Granulocyte and Macrophage Colony-Stimulating Factor from Human Placenta Conditioned Medium†

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ABSTRACT: Conditioned medium prepared from human placenta tissue stimulates in vitro colony formation by mouse and human marrow cells. At least two types of colony-stimulating activities are found and can be separated by isoelectrofocusing. The first type of colony-stimulating factor (CSF) shows heterogeneity on isoelectrofocusing with p*I* in the range of 3.6–4.7. It has been purified 10^5-fold by hydroxylapatite chromatography, preparative isoelectrofocusing, gel filtration, polyacrylamide gel isoelectrofocusing, and electrophoresis to a specific activity of 7.5×10^7 units/mg. The purified CSF shows a single band on microgel electrophoresis and stimulates mostly mouse macrophage colony formation. It has a molecular weight of 41 000 as determined by gel filtration and is sensitive to proteolytic enzyme digestion and periodate oxidation. Treatment with neuraminidase is without effect on activity. It is stable at pH 5–10 and 60 °C for 30 min. The second type of CSF appears as a single activity peak on isoelectrofocusing with a p*I* of 5.7. The molecular weight is calculated to be 27 000 from gel filtration. It is very sensitive to trypsin and periodate but inert to neuraminidase or mercaptoethanol treatment. It is stable at neutral pH but labile at 60 °C for 30 min. The purified CSF stimulates predominantly granulocytic colony formation from human marrow cells.

Studies on the regulation of granulopoiesis have been greatly facilitated by the introduction of a cloning method in soft agar (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966). In this assay system, a protein factor named colony-stimulating factor (CSF)[1] is required for the in vitro growth of granulocytic and macrophage colonies from bone marrow cells. Evidence provided from murine and human studies suggests that CSF may play an important role in the control of granulopoiesis in vivo. CSF has been found widely distributed in animal tissues and sera, and several mouse CSFs have been purified to apparent homogeneity, including that from mouse lung (Burgess et al., 1977a) and cultured L-cells (Guez & Sachs, 1973; Stanley & Heard, 1977; Waheed & Shadduck, 1979). The source of human CSF has been limited, although it has been purified from urine (Stanley et al., 1975) and recently from a cultured pancreatic carcinoma cell line (Wu et al., 1979). The purified CSF appears to stimulate colony growth in mouse marrow, but shows little or no activity in human marrow. In our search for human CSF sources for comparative biochemical studies, we have found that conditioned media prepared from human lung (Fojo et al., 1977, 1978) and placenta (Wu et al., 1978) provide relatively high CSF activity. In this report we describe the purification and characterization of two distinct CSF activities from placenta-conditioned medium.

Materials and Methods

Dulbecco's modified Eagle's medium (DME), fetal calf serum, and horse serum were obtained from Grand Island Biological Co. (New York, NY). Hydroxylapatite (Bio-Gel HTP), acrylamide, and bis(acrylamide) were from Bio-Rad (Richmond, CA). Ultrogel (AcA-44), Ampholite, Ultrodex, and preparative flat bed gel isoelectrofocusing apparatus were from LKB (Rockville, MD). Endotoxin (*Salmonella typhosa*), trypsin (type III, bovine pancreas), α-chymotrypsin (type II, bovine pancreas), subtilisin (type VII, *Bacterium amylolique faciens*), and neuraminidase (type IX, *Clostridium perfringens*) were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade. Human placentas were freshly collected from Jackson Memorial Hospital (Miami, FL).

Assay of CSF. The method for CSF assay has been described previously (Fojo et al., 1977). Mouse bone marrow cells were obtained from C57 BL/6J inbred mice, and human marrow cells were obtained from the posterior iliac crest of normal volunteers. Human marrow cells were further fractionated by the Ficolli–Hypaque method followed by removal of the adherent cells (Kurland et al., 1978). Colony counts were performed using a dissecting microscope at day 7, and aggregates of 50 cells or more were scored as colonies. A unit of CSF activity was arbitrarily defined as the amount of CSF that stimulates the formation of one colony under the specified conditions of the assay.

