Interleukin 1 Stimulates Granulocyte Macrophage Colony-stimulating Activity Release by Vascular Endothelial Cells

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Abstract

Studies designed to characterize monocyte-derived recruiting activity (MRA) a monokine that stimulates endothelial cells to produce granulocyte macrophage-colony-stimulating activity (CSA) by endothelial cells, show that it is a thermolabile protein of from 12,000 to 24,000 D which, on chromatofocusing, shows three separate peaks of eluted activity from pH 7.5 to 5.0. Because these and many other properties of MRA are identical to those of interleukin 1 (IL-1), we tested the hypothesis that MRA and IL-1 are identical. We cultured vascular endothelial cells with various concentrations of purified native and recombinant IL-1 (pI 7 form), then tested the endothelial cell supernatants for GM-CSA. Purified native IL-1 and recombinant IL-1 stimulated endothelial cells to release CSA. The MRA of native IL-1, recombinant IL-1, and unfractionated monocyte conditioned medium was neutralized by a highly specific rabbit anti-human IL-1 antiserum. Chromatofocusing fractions that contained MRA contained immunoreactive IL-1 on immunoblotting and the bioactivity was neutralized completely by treatment with the antiserum. We conclude that IL-1 induces the release of CSA by vascular endothelial cells, that IL-1 is constitutively produced by monocytes in vitro, and that MRA and IL-1 are biologically, biophysically and, immunologically identical.

Introduction

Granulocytes and monocytes are descendants of committed progenitor cells (CFU-GM),[1] which form granulocyte/macrophage colonies in semisolid media. The clonal growth of these progenitors depends upon a family of growth factors known as colony-stimulating activity (CSA) (1–3), the most well defined of which are GM-CSF (4) and G-CSF (5). CSA is produced by a variety of cell types including T lymphocytes (6–8), fibroblasts (9), and vascular endothelial cells (10–14), all of which are components of the hematopoietic microenvironment (14, 15).

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1. *Abbreviations used in this paper:* CSA, granulocyte/macrophage colony-stimulating activity; HPCM, human placental conditioned medium; HPLC, high performance liquid chromatography; IL-1, 2; interleukin 1 and 2; LAF, lymphocyte activating factor; LPS, lipopolysaccharide; MCM, monocyte-conditioned medium; MRA, monocyte-derived recruiting activity; PDGF, platelet-derived growth factor; PHA, phytohemagglutinin M; TNF-a, tumor necrosis factor alpha.

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The release of CSA and other hematopoietic growth factors by stromal cells is under the influence of a factor or factors from mononuclear phagocytes, which we have termed monocyte-derived recruiting activity (MRA). (6, 9, 13, 14, 16–18). For the past 2 yr we have carried out a series of studies designed to characterize and purify the MRA species that stimulate the release of CSA by vascular endothelial cells. In a recently published work we found that the production and release of MRA by mononuclear phagocytes in vitro requires serum, involves protein synthesis, is markedly enhanced by exposure of the monocytes to endotoxin, and that induced release is maximal at 24 h (19). The studies reported herein document that MRA is produced not only by peripheral blood monocytes but by a macrophage cell line (U937) and lung fibroblast cell line (IMR-90). In addition, MRA is sensitive to papain and leucine aminopeptidase, but resistant to neuraminidase, and is hydrophobic and thermolabile, with an estimated molecular mass of from 12,000 to 25,000 and with at least two (~7.0 and ~5.0) isoelectric points. Because these cellular and physical attributes are characteristic of the monokine known as interleukin 1 (IL-1) (20–29) we carried out studies in which endothelial cells were exposed to purified human monocyte-derived IL-1 (pI 7), or unfractionated monocyte conditioned medium in the presence and absence of a highly specific neutralizing anti-IL-1 antibody.

Methods

Experimental design. MRA assays were carried out in three steps (14, 19); (*a*) peripheral blood mononuclear phagocytes were cultured to obtain monocyte-conditioned media (MCM); (*b*) MCM were diluted serially and incubated for 3 d with endothelial cells in vitro; (*c*) colony-stimulating activity (CSA) titers were measured in the endothelial cell conditioned medium. All MCM samples were also assayed for CSA content (see below).

MCM. MCM was prepared according to methods previously described (9, 14, 16). Peripheral blood mononuclear phagocytes were prepared from heparinized peripheral blood obtained from normal consenting volunteers using Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) centrifugation. The low density cells were washed in RPMI 1640 twice and were adjusted to a concentration of 5×10^5/ml. 1 ml of suspension was pipetted into 35-mm petri dishes coated with lactoferrin-depleted fetal calf serum (FCS) (7, 12, 17) and were incubated at 37°C for 1 h. Nonadherent cells were removed by gentle washing. The cells remained on the dish for a period of 3 d in RPMI 1640 with 5% lactoferrin-depleted FCS. All incubations were carried out at 37°C in a fully humidified incubator set at 5% CO_2. MCM were assayed for MRA and/or IL-1.

Cell lines. 3-d cultures of $1–2 \times 10^5$ cells/ml of certain cell lines were used to generate conditioned media for MRA assay. In these cases, the conditioned media were serially diluted and assayed for MRA as above. As controls supernatant media were placed in "sham" culture dishes containing medium alone and cultured in parallel with the plates of endothelial cells for 3 d. Cell lines utilized in these studies included: U937 (American Tissue Culture Collection [ATCC]), IMR-90 (ATCC),

CCDS (ATCC), CCDC (ATCC), THP-1 (a gift from Dr. H. P. Koeffler, University of California at Los Angeles, UCLA), HL-60 (a gift of Dr. Robert Gallo, NIH, Bethesda, MD), and National Institutes of Health (NIH) 3T3 (ATCC).

