (Fig. 1B), that

FIG. 3. (A) Effect of addition of different CM on the stimulation of CFU-C (M+G+GM) and CFU-E by spleen cell conditioned medium (SCCM). The source of each CM is noted on the abscissa. The number of resulting colonies is expressed as a percentage of the maximum, 100% defined as that obtained from the control cultures incubated with SCCM alone (for rat marrow CFU-C) or WEHI-3B CM (for murine marrow), or with erythropoietin (for rat and murine CFU-E). Depending on the assay conditions (i.e. batch of SCCM, individual animal, etc.), the absolute number of colonies ranged from 80 to 120×10^5 bone marrow cells plated. The origin of the indicator bone marrow (i.e. rat or mouse) is designated by rCFU or mCFU respectively. Designation of CM as in Fig. 1A; *LFacid*, represents CM of LFmcm treated with acid as described in the 'Methods'. (B) Time course of appearance of inhibitory activity in CM from MF after incubation with MCM. The experimental design for generation and collection of the CM is identical to that described for Fig. 1B. In this graph, 'CFU-G' includes both CFU-G and CFU-G/M. (C) Competitive effect of CM from LF on the stimulatory (CSA) activity present in SCCM and CM of MFmcm. Values expressed as a percentage of the maximum obtained in presence of each CM alone; i.e. bars on the left side: 100% represents the colonies obtained with SCCM alone; and bars on the right side: 100% represents the total colonies obtained in the presence of CM of MFmcm. Thus, the absolute number of colonies is different, as shown in Fig. 2.

for the inhibitory activity was maximal by 24 h and continued for the seven days of the experiment (Fig. 3B). Because CM from the antigenically homogeneous primary lung cells did not support the growth of marrow colonies, its ability to compete against the CSA of stimulated marrow FB (i.e. MFmcm) could be tested. The comparison shown in Fig. 3C demonstrates that the CSA in the CM of MFmcm was overcome by the inhibitory factor(s) contained in the CM of lung fibroblasts.

The availability of recombinant human M-CSF, and the fact that it is active on rodent cells, enabled the effect of the lung-derived inhibitor to be tested directly on a defined hemopoietic growth factor. As shown in Table 2, consistent with the previous experiment, CM from primary lung cells (LF) obliterated the stimulation of CFU-M by both 100 and 200 U of rhM-CSF. However, CM from stimulated marrow cells (MFmcm) appeared to act in synergy with M-CSF to augment the number of macrophage colonies (Table 3), in a manner that was not reversed by the inhibitor(s). Addition of CM from MFmcm cultures to colony assays supported by SCCM did not inhibit CFU-M, although there was a possible trend to a decrease in CFU-GM. The support of the growth of CFU-M, but not that of CFU-GM, was decreased by pre-incubating the SCCM with antisera to M-CSF (Table 3).

The inhibitory effects on the growth of hemopoietic colonies displayed by these fibroblast products could be the result of antagonism to the activity of hemopoietic growth factors or possibly to direct inhibition of cell replication. To give an initial indication of these possibilities, the CM was added to the colony assays at different times after the marrow cells had been planted in methyl cellulose. As shown in Fig. 4, addition of the lung factor up to two days resulted in maximal inhibition of colony formation, but at day three there was less, and after day four there was not any inhibition. Because the size of the colonies doubled at least two-fold between days three and seven (when they are enumerated), it appears that the effect of the CM cannot be explained wholly by a non-specific (toxic) effect on the replication of the precursor cells at all stages in the growth of a CFU-C.

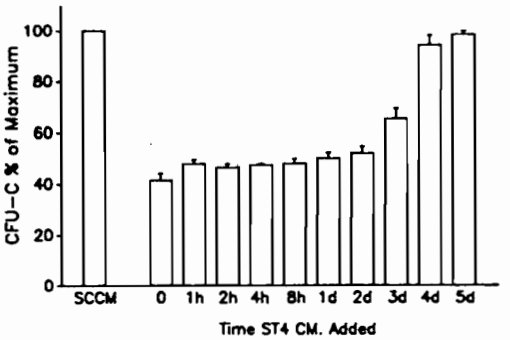


FIG. 4. Effect of delaying the time of addition of lung-derived CM on its subsequent inhibitory action on CFU-C development. Marrow cultures were initiated as in Fig. 3A, but the CM was overlayed on the methyl cellulose at the noted times. The source of CM was the 'ST4' cell line derived from lung fibroblasts.

DISCUSSION

To ensure a regulated flow of blood to the circulation, the bone marrow ecology must maintain a fine balance between influences that promote cell growth and those that retard it. We show here that the culture medium from *early passage* and macrophage-depleted primary fibroblastoid cells of rat marrow ($ST3^+/ST4^-$ phenotype) can be stimulatory or inhibitory depending on the conditions. Stromal cells from a peripheral organ such as the lung ($ST3^-/ST4^+$), however, were always inhibitory. This pattern was consistent for marrow and lung cell preparations from the same rat in more than ten independent experiments from different animals.

Although the cells studied here were selected by the culture conditions that supported their growth, and thus may not be representative of what occurs in the native organ, it is conceivable that such activity produced by lung stromal cells could function to restrict the replication of resident macrophages [20], and possibly to prevent colonization of circulating stem cells that become trapped or break through defects in the endothelial barrier. If other peripheral organs were found to produce similar inhibitors, then it would be reasonable to ask if the progressive localization of hemopoiesis into the medullary cavity during evolution might be the consequence of a unique permissive stroma [21]. Accordingly, one might predict that an inhibitory microenvironment caused by metaplasia of fibroblast subtypes, or other pathological imbalances, could predispose to hemopoietic failure.

The observation that various mesenchymal cells can be induced to make hemopoietic growth factors is not new. After initial attempts to demonstrate this were unsuccessful [22], it soon was recognized that CSFs could be removed from the CM by hemopoietic cells [23–28], that a very low basal secretion of CSFs could be augmented to detectable levels by IL-1 and TNF [9, 13, 14], and that the CSA effect could be masked by inhibitors e.g. [12, 24, 25, 28, 29]. The lack of CSA in CM from rat lung fibroblasts after macrophages had been immunodepleted differs from what has been reported in other studies using human

lung fibroblasts [13, 30, 31]. Apart from possible species variation, this might be explained if rat lung cultures did not make CSFs under these experimental conditions, or if rat cells produced a concentration of inhibitors sufficient to block CSA. We favor the latter possibility because if the inhibitory fraction is removed from the CM by ultrafiltration, stimulatory activity could be detected in the < 100 kDa fraction (unpublished observations). Thus, in this situation the inhibitory activity was able to overcome the effect of the CSA that was present.

Although inhibitory activity predominated in CM from $ST4^+$ lung cells, stimulatory activity predominated in CM from $ST3^+$ marrow cells (Fig. 1A). The conditioned medium from stimulated marrow FB (MFmcm), as the only addition to the CFU-C cultures except for the fetal calf serum in the basal medium, stimulated predominantly CFU-M. When added in combination with rhM-CSF, it increased the number of colonies above that observed at the maximum plateau concentration of either alone (Fig. 2A and Tables 2 and 3). Thus, if the CM of MFmcm contained a small amount of GM-CSF, this result would be consistent with what has been reported on the synergistic effects of combinations of M- and GM-CSF [32]. On the other hand, this same CM decreased the number of CFU-C induced by SCCM, and tended to have greater effect on the CFU-GM than on CFU-M (Table 3). This was the opposite of what was seen after the addition of antisera to M-CSF, which decreased CFU-M much more than the CFU-G/M. The inhibitor(s) contained in LF, however, almost completely prevented the growth of both CFU-M and -GM supported by either rhM-CSF, CM of MFmcm, or SCCM (Table 2, Fig. 3A and 3C). Taken together these results are consistent with the possibility that the inhibitor in the CM of stimulated marrow FB (MFmcm) is less active on M-CSF than it might be against other CSAs known to be present in SCCM (i.e. G- and GM-CSF, and/or IL-3 and IL-6 in combination). From the present data we are not able to discern whether the broader spectrum of anti-CSF activity contained in the CM of LF is due to a greater inhibitor/stimulator ratio than that in the CM of MFmcm, or if the CM from the two different sources contain different products. An answer to such questions will remain speculative until the molecules themselves are purified, and a system is available in which one may test the activity of the lung-derived inhibitor against pure rat CSFs and appropriate factor-dependent cell lines. Work to date indicates that the inhibitor is a heparin-binding glycoprotein [33] whose activity cannot be explained by beta-interferon, beta-transforming growth factor, tumor necrosis factor, or acidic interferon [34].

Finally, in the marrow ecology the demands of regulation will be much more complex than in a simplified culture system such as this. There are many possible modes of regulation that may be acting in the bone marrow to modulate the levels of both inhibitors and stimulators to finely tune the maturation of blood cells. Indeed, Lord *et al.* [35] have shown in long-term marrow cultures that the concentrations of some of these activities are cyclic. Although we have shown differences in inhibitory activity in media conditioned by different fibroblast subtypes, it is possible that the putative molecule is synthesized by both kinds of cells, but is selectively activated, degraded, concentrated, or consumed by one and not the other.

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Chapter 3 EFFECTS OF A FIBROBLAST-DERIVED INHIBITOR ON THE GROWTH OF NORMAL MARROW AND LEUKEMIC CLONOGENIC CELLS[2]

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EFFECTS OF A FIBROBLAST-DERIVED INHIBITOR ON THE GROWTH OF NORMAL MARROW AND LEUKEMIC CLONOGENIC CELLS*

HONG WANG and ARTHUR K. SULLIVAN

Department of Medicine, Division of Hematology, Royal Victoria Hospital, 687 Pine Avenue, West Montreal, Canada H3A 1A1

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Abstract—Blood cells develop in the bone marrow, controlled by a network of regulatory factors, some of which originate in the stroma. Previously, we found that most fibroblastoid (FB) cells growing in primary cultures of rat marrow bear surface antigens different from those found on FB of certain other tissues. As determined by two monoclonal antibodies ("ST3" and "ST4"), the "marrow type" is $ST3^+/ST4^-$ and releases predominantly a colony-stimulating activity (CSA) into its culture media (CM), whereas the "peripheral type" (e.g. lung) is predominantly $ST3^-/ST4^+$ and produces inhibitory activity in excess of CSA. The studies described here show that this inhibitor also is active on rat leukemic myeloblasts (the BNML cell line), but not on eight other cell lines derived from rat tumors of various origins or on the human-derived leukemic cell lines tested. It was produced without exogenous stimulation, was labile to heat and acid, was not neutralized by antisera to transforming growth factor-beta, beta-interferon, or ferritin, and had an apparent mol wt in the range of 100–120 kD (peak of activity by gel filtration). From the results obtained at this time, we are not able to ascribe this fibroblast-derived activity to any known inhibitor molecule.

Key words: Leukemia, bone marrow, stromal cells, fibroblasts, lung, colony-stimulating factors, hemopoiesis, inhibitors.

INTRODUCTION

PRECURSORS of blood cells proliferate and mature in the bone marrow under the control of a complex network of regulatory factors that include promoters [1, 2] and inhibitors of growth [3–10], some of which are produced by the stromal elements [11]. In turn, the secretory activity of fibroblasts and endothelial cells in the microenvironment [12, 13] is influenced by cytokines released by stimulated macrophages (e.g. TNF and IL-1) [14, 15 and reviewed in 16]. In previous work we have demonstrated that the predominant population of fibroblastoid cells cultured from rat bone marrow bears surface antigens different from those found on the majority of similar-appearing cells from selected non-hemopoietic organs [17]. As indicated by the binding of two monoclonal antibodies called ST3 and ST4, the marrow species is predominantly $ST3^+/ST4^-$, and the majority of those grown from lung, diaphragm, and adipose tissue stroma are $ST3^-/ST4^+$. Further comparison of the antigen-defined fibroblasts from bone marrow and lung showed that the two types differed in the spectrum of hemopoietic regulatory activities that they release into their culture medium [18]. Here we describe some of the characteristics of the lung-derived growth inhibitor that is active on both normal and leukemic hemopoietic precursor cells.

*Part of this work has appeared in abstract form. Wang H. and Sullivan A. K. (1988) An inhibitor of myeloid colonies different from beta-TGF is secreted by rat marrow stroma cells. *Blood* **72** (Suppl. 1), 138a.

Abbreviations: *CFU*, colony-forming unit; *CM*, conditioned medium; *EGF*, epidermal growth factor; *FB*, fibroblast; *LF*, lung fibroblasts, treated as per "Methods"; *LFmcm*, LF cultured with MCM; *LFNT*, LF "not treated" to remove other cells; *MCM*, macrophage-conditioned medium; *Med*, medium alone; *MF*, marrow fibroblasts, treated as per "Methods"; *MFmcm*, MF cultured with MCM; *MFNT*, MF "not treated" to remove other cells; *SCCM*, spleen cell conditioned medium.

Correspondence to: Dr A. K. Sullivan, Rm 714 McGill Cancer Centre, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6.

MATERIALS AND METHODS

Animals and cell culture

Bone marrow and other tissues were obtained from 200 to 300 g Brown Norway rats sacrificed by an overdose of inhaled ethyl ether. Unless otherwise stated, cells were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in tissue culture

flasks (T75, No. 25110-75 or T25 No. 25100; Corning) in a 37°C humidified incubator with an atmosphere of 5% CO_2/95% air. Cell lines were not cultured in the presence of antibiotics.

Assay of marrow CFU-C

Marrow colony-forming units (CFU-C) were enumerated by an adaptation of established techniques [19]. Briefly, 3–4.5 × 10^6/ml of rat marrow cells in 10% FCS were mixed with 0.8% methyl cellulose to give a final concentration of 1–1.5 × 10^5 cells/ml, 30% FCS, 5 × 10^{-5} M 2-mercaptoethanol, 5% spleen cell conditioned medium (or other stimulators as indicated) in Iscove's medium. They were then distributed into 35 mm Petri dishes, and after seven days culture in 5% CO_2 at 37°C the colonies were counted using an inverted microscope (colony defined as >50 cells). Typically, depending on the individual rat and batch of SCCM, there were between 70–120 colonies/1 × 10^5 bone marrow cells, of which 15–30% were CFU-G + CFU-CM and the remainder were CFU-M. Spleen cell conditioned medium (SCCM) was prepared as described by Johnson and Metcalf [20], except that phytohemagglutinin (PHA-L Pure, E.Y. Labs, San Mateo, CA) was used as the stimulator.

Clonogenic assay of cell lines (CFU-cell line)

The effect of various conditioned media on the colony forming ability of selected cell lines was assayed by a slight modification of the procedure described above for CFU-C. Between 0.5 and 1.0 × 10^3 cells/ml were suspended in 5% of the stated conditioned medium. The cell lines tested included the following: rat-derived lines, BNML (promyelocytic leukemia; the BNML-RS line is adapted to growth in 4% rat serum/6% FCS, BNML-FCS is adapted by growth in 10% FCS) [26], C58 (thymoma) [52], IR and Y3 (myeloma), PC12 (neuron-pheochromocytoma), C6 (glioma) [latter three referenced in the *Catalog of Cell Lines & Hybridomas*, 6th edition (1988) of the American Type Culture Collection], GN6TF and GN3TG (hepatoma, the former of known sensitivity to TGF-beta [1 ng/ml], and the latter a known resistant) [21], MT450 (breast carcinoma); human-derived lines [53], KG-1 (myeloblast), HL-60 (promyelocyte), U937 (promonoblast), Raji (lymphoblastoid); murine, WEHI 3B (myelomonoblast).

For the normal rat kidney (NRK) assay for transforming growth factors, the procedure of Roberts *et al.* was followed [22]. NRK fibroblasts (obtained from the American Type Culture Collection; NRK-49F ATCC No. CRL 1570) were cultured in RPMI 1640 medium supplemented with 10% FCS, and passaged when they appeared to be approximately 60% confluent. Stock solutions of agar (Difco Noble agar, No. 0412-01) in two times the normal concentration of 1640 medium were diluted to a final concentration of 0.5% (w/v) agar, and 10% FCS. To tissue culture dishes (60 × 15 mm, Corning No. 2540), 2 ml of 0.5% agar solution was added and allowed to solidify at room temperature for 5–10 min, after which another 2 ml of 0.5% agar containing 1–2.0 × 10^3 NRK cells were poured over the first layer. After this had solidified (5–10 min), a third agar layer of 1 ml was added, and the dishes were incubated at 37°C in a 5% CO_2 atmosphere overnight. The following day, each dish was layered with 1.0 ml of epidermal growth factor (EGF, No. 6101LA BRL Inc., final concentration 5 ng/ml), with or without TGF-beta (at a final concentration of 0.4 ng/ml), with or without fibroblast conditioned medium (final concentration 5%). These dishes were incubated at 37°C for 10–14 days, at which time the colonies, appearing as compact spheres, were enumerated.

Preparation of conditioned medium (CM): macrophage conditioned medium (MCM)

The preparation of conditioned media from macrophage and fibroblast cultures have been described in detail elsewhere [18] and will be summarized here.

Rat spleens were removed, minced, the pieces pushed through a stainless steel screen, and the cell suspension seeded into a single T75 flask. On day 4, the non-adherent cells were decanted, and those remaining were incubated for 30 min with antibodies ST3 and ST4 (murine ascitic fluid), at a pre-tested dilution that was found to be optimal for cytotoxicity, followed by 45 min with mouse complement (Low-Tox M No. CL3015, Cedarlane, Hornby, Ontario), washed, and finally replenished with 40 ml of medium. The intent of this was to deplete the cultures of fibroblast precursors [17]. By day 6 this resulted in a rich growth of macrophage-like cells staining with BN35 and ST2 anti-macrophage antibodies [described in 17], with only a rare fibroblastoid cell being identified by immunostaining with ST3 or ST4 antibodies.

Preparation of conditioned medium (CM): fibroblast conditioned media

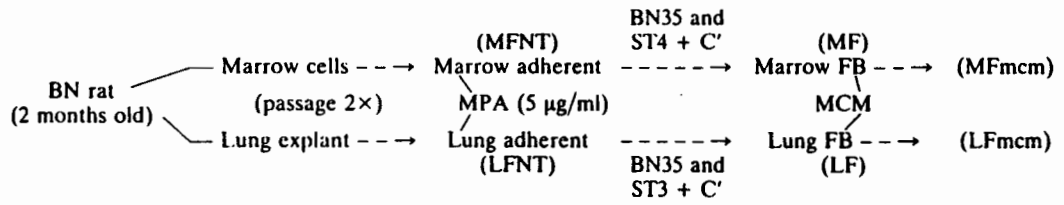
The marrow cavities were vigorously flushed with medium and the resulting cell suspension was transferred to a T75 flask in a final volume of 50 ml, after which the cultures received the following manipulations: on day 3, non-adherent cells were decanted; day 7, the adherent layer was removed by scraping with a rubber policeman and placed into a new T75 flask; day 14, cells were removed by incubation with trypsin and distributed equally into 8–10 T25 flasks; day 17, mycophenolic acid (MPA, Sigma No. M5255) was added to cultures at a final concentration of 5 μg/ml to inhibit growth of hemopoietic precursors [23]; day 21, media changed, without MPA; day 24, media changed, with MPA; day 27, media changed, without MPA; days 31 and 33, the remaining cells were treated with ST4 and BN35 antibodies in the presence of complement to deplete remaining macrophages and the minor subpopulation of "non-marrow" type fibroblasts bearing the ST4 antigen. The progeny of these cultures were called "MF"; day 35. To stimulate the fibroblasts with macrophage-derived cytokines, MCM (40% final volume) was added to individual MF cultures for 24 h. The resulting supernatants, designated as "MFmcm", were collected and used to test for inhibitory activity.

Control cultures were not grown in the presence of mycophenolic acid, and they were not exposed to anti-macrophage or anti-fibroblast antibodies; otherwise they were subjected to the same manipulations. The cells in these parallel cultures are designated as "MFNT".

Fibroblasts were prepared from the lung of the same rat by a similar strategy. The same scheme was followed, except that on days 31 and 33, the cultures were treated with antibodies BN35 and ST3 (instead of ST4) to deplete residual macrophages and "marrow type" fibroblasts. Cells of these cultures are designated "LFNT", "LF", and "LFmcm".

Cultures prepared in the manner described and used to obtain the CM from MF and LF were greater than 99.9% homogeneous by immunostaining (i.e. we did not see any $BN35^+$ or $ST3^+$ cells in 1000 lung cells, or any $BN35^+$ or $ST4^+$ cells in marrow-derived adherent cells).

The sequence of manipulations is summarized below.



In some of the later experiments, as stated in the text, the CM was obtained from an ST4$^+$ cell line that had developed spontaneously from a culture of rat lung FB. The production of CSA and inhibitory activity, both constitutive and in response to MCM, was as described for LF. These cells were used to obtain the starting product for further purification.

Further treatment of the conditioned media (CM): antibody neutralization

These experiments were designed to test the ability of various antibodies to eliminate the inhibitory activity in the CM of lung FB. For anti-TGF-beta, the optimal dilution of a neutralizing antiserum (R & D Systems Inc., No. AB-10-NA, rabbit antiserum to porcine-derived TGF-beta-1, also reactive with rat TGF-beta) was determined to be 20 μg IgG for 2.0 ng of pure TGF-beta (R & D Systems Inc.; from porcine platelets, No. 101-b1) after 1 h incubation at room temperature, as indicated by residual activity in the NRK assay (described below). Each CM was treated in a similar manner, and its effects on NRK cell growth was tested in clonogenic assays.

Similarly, an antiserum to rat beta-interferon was assessed for its ability to neutralize the inhibitory activity from LF. The activity of the antiserum to rat beta-interferon (antiserum prepared in rabbits against rat beta-interferon; Lee Biomolecular Research Laboratory Inc., San Diego, CA, No. 40032) was confirmed by demonstrating that it could reduce to baseline the maximal inhibition (75 units of pure rat beta-interferon; Lee Biomolecular; 2.5×10^4 units/ml; 2.7×10^4 units/mg; No. 40041) of clonogenic growth of rat C6 astrocytoma cells, known to be sensitive to interferon [24]. After incubation together for 1 h at room temperature, 15 U of antibody neutralized 75 U of interferon. The CM was incubated with 15 U of antiserum, and its residual activity subsequently was tested in the marrow CFU-C assay.

