Multiple Hemopoietic Lineages Are Found After Stimulation of Mouse Bone Marrow Precursor Cells With Interleukin 3

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When the murine T-lymphocyte clone L2 is stimulated with concanavalin A, it secretes at least two distinct factors that affect hemopoietic precursor cells, interleukin 3 (IL3) and granulocyte/macrophage colony-stimulating factor (GM-CSF). IL3 accounts for approximately 10% of the colony-stimulating activity in L2-cell-conditioned medium. The IL3 secreted by L2 cells is similar antigenically to the IL3 secreted by WEHI-3 cells. Like the IL3 from WEHI-3 cells, IL3 secreted by L2 cells does not bind to DEAE Sephacel and can be separated from the L2-cell GM-CSF, which does bind to DEAE. By assessment of the functional, morphologic, surface phenotypic, and cytochemical characteristics of bone marrow cells 6 days after stimulation with IL3 in liquid culture, four hemopoietic lineages were found, including macrophage, neutrophilic granulocyte, megakaryocyte, and basophil/mast cell. In addition, when bone marrow cells were stimulated with IL3 in semisolid medium, several types of colonies were found, including mixed colonies containing macrophage, megakaryocyte, and granulocyte lineages. (Am J Pathol 1984, 117:171-179)

SEVERAL TYPES of factors (colony-stimulating factor, CSF) affecting hemopoietic precursor cells have been described.[1,2] CSF-1, which is produced by fibroblasts, induces proliferation and differentiation along a single lineage, macrophage.[3] In contrast, factors derived from mitogen-stimulated spleen cells affect multiple hemopoietic lineages.[4] Since several transformed T cell lines[5-8] and T-lymphocyte clones[9-13] secrete large quantities of CSF, it is likely that T-lymphocytes represent a major source of spleen-cell-derived CSF. Conditioned medium obtained after lectin stimulation of the T-lymphocyte clone L2 contains at least two biologically and three biochemically distinct types of CSF.[14-16] The major CSF produced by L2 cells has been partially purified to a specific activity of 2–4 $\times$ 10^8 colonies/mg protein and induces granulocyte/macrophage (GM) colony formation.[16] Interleukin 3 is another potent inducer of colony formation (specific activity, 3 $\times$ 10^9 colonies/mg protein),[15] has erythroid burst promoting activity,[17] and represents a minor colony-stimulating activity found in L2-cell-conditioned medium. In this study the effects of homogeneous interleukin 3 (IL3) on hemopoietic precursor cells are described and compared with the effects of the L2-cell GM-CSF and L-cell CSF-1.

Materials and Methods

Animals

DBA/2 and CBA/J mice, 6–8 weeks of age, were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used for the maintenance of the T helper cell clone L2. CBA/J mice were used also for thymi-

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dine incorporation studies. BDF_1 mice used for the conventional colony-counting assay were purchased from Laboratory Supply, Indianapolis, Indiana.

T-Cell Clone

The derivation and maintenance of the T helper cell clone L2 has been described previously.[18,19] Briefly, L2 cells were maintained by weekly restimulation with irradiated alloantigen and secondary mixed lymphocyte culture supernatant in Dulbecco's modified Eagle's medium (DMEM, GIBCO H-21), containing 2% fetal calf serum (FCS, KC Biological, Inc., Lenexa, Kan), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and additional amino acids.[20] L2 cells (1×10^6/ml) were stimulated with concanavalin A (10 μg/ml), and the supernatant was harvested at 24 hours and fractionated with the use of ion-exchange chromatography. These supernatants contained multiple lymphokine activities and have been described in detail.[13]

Colony-Stimulating Factor Assay

A modification[11] of the method of Stanley et al[21] was used for measurement of the ability of culture supernatants to promote the growth of colonies derived from bone marrow cells. Triplicate cultures of BDF_1 bone marrow cells (7.5×10^4) suspended in 1 ml of a medium consisting of 85% alpha medium (No. 10-311, Flow Laboratories, Rockville, Md), 2% wt/vol bovine serum albumin (BSA, Leptalb-7, Armour Pharmaceutical Company, Kankakee, Ill), 10^{-7} M sodium selenite, 10^{-4} M 2-mercaptoethanol, 0.8% methyl cellulose, 15% FCS, and a source of CSF were incubated in 35 mm plastic Petri dishes (No. 5221-R, Lux, Newbury Park, Calif.) at 37 C in 5% CO_2 for 7 days. Clusters of more than 50 cells constitute colonies; and, by definition, the net formation of one colony above background equals one unit of CSF activity.