Endothelial cell cultures. Multiply passaged confluent endothelial cell cultures were exposed to various conditioned media in the MRA assay. Human umbilical vein endothelial cells were prepared as previously described (14, 16, 19), using limited collagenase (type 1, 0.1% wt/vol in phosphate-buffered saline, PBS; Worthington Diagnostic Systems, Freehold, NJ) treatment. The cells were suspended in RPMI 1640 medium supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, 20% FCS antibiotics and 250 μg/ml endothelial cell growth factor extracted from bovine hypothalami (Pel-Freeze, Rogers, AR) (14). The endothelial cells were cultured in 16-mm tissue culture wells coated with fibronectin (2 μg/cm^2 surface area; Collaborative Research, Bethesda, MD) for 3 d at 37°C in 5% CO_2 in air (16, 19). The subcultured cells exhibited the characteristic "cobblestone" appearance of endothelial cells, and 90–98% of these cells contained Factor VIII–related antigen as determined by indirect immunofluorescence using rabbit anti–Factor VIII and fluorescein-labeled goat anti–rabbit IgG (Cappel Laboratories, Cochranville, PA). The cells did not react with monoclonal antibodies, OKM1, MAC 120, or OKT3 (16). In the experiments described below, endothelial cells from 4th to 15th passage were used. Cell density ranged from 1 to 5 $\times$ 10^4 cells/cm^2. The endothelial cell culture medium was replaced with MCM or dilutions thereof or sham-conditioned media diluted with medium and the cultures were returned to the incubator. Control wells contained the same dilutions of the same media but no endothelial cells. At the end of the 3-d incubation period, the medium was aspirated and centrifuged 200 *g* for 5 min and the supernatants were assayed for CSA on the day of harvest.

CSA assay. CSA assay was carried out as described previously (9, 14, 16, 19). Low-density bone marrow cells from normal volunteers were depleted of macrophages and T lymphocytes as previously described (7, 12, 14, 17). 10^5 cells were cultured in 1-ml vol of 0.9% methylcellulose in alpha medium supplemented with 15% heat-inactivated FCS. The methyl cellulose was layered on an agar gel (0.5% wt/vol in McCoy's 5A medium) to which had been added 10% vol/vol of the samples to be assayed. In each experiment a positive CSA control consisted of 10% vol/vol human placental conditioned medium prepared as previously described (7, 31). Colonies (aggregates of >39 cells) were counted on days 7–9 of culture. Representative colonies were plucked for morphological analysis using Wright's/Giemsa and alpha-naphthyl butyrate esterase stains (Sigma Chemical Co., St. Louis, MO).

The CSA assays were performed using several dilutions of the endothelial cell-conditioned medium. The CSA content of the sample was calculated from the linear portion of the dilution curve and, in accordance with convention (17, 32, 33), 1 U of CSA was defined as that amount resulting in the formation of one granulocyte/macrophage colony. Similarly, MRA content was calculated from the linear portion of the MRA dilution curve. 1 U of MRA is defined as that amount stimulating the release of 1 U of CSA (19).

IL-1 assays. Two separate assays for IL-1 were used in these studies and represented modifications of previously published techniques (30, 34).

Thymocyte proliferation assay. Thymocytes prepared from Balb/C mice (Simonson Laboratories, Gilroy, CA) were cultured at 10 $\times$ 10^6/ml with or without 1% PHA-M (Gibco, Grand Island, NY) in a final volume of 0.2 ml in flat-bottomed 96-well microplates containing various dilutions of test medium and control supernatants added in quadruplicate. The cultures were incubated for 72 h at 37°C and 5% CO_2. 6 h before harvest, the wells were pulsed with 0.1 Ci [^{3}H]thymidine (sp act 6.7 Ci/mmol; New England Nuclear, Boston, MA) and subsequently harvested with an automated sample harvester (Flow Laboratories, Rockville, MD). Samples were counted by liquid scintillation using a Tricarb beta counter.

IL-1–dependent production of IL-2. IL-1 assays were also performed using a cell line LBRM-33-1A5, which produces IL-2 in response to IL-1 and PHA-M (34). 100 μl of LBRM-33-1A5 cells (5 $\times$ 10^5 cells/ml) were cultured in 96-well flat-bottom plates in the presence of an equal volume of tissue culture medium alone, PHA test samples, or control samples at several dilutions, or both PHA and the test material. After 24 h of incubation at 37°C and 5% CO_2, 100 μl of supernatant from the plate with the LBRM cells was transferred from each well to a duplicate plate. 100 liters of CTLL-2 cells (4 $\times$ 10^4/ml) were added to each well of the duplicate plate. The microwell cultures were incubated for an additional 20 h followed by a 4-h pulse with 0.1 μCi of [^{3}H]thymidine (sp act 6.7 Ci/mmol). The thymidine-pulsed cultures were harvested onto glass fiber filter strips. Thymidine incorporation was measured by liquid scintillation counting. Units of IL-1 activity were determined using the reciprocal dilution of the sample that caused 50% maximum indicator cell line proliferation. 1 U of activity was thus equivalent to the number of microliters present in that culture that caused 50% of maximal proliferation (34).

Immunoblotting. 2-ml fractions from the chromatofocusing column (pH 9.6) were assayed both for MRA activity and IL-1 immunoreactivity by immunoblotting. 0.75-ml samples from adjacent 2-ml fractions were pooled and the total of 1.5 ml filtered through a nitrocellulose membrane by a slot blot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). 1, 10, and 50 ng/ml of recombinant IL-1 (see below) in FCS were filtered as positive controls through nitrocellulose. The membranes were floated in buffer containing bovine serum albumin (BSA) to block protein binding sites, rabbit anti–human IL-1 anti-serum was used as the primary antibody and was bound to the membrane by incubating for 30 min. The membrane was subsequently washed. An anti–rabbit IgG alkaline phosphatase conjugate (Promega Biotec, Madision, WI) was subsequently bound, the membrane was washed again and the color reaction developed with nitroblue tetrazolium in 70% dimethyl formamide, and 5-bromo-4-chloro-3-indolyl phosphate in dimethyl formamide as substrates for 1 h. The membranes were rinsed and photographed.

Treatment of MCM. In experiments designed to investigate the properties of MRA, MCM was subjected to a variety of treatments prior to its use in cultures of endothelial cells. These treatments included heating at 56°C for 15 min, 80°C for 5 min, and 100°C for 2 min; threefold freeze/thaw cycles (−20°C) over an 8-h period; membrane filtration through high and low binding 0.22 μm filters (high binding, Millex GS; low binding Millex GV, Millipore Corp., Bedford, MA); and multiple enzyme treatments (neuraminidase [1 U/mg], alkaline phosphatase [10 μg/ml, Sigma Chemical Co., St. Louis, MO], pronase [5 mg, Sigma Chemical Co.], proteinase K [5 mg/ml, EM Laboratories, Elmsford, NY], papain [10 μg/ml], leucine aminopeptidase, [5 μg/ml, Miles Laboratories, Elkhart, IN]). Enzyme treatments were carried out for 6 h at 37°C. Following each of the above treatments, the conditioned media were centrifuged 10,000 *g* for 1 h at 4°C, the supernatants removed and serially diluted. Serial dilutions were placed upon endothelial cell monolayers (see above).

