London, Ser. B 166, 124.
Reisfeld, R. A., & Small, P. A. (1966) *Science 152,* 1253.
Schroeder, W. A., & LeGette, J. (1953) *J. Am. Chem. Soc. 75,* 4612.
Segal, D. M., Padlan, S. A., Cohen, G. H., Silverton, E. W., Davies, D. R., Rudikoff, S., & Potter, M. (1974) *Progress in Immunology II* (Brent, L., & Holborow, J., Ed.) Vol. 1, p 93, North-Holland Publishing Co., Amsterdam.
Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., & Ballantyne, D. L. (1971) *Biochemistry 10,* 4912.
Smyth, D. G. (1967) *Methods Enzymol. 11,* 214.
Strosberg, A. D., Margolies, M. N., & Haber, E. (1975) *J. Immunol. 115,* 1422.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53,* 624.
Tosi, R., & Landucci-Tosi, S. (1973) *Contemp. Top. Mol. Immunol. 2,* 79.
Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science 178,* 871.
Wu, T. T., & Kabat, E. A. (1970) *J. Exp. Med. 132,* 211.

Purification and Characterization of a Colony Stimulating Factor from Human Lung†

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ABSTRACT: Conditioned medium prepared from human autopsy lung tissue contains high level activity of colony stimulating factor which stimulates granulocytes and macrophage colony formation in both mouse and human bone marrow. The lung colony stimulating factor has been purified about 2250-fold by methods including hydroxylapatite chromatography, preparative gel electrophoresis, preparative isoelectric focusing, and gel filtration chromatography. The final specific activity was 2.7×10^6 units/mg. The purified factor has a molecular weight of 41 000 as determined by gel filtration. It is stable at the pH range of 6.5–10 and at 56 °C for 30 min but sensitive to protease digestion and periodate oxidation. On polyacrylamide gel electrophoresis, it migrates in the α-globulin post-albumin region. Upon isoelectrofocusing lung colony stimulating factor appears heterogeneous with isoelectric points of 3.7–4.3. Treatment with neuraminidase did not affect its activity, but caused a change in electrophoretic mobility and isoelectric point. Antibody produced by immunizing rabbits with partially purified lung colony stimulating factor exerted strong inhibitory activity on the factor from lung as well as on colony stimulating factor from other human sources including serum, urine, and placenta.

During the past decade, the study of the mechanism involved in the regulation of granulopoiesis has been facilitated by the introduction of in vitro assay techniques. A method for cloning bone marrow cells in soft agar cultures was reported, independently, by Pluznik & Sachs (1965) and Bradley & Metcalf (1966). When colony forming cells in the marrow are grown in this assay system, they proliferate to form colonies composed of macrophage and/or granulocytic cells. The formation and development of these colonies depend on the presence of an inducing substance which has been called colony stimulating factor or CSF[1] (Metcalf & Foster, 1967; Paran & Sachs, 1968). Hence the target cell for CSF has been termed CFU-C or colony forming unit-CSF dependent. Because of its essential role in the formation of macrophage and granulocytic colonies in vitro, CSF has been postulated to be the primary regulator of myeloid production in vivo (Metcalf, 1973).

CSF can be isolated from human serum (Foster et al., 1968) and urine (Stanley & Metcalf, 1969) and a variety of human tissues such as lung (Fojo, 1977; Fojo et al., 1977), placenta (Ratzan & Yunis, 1974), and peripheral leukocytes (Iscove et al., 1971). Medium conditioned by these tissues will stimulate granulopoiesis in vitro.

CSF has been partially purified from medium conditioned by human peripheral leukocytes (Price et al., 1975) and human placenta (Burgess et al., 1977b). It has been purified to apparent homogeneity from human urine (Stanley & Metcalf, 1973), mouse lung (Burgess et al., 1977a), and cultured mouse cells (Stanley & Heard, 1977; Guez & Sachs, 1973).

Clearly the apparent role of CSF in the control of granulopoiesis and its disorders has added great impetus to the study of this poietin. In spite of the wide distribution of CSF in various tissues, the sources of human CSF have been limited; thus, most of the studies dealing with in vitro human bone marrow culture have utilized peripheral leukocyte-feeder layer as a source of CSF. Curiously human urinary CSF stimulates CFU-C growth in mouse but is weakly active in human bone marrow (Metcalf, 1974). We have found that serum free conditioned medium prepared from autopsy human lung provides a rich source of CSF which stimulates granulocytic and macrophage growth from both mouse and human marrow (Fojo et al., 1977). In this paper we report further purification

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[1] Abbreviations used: CSF, colony stimulating factor, CFU-C, colony forming unit-CSF dependent; HLCM, human lung conditioned medium; SF-DME, serum-free Dulbecco's modified Eagle's media.

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and characterization of CSF from human lung-conditioned medium as well as the production of antibody against this factor.

Methods and Materials

Human lung tissue obtained at autopsy was kindly provided by Dr. Arkadi Rywlin and his associates at Mt. Sinai Hospital, Miami Beach, Florida. Dulbecco's modified Eagle's medium (H-21), fetal calf serum, horse serum, penicillin, and streptomycin were purchased from GIBCO, Grand Island, N.Y. Ultrogel, Ampholine, and Ultrodex were from LKB, Rockville, Md. Hydroxylapatite (Bio-Gel HTP), acrylamide and bis-(acrylamide) were from Bio-Rad, Richmond, Calif. Endotoxin (*Salmonella typhosa*), neuraminidase (type IX, *Clostridium perfringens*), α-chymotrypsin (type II, bovine pancreas), myoglobin (type III, horse heart), bovine serum albumin (fraction V), subtilisin (type VII, *Bacterium amyloliquefaciens*), and trypsin (type II, bovine pancreas) were from Sigma, St. Louis, Mo. Carrier-free ^{125}I (NEZ 033H) was from New England Nuclear, Boston, Mass. All other chemicals were of reagent grade.

