

FACTORS REGULATING MACROPHAGE PRODUCTION AND GROWTH: IDENTITY OF COLONY-STIMULATING FACTOR AND MACROPHAGE GROWTH FACTOR*

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Study of the control of growth and differentiation of macrophages has been greatly facilitated in recent years by the development of assays for granulocyte-macrophage progenitor cells (1, 2) and for factors regulating *in vitro* growth and production of macrophages. Two factors whose properties and action on mouse cells have been described in some detail are macrophage growth factor (MGF)¹ (3-5), which stimulates the proliferation of peritoneal macrophages, and colony-stimulating factor (CSF) (6-8), which stimulates the differentiation of individual progenitor cells from bone marrow, spleen, and blood to colonies of granulocytes and macrophages. Both factors have similar properties, have been found in similar sources, and require the presence of serum for their *in vitro* effects. However, there are marked differences in the assay systems for MGF and CSF. In the MGF assay, adherent peritoneal cells from mice intraperitoneally injected with soluble starch 4 days previously are cultured on glass in liquid medium. In the presence of MGF, a proportion of these cells (up to 95%) incorporate [³H]thymidine and divide. The MGF activity is determined from autoradiographs and expressed as percent of total cells labeled (3). In the CSF assay, normal mouse bone marrow cells are cultured in semisolid medium. In the presence of CSF, approximately one-two hundredth of the plated nucleated cells can form colonies, and activity is determined by colony counts. Thus, for the MGF assay system the test appears to be a proliferative response, while for the CSF assay system the test is a response involving proliferation and differentiation.

In the mouse system, MGF has been found in medium conditioned by certain mouse fibroblastic cell lines and passaged embryo cells, but not from lines of rat, hamster, rabbit, monkey, or human origin or from mouse nonfibroblastic lines (4). It is also found in the ascitic fluid of mice (9) and in trypsin digests from mitotic and S-phase L cells (10).

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CFU-C, CSF-dependent colony-forming unit; Con A, Concanavalin A; CSF, colony-stimulating factor; FCS, fetal calf serum; MEM, minimal essential medium; MGF, macrophage growth factor; PBS, phosphate-buffered saline; PEG, polyethylene glycol; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone; V_e/V_0 , elution volume to void volume ratio.

MGF in L-cell-conditioned medium is resistant to inactivation by a variety of enzymes including nucleases and proteases and to heat treatment with periodate (3). CSF has been found in similar sources and also in the serum and urine of mouse and man. CSFs in the medium conditioned by L cells (11), mouse embryo fibroblasts (12), or human urine (8) share the properties mentioned above for MGF. In addition, it has been shown that CSFs from the latter sources are sialic acid-containing glycoproteins of mol wt 45,000-70,000 (8) with ion-exchange and gel-filtration characteristics similar to MGF (4, 8, 11, 12).

In view of these physical similarities and because of the early appearance of macrophages in mouse colonies stimulated by CSF from these sources (13), it was of interest to determine whether the MGF and CSF assay systems were detecting the same molecule(s). Part of this interest stems from the observations that CSF and MGF are produced in many immunological reactions in which macrophages and possibly macrophage proliferation are important (14-16). This paper demonstrates an identity of sources, biological properties, physicochemical behaviour, and reactivity to antibody for both MGF and CSF. Methods for the purification of active material from serum-free L-cell-conditioned medium and the preparation and some properties of antibody to this material are also described.

Materials and Methods

MGF Assay. 6-12-wk-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) were injected intraperitoneally with 1.5 ml of a 2% soluble starch suspension (Mann Research Labs, Inc., Orangeburg, N. J.) in phosphate-buffered saline (PBS). After 4 days, the mice were killed with ether, and their peritoneal cavities were washed with two 4-ml vol of minimal essential medium (MEM, Auto-Pow medium, Flow Laboratories, Inc., Rockville, Md.) containing 1% heparin. The cells were centrifuged (800 *g*, 10 min), washed twice in 1 vol of PBS, resuspended in MEM containing 10% FCS, counted in leukocyte counting fluid, and seeded in Lab Tek 8 chamber slides (Miles Laboratories, Inc. Elkhart, Ind.) at 5×10^4 nucleated cells/cm². 30 min later the medium was suctioned off to reduce the numbers of lymphocytes and fibroblasts in the culture. The attached cells were washed twice with PBS and refed. 1-4 days after seeding, culture medium in wells was replaced with 0.5 ml of medium containing samples and [³H]thymidine (sp act 52.2 Ci/mmol) at 0.2 μ Ci/ml. Medium used for preparation and incubation of cultures was TES-HEPES-buffered Eagles medium pH 7.6 (17) containing 10% "pretested" (5) fetal calf serum (FCS, Flow Laboratories Inc.), penicillin (100 U/ml), and streptomycin (0.05 μ g/ml). Duplicate culture wells were set up for each sample tested. After 5 days incubation at 37°C in 10% CO₂ in air in a humidified incubator, cells were washed with PBS, fixed in Carnoy's solution for 20 min and washed with ethanol. Dry slides were dipped in Kodak NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.), dried at room temperature, and left 2 days in sealed boxes containing a dessicant at 4°C for exposure before development with Kodak D-19 developer (Eastman Kodak). The developed slides were fixed in Amfix (May & Baker LTD., Montreal, Quebec) and stained with Giemsa. The percentage of labeled cells was determined microscopically under 400 magnification by counting at least 300 cells in each culture well.

CSF Assay. The techniques and media used in the agar culture of mouse bone marrow cells and the use of such cultures to assay CSF have been reported in full elsewhere (18). Sterile samples (<0.2 ml) were each mixed in duplicate 35-mm Petri dishes (Falcon Plastics, Oxnard, Calif.) with 1 ml of agar medium containing 75,000 nucleated bone marrow cells from adult LAF₁ mice (The Jackson Laboratory). The medium used in these experiments was alpha-MEM (19) including 5% (vol/vol) horse serum, 10% (vol/vol) fetal calf serum (Flow Laboratories), 10% (vol/vol) trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.), 75 μ g/ml DEAE dextran (Pharmacia, Uppsala, Sweden), 5×10^{-5} M mercaptoethanol, 0.4% Bacto-Agar (Difco Laboratories, Detroit, Mich.) and additional asparagine (20 μ g added/ml of medium), and glutamine (0.29 mg added/ml of medium). The dishes were incubated at 37°C in a fully humidified atmosphere of 10%

CO₂ in air (pH 7.2). After 7 days of incubation colony counts were performed at 25 magnification using dark field on an Olympus dissecting microscope. Colony size ranged from 50–2,000 cells.

