MOLECULAR AND BIOLOGICAL PROPERTIES OF A MACROPHAGE COLONY-STIMULATING FACTOR FROM MOUSE YOLK SACS

G. R. JOHNSON and A. W. BURGESS

From the Cancer Research Unit, Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Victoria, 3050, Australia

ABSTRACT

A colony-stimulating factor (M-CSF) has been partially purified and concentrated from mouse yolk sac-conditioned medium (YSCM). M-CSF appeared to preferentially stimulate CBA bone marrow granulocyte-macrophage progenitor cells (GM-CFC) to differentiate to form macrophage colonies in semisolid agar cultures. By comparison, colony-stimulating factor (GM-CSF) from mouse lung-conditioned medium (MLCM) stimulated the formation of granulocytic, mixed granulocytic-macrophage, and pure macrophage colonies. Mixing experiments indicated that both M-CSF and GM-CSF stimulated all of the GM-CFC but that the smaller CFC were more sensitive to GM-CSF and that the larger CFC were more sensitive to M-CSF. Almost all developing "clones" stimulated initially with M-CSF continued to develop when transferred to cultures containing GM-CSF. In the converse situation, only 50% of GM-CSF prestimulated "clones" survived when transferred to cultures containing M-CSF. All clones initially stimulated by M-CSF or transferred to cultures stimulated by M-CSF contained macrophages after 7 days of culture. These results suggest that there is a population of cells (GM-CFC) that are capable of differentiating to form both granulocytes and macrophages, but, once these cells are activated by a specific CSF (e.g. M-CSF), they are committed to a particular differentiation pathway. The pattern of CFC differentiation was not directly related to the rate of proliferation: cultures maximally stimulated by M-CSF produced mostly macrophage colonies, but the presence of small amounts of GM-CSF produced granulocytic cells in 30% of the colonies. Gel filtration, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and affinity chromatography with concanavalin A-Sepharose indicated that M-CSF from yolk sacs was a glycoprotein with an apparent molecular weight of 60,000. There was some heterogeneity of the carbohydrate portion of the molecule as evidenced by chromatography on concanavalin A-Sepharose.

KEY WORDS fetal · colony-stimulating factor · macrophage · purification · colony-forming cell · differentiation

Cells capable of differentiating into mature hemopoietic cells can be cloned in vitro under appropriate culture conditions (1, 4). These colony-

forming cells (CFC)[1] require the presence of specific hemopoietic regulatory molecules (referred to as colony-stimulating factors [CSF]) for their in vitro proliferation and differentiation (9). There appear to be specific CSF's for each hemopoietic cell type, although the one CSF purified thus far (9), granulocyte-macrophage colony-stimulating factor (GM-CSF), controls the differentiation and proliferation of both neutrophil granulocytes and macrophages (2) from granulocyte-macrophage colony-forming cells (GM-CFC). The in vitro differentiation and proliferation of GM-CFC is regulated in part by the concentration of GM-CSF (2, 11) but can also be altered by the presence of other biologically active molecules (especially inhibitors to colony growth). For these reasons, previous studies on the properties of GM-CFC have been hampered by the poor quality of CSF used. In this report, we indicate a simple scheme for partially purifying two CSF's so that they will give maximal stimulation to the GM-CFC and enable the properties of the cells to be studied independently of the CSF concentration effect.

Recently, a macrophage colony-stimulating factor (M-CSF) was obtained from fetal CBA mouse yolk sac-conditioned medium (YSCM; reference 5). The biological and molecular properties of M-CSF have been directly compared to those of GM-CSF from mouse lung-conditioned medium (MLCM). After partial purification and concentration, both M-CSF and GM-CSF maximally stimulate bone marrow cultures but the patterns of differentiation produced are quite different. The molecular properties suggest that M-CSF is a larger molecule than GM-CSF but contains less carbohydrate than the latter. The availability of these two CSF has allowed the direct study of the factors controlling granulocyte and macrophage differentiation. The development of single clones stimulated by GM-CSF, M-CSF, or various concentrations of both and the effect of transferring a developing clone to a different stimulus have been studied.

[1] *Abbreviations used in this paper:* CFC, colony-forming cell; GM-CSF, granulocyte-macrophage colony-forming cell; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; MLCM, mouse lung-conditioned medium; YSCM, yolk sac-conditioned medium; DME, Dulbecco's Modified Eagle's Medium; DEAE, diethylaminoethyl; EBSS, Eisen's Balanced Salt Solution.

MATERIALS AND METHODS

Preparation of Cell Suspensions

C57BL/6J Wehi$_t$ and CBA/Wehi mice were used to provide all the tissues or conditioned media referred to in this report. Bone marrow cells were collected by flushing the cells from the femur with a 22-gauge needle into Eisen's Balanced Salt Solution (EBSS). Single cell suspensions were prepared by repeated aspiration with glass pipettes. Cell counting was performed with a hemocytometer, and viability was determined by eosin dye exclusion.

Preparation of Conditioned Medium

Yolk sacs were obtained from CBA mice at 14 days of gestation. (Day of vaginal plug = day 0 of gestation; reference 5.) After removal from the fetus, yolk sacs were washed several times through EBSS. Each washed yolk sac was cultured in 1 ml of serum-free Dulbecco's Modified Eagle's Medium in a round bottom plastic tube (no. 2001, BioQuest, BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md.) for 5 days at 37°C in a fully humidified atmosphere containing 10% CO_2.

After removal of the yolk sacs, the conditioned medium (YSCM) was dialyzed for 3 days against three changes of 0.03 M Tris HCl buffer, pH 7.4. Although this conditioned medium stimulated colony formation, there was insufficient M-CSF to maximally stimulate all of the CFC in the semisolid agar assay. DEAE-Sepharose CL-6B (5 ml) equilibrated with the same buffer was added to 500 ml of dialyzed conditioned medium and mixed gently for at least 4 h. (The addition of 50 μl of bromphenol blue (0.01%, wt/vol) to the dialyzed conditioned medium is useful for determining when the binding has gone to completion.) The ion exchange beads were allowed to settle and most of the medium was decanted. The beads were collected and then packed into a column (60 × 10 mm) and diluted with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl, 0.005% (wt/vol) polyethylene glycol 6,000, and 0.02% sodium azide (wt/vol). Fractions (2 ml) were collected and assayed for CSF. Most of the CSF eluted in fractions 3 and 4 whereas the bromphenol blue or phenol red eluted in fractions 5–8. This batch chromatographic procedure routinely achieved a 50- to 100-fold concentration of the CSF. This DEAE-Sepharose eluate is referred to as the standard M-CSF preparation. This preparation was slightly inhibitory when 0.1 ml was used to stimulate the conventional 1-ml agar cultures, but this inhibitory material could be removed by gel filtration chromatography (see below). Alternatively, the reagent can be used at a four- to eightfold dilution (where the CSF concentration appears to be approximately two times greater than that necessary for maximum stimulation).