Assay of Colony Stimulating Factor. The method developed by Bradley & Metcalf (1966) was used with slight modification (Ratzan et al., 1974). Mouse bone marrow cells were obtained from C57 BL/6J inbred mice and human bone marrow cells were obtained from the posterior iliac crest of normal volunteers. Cells were added to the medium-agar preparation to give 1×10^5 cells/mL (mouse) or 2×10^5 cells/mL (human) and the mixture was plated in 1-mL aliquots. CSF was added directly to the plates and was always assayed at two concentrations (0.05 mL and 0.1 mL or 0.1 mL and 0.2 mL). Plates were then incubated at 37 °C in a humidified incubator with 10% CO_2 in air for 7 days (mouse) or 10 days (human). Colony counts were performed using a dissecting microscope; aggregates of 50 cells or more were scored as colonies. A unit of CSF activity was arbitrarily defined as the amount of CSF which stimulates the formation of one colony under the specified conditions of the assay. For morphologic identification, individual colonies were aspirated with a fine tip Pasteur pipet and stained with 0.6% orcein in 60% acetic acid.

Preparation of Human Lung Conditioned Medium (HLCM). Human autopsy lung tissue, uninvolved with disease (8–18 h postmortem), was collected under sterile conditions and placed in 150 × 25 mm Falcon plastic Petri dishes, where it was minced with sterile scissors. The pieces were washed once with the serum-free Dulbecco's modified Eagle's media (SF-DME) containing 10 units of penicillin and 10 μg of streptomycin per mL and then distributed among 1-L tissue culture bottles, at approximately 0.1 g of tissue per mL of SF-DME. Endotoxin at a concentration of 50 μg/mL was also present during the incubation.

The bottles were then incubated at 37 °C in a humidified incubator gassed with 10% CO_2 in air. At the end of the incubation period (2 days), the medium was removed and centrifuged at 9000*g* for 30 min in order to remove cellular debris. The supernatant was heated at 56 °C for 30 min to inactivate complement after which it was again centrifuged at 9000*g* for 30 min. The supernatant from this last run was then dialyzed against distilled water (4 × 20 volume changes for 48 h at 4 °C). The precipitated material was removed by centrifugation at 9000*g* for 30 min and the supernatant was sterilized by filtering through millipore filters (0.45 μm) before assay.

Preparation of CSF from Other Sources. Human placental CSF was prepared by the same method used to prepare human lung conditioned medium. Placenta was obtained under sterile conditions immediately after delivery. For the preparation of urinary CSF, various urine specimens from a patient with immune neutropenia were collected, pooled, dialyzed, and concentrated after centrifugation. When tested in human marrow, this urine yielded relatively small colonies compared with human lung CSF but was considered adequate for immunological cross reactivity studies (vide infra). Human serum CSF was prepared from blood obtained from the patient with immune neutropenia. Serum was dialyzed against distilled water and extracted with chloroform to remove CSF inhibitor according to the procedure described by Granstrom (1974). Mouse lung CSF was prepared according to a procedure previously described (Ratzan et al., 1974).

Hydroxylapatite Chromatography. HLCM (628 mL) prepared by procedures described above was applied to a hydroxylapatite column (2.5 × 21 cm; bed volume, 100 mL) which was previously equilibrated with 0.01 M phosphate buffer, pH 6.5. The column was immediately washed with 500 mL of 0.06 M phosphate buffer. The "breakthrough" effluent and the buffer wash were combined and concentrated by ultrafiltration on Amicon (PM 10 membrane).

Preparative Gel Electrophoresis. The procedure of Jovin et al. (1964) was used with a Buchler preparative gel electrophoresis apparatus using 6.5% acrylamide. The active concentrated sample (15 mL) from hydroxylapatite column was dialyzed against stacking gel buffer and applied to the gel. Electrophoresis was carried out in 5 mM Tris–glycine buffer (pH 8.6) at 30–40 mA for 15 h. Eluates from the gel were collected in an automatic fraction collector (2.5 mL/fraction). Fractions were assayed for protein and CSF activity after dialysis against water.

Preparative Flat-Gel Isoelectrofocusing. All active fractions from preparative gel electrophoresis were pooled and concentrated to 3 mL by Amicon ultrafiltration using a PM 10 membrane and then dialyzed against distilled water overnight. The sample was then subjected to preparative flat-bed isoelectrofocusing in granulated gel (Ultrodex) as described in LKB Application Note No. 198 (Winter et al., 1975). The sample was applied to the Ultrodex gel bed as a narrow zone and isoelectrofocusing was conducted overnight. Each fraction was eluted with 5 mL of 0.1 M NaCl containing 0.01% Tween 20. Eluates were dialyzed against distilled water, sterilized by filtration, and assayed for CSF activity using mouse marrow. The pH of each fraction was determined prior to dialysis and absorbance at 280 nm measured after dialysis.

Gel Filtration on Ultrogel AcA44. One milliliter of concentrated material from the previous step was applied to an Ultrogel AcA 44 column (1.1 × 100 cm, bed volume 100 mL) equilibrated with a solution of 0.1 M NaCl containing 0.01% Tween 20, 5 units of penicillin, and 5 μg of streptomycin per mL. Elution was accomplished with the same buffer collecting 1-mL fractions. Protein profile was monitored by absorbance at 280 nm and CSF activity was assayed as described using mouse marrow. For molecular weight determination the column was calibrated with standard markers including Blue Dextran 2000 (V_0), bovine serum albumin (mol wt 68 000), chymotrypsinogen (mol wt 27 000), myoglobin (mol wt 16 000), and phenol red (V_R).

Radioiodination. The procedure of Greenwood et al. (1963) was used in the radioiodination of purified CSF. Purified HLCM-CSF from Ultrogel column (2.7×10^6 μm/mg of protein) was dialyzed against 0.05 M phosphate buffer pH 7.5 and concentrated to 0.1 mL by dialysis against a saturated sucrose solution. A mixture containing CSF (100 μL, 32 μg protein), carrier-free ^{125}I (5 μL, 384 mCi/mL), and Chloramine-T (100 μL, 4 mg/mL) was incubated at 0 °C for 5 min,

and the reaction was stopped by the addition of 100 μL of a 10 mg/mL solution of sodium bisulfite and 100 μL of 0.1 M potassium iodide. The iodinated CSF was then purified by gel filtration on Sephadex G-25 column (1.3 × 100 cm) equilibrated with 0.15 M NaCl, 5 mM phosphate buffer (pH 7.5) containing 0.01% Tween 20. About 50% of the CSF activity was recovered from this step.