Conditioned Media and Sources of Activity. Serum containing media conditioned by the following cell lines were used: Human WI38, normal embryonic fibroblasts (20); NS20, a cholinergic clone of mouse neuroblastoma cells (21); IC21, an SV₄₀-transformed line of peritoneal macrophages derived from C57BL/6J mice (22); L-5178Y, a mouse lymphoma induced by methylcholanthrene (23); and embryo fibroblasts from BALB/c mice (24) and L 60 T cells, a clonal isolate of mouse L cells (25) (for standard L-cell-conditioned medium). Ehrlich ascites fluid was prepared by intraperitoneal injection of 5×10^6 – 10^7 Ehrlich ascites cells (26) into 10–16-wk-old C57BL/6J male mice. The fluid was collected after 13 days, left at room temperature for 6 h, then centrifuged at 1,800 g, the supernate collected and filtered through a 0.22 μ m filter (Millipore Corp., Bedford, Mass.). Freund ascites fluid was prepared by intraperitoneal injection of 1.5 ml of complete Freund's adjuvant (CFA) (Difco Laboratories) into 10–16-wk-old C57BL/6J male mice. This ascites fluid was harvested in the same way as the Ehrlich ascites fluid.

The L-cell trypsin digest was prepared by treating confluent cultures in 160 cm² glass bottles with 0.1% trypsin (Flow Laboratories) 24 h after plating at 37°C. After 5 min, trypsinization of the cells was inhibited by addition of FCS; the medium was harvested and filtered through a 0.22 μ m filter (Millipore Corporation). Endotoxin mouse serum and normal mouse serum were separated from blood obtained from LAF₁ mice (Jackson Laboratory). For endotoxin mouse serum the mice were given an intraperitoneal injection of 5 μ g *Escherichia coli* lipopolysaccharide (Difco Laboratories) and bled 4 h later (27). Stage VI human urinary CSF was purified from human urine as described elsewhere (8) and possessed an activity of 3×10^7 colonies per mg protein. Extract of pregnant mouse uterus was prepared from pregnant BALB/c mice as described earlier (28).

Purification of CSF from L-Cell-Conditioned Medium. L cells were passaged in spinner cultures of alpha medium containing 10% FCS (Flow Laboratories). For production of serum-free-conditioned medium for purification of CSF, cells were collected from the spinner flask, washed twice in serum-free alpha medium, and seeded at 2×10^5 cells/ml in 75 cm² plastic bottles (Falcon Plastics) each containing 40 ml of serum-free alpha medium. After 7 days incubation at 37°C in 10% CO₂ in air in a humidified incubator, the medium was collected by decantation, concentrated approximately 10-fold by vacuum rotary evaporation (29), dialyzed against distilled water (3 \times 20 volume changes, 4°C), centrifuged (1,500 g, 10 min), and the precipitate discarded. The supernatant fluid was further concentrated to a small volume by vacuum rotary evaporation. 5–10 liters of conditioned medium could be made and processed in this way to yield a final volume of approximately 10 ml. This material, although substantially purified from the unconcentrated conditioned medium, was designated as starting material for the purification. The starting material was dialyzed against 0.1 M Tris-HCl pH 7.4 and chromatographed on a DEAE cellulose (Eastman Kodak) column (dimensions 107 \times 2.2 cm) using linear salt gradient (0–0.8 M NaCl in 0.1 M Tris-HCl pH 7.4) elution at 4°C (Fig. 3). Active fractions were pooled (Stage I material), concentrated, dialyzed against 0.03 M Tris-HCl, pH 7.4, and subjected to Sephadex G-200 (Pharmacia Fine Chemicals) gel-filtration (column dimensions 130 \times 2.6 cm) in that buffer at 4°C. At this stage the activity was usually found in two regions (elution volume to void volume ratio, $V_e/V_o = 1.3$ – 1.5 and $V_e/V_o = 1.7$ – 2.2) and fractions from each of these regions separately pooled (stages IIi and IIii, respectively), concentrated and dialyzed against Concanavilin A (Con A) buffer (0.1 M acetate pH 6.0 containing 1 M NaCl, 10^{-3} M MgCl₂, 10^{-3} M MnCl₂, 10^{-3} M CaCl₂, and 0.001% polyethylene glycol (PEG, 6,000). Both stage IIi and IIii material were chromatographed on Con A-sepharose (Pharmacia Fine Chemicals) (column dimensions 36 \times 1.0 cm) in Con A buffer at 4°C. After collection of the unadsorbed material, elution of material bound to the Con A-sepharose was accomplished using 0.1 M α -methyl-D-glucoside in Con A buffer (30). Activity was found in both the unadsorbed (IIIi) and bound (IIIii) fractions for pool IIi and similarly for pool IIii (IIIiii, unadsorbed; IIIiv, bound) (Fig. 3). All 4 stage III pools were concentrated to approximately 2.0 ml, dialyzed against distilled water, and stored at -20°C . Protein estimations (31) were carried out on all stages of each purification.

Each of the stage III fractions was subjected to gradient gel electrophoresis in a gradipore system (32) using preformed gradient gels (Ortec Inc., Oak Ridge, Tenn.). After electrophoresis of duplicate samples for 8 h at 75 V constant voltage, duplicate gel strips were cut out, one stained for protein with Coomassie Brilliant Blue (33), and the other fractionated using a razor blade device.

Each gel segment was eluted overnight in 1.5 ml of 0.01% PEG and the supernatant fluid assayed. Marker proteins (Schwarz/Mann, Orangeburg, N. Y.) radiolabeled with ^{125}I (34) were electrophoresed alongside samples and their position in the gel determined by counting ^{125}I in fractions.

Purification of MGF from a Tryptic Digest of L Cells. Similar methods were used for the purification of MGF from the tryptic digest of L cells. Stage I was Sephadex G-200 (Pharmacia Fine Chemicals) gel-filtration in 0.1 M Tris-HCl, pH 7.4, and stage II involved stepwise elution ion exchange chromatography on QAE Sephadex (Pharmacia Fine Chemicals), pooling material eluted with 0.3 M NaCl in 0.1 M Tris-HCl, pH 7.4.

Kinetics of Disappearance of MGF and CSF from Cultures used in their Assay. Replicate MGF cultures were set up with 20% L-cell-conditioned medium in the absence of $^3\text{HTdr}$, and the medium was removed for both MGF and CSF assays at various times afterwards. In addition, replicate 1-ml CSF cultures were overlaid with 1 ml of liquid medium and contained 20% L-cell-conditioned medium throughout. Periodically, liquid overlays were removed from cultures and assayed for MGF and CSF.

Treatments. The high molecular weight, Con A-Sepharose adherent fraction (stage IIIii L-cell CSF, 3.1×10^7 colonies/mg protein) was used for all experiments. Periodate treatment was carried out as described earlier (29). Incubation mixtures (0.9 ml) containing stage IIIii CSF (2.4 μg) and KIO_4 (0.01 M) in 0.1 M acetate-HCl, pH 5.0, were incubated for various times at 4°C in the dark. Reaction was stopped by addition of 0.1 ml of 50% sucrose. For heat inactivation, incubation tubes contained 0.8 μg of stage IIIii CSF in 250 μl of 0.03 M Tris-HCl, pH 7.3. Heating was at 90°C for various times. Treatment with α -chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.) was carried out as described earlier (35). Incubation tubes (28 μl) contained 0.8 μg of stage IIIii CSF and 16 μg α -chymotrypsin in 0.05 M CaCl_2 0.05 M Tris-HCl, pH 7.3. Incubation was at 37°C for various times, and the reaction was stopped by addition of 2.0 ml of 2% bovine serum albumin (BSA) and cooling to 4°C .