Mouse lung-conditioned medium (MLCM) was prepared using C57BL mouse lungs (14) excised 3 h after

the injection of 5 μg of endotoxin (Difco Laboratories, Detroit, Mich.). Each pair of lungs was cultured in 5 ml of serum-free Dulbecco's Modified Eagle's Medium contained in a round bottom plastic tube (no. 2001, Falcon Plastics) for 48 h at 37°C in a fully humidified atmosphere with 10% CO_2. After heating at 56°C for 30 min and centrifugation at 12,000 *g* for 15 min to remove precipitate, the MLCM was dialyzed below 8°C against 3 × 10 vol of deionized distilled water. The precipitate was removed by centrifugation, and calcium phosphate gel was stirred into the solution (1 ml of gel/10 mg of protein). After 2 h, the supernatant fluid was decanted and the gel was collected by centrifugation. The gel was washed initially with 2 vol of 0.01 M sodium phosphate buffer, pH 6.8, and then with 1.5 vol of 0.1 M sodium phosphate buffer, pH 6.8, containing polyethylene glycol 6,000 (0.005%, wt/vol) and sodium azide (0.02%, wt/vol) to elute the CSF (2). Ultrafiltration over a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass.) was used to concentrate the eluted CSF fivefold to yield the material referred to as the standard GM-CSF preparation. This concentrate still maximally stimulated agar bone marrow cultures even at 200-fold dilution. It was routinely used at dilutions between 2- and 30-fold in the work reported here. GM-CSF used for affinity chromatography on concanavalin A-Sepharose was further purified by gradient salt elution from diethylaminoethyl (DEAE)-Sepharose and concentrated by ultrafiltration (2).

Concanavalin A-Sepharose Chromatography

GM-CSF (1.5 ml) eluted from DEAE-Sepharose and equilibrated with 0.2 M sodium acetate buffer, pH 5.0, containing Triton X-100 (0.01%, vol/vol) and Ca^{++}, Mn^{++}, Mg^{++} ions (1 mM), was applied to a column containing concanavalin A-Sepharose 4B (1.5 × 2.5 cm) equilibrated with the same buffer (2). After the first peak of protein had been collected, methyl-α-D-glucopyranoside (0.05 M) was used to elute these glycoproteins.

M-CSF (1.25 ml), which had been eluted batchwise from DEAE-Sepharose, was mixed with 0.1 mg of Dextran Blue and 1.5 μl of Triton X-100 solution (10%, vol/vol) and applied to a column containing concanavalin A-Sepharose 4B (1.5 × 2.5 cm) equilibrated with 0.2 M sodium acetate buffer, pH 5.0, containing Triton X-100 (0.01%, vol/vol) and Ca^{++}, Mn^{++}, Mg^{++} ions (1 mM). After the first peak of protein had been collected, methyl-α-D-glucopyranoside (0.05 M) was added to the buffer to facilitate the elution of the glycoproteins.

Gel Filtration Chromatography

M-CSF (1.0 ml), eluted from DEAE-Sepharose or from polyacrylamide gel after electrophoresis, was applied to a column (1.5 × 18 cm) of Sephadex G-100 (superfine, Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl and Triton X-100 (0.1%, vol/vol). The flow rate was maintained at 3 ml/h, and 1.5-ml fractions were collected. M-CSF eluted from the column between 20 and 30 ml. Biologically active fractions were pooled and concentrated using a Diaflo PM-10 ultrafiltration membrane.

Polyacrylamide Gel Electrophoresis

Samples containing M-CSF were applied to a preparative (2.0 × 7.0 cm) gel electrophoresis column (3, 13). Analytical polyacrylamide electrophoresis was performed according to the method of Laemmli (6), except that the samples were equilibrated with sodium dodecyl sulfate (3%, wt/vol) for 1 h at 37°C. At the completion of the eletrophoresis, the gels were either sliced into 1.5-mm sections for subsequent elution or stained with Coomassie Blue R-250 to locate the protein bands. M-CSF was eluted from the crushed gel slices with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl. Where sodium dodecyl sulfate was present, these eluates were deionized with approximately 0.5 g of a mixed bed ion exchange resin AG-501 X8 (D) (BioRad Laboratories, Richmond, Calif.). Protein staining was recorded photographically or by densitometry using a Canalco model J microdensitometer (Ames Co., Div. of Miles Lab. Inc., Elkhart, Ind.). M-CSF was located by assaying the eluates (0.1 ml) from each fraction by the soft agar cloning technique.

Agar Cultures

The techniques used to prepare the semisolid agar cultures were similar to those described by Metcalf (8). Aliquots (0.1 ml) of the appropriate dilutions of the granulocyte and/or macrophage CSF were placed in four replicate plastic Petri dishes (35 mm). Cell suspensions were diluted to the required cell concentrations in not more than 0.4 ml of EBSS and added to an equal volume of the agar medium (prepared by mixing equal volumes of double strength Dulbecco's Modified Eagle's Medium and 0.6% agar). The composition of the double-strength nutrient medium had been slightly altered since earlier studies: Dulbecco's Modified Eagle's Medium HG Instant Tissue Culture Powder H16 (10.0 g; Grand Island Biological Co., Grand Island, N. Y.), metal-distilled deionized water, 390 ml, 3 ml of L-asparagine (20 μg/ml), 1.5 ml of DEAE-Dextran (75 μg/ml; Pharmacia Fine Chemicals, mol wt = 2×10^6, η = 0.70), 0.575 ml of penicillin (200,000 U/ml), 0.375 ml of streptomycin (200 μg/ml), 4.9 g of NaH CO_3, 63 ml of horse serum (CSL, Melbourne Batch no. 1413), 187 ml of fetal calf serum (Laboratory Services Ltd., Auckland, N. Z.). All ingredients were sterilized by filtration through 0.45-μm cellulose acetate membranes (Millipore Corp., Bedford, Mass.). Cell suspensions in agar medium (1 ml) were added to each Petri dish and mixed with the stimulus. After gelling at room temperature, the cultures were incubated at 37°C for 7 days in a

fully humidified atmosphere containing 10% CO_2 in air. In this culture system, maximally stimulated CBA mouse bone marrow cells yielded 180–250 colonies per 10^5 cells plated when estimating the frequency of CFC in a given cell suspension. Cultures always contained sufficient CSF to maximally stimulate the cultures. Colony counts were made with an Olympus dissection microscope (X 35) with indirect lighting. Discrete aggregates of more than 50 cells were scored as colonies, and aggregates containing 3–50 cells were scored as clusters. For cytological identification of granulocyte and macrophage colonies, colonies were transferred to microscope slides, allowed to dry, overlaid with a coverslip, and stained with 0.6% orcein in 60% acetic acid (10).