When determining cell types within colonies, bone marrow cells (5×10^4) were grown in 0.3% agar with the same culture medium and under the same conditions as described above. After 7 days, the cultures were fixed with 2% glutaraldehyde for 30 minutes at room temperature. The agar was dried onto a slide and stained with Giemsa.

Bone Marrow Thymidine Incorporation Assay

The method of Prystowsky et al was used.[22] Briefly, femurs were taken from CBA/J mice, and bone marrow cells were removed as a core with a 25-gauge needle. Bone marrow cells were separated with Ficoll/Hypaque density gradient centrifugation; the interface cells were resuspended in culture medium consisting of alpha medium supplemented with 15% FCS, 2% BSA, 10^{-7} M sodium selenite, 100 U/ml of penicillin, 100 μg/ml of streptomycin and sodium bicarbonate (2.2 mg/ml). After the cells were washed once, the concentration was adjusted to 5×10^5 cells/ml. One hundred microliters of the cell suspension was then added to 100 μl of sample containing factor or medium alone in 96-well flat-bottom microwells (No. 3596, Costar, Cambridge, Mass). Six to eight threefold serial dilutions of each factor were tested in triplicate. After 116 hours in culture in a humidified incubator at 37 C in 5% CO_2, the cultures were pulsed with 0.5 μCi of ^{3}H-thymidine (25 μl, 2 Ci/mmol) for 4 hours, harvested onto glass microfiber filters (934-AH, Whatman, Clifton, NJ) with a PHD cell harvesting system (Cambridge Technology, Cambridge, Mass), and assayed for radioactivity with the use of a Searle liquid scintillation counter. Counts per minute were converted to disintegrations per minute (dpm) with the channels ratio method, background incorporation (medium control, usually less than 1000 dpm) was subtracted from the experimental values, dose-response curves were constructed, and concentrations causing 50% maximal stimulation (ED_{50}) were determined.

When determining cell types in liquid cultures, we collected the cells from individual culture wells using a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa) and stained with Wright's stain.

IL3 Assay

Thymidine incorporation by the IL3-dependent cell line FDC-P1 was used for measurement of IL3 activity.[23]

Phagocytosis

Bone marrow cells were cultured in the presence of CSF at 37 C in 5% CO_2 for 6 days in 17-mm wells (Linbro, 76-033-05, Flow Laboratories, Inc., McLean, Va) with a 12-mm round glass coverslip on the bottom of the well. Cells that were adherent to the coverslip were incubated with immunoglobulin (Ig)-coated sheep erythrocytes for 1-2 hours at 37 C in 5% CO_2. Extracellular red blood cells were lysed by a 30-second exposure to distilled water, and the adherent cells were stained with Wright's stain.

Anti-IL3 Antiserum

An antiserum against IL3 was prepared by immunizing a rabbit with multiple inoculations of homogeneous IL3 in Freund's complete and incomplete adjuvant

(J. N. Ihle and T. Bowlin, manuscript in preparation). Antibody response was monitored by a radioimmune precipitation assay utilizing iodinated IL3. Fractions enriched for Ig were used in all experiments, and these fractions consisted of the portion of immune serum or normal rabbit serum that bound to protein A-Sepharose and was eluted with 1 M acetic acid.

Sources of CSF

Interleukin 3 was purified to homogeneity from WEHI-3-conditioned medium as described by Ihle and coworkers.[23] A T-lymphocyte GM-CSF was partially purified (approximately 2–4 × 10^8 colonies/mg of protein) from L2-cell-conditioned medium; this GM-CSF contained less than 0.1 U/ml of interleukin 2 and less than 1 U/ml of IL3 activities.[16] Another preparation of GM-CSF derived from the same T-lymphocyte clone (L2) was prepared with a combination of DEAE-Sephacel chromatography[15] and HPLC reverse-phase chromatography.[16] Conditioned media from cultures of L929 cells were used as a source of L-cell CSF.[1]