Fractionation Experiments. Stage IIIii L-cell-conditioned medium CSF (3.1×10^7 colonies/mg protein) was used for all experiments. In the gel-filtration experiments, trypsin treatment was effected by incubation of 15 μg stage IIIii CSF with 12.5 μg L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp.) in 0.05 M CaCl_2 0.05 M Tris-HCl pH 7.3 buffer (0.4 ml) in the presence of a trace amount of ^{125}I -BSA. A duplicate incubation was set up excluding trypsin. After 4 h incubation at 40°C , 0.2 ml of 10% BSA was added to each tube before their separate chromatography on Sephadex G-200 (column dimensions, 90×1.6 cm, conditions as for stage II of purification). Fractions were counted for ^{125}I before assay to establish that the trypsin was active under the conditions employed. Polyacrylamide disk electrophoresis of 5.0 μg stage IIIii CSF was performed in 7.8% polyacrylamide at a running pH of approximately 9.4 (36). Gradient gel electrophoresis of 12 μg (20 μg for staining) of stage IIIii CSF, of 200 μg of stage IIIii CSF, and the fractionation and assay of gels from the disk electrophoresis were carried out as described for stage III fractions from the purification.

Preparation and Testing of Rabbit Sera. An outbred rabbit was bled twice by heart puncture to provide 50 ml of prebled serum. It was then injected at multiple intradermal sites with stage IIIii L-cell-conditioned medium CSF (0.14 mg) emulsified in an equal volume of CFA (Difco Laboratories) (total injected volume 0.4 ml). 2 wk later an identical booster injection and thereafter further boosters of 0.14 mg of stage IIIii material were given in various ways at 1-2-wk intervals. At the same time, small blood samples were collected via the ear veins for determination of the serum neutralization titre. Testing of the antiserum was carried out by mixing preparations of MGF or CSF with the antiserum or the PBS-diluted antiserum and incubating at room temperature for 30 min before assaying samples in both assay systems.

Results

Dose Response Curves. Typical dose response curves for the standard L-cell-conditioned medium in both MGF and CSF assay systems are presented in Fig. 1. The CSF assay system at certain concentrations was approximately twice as sensitive as the MGF assay system. A sample causing almost maximal response in the CSF assay could show no or low response in the MGF assay because of the sigmoidal nature of the dose-response curve in the latter assay. Inhibition at

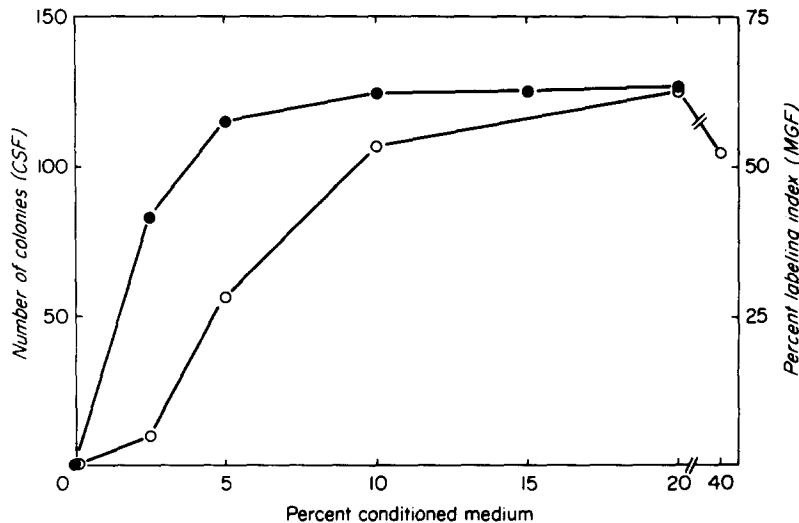


FIG. 1. Response to varying concentrations of L-cell-conditioned medium in the CSF assay (●) and the MGF assay (○). Each point represents the mean of counts on two plates or wells. Data for the MGF assay are plotted here and elsewhere as percent labeling index after subtraction of background labeling determined from cultures containing no added factor.

high doses of conditioned medium could be seen in the MGF assay dose response (4). These differences between the two assay systems are important for interpretation of the results of some of the following experiments.

Sources of Activity. A number of sources for both factors have been reported. Some sources such as ascites fluid and endotoxin mouse serum had been tested in only one system. Others such as normal mouse serum were only active in the CSF assay. To clarify the questions raised by these data, various sources that had been shown to be active or inactive in one of the two systems were tested at the same time for both CSF and MGF activity at different concentrations. The results are summarized in Table I. There was a complete correlation between assays for the presence or absence of activity in all of the various source materials assayed except for normal mouse serum where activity was low in the CSF assay and undetectable in the MGF assay.

Kinetics of Depletion of MGF and CSF Activities in both Culture Systems. One biological test for identity of MGF and CSF is to compare the kinetics of disappearance of both activities in the different culture systems. Activities of MGF and CSF in their respective assay systems have been shown to decrease with time of culture (5, 18). Transfer of medium from a test culture to fresh cells results in decreased activity in the recipient culture. Conditioned medium was incubated with either activated macrophages or mouse bone marrow cells, and the rate of disappearance of both CSF and MGF activities was measured. The results are summarized in Fig. 2. There was no significant difference between the kinetics of loss of CSF and the kinetics of loss of MGF during incubation with either cell population. Previous studies in both systems (5, 18) have shown that under the same incubation conditions in the absence of cells, both activities remain at initial levels.

TABLE I
Coincidence of MGF and CSF in Various Sources

Source	Activity	
	MGF	CSF
Human WI38 CM*	-	-
Mouse S-20 CM	-	-
Mouse IC-21 CM	-	-
Mouse L-5178Y CM	-	-
Mouse embryo fibroblast CM	+	+
Ehrlich ascites fluid	+	+
Freund adjuvant ascites fluid	+	+
L-cell trypsin digest	+	+
Endotoxin mouse serum	+	+
Normal mouse serum	-	±‡
Stage VI human urinary CSF (8)	+	+

* CM, conditioned medium.

‡ Low activity, below detectability in the MGF assay.