MCM was dialyzed against phosphate-buffered saline (PBS) at 4°C for 3 d (four exchanges, 3,000 D, and 10,000–12,000-D cutoff membranes). The material remaining in the dialysis membranes was assayed for MRA on endothelial cells as above. MCM was further characterized biophysically by molecular-sieving, high performance liquid chromatography, and chromatofocusing.

High performance liquid chromatography. MCM was injected into a ^{125}I-protein column (0.8 $\times$ 30 cm, Waters Associates, Milford, MA) at 1,200 psi at a flow rate of 4 ml/min. Retention time and absorbance at 254 and 280 nm were recorded and compared with molecular weight standards (BSA [67,000], ovalbumin [45,000], trypsinogen [24,000], lysozyme [14,300]). Pooled samples were assayed for MRA and CSA.

Chromatofocusing. 25 ml of fresh MCM (50–200 mg protein) was concentrated to 2 ml by dialysis at room temperature in Spectropor 3 dialysis tubing (3,500-D cutoff) against 25% wt/vol polyethylene glycol (8,000 mol wt, Fisher Scientific, Santa Clara, CA). The concentrated MCM sample in the same tubing was then further dialyzed for 24 h against 0.025 M imidazole buffer (pH 7.4, 4°C, four exchanges). A poly-buffer exchanger column (Pharmacia Fine Chemicals) of 1 cm internal diameter and bed volume of 10 ml was equilibrated with 0.025 M imidazole (Sigma Chemical Co.) buffer at pH 7.4. The sample of MCM was applied to the column and was eluted with poly-buffer 74, diluted

1:8 with water and adjusted to pH 4.0. Fractions of 3 ml each were collected at 0.5 ml/min for 2–3 h. The pH of each fraction was measured and the fractions filtered through 0.22 μm low-binding filters. Aliquots of each fraction were applied to monolayers of endothelial cells and to sham cultures containing medium only.

Purified monocyte-derived IL-1 and anti-IL-1. IL-1 (pI 7 form) was purified from adherent human blood monocytes as previously described (30). The anti-IL-1 used in these studies was prepared from rabbits immunized with human monocyte-derived IL-1 as previously described (30, 31). For these studies, the antiserum had been adsorbed against human peripheral blood mononuclear cells for 4 d at 4°C and then precipitated with ammonium sulfate. The maximal inhibitory activity of this neutralizing immunoglobulin was identified by incubation of purified monocyte-derived IL-1 (1 U/ml) with the antibody for 16 h at 4°C, centrifugation at 2,000 *g* for 10 min, then utilizing the mixture (with the appropriate controls) in the lymphocyte activating factor (LAF) assays as previously described (35). The final concentrations of immunoglobulin in the LAF experiment included 0.5, 0.05, and 0.005% vol/vol. Because the 0.5% final concentration of antibody was optimally inhibitory in the LAF assay (see Fig. 2), all subsequent antibody neutralization studies for MRA analyses were performed using this dilution. Control studies were performed to test the effect of the antibody alone at this concentration on the proliferation of T cells, the production of IL-2, the release of CSA, and the proliferation of normal human bone marrow cells. No endotoxin contamination of MCM or ECM was detectable using the limulus amebocyte assay (Sigma Chemical Co.). Recombinant IL-1 contained 40 pg endotoxin/mg IL-1.

Recombinant IL-1. Recombinant human (pI 7) IL-1 was expressed in *Escherichia coli* from amino acid 112 to 269 of the IL-1 precursor (29). The IL-1 was extracted and purified to homogeneity as previously described (35). The purified material was suspended in PBS and 1% FCS. The FCS used contained no bioactive or immunoreactive native IL-1. Confluent endothelial cells in RPMI with 10% ECS or cell-free medium were exposed to 1, 10, 50, and 100 μg/ml recombinant IL-1 for 3 d in vitro. The medium was harvested and tested in CSA assays as above.

Results

Of the cell lines tested for MRA activity, only two, U937 (a line of human monocyte-like cells) (36) and IMR-90 cells (human lung fibroblasts) produced detectable amounts. As shown in Table I, lines of other fibroblastoid cells failed to produce IL-1 as did the human monocytic leukemia cell line THP-1 and the human progranulocytic leukemia cell line HL-60. Neither of these latter two cell lines was induced to differentiate in vitro when studied. All of the cell lines were studied with and without exposure to lipopolysaccharide (LPS). The cell lines failing to constitutively produce MRA also failed to release MRA following stimulation with LPS. LPS enhanced MRA produced by the U937 cell line both temporally and quantitatively (data not shown). Colonial cell morphology included neutrophils and mononuclear phagocytes. No eosinophil colonies were noted when cell line conditioned media or MCM were used to stimulate CSA release by endothelial cells.

*Table I. Cell Line Sources of MRA**

Cell type	CSA	MRA
	μm/ml	*μm/ml (±SD)*
U937	7±2	1,800±380
IMR-90	0	4,060±500
CCDS	0	0
NIH-3T3	0	0
THP-1‡	0	0
HL-60‡	0	0

0, none detected.
* MRA detected ("+") in supernatants of 3-d cultures of 10^5 cells/ml.
‡ Uninduced.

Table II. Effects of Heat, Freeze Thawing, Membrane Filtration, and Enzyme Treatments of MRA

Treatment	Residual MRA activity*
	% control
Heat	
56°C, 15 min	100±6
80°C, 5 min	38±4
100°C, 2 min	0
Freeze/thaw ×3 in 8 h	33±14
Membrane filtration	
0.22 μm, Millipore (Millex GS) 22°C	34±16
0.22 μM, Millipore (Millex GV) 22°C	80±21
Enzyme‡	
Neuraminidase	100±14
Alkaline phosphatase	99±4
Papain	43±29
Leucine aminopeptidase	44±8

* Mean value for three experiments±SD. (100% MRA activity in three experiments: 3,840 and 6,080, and 3,040 U/ml)
‡ Enzyme treatments were each for 6 h at 37°C.