Detection of IgE Fc Receptors

Receptors for the Fc portion of IgE were detected using a three-step indirect immunofluorescence staining procedure. One million cells were incubated for 15 minutes on ice with 25 μl of a 1:5 dilution of a monoclonal mouse IgE (HI DNP-e-26-82, undiluted, 5.2 mg/ml; a gift from Dr. Henry Metzger). Following two washes with PBS containing 5% FCS and 0.05% sodium azide, cells were incubated for 15 minutes on ice with 25 μl of a 1:5 dilution of affinity-purified rabbit antiserum to mouse IgE (AMO-19, undiluted, 0.5 mg/ml; a gift from Dr. Henry Metzger). After two washes the cells were incubated for 15 minutes on ice with 25 μl of a 1:5 dilution of fluorescein-conjugated guinea pig antiserum to rabbit IgG (a gift from Dr. Michael Loken). Staining controls were prepared by using washing medium instead of mouse IgE in the first step. Propidium iodide (0.2 μg/ml) was added to each sample, and the relative fluorescence intensity was determined with an FACS IV (Becton Dickinson, Mountain View, Calif). Only viable cells which excluded propidium iodide were analyzed.

Cytochemical Reactions

Methods that have been reported previously were used for determination of the presence of the following intracellular enzymes: alpha naphthyl acetate (ANA) esterase,[24] naphthol AS-D chloroacetate (NCA) esterase,[25] peroxidase,[26] and cholinesterase.[27] To insure that the cytochemical reactions were working properly, we used human peripheral blood and bone marrow as control tissues for all stains except for the reaction for choiinesterase, where rectal myenteric plexus was used.

Table 1 – Effect of Anti-IL3 Antiserum on Factor-Induced Thymidine Incorporation*

Factor	Anti-IL3 antiserum† (dpm)	Control serum (dpm)
Experiment 1		
WEHI-3-cell IL3	3,400 ± 300	13,100 ± 1,970
L2-cell GM-CSF‡	19,800 ± 1,000	14,800 ± 2,050
Experiment 2		
DEAE effluent	1,990 ± 140	13,900 ± 3,170
DEAE eluate	20,300 ± 3,840	22,000 ± 3,470

* Background thymidine incorporation was 1,500 ± 110 dpm for Experiment 1 and 350 ± 50 DPM for Experiment 2.

† The same lot and concentration of anti-IL3 antiserum and control serum was used for both experiments.

‡ This preparation of L2-cell GM-CSF has been described previously.[16]

Results

It has been shown previously that the T-lymphocyte clone L2 secretes at least two factors affecting hemopoiesis, ie, IL3 and GM-CSF.[15] These two factors can be separated with DEAE chromatography.[15,28] L2-cell-conditioned medium was concentrated with the use of a centrifugal vacuum concentrator and fractionated with DEAE chromatography. Approximately 67% of the input CSF activity loaded onto the gel was recovered in the effluent and eluate fractions. Ninety-two percent (1.6 × 10^7 units) of the recovered CSF activity was in the eluate fraction. Ninety-three percent (2100 units) of the recovered IL3 activity was found in the effluent fraction, and 7% was in the eluate fraction. As can be seen from the bioassay data, there is a small amount of IL3 contaminating the GM-CSF eluate fraction; this contaminant represents <1% of the CSF activity in that fraction. Neither the CSF nor the IL3 assay allowed us to determine whether the effluent IL3 fraction was contaminated with or free of GM-CSF.

Anti-IL3 antiserum raised against WEHI-3-produced IL3 abrogates the WEHI-3 IL3-induced proliferation of bone marrow cells as measured by thymidine incorporation but does not inhibit the L2 cell GM-CSF-stimulated thymidine incorporation.[15] As shown in Table 1, anti-IL3 antiserum not only inhibited the effects of WEHI-3-produced IL3 but also inhibited thymidine incorporation stimulated by L2-cell-produced IL3. This indicates that IL3 secreted by the T-lymphocyte clone L2 and that produced by the myelomonocytic cell line WEHI-3 are antigenically simi-