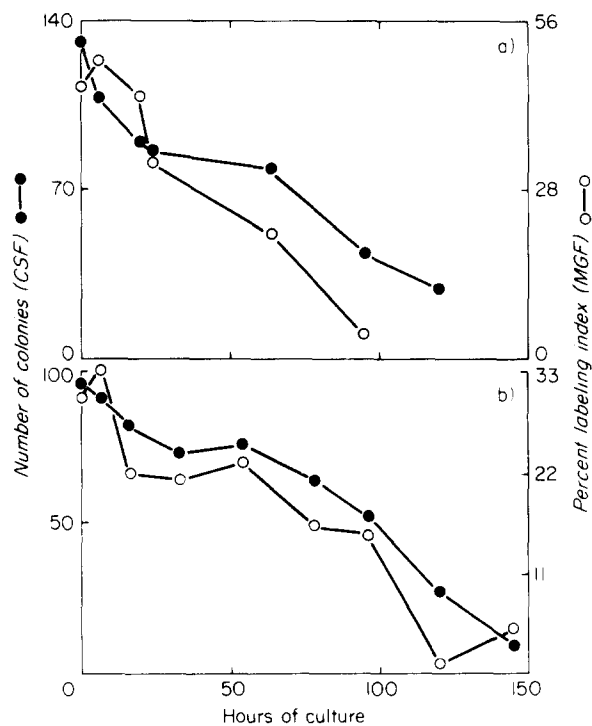


FIG. 2. Kinetics of disappearance of L-cell medium CSF (●) and MGF (○) during culture in the MGF (a) and CSF (b) assay systems. Each point represents the mean of counts on two plates or wells.

Copurification of CSF and MGF from L-Cell-Conditioned Media. Fractionation of L-cell-conditioned medium pools was carried out as summarized in Fig. 3. Activity of fractions was followed using the CSF assay. In two of three purifications, two peaks of activity were found at stage II, one of higher

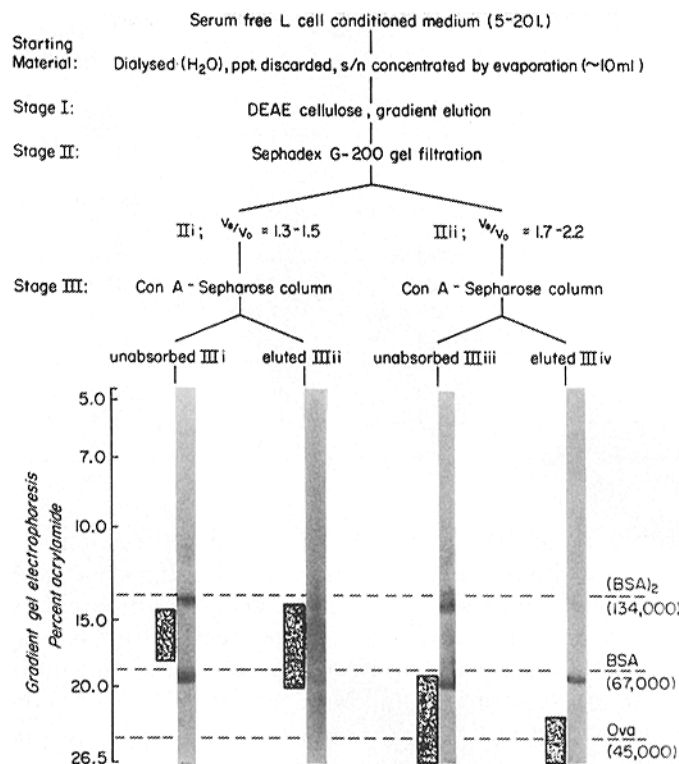


FIG. 3. Purification of CSF from serum-free L-cell-conditioned medium and analysis of the four active products by gradient gel electrophoresis. The banding of colony-stimulating activity for each of the stage III fractions is represented by the filled bands adjacent to the photographs of the stained sections of the gel slab. The migration of marker proteins included in the run is also indicated (Ova, ovalbumin; BSA, bovine serum albumin; (BSA)₂, BSA dimer).

apparent molecular weight (*i*) containing most of the activity and the other of lower apparent molecular weight (*ii*). Passage of stage II pools through Con A-sepharose columns resulted in further fractionation. In all cases most of the activity was adsorbed to Con A-sepharose. Material that was unadsorbed (stages III*i* and III*iii*) failed to bind when passed through newly prepared Con A-sepharose columns. Differences in the size characteristics of the activity and stainable protein in the four active fractions resulting from the fractionation can be seen in the gradient gel electrophoretograms presented in Fig. 3. Separation under the conditions used was almost entirely due to apparent size differences (32). The banding of activity in each electrophoretogram was broad and polydisperse compared with the banding of marker proteins. Results of a typical purification are summarized in Table II. It can be seen that most of the recovered activity was found in fraction III*ii* (high molecular weight, Con A adherent), that it represented a substantial proportion of the starting activity, and was the most purified fraction with respect to protein. This fraction was used in the inactivation experiments, in the fractionation experiments, and to raise antibody for the neutralization experiments.

TABLE II
Purification of L-Cell CSF from 5 Liters of Serum-Free-Conditioned Medium

Stage	Total volume	Total activity colonies $\times 10^{-4}$	Total protein	Yield	Specific activity
	<i>ml</i>		<i>mg</i>	% starting activity	colonies/mg $\times 10^{-5}$
SM*	12.00	1,780	30.35	100.0	5.9
I	6.50	1,530	5.50	86.0	27.8
IIi	2.45	1,320	3.19	74.2	41.4
IIIi	1.75	29	1.23	1.6	2.4
IIIii	1.34	72	2.68	4.0	2.7
IIIiii	1.75	1,090	≤ 0.35	61.2	≥ 311.4
IIIiv	0.28	6.4	≤ 0.50	0.4	≥ 1.3
IIIiv	0.31	6.5	≤ 0.40	0.4	≥ 1.6

* SM, starting material.

Results of simultaneous assays for MGF and CSF on fractions from purifications from L-cell-conditioned medium and a tryptic digest of L cells are presented in Table III. It can be seen that, considering the inherent variability of the assays, there was a close correlation between results from both systems. For these experiments fractions were diluted for assay with α -medium which was shown to result in loss of both CSF and MGF activity. This loss of activity could be prevented by dilution in 0.01% PEG (8), e.g., stage IIIii in α -medium, 460 colonies/ml, and in PEG, 23,200 colonies/ml.

Kinetics of Inactivation of Purified L-Cell Material. Previous studies on MGF (3) and CSF (12) from mouse sources have shown that they are inactivated by heat or periodate treatment and relatively resistant to treatment with α -chymotrypsin. There was no significant difference in the kinetics of inactivation between the MGF and CSF activities of stage IIIii material during incubation with α -chymotrypsin or periodate (Fig. 4) and during heating at 90°C (not shown). In contrast to results obtained with human urinary CSF (35), neither activity was lost on incubation with the high concentrations of α -chymotrypsin used. Similar kinetics to those for the inactivation by periodate were observed for the inactivation by heating at 90°C, 50% of both activities being lost after 45 min.