Liquid Culture of Bone Marrow Cells

Bone marrow cells (1×10^5 or 2×10^5/ml) were cultured in Dulbecco's Modified Eagle's Medium (DME), identical in composition to that used for the agar cultures except that an equal volume of double-distilled water was added to the DME instead of 0.6% agar. 1-ml aliquots of cells in liquid DME plus 0.1 ml of a maximal stimulus of GM-CSF were layered over previously prepared agar underlayers containing DME, 0.5% agar, and an identical concentration of GM-CSF in 35-mm Petri dishes. Identical cultures were established with optimal concentrations of M-CSF. After 1 or 3 days of incubation at 37°C in a fully humidified incubator containing 10% CO_2 in air, cells were harvested, and viable nucleated cell counts were determined, and then the cells were cultured for 7 days in replicate agar cultures containing either GM-CSF, M-CSF, or normal saline (0.15 M).

"Clone" Transfer Studies

Agar cultures ("donor cultures") were established with low numbers of CBA bone marrow cells (5,000–10,000/1 ml of culture) optimally stimulated with either GM-CSF or M-CSF. At the same time, identical "host" cultures were prepared which contained 1 ml of agar medium and optimal concentrations of M-CSF, GM-CSF, or 0.1 ml of 0.15 M NaCl but no cells. After incubation for a period of 2 or 3 days, single developing clones were removed from the donor cultures with a fine glass pipette and transferred to marked areas in the host culture dishes. Transferred clones were placed into "host" culture medium containing the same stimulus as the donor culture, the opposite stimulus, or normal saline. Routinely, five developing clones were transferred to each host culture dish. Host cultures were incubated in the normal way so that the total culture period was 7 days.

Sedimentation Velocity

Separation of cells by sedimentation at unit gravity was carried out by the technique of Miller and Phillips (12). Cell suspensions were prepared in EBSS containing 3.3% fetal calf serum and applied to a step-gradient of fetal calf serum (7.5–25%, vol/vol) in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl. After sedimentation in a glass chamber (11-cm diameter, Johns Scientific, Toronto, Canada) at 4°C for 2 h, the cone volume was discarded and 10-ml fractions were collected. Each fraction was centrifuged and the cells were resuspended in 2 ml of 0.01 M sodium phosphate buffer, pH 7.3, 0.15 M NaCl. Viable cell counts were made using eosin dye. CFC numbers were determined in four replicate soft agar cultures of appropriate numbers of cells optimally stimulated by either M-CSF or GM-CSF.

RESULTS

Initially, YSCM was prepared by incubating a single yolk sac from a 14-day CBA mouse fetus for 5 days in 3 ml of serum-free DME at 37°C in a fully humidified incubator containing 10% CO_2 in air. This conditioned medium (0.1 ml) usually stimulated 80–90 colonies/10^5 CBA bone marrow cells in the standard semisolid agar assay system. When the yolk sacs were cultured in 1 ml of medium, 0.1 ml of the YSCM was capable of stimulating 180 colonies/10^5 CBA bone marrow cells—this being equivalent to maximal stimulation by concentrates of GM-CSF. The volume of conditioned medium used for the culture of the yolk sacs could be varied from 1 to 3 ml without alteration in the total amount of M-CSF produced. The small volume (1 ml) of culture medium was chosen for most of these studies to provide a more concentrated starting pool of M-CSF for the partial purification and characterization. YSCM was pooled and stored frozen until 300 ml was available for dialysis and batch adsorption onto DEAE-Sepharose 6B-CL. The M-CSF eluted from DEAE-Sepharose stimulated colony formation from CBA mouse bone marrow cells at all dilutions between 1:1 and 1:128 (Fig. 1). At dilutions between 1:3 and 1:10, the maximal number of colonies appeared to form, but at higher concentrations (1:2 and 1:1) there appeared to be some inhibition of colony growth (Fig. 1). Gel filtration chromatography of the DEAE-Sepharose concentrate on Sephadex G-100 (s) removed this inhibitory material so that concentration of the active fractions yields M-CSF which is able to maximally stimulate colony growth at all dilutions between 1:1 and 1:20 (Fig. 1). Maximal stimulation by either M-CSF or GM-CSF resulted in identical colony numbers in cultures of CBA bone marrow cells (Table I). However, maximally stimulated M-CSF cultures rarely contained granulocytic colonies, whereas GM-CSF cultures contained up to

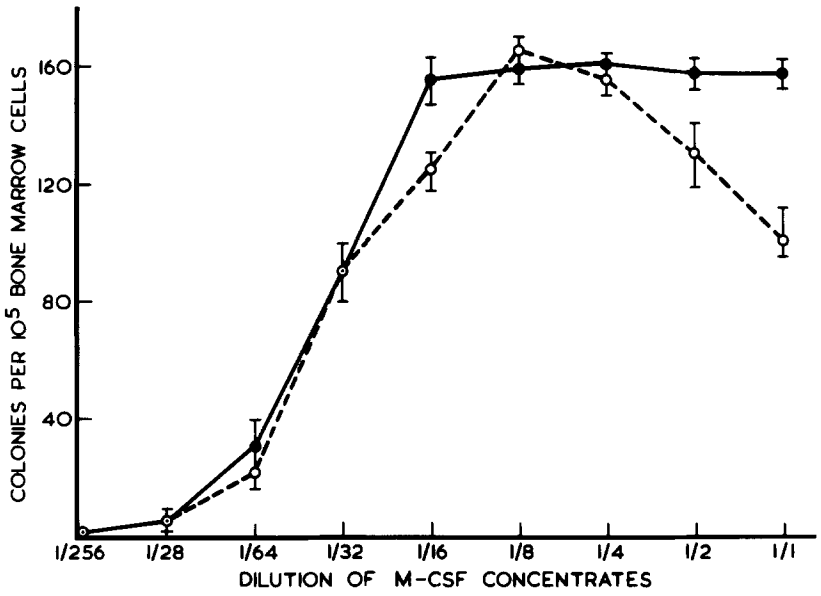

FIGURE 1 Effect of dilution of M-CSF derived from YSCM on colony incidence in cultures of mouse bone marrow. Cultures were stimulated by M-CSF eluted from DEAE-Sepharose (O- -O) or M-CSF purified further using Sephadex G100 (●—●). All cultures contained 40,000 CBA bone marrow cells and 0.1 ml of the appropriate stimulus (see Materials and Methods) at varying dilutions. Mean ± SD data from four replicate cultures at day 7 of incubation.