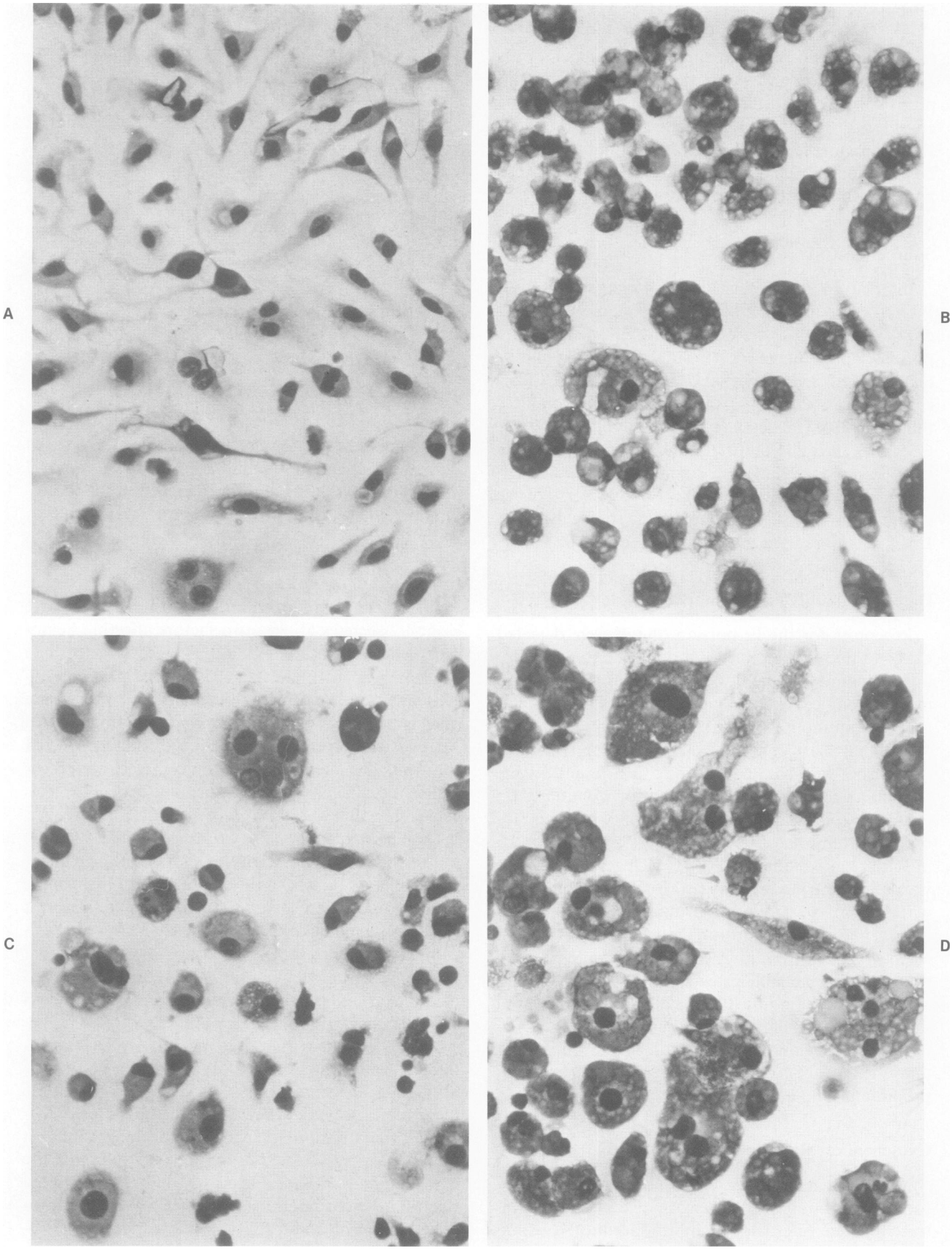

Figure 1 — Adherent cells found after stimulation of bone marrow with IL3- or L929-cell-conditioned medium (CSF-1). Bone marrow cells were cultured with CSF-1 (**A** and **B**) or IL3 (**C** and **D**) for 6 days. Cells adhering to a glass coverslip on the bottom of the culture well were either stained with Wright's stain (**A** and **C**) or incubated with Ig-coated erythrocytes before staining with Wright's stain (**B** and **D**). (×365)