Morphological Analysis. The study of colony morphology in assay plates was conducted by a new technique recently developed in our laboratory (A. M. Miller, unpublished results). The soft agar plates containing colonies were first fixed with 30% acetic acid in absolute ethanol for 30 min at room temperature. Each plate was then rinsed sequentially with 100, 80, and 50% ethanol. The fixed agar layer was removed by floating in water and dried at 60 °C for 2 h. The dried thin film was stained with alum hematoxylin for 15 min and then rinsed with running water to remove excess dye. The film was then air-dried and examined under the microscope for individual cell morphology within the colonies.

Preparation of Human Placenta Conditioned Medium (HPCM). Normal human placentas were obtained immediately after delivery and inspection. Each placenta was placed in a large sterile dissecting plan, and under sterile conditions the cotyledon mesoderm was removed using scissors and forceps. Placental tissues were cut into slices and placed in a 2-L sterile beaker and washed twice with Hank's balanced salt solution (HBS). Sections were then transferred to 100-mm

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[1] Abbreviations used: CSF, colony-stimulating factor; p*I*, isoelectric point; DME, Dulbecco's modified Eagle's medium; HPCM, human placenta conditioned medium; HBS, Hank's balanced salt solution; Con A, concanavalin A.

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plastic petri dishes and minced with sterile scissors. The pieces were again washed with HBS solution and then distributed in 1-L tissue culture bottles at ~0.1 g of tissue per mL of CSF-DME medium. Endotoxin at a concentration of 50 μg/mL and phenylmethanesulfonyl fluoride at 10 μg/mL were present during the incubation.

The bottles were incubated at 37 °C in a humidified incubator with 10% CO_2 in air for a period of 3 days. The medium was then removed and filtered through gauze to remove debris. The supernatant was centrifuged at 1000*g* for 30 min. This medium was heat inactivated at 57 °C for 30 min and then dialyzed against distilled water (3×). The human placenta conditioned medium was then stored at −20 °C after adding Tween 20 (0.01%) and sodium azide (0.01%). No loss of CSF activity was observed for 1 year under the storage conditions.

Purification. The purification procedures including hydroxylapatite chromatography, preparative flat-bed isoelectrofucusing, gel filtration chromatography, and gel electrophoresis have been described previously in the purification on CSF from human lung conditioned medium (Wu et al., 1979; Fojo et al., 1978). The protein profiles in all the purification procedures are monitored by measuring the A_{280}. Protein concentrations of the pooled samples are determined by the procedures of Lowry et al. (1951) and Shaffner & Weismann (1973) using bovine serum as a standard. To examine the purity of the purified CSF, microgel electrophoresis was used as described previously (Wu et al., 1979).

Results

Secretion of Colony-Stimulating Factors by Placenta Tissues. CSFs are secreted into the conditioned medium continuously up to 3 days. After that, the activities reached a plateau. The crude human placenta conditioned medium thus prepared is active in both human and mouse bone marrow with a specific activity of 300–800 units/mg for human and 500–1500 units/mg for mouse marrow.

Purification of Colony-Stimulating Factors. Separation of Two Activities by Isoelectrofocusing. Both of these activities can be absorbed by hydroxylapatite and eluted by 0.06 M phosphate buffer, pH 6.5, with 70–80% recovery and three- to sixfold purification similar to the purification of human lung CSF (Fojo et al., 1977).

The active concentrated sample (4.5 mL) from the hydroxylapatite column was dialyzed overnight against distilled water (3 times). The sample was then subjected to preparative flat-bed isoelectrofocusing in granulated gel (Ultrodex) as described by Winter et al. (1975). The sample was applied to the 150-mL Ultrogel bed as a narrow zone, and isoelectrofocusing was conducted overnight. The gel bed was fractionated and eluted with phosphate-buffered saline containing 0.01% Tween 20 and 0.1% sodium azide. The protein concentration profile was determined by measuring A_{280}, and CSF activity was assayed after dilution (1:100) with 5% fetal calf serum and dialysis against distilled water overnight (3 times).