Polyacrylamide Gel Electrophoresis. Analytical gel electrophoresis was carried out in a Canalco Model 1200 apparatus according to the procedure of Davis (1964). Protein bands were located by staining the gel with 0.25% amido black in 7.5% acetic acid and destaining the background in 7.5% acetic acid. To locate the radioactivity and CSF activity, the gel was cut into 2-mm slices. The gel slices were counted in a Nuclear Chicago γ counter for radioactivity and then extracted with 0.1 M NaCl containing 0.01% Tween 20, penicillin (40 units/mL), and streptomycin (40 μg/mL) for 24 h. The extracts were then dialyzed and assayed.

Protein Concentration. Protein concentrations were determined by the method of Lowry et al. (1951) or that of Schaffner & Weissman (1973). Bovine serum albumin was used as a protein standard.

Characterization. The following characterization experiments were performed using a CSF pool consisting of the most active fractions obtained from preparative gel electrophoresis (specific activity 1.8×10^5 units/mg). CSF assays were done using mouse marrow cells.

Heat Stability. One milliliter of HLCM-CSF was incubated at the desired temperatures (25–90 °C) for 30 min or 100 °C for 5 min. The samples were then chilled in ice and centrifuged at 9000*g* for 15 min in order to remove any precipitated material. Each sample was then sterilized and assayed.

pH Stability. The stability of HLCM-CSF at various pH values was determined by incubating 1 mL of the CSF solutions with 0.1 mL of a buffer solution containing 27.44 g of NaOH, 7.08 g of boric acid, 12.8 g of citric acid, and 8.5 mL of phosphoric acid in 200 mL final volume and adjusted to the appropriate pH by either HCl or NaOH. The samples were incubated for 24 h at 4 °C after which no significant changes in pH were noted. Their pH was then adjusted to 7.4 by extensive dialysis against 0.1 M Tris-HCl buffer, pH 7.4, followed by dialysis against water. Samples were then sterilized and assayed.

Stability to Protease, Neuraminidase, and Periodate. The stability of HLCM-CSF was determined by incubating 1 mL of the CSF solutions for 3 h at 37 °C with the appropriate agent. In each case the protein ratio of the CSF solutions to enzyme was 1:1 and 1:10. For trypsin (150 units/mg), chymotrypsin (60 units/mg), and subtilisin (9 units/mg), 0.01 M Tris-HCl buffer with 0.01 M $CaCl_2$ (pH 8.0) was used. Neuraminidase (1 unit, 32 units/mg) and 5 mM sodium periodate were incubated in 0.04 M acetate buffer, pH 5.0. Reactions were stopped by immediately lowering the temperature to 5 °C and the addition of 100 μL of fetal calf serum. The pHs of the fractions were adjusted to pH 7.4 by dialysis against 0.1 M Tris-HCl buffer, pH 7.4. The samples were then dialyzed against distilled water, filtered, and assayed. With each enzyme tested, three controls were included: (a) enzyme alone, incubated for 3 h at 37 °C in the appropriate buffer; (b) CSF alone, similarly incubated; and (c) enzyme and CSF added simultaneously to the culture plates prior to the addition of bone marrow cell–agar mixture.

Isoelectric Focusing of Neuraminidase Treated HLCM. A four-hundred microliters sample of HLCM-CSF equilibrated with 0.04 M acetate buffer, pH 5.0, was incubated at 37 °C for 4 h in the presence of 1 unit of neuraminidase activity (32 units/mg). Control was treated in the same way without enzyme. After the incubation, all samples were dialyzed against distilled water and isoelectric focusing on 7.5% polyacrylamide gel was conducted according to the method of Wrigley (1971). The gels were removed from the tubes and cut into 5-mm slices which were extracted with 0.5 mL of distilled water containing bovine serum albumin (1 mg/mL), penicillin (10 units/mL), and streptomycin (10 μg/mL) for 24 h. The extracts were dialyzed against distilled water and assayed for activity using mouse bone marrow. In order to determine the position to which neuraminidase migrated, the gel slices were also tested for neuraminidase activity by the method of Warren (1959).

Antibody Production. HLCM-CSF (1.8×10^5 units/mg) was used to immunize one male New Zealand rabbit weighing approximately 4 kg. Weekly injections, administered subcutaneously and intraperitoneally, consisted of 1 mL containing 150 000 units of CSF mixed with 2 mL of Freund's adjuvant. After 4 weeks of such immunizations the rabbit was bled from the central ear artery. Subsequently, the animal was boostered weekly followed by bleeding. Seven unimmunized rabbits were also bled and their sera pooled to be used as control.

Partial Purification of the IgG Fraction from Rabbit Serum. Sera from control and immunized animals were heated at 56 °C for 30 min and the globulin fraction was isolated by precipitation in 33% ammonium sulfate. This was further fractionated by DEAE-cellulose chromatography to obtain an enriched IgG fraction (Fahey, 1967).

Inhibition of CSF Activity by Antibody. The enriched IgG peaks obtained by DEAE-cellulose chromatography from both control and immunized rabbit sera were tested for inhibitory activity. Increasing volumes of the IgG fraction were added to a constant amount of CSF in the assay plate and the final volumes kept constant by the addition of sterile saline. This mixture was incubated for 5 min before the addition of the DME–agar–cell mixture. All CSF preparations were adjusted to approximately 1000 units of CSF activity per mL before testing.

Results

Purification of Human Lung CSF. A typical purification profile is summarized in Table I. Mouse marrow was used routinely for assay. However, the final pool from each step was also assayed in human marrow.