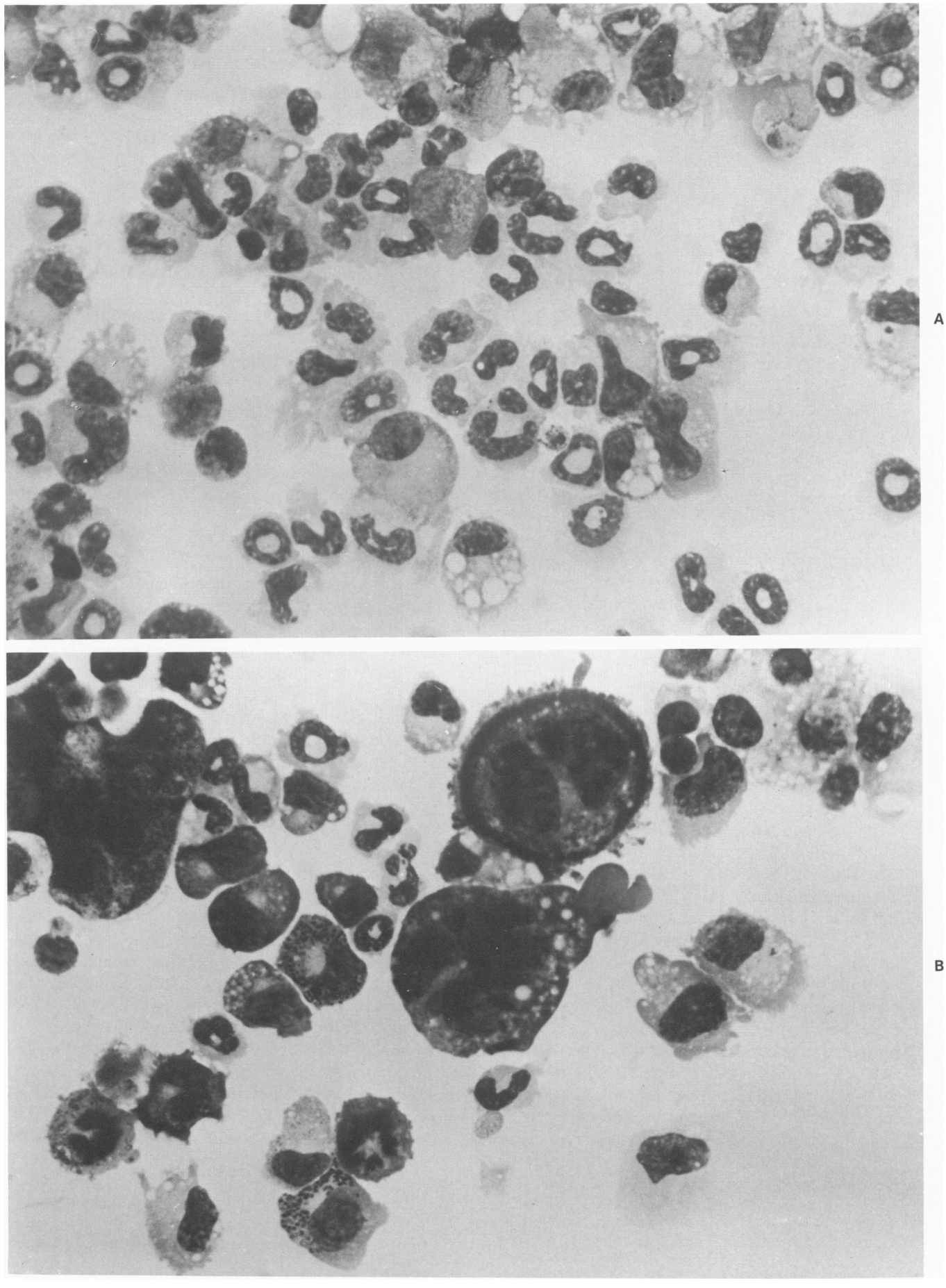

Figure 2 — Nonadherent cells found after stimulation of bone marrow cells with GM-CSF or IL3. Bone marrow cells were incubated for 6 days with GM-CSF derived from L2 cells[16] (**A**) or IL3 derived from WEHI-3 cells[23] (**B**). Nonadherent cells from individual culture wells were harvested, and cytocentrifuge preparations were made. Cultures containing GM-CSF have granulocytes and macrophages; cultures containing IL3 consist of granulocytes, macrophages, megakaryocytes, and cells with large granules. (Wright's, × 900)