The preparative isoelectrofocusing profile is shown in Figure 1. Two distinct types of activity were observed. The first (type I, peaks I–III) had greater activity when assayed with mouse than with human marrow and exhibited a polydisperse pattern with a p*I* range of 3.6–4.7. The second (type II) was more active when assayed with human than with mouse marrow and migrated at the end of the profile with a p*I* of 6.0. When this peak was pooled and resubjected to isoelectrofocusing on a pH 4–6.5 gel bed, it was resolved as a single peak with a p*I* of 5.7 (Figure 2).

Gel Filtration Chromatography. The two types of CSF activity were further purified by gel filtration chromatography

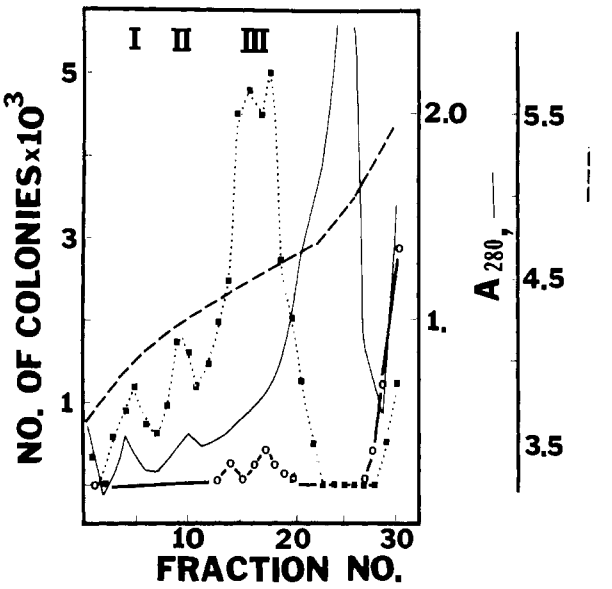

FIGURE 1: Flat-bed preparative isoelectrofocusing on Ultrodex gel. The 0.06 M phosphate buffer eluate from the hydroxylapatite column was concentrated to 4.5 mL by Amicon and dialyzed overnight against distilled water. The sample was applied as a narrow zone to the Ultrodex gel bed (150 mL), and isoelectrofocusing was then conducted overnight in the cold room using a pH gradient of 3.5–5.0 Ampholine. The gel bed was fractionated and fractions were eluted and dialyzed. The protein profile was monitored with A_{280}, and CSF activities were assayed both in mouse (■) and human (○) marrow after 1:100 dilution with 5% fetal calf serum.

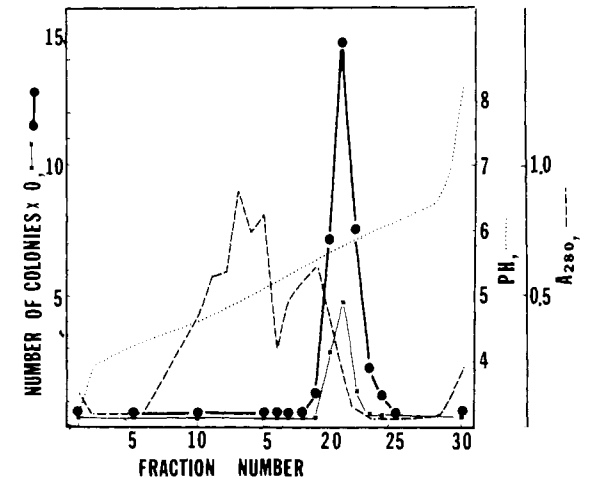

FIGURE 2: Reisoelectrofocusing of human CSF. Fractions 28–30 of Figure 1, which contained most of the human CSF activity, were pooled and concentrated. The smple was rerun on flat-bed isoelectrofocusing with a pH gradient of 4 to 6.5 under the same conditions. Fractions were eluted from the gel bed, and protein and CSF activities were measured as in Figure 1: CSF activity on mouse marrow (■) and human marrow (●).

on Ultrogel AcA 44 (1.1 × 100 cm, bed volume 100 mL) which was 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. The three major peaks of mouse activity (type I) were each pooled separately and applied to the same column. The activity profiles revealed that they have molecular weights of 50 000, 45 000, and 41 000, respectively, for peaks I, II, and III of Figure 1. The gel filtration profile of peak I is shown in Figure 3. However, treatment of these peaks with neuraminidase reduced them to a single peak with a p*I* of 4.7 and a mol wt of 41 000, indicating that the heterogeneity of type I CSF both on isoelectrofocusing and on gel filtration is due to different contents of sialic acid.