Table II summarizes the effects of heat, freezing and thawing, membrane filtration, and enzyme treatments of MCM on MRA activity. Substantial reductions of MRA activity were noted after heating for 5 min at 80°C and after freezing and thawing thrice in 8 h. Complete inactivation of MRA activity was noted after treatment at 100°C but MRA resisted heating at 56°C for 15 min. One filtration of MCM at 22°C with a Millex (GS) filter resulted in a 66% reduction in MRA activity but filtration of MCM through a "low-binding" hydrophobic membrane (Millex GV) resulted in only 20% loss of MRA activity. In the enzyme-treatment studies, neither alkaline phosphatase nor neuraminidase treatment altered the MRA titer of MCM. Pronase and proteinase K treatment, however, induced endothelial cell damage as detected morphologically and with trypan blue staining (>50% trypan blue positive cells, data not shown). Moreover, control studies detected substantial degradation of CSF using these two enzymes so that results of the MCM treatment could not be evaluated. Such was not the case, however for papain and leucine aminopeptidase, both of which were partially inhibitory (57 and 56%, respectively).

High performance liquid chromatography studies, a representative example of which is shown in Fig. 1, showed elution of activity in the fractions ranging from 12 to 24,000 D. Minor peaks of activity were also observed at lower molecular weight ranges in some experiments. Five separate studies of MCM using chromatofocusing columns with a pH range of 7.5 to 4.0 consistently showed three peaks of activity between pH 7.0 and 5.0 (Fig. 2). The major peak of activity ranged from 6.9 to 7.2. Minor peaks of activity were seen at pI 5.0–5.5 and 5.2–6.2. Often a fourth peak at pH 4.3 to 4.7 was also seen. This peak is not shown in Fig. 2.

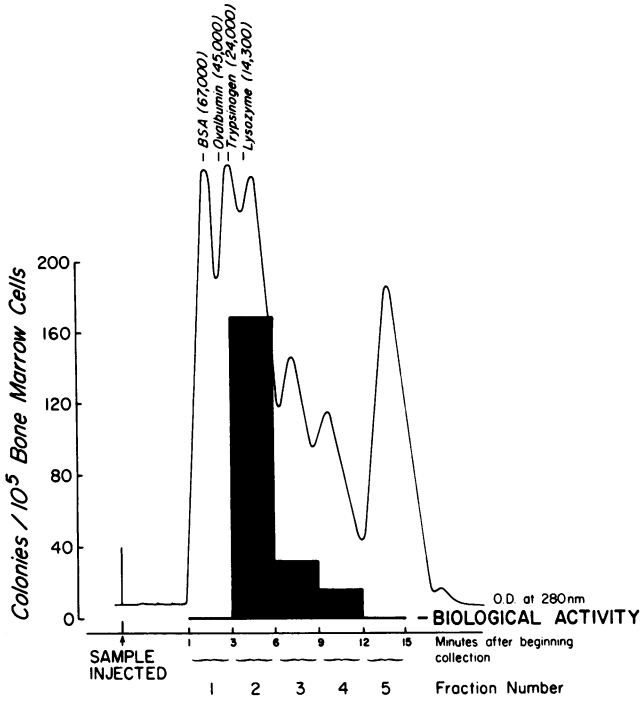

Figure 1. Estimated molecular weight of MRA. MCM prepared in 2.5% FCS was injected into a Waters ^{125}I column (0.8 × 30 cm) at 1,200 psi and at a flow rate of 4 ml/min. In this experiment, five fractions were collected every 3 min and each of the fractions was analyzed for MRA and CSA content. The unbroken curve represents continuous OD 280 of MCM. Molecular weight standards are shown. Each fraction was diluted 1:1 with complete (RPMI) medium with 5% FCS, then was used to completely replace medium in which confluent endothelial cells had been cultured. After 3 d of culture, the endothelial cell–conditioned media, along with appropriate controls containing sham-conditioned medium, were analyzed for CSA content after having been diluted 1:10 in complete medium. CSA content of 10% endothelial cell–conditioned media is expressed as colonies/10^5 auxiliary-cell-depleted bone marrow cells. Each bar represents the mean of four replicate plates. The fraction of MCM with the majority of the MRA corresponded to a molecular mass range of 12,000 to 24,000.

Serial dilutions of MCM were either placed on endothelial cells for 3-d cultures prior to MRA assays or directly analyzed for IL-1 content. As shown in Fig. 3, the activity of these serially diluted samples in both assays were comparable. The half maximal activity in both assays was detected at concentrations of 10% MCM. The half maximal activity in the thymocyte assay was higher (20%).

Virtually all of the biological and biophysical characteristics described above for MRA are shared by IL-1 (20–30, 37–39). Therefore, we carried out studies designed to test the hypothesis that MRA and IL-1 are identical molecules. We noted that on immunoblot analysis of the chromatofocused MCM only the active fraction contained immunoreactive IL-1 (Fig. 4). To identify the appropriate dose of antibody for MRA neutralization studies, we serially diluted the IL-1 antibody (see Methods) and analyzed its ability to neutralize the LAF activity of monocyte-derived IL-1 (1 U/ml). The results are shown in Fig. 5 and indicate that a 1:100 dilution of IL-1 antibody neutralized >95% of the IL-1 activity. Accordingly, all further studies using this antibody in the treatment of MCM for MRA and LAF assays were carried out using this dilution of the antibody. 16 experiments were performed in which multiple doses of purified monocyte-derived IL-1 were treated with the rabbit immunoglobulin or control sera, then exposed to endothelial cells or incubated in sham cultures which were analyzed for CSA content 3 d later. These dose-response curves were compared to similarly treated unfractionated MCM at two dilutions. The results are shown in Fig. 6. IL-1 induced the release of CSA from endothelial cells. The half-maximal activity of IL-1 in this assay was 10 U/ml (Fig. 6 *a*). This activity was completely neutralized by treatment with the antibody in vitro (Fig. 6 *a, B*). IL-1 had no CSA activity in sham culture experiments (Fig. 6 *a, C*). MCM, which also lacked detectable CSA activity (Fig. 6 *a, C*), was potent in stimulating CSA release. Marked reduction in CSA release was noted when antibody-treated MCM was used in endothelial cell cultures (Fig. 6 *b, B*). The antibody neither induced CSA release nor neutralized CSF activity in control studies in which human placental-conditioned medium (HPCM) was incubated with the antibody (not shown). The antibody did not inhibit granulocyte colony growth in control studies using HPCM as a stimulator (data not shown).

Finally, as shown in Fig. 7, recombinant (pI 7) IL-1 stimulated the release of CSA by endothelial cells but was incapable of directly inducing GM colony growth. As is the case with MRA, there was a direct relationship between IL-1 dose and CSA released.