Finally, rabbit antiserum to murine liver ferritin was obtained as a gift from Dr P. Ponka (Lady Davis Research Institute, Montreal). It was shown by Ouchterlony immunodiffusion to cross-react with ferritin obtained from both rat liver (Sigma) and rat heart (further characterization in legend to Table 2). For inhibitor neutralization experiments it was used at a dilution (1/10) that had been shown to reverse the inhibitory activity of rat heart ferritin on marrow CFU-C (Table 2).

Further treatment of CM: removal of inhibitory activity by incubation with cells

Experiments to test the ability of different types of cells to remove the inhibitory activity by absorption were performed separately on the CM of LF, control media (RPMI 1640), 1% BSA (carrier protein in which TGF-beta was supplied), and control media to which 20 ng/ml TGF-beta had been added. For absorption with hemopoietic cells, peripheral blood was obtained by cardiac puncture, and erythrocytes were recovered after leukocytes had been separated above a barrier of Ficoll–Paque (Pharmacia). Bone marrow was obtained as described above, and the nucleated cells were recovered above a one-step barrier of Percoll (1.095 g/ml; Pharmacia). Lymphocytes were obtained from spleen cell suspensions that had been depleted of macrophages by incubation on plastic culture dishes at 37°C for 60 min, rinsed off, and reincubated. After being washed three times, 1.0×10^7 cells were suspended in 1.0 ml of CM of LF, 1% BSA, TGF-beta solution, or control supernatant (1640 medium with 10% FCS), and incubated for 30 min at room temperature; after being mixed once and equilibrated with 5% CO_2, they were incubated for another 30 min. They were then removed by centrifugation (1000 *g* for 15 min), and the absorption was repeated twice more with fresh cells. The resulting supernatant was then assessed for inhibitory activity in clonogenic assays.

Further treatment of CM: heat and acid

In some experiments, samples of CM were heat-treated by boiling for 5 min prior to being tested for activity in the clonogenic assay. To test for acid stability, aliquots of CM were acidified to pH 1.5–2.0 with 1 M HCl at 4°C for 60 min, brought to pH 8.0 with 1 M NH_4Cl, and then immediately frozen and lyophilized. Before further use, the sample was reconstituted in the original volume of distilled water. The activity of pure porcine TGF-beta in the NRK assay was not inactivated by this treatment. In control experiments, the inhibitory activity in CM of LF was stable to freezing and thawing at least once.

Partitioning of inhibitory activity by membrane and gel filtration

Conditioned medium from the ST4$^+$ cell line grown in RPMI 1640 supplemented with 10% Nu-Serum (which contains 2.5% FCS; Collaborative Research, Inc., Lexington, MA, U.S.A.; No. 50007), was collected and passed through a series of membrane filters to obtain fractions of proteins that partition in different ranges of molecular weight. The filters used had exclusion limits of 100, 50, and 10 kD (XM100, XM50, and UM10; Amicon, Lexington, MA, U.S.A.). The initial sample was concentrated to approximately 20% of its original volume over a membrane of >100 kD retention limit; the ultrafiltrate from this step was added to another chamber containing a membrane of >50 kD exclusion limit and concentrated to approximately 20% of the added volume; the ultrafiltrate from this step was added to another chamber containing a membrane of >10 kD exclusion limit; this ultrafiltrate was analyzed for the presence of inhibitors of CFU-C. After the initial sample had been concentrated over the 100 kD filter, the

retained material was reconstituted to the original volume with RPMI 1640 and reconcentrated, and the process was repeated again to wash through residual molecules of lower molecular weight. This was performed for each of the initial concentrates of the different size filters, and the ultrafiltrates of the washes were discarded. The retained material from each chamber was then reconstituted with RPMI 1640 to the original volume that had been added, and then was used to assay inhibitory activity.

Preparation of rat heart ferritin

Rat heart ferritin was prepared as described by Penders and De Rooij-Dijk [25]. Briefly, six rat hearts were homogenized in water, the resulting suspension was heated at 80°C for 15 min, filtered through fiberglass filter (Whatman), and the filtrate was then centrifuged at 78 000 *g* for 60 min. The supernatant was discarded and the brown sediment was suspended in water and re-centrifuged at 7000 *g* for 60 min. A small precipitate was discarded, and the supernatant was centrifuged at 95 000 *g* for another 60 min; this sediment was resuspended, centrifuged, resuspended, and used in subsequent studies.

RESULTS

Inhibitory action of CM on rat leukemic myeloblasts

To determine if the action of the marrow and lung-derived inhibitors extended beyond normal hemopoietic precursors to cells that are not wholly dependent on exogenous factors for replication (other than those in serum), we studied the response of the rat promyelocytic leukemia line BNML-FCS [26] and showed that CM from lung FB decreased its clonogenicity in a dose-dependent manner (Fig. 1A). The relative pattern of inhibition by CM from LF and MF observed on these cultured leukemic cells was similar to that previously seen on normal marrow colonies [18], in that lung-derived FB produced an inhibitory CM constitutively but marrow-derived FB did so only after they had been stimulated with macrophage products. Macrophage CM (MCM) alone did not have any effect (Fig. 1B). Because the inhibitory activity found in the CM of lung FB was produced without exogenous stimulation and appeared to be more potent than that from marrow FB, we then focused our efforts on characterization of the LF product.

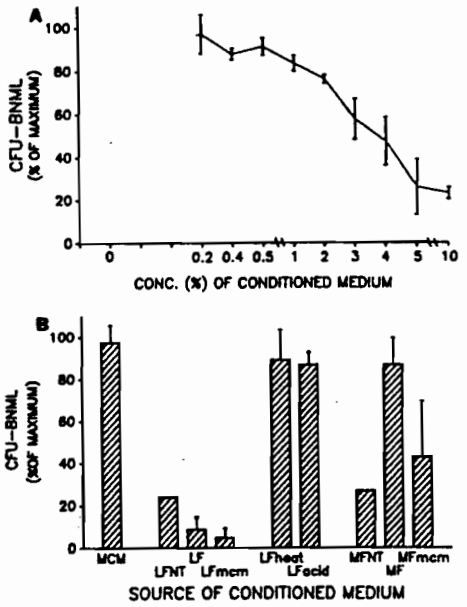


FIG. 1. (A) *Dose–response curve of CM from lung fibroblasts on BNML-FCS cells.* Values expressed as a percentage of maximum (100%) colonies grown in the absence of added CM. The cloning efficiency of BNML cells is between 40 and 60%; from 0.5 to 1.0 cells/dish, 200–600 colonies would result. On the abscissa is the final concentration expressed as a percent (v/v) of CM in which cells were grown. (B) *Effect of different CM on the cloning efficiency of rat leukemic promyelocytes (BNML-RS).* The source of each CM is noted on the abscissa. The number of resulting colonies are expressed as a percentage of the maximum, 100% defined as that obtained from control cultures grown in the presence of growth medium alone. The sources of the CM are as follows: *MCM*, macrophage-conditioned medium; *LFNT*, CM from adherent lung FB that had not been treated to remove hemopoietic precursors, macrophages, or "other" type FB; *LF* (lung FB), CM from LFNT cultures that had been treated to deplete macrophages and "other" FB, as described in the "Methods"; *LFmcm*, CM from LF incubated with MCM; *LFheat and LFacid*, LF that had been treated with acid or by boiling as described in the "Methods"; *MFNT*, *MF*, *MFmcm*; analogous cultures of marrow FB.

Action of lung fibroblast CM on other cell lines

The spectrum of inhibitory activity was further tested for its effect on the clonal growth of various cell lines. Summarized in Table 1 are the results showing that of ten lines of rat origin, only BNML promyelocytes were inhibited by the CM of LF at a concentration that prevented the growth of marrow hemopoietic colonies [18]. This inhibitor was not active on four human leukemic lines, and in agreement with previous observations that it did not inhibit normal murine CFU-C [18], it did not decrease the clonogenic growth of murine WEHI-3B myeloid cells.

Biochemical characteristics of the inhibitory activity from LF

To determine some of the physical characteristics of the inhibitory molecule(s), CM of LF was subjected to various manipulations and the remaining activity was tested. The inhibitory activity was not diminished after it was stored at 4°C for at least eight weeks, or after it was heated to 56°C for 30 min (not

TABLE 1. INHIBITION OF THE CLONING EFFICIENCY OF CELL LINES BY CM*

Cell line	Addition to the culture: CM of LF	TGF-beta: 1 ng/ml	TGF-beta: 4 ng/ml
BNML-RS	34 ± 16†	94 ± 5†	70‡
BNML-FCS	9 ± 6	87 ± 14	
GN3TG	100 ± 6	97 ± 3	
GN6TF	99 ± 1	79 ± 2	
U937	99 ± 2	70 ± 5	18
WEHI-3B	92 ± 6	67 ± 8	

* Cloning efficiencies of cell lines cultured in the presence of the noted CM, expressed as a percentage of that observed in medium alone (100%).

† Values of the mean of three independent experiments ± 1 SD, performed with three different CM.

‡ Average of two independent determinations.

Other cell lines tested that were not inhibited by the CM of lung FB were: (rat origin) C58, IR, Y3, MT450, PC12, C6; (human origin) KG-1, HL-60, and Raji (range of values for percent growth was 97–109% ± 3).

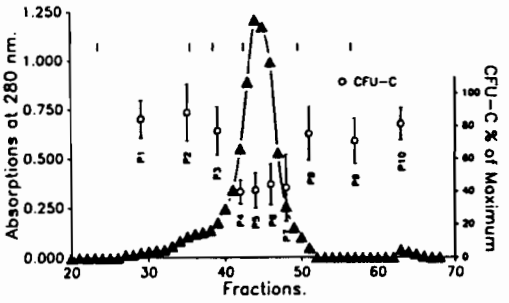


FIG. 2. Elution profile from BioGel A-1.5 m (200–400 mesh, Bio-Rad, Mississauga, ON) of inhibitor activity in concentrated CM of LF retained by the membrane with >100 kD mol wt cut-off. Column dimensions: total volume, 310 cm^3 (r = 1.35 cm; h = 54 cm); flow rate, 40 ml/h; fraction size, 5.0 ml/fraction; running buffer, PBS. Fractions pooled and tested for activity in the rat marrow CFU-C assay are denoted as P1, etc. P1, fractions 26–32; P2, 33–38; P3, 39–41; P4, 42–43; P5, 44–45; P6, 46–47; P7, 48–49; P8, 50–52; P9, 53–59; P10, 60–66. Vertical lines at top of graph refer to mol wt standards (Standard kit, Pharmacia) in order: Blue dextran (2 000 000 kD), thyroglobulin (669 000), ferritin (440 000), catalase (232 000), ovalbumin (43 000), and cytochrome C (12 000). Inhibitory activity (open dots) in the stated fractions were assayed by the CFU-C assay using rat bone marrow stimulated with SCCM. Values are expressed as a percentage of the number obtained with SCCM alone (100%) in the absence of other CM. The absolute number of colonies in the 100% value ranged from 70 to 120, depending on the individual rat or preparation of SCCM. Error bars are the mean value ± 1 SD of three separations of three independent samples of CM. Solid triangles represent the absorption at 280 nm.

shown). Conversely, boiling for 5 min, or exposure to acid (pH 2) destroyed the activity tested on BNML cells (Fig. 1B). The fact that it was destroyed by incubation of the CM with trypsin (not shown) indicated that it is a protein.

Sequential passage of the CM through ultrafiltration membranes of different pore sizes showed that only the material retained by the filter with a >100 kD exclusion limit significantly inhibited marrow CFU-C. The percentages of CFU-C, relative to that formed in the presence of SCCM (100%) were as follows (mean of three independent supernatants ± SD): >100 kD fraction, 41 ± 16; 50–100 kD fraction, 92 ± 8; 10–50 kD fraction, 83 ± 5; and <10 kD fraction, 95 ± 2. Gel filtration demonstrated that the peak of the major inhibitory activity in the CM was in the range of 100–120 kD mol wt (Fig. 2).

Removal of inhibitory activity from CM by sensitive and resistant cells

If the inhibitory activity of the CM was due to a distinct protein molecule that acts on a target cell receptor, and it was present in low concentration, as might be anticipated for such factors, it should adsorb to responsive cells and be removed. The results of experiments shown in Fig. 3A demonstrate that the activity against both bone marrow CFU-C and BNML cells was decreased after incubation with sensitive rat BNML cells, but not with insensitive human HL-60 promyelocytes or U937 promonocytes. To assess if more mature blood elements had a similar capability, mononuclear cells from marrow and spleen, and peripheral blood erythrocytes were tested for their capacity to decrease the inhibitory activity from CM. The percentages of CFU-C, relative to that formed in the presence of SCCM (100%) were as follows (mean of three independent experiments ± SD): CM of lung fibroblast line, 34 ± 6; CM absorbed with BNML cells, 81 ± 10; absorbed with peripheral erythrocytes, 41 ± 5; absorbed with spleen cells depleted of macrophages, 42 ± 5; absorbed with bone marrow cells, 43 ± 4. Thus, only the BNML cells were able to remove the activity to an extent that was significant.

Discrimination of the rat lung FB inhibitor from other known hemopoietic inhibitors

Transforming growth factor-beta has been reported to inhibit the growth of marrow CFU-C [7, 8]. In the assay that measures TGF-beta by its ability to potentiate anchorage-independent growth of normal rat kidney (NRK) cells, the CM of LF appeared to contain a small amount of activity that was decreased by a neutralizing antiserum to TGF-

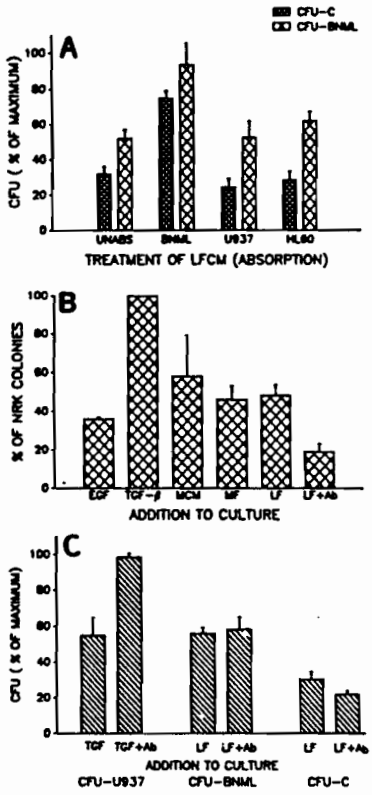


FIG. 3. (A) *Effect of absorption of CM from lung FB with cell lines.* Aliquots of CM from cultures of LF were incubated with BNML cells (sensitive to LF inhibitor, but only slightly to TGF-β), human HL-60 promyelocytes, or U937 monoblasts (both human lines sensitive to TGF-β, but not to LF inhibitor). The recovered CM was assayed for inhibitory activity on both BNML cells and rat marrow CFU-C. The number of colonies is expressed as the percentage of the maximum; for BNML, 100% refers to colonies obtained in growth medium alone; for CFU-C, 100% refers to colonies obtained in the presence of optimal concentrations of SCCM (absolute number of colonies ranged from 70 to 120/10^5 cells). The notation of the bars refer to the absorbing cell line: Unabs, control CM, not absorbed. The error bars indicate the mean ± 1 SD of at least three independent experiments. For each data point, colonies were enumerated in duplicate culture dishes. At least two independent preparations of CM from lung fibroblasts were used. (B) *Effect of different CM on colony formation of NRK cells.* The number of colonies are expressed relative to 100%, i.e. colonies formed in presence of EGF + 0.4 ng/ml TGF-β. Additions to the culture in order of bars on graph were: *EGF*, epidermal growth factor; *TGF-β*, EGF + TGF-β (0.4 ng/ml); *MCM*, EGF + MCM; *MF*, EGF + CM of marrow FB; *LF*, EGF + CM of lung FB; and *LF + Ab*, EGF + LF preincubated with antisera to TGF-β at a dilution that neutralized activity of TGF-β in control assays. (C) *Effect of treatment of CM from lung FB with neutralizing antisera to β-TGF.* The number of colonies are expressed relative to 100%, as in (A). Results shown in bars labeled "CFU-U937" show the effect of TGF-β (2 ng/ml) without and with anti-TGF-β (TGF + Ab); CFU-BNML shows the effect of CM from lung FB with and without anti-TGF; CFU-C, shows the effect on rat marrow colonies.

beta (Fig. 3B). However, the clonogenic growth of U937 cells was decreased by 30% by 1 ng/ml of pure TGF-beta and by 80% by 4 ng/ml (Table 1), but it was not decreased by the CM of LF at the same concentration that diminished the growth of BNML cells by 70–90%. Conversely, growth of BNML cells was only minimally (if at all) decreased by 1 ng/ml. The antiserum that was able to reverse the inhibitory effect of at least 2 ng/ml on U937 did not change the inhibitory activity of CM of LF on either BNML cells or marrow CFU-C (Fig. 3C). Further comparison of the effects of CM of LF and TGF-beta on other cell lines confirmed that the two inhibitors did not act in a concordant manner (Table 1); murine WEHI-3B, human U937, and rat GN6TF lines all were inhibited to some degree by TGF-beta, but not by CM of LF.

Another fibroblast-derived product reported to have negative regulatory effects on hemopoietic cells, and to act in a species-restricted manner is beta-interferon. Control experiments confirmed that C6 rat astrocytoma cells were inhibited by pure rat beta-interferon, and that an antiserum to it reversed the effect [24]. However, preincubation with this same antiserum did not decrease the inhibitory activity of the CM of LF on the growth of marrow hemopoietic colonies (not shown).

The acidic form of ferritin composed of the H subunit can inhibit the growth of hemopoietic colonies (ref. [4]; Table 2). A rabbit antiserum prepared to murine hepatic ferritin that was shown to cross-react with ferritin of both rat heart (enriched in the H subunit) and liver (enriched in the L subunit) reversed >95% of the negative effect of heart ferritin on rat CFU-C, but decreased the inhibitory activity in the CM of LF by only 20–30%. However, the same antiserum did not have any effect on the inhibitory activity in the 100–120 kD fraction obtained by gel filtration (Table 2).

DISCUSSION

These experiments and other studies published elsewhere [18], demonstrate that culture medium conditioned by rat lung fibroblastoid cells contains an activity that is inhibitory to normal rat hemopoietic precursors and to a myeloid leukemia-derived cell line (BNML). This inhibitor did not retard the growth of a murine myeloid leukemic line (WEHI-3B) or three human leukemic lines. As more cell lines from other hemopoietic lineages become available to us, and as the molecule(s) become better defined, the issue of possible species specificity can be examined with greater precision. An alternate possibility might be that, except for BNML myeloblasts, all of the tumor lines we tested had become resistant to the

TABLE 2. EFFECT OF FERRITIN AND ANTI-FERRITIN ON HEMOPOIETIC COLONIES

Addition to culture:	Exp:	Number of CFU-M + CFU-GM (% of Max in SCCM alone) No. 1	No. 2	No. 3	No. 4
Medium alone		10	8	10	11
SCCM		100	100	100	100
SCCM + anti-ferritin		103	104	90	96
SCCM + ferritin*		67	65	59	ND
SCCM + ferritin + anti-ferritin		100	93	96	ND
SCCM + CM of ST4 line		42	44	35	32
SCCM + CM of ST4 line + anti-ferritin		58	60	49	ND
SCCM + Fx100–120†		ND	ND	25	25
SCCM + Fx100–120 + anti-ferritin		ND	ND	26	26

* Ferritin was purified from rat heart as described in the "Methods"; 5 μl of stock solution containing 1.9 mg/ml was added to 1 ml of methyl cellulose mixture for the colony assay in experiment No. 1 and 10 μl in experiment Nos 2 and 3 (i.e. a 1/200 and 1/100 dilution; 9.5 and 19 μg/ml final concentration). By Ouchterlony immunodiffusion (not shown) the rabbit anti-mouse ferritin produced a very strong precipitin line with 1.9 mg/ml rat heart ferritin and a faint line with CM from "ST4" cell line that had been concentrated 10-fold to bring it to 200 times the concentration used in the CFU-C assay (so that equivalent ratios of ferritin and CM were used in the immunodiffusion and CFU-C assays). Thus, by immunodiffusion there was less ferritin in the CM than there was in the partially purified preparation from rat heart used in the CFU-C experiments.

† Fx100–120 is the pooled 100–120 kD fraction obtained by gel filtration, as shown in Fig. 2.

inhibitor's action after they had been established in culture.

Several molecules [reviewed in 27] reported to inhibit the clonal growth of hemopoietic cells in culture include prostaglandins [3], lactoferrin [5], acidic isoferritin [4], tumor necrosis factor [6], interferons [9, 10], and transforming growth factor-beta [7, 8], some of which can be secreted by stromal elements. Also, other substances from various sources have yielded products that can inhibit different lineages or cell subsets. Del Rizzo *et al.* have purified a 79 kD protein inhibitory to BFU-E that is released into the CM of marrow cultures of the B6 mouse [28]. The leukemia inhibitory factor (LIF) identified by Metcalf and co-workers [29–31] has a mol wt of 58 kD, and induces differentiation of some murine myeloid leukemia cell lines, but does not reverse the effect of G-, GM- or multi-CSF on normal bone marrow cells. A CFU-S proliferation inhibitor of 50–100 kD from marrow macrophages of both mice and humans appears not to act on committed precursors in the CFU-C assay of either species [32–34]. Possibly related to this activity is the "stem cell inhibitor", or the macrophage inflammatory protein (MIP-1α) that is a polypeptide of 8 kD inhibitory to primitive cycling stem cells but not to CFU-GM [35]. In other assays hemoinhibitory activity has been noted for two different peptides, composed of four or five amino acids, that have been purified from homogenates of porcine marrow and leukocyte CM. These molecules can act across species barriers, and may enhance binding of stem cells to the stroma [36, 37]. Another factor of <50 kD derived from murine monocytic leukemia cells has strong inhibitory activity on CFU-C stimulated by M-CSF, and was stated to be different from other known cytokines [38]. In addition to these, several other less characterized inhibitors have been identified in exudates, CM, or in agar overlay culture systems [11, 13, 39–43]. Finally, Zipori found an activity produced by a stromal cell line that appeared to have its maximal effect on cultured myeloma cells [44], and has advanced the notion of a family of factors called "restrictins" [45] that could function to limit proliferation of a given lineage in specific regions of the marrow.