Table I: Summary of the Purification of Type I HPCM-CSF

methods	total protein (mg)	total act. (U)	sp act. (μg/mg)	x-fold purification	recovery (%)
1. gel filtration[a]	0.52	80 000	1.5×10^5	1	100
2. gel isoelectrofocusing	0.031	65 000	2.1×10^6	14	82
3. gel electrophoresis	2.7×10^{-4}	20 000	7.5×10^7	500	26

[a] Starting sample is peak I of preparative isoelectrofocusing after gel filtration chromatography (Figure 3).

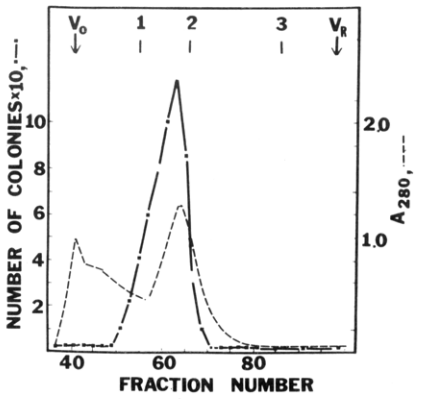

FIGURE 3: Gel filtration of peak I CSF of type I on Ultrogel AcA 44 column. Peak I of Figure 1 was pooled and concentrated to 1.5 mL. The sample was then applied to an Ultrogel AcA 44 column (1.1 × 100 cm, column volume 100 L) previously equilibrated with 0.1 M NaCl solution containing 0.01% Tween 20, 0.01% NaN_3, 5 μg mL of streptomycin, and 5 units/mL of penicillin. Fractions were dialyzed against distilled water and assayed on mouse marrow. Standard marker proteins are (1) bovine serum albumin (68 000), (2) ovalbumin (45 000), and (3) chymotrypsinogen (26 000).

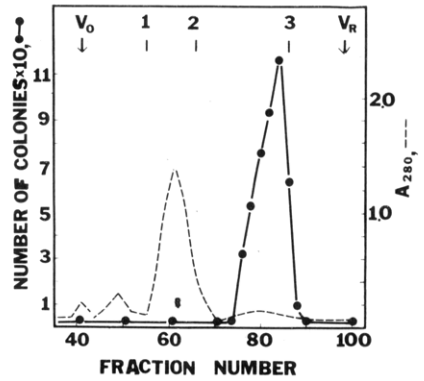

FIGURE 4: Gel filtration of type II CSF on Ultrogel AcA 44 column. The type II CSF (human active CSF from isoelectrofocusing on Figure 2 (fractions 19–24) was concentrated to 1.5 mL and subjected to gel filtration as described in Figure 3. Samples from each fraction were assayed on human marrow cells.

The human CSF activity, type II, when filtered on Ultrogel AcA 44 emerges as a single peak with a mol wt of 27 000 (Figure 4).

Further Purification of Type I CSF. Since peak I of the mouse activity had the lowest p*I* and hence the highest sialic acid content, advantage was taken of the change in p*I* after neutraminidase treatment to further purify this CSF. Thus, CSF from peak I was first filtered on Ultrogel (Figure 3) and the active fractions were pooled and concentrated to 1 mL. The sample was adjusted to 0.05 M sodium acetate buffer, pH 5.0, and incubated with Sepharose–neuraminidase (Sigma Chemicals) in the same buffer. The enzyme was removed by filtration, and the CSF was applied to polyacrylamide gel isoelectrofocusing according to the method of Wrigley (1971). The CSF activity profile from this step is shown in Figure 5. Treatment with neuraminidase caused a shift of p*I* from 3.8 to 4.7 resulting in further separation and purification of CSF.