Hydroxylapatite Chromatography. In preliminary work it was demonstrated that CSF could be adsorbed to hydroxylapatite gel and eluted with 0.06 M sodium phosphate. This step resulted in a 4.7-fold purification and 70–90% yield. In this particular preparation the specific activity from the hydroxylapatite step was 5925 units/mg compared with 1200 units/mg in the starting material (~5-fold purification).

Preparative Gel Electrophoresis. Although some fractions from this step had a high specific activity representing as much as 100-fold purification (Fojo, 1977), it was necessary to sacrifice purity in order to maintain a workable yield. Thus, the fractions containing part of the large albumin peak were included in the pool resulting in only 15-fold purification. Nevertheless, preparative gel electrophoresis removed a considerable amount of contaminant protein and contributed significantly to the excellent results obtained in subsequent purification steps.

Preparative Isoelectrofocusing. A typical profile of this step is shown in Figure 1. The area of CSF activity falls in the pH range of 3.7–4.5 with partial overlap with a large protein peak. The latter consists predominantly of a protein with the electrophoretic mobility of albumin. The active fractions excluding

TABLE I: Purification of HLCM-CSF.

sample	source of marrow cells for assay	total protein (mg)	total act. (units)	specific act. (units/mg)	fold purification	recovery (%)
1. crude HLCM-CSF	mouse	565.4	678 500	1 200	1	100
	human		373 164	660	1	100
2. hydroxylapatite chromatography	mouse	103.0	610 650	5 925	5	90.0
	human		311 266	3 022	4.6	83.4
3. preparative gel electrophoresis	mouse	25.8	444 300	17 200	14.3	65.5
	human		279 569	10 836	16.4	74.9
4. preparative isoelectrofocusing	mouse	0.12	124 875	1.04×10^6	867	18.4
	human		79 200	0.66×10^6	1000	21.2
5. Ultrogel AcA44	mouse	0.032	86 000	2.7×10^6	2250	12.7
	human		57 600	1.8×10^6	2727	15.4

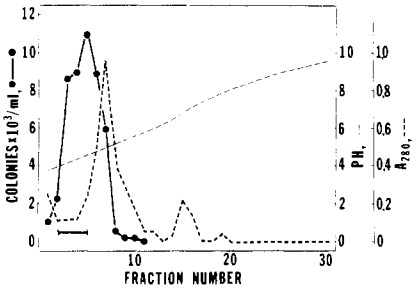

FIGURE 1: Preparative flat bed isoelectrofocusing. Three milliliters of HLCM-CSF sample from preparative gel electrophoresis was applied to isoelectrofocusing as described in Materials and Methods. Fractions were dialyzed against distilled water and 0.1 mL of the sample was diluted to 1.0 mL with 5% fetal calf serum (FCS) and then 0.2 mL was assayed in mouse marrow.

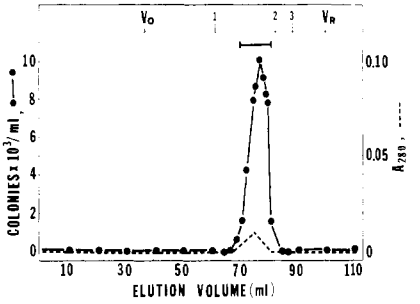

FIGURE 2: Gel filtration on Ultrogel AcA44. Concentrated sample (1 mL, 0.12 mg, 124 875 units) from preparative isoelectrofocusing was loaded on an Ultrogel AcA44 column (1.3 × 100 cm, column volume = 100 mL) for fractionation as described in Materials and Methods. Samples from each fraction were diluted 1 to 10 with 5% FCS, and 0.1 mL of each of the diluted samples were then assayed in mouse marrow. Standard proteins were: (1) bovine serum albumin; (2) chymotrypsinogen; (3) myoglobin.

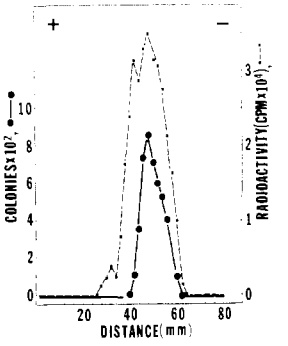

FIGURE 3: Gel electrophoresis of purified HLCM-CSF. One hundred microliters of ^{125}I-iodinated purified CSF (8600 units, 1.6×10^6 cpm) was subjected to gel electrophoresis in 7.5% gel as described in text. Assay of radioactivity and CSF activity were carried out as described in Materials and Methods.

the "albumin" peak were pooled and concentrated. This step resulted in 60-fold purification with an 18% yield.

Gel Filtration. A typical gel filtration profile is shown in Figure 2. This step resulted in approximately 3-fold purification. The overall purification from the starting material was about 2250-fold with a final specific activity of 2.7×10^6 units/mg as assayed in mouse marrow and 1.8×10^6 units/mg as assayed in human marrow. The purity of the final HLCM-CSF preparation was examined in polyacrylamide gel electrophoresis after radioiodination with ^{125}I. As shown in Figure 3, three radioactivity peaks were observed with the major one superimposed on the CSF activity peak suggesting 40–60% purity of the CSF.

Morphology of Colonies. HLCM-CSF (1.8×10^5 units/mg) stimulated the growth of both mouse and human CFU-C yielding a sigmoidal dose response pattern as crude HLCM-CSF shown previously (Fojo et al., 1977; Fojo, 1977).

When the cells from different colonies were examined, three kinds of colonies—granulocytic, macrophagic, and mixed—were observed. Similar to CSF from other sources (Burgess et al., 1977a), HLCM-CSF when assayed at high concentrations, in mouse or human marrow, stimulated the formation of predominantly granulocyte colonies. However, a reduction in the CSF concentration caused an increase in the proportion of macrophage colonies (Table II). There was no apparent difference in the types of colonies stimulated by crude HLCM-CSF vs. purified HLCM-CSF.

Molecular Weight. As calculated from gel filtration on Ultrogel AcA44 shown in Figure 2, the estimated molecular weight of HLCM-CSF was about 41 000. This value is in agreement with a molecular weight of 42 000 obtained from sucrose density sedimentation techniques (Fojo, 1977).