From the results obtained at this time we are not able to ascribe the fibroblast-derived inhibitor of CFU-C to any of the known molecules discussed above. Biochemical studies in progress indicate that it is a heparin-binding glycoprotein which remains active in partially purified form (10 000 ×) [51]. The fact that it was derived from fibroblasts (as opposed to macrophages), and was not active on human HL-60 or U937 cells or murine CFU-C is against it being tumor necrosis factor [46]. The apparent species specificity suggests that it might be beta-interferon, but the acid lability is not consistent with what has been reported for this class of compounds derived from the rat [24, 47, 48], and antisera to rat interferon did not reverse its effect. Further experiments were

designed to eliminate the possibility that it might be TGF-beta. The LF-derived activity was heat and acid labile, it did not inhibit cell lines confirmed to be sensitive to TGF-beta, it inhibited cell lines not sensitive to TGF-beta, it gave minimal stimulation in the NRK assay, and it was not blocked by an antiserum to TGF-beta. The high apparent mol wt of this rat factor suggests it might be acidic isoferritin. We consider that not to be likely because by gel filtration the peak activity was found in the 100–120 kD range—much lighter than the >400 mol wt of the H-subunit isoferritin multimer [49, 50]; neither was it inactivated by antisera to ferritin. Furthermore, were the observed effects simply due to trivial "non-specific toxicity", one would not expect its action to have been so selective on the different cell lines or that it would have been absorbed by sensitive cells. This is consistent with previous observations that the inhibitor is active on CFU-C and CFU-E of the rat, but not those of the mouse [18].

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Chapter 4 INHIBITORS OF HEMOPOIETIC COLONIES ARE PRODUCED BY CERTAIN RAT FIBROBLASTOID CELL LINES AND ARE MODULATED BY CORTICOSTEROIDS[4]

[4]This chapter is a duplication of the paper by H. Wang and A.K.Sullivan, which has been accepted for publication by Experimental Hematology.

ABSTRACT.

Both stimulatory (CSA) and inhibitory (INH) factors may contribute to hemopoietic regulation, but little is known about how their physiologic balance is maintained. Previously we have shown that antigen-defined fibroblastoid cells cultured from rat lung (ST3-/ST4+) constitutively produce INH, and those derived from bone marrow (ST3+/ST4-) respond to macrophage cytokines to release both CSA and INH into their conditioned media (CM). Here we show that this pattern was maintained in cell strains ("ST3" and "ST4") propagated from the primary cultures, and that the presence of CSA was measured in "ST4" CM if the inhibitory >100 kDa fraction was removed. Two subclones of the "ST3" line, called 2A and 9D, were selected for high or low expression of the ST3 antigen respectively. Both produced CSA, but only 9D produced the >100 kDa inhibitor. In the conditioned medium of cells cultured in the presence of hydrocortisone there was less INH detected but CSA was not changed. From these data, however, we can not assess how many individual cell products might be contributing to the INH activity. These results demonstrate that the appearance of inhibitory activity in the growth media differs among fibroblast subpopulations, and that it can be modified by natural regulators such as corticosteroids.

INTRODUCTION.

Growth of hemopoietic cells in vitro is influenced by several stimulatory [1] and inhibitory [2] molecules, but the source and physiological regulation of these factors are not well defined.

In previous experiments designed to dissect stromal cell populations and study their functional differences we have shown that the predominant fibroblastoid (FB) cell growing in primary cultures of rat bone marrow bears a surface antigen phenotype different from that expressed by most similar-appearing cells from certain non-hemopoietic organs (e.g., lung). As indicated by the monoclonal antibodies ST3 and ST4, prepared to subpopulations of fibroblastoid cells in cultured rat bome marrow, the "marrow type" is predominantly ST3+/ST4- and the "peripheral type" is predominantly ST3-/ST4+ [3]. Using primary cultures of marrow and lung FB we found that the marrow type secreted colony-stimulating activity into the conditioned medium (CM) in response to stimulation by macrophage products, but the lung type did not produce any detectable activity. Subsequent work revealed that lung cells constitutively produced an inhibitor of CFU-M,-G/M, and -E, but marrow cells did so only after they had been stimulated [4].

The inhibitory activity from lung FB CM has the following characteristics [5]: (a) It also inhibits the clonogenic growth of BNML rat promyelocytic leukemia cells, but not that of eight other rat lines of various origins, human hemopoietic lines (HL60, U937, Raji),

murine WEHI-3B, or murine myeloid and erythroid precursors. (b) It can be removed from the CM by incubation with BNML cells under conditions that it is not removed by lines that are not inhibited by it. (c) It does not inhibit rat lines shown to be sensitive to inhibition by TGF-beta or beta-interferon, and its activity was not inhibited by neutralizing antibodies to these agents or to acidic isoferritin. (d) It was acid and heat labile, but trypsin sensitive. (e) By gel filtration of lung FB CM, the activity migrated in the 100-120 kDa range. (f) Partial purification by ion exchange and affinity chromatography (synthetic dye ligands, heparin, and wheat germ lectin) resulted in a 10,000-fold enrichment of specific activity [6].

These studies, having shown functional differences between primary FB of lung and marrow, raised the question of whether the relative balance between inhibitory and stimulatory activities was a stable aspect of their phenotype and whether the two could be regulated independently. Here we further examine the behavior of cell lines obtained from antigen-defined fibroblasts from marrow and lung.

METHODS.

Animals and cell culture. Bone marrow and other tissues were obtained from 200-300 gm Brown Norway rats sacrificed by overdose of inhaled ethyl ether. Unless otherwise stated, cells were cultured by standard techniques in RPMI 1640 medium supplemented with 10% fetal bovine serum (FCS), 2 mM l-glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin in tissue culture flasks in a 37^{o} C humidified incubator with an atmosphere of 5% CO2/95% air.

Derivation of the cell lines. The derivation of homogeneous primary FB cultures from marrow and lung has been described elsewhere [4]. Briefly, adherent cell cultures grown in RPMI 1640/10% FCS were treated with the BN(MB)35 anti-rat myeloid antibody [3] and complement, mycophenoloic acid to deplete hemopoietic precursors, and either ST3+C' (lung cultures, LF) or ST4+C' (marrow cultures, MF) to deplete residual FB of the "other" type. By culture day 30, >99.9% of cells in the MF or LF preparations stained by the immunoperoxidase technique for ST3 or ST4 antigen respectively. The cells then were cultured continuously in T25 flasks and passaged when they had become 70-90% confluent by visual examination (approximately weekly). After four weeks the cell proliferation rate in some of the flasks had increased dramatically, and a few "cell clumps" were observed suspended in the supernatant. A single clump was tranferred to another flask and propagated. When growth of a new adherent cell layer had become confluent, samples

were taken for immunohistological staining and shown to react appropriately with the ST3 and ST4 antibodies. This was defined as "pass #1" and the cells were named "ST3" and "ST4" accordingly.

Derivation of subclones. Cells from "pass 1" of the "ST3" strain were distributed into chambers of 96 well plates at 10 cells/well. When growth was established, the cells of each well were removed by trypsinization and passaged into the corresponding well of another 96 well plate. One plate was used to measure the concentration of the ST3 antigen on the cells in each well by the ELISA method [3]. The cells in the wells corresponding to the highest and lowest values then were expanded, replated into 96 wells, assayed as in the first cycle, and again the wells with the extreme values of ST3 antigen expression were selected. Their progeny then were cloned twice on fragments of glass coverslips, and the clones with the highest and lowest staining were selected; the "high ST3" clone was called "2A" and the "low ST3" clone 9D. After expansion in T25 flasks, the progeny were defined as "pass #1".

Measurement of growth kinetics of cell lines. Cells of either the "ST3" or "ST4" strain were removed by trypsinization and pipetted into 24-chamber culture dishes at 1 or 0.5 x 105 cells/well respectively. The cells were collected daily from replicate wells by trypsinization and viable cells (trypan blue) were counted; alternatively,

the cells in the wells were washed with PBS, solublized with 1% SDS, and the protein content estimated by the absorbance at 280 nm in a Beckman BU-65 spectrophotometer.

For other experiments testing the effect of lung FB conditioned media on the growth of BNML rat myeloid leukemia cells, the XTT assay was used to measure cell number. BNML-FCS cells were cultured routinely in RPMI 1640/10% FCS and were harvested in mid log phase, washed twice in RPMI 1640 without serum, resuspended in RPMI 1640/10% FCS with or without added CM, distributed into quadruplicate wells of a 96-well culture dish at 5, 10, or 20x103/well, and incubated at 37o C. At 24, 48, or 96 h the cells growing in the wells were enumerated by the XTT assay as described by Scudiero, et al [7]. Briefly, the solution of XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; Polysciences} and PMS (phenazine methosulfate; ICN Flow Canada, Mississauga, ON) was added directly to the wells. After the cells had been incubated for three hours at 37o C, the absorbance was measured with a Biorad microplate reader (blank consisted of reagents without cells). A standard calibration curve was prepared at the time using cells counted directly under a hemocytometer.

Preparation of conditioned media (CM). This is described in detail elsewhere [4,5]. Briefly, for macrophage conditioned media (MCM) rat spleens were cultured in a T75 flask, and on day 4 the non-adherent cells were decanted and the remaining cells were treated with a mixture of ST3 and ST4 antibody in the presence of

complement (Low-Tox M #CL3015, Cedarlane, Hornby, Ontario). By day 6 when a rich growth of macrophages was evident, the medium was changed, and at day 8 it was harvested for co-culture with FB.

Medium from cultures of fibroblasts (prepared as described above) at approximately 60-80% confluence was harvested for use in the CFU-C assays. To observe the effect of MCM, the medium was removed from the FB cultures and replaced with 40% MCM in fresh medium; after 24 h the CM was harvested.

To assess the effect of hydrocortisone on the CM, FB were cultured in 6-well culture dishes until approximately 60% confluent, at which time hydrocortisone (#H-4001, Sigma Chemical Co., St. Louis, MO) at the noted concentration was added to the culture. Cells were then cultured for another 4 days, the medium was changed, and one day later the supernatant was collected.

Partitioning of inhibitory activity by membrane filtration. Conditioned medium from cell lines was collected and passed through a series of membrane filters. For example, the ultrafiltrate of 100 kDa was placed into the chamber of the next smaller pore membrane. The filters used had exclusion limits of 100, 50, and 10 kDa (XM100, XM50, and UM10; Amicon, Lexington, MA, USA). At each step, 20% of the starting volume of the retained material was reconstituted to 100% with RPMI 1640 without serum, and the process repeated twice more. Each of the retentate and ultrafiltrate fractions were reconstituted to the initial volume, and used to assay for activity as described below.

Clonogenic assays: marrow colony forming units (CFU-C). These were enumerated by an adaptation of established techniques [8]. Briefly, $3.0\text{-}4.5 \times 10^6$/ml of rat marrow cells in 10% FCS were mixed with 0.8% methyl cellulose to give a final concentration of $1.0\text{-}1.5 \times 10^5$ cells/ml, 30% FCS, 5×10^{-5} M 2-mercaptoethanol, 5% spleen cell conditioned medium (or other stimulators as indicated) in Iscove's medium. They were then distributed in 1 ml into 35 mm Petri dishes, and after seven days culture in 5% CO_2 at 37^{o} C the colonies were enumerated using an inverted microscope (colony defined as >50 cells). Spleen cell conditioned medium (SCCM) was prepared as described by Johnson and Metcalf [9], except that phytohemagglutinin (PHA-L Pure, E.Y Labs, San Mateo, CA) was used as the stimulator. Depending on the batch of SCCM and the individual animal, under these conditions cultures give rise to 60-120 CFU-C/10^5 cells plated. This is a plating efficiency of approximately 0.1%, which is in the range generally reported.

To assay for inhibitory activity in the different CM, the CFU-C assay was performed as described, except that the CM from the fibroblast culture was added at a final concentration of 5% in the presence of SCCM. Controls for the effect of residual MCM alone and in combination with each CM were added when appropriate, and did not differ significantly from background (not shown). Because of the range of variation in the total colonies produced in the

CFU-C assay in the presence of SCCM, the data presented for the inhibitor assays are expressed as a percentage of the maximum derived from SCCM control cultures.

To measure CFU-E, bone marrow cells were separated by flotation on a barrier of Ficoll-Paque before being suspended in media containing 20% FCS/1% BSA with a final concentration of 2 U/ml erythropoietin (step III, purified from sheep; Connaught Laboratories, Willowdale, Ontario; #1501-5-7); the colonies were counted on day two. Depending on the different bath of erythropoietin and individual animal, cultures give rise to 117-200 CFU-E/105 cells plated, which is a plating efficiency of about 0.2%. Inhibition by CM was performed as for CFU-M and -GM, except that erythropoietin was the stimulator instead of SCCM.

In some experiments the effect of CM on the clonogenic growth of BNML cells was measured, as described previously [5]. Briefly, BNML cells growing in suspension culture in RPMI 1640/10% FCS were harvested, washed twice in medium, and then 5x10**6** were resuspended in 5 ml and recultured in 35 mm Petri dishes in either medium alone, medium + 5% CM, or 100% CM at 37**o** C for 4 h. The cells were then washed twice with RPMI/10% FCS, resuspended in media, and incubated for another 1 h at 37**o**. Aliquots were taken for the clonogenic assay in methyl cellulose, in the presence or absence of 5% CM as previously described [5], and enumerated on day 7. The plating efficiency of the BNML-FCS line ranges between 30-50%.

RESULTS.

Growth and phenotypic characteristics of marrow and lung-derived lines. After several weeks of propagation at high density, two isolates of primary adherent cells from lung and marrow increased their rate of proliferation and became capable of being subcultured at low density. The growth curves shown in Figure 1 demonstrate that the lung-derived strain called "ST4" grew more rapidly than the marrow-derived strain called "ST3". The decrease in the number of "ST4" cells after day 4 is due to overgrowth and cell death. Both types stained with antibodies to collagen I and III, but not to von Willebrand's factor (not shown). Initially, the ST3 or ST4 antigens continued to be expressed on the respective cell types, but after passage 30-40 the concentration of both antigens diminished to a level barely detectable by immunostaining.

Also, the two cell types displayed morphological differences (Fig. 2). The "ST3" strain, similar to primary marrow cells, appeared more flat, spread-out, and arranged in a random fashion over the surface of the culture dish, whereas the lung-derived ("ST4") cells appeared more fusiform and grew in a more organized reticular pattern. After continued growth of "ST3" many large flattened cells become evident. For comparison is shown a typical flat fibroblastoid cell from a 21 day primary culture of rat bone marrow grown in liquid culture in the presence of 10% FCS, under which several "phase-dull" cells can be seen.

Stimulatory and inhibitory activities in the conditioned media of the different cell types. The behavior of the cell lines was similar to what we had observed previously for primary FB - marrow-derived cells produced detectable colony-stimulating activity (CSA) in the CM only after they had been cultured in the presence of MCM. Under those conditions CSA was measured in the CM of "ST3" cells but not in CM of "ST4" (Fig. 3A). Likewise, inhibitory activity was minimal in the CM of "ST3" until the cells had been cultured with MCM (Fig. 3B), after which its effect was found to be more pronounced on CFU-G/M than on CFU-E. Again, the lung-derived FB behaved differently, constitutively releasing inhibitors into the CM without any further increase after exposure to MCM. Although "ST4" cells had markedly decreased its expression of ST4 antigen by passage #30, they continued to produce the inhibitory activity at the usual level (not shown). Production of such activity may not be a general feature of cultured fibroblasts, because it was not found in the CM of normal rat kidney (NRK) FB, which continued to produce detectable CSA (Table 1).

The apparent lack of CSA in the CM of "ST4" could be due to a failure of factor production or its being masked by inhibitors. Similarly, if the inhibitor(s) acted in a competitive fashion to CSA, then the failure to detect it in CM of "ST3" could be due to a higher relative effect of the stimulators. Because the inhibitory activity partitions in the 100-120 kDa range by membrane or gel filtration, it can be separated from most of the CSF's

that are of a lower m.w. As shown in Figure 4a, the 50-100 kDa fraction of the CM of both "ST3" and "ST4" lines contained CSA, which MCM caused to increase only in "ST3". The results in Figure 4b show that there were inhibitors in the >100 kDa fraction of both cell strains.

Activities produced by subclones of the "ST3" line. Although almost all of the "ST3" cells bore the ST3 antigen, other experiments had indicated (not shown) that there was a variation in its quantity of expression. This and the variable potency of inhibitory activity in different batches of CM from "ST3", in contrast to CM of primary marrow FB, suggested that there might be fluctuating ratios of cell subsets within the parent population. To explore this possibility we derived clones from "ST3" cells that differed in their content of ST3 antigen. Clone 2A was selected for a relatively high level, and clone 9D for a low level of expression (see "Methods"), and then the CM of their progeny were tested for stimulatory and inhibitory activities. As shown in Figure 5a both of these cell lines produced CSA in response to MCM; however, clone 9D produced inhibitors but clone 2A did not (Fig. 5b).

Modulation of factors by corticosteroids. Although corticosteroids have been used to optimize the growth of hemopoietic cells in long-term marrow cultures [10], it is not known how these hormones might modify factor production and other aspects of the microenvironment. Shown in Figures 6 and 7 is the effect of hydrocortisone (OHC) on the level of stimulatory and inhibitory

activities in the CM of the cell lines described above. At concentrations up to 10-5 M, OHC did not significantly alter the induction of CSA in any of the lines tested (Fig. 6a), including that found in the 50-100 kDa fraction of the "ST4" strain (Fig. 7a). However, incubation of cells at this concentration blocked the MCM-induced release of inhibitor from ST3-clone 9D (Fig. 6b), and decreased the constitutive production by both primary lung FB and the "ST4" strain in a concentration-dependent manner (Figs. 6b, 6c, and 7b).

Comparison of the effect of CM on leukemic cells in liquid and clonogenic cultures. The action of the inhibitory molecule(s) could be mediated through cells in the bone marrow other than those which are measured directly in the clonogenic assay. Alternatively, it could be a direct effect, but due to toxicity and death of the clonogenic precursors. As we have shown previously, the inhibitory action of the CM was seen also on cells of a homogeneous type, the BNML rat myeloid leukemia line [5]. If this were due to "non-specific" toxicity, then one would expect that increasing the concentration of CM would result in a greater degree of kill. To test this, BNML cells were incubated for 4 h in either growth medium alone, 5% ST4 CM, or 100% ST4 CM ("Primary incubation") and then an aliquot was cultured in methyl cellulose to measure the effect on the clonogenic capacity in the presence or absence of ST4 CM ("Secondary incubation"). As shown in Table 2, the cloning efficiency was not decreased any further by incubation in 100% CM than it was in 5% CM.

The viability determined by trypan blue exclusion did not decrease after the 4 h primary incubation or the 1 h recovery period. Thus, if the inhibition were due to "non-specific" toxicity it is not dose related and does not affect the ability of the cell membrane to exclude dye. However, removing the inhibitory CM and incubating in growth medium for 1 h did not result in recovery of the full clonogenic potential, suggesting that the action of the molecules are sustained for a longer period.

This later observation might also be explained by toxicity. Such "non-specific" agents, however, would be expected to exert their effect on cells growing in suspension culture as well as in the clonogenic assay. As shown in Table 3, the growth of BNML cells in suspension culture was not diminished by the CM that inhibited BNML colonies. This was consistent for three different initial concentrations of cells, and for three different concentrations of CM cultured for 24, 48, and 96 h. In fact, under these conditions the growth was augmented over that observed for cells cultured in RPMI 1640/10% FCS alone. Thus, explanations other than "non-specific" killing must be sought to explain the inhibitory effects of these CM. After the molecules have been purified to homogeneity, their effects on specific cell cycle parameters can be studied directly.

DISCUSSION.

These results show that fibroblastoid (FB) cell strains propagated from primary isolates of rat marrow and lung continue to express their respective ST3 or ST4 antigens, but exhibit dissimilar morphology and rates of growth. Although the concentration of both antigens decreased after further culturing, the cells maintained the parental pattern of CSA and inhibitor release. Thus, it appears that neither of the surface molecules is necessary for cell replication or production of the factors.

As has been reported by several investigators, most tissues secrete CSA in the form of G-, M-, and GM-CSF, and the sources are thought to be macrophages, endothelial cells, and fibroblasts [11,12]. Our results with FB of lung and marrow are in agreement, in that we found low concentrations of CSA in the conditioned media of all cell lines that had been stimulated with MCM, although the biological activity in the CM of "ST4" cells was detected only after the >100 kDa inhibitor(s) had been removed (Fig. 4a). In contrast to the uniformity observed for growth factor production, inhibitory activity was not detected in all CM. Whereas "ST4" cells, like primary lung FB, appeared to produce it spontaneously, "ST3" marrow cells yielded a measurable concentration only after they were stimulated with MCM (Fig. 3b for cells at passage #5) or after the >100 kDa fraction was separated from the CSA (Fig. 4b for cells at passage #10). More rigorous analysis of the production of these competing factors, however,

must await the availability of specific assays for their quantitation in the CM and appropriate probes to assess the cellular content of their RNA's.

Although we did not attempt to isolate variant clones of the "ST4" strain, what appears to be constitutive production of inhibitors by lung-derived cells conceivably might reflect stimulation by another population. Thus, it is possible that this apparent difference in secretion pattern might be due to autostimulation by FB subpopulations within the culture. For example, IL-1 may be produced by some types of FB and not others [13-15]. Although at this time we can not exclude the possibility that such an association is coincidental or an artifact of cell culture, the fact that clones 2A and 9D were selected according to their level of ST3 antigen expression supports the notion that subpopulations of fibroblasts can arise and possibly diversify into forms capable of different functions.

As we have shown before [4], isolated primary marrow FB continued to produce inhibitors for seven days after being stimulated with MCM, and such a prolonged release of growth factor antagonists might not reflect any physiologic sequence of events in the bone marrow. Thus, it would be reasonable to anticipate that if the inhibitors were part of a regulatory network, there might be other factors that could dampen their production. Corticosteroids, which dampen many aspects of the inflammatory response including secretion of IL-1 and TNF [16,17], and improve the growth of hemopoietic cells in

long-term bone marrow cultures [10], also decreased the appearance of the lung-derived inhibitor. The lack of effect on CSA production by isolated rat FB differs from the findings of Hirata et al using heterogeneous adherent marrow cells of human origin [18]. Conceivably, the apparent increase in CSA that they observed could have been due to a decrease in inhibitor production by the FB without any change in the release of CSF's by the other types of cells present in their cultures.