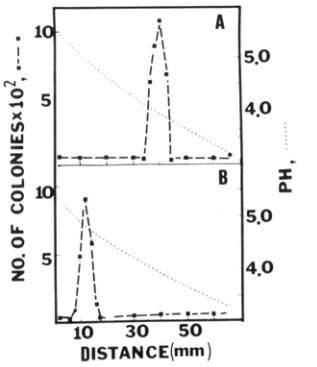

FIGURE 5: Isoelectrofocusing of neuraminidase-treated type I CSF on polyacrylamide gel. The active fractions of Figure 3 were pooled, concentrated, and treated with neuraminidase. The treated sample was subjected to acrylamide gel isoelectrofocusing using an ampholine pH gradient of 3.5–5.0. Gels were sliced, extracted, and assayed on mouse marrow. The detailed procedures are as described in the text: (A) type I CSF without neuraminidase treatment; (B) CSF activity from Figure 3 treated with neuraminidase.

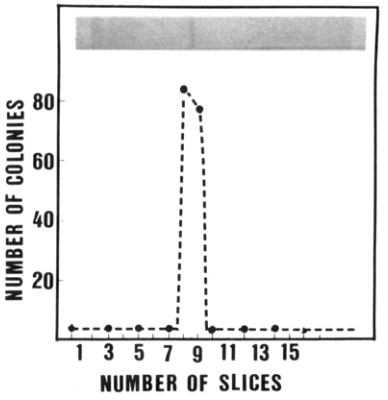

FIGURE 6: Microgel electrophoresis of purified type I CSF. Two gels (about 100 ng of protein each) were run simultaneously. One was stained with coomassie blue and the other was sliced and assayed for CSF activity on mouse marrow.

The CSF peak from Figure 5B was extracted 2 times with phosphate-buffered saline containing 0.01% Tween 20 and 0.1% sodium azide and subjected to polyacrylamide gel electrophoresis. Extracts from the active gel slices were pooled and analyzed on microgel electrophoresis as described previously (Wu et al., 1979). The apparent homogeneity of the purified CSF with an activity peak superimposed on a single coomassie blue stained protein band is shown in Figure 6. The purification of type I CSF is summarized in Table I.

Characterization of Types I and II CSF. The following characterization experiments were performed using a CSF pool consisting of the most active fractions obtained from gel filtration (Figures 3 and 4).

Heat Stability. One milliliter of both types of HPCM-CSF was incubated at the desired temperatures (25–100 °C) for 30 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. The results are shown in Figure 7. Type I activity was stable at 60 °C for 30 min, and 30% of the activity was recovered after

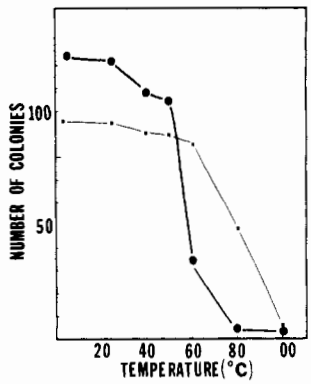

FIGURE 7: Thermostability of types I and II CSF. Types I and II, after the gel filtration purification step, were incubated at different temperatures for 30 min. Samples were cooled in ice and then assayed. Type I CSF (■) was assayed on mouse marrow and type II CSF (●) was assayed on human marrow.

Table II: Sensitivity of CSF to Proteases, Neuraminidase, Periodate, and Mercaptoethanol

treatment	no. of colonies	
	type I CSF	type II CSF
control	88 ± 4	101 ± 15
trypsin	67 ± 13	5 ± 4
chymotrypsin	0	0
periodate	0	0
neuraminidase	91 ± 3	111 ± 15
mercaptoethanol	82 ± 6	91 ± 6

treatment at 100 °C for 15 min. In contrast, type II activity was heat labile at 60 °C with loss of most of the activity in 30 min.