TABLE I

Effect of GM-CSF on the Differentiation of CBA Mouse Bone Marrow GM-CFC in the Presence of Optimal Concentrations of M-CSF

M-CSF dilution	GM-CSF dilution	Mean no.* of colonies per culture	Percent colonies‡		
			Granulocytic	Mixed	Macrophage
1:8	0	102 ± 6	0	5	95
0	1:20	99 ± 7	40	29	31
1:8	1:3	95 ± 9	49	25	26
1:8	1:9	131 ± 16	52	23	25
1:8	1:27	111 ± 12	60	18	22
1:8	1:81	118 ± 12	49	20	31
1:8	1:243	118 ± 12	33	17	50
1:8	1:729	129 ± 10	26	13	61
1:8	1:2,189	111 ± 10	22	7	71

All cultures stimulated by 0.1 ml of the standard GM-CSF or M-CSF preparations at the given dilutions (see Materials and Methods).

* Mean colony numbers ± SD from four replicate cultures each with 40,000 CBA bone marrow cells.

‡ Colony morphology determined by staining 45 sequential colonies from each culture.

40% granulocytic colonies (Table I). To determine whether the different colony growth patterns were due to preferential stimulation of different subpopulations of CFC, mixing experiments were performed. With cultures containing optimal concentrations of both M-CSF and GM-CSF, total colony numbers were comparable to those of cultures containing either stimulus alone (Table I). Colony morphology in mixed cultures, however, was always the same as with GM-CSF alone, although when suboptimal concentrations of GM-CSF were mixed with optimal concentrations of M-CSF, an increase in the proportion of macrophage colonies was observed (Table I).

Bone marrow cells from CBA mice were separated into 20 fractions by velocity sedimentation.

Cells from each fraction were then cultured in replicate with optimal concentrations of GM-CSF or M-CSF; the results of two separate experiments are shown in Fig. 2. Both M-CSF and GM-CSF stimulated CFC sedimenting between 2 and 10 mm/h. The smaller CFC (sedimentation velocity 2–4 mm/h) produced more colonies with GM-CSF, whereas the larger CFC (sedimentation velocity = 6–9 mm/h) were more responsive to M-CSF. The ratio of the total number of colonies stimulated by M-CSF (i.e. the sum of the number of CFC present in all fractions) to the total number of colonies stimulated by GM-CSF was 1.04.

Liquid Cultures

The relationships between the CFC stimulated by M-CSF and GM-CSF were also investigated by preparing liquid cultures of CBA bone marrow cells containing either M-CSF or GM-CSF and subsequently harvesting the cells to analyze the CFC by the semisolid agar cloning technique. In the presence of either CSF, the total number of cells decreased during the first 3 days of liquid culture, although the frequency of CFC increased (Table II). There were more cells present in the cultures containing GM-CSF (compared with M-CSF-stimulated cultures) after both 1 and 3 days of liquid culture, but after 3 days there were slightly more CFC in the liquid cultures stimulated by M-CSF. Cells initially stimulated in liquid culture by M-CSF and cultured in the presence of M-CSF gave rise to only macrophage colonies (Table II). Even 24-h exposure to M-CSF appeared to commit most of the CFC to the macrophage differentiation pathway, because subsequent culture in the presence of GM-CSF yielded over 60% macrophage colonies, compared with 30% when GM-CSF was used to stimulate the liquid cultures (Table II). After 3 days in liquid culture in the presence of M-CSF, all of the surviving CFC appeared to be committed to macrophage differentiation even when GM-CSF was used to stimulate the agar cultures.

Clone Transfer Experiments

Liquid culture experiments do not permit analysis of the progeny of any particular cell. For this reason, experiments were performed with bone marrow cells cloned in agar to study the influence of GM-CSF and/or M-CSF on clonal differentiation. After 7 days of culture, the morphology of individual colonies was found to be dependent upon the type of CSF used (Table III); developing clones were therefore analyzed at earlier timepoints to determine at what time clonal morphology was altered. After stimulation of bone marrow cells in agar culture for 2 days with GM-CSF, in

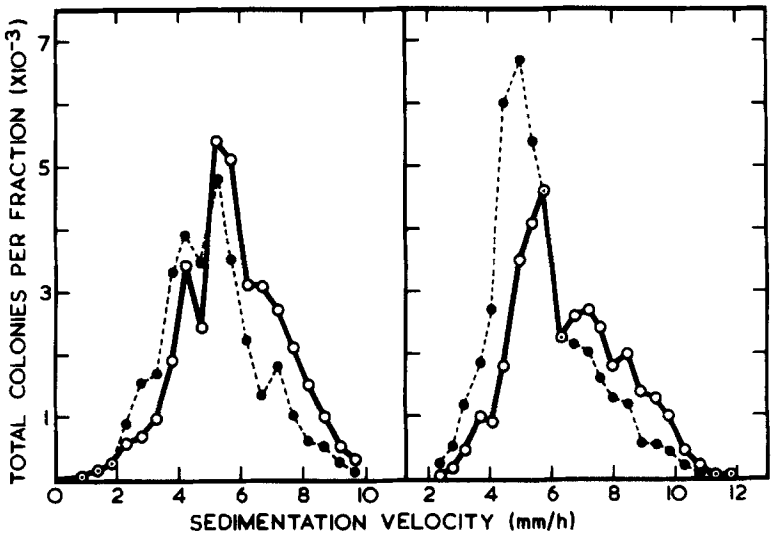

FIGURE 2 Distribution of CFC in CBA bone marrow separated by velocity sedimentation. The two panels are from separate experiments performed under identical conditions. All cultures were stimulated by 0.1 ml of the standard GM-CSF preparation diluted 1:20 (●- -●) or the standard M-CSF preparation diluted 1:8 (○—○).