Temperature Stability. As illustrated in Figure 4, HLCM-CSF was stable at 50 °C for 30 min but lost approximately 50% of activity at 60 °C and most of the activity at 70 °C.

pH Stability. Figure 5 demonstrates the stability of HLCM-CSF at various pH conditions. It is stable over the pH range of 6.5–10.5. At pH 4.5, 45% of the activity still remained after a period of 24 h; however, a sharp decrease in the stability was observed at the pH above 10.5.

TABLE II: Effect of Varying Concentrations of HLCM-CSF on the Morphology of Human Bone Marrow Colonies.[a]

CSF source	amount of CSF (mL)	mean no. of colonies	% of colonies		
			granulocytic	mixed	macrophage
crude HLCM-CSF	0.20	96 ± 5	57	23	20
	0.15	68 ± 6	45	26	29
	0.10	34 ± 2	34	26	40
	0.05	21 ± 2	26	23	52
purified HLCM-CSF	0.20	101 ± 4	55	23	22
	0.15	71 ± 2	45	20	35
	0.10	40 ± 3	34	22	44
	0.05	21 ± 3	27	20	53

[a] Crude and purified HLCM-SCF (1.8×10^5 units/mg) were assayed at varying concentrations in human bone marrow cultures. Colonies were counted and analyzed (by acetoorcein stain) on day 10 of incubation. Mean number of colonies for three replicate cultures ± standard deviations are shown. At least 30 colonies were analyzed from each type of culture.

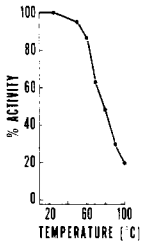

FIGURE 4: Temperature stability of purified HLCM-CSF. HLCM-CSF was incubated at the indicated temperatures for 30 min and then assayed in mouse bone marrow cultures using 0.1 mL of CSF per plate. One hundred percent activity was equivalent to 142 colonies.

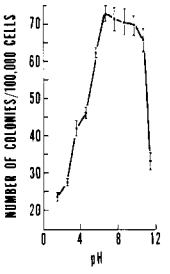

FIGURE 5: pH stability of HLCM-CSF. HLCM-CSF was incubated at the indicated pHs for 24 h as described in Materials and Methods. Each sample was then assayed in mouse bone marrow cultures using 0.1 mL of CSF per plate. Bars represent the deviation of duplicate plates from the mean number of colonies.

Stability to Proteolytic Enzymes, Neuraminidase, and Periodate. The effects of proteolytic enzymes, neuraminidase, and periodate on HLCM-CSF are summarized in Table III. The CSF activity was destroyed by incubation with subtilisin, chymotrypsin, and periodate but was not affected by treatment with neuraminidase.

Isoelectric Focusing on Neuraminidase Treated HLCM-CSF. The migration patterns of CSF on isoelectric focusing, before and after treatment with neuraminidase, are shown in Figure 6. Untreated CSF migrated to values in the pH range of 3.2–4.3, and to a position 3.5–5.5 cm into the gel. However, after treatment with neuraminidase, a shift in the location of activity became evident: most of the activity migrated to a position 3.5 cm into the gel and to an isoelectric pH value of 4.3. Neuraminidase was found to migrate separately, at a position 2.5 cm into the gel and a pH value of 4.6 (not illustrated).

Anti-HLCM-CSF Antibody and Its Cross-Reactivity with

TABLE III: Stability of Purified HLCM-CSF to Proteases, Neuraminidase, and Periodate.

	% act. of enzyme:CSF	
	1:1	1:10
control[a]	100	100
subtilisin	0	25
chymotrypsin	0	15
trypsin	10	42
neuraminidase	101	102
periodate	0	0

[a] Two controls were included: (1) CSF incubated without enzyme; (2) CSF and enzyme added simultaneously to the culture plate prior to the addition of bone marrow cell in agar mixture. Note: The addition of enzyme alone to the culture plate does not stimulate colony growth.

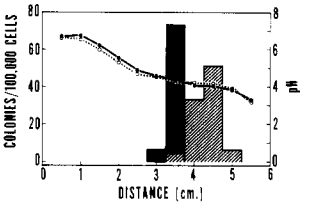

FIGURE 6: Isoelectric focusing of neuraminidase treated CSF. HLCM-CSF incubated in the presence or absence of neuraminidase (1 unit/mL) was subjected to isoelectric focusing as described in Materials and Methods. pH gradient: (O- - -O) with neuraminidase; (●—●) without neuraminidase. CSF activity: (■) solid area with neuraminidase; (▨) shaded area without neuraminidase.

CSF from Other Sources. The concentrated IgG fractions from both control and immunized rabbit sera were tested for their inhibitory activity against HLCM-CSF (Figure 7). Virtually complete inhibition of CSF activity by the "immune" IgG was observed in both mouse and human marrow. No inhibition was seen from control IgG. The antibody was equally inhibitory against CSF of human serum, urine, and placenta as assayed in both human (Figure 8) and mouse bone marrow (Figure 9). There was little or no cross-reactivity with mouse lung. Preincubation of bone marrow cells with antibody for 1 h followed by washing had no effect on CFU-C growth (not illustrated).

The antibody to CSF prepared in these studies, while inhibitory to CSF activity, did not produce a precipitin reaction

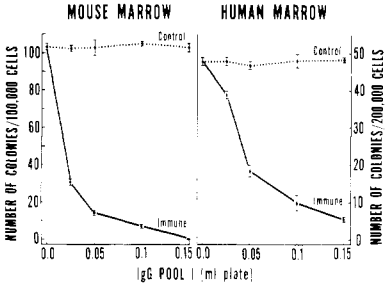

FIGURE 7: Inhibition of CSF activity by IgG fraction. Increasing volumes of IgG fraction from DEAE-cellulose were added to a constant amount (50 μL) of HLCM in the assay plates. Final volumes were kept constant by the addition of sterile saline. Both "immune" and control IgG fractions had a protein concentration of 13.5 mg/mL.