Discussion

Not long after the semisolid culture systems for clonal proliferation of hematopoietic cells were developed (40, 41) it became

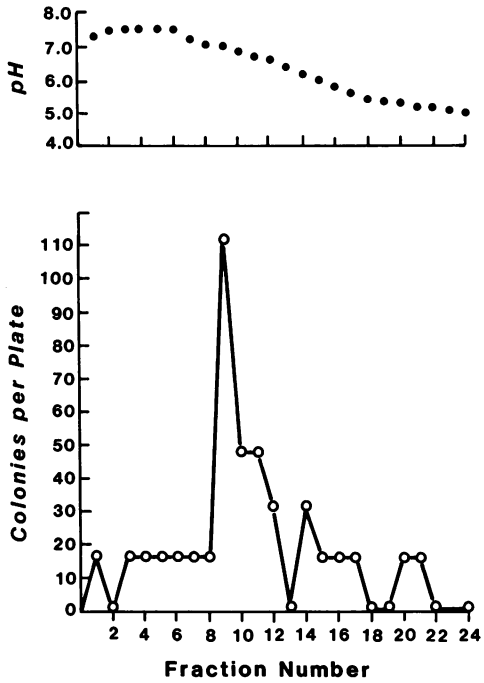

Figure 2. Chromatofocusing of MCM. 12-fold concentrated MCM was dialyzed against 0.025 M imidazole buffer (pH 7.4, 4°C) and was then applied to a poly-buffer exchanger column. The sample was eluted with poly-buffer 74 that had been diluted 1:9 with water and adjusted to pH 4.0. Each 3-ml fraction was analyzed for both MRA and CSA content. In this representative one of five experiments, the upper curve represents the pH of the eluted fraction. No CSA was detected between pH 7.5 and 5.0. MRA eluted in four peaks in this range. The major peak was found at pH 7.0.

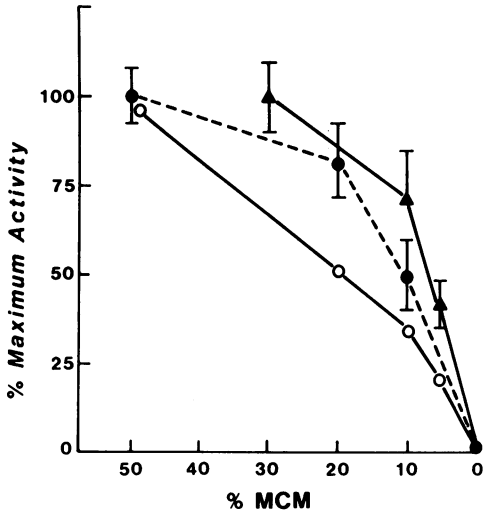

Figure 3. Serial dilutions of MCM in IL-1 and MRA assays. Dilutions of MCM shown (x axis) represent the final concentration of MCM in endothelial cell cultures (MRA assay) or in LAF assays (IL-1). MRA (U CSA/ml) and IL-1 content (cpm [^{3}H]thymidine incorporated) are expressed as percent maximal stimulation. The dilution curve for MRA (mean [CSA] in three experiments±SD) is shown by the dashed line. Four separate experiments were done to define the dilution curve for IL-1 (——, n = 3): three (▲, n = 3) utilized the LBRM assay (half maximal activity 5–10%), one (○, n = 1), the murine thymocyte assay.

clear that adherent cells, in particular mononuclear phagocytes, while incapable of forming colonies themselves, played an important "accessory" role in the formation of granulocyte/macrophage colonies in vitro. Initially, mononuclear phagocytes were thought to provide a humoral factor, CSA, which stimulated the proliferation of the granulocyte/macrophage colony-forming unit (CFU-GM) (42, 43). Subsequent studies from our own laboratory and, more recently, other groups (13), have demonstrated that monocytes require accessory cells to exert their colony-stimulating effects. Specifically, we have shown that mononuclear phagocytes produce and release into culture medium, a factor or factors, which we have operationally termed MRA. MRA stimulates endothelial cells and fibroblasts to release CSA (9, 14, 19) and enhances CSA production by T lymphocytes (6). Macrophages are also important auxiliary cells in clonal growth of other committed hematopoietic progenitor cells as well. For example, Zuckerman reported that erythroid burst growth depended on monocytes in vitro (44), results that were later confirmed by Levitt et al. (45). Recently, one mechanism by which monocytes effect their accessory role in other hematopoietic lineages has been described and resembles the effects of monocytes on granulopoiesis. Specifically, three collaborating groups, including our own, have shown that monocyte culture supernatants induce the release of factors into endothelial cell–conditioned medium, which stimulates the clonal proliferation of erythroid burst forming units (16, 17), megakaryocytic colony forming units, and multipotent colony forming units (18). Because macrophages, fibroblasts, endothelial cells, and colony forming cells are each components of the hematopoietic marrow, we subscribe to the point of view that multilineage hematopoiesis in hematopoietic microenvironments might be under the regulatory control of mononuclear phagocytes.

For the past two years we have carried out a series of studies designed to characterize and purify these monokines. We began with the granulopoietic regulatory monokine using the endothelial cell CSA assay (see Methods) and in recently reported studies found that MRA production by monocytes requires serum in the culture medium, declines after treatment with protein synthesis inhibitors, and is substantially enhanced both quantitatively and temporally by exposure of the monocytes to LPS; the maximal induced secretory response occurring at 24 h (17 [Table I]). Therefore, MRA could represent a novel monokine or a novel activity of a previously reported monokine with the above attributes. Such monokines include (*a*) tumor necrosis factor alpha (TNF-a) (46–48), (*b*) platelet-derived growth factor (PDGF) (49), and (*c*) IL-1 (20, 21).

Further studies suggested that MRA shared more biological similarities with IL-1 than with PDGF or TNF-a. The pattern of cellular sources is more compatible with that reported for IL-1 (20, 26, 27) than with TNF (not constitutively produced by U937 cells [50]) or PDGF (not constitutively produced by monocytes in vitro [49]). More importantly, PDGF (28,000–32,000 D [51]) has a higher molecular mass, higher pI (10 [52]) and is markedly thermostable (49). TNF (17,000 D [46, 53]) has a pI of 5.3 (47) and is more thermostable than IL-1 (50). Taken together, these studies indicated that of the three known monokines, IL-1 shared the greatest number of biological and biophysical features with MRA. Other attributes of MRA shared with IL-1 are its thermolability, sensitivity to freezing and thawing (28), neuraminidase resistance and papain sensitivity (20, 22, 25), complex chromatofocusing profile (30) and its tendency to bind to nonhydrophobic filtration membranes (21). For these reasons, we carried out a series of experiments designed to determine with certainty whether IL-1 exhibited MRA.