In conclusion, the ability of cytokines and corticosteroid hormones to modulate the appearance of inhibitor activity in the culture medium, along with the variation in production by different cell lines, makes it reasonable to speculate that such "negative" acting molecules might have a physiologic role in dampening the effects of hemopoietic growth factors at different anatomic sites.

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TABLE 1.

ACTIVITIES IN CM OF NORMAL RAT KIDNEY FIBROBLASTS

	STIMULATOR: No. CFU-C	INHIBITOR: % Max CFU-C
Experiment #1:		
Medium (or SCCM)	**5**	**100**
CMNRK	**14**	**107**
CMNRKm	**20**	**98**
Experiment #2:		
Medium (or SCCM)	**6**	**100**
CMNRK	**10**	**90**
CMNRKm	**12**	**94**

CMNRK or CMNRKm refer to conditioned medium from NRK cells that had not been cultured with macrophage conditioned medium, and those that had.

TABLE 2.

EFFECT OF PRE-INCUBATION OF BNML-FCS CELLS WITH ST4 CM ON SUBSEQUENT CLONING EFFICIENCY.

CONTENT OF PRIMARY INCUBATION	CONTENT OF SECONDARY INCUBATION	RELATIVE CLONING EFFICIENCY (% OF MAX ± SD)
a) MEDIA	MEDIA	100
b) MEDIA	MEDIA + 5% ST4 CM	63 ± 8
c) 5% ST4 CM	MEDIA	59 ± 29
d) 5% ST4 CM	MEDIA + 5% ST4 CM	48 ± 13
e) MEDIA	MEDIA	100
f) MEDIA	MEDIA + 5% ST4 CM	64 ± 12
g) 100% ST4 CM	MEDIA	59 ± 17
h) 100% ST4 CM	MEDIA + 5% ST4 CM	35 ± 8

Mean ± SD of four experiments in which clonogenic assays were performed in duplicate.

Statistical analysis performed on the paired samples of each individual measurement in each of the four experiments (two tailed t test) indicate the following:

a vs b: $p < 0.01$;
c vs d: not significant;
e vs f: $p < 0.01$;
g vs h: $p < 0.05$

TABLE 3.

EFFECT OF LUNG FIBROBLAST CONDITIONED MEDIUM ON THE GROWTH OF BNML-FCS CELLS IN SUSPENSION CULTURE.

	NUMBER OF CELLS PLATED		
	5x103	10x103	20x103
	PERCENT INCREASE OVER CELLS PLATED		
EXPERIMENT #1 (24 h culture)			
MED/FCS		137	135
ST4 CM or [LF CM]			
5%		160 [151]	184 [173]
10%		188 [130]	209 [165]
20%		160 [146]	187 [162]
EXPERIMENT #2 (48 h culture)			
MED/FCS		139	138
ST4 CM or [LF CM]			
5%		251 [158]	265 [197]
10%		233 [194]	286 [214]
20%		305 [173]	265 [220]
EXPERIMENT #3 (4 day culture)			
MED/FCS	187		
ST4 CM or [LF CM]			
5%	590 [701]		
10%	656 [640]		
20%	666 [533]		

The values shown are the average of quadruplicate determnations made for each point.
The first is that obtained for cells cultured in the presence of CM from the ST4 cell line, and the next in brackets [] is that obtained in CM of primary lung FB.

LEGEND TO FIGURE 1.

Representative growth curves expressed as cells/well and protein/well. Panel A: "ST3" (marrow-derived) cell strain; Panel B: "ST4" (lung-derived) strain. Cells were seeded into wells of a 24-chamber culture dish at 1.0 or 0.5x105 cells/well respectively; each point is the mean of triplicate wells.

LEGEND TO FIGURE 2.

Photomicrographs of cell lines. a: "ST3" strain, passage 5-10, microscopic magnification 80x; b: "ST3", 160x; c: "ST3", passage 30-40, 160x; d: primary marrow fibroblast showing smaller "phase dull" cells under its flattened cytoplasm, 160x; e: "ST4" strain, pasage 5-10, 80x; f: "ST4", 160x; g: ST3-clone 2A, 160x; h: ST3-clone 9D, 160x. Phase contrast.

LEGEND TO FIGURE 3.

Effect of CM from "ST3" and "ST4" cell lines on hemopoietic colonies of normal rat bone marrow, and the effect of stimulation of FB with macrophage CM (MCM) as described in the "Methods". Panel A: Colony stimulatory activity (CFU-M and -G/M) in CM of cell lines. The source of the CM is noted ("ST3" or "ST4"); when co-cultured with MCM ("ST3m" and "ST4m"). In the presence of medium alone, without added CM, 4-8 colonies appeared. Panel B: Inhibitory activity of CM on the stimulation of myeloid (CFU-C) or erythroid (CFU-E) colonies by spleen cell conditioned medium (SCCM) or erythropoietin. Depending on the batch of SCCM and the individual animal, under these conditions cultures give rise to 60-120 CFU-C/105 cells plated. Results normalized to 100%, which was that obtained in the presence of SCCM or Epo alone. As described in the "Methods", 1x105 bone marrow cells were cultured in methyl cellulose in duplicate 35 mm Petri dishes. At least three independent preparations of each CM were tested and found to behave similarly. In this and subsequent graphs, unless othrwise stated, the values illustrated are the mean value + 1 SD (error bar shown) of at least three independent experiments performed with cells at passage #5.

Legend to figure 3 continued.

Student t test: Panel A: ST3 vs ST3m: $p < 0.01$;
ST4 vs ST4m: not significant;
Panel B: ST3 vs SCCM/Epo: not significant;
ST3m vs SCCM/Epo: $p < 0.01$;
ST4 vs SCCM/Epo: $p < 0.01$;
ST4m vs SCCM/Epo: $p < 0.01$;

LEGEND TO FIGURE 4.

Partitioning of stimulatory and inhibitory activities in CM by membrane filtration; cell lines as designated in legend to Fig. 3. Panel a: stimulatory activity in fractions obtained from each range of molecular size (kDa) excluded by the membranes. Panel b: inhibitory activity. UF: unfractionated CM. Performed with cells at passage #10.

Student $\underline{t}$ test:

Panel A: vs medium (8 $\pm$ 1, not shown in figure):

UF, ST3m, $p < 0.01$;
ST3, ST4 and ST4m, not significant.

>100K, ST3, ST3m, ST4 and ST4m, not significant.

50-100K, ST3, ST3m, ST4 and ST4m, $p < 0.01$.

Panel B: vs SCCM alone (100):

UF, ST3 and ST3m, $p < 0.05$;
ST4 and ST4m, $p < 0.01$.

>100K, ST3, ST3m, ST4 and ST4m, $p < 0.01$.

50-100K, ST3, $p < 0.05$;
ST3, ST4 and ST4m, not significant.

LEGEND TO FIGURE 5.

Effect of CM from subclones of the "ST3" line on hemopoietic colonies of normal rat bone marrow. Panels a and b as designated in Figure 3. Performed with cells at passage #5.

Student t test:

Panel A: c2A vs c2Am: $p < 0.01$;
c9D vs c9Dm: $p < 0.01$.

Panel B: c2A vs SCCM/Epo, not significant;
c2Am vs SCCM/Epo, $p < 0.05$;
c2A vs c2Am, not significant;
c9D vs SCCM/Epo, $p < 0.01$;
c9Dm vs SCCM/Epo, $p < 0.01$;
c9D vs c9Dm, $p < 0.01$.

LEGEND TO FIGURE 6.

Effect of hydrocortisone on stimulatory and inhibitory activities in the CM of "ST3" and "ST4" strains, and of "ST3" subclones. Panel a: stimulatory activity. Panel b: inhibitory activity. Open bars: without addition of hydrocortisone; solid bars: FB incubated in presence of 10-5 M hydrocortisone. Panel c: effect of concentration of hydrocortisone on the production of inhibitory CM by "ST4" cells. Performed at passage #10. CM obtained from lung fibroblasts (LF) and marrow fibroblasts stimulated with MCM (MFm) are shown for comparison.

Student t test:

Panel A: with vs withou OHC:

All are not significant.

Panel B:with vs without OHC:

MFm, not significant;

c2Am, not significant;

c9Dm, $p < 0.01$;

LF, $p < 0.01$;

LEGEND TO FIGURE 7.

Stimulatory and inhibitory activities in fractions of CM of "ST4" cells cultured in the presence of hydrocortisone. Partitioning of stimulatory and inhibitory activities in CM by membrane filtration as described in Fig. 4. Panel a: stimulatory activity. Panel b: inhibitory activity. Performed at passage #10.

Student t test:

Panel A: ST4 vs ST4OHC

UF, not significant;

>100K, not significant;

50-100K, not significant;

Panel B: ST4 vs ST4OHC:

UF, $p < 0.01$;

>100K, $p < 0.01$;

50-100K, not significant;

Figure 1.

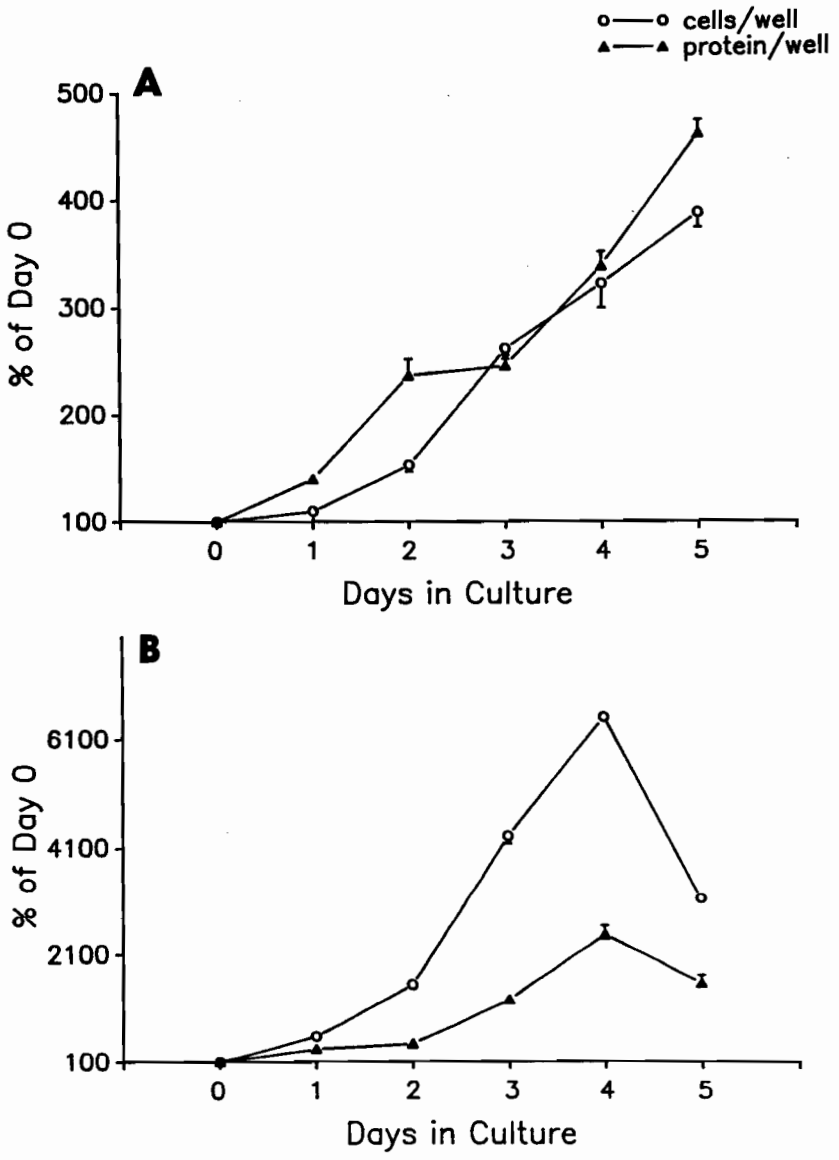

o—o cells/well
▲—▲ protein/well
A
500
400
300
200
100
% of Day 0
0
1
2
3
4
5
Days in Culture
B
6100
4100
2100
100
% of Day 0
0
1
2
3
4
5
Days in Culture

Figure 2.

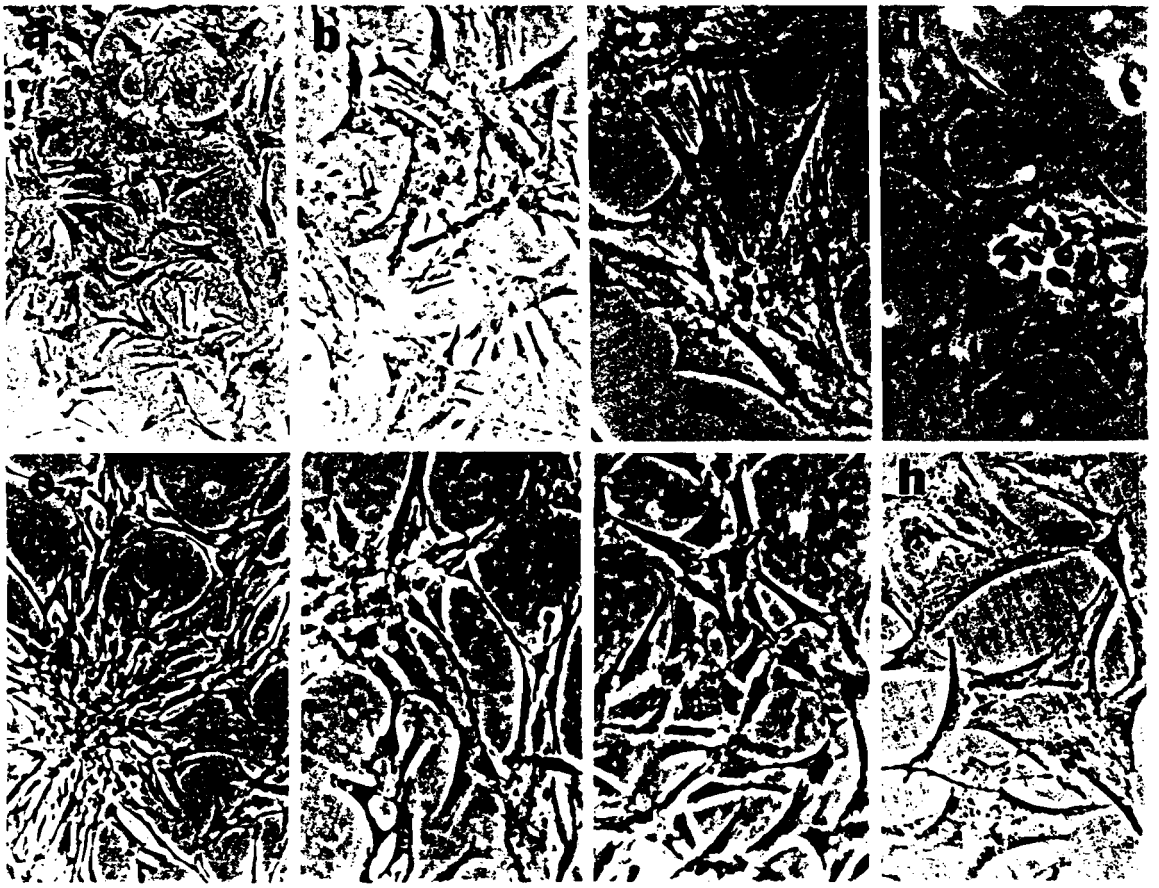


Figure 3.

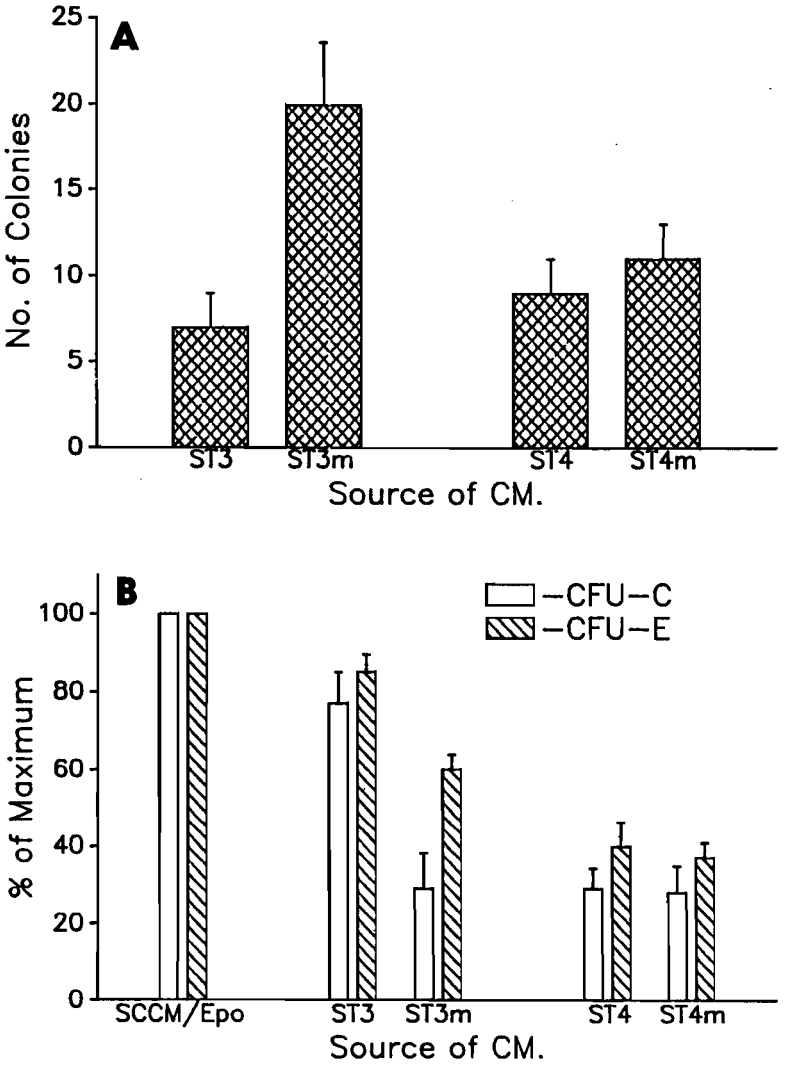

A
No. of Colonies
25
20
15
10
5
0
ST3
ST3m
ST4
ST4m
Source of CM.
B
% of Maximum
100
80
60
40
20
0
-CFU-C
-CFU-E
SCCM/Epo
ST3
ST3m
ST4
ST4m
Source of CM.

Figure 4.

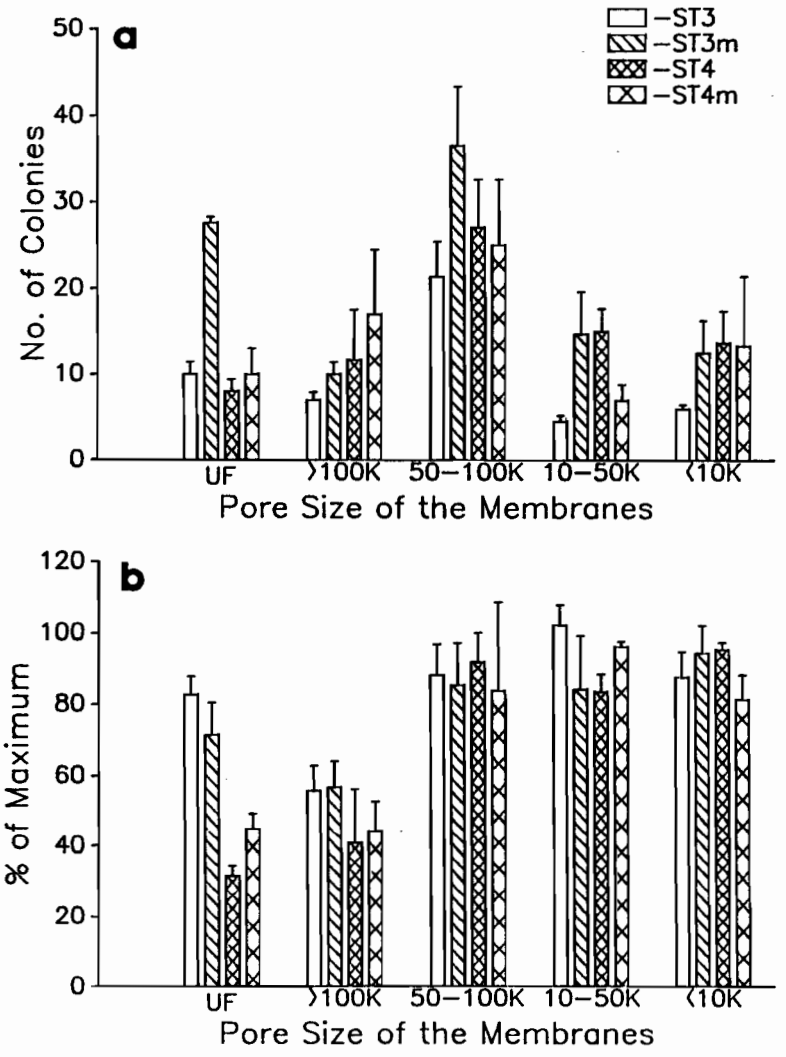

a
-ST3
-ST3m
-ST4
-ST4m
No. of Colonies
0
10
20
30
40
50
UF
>100K
50-100K
10-50K
<10K
Pore Size of the Membranes
b
% of Maximum
0
20
40
60
80
100
120
UF
>100K
50-100K
10-50K
<10K
Pore Size of the Membranes

Figure 5.