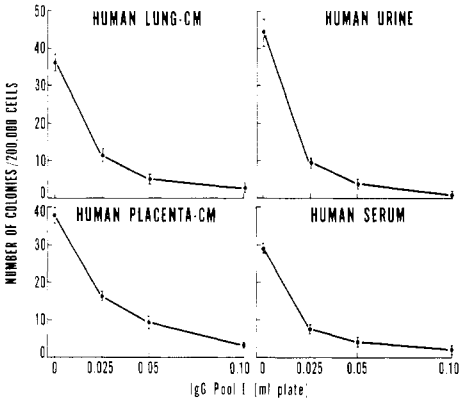

FIGURE 8: Cross-reactivity of anti-human lung CSF IgG with CSF from other sources as assayed in human bone marrow. HPCM-CSF, human urinary CSF, and human serum CSF were all tested for cross reactivity with antibody against HLCM-CSF as assayed in human bone marrow cultures. Increasing volumes of IgG fraction from DEAE-cellulose were added to a constant amount (50 μL) of the CSF preparation in the assay plates. Final volumes were kept constant by the addition of sterile saline.

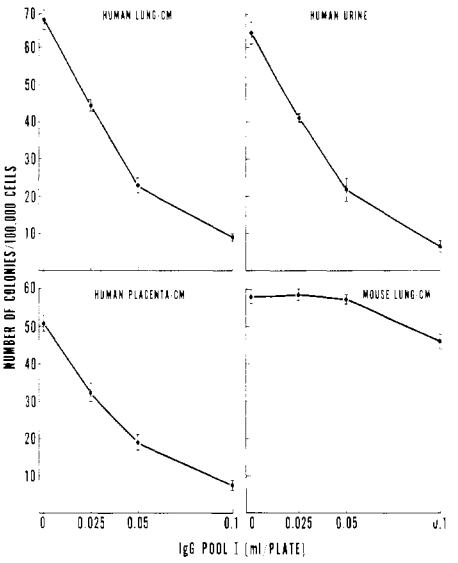

FIGURE 9: Cross-reactivity of anti-human lung CSF IgG with CSF from other sources as assayed in mouse bone marrow. HPCM-CSF, MLCM-CSF, and human urinary CSF were tested for cross-reactivity with antibody against HLCM-CSF in mouse bone marrow cultures. Increasing volumes of IgG fraction from DEAE-cellulose were added to a constant amount (50 μL) of the CSF preparation in the assay plates. Final volumes were kept constant by the addition of sterile saline.

TABLE IV: Binding of [^{125}I]CSF to Its Antibody.

	CSF act. in supernatant (no. of colonies/ 0.2 mL)	radioact. (cpm)	
		precipitate	supernatant
control (PBS)	73 ± 5	1 012 ± 43	10 145 ± 89
control IgG	64 ± 4	1 890 ± 36	9 709 ± 107
immune IgG	8 ± 2	10 214 ± 102	2 378 ± 53

[a] Iodinated HLCM-CSF was incubated with "immune" or control IgG followed by precipitation with ammonium sulfate. The precipitate and supernatant were then assayed for radioactivity and the supernatant for CSF activity.

when added to the antigen. Thus, in order to further demonstrate the specific nature of the antibody, the following experiment was performed. Iodinated HLCM-CSF (0.8 mL; 400 units; 1.2×10^4 cpm) was incubated in 0.2 mL of phosphate-saline solution containing "immune" or control IgG (8.6 mg/mL) for 2 h at 37 °C. Saturated ammonium sulfate solution was then added to a final concentration of 33%. The total radioactivity of the precipitate and supernatant was determined. The supernatant solution was then dialyzed against distilled water and assayed for CSF activity. The results shown in Table IV indicate that when CSF is incubated with its antibody greater than 80% of the radioactivity was recovered in the ammonium sulfate precipitate and the supernatant contained only about 10% of the total CSF activity. By contrast CSF activity and radioactivity remained in the supernatant in control samples.

Discussion

Medium conditioned by human autopsy lung has been found to stimulate the in vitro proliferation of specific progenitor cells to form colonies of granulocytes, macrophages, or both. This finding provides all interested with a rich and readily available source of human CSF. Human lung conditioned medium had a specific activity of 400–1000 units/mg as assayed in human marrow and 600–1500 units/mg as assayed in mouse marrow. This specific activity is higher than that of human placenta conditioned medium (Burgess et al., 1977b), human urine (Stanley et al., 1975), and approximately the same specific activity as in human peripheral leukocyte conditioned medium (Price et al., 1974). The lung cell type responsible for the production of CSF is unknown; however, a likely candidate is the alveolar macrophage which has previously been shown to be a rich source of CSF (Golde et al., 1972).

The combined procedures of hydroxylapatite chromatography and preparative gel electrophoresis provided excellent initial steps in the purification as previously reported (Fojo et al., 1977). Although purification by the preparative gel electrophoresis step amounted to only threefold, this step indeed removed most of the slower migrating proteins and thus made the next step, preparative isoelectrofocusing, more effective in further purification. The preparative isoelectrofocusing not only had a high resolution power but also a very high loading capacity; up to 300 mg of protein in 4.5 mL of solution could be applied in a single run.

Gel filtration on Ultrogel AcA44 has been used by Burgess et al. (1977a) in the purification of mouse lung CSF. We have also used this method with good results. Purified HLCM-CSF

obtained from this final step had a specific activity of 2.7×10^6 units/mg protein as assayed on mouse marrow and 1.8×10^6 units/mg as assayed on human marrow cells. Although the final HLCM-CSF preparation was not homogeneous as indicated by radioiodination and gel electrophoresis, this is the highest specific activity ever reported for a human CSF which stimulates granulocyte and macrophage colony growth from both mouse and human marrow cells. Burgess et al. (1977b) have reported the purification of CSF from human placenta conditioned medium (HPCM-CSF) 1800-fold with a specific activity of 2.5×10^5 units/mg. Price et al. (1975) have also reported the purification of CSF from medium conditioned by human peripheral leukocytes. The highest specific activity they obtained was 9×10^5 units/mg.