TABLE II

Effect of Short-Term Liquid Culture on Agar Colony Morphology

Initial liquid culture			Final agar culture		Colony morphology		
Time	Stimulus	No. cells per culture* ($\times 10^{-5}$)	Stimulus	No. colonies‡ per culture	Granulocytic	Mixed	Macrophage
days					%	%	%
1	M-CSF	1.1	M-CSF	1,200	0	0	100
1	M-CSF	1.1	GM-CSF	400	27	9	64
1	GM-CSF	1.8	M-CSF	1,000	7	14	79
1	GM-CSF	1.8	GM-CSF	800	39	27	34
3	M-CSF	0.72	M-CSF	2,400	0	2	98
3	M-CSF	0.72	GM-CSF	1,300	0	2	98
3	GM-CSF	0.89	M-CSF	2,000	7	16	77
3	GM-CSF	0.89	GM-CSF	1,200	21	27	52

* Cultures were initiated with 10^5 CBA bone marrow cells in 1-ml cultures and stimulated with 0.1 ml of the standard GM-CSF preparation (diluted 1:20) or the standard M-CSF preparation (diluted 1:8). Nucleated cells were counted on a hemocytometer, using eosin dye exclusion to determine the viable cells.

‡ Colonies were counted after 7 days in agar culture, and numbers indicate the total number of CFC in each initial 1 ml of liquid culture.

TABLE III

Morphology of Early Clones Stimulated by GM-CSF or M-CSF

Time of incubation	Stimulus*	Total clones‡ assayed	Morphology of clones			
			Granulocytic	Mixed	Macrophage	Mononuclear
days			%	%	%	%
2	GM-CSF	58	60	24	2	14
2	M-CSF	67	19	27	8	46
3	GM-CSF	42	81	10	2	7
3	M-CSF	42	14	21	10	55
4	GM-CSF	31	91	3	3	3
4	M-CSF	31	19	16	52	13
7	GM-CSF	45	38	22	40	0
7	M-CSF	45	0	7	93	0

* Cultures (1 ml) were stimulated with 0.1 ml of the standard GM-CSF preparation (diluted 1:20) or the standard M-CSF preparation (diluted 1:8). Day 7 data obtained from clones of >50 cells.

‡ CBA mouse bone marrow cells (40,000) were cloned in semisolid agar in the presence of the appropriate stimulus.

58 clones analyzed the mean number of cells per clone was found to be 11 and 60% of developing clones were pure granulocytic (Table III). After stimulation with M-CSF for 2 days, <20% of developing clones contained only granulocytes (Table III), and the mean number of cells per clone (determined from 67 analyzed, of all types) was found to be 8. Similar differences were also observed after 3 days of culture. Stimulation by GM-CSF produced clones of which 81% contained only granulocytes (Table III), the mean cell number of all clones being 42; with M-CSF stimulation, 14% of clones contained only granulocytes (Table III), and the mean cell number, of all clones, was 13 per clone.

To determine whether obvious differences (Table III) in clone morphology and cell numbers observed at days 2, 3, and 4 of development reflected a commitment to neutrophil or macrophage differentiation, clone transfer experiments were performed. The results of these experiments are summarized in Table IV. After 2 or 3 days in the presence of the initial stimulus, in all cases except one (day 3, GM-CSF–GM-CSF: only 72% survival), more than 87% of transferred clones survived if transferred to the same stimulus type in the secondary cultures. When clones initiated in M-CSF-stimulated cultures were transferred to secondary cultures containing GM-CSF, more than 97% of clones survived. Upon subse-

quent culture for a total period of 7 days, day 2-transferred aggregates produced clones of which 97% were composed of macrophages (Table IV). No pure granulocytic clones were observed. When clones were initiated in GM-CSF-stimulated cultures, and then transferred at day 2 or day 3 to M-CSF-stimulated cultures, only 58 and 51% of transferred clones, respectively, survived. Of those clones that survived transfer, more than 70% were composed entirely of macrophages (Table IV). In control experiments in which clones were transferred to secondary cultures containing normal saline instead of CSF, no clones survived the total 7-day culture period.

Chemical Characterization of M-CSF

Although the DEAE-Sepharose batch absorption and elution only leads to a preparation of M-CSF with a specific activity slightly higher than that of the starting conditioned medium (Table V), the yield of colony-stimulating activity was approximately 95% and the volume in which the M-CSF was contained had decreased 60-fold. Chromatography of M-CSF from DEAE-Sepharose on Sephadex G-100 yielded two major peaks of protein—one at the void volume and the other close to albumin (Fig. 3). A single peak of colony-stimulating activity eluted at a position similar to that of albumin (although the activity elution profile was rather broad). From the linear plot of the logarithm of the molecular weight against the elution volume for a set of proteins of known size, the apparent molecular weight of M-CSF was determined to be 60,000 (Fig. 4). Even though it was possible to recover 80% of colony-stimulating activity after chromatography on Sephadex G-

TABLE IV

Effect of Short-Term Exposure in Agar Culture to a Specific CSF on Clonal Differentiation

Initial culture*	Initial stimulus‡	Final stimulus§	No. of clones transferred	Viable clone recovery	Average cells per recovered clone	Clone morphology		
						Granulocytic	Mixed	Macrophage
				%			%	
2	GM-CSF	GM-CSF	52	87	147	17	52	31
2	GM-CSF	M-CSF	40	58	106	4	14	82
2	M-CSF	M-CSF	30	93	57	0	0	100
2	M-CSF	GM-CSF	30	97	30	0	3	97
3	GM-CSF	GM-CSF	50	72	57	56	22	22
3	GM-CSF	M-CSF	77	51	92	10	21	69
3	M-CSF	M-CSF	102	98	56	2	0	98
3	M-CSF	GM-CSF	80	95	48	0	0	100

* Cultures (1 ml) were stimulated with 0.1 ml of the standard GM-CSF preparation (diluted 1:20) or the standard M-CSF preparation (diluted 1:8).

‡ CBA mouse bone marrow cells (40,000) were cultured in agar in the presence of the initial stimulus.

§ At the time indicated, developing clones were transferred to a second agar culture containing the final stimulus, and the culture was continued until day 7.

TABLE V

Purification of M-CSF from YSCM

Stage		Volume	No. of colonies*	Amount of protein	Specific activity ($\times 10^{-3}$)	Yield
		ml	*$\times 10^{-5}$*	*mg*	*colonies/mg*	%
YSCM		300	5.8	55	10	100
DEAE-sepharose		5.0	5.6	40	14	95
Concanavalin A-	I	5.5	2.2	21	10	40
Sepharose	II	4.0	1.0	4.5	22	20
Electrophoresis	I	0.9	1.5	1.2	125	25
	II	0.9	0.4	0.3	135	7

* Calculated from the linear portion of the titration curve per 10^5 CBA mouse bone marrow cells in the standard semisolid agar cloning system. I, the protein fraction passing through the concanavalin A-Sepharose. II, the protein bound to concanavalin A-Sepharose and eluted with 0.05 M methyl-α-D-glucopyranoside.