First, similar MCM dose-response curves were described in both MRA and IL-1 assays (Fig. 3). We next used purified monocyte-derived IL-1 and recombinant (pI 7) IL-1 in our MRA assay system. We found that these IL-1's induced the release of CSA from multiply subcultured human umbilical vein endothelial cells (Figs. 6 and 7), and that treatment of both monocyte-

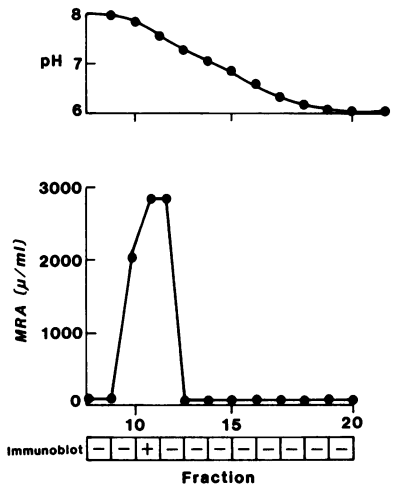

Figure 4. MCM was prepared as in Fig. 2 and was applied to a polybuffer exchanger column with a range of pH 9 to 6. Each 2-ml fraction was analyzed for MRA prior to and after 18 h exposure to the anti-IL-1 antibody. 1.5-ml pools of two adjacent fractions were filtered through nitrocellulose for immunoblotting. Immunoreactive material is denoted "+." Nonreactive pools are marked "−." The pool of two fractions both positive in MRA assays was also positive for IL-1 on immunoblotting.

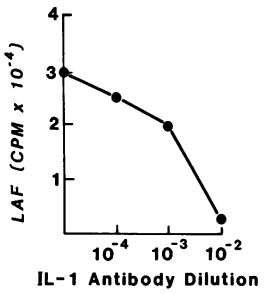

Figure 5. The IL-1 neutralizing effect of the rabbit anti-human IL-1 immunoglobulin fraction. 1 U/ml of purified monocyte-derived IL-1 was incubated with several dilutions of the antibody for 16 h at 4°C. The mixture was centrifuged, the supernatant was diluted 1:1 with complete medium, and was then used in the LAF assay (see Methods). The IL-1 activity is expressed as the mean [^{3}H]TdR incorporation (cpm) in three replicate wells.

derived purified IL-1 and unfractionated MCM with a neutralizing rabbit anti-human IL-1 immunoglobulin (30) abrogated this effect (Fig. 6). This antibody also recognized the active fractions from a chromatofocusing column by immunoblot analysis. The half maximal activity of purified IL-1 in MRA assays (10 U/ml [see Fig. 6]) was identical to its activity in LAF assays (30). Both IL-1 and MCM failed to stimulate colony growth by auxiliary cell depleted normal marrow (Fig. 6) (6, 9, 14, 19). In most experiments the treatment of a 1:10 dilution of MCM with the anti-IL-1 immunoglobulin abrogated completely its MRA. The 1:2 dilutions of MCM retained some residual activity (Fig. 6). This finding is compatible with the point of view that the 1:2 dilution represented antigen excess. In support of this view are our findings (Fig. 6) that maximum colony growth was achieved in all eight experiments with this particular dose. An alternative interpretation is that IL-1 has MRA but a minor species of non-IL-1 MRA exists in MCM. Therefore, at this time we cannot state that all MRA in this particular assay system

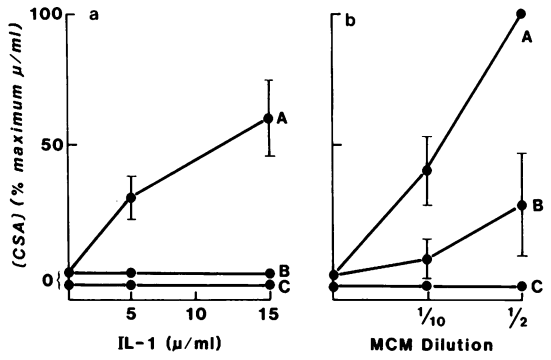

Figure 6. The effect of MCM and IL-1 on release of CSA by endothelial cells in vitro. Various doses of purified monocyte-derived IL-1 were assayed for MRA activity before (*a, A*) and after (*a, B*) treatment with the neutralizing antibody. These dilutions were also analyzed for CSA activity (*a, C*). IL-1 had no CSA activity but did stimulate the release of CSA from endothelial cells, an effect that was entirely abrogated by treatment of IL-1 with the neutralizing antibody. In parallel studies dilutions of MCM were analyzed for CSA activity (*b, C*), and MRA activity before (*b, A*) and after (*b, B*) treatment with the same dilution of the antibody. We found that MCM contained no detectable CSA but contained MRA, which in most experiments at the 1:10 dilution was completely neutralized by treatment with the antibody. There was substantial (70%) but incomplete neutralization of MRA activity in antibody-treated MCM at the 1:2 dilution. Each point represents mean CSA release (±SD) in 16 separate experiments. Each of the two dilution points in both curves H are significantly different than the corresponding points of curves *B* ($P < 0.001$, Student's *t* test).

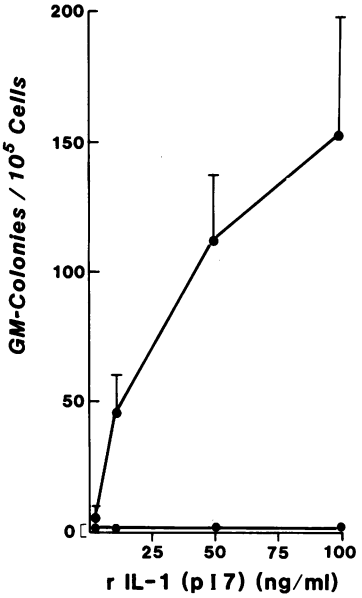

Figure 7. The effect of recombinant IL-1 (pI 7) on release of CSA by endothelial cells in vitro. This graph represents one of four experiments with similar results. As in Fig. 5, confluent endothelial cells were exposed to multiple doses of IL-1. 5% dilutions of endothelial cell conditioned media were added to marrow cell cultures in CSA assays. In no case did endothelial cells release detectable CSA in the absence of IL-1 (not shown). IL-1 induced the release of substantial quantities of CSA in all four cases (upper curve). That CSA was derived from endothelial cells was confirmed in sham cultures of IL-1 that indicated that even at 100 μg/ml IL-1 has no detectable CSA (lower curve). Data points shown represent mean colonies/10^5 cells in quadruplicate cultures±SD. Each point on the upper curve 10 μg/ml and above is significantly different ($P < 10^{-6}$) than control sham cultures.

is IL-1. Nor can we yet propose that the MRAs that induce the production by endothelial cells of other hematopoietic growth factors (16, 18) are also IL-1.