The gross morphology of colonies from human marrow cells stimulated by HLCM-CSF was similar to that of colonies from mouse marrow cells. At high concentrations, human lung CSF stimulated the formation of predominantly granulocytic colonies in human marrows. However, a reduction in the amount of CSF caused a fall in the proportion of granulocytic colonies. These results are similar to those reported by Burgess et al. (1977a) and Metcalf & Moore (1972) in their work with CSF from mouse lung and human urine.

The purified HLCM-CSF had a molecular weight of 41 000 as determined by gel filtration and 42 000 by sucrose gradient sedimentation method. This value is similar to the 45 000 reported for human urinary CSF (Stanley & Metcalf, 1971a). It is considerably larger than mouse lung CSF (mol wt 29 000) but smaller than mouse L cell CSF (mol wt 70 000) (Stanley & Heard, 1977). The thermostability of HLCM-CSF is similar to that of mouse lung CSF (Sheridan & Metcalf, 1973) and human urinary CSF (Stanley & Metcalf, 1969). The pattern of sensitivity of HLCM-CSF to proteolytic digestion is similar to that of human urinary CSF (Stanley & Metcalf, 1971b), mouse lung CSF (Sheridan et al., 1974), and human peripheral leukocyte CSF (Price et al., 1975).

Human lung CSF, like CSF from human urine (Stanley & Metcalf, 1969), was inactivated by incubation with 5×10^{-3} M periodate suggesting that it possesses a carbohydrate moiety. However, more definitive evidence that human lung CSF is a sialic acid containing glycoprotein was provided by the neuraminidase experiment. Treatment of HLCM-CSF with this enzyme altered the polydisperse pattern of migration on isoelectrofocusing reducing it to a single peak indicating that the factor is a glycoprotein. The sialic acid residues appeared to be nonessential for in vitro activity. Similar observations have been made by Metcalf (1973) and Sheridan et al. (1974), on human urinary CSF and mouse lung CSF, and by Dorado et al. (1972) on erythropoietin.

Our studies demonstrated that HLCM-CSF was immunogenic in rabbits and that the antibody produced was inhibitory to CSF activity. In order to demonstrate the specific inhibitory activity of the antiserum on CSF, the antigen, it was necessary to purify the γ-globulin fraction from the antiserum to remove nonspecific inhibitors (Stanley et al., 1968). Also the corresponding enriched IgG fraction from sera of nonimmunized rabbits was used as control and shown to be noninhibitory to CSF activity. Finally the specificity of the antibody for its antigen was established by demonstrating the binding of radioiodinated antigen to the immune globulin fraction and its precipitation with ammonium sulfate.

Cross inhibition studies showed that antihuman lung CSF antibody was equally inhibitory to CSF from other human sources, namely, serum, urine, and placenta, but had little or no effect on mouse lung CSF. This cross-reactivity pattern is in sharp contrast to what has been observed in mouse in that antimouse lung CSF did not inhibit the activity of CSF from other mouse tissues (Sheridan et al., 1974).

References

Bradley, T. R., & Metcalf, D. (1966) *Aust. J. Exp. Biol. Med. Sci. 44,* 287.

Burgess, A. W., Camakanis, J., & Metcalf, D. (1977a) *J. Biol. Chem. 252,* 1998.

Burgess, A. W., Wilson, E. M. A., & Metcalf, D. (1977b) *Blood 49,* 573.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121,* 305.

Dorado, M., Langton, A. A., Brandan, M. C., & Espada, J. (1972) *Biochem. Med. 6,* 238.

Fahey, J. (1967) *Methods Immunology Immunochem. 1,* 321.

Fojo, S. S. (1977) Ph.D. Thesis, University of Miami.

Fojo, S. S., Wu, M.-C., Gross, M.-A., & Yunis, A. A. (1977) *Biochim. Biophys. Acta 494,* 92.

Foster, R., Metcalf, D., Robinson, W. A., & Bradley, T. R. (1968) *Br. J. Haematol. 15,* 147.

Golde, D. W., Finley, T. N., & Cline, M. J. (1972) *Lancet 2,* 1397.

Granstrom, M. (1974) *Exp. Cell Res. 87,* 307.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89,* 114.

Guez, M., & Sachs, L. (1973) *FEBS Lett. 37,* 149.

Iscove, N. M., Senn, J. S., Till, J. E., & McCulloch, E. A. (1971) *Blood 37,* 1.

Jovin, R., Chrambach, A., & Naughton, H. (1964) *Anal. Biochem. 9,* 351.

Lowry, O. H., Bird, J., Rosebrough, A., Farr, L., & Randall, R. (1951) *J. Biol. Chem. 193,* 265.

Metcalf, D. (1973) in *Humoral Control of Growth and Differentiation* (LoBue, J., & Gordon, A. S., Eds.) Vol. I, pp 91-118, Academic Press, New York, N.Y.

Metcalf, D. (1974) *Exp. Hematol. 2,* 157.

Metcalf, D., & Foster, R. (1967) *Proc. Soc. Exp. Biol. Med. 126,* 758.

Metcalf, D., & Moore, M. A. S. (1972) in *Haemopoietic Stem Cells* (Wostenholme, G. E. W., & O'Connor, M., Eds.) p 157, Associated Scientific Publishers, Amsterdam.

Paran, M., & Sachs, L. (1968) *J. Cell. Physiol. 72,* 247.

Pluznik, D. H., & Sachs, L. (1965) *J. Cell. Physiol. 66,* 319.

Price, G. B., Senn, J. S., McCulloch, E. A., & Till, J. E. (1974) *J. Cell Physiol. 84,* 383.

Price, G. B., Senn, J. S., McCulloch, E. A., & Till, J. E. (1975) *Biochem. J. 148,* 209.

Ratzan, R. J., & Yunis, A. A. (1974) *Clin. Res. 22,* 402A.

Ratzan, R. J., Moore, M. A. S., & Yunis, A. A. (1974) *Blood 43,* 363.

Sheridan, J. W., & Metcalf, D. (1973) *J. Cell. Physiol. 81,* 11.

Sheridan, J. W., Metcalf, D., & Stanley, E. R. (1974) *J. Cell Physiol. 84,* 147.