Fractionation of the CSF and MGF Activities of Purified L-Cell Material. The most purified fraction (stage IIIii) was used for these experiments. On Sephadex G-200 gel-filtration (Fig. 5 a) the band of MGF lay under the band of CSF. Austin et al. (11) reported an increase in elution volume on gel filtration of L-cell-conditioned medium CSF previously treated with trypsin. This finding was confirmed in experiments in which stage IIIii material was treated with trypsin before gel-filtration. Furthermore, trypsin treatment increased coordinately the elution volume of CSF and MGF activities (Fig. 5 b). Higher resolution analyses of the two activities in stage IIIii material were performed, separating according to charge by polyacrylamide disk gel electrophoresis and according to size by gradient gel electrophoresis. Cochromatography of MGF and CSF was observed on 6% polyacrylamide gel electrophoresis (Fig. 5 c) and on

TABLE III
 Copurification of MGF and CSF Activities from both L-cell-
 Conditioned Medium and a Trypsin Digest of L Cells

Stage	CSF	MGF
	colonies \times dilu- tion ⁻¹	% LI* \times dilu- tion ⁻¹
L-cell-conditioned medium		
I	220,200	≥ 800
IIi	42,200	≥ 300
IIIi	7,800	128
IIIi	17,300	254
IIIii	5,200	91
IIIiii	4,400	105
IIIiv	16,200	546
Tryptic digest of L cells‡		
SM§	ND	1,850
I	4,800	360
II	16,000	700

* LI, labeling index.

‡ Assayed independently of the L-cell-conditioned medium fractions; comparisons can be made within but not between the purifications.

§ SM, starting material.

|| ND, not done.

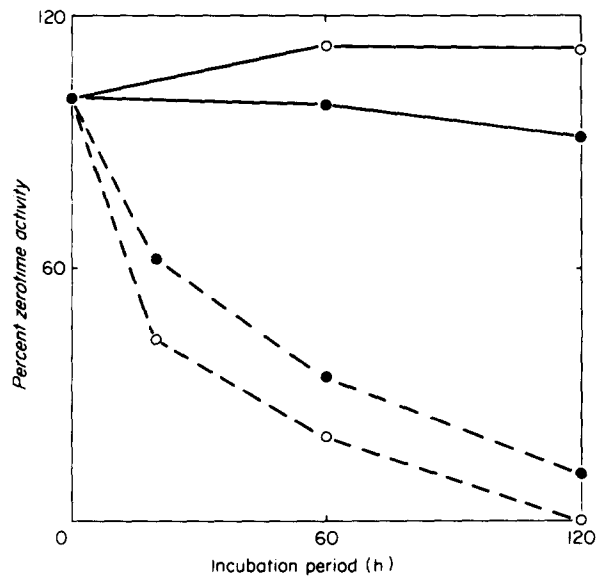


FIG. 4. Kinetics of inactivation of MGF (○) and CSF (●) activity of stage IIIii material (high molecular weight, Con A adherent) during incubation with α -chymotrypsin (continuous lines) and periodate (broken lines). Each point represents the mean of counts on two plates or wells.

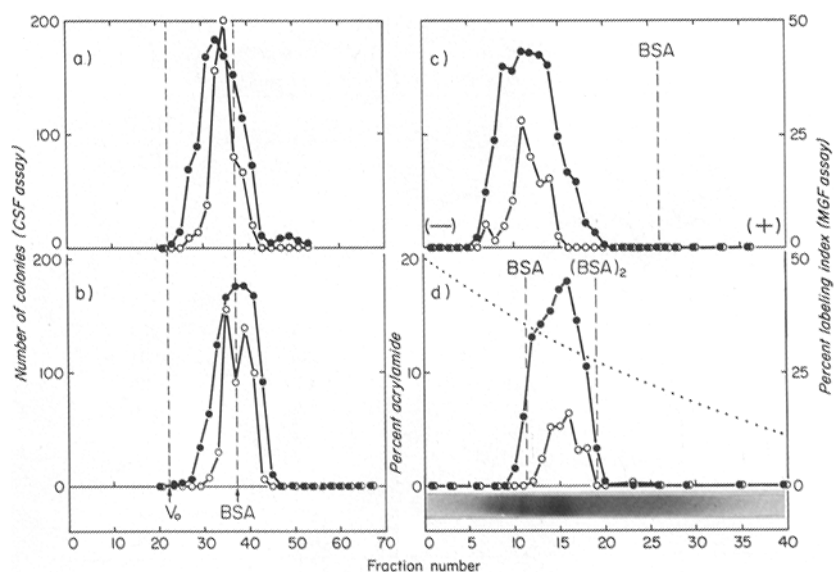


FIG. 5. Cochromatography of the CSF and MGF activities of stage III_{ii} material (high molecular weight, Con A adherent). The banding of MGF (○—○) and CSF (●—●) on gel-filtration on Sephadex G-200 before (a) and after (b) treatment with trypsin; on 7.8% polyacrylamide gel electrophoresis pH 9.4 (c) and on gradient gel electrophoresis (d). The positions of the peaks of marker proteins, bovine serum albumin (BSA; 67,000 daltons), and BSA dimer ((BSA)₂, 134,000 daltons) are also indicated. The banding of biological activity on gradient gel electrophoresis is shown against the Coomassie Brilliant Blue staining pattern for stage III_{ii} material.

gradient gel electrophoresis (Fig. 5 d) where the polydisperse activity bands coelectrophoresed with a polydisperse protein stained band. In addition, the MGF and CSF activities of stage III_{ii} material (high molecular weight, Con A nonadherent) were also shown to coelectrophorese on both disk electrophoresis and gradient gel electrophoresis (results not shown).

Kinetics of Antibody Production. Repeated injection of 0.14 mg of stage III_{ii} material with adjuvant into a rabbit produced an antiserum with neutralizing activity for L-cell-conditioned medium CSF and MGF and for human urinary CSF. The kinetics of production of the neutralizing activities of the antiserum are shown in Fig. 6. Neutralizing activity for CSF and MGF of L-cell-conditioned medium developed simultaneously. In contrast, the neutralizing activity for human urinary CSF took a longer time to develop and attained a lower but quite significant titre. There was no inhibitory effect of the prebled rabbit serum on the CSF and MGF assays at the highest concentration of antiserum used. In addition, the untreated antiserum at the highest concentration used had no direct effect on colony formation if preincubated with the target cells for 1 h at 0°C in the presence or absence of guinea pig serum (as a source of complement), indicating that the neutralizing activity produced was directed against the factors and not the cells.