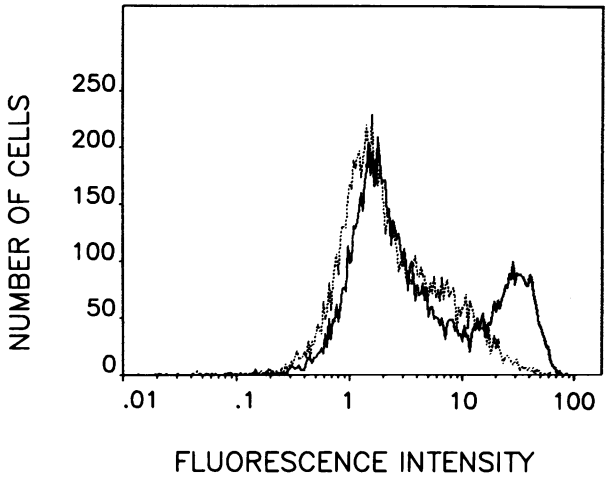

Figure 3—IL3-stimulated bone marrow cells express receptors for IgE. After bone marrow cells were cultured with IL3 for 6 days, they were incubated sequentially with mouse IgE (——) or medium (---), affinity-purified rabbit anti-mouse IgE, and fluorescein-conjugated guinea pig anti-rabbit IgG. Propidium iodide (0.2 μg/ml) was added to each sample, and only viable cells were analyzed.

lar. In addition, since greater than 85% of the effluent activity is inhibited by the anti-IL3 antiserum, most, if not all, of the CSF activity found in this fraction can be attributed to IL3 and not to GM-CSF. The eluate fraction containing GM-CSF, which accounts for most of the CSF activity secreted by L2 cells, is not inhibited by anti-IL3 antiserum (Table 1).[15]

The liquid culture system established for the thymidine incorporation assay was used for examination of the cells induced by IL3 from WEHI-3 cells,[22] GM-CSF from L2 cells,[16] and CSF-1 from L929 cells (Figure 1). For these experiments 5×10^5 to 10^6 Ficoll-Hypaque separated bone marrow cells were grown in 17-mm wells in 1 ml of culture medium for 6 days. Twelve-millimeter coverslips were placed in wells for study of the adherent cell population, and cytocentrifuge preparations were used for study of the nonadherent cells. In the absence of inducing factors, very few cells were present on Day 6. That L-cell-conditioned medium, as shown by Stanley et al,[3] induced the appearance of an adherent cell population. Greater than 95% of these cells were spindle-shaped macrophages (Figure 1A) that could phagocytize Ig-coated erythrocytes (Figure 1B). Both the partially purified GM-CSF and IL3 induced growth of adherent and nonadherent cells. There was greater size variation in the adherent cells produced by these factors than by CSF-1, and these adherent cells are rounder than the CSF-1-induced macrophages (IL3-induced macrophages are shown in Figure 1C). Like the CSF-1-induced macrophages, the GM-CSF-induced and IL3-induced macrophages could phagocytize Ig-coated erythrocytes (IL3-induced macrophages, Figure 1D). The nonadherent cells present after 6 days in culture with the L2-cell GM-CSF consisted of granulocytes and macrophages (Figure 2A). This GM-CSF also induced granulocyte-macrophage colonies in methyl cellulose, indicating that the major T-cell CSF is a GM-CSF.[16] Unlike the GM-CSF, IL3 induced multiple hemopoietic lineages in the liquid culture system, which, as shown in Figure 2B, included macrophages, neutrophilic granulocytes, megakaryocytes, cells with large granules, and immature cells.

We have confirmed the presence of multiple hemopoietic lineages after stimulation with homogeneous IL3 by using a variety of cytochemical stains. In three experiments, an average of ~50% of the cells contained NCA esterase and 30–50% contained peroxidase, indicating granulocytic differentiation; ~60% contained diffuse ANA esterase, suggesting granulocyte/monocyte/macrophage differentiation; 3–7% of the cells were large and contained cholinesterase, an enzyme found in mouse megakaryocytes[29]; and 35–50% of the cells were granulated and stained with alcian blue at pH 1, suggesting basophil/mast cell differentiation.

Since it was possible that the alcian blue staining cells represented granulocyte precursors, rather than basophil/mast cells, the number of cells bearing IgE Fc receptors was quantified (Figure 3). In two experiments, IgE Fc receptors were present on 18% and 26% of bone marrow cells 6 days after stimulation with IL3, indicating that roughly one-half of the alcian-blue-stained cells were basophil/mast cells. When taken together, the functional, morphologic, cytochemical, and surface phenotypic data indicate that at least four hemopoietic lineages, macrophage, neutrophilic granulocyte, megakaryocyte, and basophil/mast cell, were present after bone marrow precursor cells were stimulated with IL3 in liquid culture.