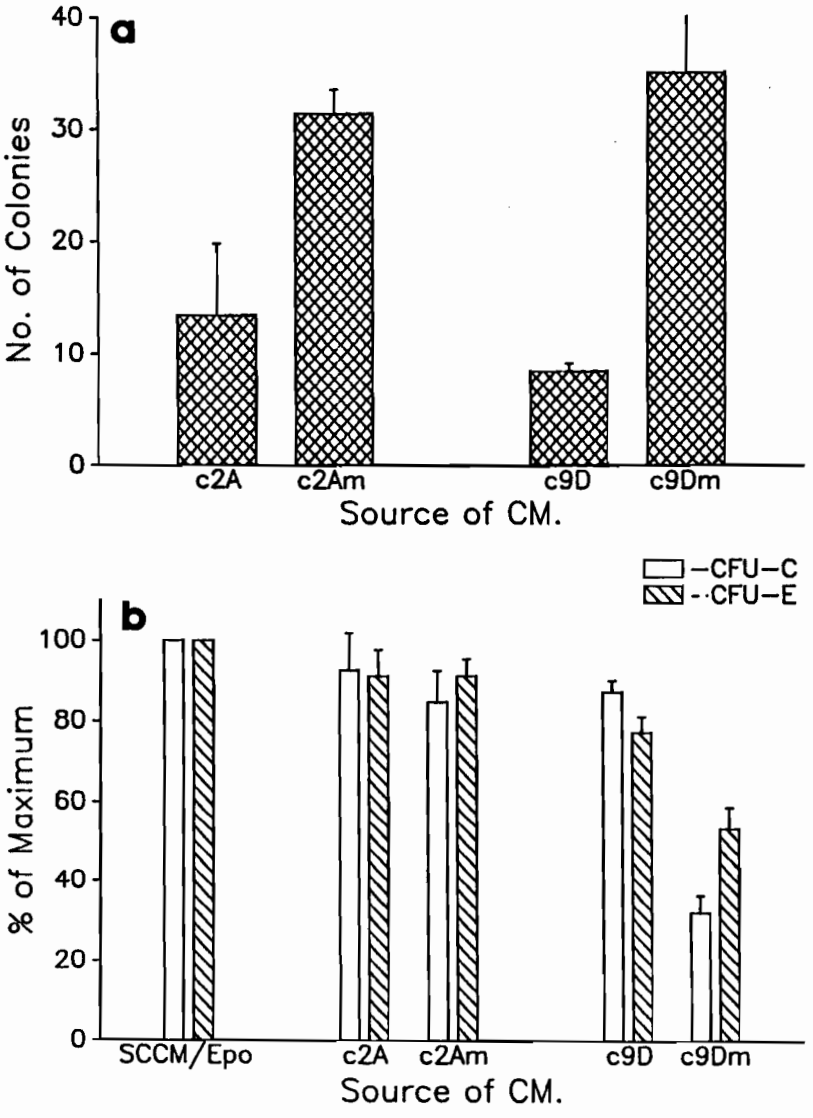

a
40
30
20
10
0
No. of Colonies
c2A
c2Am
c9D
c9Dm
Source of CM.
-CFU-C
-CFU-E
b
100
80
60
40
20
0
% of Maximum
SCCM/Epo
c2A
c2Am
c9D
c9Dm
Source of CM.

Figure 6.

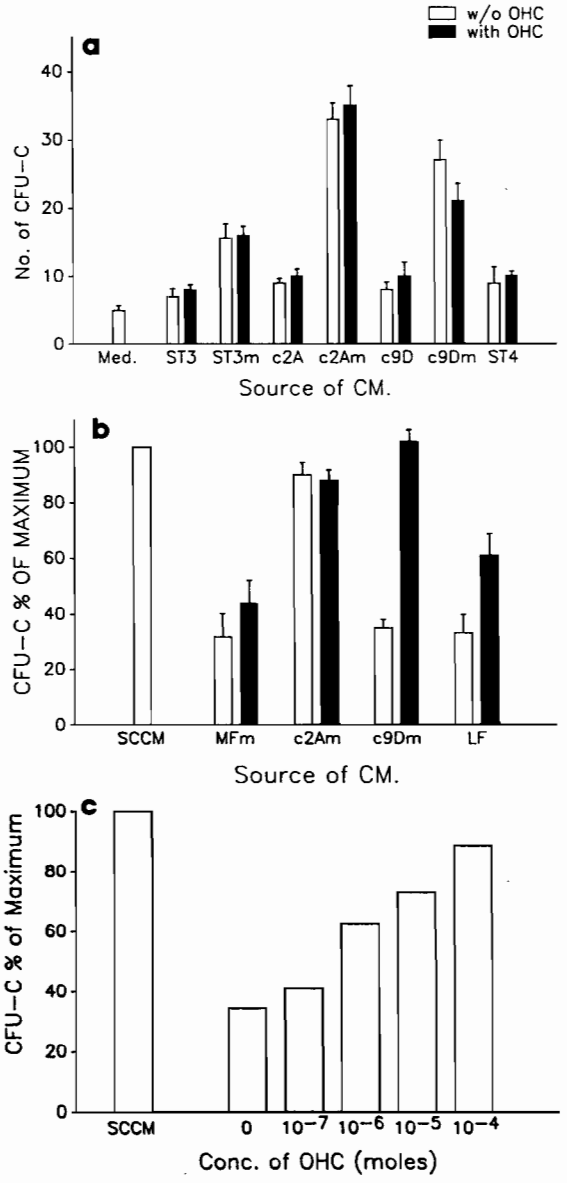
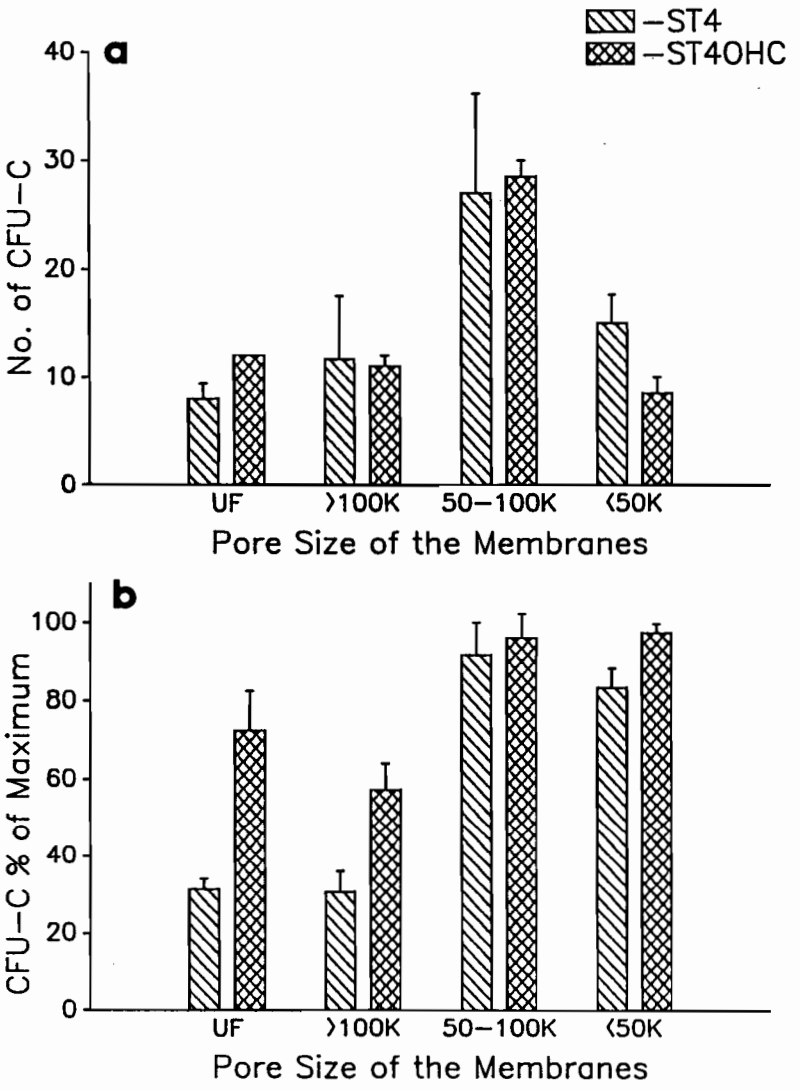

a
w/o OHC
with OHC
No. of CFU-C
0
10
20
30
40
Med.
ST3
ST3m
c2A
c2Am
c9D
c9Dm
ST4
Source of CM.
b
CFU-C % OF MAXIMUM
0
20
40
60
80
100
SCCM
MFm
c2Am
c9Dm
LF
Source of CM.
c
CFU-C % of Maximum
0
20
40
60
80
100
SCCM
0
10-7
10-6
10-5
10-4
Conc. of OHC (moles)

Figure 7.


a
−ST4
−ST4OHC
No. of CFU−C
40
30
20
10
0
UF
>100K
50−100K
<50K
Pore Size of the Membranes
b
CFU−C % of Maximum
100
80
60
40
20
0
UF
>100K
50−100K
<50K
Pore Size of the Membranes

Chapter 5 PARTIAL PURIFICATION OF A HEMATOPOIETIC INHIBITOR PRODUCED BY RAT LUNG FIBROBLASTS[5]

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[5]This chapter is a duplication of the paper by H. Wang and A.K.Sullivan, which has been published in Ann. NY. Acad. Sci. 628:310 (1991).

Partial Purification of a Hematopoietic Inhibitor Produced by Rat Lung Fibroblasts[a]

HONG WANG AND ARTHUR K. SULLIVAN[b]

McGill Cancer Centre, and the
Department of Medicine
Division of Hematology
Royal Victoria Hospital
Montreal, Quebec, Canada H3G 1Y6

In previous work[1] we demonstrated that the predominant fibroblastoid (FB) cell that grows from rat bone marrow bears a surface antigen phenotype different from that expressed by most similar-appearing cells from other nonhematopoietic organs (e.g., lung). Subsequently we found that lung FB constitutively secreted a potent growth inhibitor of both normal and leukemic blood cells.[2] This activity had the following characteristics: (a) It inhibited the clonogenicity of BNML (Brown Norway myeloid leukemia) promyelocytic leukemia cells, but not that of eight other rat lines of various origins, human hematopoietic lines (HL60, U937, Raji), murine WEHI-3B, or murine myeloid and erythroid precursors. (b) It was removed from the conditioned medium (CM) by BNML cells, but not by lines that were not inhibited by it; it was not removed by mature erythrocytes, unseparated marrow cells, or splenic lymphocytes. (c) It did not inhibit rat lines shown to be sensitive to inhibition by transforming growth factor-beta or beta-interferon, and its activity was not inhibited by neutralizing antibodies to these agents or to acidic isoferritin. (d) It was acid and heat labile, and trypsin sensitive. (e) By gel filtration of CM, the activity migrated in the 100-120 kDa range.[2] We report here the initial steps toward purification of the protein.

The cell lines, culture methods, clonogenic assays, and characterization of the fibroblast-derived inhibitory activity is reported in detail elsewhere.[1,2] Biochemical methods are outlined in the note to TABLE 1 and the legend to FIGURE 1.

After completing initial experiments to determine the binding characteristics of the inhibitory protein(s), the series of manipulations shown in TABLE 1 resulted in enrichment of activity approximately 10,000-fold. FIGURE 1a and 1b illustrates the binding to DEAE-Sephacel and the inhibitory capacity of the different pools. In both the dye ligand (not shown) and heparin-Sepharose affinity chromatography procedures (FIG. 1c), only a small portion of the total proteins bound. Also shown in FIGURE 1c is the inhibition of rat marrow colony-forming units in culture (CFU-C) and thymidine incorporation by BNML (promyelocytic leukemia) cells but not by C6, an interferon-sensitive astrocytoma cell line. Optimal binding of the activity to lectins occurred after the initial purification steps, and was considerably more with wheat germ than with concanavalin A (FIG. 1d). After these five steps, although there was a significant degree

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[b]Address for correspondence: Dr. A. K. Sullivan, Room 714, McGill Cancer Centre, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6.

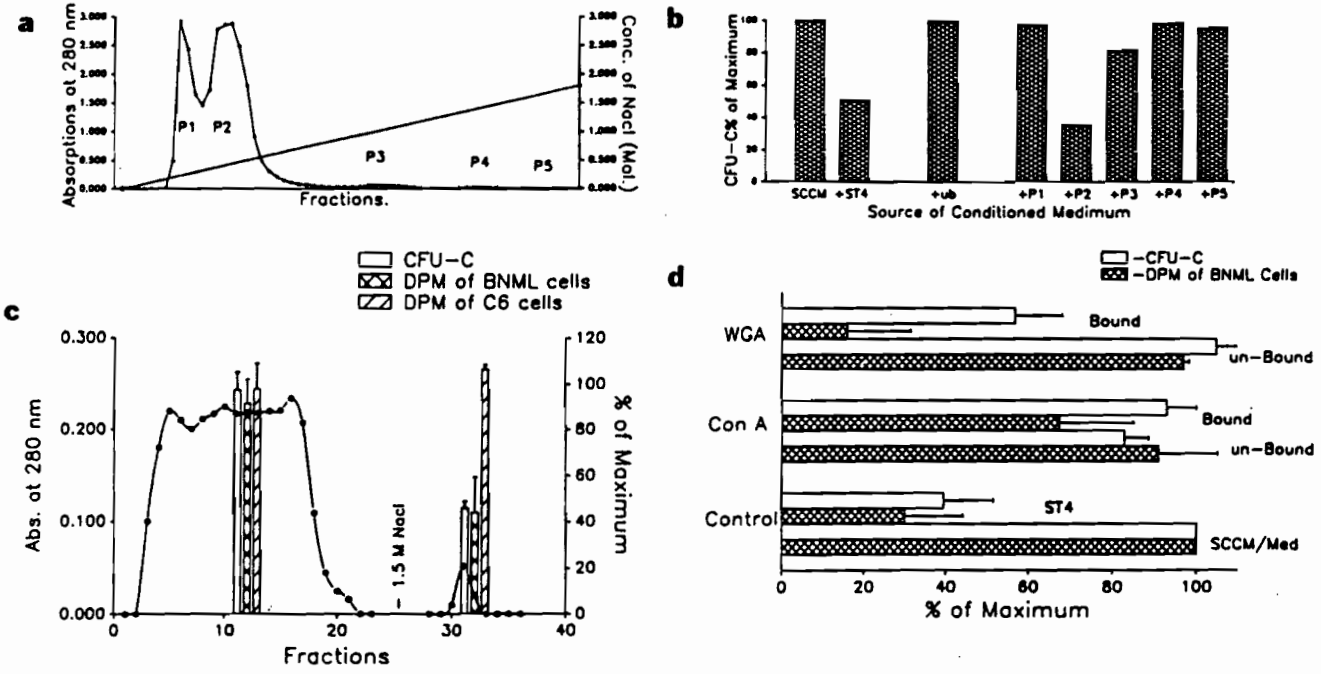

FIGURE 1. (a) Profile of proteins bound and eluted from DEAE-Sephacel (Pharmacia). Total volume applied: 37 ml. Most of the inhibitory activity eluted between 0.35-0.50 M NaCl in 20 mM phosphate buffer at pH 8.5. (b) Assay of inhibitory activity of fractions (P1-P5) noted in panel a. SCCM: 100% of colonies supported by spleen cell-conditioned medium. ST4: colonies formed in presence of SCCM and CM from ST4 lung fibroblast line. UB: SCCM + fraction not bound to DEAE. P1-P5: SCCM + aliquot of noted fraction. (c) Profile of protein concentration (OD_{280}) and inhibitor activity in fractions bound and not bound to heparin-Sepharose C1-6B (Pharmacia). Volume applied: 13 ml; running buffer: PBS; flow rate: 25-30 ml/hr; eluting buffer: 1.5 M NaCl in phosphate buffer. (d) Inhibitory activity in marrow CFU-C and BNML thymidine uptake assays. Samples were applied in PBS for both lectin columns, and were eluted with either 200 mM alpha-methyl mannoside (for Con A) or 120 mg/ml *N*-acetylglucosamine in PBS (for wheat germ).

of purification, SDS-PAG electrophoresis revealed more than ten bands (not shown), of which no single one was predominant.

The hematopoietic inhibitor found in media conditioned by rat lung fibroblasts remained stable for several weeks at 4 °C, tolerated the manipulations described, and appears to be present in less than micromolar concentration (assuming a molecular mass of 100 kDa estimated by gel filtration). Its lectin binding properties suggests that the inhibitor is a glycoprotein bearing *N*-acetylglucosamine or sialic acid, but not mannose in a form accessible to concanavalin A. Although these five steps resulted in a several thousand-fold increase in specific activity, there must be extensive further purification before it will be homogeneous.

TABLE 1. Representative Tabulation of Activity and Yield

Sample	Protein[a] (mg)	Activity[b] (Units)	Specific Act. U/mg)	Purification Factor	Yield (%)
Crude CM	48,000	200,000	4.2		100
Step #1	22,800				
2	15	66,000	4,300	1,000	33
3	1.0	27,000	27,000	6,400	13
4	0.6	20,000	34,000	8,100	10
5	0.4	18,000	45,000	10,700	9

NOTE: Starting material: 10 L of CM from ST4 cells grown in roller bottles in 10% Nu-Serum (a semidefined growth support that contains 2.5% fetal calf serum). Step #1: Concentration and membrane filtration through Amicon filter with a 100-kDa exclusion limit; retained material to Step #2: Ion exchange chromatography on DEAE-Sepharose. Sample loaded in 0.35 M NaCl/ 20 mM phosphate buffer at pH 8.5 and batch eluted with 0.5 M NaCl/20 mM phosphate, pH 8.5; eluate to Step #3: Dye-ligand affinity chromatography with Matrix Gel Blue B (Amicon); sample was loaded in PBS and eluted with 1.5 M NaCl in PBS; eluate to Step #4: Affinity chromatography with Heparin-Sepharose; eluate to Step #5: Affinity chromatography with wheat germ-Sepharose.

[a] Protein concentration measured by the colloidal gold method.[3]

[b] One unit arbitrarily defined as that required to inhibit SCCM-stimulated CFU-C by 60%.

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2. WANG, H. & A. K. SULLIVAN. 1991. Fibroblastoid cells of rat bone marrow and lung differ in their release of activities that are stimulatory or inhibitory to hemopoietic precursors. Submitted for publication.
3. STOSCHECK, C. M. 1990. Quantitation of protein. Methods Enzymol. 182: 63-65.

Chapter 6 DISCUSSION[6]

[6]The references cited in this chapter are listed in "References" from Page 157 to Page 195.

6.1 Summary of the results.

The results obtained relate first to the different patterns of soluble factors released by two antigen-defined fibroblastoid cell subpopulations, and second to the characteristics of what may be a novel protein that possesses activity inhibitory to the growth of normal and leukemic blood cell precursors.

A. Activities produced by the cell subpopulations.

1. Two subpopulations of fibroblastoid cells were derived, one from the bone marrow and the other from the lung. Using the antibodies to obtain homogeneous cell strains, the primary cultures proved to be ST3+/ST4- and ST3-/ST4+ respectively. Conditioned media (CM) were obtained from these cultures and assayed for their ability to support hemopoietic colonies (CFU-C) of rat bone marrow. After they had been co-cultured with macrophage products, the fibroblasts derived from the marrow, but not those from the lung, produced CM with CSA. Typical of the growth pattern of rat marrow, most of the colonies were CFU-M, and CSA was neutralized by antisera to M-CSF.

2. The subpopulations of fibroblasts differed from each other not only in their release of CSA, but also in the inhibitory activity they produced. The lung-derived cells constitutively released a factor (or factors) that inhibited myeloid (CFU-GM and CFU-M) and erythroid (CFU-E)

colonies that had been maximally stimulated by a mixture known to contain the major growth factors, whereas the marrow-derived fibroblasts produced the activity only after they had been stimulated by macrophage products. Also, the inhibitor decreased the growth of leukemic cells (the BNML promyelocytic leukemia cell line) in both clonogenic and liquid culture assays.

3. Cell lines were established from the primary cultures of marrow and lung fibroblasts, and both of them maintained the described pattern of release of CSA and the inhibitor into the CM.

Two subclones were then derived from the ST3+ marrow cell line, based on high and low expression of the antigen, and were named clones "c2A" and "c9D" respectively. Clone c2A grew faster than c9D, both released CSA in response to stimulation by macrophage products, but only c9D released the inhibitory activity.

4. As determined by membrane ultrafiltration, CSA and the inhibitory activity partitioned at different apparent molecular weights, the former below 100 kDa and the latter above 100 kDa. This enabled independent assessment of each in the different CM, so that it was demonstrated that the ST4+ lung-derived cell line produced CSA that was masked by the inhibitor.

5. Because hydrocortisone must be present for optimal growth of hemopoietic cells in the LTBMC, its effects on the release of CSA and inhibitors by the fibroblasts were assessed. In a dose-dependent manner, this hormone decreased the release of the inhibitory activity, but did not alter the concentration of CSA.

B. Characteristics of the inhibitory activity.

1. The inhibitory activity appears to be species and lineage restricted, in that it did not have any effect on the growth of murine marrow colonies, and in a panel of tumor-derived cell lines retarded the growth of only the myeloid leukemia (BNML).

2. The fact that the inhibitory activity could be removed by cells sensitive to it, but not by those resistant, suggests that it may act on a target cell receptor.

3. To distinguish this inhibitor from others, a series of antibody neutralization experiments were performed. Antibodies to TGF-beta, interferon-beta, and isoferritin did not alter the lung fibroblast-derived activity.

4. The physical and biochemical characteristics include the following:

- It was inactivated by boiling for 5 min but not by heating at 56o C for 30 min; it was stable on storage at 4oC for at least 8 weeks.

- It was inactivated at pH 2, and by trypsin.

- The form released into CM migrated in the range of 100-120 kDa by gel filtration, and bound to immobilized heparin and wheat germ agglutinin (WGA).

- Initial steps in purification have enabled a 10,000-fold enrichment of specific activity with 9% yield. These included membrane filtration (100 kDa exclusion), ion exchange chromatography (DEAE-Sephacel), dye-ligand affinity chromatography (Matrix Gel Blue B), heparin-Sepharose affinity chromatography, and wheat germ-Sepharose affinity chromatography.

6.2 Relation to published studies.

6.2.1 Functional heterogeneity of fibroblast subpopulations.

We found that antigen-defined fibroblast subpopulations behaved differently in their pattern of production of activities that could stimulate or inhibit the growth of bone marrow cells. This extends evidence from studies from other laboratories indicating that there may be heterogeneity among different fibroblasts of different organs.