Nonetheless, we have shown that the biological effects, cellular sources and biophysical properties (including molecular weight estimates and isoelectric points) of the activities we have termed MRA are identical to those of IL-1. In addition we find that both purified monocyte-derived IL-1 and recombinant IL-1 induce CSA release by endothelial cells in vitro and that the majority, if not all, MRA in MCM is neutralized by a highly specific neutralizing IL-1 antibody. Taken together, our results indicate that what we originally described as MRA represents a novel hematopoietic activity of IL-1. In view of these results, and the recently reported observation that IL-1 stimulates release of CSA by a cell line of human fibroblasts (54), the known neutrophilia-inducing effects of IL-1 in the whole animal (20, 40), and the knowledge that both macrophages and IL-1–responsive stromal cells are components of the hematopoietic microenvironment, we propose that IL-1 may represent an important mechanism by which granulopoiesis is regulated in vivo.

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References

1. Burgess, A. W., and D. Metcalf. 1985. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood.* 56:947–958.

2. Stanley, E. R. 1981. Colony stimulating factors. *In* The Lymphokines. W. E. Stewart and J. W. Hadden, editors. Humana Press, Clifton, NJ. 101–132.

3. Das, S. K., E. R. Stanley, L. J. Guilbert, and L. W. Forman. 1981. Human colony stimulating factor (CSF-1) radioimmunoassay. Resolution

of the subclasses of human colony-stimulating factors. *Blood.* 58:630–641.

4. Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, D. P. Luxenberg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang, and S. C. Clark. 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science (Wash. DC).* 228:810–815.

5. Souza, L. M., T. C. Boone, J. Gabrilove, P. H. Lai, K. Zsebo, D. C. Murdock, V. R. Chazin, J. Bruszewski, H. Lu, K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelsmann, and K. Welte. 1986. Recombinant human granulocyte colony stimulating factor: effects on normal and leukemic myeloid cells. *Science (Wash. DC).* 232:61–65.

6. Bagby, G. C., V. D. Rigas, R. M. Bennett, A. A. Vandenbark, and H. S. Garewal. 1981. Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. *J. Clin. Invest.* 68:56–63.

7. Ruscetti, F. W., and P. A. Chervenick. 1975. Release of colony stimulating activity from thymus-derived lymphocytes. *J. Clin. Invest.* 55:520–527.

8. Cline, M. J., and D. W. Golde. 1974. Production of colony stimulating activity by human lymphocytes. *Nature (Lond.).* 248:703–704.

9. Bagby, G. C., E. McCall, and D. L. Layman. 1983. Regulation of colony stimulating activity production. Interactions of fibroblasts, mononuclear phagocytes and lactoferrin. *J. Clin. Invest.* 71:340–344.

10. Knudtzon, S., and B. T. Mortenson. 1975. Growth stimulation of human bone marrow cells in agar culture by vascular cells. *Blood.* 46: 937–943.

11. Quesenberry, P. J., and M. A. Gimbrone. 1980. Vascular endothelium as a regulator of granulopoiesis. Production of colony-stimulating activity by cultured human endothelial cells. *Blood.* 56:1060–1067.

12. Ascensao, J. L., G. M. Vercellotti, H. S. Jacob, and E. D. Zanjani. 1984. Role of endothelial cells in human hematopoiesis: modulation of mixed colony growth in vitro. *Blood.* 63:553–558.

13. Gerson, S. L., H. M. Friedman, and D. B. Cines. 1985. Viral infection of vascular endothelial cells alters production of colony-stimulating activity. *J. Clin. Invest.* 76:1382–1390.

14. Bagby, G. C., E. McCall, K. A. Bergstrom, and D. Burger. 1983. A monokine regulates colony-stimulating activity production by vascular endothelial cells. *Blood.* 62:663–668.

15. Keating, A., J. W. Singer, P. D. Killen, G. E. Striker, A. C. Salo, J. Sanders, E. D. Thomas, D. Thorning, and P. J. Fialkow. 1982. Donor origin of the in vitro haematopoietic microenvironment after marrow transplantation in man. *Nature (Lond.).* 298:280–282.

16. Zuckerman, K. S., G. C. Bagby, E. McCall, B. Sparks, J. Wells, and V. Patel. 1985. A monokine stimulates production of human erythroid burst promoting activity by endothelial cells in vitro. *J. Clin. Invest.* 75:722–725.

17. Broudy, V., K. Zuckerman, S. Jetmalani, J. H. Fitchen, and G. C. Bagby. 1984. The production of burst promoting activity (BPA) and colony stimulating activity (CSA) by adherent fibroblastoid marrow cells (F) is regulated by autologous monocytes. *Clin. Res.* 32:305a. (Abstr.)

18. Segal, G. M., E. McCall, T. Stueve, and G. C. Bagby. 1985. Monokine stimulated endothelial cells promote human megakaryocytic and mixed cell colony growth in vitro. *J. Immunol.* (In press).

19. McCall, E., and G. C. Bagby. 1985. Monocyte-derived recruiting activity. Kinetics of production and effects of endotoxin. *Blood.* 65:689–695.

20. Dinarello, C. A. 1984. Interleukin-1. *Rev. Infect. Dis.* 6:51–95.

21. Lachman, L. B. 1983. Human interleukin-1: Purification and properties. *Fed. Proc.* 42:2639–2645.

22. Wood, D. D. 1979. Purification and properties of human B cell activating factor. *J. Immunol.* 79:2395–2399.

23. Luger, T. A., B. M. Stadler, B. M. Luger, B. J. Mathieson, M. Mage, J. A. Schmidt, and J. J. Oppenheim. 1982. Murine epidermal cell-derived thymocyte-activating factor resembles murine interleukin-1. *J. Immunol.* 128:2147–2152.

24. Simon, P. L., and W. F. Willoughby. 1981. The role of subcellular factors in pulmonary immune function: physical-chemical characterization of two distinct species of lymphocyte-activating factor produced by rabbit alveolar macrophages. *J. Immunol.* 126:1534–1541.