Neutralization by Antiserum. The results of a neutralization titration of high titre antiserum against L-cell-conditioned medium CSF and MGF and

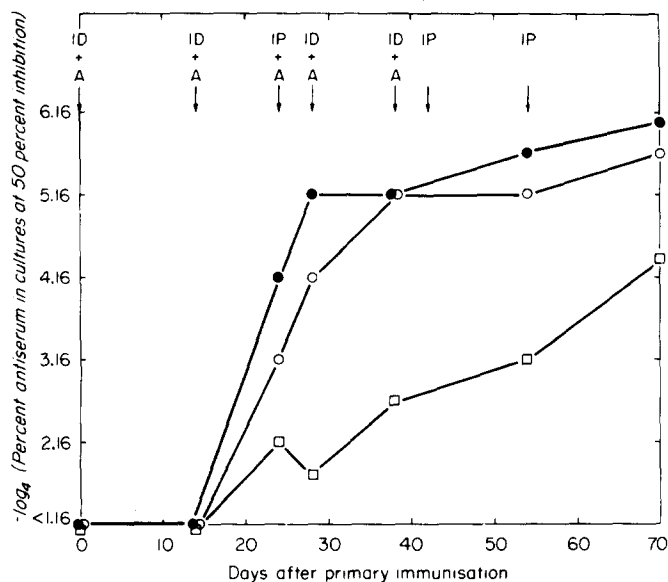


FIG. 6. Kinetics of antibody production in a rabbit given 0.14 mg of stage III_{iii} material (high molecular weight, Con A adherent) at the times indicated (ID, intradermally; IP, intraperitoneally; A, in 50% CFA). Ordinate expresses the final concentration of antiserum in cultures (vol/vol) required for neutralization of 50% of the activity of a near optimal dose of L-cell-conditioned medium (○—○, MGF assay; ●—●, CSF assay) or human urinary CSF (□—□).

human urinary CSF are shown in Fig. 7. Titration curves for L-cell medium CSF and MGF were coincidental and distinct from the curve for human urinary CSF.

Action of Antiserum on Murine Sources of CSF. The neutralizing activity of the antiserum for a number of sources of murine CSF was tested. With the exception of stage III_{iii} (low molecular weight, Con A nonadherent), all the stage III purification fractions (including trypsin-treated stage III_{iii}) were neutralized at equivalent concentrations of antiserum (0.0001% antiserum (vol/vol) for 50% reduction of colony numbers). There was only partial neutralization (40%) of stage III_{iii} material by the antiserum even at concentrations as high as 0.05%. The stimulating activities of mouse embryo-conditioned medium (24), extract of pregnant uterus (28), serum from mice injected with endotoxin (13), the Ehrlich ascites fluid, and the partially purified trypsin digest of L cells were completely neutralized by the antiserum. They exhibited identical neutralization curves with 50% reduction end points at 0.0001% antiserum.

Colony Formation by Peritoneal Cells. Lin and Stewart (37, 38) reported that thioglycollate-induced peritoneal cells form macrophage colonies in agar in the presence of L-cell-conditioned medium. These colonies were distinct from the CSF-dependent colony forming units (CFU-C) seen in bone marrow cultures, being smaller and appearing later by approximately 3 wk of culture. To test whether the factor stimulating colony formation by peritoneal cells was related to CSF, starch-induced and thioglycollate-stimulated (38) peritoneal cells were

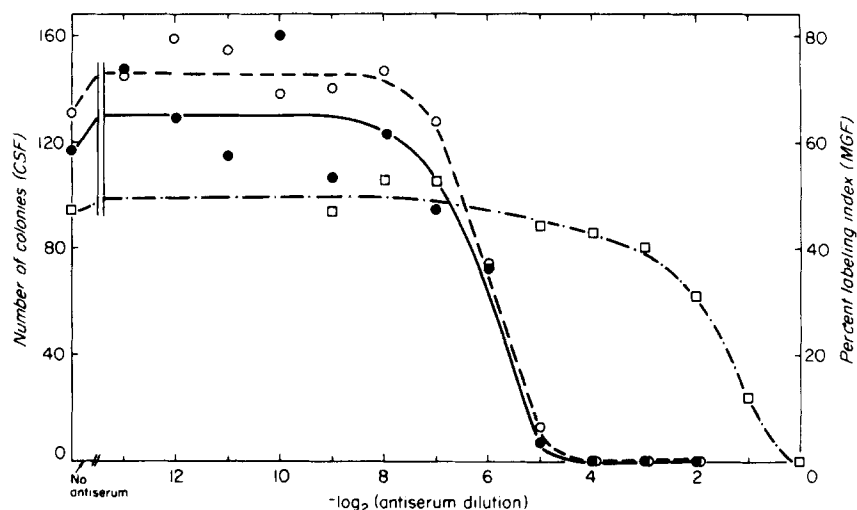


FIG. 7. Neutralization by antiserum to stage IIIii material (bled day 54). Abscissa expresses dilution of antiserum (1 vol) in tubes containing a fixed concentration of standard L-cell-conditioned medium or human urinary CSF (2 vol). Samples from tubes were assayed at 7.5% (CSF assay) or 15% (MGF assay). L-cell-conditioned medium MGF assay (○—○), CSF assay (●—●); human urinary CSF (□—□).

TABLE IV
*Colony Formation by Peritoneal Cells in Presence of Stage IIIii CSF**

Cell source	Cell no. plated/ ml	1-wk colo- nies‡	3-wk colo- nies§
<i>CFU-C</i>			
Starch-induced peritoneal cells	5×10^3	0	35
Thioglycollate-induced peritoneal cells	5×10^3	0	32
Bone marrow	7.5×10^4	180	ND

* Colonies did not appear in peritoneal cell or control bone marrow cultures in the absence of stage IIIii CSF.

‡ 500-2,000 cells/colony.

§ 50-100 cells/colony.

|| ND, not done.

plated with the most purified CSF fraction, stage IIIii. The results (Table IV) indicate that purified CSF stimulates colony formation by both sources of peritoneal cells, and that there was no colony formation of the CFU-C type observable at 1 wk of culture. In addition, quantitatively similar results to those shown in Table IV were obtained using an unpurified L-cell-conditioned medium.

Discussion

Very close similarity of CSF and MGF has been demonstrated by a number of biological, physicochemical, and immunological criteria. In no instance could

any significant difference between the two activities be demonstrated.

They were coordinately present or absent in the same sources. There was coordinate depletion or stability of activity of conditioned medium during incubation with peritoneal macrophages or bone marrow and after inactivation with heat, periodate, and α -chymotrypsin. Pooled, purified fractions from CSF and MGF purifications were active in both systems. Profiles of MGF and CSF activities of purified CSF from polyacrylamide gel electrophoresis and gradient gel electrophoresis were coordinate, as were elution profiles from G-200 chromatography before and after trypsin treatment. Antibody made in rabbits against the most purified material available appeared against CSF and MGF at the same time. In addition, the neutralization curves of CSF and MGF were identical using this antiserum. While absolute proof of identity must await chemical analysis of purified material, the data indicate that the MGF and CSF assays are probably detecting the same molecule. The chance that any two different glycoproteins would share the same source, heterogeneity on fractionation, physicochemical, biological, and antigenic properties, as do the molecules detected by the MGF and CSF assays, is quite remote. However, it is apparent that the CSF assay is more sensitive than the MGF assay, and this probably explains earlier findings (4) that indicated that certain preparations expected to contain CSF were inactive in the MGF assay.