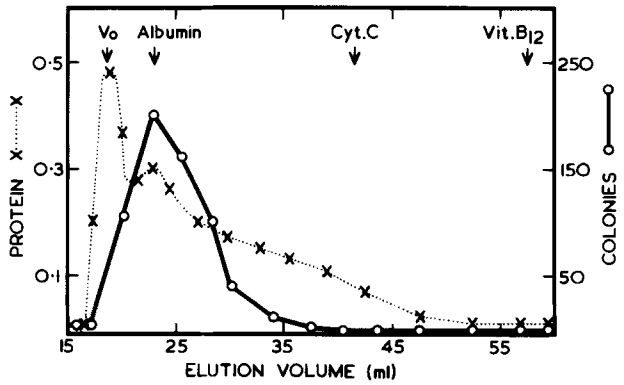

FIGURE 3 Sephadex G100 gel filtration chromatography of M-CSF eluted from DEAE-Sepharose. The protein profile was determined by optical density at 280 nm using a 1-cm cell. The colony-stimulating activity (O—O) was measured using 0.1 ml from each fraction in the standard semisolid agar system.

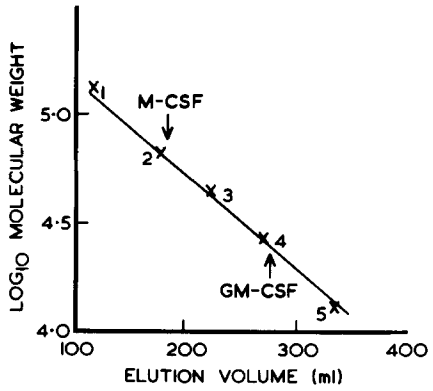

FIGURE 4 Apparent molecular weight of M-CSF and GM-CSF using gel filtration on Sephadex G100. Proteins of known molecular weight were used to calibrate the column. (*1*) Immunoglobulin G; (*2*) bovine serum albumin; (*3*) ovalbumin; (*4*) α-chymotrypsinogen; and (*5*) myoglobin.

100, there was only a 1.5-fold increase in specific activity. This step was not routinely incorporated into the initial purification scheme; however, the concentrated pool from Sephadex G-200 was capable of maximally stimulating CBA bone marrow cells over a 20-fold concentration range. M-CSF eluted from DEAE-Sepharose was chromatographed on concanavalin A-Sepharose. The results are compared to those in a similar experiment with GM-CSF which was also eluted from DEAE-Sepharose (Fig. 5). As expected, when GM-CSF was chromatographed, most of the protein failed to bind to the column, but almost 80% of the GM-CSF bound and was specifically eluted with methyl-α-D-glucopyranoside (0.05 M). Although the protein profile was similar for M-CSF, over 70% of the colony-stimulating activity failed to bind to the concanavalin A-Sepharose column (Table V). The M-CSF eluted from concanavalin A-Sepharose with methyl-α-D-glucopyranoside (0.05 M) had a specific activity of 2.2×10^4 colonies/mg protein (Table V).

Further purification of M-CSF was attempted using a preparative polyacrylamide gel electrophoresis column (20 × 70 mm). The electrophoresis was performed at pH 8.6 with an acrylamide concentration of 10% for 9 h at 4 mA. A portion of the gel was stained for protein with Coomassie Blue R-250, and the remainder was sliced into 1.5-mm slices for elution with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl and 0.005% polyethylene glycol 6,000 (2 ml). After sterilizing the eluate by membrane filtration, 0.1-ml aliquots from each fraction were assayed for CSF. The colony-stimulating activity and protein profiles for the electrophoretogram of the protein and CSF, which fails to bind to concanavalin A-Sepharose, are shown in Fig. 6. A single peak of colony-stimulating activity (mobility relative to bromphenol blue equal to 0.15) was observed, but over 20 protein bands were apparent. The specific activity of the eluted M-CSF was over 10-fold higher than in the original YSCM. Fewer protein bands were observed when the M-CSF eluted from concanavalin A-Sepharose was subjected to electrophoresis under the same conditions. Both the unbound and bound M-CSF from concanavalin A-Sepharose chromatography migrated in an identical manner on electrophoresis. The recovery of M-CSF from the electropho-

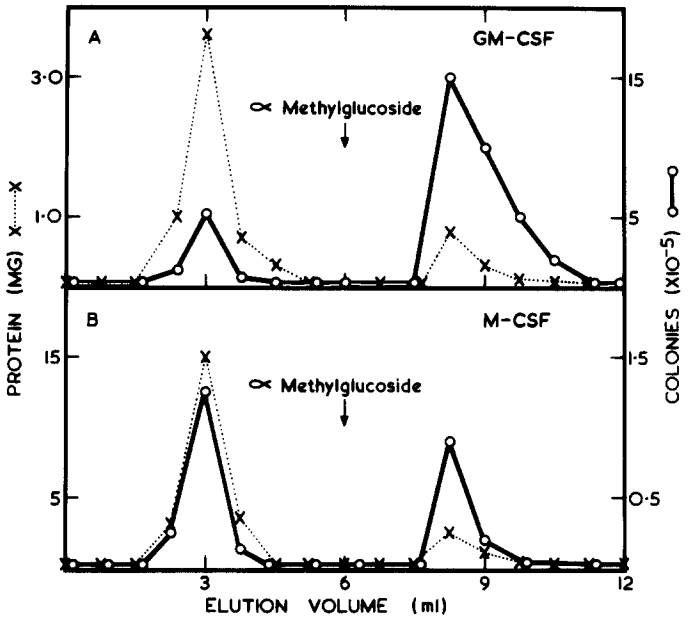

FIGURE 5 Concanavalin A-Sepharose chromatography of GM-CSF and M-CSF.

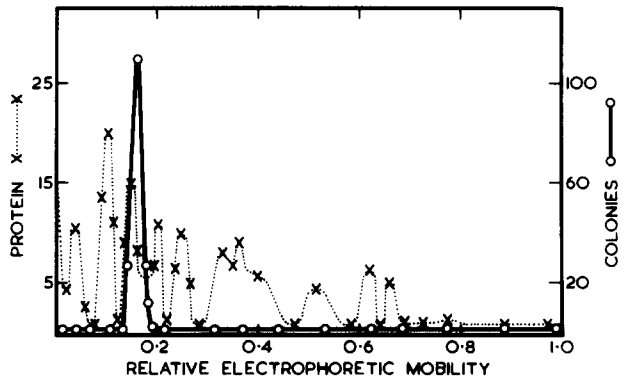

FIGURE 6 Polyacrylamide gel electrophoresis at pH 8.6 of M-CSF eluted from DEAE-Sepharose but which fails to bind to concanavalin A-Sepharose. The protein profile was determined by densitometry of the Coomassie Blue-stained bands, and the units on that axis are arbitrary. The CSF profile was determined by assaying 0.1 ml of the eluate from the gel slices in the standard semisolid agar system. Colony numbers are expressed per 10^5 CBA bone marrow cells.

resis of the protein bound to concanavalin A-Sepharose was <10% of the original activity (Table V).