25. Mizel, S. B. 1979. Physicochemical characterization of lymphocyte activating factor (LAF). *J. Immunol.* 122:2167–2172.

26. Amento, E. P., J. T. Kurnick, A. Epstein, and S. M. Crane. 1982. Modulation of synovial cell products by a factor from human cell line: T-lymphocyte induction of a mononuclear cell factor. *Proc. Natl. Acad. Sci. USA.* 79:5307–5311.

27. Mizel, S. B., and B. J. Andersen. 1983. Interleukin-1 production by a human monocytic leukemia cell line, THP-1. *In* Interleukins, Lymphokines and Cytokines: Proceedings of the Third International Lymphokine Workshop. J. J. Oppenheim and S. Cohen, editors. Academic Press, Orlando, FL. 401–407.

28. Cannon, J. G., and C. A. Dinarello. 1985. Increased plasma interleukin-1 activity in women after ovulation. *Science (Wash. DC).* 227: 1247–1249.

29. Gowen, M., D. D. Wood, and R. G. G. Russell. 1985. Stimulation of the proliferation of human bone cells in vitro by human monocyte products with interleukin-1 activity. *J. Clin. Invest.* 75:1223–1229.

30. Dinarello, C. A., H. A. Bernheim, J. G. Cannon, G. LoPreste, S. J. C. Warner, A. C. Webb, and P. E. Auron. 1985. Purified, ^{35}S-MET, ^{3}H-LEU-labelled human monocyte interleukin-1 (IL-1) with endogenous pyrogen activity. *Br. J. Rheumatol.* 24(Suppl):59–64.

31. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, S. M. Wolff, and C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* 81:7907–7911.

32. Schlunk, T., and M. Schleyer. 1980. The influence of culture conditions on the production of colony stimulating activity by human placenta. *Exp. Hematol.* 8:179–184.

33. Metcalf, D., H. R. MacDonald, and H. M. Chester. 1975. Serum potentiation of granulocyte and macrophage colony formation in vitro. *Exp. Hematol.* 3:261–270.

34. Wagner, C. R., R. M. Vetto, and D. R. Burger. 1985. Expression of I-region-associated antigen (Ia) and interleukin-1 by subcultured human endothelial cells. *Cell. Immunol.* 93:91–104.

35. Dinarello, C. A., J. G. Cannon, J. W. Mier, H. A. Bernheim, G. LoPreste, D. L. Lynn, R. N. Love, A. C. Webb, P. C. Auron, R. C. Reuben, A. Dick, S. M. Wolff, and S. D. Putney. 1986. Multiple biological activities of human recombinant interleukin-1. *J. Clin. Invest.* 77:1734–1739.

36. Sundstrom, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U937). *Int. J. Cancer.* 17:565–577.

37. Kaye, J., S. Gillis, S. B. Myzel, E. M. Sehevach, T. R. Malek, C. A. Dinarello, L. B. Lachman, and C. A. Janeway. 1982. Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin-1 is required for the expression of receptors for interleukin-2. *J. Immunol.* 133:1339–1345.

38. Duff, G. W., and E. Atkins. 1982. The inhibitory effect of polymyxin B on endotoxin-induced endogenous pyrogen production. *J. Immunol. Methods.* 52:333–340.

39. Oppenheim, J. J., B. M. Stadler, R. P. Siraganian, M. Mage, and B. Mathieson. 1982. Lymphokines: their role in lymphocyte responses. Properties of interleukin 1. *Fed. Proc.* 41:257–262.

40. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells in vitro. *Aust. J. Exp. Biol. Med. Sci.* 44:287–299.

41. Pluznik, D. H., and L. Sachs. 1965. The cloning of normal "mast" cells in tissue culture. *J. Cell. Comp. Physiol.* 66:319–324.

42. Golde, D. W., and M. J. Cline. 1972. Identification of colony-stimulating cells in human peripheral blood. *J. Clin. Invest.* 51:2981–2983.

43. Chervenick, P. A., and A. F. LoBuglio. 1972. Human blood monocytes, stimulators of granulocyte and mononuclear colony formation in vitro. *Science (Wash. DC).* 178:164–166.

44. Zuckerman, K. S. 1981. Human erythroid burst-forming units.

Growth in vitro is dependent on monocytes, but not T lymphocytes. *J. Clin. Invest.* 67:702–709.

45. Levitt, L., T. J. Kipps, E. G. Engleman, and P. L. Greenberg. 1985. Human bone marrow and peripheral blood T-lymphocyte depletion: Efficacy and effects of both T-cells and monocytes on growth of hematopoietic progenitors. *Blood.* 65:663–679.

46. Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, and G. E. Nedwin. 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.).* 312:724–729.

47. Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel, and R. N. Harkins. 1985. Human tumor necrosis factor: production, purification, and characterization. *J. Biol. Chem.* 260:2345–2354.

48. Mannel, D. M., R. N. Moore, and S. E. Mergenhagen. 1980. Generation and characterization of a lipopolysaccharide. Induced and serum derived cytotoxic factor for tumor cells. *Infect. Immun.* 28:204–211.

49. Martinet, Y., P. B. Bitterman, J-F. Mornex, G. R. Grotendorst, G. R. Martin, and R. G. Crystal. 1986. Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF like activity. *Nature (Lond.).* 319:158–160.

50. Ruff, M. R., and G. E. Gifford. 1981. Tumor Necrosis Factor. *Lymphokines.* 2:235–272.

51. Van Zoelen, E. J. J., W. J. M. Van de Ven, H. J. Franssen, T. M. J. Van Oostwaard, P. W. Van der Saag, C-H Heldin, and S. W. de Laat. 1985. Neuroblastoma cells express c-sis and produce a transforming growth factor antigenically related to the platelet-derived growth factor. *Mol. Cell. Biol.* 5:2289–2297.

52. Deuel, T. F., and J. S. Huang. 1984. Platelet derived growth factor, structure function, and roles in normal and transformed cells. *J. Clin. Invest.* 74:669–676.

53. Wang, A. M., A. A. Creasey, M. B. Ladner, L. S. Lin, J. Strickler, J. N. Van Arsdell, R. Yamamoto, and D. F. Mark. 1985. Molecular cloning of the complementary DNA for Human tumor necrosis factor. *Science (Wash. DC).* 228:149–154.

54. Zucali, J. R., C. A. Dinarello, D. J. Oblon, M. A. Gross, L. Anderson, and R. S. Weiner. 1986. Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony stimulating activity and prostaglandin E_2. *J. Clin. Invest.* 77:1857–1863.