In murine-derived CFU-F, Thy-1 was expressed by the fibroblasts from spleen, thymus, blood, and skin, but not by those from marrow; the T200 antigen (known to be present on immature hemopoietic cells) was detected on those from marrow, spleen, and thymus, but not on those from blood and skin [Piersma 1985a]. Human fetal and tumor-associated fibroblasts differed from those derived from the normal adult tissues that were studied, as indicated by an antibody (MoAb VIF3) made to malignant fibrous histiocytoma cells [Bartal 1986]. In another report, human-derived fibroblasts from different organs were observed to bear different antigens. The fibroblasts from skin reacted with antibody J5 (CD10/common acute lymphoblastic leukemia antigen), whereas the fibroblasts from bone marrow reacted with HNK1 (CD57, reactive with human natural killer cells); the fibroblasts from embryonic lung reacted with neither of

these two antibodies [Ndumbe 1985]. The fibroblasts derived from lung and skin also could be distinguished by their differential capacity to bind and respond to dexamethasone [Kondo 1985]. Although it has been suggested that different fibroblasts might interact with the hemopoietic system in different ways, it has not previously been documented by experiments designed to test the possibility directly.

6.2.2 The discrepancy between different species in the production of growth modulators by lung derived fibroblasts.

The observation that fibroblasts can be induced by monocyte products to produce CSF is not new. As has been extensively discussed in Section 1.4.3, cultured monocytes, macrophage conditioned medium [Broudy 1986b], IL-1 [Zucali 1987], and TNF [Zucali 1988] all were shown to induce fibroblasts to produce GM-, M- [Kaushansky 1988], and G-CSF [Fibbe 1988a]. Human fibroblasts derived from both lung [Munker 1986] and marrow [Rennick 1987a] could augment the production of CSF in response to stimulation by TNF or IL-1.

The observations presented here that lung-derived fibroblasts produced an inhibitory CSA might at first inspection appear to differ from the results of others using murine or human-derived fibroblasts. In the majority of papers published before 1985 that were reviewed, fibroblastoid cells were reported to release a

predominantly stimulatory activity for hemopoietic precursors in vitro, and the presence of inhibitors was seldom noted. However, none of the experiments was performed in the manner of those presented here, in which early passage primary cells were made homogeneous and depleted of extraneous macrophages by an immunoselection process. As is shown in the experiments described in Chapter 2, when heterogeneous cell populations similar to those reported in the older literature were used, the results also were similar.

In working with systems that attempt to approximate the physiologic state, such as conditioned culture media, the complexity must be duly considered. The final activity, be it stimulator or inhibitor, is dependent upon multiple factors. The state of homogeneity of the culture (absence of macrophages or other fibroblast subpopulations) is of utmost importance because even a small number of macrophages can produce factors that can synergize with others to change the apparent spectrum of target cell activity [Bot 1988]. Even cultures expanded from CFU-F, long considered to be "pure" by definition, contain contaminating macrophages and endothelial cells [Perkins 1990; Wang 1990]. Furthermore, even clonal lines can be modulated by other cells and cytokines to vary in the end products that the cells release into the surrounding medium. For example, such functional plasticity is reflected in the ability of the same cells to support either lymphopoiesis or myelopoiesis under different conditions [Hunt 1987; Collins 1987].

Furthermore, when cells have been in culture sufficiently long to be spontaneously transformed or have been transformed by a virus, their behavior may vary unpredictably and may not reflect their normal origin. For example, cells that did not secrete CSA without stimulation (except for a low level of M-CSF) began to release significant activity constitutively after they had been selected for anchorage independence [Nemunaitis 1989]. Such variation in uncontrolled variables may explain other apparent discrepancies in the literature. For example, GM-CSF is produced constitutively by lines derived from human embryonic lung (WI38) [Koeffler 1988] and certain stromal isolates [Yang 1988], but in other adult lung-derived lines (e.g., CCL202) it is produced only after stimulation [Zucali 1988].

The assay system also may influence the dominant effect of an inhibitor/stimulator mixture. Indeed, in most of the papers published studying these phenomena there was not any attempt to measure inhibitors, and thus only the stimulatory activities in excess of the total inhibitors would have been detected. This would lead to under-estimation, but not total "invisibility", of the CSA. In those situations, as is clearly shown in the experiments in Chapter 2, it may be essential to maximally stimulate the target hemopoietic cells with exogenous CSF's before the inhibitory effect could become evident. Also, the indicator cell is relevant in that IL-1, which induces fibroblasts and endothelial cells to produce a

myelo-stimulatory conditioned medium, causes the release of inhibitory factors for murine pre-B cells [Billips 1990].

Conversely, a few reports have been published in which the experimental design was appropriate to detect inhibitors. In most of them the presence of inhibitors was evident from inspection of the data, although the authors chose not to comment on it. Indeed, such activities have been found in the conditioned media of early passage primary human marrow-derived fibroblastoid cells [Greenberg 1981; Gordon 1981], from an established monkey fibroblast line [Bot 1988], from the adherent layer of both hamster and murine long-term marrow cultures [Eastment 1985; Naparstek 1985; Shadduck 1983], from a fibroblastoid line established from murine long-term marrow cultures [Garnett 1982], as well as from human endothelial cells [Ascensao 1984; Quesenberry 1980]. Finally, it is relevant to point out, lest the early experiments be misinterpreted, that the mouse lung conditioned medium used as one of the first sources of CSF's was derived from a crude mixture of lung fragments containing macrophages, fibroblasts, endothelial cells, and pneumocytes [Burges 1977] - very different from the more refined cell strains used in the work described here.

Another source of apparent conflict has arisen from the difficulty in detecting certain stimulatory and inhibitory activities in a conditioned medium. Some investigations have required double layer assays to detect them [e.g., Peschel 1989]. Such apparent differences might

be due to the ability of the different lines to degrade or inactivate an inhibitor. Alternatively, a stromal cell might produce both activities, but the ECM might differ, permitting the release the factors into the CM around one type of cell while retaining it around another. This might give the appearance of species differences and account for the greater ease of detecting CSF's by immunoassay than by assays of biological activity [Shadduck 1983]. Thus, the difficulty has not been in demonstrating inhibitors in cultures derived from species other than rat, but in standardization of conditions in complex cell culture experiments.

Finally, one must consider the possibility that the rat differs from even the mouse in the way that these cells synthesize, secrete, or release factors into the surrounding medium. While it is generally assumed that essential regulatory controls are maintained throughout evolution, the location of the hemopoietic system varies greatly among fish, reptiles, birds, and mammals [Tavassoli 1986]. Even among mammals, more subtle variations have been noted <u>in vitro</u> that may or may not reflect the <u>in vivo</u> state. For example, murine long-term marrow cultures can be propagated more easily and for a longer time than those of humans. Also, human-derived CFU-M do not grow in response to M-CSF as readily as do those from the mouse. This has been shown to be due to an inhibitory effect of marrow macrophage or possibly other myeloid cells in humans [Rosenfeld 1990].

Although the aim of this work was not to reproduce the old experiments or to compare the relative abundance of inhibitors and stimulators in the conditioned medium of different fibroblasts of different tissues of different species, some of the apparent discrepancies may be explained by the fact that we sought to use homogeneous fibroblastoid cell strains of low passage. In this manner it was demonstrated that sublines of marrow-derived cells have a different capacity to produce an inhibitory conditioned medium, and that the conditioned medium from lung-derived fibroblasts could be stimulatory until the macrophages and minor population of ST3+ fibroblastoid cells had been depleted. Conditioned media from lung and marrow-derived fibroblasts cultures treated in that manner clearly differed, and furthermore, this difference was maintained in established lines derived from the primary growths. Because the dominant activity (stimulator or inhibitor) in the different CM probably is the product of multiple factors acting in synergy and in competition, further effort was directed to separating the activities in the different CM. Accordingly, it was found that both types of cells produced both activities. Further clarification of the issue ultimately will depend on purification of the hemo-inhibitory molecules and examining their behavior alone and in combination with other cytokines. To this end initial steps were begun to purify the predominant inhibitory activity in the CM of lung fibroblasts. Although this was accomplished to 10,000-fold (sufficient purity for many enzymes), it

remained impure and may require purification to the level of greater than 100,000 (typical for many growth factors). This work is being continued by others in Dr. Sullivan's laboratory.

In summary, taken together the results presented in this thesis do not necessarily conflict with established concepts, but in fact offer both evidence and a partial explanation for why many of the older publications are in conflict with one another.

6.2.3 Relation of the inhibitor to others that have been reported.

The hemopoietic inhibitory activity identified in this thesis can not be ascribed to any of the molecules discussed in section 1.5. However, only after purification of the protein to homogeneity, and molecular cloning of the gene will we know with certainty what it is.

Transforming growth factor-beta (TGF-beta) is an inhibitor of hemopoietic precursors (such as BFU-E, CFU-GM, and CFU-G). Although both TGF-beta [Roberts 1981] and the inhibitory activity were produced by the rat fibroblasts studied here, the lability to acid and heat (TGF-beta is resistant to both acid and heat), the species specificity (TGF-beta has broad cross species reactivity), the differential sensitivity in a panel of cell lines (e.g., U937, KG-1 and WEHI-3B are sensitive to TGF-beta

but resistant to the inhibitory activity we observed), and the antibody neutralization studies all support the conclusion that the two activities are not identical.

Tumor necrosis factor (TNF), depending on the alpha or beta form, is produced primarily by macrophages or lymphocytes, but can be made by other cells. The inhibitor observed here is a fibroblast product, and does not alter the clonogenic growth of cell lines (e.g., U937 and HL60) known to be sensitive to TNF.

The apparent species specificity suggested that it might be interferon-beta. Rat interferon, however, is acid and heat stable [Wiedbrauk 1986], and can also inhibit C6 cells [Poindron 1981], which are not affected by the rat fibroblast-derived inhibitory activity. Furthermore, the antibodies against interferon did not neutralize its activity.

The high apparent mol wt of the inhibitory activity required that acidic isoferritin be considered. However, the inhibitor's activity was not neutralized by the antisera to ferritin. The negative effect of lactoferrin on hemopoietic progenitors is mediated through the inhibition of the production of endogenous CSA.

Several other molecules have been reported that can inhibit the <u>in vitro</u> growth of hemopoietic cells. These include the group of CFU-S specific inhibitors such as that originally observed by Lord [1976] and Cork [1981], which may be accounted for by the macrophage inflammatory protein family [MIP] [Graham 1990], and the small peptides isolated from marrow [Lefant 1989; Paukovits 1987]. Most

of them, however, are CFU-S specific and do not act on more mature hemopoietic progenitors or on leukemic cells. A BFU-E specific inhibitor (superoxide dismutase) also was observed [Axelard 1981]. It does not have any effect on other progenitors or on leukemic cells. Another glycoprotein (restrictin P), identified in the conditioned medium of a fibroblast-like cell line, suppresses the proliferation of plasmacytoma cells but does not influence normal hemopoietic progenitors [Zipori 1985, 1986]. Among the hemopoietic inhibitors derived from leukemic cells, some can modulate only the growth of leukemic cells (i.e., LIF) [Gearing 1987]); others tend to be more active on normal hemopoietic precursors than on leukemic cells (e.g., hairy cell leukemia-derived inhibitor) [Lauria 1987].

6.3 Possible biological relevance.

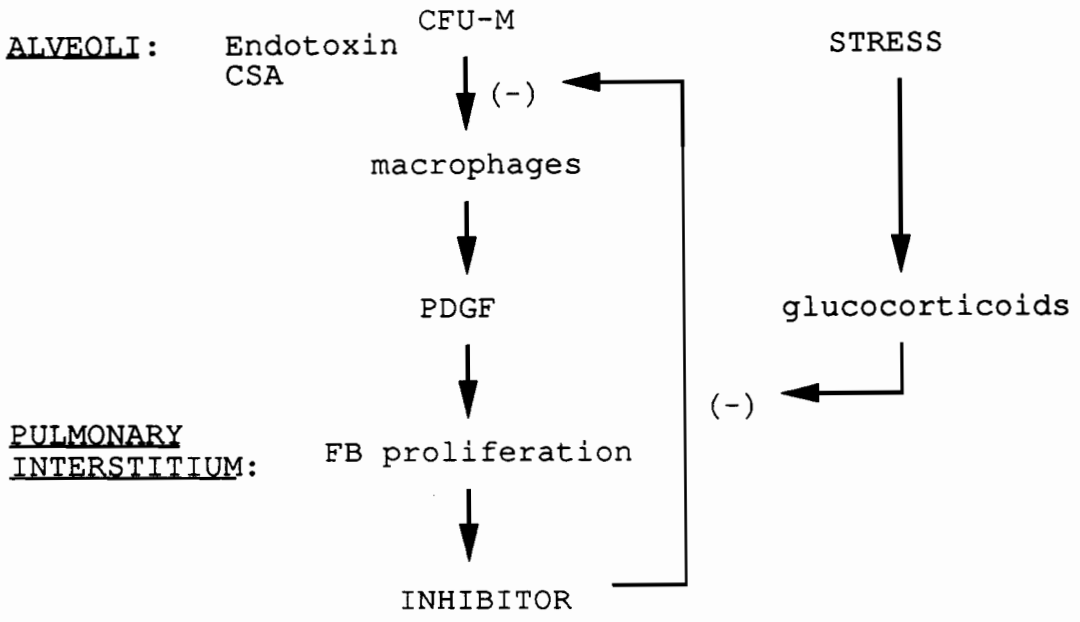
Any discussion or speculation related to the biological relevance of observations made in a complex *in vitro* model such as this must proceed with caution. Indeed, the true function of most of the known cytokines and colony stimulating factors remain uncertain, although many have been purified, are available in recombinant form, and are active *in vivo* in pharmacological doses. Evidence has been presented here that isolated fibroblasts derived from the bone marrow produce a conditioned medium that is stimulatory to hemopoietic colonies *in vitro*, but after they have been cultured in the presence of macrophages or their CM, the inhibitory activity predominates. In contrast, fibroblasts from the lung cultured under identical conditions constitutively produce an inhibitory CM. At present we do not know if the molecules mediating these actions in the CFU-C assay are the same for lung and marrow. With the caveat that these cells may be acting in a totally different manner within the natural organ, surrounded by their own ECM and maintained in their native homeostatic environment, we will proceed to envision how this might relate to normal physiology.

Not limiting our perspective to hemopoiesis, but considering also the function of the lung, further insights might arise. One of the major regulatory loops in the maintenance and remodeling of this tissue is the interaction between macrophages and fibroblasts. The

pulmonary macrophage provides an early line of defence against inspired organisms, and is replenished from a pool of committed precursors resident in the alveoli. In the activated state it releases products that stimulate the proliferation of fibroblasts. It also may act to dampen the proliferation of fibroblasts (and subsequent fibrosis) by its secretion of collagenase [Bauman 1987; Jordana 1987; Goldstein 1986; Freundlich 1986]. In the lung, the hemo-inhibitory activity of the fibroblast may be one of the ways that the proliferation of macrophages is dampened in the sea of M-CSF. Without a means to break the macrophage-fibroblast stimulatory loop, one would expect uncontrolled fibrosis and destruction of alveoli, as occurs in the bleomycin model of pulmonary fibrosis [Doherty 1992]. Moreover, it has been proposed that fibrosis of the marrow in myeloproliferative disorders may result from a relative deficit in the macrophages needed to counteract the proliferation of fibroblasts stimulated by PDGF released from neoplastic megakaryocytes [McCarthy 1984].

One approach taken to try to identify the biological significance of a molecule found to have activity in vitro is to demonstrate whether it might fit into any of the known regulatory networks. Because the glucocorticoid hormones are one of the major regulators of the stress/inflammation response, decreasing the expression of IL-2 and IF by T lymphocytes and blunting the TNF-stimulated secretion of GM-CSF by fibroblasts

[Tobler 1991], one might expect that such a negative hemo-inhibitor also might be under their influence. Showing that it is regulated would suggest, although not prove, that it contributes to the local regulation of *in vivo* cell turnover. The experiments shown in Chapter 4 indicate that lung fibroblasts, when cultured in the presence of hydrocortisone, release less inhibitory activity into the CM. One might envision the following pathway:



This model proposes that the pulmonary interstitial fibroblast constitutively produces an inhibitor of macrophage expansion as part of the normal homeostatic

process, but under conditions of acute stress, the negative component is released so that the macrophage precursor pool can proliferate.

In the bone marrow the requirements would be different. Here both hemopoiesis and remodeling of bone must be considered. The predominant ST3+ FB does not produce much activity until it has been stimulated, after which it produces a conditioned medium that appears to be stimulatory for hemopoietic precursors in the presence of a submaximal concentration of CSA. However,under conditions of a maximal concentration of multiple synergistic factors, the fibroblast conditioned medium in inhibitory.

The production of both activities in response to macrophage products is puzzling. However, one could speculate that the position of different target cells within the marrow architecture might enable them to respond differently to different stimulator/inhibitor ratios. In this manner diffusion gradients and differential absorption to ECM of variable composition would be relevant. Also, as shown in Chapter 2, 2-3 days after stimulation the increased CSF production by marrow fibroblasts has returned to baseline, but that of the inhibitor remained elevated for at least 5 days. This again could contribute to the feedback loops that control proliferation of hemopoietic cells.

Another cell of the bone marrow-derived hemopoietic origin is the osteoclast. Because it has been shown that glucocorticoids directly stimulate maturation of

osteocytes from their precursors [Bellows 1990], one could envision a mechanism permissive to bone remodelling through which steroid-mediated repression of the inhibitor also would allow increased production of osteoclasts.

6.4 Implications and future directions of this work.

1. The inhibitor identified and partially purified by the candidate appears not to be any of the factors recognized to have a similar activity, such as TGF-beta, TNF, IFN, acidic isoferritin, or others that have not been as well characterized. Thus, it may either be a novel cytokine or at least a novel aspect of a known molecule. Although a 10,000-fold enrichment of specific activity was obtained by the initial five purification steps (membrane filtration, ion exchange, and dye-ligand, heparin, and WGA affinity chromatography), further purification is required. This will involve additional chromatographic steps, generation of a monoclonal antibody, partial sequence determination, and molecular cloning from a cDNA library from a producer cell line.

2. Lung-derived fibroblasts (ST3-/ST4+), in contrast to those derived from the marrow (ST3+/-), constitutively release the inhibitory activity. If this cell type proves to be representative of the predominant type of mesenchymal cell in non-hemopoietic tissues, then one could speculate that they might have a role in counteracting the ubiquitous CSA to which circulating hemopoietic precursors might be exposed. Such a mechanism also might be part of the explanation for why the bone marrow stroma is permissive to the growth of blood cells.

3. This inhibitory activity suppressed the growth of both normal and leukemic hemopoietic precursors. However, its mechanism of action on normal or leukemic cells, and its relationship to the other hemopoietic inhibitors described remains to be determined. Such studies must await the availability of a pure molecule. After a gene has been obtained from rat DNA, then the human analog can be sought by cross hybridization.

4. The inhibitory activity is a heparin-binding protein. It is known that proteoglycans of the extracellular matrix of marrow stroma [Wight 1986] can concentrate different CSFs, some of which can bind to heparin [Lobb 1988; Gordon 1987]. With an antibody to the molecule, one can perform histological studies to determine if it might contribute to the control of local hemopoietic microenvironment.

5. Beyond possible relevance to the hemopoietic system, these observations raise the speculation that mesenchymal elements also might undergo organ-specific differentiation. Although, for the most part, all fibroblasts appear similar, they might serve functions specific to the organ in which they develop.

Chapter 7 ORIGINAL CONTRIBUTIONS OF THIS WORK

1. Antibody-defined fibroblasts from two different organs, one hemopoietic and the other non-hemopoietic, differ in the pattern of stimulatory and inhibitory activities that they produce.

2. In cultures of lung-derived fibroblasts, an inhibitory activity predominates, whereas in cultures of marrow-derived fibroblasts inhibitors are detected only after the cells had been stimulated with macrophage-derived products.

3. The production of the inhibitors by lung-derived fibroblasts is decreased by hydrocortisone.

4. An activity inhibitory to both normal and leukemic myeloid cells was characterized and partially purified; after final purification, this may prove to be a novel cytokine.

All of the experiments described in this thesis were performed by the candidate, although the guidance and supervision of Professor Sullivan was present throughout.

Appendix 1 THE PRESENCE OF INHIBITORY ACTIVITY IN RAT PLASMA AND SERUM.

ASSAY FOR THE PRESENCE OF INHIBITORY ACTIVITY IN RAT PLASMA AND SERUM.

	% of Maximum	
	EXPERIMENT #1	EXPERIMENT #2
Medium alone	11	8
SCCM (5%)	100	100
SCCM + RPMI/10% FCS (5%)	86	100
SCCM + ST4 CM (5%)	57	58
SCCM + rat plasma (5%)	77	84
SCCM + rat serum (5%)	38	44

[Assays performmed in duplicate plates in each experiment; the two experiments were performed with two separate CM and different rats on different days].

To test whether there might be circulating inhibitory activity in rat plasma, the standard assay was performed. As can be seen plasma contained minimal activity. However, serum inhibited to a greater extent than did the ST4 CM. Because platelets are a major depot for TGF-B it is possible that this cytokine might be responsible for the inhibitory effect. However, it also is possible that platelets contain the fibroblast factor which we have shown not to be the usual form of TGF-b. Proof must await future studies to be done when a specific immunoassay is available for this molecule.

Appendix 2 CELLULAR COMPOSITION OF RAT BONE MARROW STROMA

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LABORATORY INVESTIGATION

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Cellular Composition of Rat Bone Marrow Stroma

Antigen-Defined Subpopulations

ARTHUR K. SULLIVAN, DAVID CLAXTON, GENE SHEMATEK, AND HONG WANG

McGill Cancer Centre, Montreal, Quebec, Canada, H3G 1Y6, and the Division of Hematology, Royal Victoria Hospital, Montreal, Canada

Although stromal cells establish the architecture of mammalian bone marrow and organize hemopoiesis, the interrelationships among their macrophage, fibroblastic, endothelial, and adipocyte-like components are not wholly understood. Using murine monoclonal antibodies to cultured adherent cells of rat bone marrow, we observed that the predominant fibroblastoid cells grown from marrow differed from those of non-hemopoietic organs. The marrow type bore a detectable quantity of the ST3 but not ST4 antigen, whereas those from lung, diaphragm, and epididymal fat pad, bore more ST4 than ST3. Those from spleen were an equal mix of both types. Although the tissue distribution of the ST3 antigen was similar to that of Thy-1, it was not identical, and in the brain, the two structures were localized in different areas. While none of the ST3, ST4 (fibroblast directed), or BN(MB)35 (myeloid directed) antibodies recognized fat cells cultured from marrow, the ST10 antibody, selected for binding to marrow derived fat cells, stained peripheral adipose cells, unidentified aglobular cells in areas of fat cell formation, and macrophages, but not fibroblasts. On the basis of these observations, we suggest that the fibroblastoid cells of the marrow are different from those of non-hemopoietic tissues.