The heterogeneity of the colony-stimulating activity of L-cell-conditioned medium may be related to its *in vitro* production, since other sources of CSF produced *in vitro* possess greater heterogeneity than CSF's from body fluids (12). L-cell-conditioned medium could be fractionated into four quite different molecular species described by their size and ability to bind Con A. While the lower molecular weight species (stages III_{iii} and III_{iv}) represented less than 5% of the total recovered activity its presence in L-cell -conditioned medium has not been previously reported. The relation of this fraction to the higher molecular weight fractions (stages III_i and III_{ii}) is not understood. Its gel-filtration behaviour is similar to the low molecular weight CSF produced *in vitro* by mouse lungs in the presence of endotoxin (39-41). The specific activity of the most purified fraction, stage III_{ii}, (approximately 90% of the total activity recovered at stage III) was significantly higher than that reported for other mouse sources of CSF and MGF (5, 11, 12), except for the low molecular weight material in the medium conditioned by mouse lung in the presence of endotoxin (40). The gradient gel electrophoresis experiment indicates that stage III_{ii} material is not completely purified, although a protein band coelectrophoresed with the peak of activity. This band possessed an apparent mol wt of 70,000-120,000 daltons, whereas the high molecular weight, Con A-adherent fraction from another purification had an apparent mol wt of approximately 60,000 daltons. Both fractions have MGF activity and the 70,000-120,000 dalton band is the result of an association of the 60,000 dalton CSF with protein(s) (E. R. Stanley and P. M. Heard, manuscript in preparation). Similar behaviour has been observed for human urinary CSF (42).

The activity of highly purified human urinary CSF in the MGF assay is not surprising when one considers its physicochemical and immunological similarity to L-cell-conditioned medium CSF. These findings appear to enlarge the reported species specificity of MGF (4).

Our studies have been concerned with a particular type of CSF and should not be taken to indicate that all CSF's possess MGF activity. In human urinary CSF and L-cell-conditioned medium CSF-stimulated murine marrow cultures there is an early appearance of macrophages in colonies (13). Furthermore, L-cell-conditioned medium CSF and human urinary CSF in their most purified states are glycoproteins of mol wt 45,000-70,000 which migrate electrophoretically with the α -globulins and albumin. They are physicochemically distinct from the CSF stimulating eosinophilic colony formation (43) which migrates electrophoretically with the γ -globulins, a low molecular weight CSF (44, 45), and a high molecular weight CSF (46) (Stanley, unpublished observations) stimulating human granulocytic colony formation. Inasmuch as stage III β material stimulates the formation of small macrophage colonies (37, 38) from thioglycollate- or starch-induced peritoneal cells, it is probable that the factor in L-cell-conditioned medium responsible for stimulation in these systems is CSF (MGF). The relation of CSF (or MGF) to the factors in L-cell-conditioned medium responsible for stimulating the growth of alveolar macrophages in liquid cultures (47) has not been investigated, but it is possible that the one factor is responsible for growth of macrophages in all culture systems.

The results presented here indicate that macrophages, from an undifferentiated precursor cell to the mature differentiated macrophage, are under regulation by the same molecular species. If the molecular species responsible for CSF and MGF activity are the same, the difference then appears to reside in the test cells. Activated macrophages have a longer doubling time than CFU-C in the presence of the factor, and they respond maximally at twice the factor concentration required for maximal response of CFU-C. If receptor molecules are involved in the stimulation by CSF or MGF, an attractive hypothesis would be that their distribution or density change as the target cells progress to the final macrophage state. The differences in response would then not be due to different substances, but to cells of the same lineage, which, at different stages of their maturation, are programmed to respond by proliferating according to different concentrations of the same group of substances.

Positive regulation of macrophage production ought to be homeostatically linked to the requirements of the organism. In this context, it is of interest to consider the release of CSF and MGF. Peritoneal macrophages from unstimulated mice are a good source from which the release of CSF is largely antigen dependent (48). Maximal release of CSF from adherent macrophages occurs within 3 h of exposure to endotoxin, and the degree of release is related to endotoxin concentration (48). The release is only slightly inhibited by puromycin (49) compatible with a cell surface localization of CSF, as reported for MGF on L cells (10). It is attractive to consider the regulation of macrophage differentiation in terms of positive feedback via the antigen concentration-dependent release of a regulator from mature macrophages.

The CSF-MGF system offers a potentially valuable means of analyzing the precise operation of a regulator molecule on eukaryote cell differentiation and proliferation. In addition, since CSF levels are elevated quickly and dramatically on antigen stimulation, it is pertinent to the study of immunological systems and disease states.

Summary

The activities of a colony-stimulating factor (CSF), which stimulates granulocyte-macrophage colony formation by mouse hemopoietic cells, and macrophage growth factor (MGF), which stimulates proliferation of activated peritoneal macrophages, have been demonstrated by various criteria to reside in the same molecular species. These criteria include occurrence in various sources and copurification of the activities in mouse L-cell-conditioned medium as well as the biological, physicochemical, and antigenic properties of the activities of L-cell-conditioned medium. CSF and MGF activities of L-cell-conditioned medium are ascribable to a glycoprotein of mol wt approximately 60,000 which migrates electrophoretically with α -globulin. Human urinary CSF, which also possesses MGF activity, has similar properties and can be neutralized by antiserum to highly purified L-cell medium CSF. A procedure is described for the partial purification of material from L-cell medium that has activity at 1 ng/ml in both MGF and CSF assays.

Note Added in Proof. Further purification of L-cell-conditioned medium CSF has resulted in a preparation that is pure as assessed by isoelectric focusing, SDS polyacrylamide gel electrophoresis, and immunological criteria. The purified CSF possesses MGF activity and stimulates colony formation by thioglycolate-induced peritoneal cells (E. R. Stanley and P. M. Heard, manuscript in preparation).

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References

1. Pluznik, D. H., and L. Sachs. 1965. The cloning of normal "mast" cells in tissue culture. *J. Cell. Physiol.* 66:319.
2. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* 44:287.
3. Virolainen, M., and V. Defendi. 1967. Dependence of macrophage growth *in vitro* upon interaction with other cell types. *Wistar Inst. Symp. Monogr.* 7:67.
4. Mauel, J., and V. Defendi. 1971. Regulation of DNA synthesis in mouse macrophages. I. Sources, action and purification of the macrophage growing factor (MGF). *Exp. Cell Res.* 65:33.
5. Mauel, J., and V. Defendi. 1971. Regulation of DNA synthesis in mouse macrophages. II. Studies on mechanisms of action of the macrophage growth factor. *Exp. Cell Res.* 65:377.
6. Metcalf, D., and M. A. S. Moore. 1971. *Haemopoietic Cells*. North-Holland Publishing Company, Amsterdam.
7. Metcalf, D. 1973. Regulation of granulocyte and monocyte-macrophage proliferation by colony stimulating factors (CSF): a review. *Exp. Hematol.* 1:185.
8. Stanley, E. R., G. Hansen, J. Woodcock, and D. Metcalf. 1975. Colony stimulating factor and the regulation of granulopoiesis and macrophage production. *Fed. Proc.* 34:2272.
9. Cifone, M., P. Mocarelli, and V. Defendi. 1975. *In vivo* production of a macrophage growth factor. *Exp. Cell Res.* 96:96.
10. Cifone, M., and V. Defendi. 1974. Cyclic expression of a growth conditioning factor (MGF) on the cell surface. *Nature (Lond.)* 252:151.