Polyacrylamide gel electrophoresis (10%, total acrylamide) in the presence of sodium dodecyl sulfate (3%) of M-CSF specifically eluted from concanavalin A-Sepharose was used to estimate the molecular weight of M-CSF under dissociating conditions. After electrophoresis, the gel was sliced into 1.5-mm segments, each of which was analyzed for protein and colony-stimulating activity. (*NB:* Sodium dodecyl sulfate must be removed before biological assay because the detergent is toxic to bone marrow cells even at concentrations of 10^{-7} M; see Materials and Methods.) The distribution of colony-stimulating activity was rather broad, but there was a single peak of activity with a mobility relative to bromphenol blue equal to 0.33 (Fig. 7). This corresponds to an apparent mol wt of 60,000.

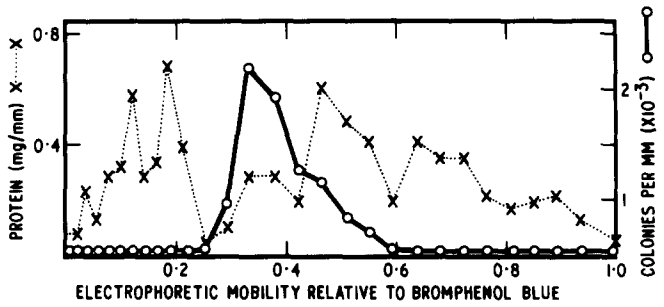

FIGURE 7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis at pH 8.6 of M-CSF specifically eluted from concanavalin A-Sepharose with methyl-α-D-glucopyranoside (0.05 M). Protein was measured colorimetrically using the method of Lowry et al. (7). CSF was measured in the standard semisolid agar system. Colony numbers are expressed per 10^5 CBA bone marrow cells.

DISCUSSION

Several reports have indicated that CSF may be obtained from various adult tissues, but the only source of fetal CSF at days 11–14 of gestation appears to be yolk sac (5). It was shown earlier that YSCM is able to stimulate colony formation from both fetal liver and adult mouse bone marrow cells (5). Although several of the adult CSF's described previously have a tendency to stimulate macrophage differentiation in preference to granulocyte differentiation, most of these studies have been complicated by the dependence of final colony morphology on the concentration of CSF used to stimulate colony formation in the semisolid agar assay (11). At low concentrations of CSF (or in the presence of inhibitors that suppress colony formation), CFC appear to favor differentiation into the macrophage line, whereas at higher concentrations of the same CSF, e.g. from MLCM, granulocyte differentiation is preferred (2, 11). Almost all extracts, tissue fluids, or conditioned media contain low concentrations of CSF or have sufficiently high levels of inhibitors to prevent maximal stimulation of mouse CFC. Without further purification or concentration, most of these sources of CSF cannot be used directly to study the cellular basis of, or commitment to, granulocyte or macrophage differentiation. M-CSF has been partially purified and concentrated (to remove some of the material inhibitory to colony growth) from YSCM concentrates. M-CSF can be used to stimulate maximal colony numbers from CBA bone marrow. Once the morphological distribution of colonies is independent of CSF concentration, it is possible to determine whether macrophage and/or granulocytic CFC as well as GM-CFC are present in a given cell suspension.

CFC that differentiate into macrophages tend to be larger than those cells that can be stimulated to form granulocytes and are also more responsive to submaximal GM-CSF stimulation (11). The size distribution for the CFC stimulated by M-CSF overlaps considerably with the size distribution of cells stimulated by GM-CSF. The sedimentation velocity of the fraction with the highest number of CFC stimulated by M-CSF was 5.7 mm/h which corresponds to the velocity of the cluster-forming cells stimulated by GM-CSF and the size of the cells stimulated to form colonies by low concentrations of human urine CSF (11).

These findings suggest that the more rapidly sedimenting cells, forming clusters with GM-CSF, are capable of colony formation when stimulated by M-CSF. Similarly, the increased number of colonies stimulated by GM-CSF, compared to M-CSF, from the small CFC could be due to either the presence of cells precommitted to granulocytic differentiation (e.g. multiple granulocytic CFC [11]) or to an increased rate of proliferation of the smaller CFC in the presence of GM-CSF.

When cultures are maximally stimulated by M-CSF, the presence of low concentrations of GM-CSF leads to the appearance of granulocytic colonies. Although the total number of colonies is not changed by the addition of GM-CSF (Table I), the morphological distribution changes toward granulocytic differentiation. This suggests that the same CFC are being stimulated by the two stimuli, but that there are receptors on some of the CFC that are available to GM-CSF but not to M-CSF. When these receptors are activated, the cell is directed toward granulocytic differentiation even in the presence of an excess concentration of the macrophage stimulator M-CSF. If a separate set of CFC committed to granulocyte differentiation

were present, the total colony numbers would be expected to increase at least two-fold in the cultures containing 60% granulocytic colonies in the presence of optimal concentrations of both stimuli (M-CSF and GM-CSF).

Short-term liquid culture of bone marrow cells in the presence of either GM-CSF or M-CSF alters the subsequent differentiation pattern of CFC in agar. Even a 24-h exposure of the CFC to M-CSF increases the commitment to macrophage differentiation, suggesting either that some of the granulocytic CFC die (e.g. multiple granulocytic CFC), that there is an increase in the number of macrophage CFC, or that the granulocytic surface receptors normally activated by GM-CSF are lost. However, in the presence of either M-CSF or GM-CSF, the number of CFC appears to remain constant during the first 24 h of liquid culture. Thus, it is more likely that the existing CFC become committed to macrophage differentiation in the presence of M-CSF. The morphology of cells within the early (2 day) clones stimulated by GM-CSF is different from that of cells stimulated by M-CSF. Cells stimulated by M-CSF give rise to clones comprised mainly of mononuclear cells even after days 2, 3, and 4 of culture, whereas the cells appearing as a result of GM-CSF stimulation appear to be mainly granulocytic, at least until day 4 of culture. This suggests that M-CSF is able to stimulate the CFC toward macrophage differentiation more quickly than GM-CSF. GM-CSF appeared to stimulate an initial burst of granulocyte differentiation which is gradually replaced by macrophage differentiation as the time of the culture is extended beyond 6 days. Experiments in which 2- and 3-day clones stimulated by M-CSF were transferred to agar cultures containing GM-CSF (Table III) indicated that GM-CSF was able to support the growth of all of the developing clones but that the clones had become committed to macrophage differentiation. The presence of GM-CSF in the initial agar cultures appeared to commit some of the CFC to granulocytic differentiation, as only 50% of the clones transferred to agar cultures containing M-CSF survived. After 2 days in agar culture, 80% of the GM-CSF-stimulated clones contained mostly granulocytic cells, yet by day 7 only 50% of the transferred clones appeared to be granulocytic. This supports the hypothesis suggested by Metcalf (9) that granulocytic cells may develop to form macrophages. The possibility that the few monocytic cells present in the 2-day clones proliferate to form macrophages whereas the granulocytic cells die cannot yet be excluded.