Additional key words: Fibroblasts, Macrophages, Adipocytes, Hemopoiesis, Monoclonal antibodies.

In the mammalian bone marrow, blood develops within a complex regulatory network of humoral, cellular, and matrix factors (15, 40, 49, 51, 57). This stromal compartment has a major role in marrow organization, as indicated by experiments demonstrating regions of lineage-restricted proliferation (34, 56), and growth of hemopoietic tissue at ectopic loci where permissive accessory cells have been implanted (12, 40, 48). From evidence accumulated through morphologic studies (61) and observations of long-term bone marrow cultures (LTBMC) (2, 29), it has been proposed that "reticular" fibroblasts, adipocytes, endothelium, and macrophages comprise the major cellular elements. Although it has been suggested that stromal cells may share a common precursor with blood (27, 28, 50), it remains uncertain what pathway they follow in differentiation, and what relationship they may have to analogous components of other tissues. The work reported here describes our initial attempts to identify the supporting cell populations of rat bone marrow by objective criteria using monoclonal antibody reagents.

EXPERIMENTAL DESIGN

To establish more objective criteria for studying the structure and function of the bone marrow, we developed a series of murine monoclonal antibodies to the major cellular elements of cultured rat marrow stroma. We then used these reagents and immunohistologic techniques to compare cells from the marrow with those with similar morphology from other selected tissues. Finally, to form a basis for further experiments on the possible role of stromal cells in the control of hemopoiesis, we analyzed the effects of the antibodies on precursor cells in standard clonogenic assays.

RESULTS AND DISCUSSION

ANTIGEN MARKERS OF THE MAJOR STROMAL CELL POPULATIONS OF CULTURED RAT MARROW

The cells in a 7 day culture of rat femoral bone marrow, manipulated as described above, appeared to be predominantly round and dendritic macrophages (Fig. 1*a*), mixed with an occasional isolated fibroblast, and a few tightly packed nodules out of which fibroblastoid cells later grew. Cells from macrophage colonies (CFU-M), after transfer to liquid culture for 24 to 48 hours, resembled those in the predominant 7 to 14-day population and gave a positive cytochemical reaction for esterase (Fig. 1*b*.); cells propagated from a fibroblast colony (CFU-F) did not stain for esterase (Fig. 1*c*).

FIG. 1. *a*, Typical appearance of adherent marrow cells between the first and second weeks. Wright's stain. *b*, Progeny of macrophage colony (CFU-M), replated in liquid media. Esterase stain. *c*, Progeny of fibroblast colony (CFU-F) after replating for several days. Esterase stain. Enlargement, microscopic: *a*, × 250; *b*, × 320; *c*, × 200; photographic: × 2.6 for all.

Over the next 2 to 3 weeks in media supplemented with only 10% fetal calf serum (FCS), the number of macrophages diminished and fibroblastoid cells became predominant. When the medium was supplemented with horse serum and hydrocortisone (HC), islands containing cells resembling multiloculated adipocytes developed. All of these cells appeared similar to those described for mouse bone marrow grown under comparable conditions (22, 46).

With antibody markers, it was possible to further distinguish these morphologically-defined cell types. As shown in Figure 2*a* and *b*, ST2 and BN(MB)35 reacted with macrophages but not with cells of fibroblastic morphology, whereas ST3 stained fibroblasts but not macrophages (Fig. 2*c* and *d*). Antibody ST4, although derived from mice immunized with marrow cells, stained fibroblastoid cells from all other tissues tested, but only a rare cell from marrow (Fig. 2*e*). In similar cultures of adherent spleen cells, macrophages of the ST2+/BN(MB)35+/ST3- phenotype predominated and, as noted with bone marrow, further culture resulted in a predominant growth of fibroblasts. Unlike marrow, however, these cells were a mixture of those reacting with ST3 and ST4 (not shown).

Expression of these antigens on fresh femoral marrow was analyzed by three parameter flow cytometry which includes (a) small-angle light scatter reflecting size, (b) 90° light scatter reflecting internal structure, and (c) intensity of fluorescent staining. With this technique erythroid, lymphoid, and granulocytic cells segregate into distinct areas of a computer-generated scattergram (52). The macrophage-reactive antibodies stained large cells with low and intermediate 90° scatter, consistent with monocytes and developing granulocytes. Neither ST3 nor ST4 reacted with cells in the "granulocyte window", but ST3 bound to a subset of smaller cells in the "lymphocyte window" (Table 1). Analysis of peripheral blood indicated that ST2 stained most polymorphic neutrophils (PMN), BN35 weakly stained a minority of PMN, and ST3 bound to only a small subpopulation of lymphoid cells (not shown).

To assess the expression of the markers on differentiated macrophages, marrow colonies were grown in agar, picked with a pipette, placed in chamber slides, and stained after overnight culture. On these terminally matured cells, both ST2 and BN(MB)35 gave a clear positive reaction. These results confirm that cultured stroma from rat bone marrow consists of macrophage and fibroblastoid species that bear distinct antigen markers recognized by the antibodies used in this study.

ANTIGEN HETEROGENEITY OF FIBROBLASTS

As determined by immunostaining of chamber slide cultures, the ST3, but not the ST4, antigen was detected on almost all fibroblastoid cells (Fig. 2*d* and *e*). Cells of similar morphology grown from other organs (*e.g.*, skin, diaphragm, lung, and epididymis) were a mixture of ST3+ and ST4+ types, with ST4+ varying between 30 and 90% of the total. Those from the lung were almost exclusively ST4+, whereas those from the spleen were a balanced mixture of each (Fig. 2*f* and *g*). Only in a sporadic manner that could not be related to culture conditions, source of flasks, or lot of serum, did the ST3+ cells predominate in cultures from organs other than marrow.

Because the Thy-1.1 antigen had been reported to be present on fibroblasts (14), ST3+ cells from marrow and ST4+ cells from lung were tested for 0x 7 binding (anti-Thy-1.1). Since both types of cells stained with equal intensity (not shown), it is not likely that either ST3 or ST4 antibodies are against epitopes recognized by 0x 7.

To obtain quantitative confirmation of the histologic impressions, early fibroblast enriched (>95% by inspection) cultures were replated at low density into 96 well dishes, and the relative concentrations of ST3 and ST4 antigens were determined by an enzyme-linked immunosorbent assay (ELISA) assay. The graphs in Figure 3 show the frequency distribution spectra of antibody binding. These measurements are consistent with the morphologic observations that fibroblastoid cells from marrow predominantly express the ST3 antigen, but those grown from peripheral, non-hemopoietic organs express more ST4. The spleen, an organ capable of supporting hemopoiesis under certain conditions, gave rise to an equal mix of both ST3+ and ST4+ types. These results indicate that, in the tissues examined under these conditions, there are at least two major species of fibroblastoid cells, a type predominant in the marrow (ST3+/

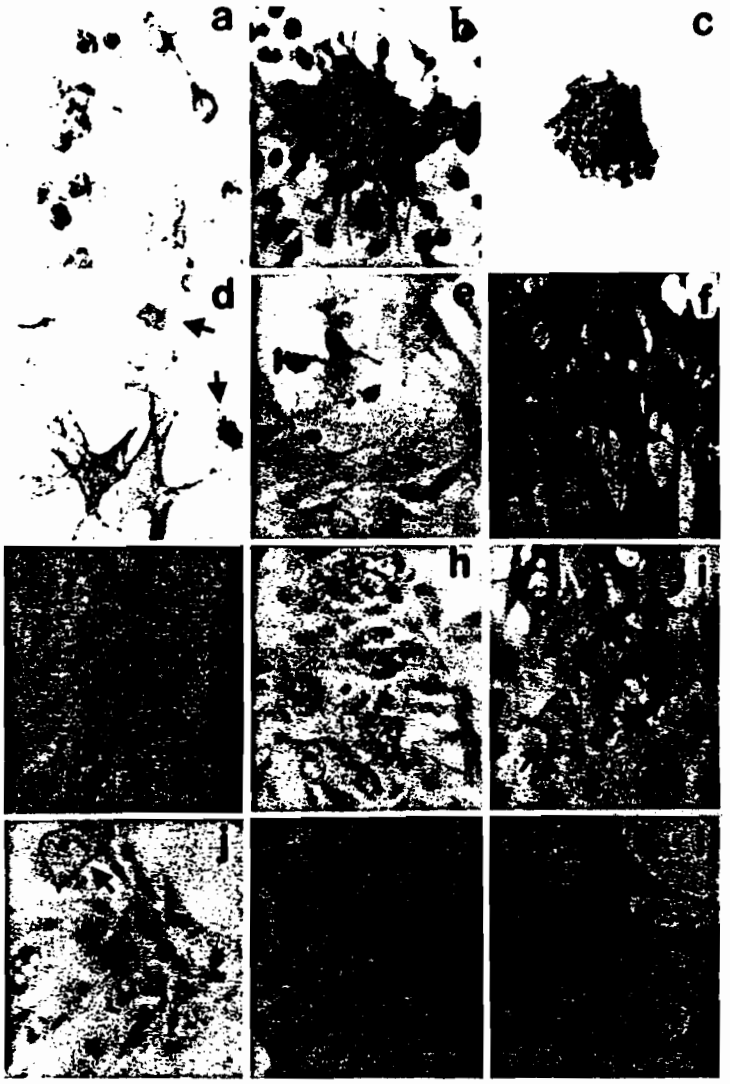


FIG. 2. Immunostaining of cells cultured in chamber slides. AEC substrate gives a red-orange color. Mayer's hematolylin counterstain gives a blue-grey color in absence of a "specific" reaction. *a* to *c*, Bone marrow adherent cells typical to cultures during second to 3rd week. *a*, ST2 staining round and dendritic macrophages. *b*, BN(MB)35 staining round and dendritic macrophages, but not a fibroblast colony in the background. *c*, ST3 staining cells in nodule, similar to those which develop into typical fibroblast colonies, as seen in *b*; surrounding macrophages do not stain. *d* to *e*, Marrow, 3rd to 4th week. *d*, ST3 staining fibroblastoid cells but not macrophages (*arrows*). *e*, ST4 staining neither type of cell. *f* and *g*, Fibroblastoid cells from lung. *f*, Stained with ST4. *g*, Most cells fail to stain with ST3; a field selected with some reactive cells for comparison. *h* to *k*, Marrow cultured in HC for growth of fat cells. *h*, Stained with Ox 1 *h* to *k*, Marrow cultured in HC for growth of fat cells, *h* Stained with Ox 1 (anti-leukocyte common antigen); all cells negative. *i*, ST10 staining fat-laden cells but not fibroblastoid cells. *Arrow* denotes unidentified "ghost" cell. *j*, ST11. *Arrow* points to "ghost" cell near area of negative fat cells. *k*, ST3 staining fibroblasts around negative fat cells. *l*, Epididymal fat stroma cultured to promote adipogenesis. Adipose-like cells stained by ST10. Enlargement, microscopic: *a*, × 360; *f*, *g*, *i*, *l*, × 320; *c*, *d*, *e*, *h*, *j*, × 250; *b*, *k*, × 200; photographic: 1.6x for all. The staining patterns shown in these selected photographs were qualitatively consistent with those of more than 20 experiments with marrow, at least five with lung, and three with epididymal fat stroma.

ST4-), and another common to most peripheral tissues (ST3-/ST4+).

PRESENCE OF ANTIGENS ON PRECURSOR CELLS

To determine if the macrophage and fibroblast related antigens are expressed on clonogenic precursors at a level detectable by cytotoxicity, we examined the effect of the BN(MB)35 and ST3 antibodies on the subsequent development of colonies. Fresh bone marrow was exposed to antibody and complement prior to culturing, and then was assayed for colony formation (CFU-C and CFU-F). As indicated in Fig. 4, BN(MB)35 suppressed the ap-

TABLE 1. SUMMARY OF ANTIBODIES AND STAINING OF CELLS FROM UNCULTURED BONE MARROW

Antibody	Subclass	Immunogen	Percentage of nonerythroid cells staining		
			Total	Granulocyte window (53%)	Mononuclear window (24%)
D46 (negative	IgM	Human HL60-D[a]	1.6	0.8	0.8
K89 controls)	IgG	Human K562[b]	1.4	0.7	0.7
ST2	IgG1	BM[c]	18	16	2.2
BN35	IgM	BNML/RS	25	22	2.5
ST3	IgM	BM[c]	10	1.4	7.4
ST4	Ig2a	BM[c]	2.3	1.4	0.9
ST10	Ig2a	OHC[d]	2.6	1.3	0.7
ST11	IgM	OHC[d]	1.5	0.7	0.8

Microscopic differential count of erythrocyte-depleted marrow: eosinophil 7%; promyelocyte/myelocyte 7%; polymorphic neutrophil 38%; lymphoid 17%; monocytoid 2%; nucleated erythroid 28%.

[a] HL60-D is a blastic subline of the promyelocytic leukemia line.

[b] K562 is a line with pronormoblastic features derived from a patient with blast phase chronic granulocytic leukemia.

[c] Marrow stroma cultured as described in the "Methods" section.

[d] From marrow cultured in the presence of hydrocortisone.

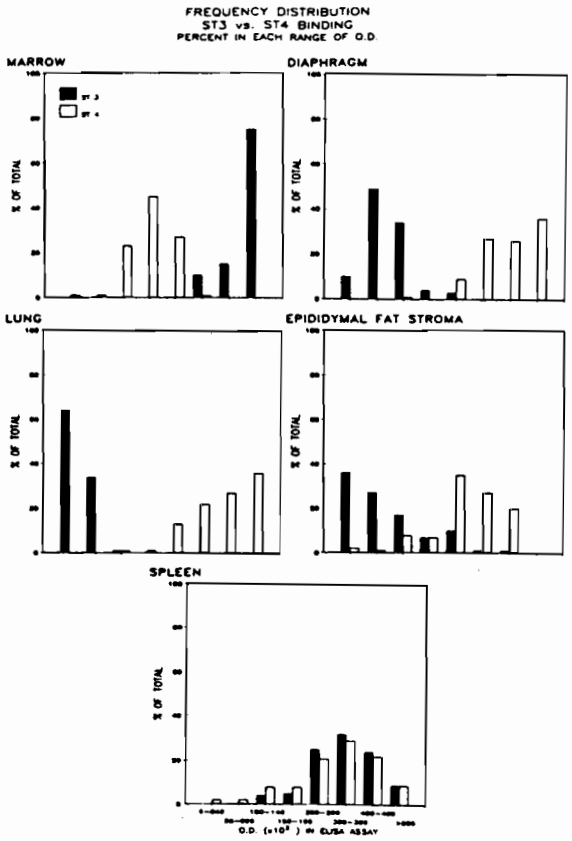


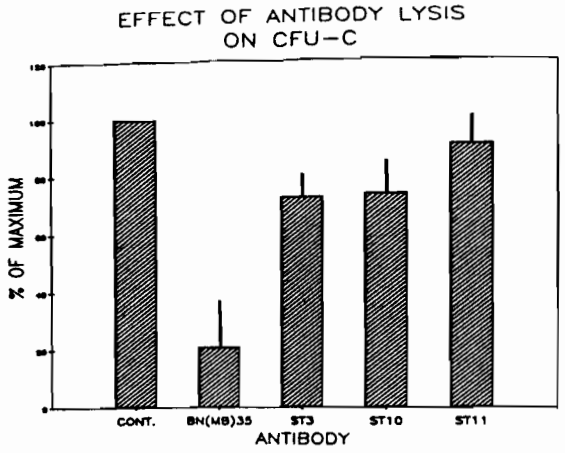
FIG. 3. Distribution of densitometry readings in the ELISA assay, expressed as the percentage of total wells in each given range. The *graphs* shown are representative profiles of one of three independent and consistent experiments. Separate 96-well plates were tested for ST3 or ST4 antibody binding. The chi-square test was performed on the distributions of all experiments; the difference between ST3 and ST4 was significant at the $p < 0.001$ level for all tissues tested, except for spleen, which was not significant in any experiment.

pearance of macrophage and granulocyte colonies, but not of fibroblasts. It also ablated (not shown) progenitors of erythroid cells (CFU-E). Although this finding suggests that the epitope is present on immature myeloid cells, these CFU-C assays do not give information on the state of more primitive stem cells (*i.e.*, CFU-S equivalent). On the other hand, treatment of marrow with ST3 resulted in a marked diminution of CFU-F, and in a lesser (but statistically significant) decrease in CFU-C. In contrast to the other observations, the effect of ST3 on CFU-C was not complement-dependent. The ST2 antibody is not of a complement binding subclass and could not be evaluated in this manner. From these experiments, we conclude that the ST3 and BN35 antibodies are expressed on precursors of independent populations of clonogenic marrow cells.

DISTRIBUTION OF ANTIGEN-BEARING CELLS IN TISSUES

The results of a histologic survey of antigen expression in other organs is summarized in Table 2. In preparations of frozen tissue sections, both BN(MB)35 and ST2 stained areas where macrophages are known to reside. Although the spleen stained with both antibodies in the interfollicular areas (Fig. 5*a* and *b*), scattered cells in the thymic medulla stained with BN(MB)35 but not ST2. Other macrophage derivatives, such as Kupffer cells of the liver, Langerhan's cells of the skin, and glial cells of the brain were not detected with either reagent. In perivascular adventitia, beneath epithelial surfaces (*i.e.*, endometrium, and small intestinal villi), and alveoli, both antigens were present in scattered areas, with ST2 expressed more strongly than BN35.

In most tissues, the fibroblast-related antibodies either did not react, or gave a weak scattered reaction. Brain and thymus were the major sites of ST3 concentration, whereas the spleen was the only organ in which ST4 was clearly evident. Thymic cortex and interlobular septae were strongly stained by ST3, and a few scattered cells in the medulla were weakly stained (Fig. 5*c* and *d*). In the brain, areas of neuronal binding were apparent in a



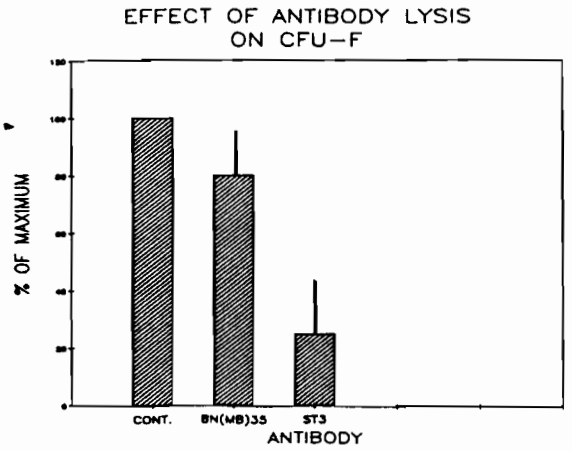


FIG. 4. Effect of pretreatment of marrow with antibody and complement on subsequent development of myeloid (CFU-C, *top panel*) and fibroblast (CFU-F, *bottom panel*) colonies. Control antibody shown (100% value), a monoclonal IgM to human myeloid cells not reactive with rat tissues, did not decrease the colony number from that observed with an irrelevant IgG (from the P3X63 Ag8 myeloma line), or complement alone. *Narrow bars* above the data bars represent 1 SD. The results are the mean of three independent experiments, except for CFU-C exposed to BN(MB)35 and ST3, which are the mean of five. Differences from the control was assessed by the one-tailed t test. For CFU-C: BN(MB)35, $p < 0.001$; ST3, $p < 0.005$; ST10, $p < 0.025$; ST11, not significant (NS). For CFU-F; BN(MB)35, NS; ST3, $p < 0.01$.

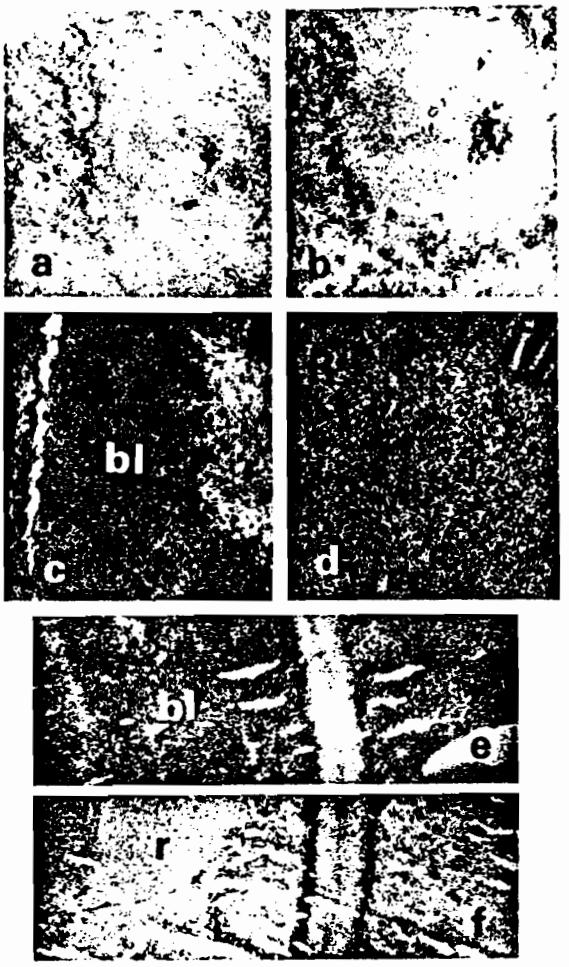


FIG. 5. Immunostaining of frozen sections of selected tissues. BCIG substrate gives a blue reaction product and stain; safranin O counterstain in absence of specific reaction appears red; intensity varies with nuclear density. *a*, Spleen stained with ST2, interfollicular reaction product. *b*, Spleen × BN(MB)35, interfollicular reaction product. *c*, Thymus × ST3. Cortical and medullary areas delineated. *d*, Thymus × ST4. *R* indicates dark red; *r* indicates light red (negative reactions); *bl* indicates blue. *e*, Brain (section through cortex with surface oriented to *right side*) × ST3. *f*, Brain × Ox 7 (anti-Thy-1). Sequential microtome cut to that of ST3; photographic print made with negative turned over to demonstrate alignment of staining areas. Enlargement, microscopic: *a* to *d*, × 60; *e* and *f* × 36; photographic: × 2.6 for all.