11. Austin, P. E., E. A. McCulloch, and J. E. Till. 1971. Characterization of the factor in L-cell conditioned medium capable of stimulating colony formation by mouse marrow cells in culture. *J. Cell. Physiol.* 77:121.
12. Stanley, E. R., T. R. Bradley, and M. A. Sumner. 1971. Properties of the mouse embryo conditioned medium factor(s) stimulating colony formation by mouse bone marrow cells grown *in vitro*. *J. Cell. Physiol.* 78:301.
13. Metcalf, D. 1969. Studies on colony formation *in vitro* by mouse bone marrow cells. I. Continuous cluster formation and relation of clusters to colonies. *J. Cell. Physiol.* 74:323.
14. McNeill, T. A. 1973. Release of bone marrow colony stimulating activity during immunological reactions *in vitro*. *Nat. New Biol.* 244:175.
15. Parker, J. W., and D. Metcalf. 1974. Production of colony-stimulating factor in mixed leucocyte cultures. *Immunology.* 26:1039.
16. Parker, J. W., and D. Metcalf. 1974. Production of colony-stimulating factor in mitogen-stimulated lymphocyte cultures. *J. Immunol.* 112:502.
17. Eagle, H. 1971. Buffer combinations for mammalian cell culture. *Science (Wash. D. C.)*. 174:500.
18. 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.
19. Stanners, C. P., G. L. Eliceiri, and H. Green. 1971. Two types of ribosome in mouse-hamster hybrid cells. *Nat. New Biol.* 230:52.
20. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25:585.
21. Amano, T., F. Richelson, and M. Nierenberg. 1972. Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci.* 69:258.
22. Mauel, J., and V. Defendi. 1971. Infection and transformation of mouse peritoneal macrophages by Simian Virus 40. *J. Exp. Med.* 134:335.
23. Fischer, G. A. 1958. Studies of the culture of leukemia cells *in vitro*. *Ann. N. Y. Acad. Sci.* 76:673.
24. Bradley, T. R., and M. A. Sumner. 1968. Stimulation of mouse bone marrow colony growth *in vitro* by conditioned medium. *Aust. J. Exp. Biol. Med. Sci.* 46:607.
25. Till, J. E., G. F. Whitmore, and S. Gulyas. 1963. Deoxyribonucleic acid synthesis in individual L-strain mouse cells. II. Effects of thymidine starvation. *Biochim. Biophys. Acta.* 72:277.
26. Loewenthal, H., and G. Jahn. 1932. Übertragungsversuche mit carcinomatöser Mäuse-Ascites flüssigkeit und ihr Verhalten gegen physikalische und chemische Einwirkungen. *Zeitsch. F. Krebsforsch.* 37:439.
27. Metcalf, D. 1971. Acute antigen-induced elevation of serum colony stimulating factor (CSF) levels. *Immunology.* 21:427.
28. Bradley, T. R., E. R. Stanley, and M. A. Sumner. 1971. Factors from mouse tissues stimulating colony growth of mouse bone marrow cells *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* 49:595.
29. Stanley, E. R., and D. Metcalf. 1969. Partial purification and some properties of the factor in normal and leukaemic human urine stimulating mouse bone marrow colony growth *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* 47:467.
30. Asperberg, K., and J. Porath. 1970. Group-specific adsorption of glycoproteins. *Acta Chem. Scand.* 24:1839.
31. 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.
32. Margolis, J., and K. G. Kenrick. 1968. Polyacrylamide gel electrophoresis in a continuous molecular sieve gradient. *Anal. Biochem.* 25:347.

33. Chrambach, A., R. A. Reisfeld, M. Wyckoff, and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Anal. Biochem.* 20:150.
34. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ¹³¹I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114.
35. Stanley, E. R., and D. Metcalf. 1971. Enzyme treatment of colony stimulating factor: evidence for a peptide component. *Aust. J. Exp. Biol. Med. Sci.* 49:281.
36. Davis, B. J. 1964. Disc electrophoresis. III. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121:404.
37. Lin, H-S., and C. C. Stewart. 1973. Colony formation by mouse peritoneal exudate cells *in vitro*. *Nat. New Biol.* 243:176.
38. Lin, H-S., and C. C. Stewart. 1974. Peritoneal exudate cells. I. Growth requirement of cells capable of forming colonies in soft agar. *J. Cell. Physiol.* 83:369.
39. 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.
40. Sheridan, J. W., and D. Metcalf. 1974. Purification of mouse lung-conditioned medium colony stimulating factor (CSF). *Proc. Soc. Exp. Biol. Med.* 146:218.
41. Sheridan, J. W., D. Metcalf, and E. R. Stanley. 1974. Further studies on the factor in lung-conditioned medium stimulating granulocyte and monocyte colony formation *in vitro*. *J. Cell. Physiol.* 84:147.
42. Stanley, E. R., and D. Metcalf. 1971. The molecular weight of colony-stimulating factor (CSF). *Proc. Soc. Exp. Biol. Med.* 137:1029.
43. Metcalf, D., J. Parker, H. M. Chester, and P. W. Kincade. 1974. Formation of eosinophilic-like granulocytic colonies by mouse bone marrow cells *in vitro*. *J. Cell. Physiol.* 84:275.
44. Price, G. B., E. A. McCulloch, and J. E. Till. 1973. A new human low molecular weight granulocyte colony stimulating activity. *Blood.* 42:341.
45. Price, G. B., J. S. Senn, E. A. McCulloch, and J. E. Till. 1974. Heterogeneity of molecules with low molecular weight isolated from media conditioned by human leukocytes and capable of stimulating colony formation by human granulopoietic progenitor cells. *J. Cell. Physiol.* 84:383.
46. Price, G. B., J. S. Senn, E. A. McCulloch, and J. E. Till. 1975. The isolation and properties of granulocytic colony-stimulating activities from medium conditioned by human peripheral leucocytes. *Biochem. J.* 148:209.
47. Soderland, S. C., and Y. Naum. 1973. Growth of pulmonary alveolar macrophages *in vitro*. *Nature (Lond.)*. 245:150.
48. Eaves, A. C., and W. R. Bruce. 1974. *In vitro* production of colony-stimulating activity. I. Exposure of mouse peritoneal cells to endotoxin. *Cell Tissue Kinet.* 7:19.
49. Sheridan, J. W., and D. Metcalf. 1973. CSF production and release following endotoxin. In *Hemopoiesis in Culture*. W. A. Robinson, editor. U. S. Government Printing Office, Washington, D. C. 135.