Stanley, E. R., & Heard, P. M. (1977) *J. Biol. Chem. 252,* 4305.

Stanley, E. R., & Metcalf, D. (1969) *Aust. J. Exp. Biol. Med. Sci. 47,* 467.

Stanley, E. R., & Metcalf, D. (1971a) *Proc. Soc. Exp. Biol. Med. 137,* 1029.

Stanley, E. R., & Metcalf, D. (1971b) *Aust. J. Exp. Biol. Med. Sci. 49,* 281.

Stanley, E. R., & Metcalf, D. (1973) in *Hemopoiesis in Culture* (Robinson, W. A., Ed.) p 102, U.S. Government Printing Office, Washington, D.C.

Stanley, E. R., Robinson, W. A., & Ada, G. L. (1968) *Aust. J. Exp. Biol. Med. Sci. 46,* 715.
Stanley, E. R., Hansen, G., Woodcock, J., & Metcalf, D. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 2272.
Warren, L. (1959) *J. Biol. Chem. 234,* 1971.
Winter, A., Perlmutter, H., & Davies, H. (1975) *LKB Appl. Note,* 198.
Wrigley, C. W. (1971) *Methods Enzymol. 22,* 559.

Biochemistry of Terminal Deoxynucleotidyltransferase: Mechanism of Inhibition by Adenosine 5′-Triphosphate†

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ABSTRACT: The polymerization of deoxyribunucleoside triphosphate catalyzed by terminal deoxyribonucleotidyltransferase (TdT, EC 2.7.7.31) is severely inhibited by the addition of ribonucleoside triphosphates, ATP being the most potent inhibitor. Examination of the inhibitory effect of ATP using oligo(dA)$_{12-18}$ as well as activated DNA as primers revealed that (a) ATP inhibition is not due to its addition onto a 3′-OH primer terminus as judged by the lack of incorporation of labeled ATP, although under similar conditions incorporation of GTP can be demonstrated, (b) a consistent degree of inhibition was noted independent of primer or enzyme concentration; (c) addition of ATP to an ongoing reaction promptly reduces the rate of polymerization; (d) kinetic studies indicate a competitive (with respect to substrate deoxy triphosphate) pattern of inhibition; (e) addition of excess deoxyribotriphosphate promptly relieves the inhibition. Unlike ATP, other ribotriphosphates yield a mixed pattern of inhibition partly mediated by competitive mechanisms. GTP and CTP and to a minor extent UTP are incorporated into DNA in the presence or absence of deoxy triphosphate. Furthermore, addition of ATP also inhibits incorporation of GTP and CTP.

The confinement of terminal deoxynucleotidyltransferase (TdT,[1] EC 2.7.7.31) to the thymus has been well established (Chang, 1971; Bollum, 1974; Kung et al., 1976). However, it was not until the demonstration of its presence in certain types of leukemic lymphocytes that the interest in the biology and function of this enzyme was renewed vigorously (McCaffrey et al., 1973; Sarin and Gallo, 1974; Gallo, 1975; Coleman et al., 1976; Sarin et al., 1976). The enzyme activity present in leukemic leukocytes clearly suggested a linkage of these cells to thymus and thereby has provided a diagnostic marker for the classification of leukemias as well as marker for T cell differentiation. However, biological function of this enzyme has remained unknown. Baltimore (1974), based on its exclusive presence in immunogenic organ, e.g., thymus and products thereof, has proposed that TdT may be a somatic mutagen and therefore may be responsible for the generation of immunoglobulin diversity. In the absence of any corroborating experimental evidence, this interesting theory remains unproven.

A detailed study of the in vitro properties exhibited by a purified enzyme may be used to infer at least some suggestive in vivo role for that enzyme. So far, demonstration of any specific homo- or heteropolymeric DNA sequence in the cells containing TdT has been lacking. It is therefore difficult to correlate DNA-synthesizing activity of TdT with plausible biological utility. During our studies on the active site of TdT (used as a model system for DNA polymerases) using pyridoxal phosphate as an active-site-specific reagent (Modak, 1976a), it was observed that ribotriphosphates exhibited a strong inhibitory action, ATP being the most powerful inhibitor of the four triphosphates (Modak, manuscript in preparation). Indeed, Bollum and colleagues have described the inhibition of this enzyme by ATP over a decade ago (Kato et al., 1967). Roychoudhury (1972) demonstrated that one or two ribonucleotides could be added to DNA primers and continued his efforts to optimize and characterize the conditions with respect to substrate and primer concentrations and divalent cation requirement for these reactions (Roychoudhury et al., 1976). Admittedly, such ribonucleotide additions provide a very useful tool for the primer extension (Roychoudhury and Kossel, 1971; Roychoudhury, 1972; Padmanabhan et al., 1974; Sekiya et al., 1974), 3′-end labeling (Kossel and Roychoudhury, 1971; Bertazoni et al., 1974), and DNA sequence analysis (Kossel et al., 1974; Wu et al., 1973; Jay et al., 1974). However, these studies do not provide any clue to the in vivo functionality of TdT. The selective inhibitory effect of ATP on TdT in contrast to other replicative DNA polymerases (Bhalla et al., 1977) and the fact that ATP is one of the important cellular micromolecules appears to suggest some (regulatory?) connection between TdT and ATP. We have, therefore, reinvestigated the mechanism of ATP inhibition of this enzyme, and results of this investigation are presented.

Materials and Methods

Enzymes. Terminal deoxynucleotidyltransferase was prepared from calf thymus following the procedure of Chang and Bollum (1971). The final preparation consisted of two subunits with molecular weights of ca. 26 000 and 8000, as judged by polyacrylamide gel electrophoresis. The enzyme is at least 98% pure on the basis of intensity of staining of the bands in the gel. The enzyme preparation is completely free of DNA poly-

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[1] Abbreviations used: rNTP, ribonucleoside triphosphate; rNMP, ribonucleoside monophosphate; dNTP, deoxyribonucleoside triphosphate; TdT, terminal deoxyribonucleotidyltransferase; DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

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