TABLE 2. SUMMARY OF ANTIBODY STAINING PATTERNS

Antibody	Cells in culture	Tissues			
		Spleen	Thymus	Brain	Kidney
BN(MB)35	Macrophage	Interfollicular	Medullary	Neg	Neg
ST2	Macrophage	Interfollicular	Neg	Neg	Diffuse glomerular
ST3	Fibroblastoid	Neg	Cortical	Cortical laminar	Centriglomerular
ST4	Fibroblastoid	Neg/weak reticular	Neg	Neg	Neg/weak reticular
ST10	Macrophages, fat cells	Interfollicular	Neg	Neg	Neg
ST11	Macrophages	Neg	Neg	Neg	Neg

multilaminar pattern (Fig. 5*e*). In the kidney, ST3 stained the central glomerular area where mesangial cells frequently are concentrated. In view of the observation that the ST3 antigen was found in cells and organs known to contain Thy-1 (*i.e.*, thymocytes, fibroblasts, renal mesangial cells, and brain), adjacent microtome cuts of brain were stained with ST3 and Ox 7 (anti-Thy-1.1); the photographs shown in Figure 5*e* and *f* demonstrate that the resulting patterns were different, suggesting a reciprocal relationship in some areas.

ANTIGENS ON FAT-CONTAINING CELLS IN CULTURE

A third major component of the marrow stroma, both *in vivo* and *in vitro*, are cells that accumulate lipid and contain fat globules. As illustrated in Figure 2*h* and *k* (for Ox 1 and ST3), Ox 1, ST3, ST4, and BN(MB)35 antigens were not detected on fat-containing cells from rat marrow developing in media supplemented with HC. Although the ST2 antibody gave an equivocal reaction, control cultures of macrophages grown in the presence of HC also showed decreased ST2 staining. Because these antibodies did not yield any positive indication of the origin or lineage of marrow fat cells, further reagents were sought which would bind to adipocytes. A subsequent antibody, ST10, strongly stained fat-like cells in marrow cultures (Fig. 2*i*), whereas ST11, selected for staining of aglobular cells, did not (Fig. 2*j*). When stromal cells from epididymal fat pads were isolated, cultured in a similar fashion until typical fat globules appeared, and then incubated with the antibodies, the fat cells stained strongly with ST10 but not with ST11 (Fig. 2*b*), ST3, or ST4. Furthermore, neither ST10 nor ST11 antibodies bound to bone marrow fibroblasts, or to other cells from fresh marrow or peripheral blood (Table 1). On the other hand, ST10 strongly stained macrophages from marrow, cultured both with and without hydrocortisone (not shown), as well as interfollicular areas of frozen sections of spleen; ST11 reacted with cultured macrophages, but did not stain sections of spleen. As shown in Figure 4, treatment of marrow with ST10 resulted in a complement-dependent decrease (34%) in CFU-C, but not to the extent caused by BN(MB)35. This effect was observed on CFU-M but not on CFU-G (not shown); ST11 did not alter the growth of either type of colony. Thus, it appears that both of these antigens may be acquired late in macrophage development, and possibly only in a subpopulation.

The cellular composition of the islands of fat-containing cells grown in vitro from rat bone marrow was of further interest. Although the fat cells themselves did not stain with ST3, they were usually surrounded by ST3+ fibroblastoid cells to which macrophages often adhered. In these islands, an unidentified type of flattened cell frequently was seen which was smaller than a fibroblast, stained with both ST10 and ST11 (Fig. 2*i* and *j*), lacked fat globules, and contained an adipocyte-like nucleus.

STROMAL CELLS IDENTIFIED BY MORPHOLOGY AND ANTIGEN EXPRESSION

In the work presented here we sought to develop objective criteria and techniques with which to study the stroma of rat bone marrow. Results of initial experiments with a series of antibodies indicate that the predominant fibroblastoid cells cultured from this tissue differ from those of other organs, and are of a lineage different from the committed clonogenic precursors of marrow macrophages. Although fat-containing cells of the marrow bore some antigens common to cultured macrophages, and were not recognized by antibodies to fibroblast-related markers or leukocyte-common antigen, their lineage relationship to other stromal cells is still not clear.

Previously, other methods have been used to study the complexity of marrow regulation including electron microscopy of intact tissue (61), long-term stromal cell cultures (9), cloned stromal cell lines (20, 35, 43, 62), and implantation of stromal elements at ectopic sites (e.g., beneath the renal capsule) (3, 48). From these and other studies suggesting that neither macrophages (7, 8, 16, 36, 44) nor vascular-type endothelial cells (8, 10, 38) are essential for maintaining a LTBMC, it appears that the most significant supporting elements are fibroblastoid cells ("reticular" or "reticulo-fibroblastoid"), defined by production of collagen types I, III (5, 8) and occasionally IV (38).

In our studies with rats, as other workers have found with mice, the predominant cells growing from bone marrow in cultures containing FCS appeared to be macrophages and fibroblasts, with the latter predominating after four weeks of culture (22, 46). Using the ST3 and ST4 antibodies, we observed that the marrow-derived fibroblastoid cell was almost totally of the ST3+/ST4- phenotype, while those from other tissues were mostly ST4+. With the antibody panel we developed, we showed that the BN(MB)35 marker was on a precursor of erythroid and myeloid cells, including adherent macrophages, but was not on progenitors of fibroblastoid cells measured in the CFU-F assay; also, the ST3 marker was on fibroblastoid, but not myeloid precursors. Although it was not surprising to find that these two cells grew from different clonogenic precursors, the results suggest that either the ST3+ fibroblast is not equivalent to the common stromal-hemopoietic cell described by Singer *et al.* (50), or that the two lineages from which they develop diverge at a point before appearance of the antigens. Although further studies will be required to determine what role these antibody-defined subpopulations might have in the control of hemopoiesis, work in progress suggests that the ST3+ but not the ST4+ cell, can be stimulated to secrete some hemopoietic growth factors (60).

FIBROBLAST HETEROGENEITY

The results of the present study show that there are at least two antibody-defined populations of fibroblastoid cells which appear in different ratios in cultures from different rat tissues. Although the microplate assay indicated that there was a more restricted expression of ST3 or ST4 on cells from any given organ than did histologic preparations, this method could have exaggerated the difference between the two cell types if both epitopes were not expressed in equal concentrations. A two-fold difference in intensity of staining by one antibody over that of the other, which may not be obvious

visually, could explain the apparent discrepancy between estimates of positive cells by the two methods. Also, it appeared that the antigens were expressed more strongly on fibroblasts in culture than in most tissues examined as frozen sections, as might occur if the epitopes were borne on structures related to replication or activation. At present, we can not be certain whether or not the two forms of fibroblastoid cell are interconvertible under appropriate stimulation, whether they both arise from a common precursor, whether the ST3 and ST4 antigens can be co-expressed on the same cell, or if there is further heterogeneity within these subsets.

Antigen heterogeneity among fibroblasts from different murine and human tissues has been reported in surveys using antibodies made against other types of cells. In mice, for example, Thy-1 has been found on fibroblasts from skin and spleen but not from marrow, and T200 (leukocyte common antigen) has been detected on fibroblasts of marrow and spleen but not of skin (44, 45). This is consistent with the observation of Boswell *et al* (7) who found that anti-Thy-1 and complement did not decrease fat cell development of hemopoiesis in LTBMC. Van Vliet *et al.* (59) showed that the antibody ER-TR7, staining specific areas in sections of spleen, reacted weakly with marrow in a diffuse distribution. Ploemacher, Piersma, and Brockbank (45) showed that antibody ER-HR1 reacted with "fibroblastic reticular" cells from murine spleen and marrow but not with those from the skin.

Studies with human cells have led to similar results. Fetus and tumor-derived fibroblasts were found to bear antigens distinct from most normal adult types (4), and fibroblastoid cells derived from skin and lung were noted to differ in their quantity of dexamethasone binding sites (33). Surveying fibroblasts from various organs with a panel of antibodies, Ndumbe and Levinsky (42) found that the marrow type was positive for HNK-1 and J5 (CALLA, common acute lymphoblastic leukemia antigen), those from skin were J5+/HNK-, and those from embryonic lung were negative for both. Lim et al (38) reported a colon-derived "reticulofibroblastoid" cell from LTBMC which produced collagen types I, III, and IV, lacked CALLA, And in the presence of HC, produced lipid-laden cells. Abboud (1) developed an antibody reactive with both fibroblasts and endothelial cells which, in the presence of complement, could eliminate CFU-F and prevent a stromal layer from forming in LTBMC. However, from studies using antibodies to an epitope found on smooth muscle actin (9), it was concluded that the stromal cell responsible for hemopoietic support is independent from the fibroblasts examined under the usual culture conditions, such as those used here.

Fat-Containing Cells of Marrow

Although the marrow adipocyte was proposed to be a significant participant in the LTBMC, the question of its lineage, origin, and function remains controversial (54, 55). Reviewing the information available, Tavassoli (54) concluded that the fat-containing cells obtained directly from marrow differ from those grown in LTBMC or from peripheral tissues, and asserted that, in spite of problems of identification and a lack of objective criteria, the "cellular origin appears to be the adventitial cells of the bone marrow." Furthermore, as demonstrated by Bainton *et al.* (3), there may be two independent adipose compartments within the marrow, one described as red, the other yellow. In that study, the associated fibroblasts appeared identical, but when implanted under the renal capsule of a rabbit, the former gave rise to blood islands, whereas the latter produced only fat. They concluded that the bone marrow stromal cell may be an immature or fetal fibroblast.

Likewise, the origin of the peripheral adipocyte remains controversial. Candidates include cells described as macrophages or precursors, fibroblast-like perivascular cells, and perivascular mesenchymal cells. Part of the controversy lies in the definition itself, for all cells containing globules are not true fat cells. Indeed, Zucker-Franklin showed that monocytes can give rise to "foam cells" with small, ectopic nuclei (63). However, in most publications that illustrate the morphology of stromal cells, there has not been clear evidence to prove that there is a precursor relationship between either of these cells types; indeed, in one study, human marrow fat cells were shown to lack a macrophage associated antigen (47). On the other hand, hemopoietic progenitors and preadipocytes may be connected embryologically, according to Hausmann's claim that the "the mesenchymal cells of blood islands could be early progenitors of cells destined to become adipocytes." (24). In vitro studies examining the stromal fraction of adult peripheral adipose tissue, supported by other in vivo investigations, indicate that fibroblastoid precursors producing collagens type I and III can be induced to form fat-laden cells (6, 13, 58). As with marrow, peripheral adipoblasts can be stimulated by steroid hormones, possibly by activation of the differentiation program (23) through the action of a steroid-dependent regulatory factor (11).

Cell culture studies appear to be consistent with these impressions. Martinez observed that human marrow fibroblasts, but not those from skin, can transform into fat cells if their growth has been retarded, and he proposed that the process might be mediated by an "adipogenic factor" (39); HC potentiates this effect. Other workers (41) have shown that colonies with fibroblastic morphology that grew from human marrow stroma in response to platelet-rich plasma and platelet-derived growth factor gave rise to adipocytes in the presence of HC. From this experiment they concluded that fat cells and fibroblasts of marrow have a common precursor. However, because this was not a semi-solid system in which the colonies were immobilized, it is possible that a small number of other cells could have given rise to the fat cells. Nevertheless, several cloned fibroblastoid lines have been reported which can transform into fat-containing cells (reviewed in 25, 26), some of which could support LTBMC (21, 31). Conversely, others have claimed that adipose conversion is not a factor essential to blood cell proliferation (37).

The results presented here do not solve the controversy. None of the two fibroblast-associated antigens (ST3 and ST4), the myeloid antigen found on CFU-M (BN(MB)35), or the leukocyte-common antigen (Ox 1) were detected on marrow or peripheral fat cells. On the

other hand, the ST10 antigen, common to adipocyte-like cells cultured from both marrow and peripheral fat, was found on cultured marrow macrophages, but not on fibroblasts. An intriguing possibility could be that the undefined flat "ghost" cell with the ST10+/ST11+/BN(MB)35-/ST3- phenotype, observed only in cultures permissive to adipogenesis and in proximity to foci of fat cell growth, might be a transitional form.

METHODS

MARROW CULTURE AND ANTIBODY PRODUCTION

To produce antibodies ST2, ST3, and ST4, bone marrow was obtained from 200 to 300 gm Brown-Norway rats. The animals were sacrificed by overdose of inhaled ethyl ether, femora and tibiae were removed from both legs, the marrow was flushed with 30 ml of RPMI 1640 medium, and the cavity was curetted with an 18-gauge needle. The resulting cell suspension was distributed into four T75 flasks (Corning #25110-75 through Fisher Scientific, Montreal, Quebec), each containing 50 ml of culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin). After 72 hours, the flasks were gently agitated, the nonadherent cells were decanted, and on day 7, the adherent cells from a single T75 flask were removed by scraping. This total cell population was injected intravenously into the lateral tail vein of a Balb/c mouse. After a single boost 10 to 14 days later with an identical cell preparation, the mice were sacrificed by cervical dislocation, the spleen removed, and the lymphocytes hybridized to SP2-0 murine myeloma cells. Monoclonal antibodies were produced according to standard techniques (32), and the resulting hybrids were screened for antibody production by indirect immunofluorescence using cells prepared as described above. Antibody ST2 was selected for reactivity with macrophage-like cells, ST3 for staining of the predominant larger cell (*i.e.*, fibroblastoid) population, and ST4 for strong reactivity with large cells found in very low frequency (less than 1/100). Subsequent assays were performed with ascitic fluid produced in mice primed with pristane (Aldrich Chemical Company, Milwaukee, Wisconsin), 10 days before intraperitoneal injection of thrice-cloned hybridoma cells.

Antibodies ST10 and ST11 were derived by similar techniques using as the immunogen adherent marrow cells prepared according to the method described above, except that they had been cultured in the presence of RPMI 1640 medium, 10% FCS, 10% horse serum HS, and 10^{-5} M HC (#H-4001 Sigma Chemical Company, St. Louis, Missouri). In these cultures, the nonadherent cells were decanted on days 3 and 7, and the adherent cells were incubated in fresh medium until the fourth week, at which time many islands of cells had appeared containing large multilocular globules stainable by oil red O. (These will be called "fat cells" for convenience, acknowledging the controversy; see discussion),. The resulting supernatants were assessed for activity by immunostaining of 96 well culture dishes containing fat cell islands (AEC technique described below for slides), and inspecting with an inverted microscope. Whereas antibody ST10 was selected for its reactivity with cells in fat islands, ST11 was selected for its staining of nonfibroblastoid cells that lacked lipid globules, in proximity to the fat cells.

Antibody BN(MB)35, previously described (53), was obtained by standard monoclonal techniques after immunizing with the BNML-RS promyelocytic cell line as immunogen (19). The Ox 1 antibody to leukocyte common antigen, and Ox 7 to rat Thy-1.1 antigen were gifts from Dr. A. Williams, Sir William Dunn School of Pathology, University of Oxford.

HISTOLOGIC METHODS AND IMMUNOLOGIC ASSAYS

To visualize antigens on different cell types, cells were grown in culture chambers mounted on plastic microscope slides (Lab-Tek 4 chambered tissue culture chamber/slides, #4804, Miles Scientific, Rexdale, Ontario). These cultures were started either directly from marrow, or after transfer from flasks manipulated as described above. For staining, the media were removed from the chambers, and replaced by cold phosphate-buffered saline (PBS)/azide (0.8 mM). After 30 minutes at ice temperature, the PBS was replaced by the antibody solution at a predetermined dilution (RPMI 1640 medium with 5% FCS/0.8 mM azide), and allowed to sit for a further 30 minutes on ice. This was followed by 15 to 20 minutes incubation with PBS/0.1% gelatin/azide and two rinses with cold PBS/azide. The slides were then fixed with cold 100% methanol for 15 minutes on ice, and rinsed once with gelatin/PBS and twice with PBS/azide. The second antibody (affinity purified, peroxidase-labeled goat anti-mouse IgG + IgM, heavy and light chains, #041809 Kirkegaard Perry through Mandel Scientific Co., Rockwood, Ontario) in PBS/azide, at a pretitred concentration, was added for 30 minutes. After three washes with azide-free PBS, AEC was added for 40 minutes at room temperature; the slides were rinsed once in PBS, counterstained with Mayer's hematoxylin for 30 minutes, rinsed with warm tap water, and mounted in PBS/glycerol (30% in 2 mM azide) (adapted from 18).

In the experiments with fat cells, marrow was plated into the chamber slides from fat cell cultures grown as described above. After detachment by trypsin (0.05%/0.53 mM EDTA, Gibco #610-5400), cells grown to 3/4 confluence were transferred from T75 flasks into eight wells of two four-chambered slides. When fat cell areas were prominent, usually in approximately four to seven days, the slides were prepared as described above.

For analysis of antigens in frozen tissue sections, animals were sacrificed, selected organs were removed immediately, and pieces placed on blocks and frozen in OCT matrix (Ames Division, Miles Laboratories, Elkhart, Indiana). Once sectioned and mounted on slides, they were stored at −20° C until use, usually within 1 to 8 weeks. After removal from the freezer, the slides were placed in cold 100% acetone at 4° C for 10 minutes, and air dried at room temperature for at least 1 hour. The first antibody, diluted in RPMI 1640 medium/5% FCS, was applied and incubated for 18 hours in a humidified airtight container. After decanting, the slides were incubated 30 minutes with PBS/0.1% gelatin, and then washed twice with PBS for 5 minutes each. The second antibody, biotin-conjugated sheep anti-mouse IgG (#RPN.1061, Amersham Canada Ltd., Oakville, Ontario), diluted in PBS/0.1% BSA, was applied to the slides for two h, and then removed by washing twice in PBS/gelatin. The process was completed by the addition of preformed streptavidin-biotinylated β-galactosidase complex (#RPN.1053, Amersham) in PBS/0.1% BSA for 45 min, and color developed overnight with substrate BCIG (5-bromo-4-chloro-3-indoyl-galactoside, Sigma # B425), according to supplier's protocol (Amersham, "Protocol for use with Biotin-streptavidin system"). After rinsing with PBS, the slides were counterstained for 1 min with safranin O, gently washed with tap water, air dried, and mounted in Permount (Fisher Scientific, #50-P-15).

Histochemical assay for α-naphthyl acetate esterase was according to the method accompanying the reagent kit (Histozyme Kit No. 90-A1 Sigma).

To allow an estimate of the quantity of each antigen studied on cells cultured from explants of different organs, fibroblastic cells were replated from flasks into 96 well culture dishes. Cells transferred from two T75 flasks at 3/4 confluence resulted in 3/4 confluence in wells by 12–16 days. Cells from the epididymal fat stroma preparation (see below) were plated directly

into 96-well dishes, without intermediate passage in flasks. At time of examination, the media were aspirated, wells washed thrice with PBS, and the cells fixed in 0.025% glutaraldehyde in PBS for 10 min. After washing, the cells were prepared and analyzed by ELISA assay according to standard methods, modified as previously described (18). Since wells did not contain an equal number of cells, the results are expressed as a frequency distribution.

For flow cytometric analysis, the marrow was sedimented over Percoll (1.095 gm/ml, Pharmacia) to remove erythrocytes, and processed as described (18).

Colony assays

Marrow was obtained from 2- to 3-month-old rats as described above and prepared for enumeration of colony forming units (CFU-C) by an adaptation of standard techniques (17). Briefly, a cell suspension (3-4.5 × 10^6 /ml), prepared in 10% FCS, was mixed with 0.8% methyl cellulose to give a final concentration of 1-1.5 × 10^5 cells/ml, 30% FCS, 5 × 10^{-5} M 2-mercaptoethanol, 5% spleen cell conditioned medium in Iscove's medium. Cells were plated into 35 mm Petri dishes and, after 7 days culture in 5% CO2 at 37° C, the colonies were counted using an inverted microscope (colony defined as >50 cells). Spleen cell conditioned medium was prepared as described by Johnson and Metcalf (30), except that phytohemagglutinin (PHA-L Pure, E.Y Labs, San Mateo, California) was used as stimulator. The CFU-E were measured by culturing bone marrow in media containing 20% FCS/1% BSA with a final concentration of 1 unit/ml erythropoietin (step III, purified from sheep; Connaught Laboratories, Willowdale, Ontario; #1501-5-7); the colonies were counted on day 4.

Fibroblast colonies (CFU-F), were measured according to the method of Castro-Malaspina, as modified by McIntyre and Bjornson (41). Briefly, marrow cell suspensions were adjusted to a concentration of 5-10 × 10^5 /4 ml in RPMI 1640 medium containing 5% rat platelet-rich plasma/1% BSA, and cultured at 37° C in 60-mm polystyrene Petri dishes. After 10 days, the medium was decanted, the cells were visualized by Wright's stain, and the colonies were counted using an inverted microscope; aggregates of >50 cells were enumerated as a colony. Platelet-rich plasma was prepared from rat blood by the following steps: cardiac puncture, removal of leukocytes by sedimentation at 150 × *g*, freezing at −20° C and thawing once, clearance of large debris by sedimentation at 1000 × *g*, and final filter sterilization.

In experiments where the effect of antibodies on clonogenic precursors was evaluated, aliquots of marrow were first incubated for 30 minutes with diluted antibody, and then with a source of rabbit complement (#CL 3331, Low-Tox-H, Cedarlane Laboratories, Hornby, Ont., from a lot chosen for low background toxicity) for a further 45 to 60 minutes before being washed and prepared for CFU-C or CFU-F assay. Each time, control incubations with a cell line and a reactive antibody were performed in parallel to verify complement activity.

Preparation of Epididymal Fat Cells

Rats were killed and the epididymal fat pads were exposed, excised, and the stroma prepared as described by Bjorntorp *et al* (6). Briefly, after treatment with 150 units/ml collagenase (collagenase, crude Type I #LS04196, Cooper Biomedical, Malvern, Pennsylvania), the mature adipocytes were removed by flotation and the sedimented stromal fraction was plated into flasks, and cultured as described for the growth of marrow fat cells. Lipid-laden cells were abundant within 21 to 28 days.

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Address reprint requests to: Dr. A.K. Sullivan, Rm. 714, McGill Cancer Centre, 3655 Drummond St., Montreal, Quebec, Canada, H3G 1Y6.

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