Only the CFC committed to macrophage differentiation or capable of conversion to macrophages in the presence of M-CSF were supported by M-CSF stimulation in the secondary cultures. The clones transferred from the initial cultures contained cells with differentiation states that are between the CFC and the mature cells. These activated CFC are no longer capable of differentiation toward both granulocytes and macrophages, but appear to be committed to one or the other of the differentiation pathways. However, it needs to be emphasized that genetic factors may also play a role because all of the present data were obtained with CBA mice. When the same preparations of M-CSF are used on C57Bl bone marrow cells, approximately 20% of day-7 colonies are composed of granulocytes.[2]

On gel filtration, the molecular weight of M-CSF eluted from DEAE-Sepharose appeared to be 60,000. However, this molecular weight determination is complicated by possible interactions with contaminating proteins. Electrophoresis under dissociating conditions indicated that the apparent molecular weight of this fetal CSF was indeed close to 60,000. This value is considerably higher than the 23,000 mol wt determined for GM-CSF (2) but is similar to the value reported for mouse L-cell CSF (16). Only a small proportion of the M-CSF eluted from DEAE-Sepharose appears to bind to concanavalin A-Sepharose. This indicated that the M-CSF was only partially glycosylated—a phenomenon frequently observed with glycoprotein hormones produced in vitro. Other CSF's have also been reported to be heterogeneous with respect to their ability to bind to concanavalin A-Sepharose (2, 15). The electrophoretic mobility and the molecular weight on gel filtration of both the unbound and bound colony-stimulating activity were the same, indicating that the molecules had quite similar physicochemical properties in spite of the difference in carbohydrate content. Although M-CSF eluted from concanavalin A-Sepharose had a considerably higher specific activity than the unbound M-CSF after purification by polyacrylamide gel electrophoresis, both fractions had similar specific activity. Thus, concanavalin A-Sepharose appears to be a useful analytical tool for the study of the glycosylation of M-CSF, but it is not an effective step during the initial purification of the molecule. In vitro culture conditions can affect the level of glycosylation,

[2] Johnson, G. R., and D. Metcalf. Unpublished observations.

and we are currently investigating these conditions to see whether the level of glycosylation can be increased.

The small amount of M-CSF likely to be present in YSCM will make the complete purification of this molecule difficult. However, highly active preparations will be available for the study of its action both in vitro and in vivo. The availability of this M-CSF should be particularly useful for the establishment of the relationships between granulocyte-macrophage, granulocyte, multiple granulocytic cells, and their corresponding progeny (cluster-forming cells) (11) in both bone marrow and fetal liver cell populations.

We are grateful for the help and guidance given to us by Dr. D. Metcalf. Excellent technical assistance has been provided by Mrs. Sue Delves, Mr. Rod Mitchell, and Ms. Kerry Haynes.

This work was supported by the Anti-Cancer Council of Victoria, the National Cancer Institute, U. S. A., contract no. NO1-CB-33854, National Health and Medical Research Council of Australia, Australian Research Grants Committee, and a Queen Elizabeth II Postdoctoral Fellowship awarded to Dr. A. W. Burgess.

Received for publication 19 August 1977, and in revised form 21 December 1977.

REFERENCES

1. BRADLEY, T. R., and D. METCALF. 1966. The growth of mouse bone marrow *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* **44**:287–300.
2. BURGESS, A. W., J. CAMAKARIS, and D. METCALF. 1977. Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *J. Biol. Chem.* **252**:1998–2003.
3. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Acad. Sci.* **121**:404–427.
4. ICHIKAWA, Y., D. H. PLUZNICK, and L. SACHS. 1966. *In vitro* control of the development of macrophage and granulocyte colonies. *Proc. Natl. Acad. Sci. U. S. A.* **56**:488–495.
5. JOHNSON, G. R., and D. METCALF. 1977. Sources and nature of granulocyte-macrophage colony stimulating factor in fetal mice. *J. Exp. Hematology*. In press.
6. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680–685.
7. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
8. METCALF, D. 1970. Studies on colony formation *in vitro* by mouse bone marrow cells. II. Action of colony stimulating factor. *J. Cell. Physiol.* **76**:89–100.
9. METCALF, D. 1974. Regulation by colony stimulating factor (CSF) of granulocyte and macrophage colony formation *in vitro* by normal and leukemic cells. *In* Control of Proliferation in Animal Cells. B. Clarkson and R. Baserga, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 887.
10. METCALF, D., T. R. BRADLEY, and W. ROBINSON. 1967. Analysis of colonies developing *in vitro* from mouse bone marrow cells stimulated by kidney feeder layers or leukemic serum. *J. Cell. Physiol.* **69**:93–108.
11. METCALF, D., and H. R. MACDONALD. 1975. Heterogeneity of *in vitro* colony- and cluster-forming cells in the mouse marrow: segregation by velocity sedimentation. *J. Cell. Physiol.* **85**:643–654.
12. MILLER, R. G., and R. A. PHILLIPS. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73**:191–201.
13. ORNSTEIN, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* **121**:321–349.
14. SHERIDAN, J. W., and D. METCALF. 1973. A low molecular weight factor in lung conditioned medium stimulating granulocyte and monocyte colony formation *in vitro*. *J. Cell. Physiol.* **81**:11–24.
15. STANLEY, E. R., M. CIFONE, P. M. HEARD, and V. DEFENDI. 1976. Factors regulating macrophage production and growth: identity of colony stimulating factor and macrophage growth factor. *J. Exp. Med.* **143**:631–647.
16. STANLEY, E. R., G. HANSEN, T. WOODCOCK, and D. METCALF. 1974. Colony-stimulating factor and the regulation of granulopoiesis and macrophage production. *Fed. Proc.* **34**:2272–2278.