Several hemopoietic lineages were also present when bone marrow cells were stimulated with IL3 in semisolid medium. In addition to disperse macrophage colonies, other types of colonies were present; two are shown in Figure 4. A colony containing granulocytes and megakaryocytes is shown in Figure 4A and 4B; a colony containing megakaryocytes, granulocytes, and macrophages is shown in Figure 4C and 4D. Colonies containing cells with large granules were also present; however, mixed colonies with erythroid cells were only seen in the presence of erythropoietin.[17]

Discussion

Large amounts of colony-stimulating factor(s) that affect several hemopoietic lineages are found in lectin-stimulated, spleen-cell-conditioned medium, a com-

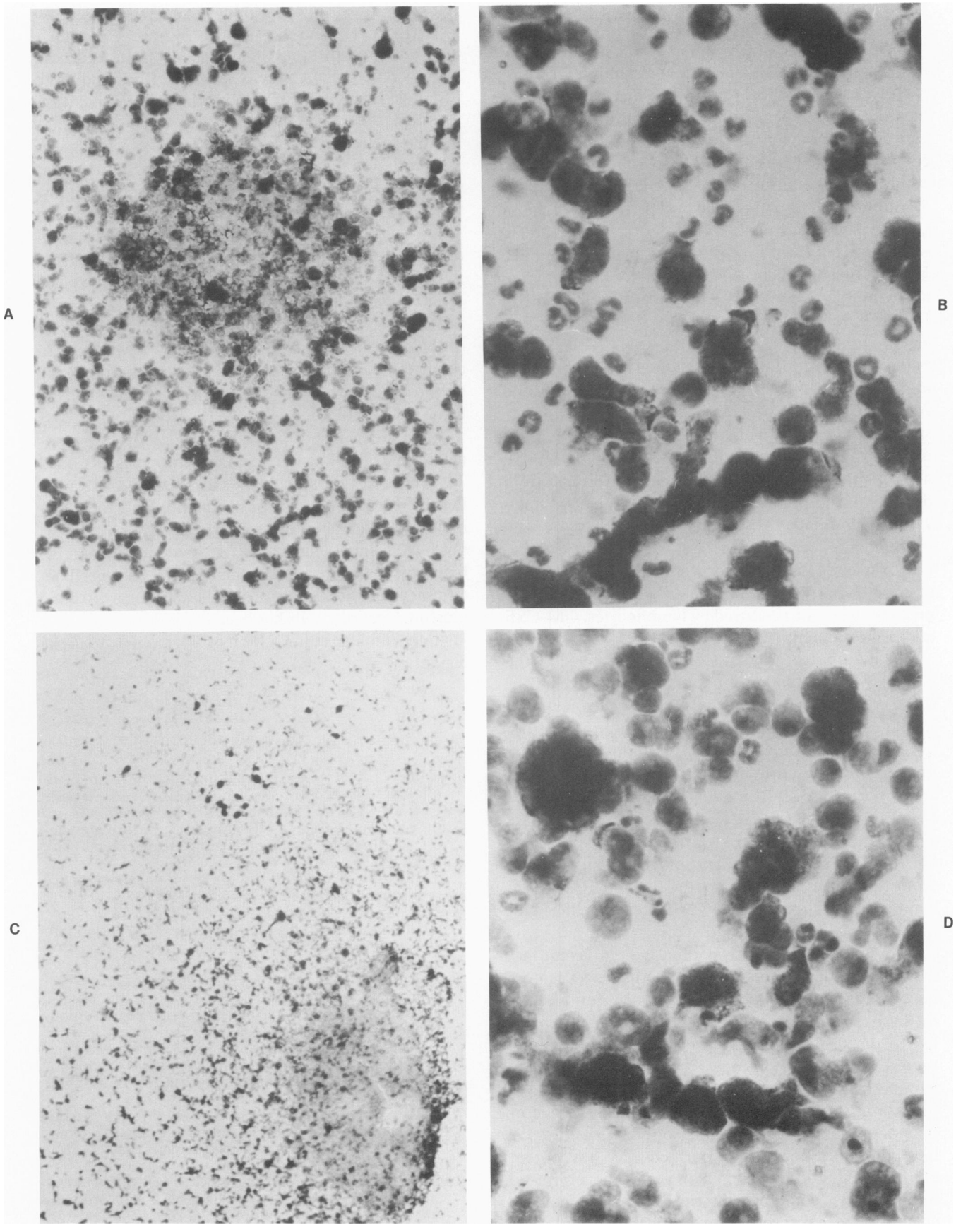

Figure 4 — IL3 stimulates the production of mixed hemopoietic colonies. IL3-stimulated bone marrow colonies were grown in 0.3% agar for 7 days. The cultures were fixed with 2% glutaraldehyde, dried onto a glass slide, and stained with Giemsa. A granulocyte-megakaryocyte colony (**A** and **B**) and a granulocyte-megakaryocyte-macrophage colony (**C** and **D**) are shown. (**A** and **C**, ×60; **B** and **D**, ×365)

monly used source of CSF.[4,30] Most of the CSF found in spleen-cell-conditioned medium is not CSF-1.[31,32] It is likely that most of the CSF in spleen-cell-conditioned medium is produced by T-lymphocytes, because CSF with properties similar to those found in spleen-cell-conditioned medium are also found in medium conditioned by transformed T-cell lines[5-8] and "normal" T-lymphocyte clones.[11-13] Regardless of the source of CSF, two biologically distinct factors can be separated on the basis of their ionic properties from conditioned medium of spleen cells,[4,28,30] a T-cell hybridoma,[33] and a T-lymphocyte clone.[15,16] One type of CSF binds to DEAE in low-ionic-strength buffers, has a lower isoelectric point than the second type of CSF, is present in higher quantities than the second type, and induces mainly granulocyte/macrophage colonies. The second type of CSF does not bind to DEAE in low-ionic-strength buffers, has a higher isoelectric point than the first type of CSF, accounts for less than 10% of the CSF activity in T-lymphocyte clone- or spleen cell-conditioned medium, stimulates the production of granulocyte, macrophage, eosinophil, megakaryocyte, and basophil/mast cell lineages, and stimulates proliferation of spleen colony-forming stem cells (CFU-S). Thus, at least two types of factors affecting hemopoietic precursor cells are produced by lymphocytes, GM-CSF and a multipotent type of CSF.

The multipotent type of CSF that is secreted by the cloned T-lymphocyte L2 or found in spleen-cell-conditioned medium is biologically and biochemically indistinguishable from IL3 that has been purified from WEHI-3-conditioned medium. In addition, anti-IL3 antiserum prepared by immunizing rabbits with IL3 from WEHI-3 cells abrogates the biologic effects of IL3 from L2 cells, indicating that these two factors are similar antigenically. In contrast, the GM-CSF secreted by L2 cells is biologically and biochemically distinct from IL3 and is not inhibited by the anti-IL3 antiserum, indicating that this GM-CSF and IL3 differ biologically, biochemically, and serologically.

Iscove et al have described a lineage-indifferent factor affecting pluripotential cells.[34] Other investigators have used lectin-stimulated spleen-cell-conditioned medium to stimulate hemopoietic stem cells, which has led to the production of mixed colonies containing several hemopoietic lineages.[35,36] Homogeneous IL3 stimulated the production of mixed hemopoietic colonies containing neutrophilic granulocytes, macrophages, megakaryocytes, and basophil/mast cells. We have recently shown that purified IL3 has potent erythroid-burst-promoting activity.[17] The erythroid burst colonies contained multiple lineages, including erythroid, megakaryocyte, macrophage, granulocyte, and basophil/mast cell. We saw the erythroid lineage only when erythropoietin was present. In addition, hemopoietic precursor cells capable of responding to CSF-1 are increased by IL3, indicating that IL3 increases the stem cell pool for at least two different lineage specific factors, CSF-1 and erythropoietin.[37] Finally, preincubation of bone marrow cells with IL3 increases the number of CFU-S as measured in the spleen colony-forming assay.[17] All of these data indicate that IL3 can act on a pluripotential stem cell. In long-term bone marrow cultures, IL3 has a lineage specific effect, causing the growth and differentiation of a particular type of mast cell.[38] In short-term bone marrow cultures, it is not clear at this time whether IL3 directly affects all of the other hemopoietic lineages or whether it acts in concert with other exogenous/endogenous factors like erythropoietin to stimulate the formation of mixed hemopoietic colonies.

T-lymphocytes, both *in vivo* and *in vitro*, are a potent source of factors that regulate hemopoiesis. Cloned T-lymphocytes provide an excellent model for studying the regulation of factor production and for determining the number of different types of CSF that T cells produce. When the array and relative quantities of T-lymphocyte factors affecting hemopoiesis has been determined and the interactions of these factors with bone marrow precursor cells has been defined, we will begin to understand the contribution of T-lymphocytes in the regulation of hemopoiesis.

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