Sensitivity to Proteases, Periodate, and Neuraminidase. Aliquots of both types of HPCM-CSF were incubated with the appropriate agent for 2 h at 37 °C. In each case the protein ratio of the CSF aliquots to enzyme was 1:10. For trypsin (150 units/mg) and chymotrypsin (60 units/mg) 0.01 M Tris-HCl buffer with 0.01 M $CaCl_2$ (pH 8.0) was used. Sodium periodate (4 mM) and neuraminidase (1 unit, 32 units/mg) were incubated in 0.04 M acetate buffer, pH 5.0. Reactions were stopped by immediately lowering the temperature to 0 °C and adding 100 μL of fetal calf serum. The results are shown in Table II. Type I was relatively resistant to trypsin digestion but was very sensitive to chymotrypsin and periodate. In contrast, type II CSF was very sensitive to trypsin. Neither type I or II activity was affected by neuraminidase or mercaptoethanol (1 mM) treatment.

Binding to Concanavalin A. Both CSF activities were tested for their ability to bind to a Con A–Sepharose column according to published procedures (Stanley & Heard, 1977; Waheed & Shadduck, 1979). Type I activity bound readily to Con A and could be eluted by 0.1 M methyl α-D-mannoside. In contrast, the human-active or type II CSF did not bind to Con A and came through in the breakthrough fraction.

Morphology of Colonies. The types of colonies grown in the presence of CSF I and II are listed in Table III. Type I (the mouse-active CSF), when assayed in mouse marrow, yielded predominantly macrophage colonies (~90%). In contrast, the human-active or type II CSF yielded predominantly granulocytic colonies (~80%).

Table III: Morphology of Colonies

source of CSF	source of marrow	no. of colonies		
		granulocyte	macrophage	mixed
type I	mouse	5	221	15
type II	human	201	28	22

Discussion

Conditioned medium prepared from human placenta appears to offer a good source of human CSF. The specific activity varies from one preparation to the other but does not appear to be related to the sex of the newborn. The addition of endotoxin (50–100 μg/mL) during incubation does not increase CSF production. The specific activity of HPCM is slightly lower than human lung conditioned medium, but placenta tissue is more readily available in large quantities.

Since Ratzan & Yunis (1974) first reported the preliminary observations, several articles have appeared describing the preparation and purification of CSF from placental tissue. Burgess et al. (1977b) reported that HPCM does not stimulate colony formation by mouse bone marrow, a finding at variance with our results. The only difference in the preparation procedures is that we used serum-free DME, while they used RPMI-1640 containing 5% fetal calf serum. We consistently find two types of CSF separable by isoelectrofocusing. Type I is more active in mouse marrow and appears polydisperse on isoelectrofocusing with at least three components with p*Is* ranging from 3.6 to 4.7 and molecular weights of 50 000, 45 000, and 41 000, respectively. This heterogeneity appears to be due to different contents of sialic acid, since pretreatment with neuraminidase causes a shift to a single peak with a p*I* of 4.7 and a mol wt of 41 000. No further reduction in molecular weight is observed by treatment with mercaptoethanol (0.3 M NaCl, 0.1 M phosphate buffer, and 1 mM mercaptoethanol, pH 7.5). Thus, this activity is probably different from mouse lung CSF and CSF from other mouse tissues, which has mol wt of 23 000 (Nicola et al., 1979).

Since peak I of type I has the highest sialic acid content and hence the greatest shift in position upon neuraminidase treatment, we have chosen it for further purification. Thus, treatment with neuraminidase followed by a second isoelectrofocusing and gel electrophoresis yields an apparently homogeneous preparation as indicated by the microgel electrophoresis. This latter technique has proven very useful in monitoring CSF purification, since only about 10 ng of protein is required.

Type II CSF appears as a single active peak on isoelectrofocusing with a p*I* of 5.7 and a mol wt of 27 000. In contrast to type I CSF, type II CSF does not bind to Con A. The highest specific activity we have obtained after isoelectrofocusing and gel filtration is 2.8×10^5 units/mg of protein as assayed on human bone marrow. The recent report by Nicola et al. (1978) indicated that the HPCM-CSF also has eosinophil colony-stimulating activity. Thus far, we have not tested our purified CSF fractions for this activity.

In addition to differences in p*I* and molecular weight, types I an II CSF exhibit other differences in properties. Type I is much more heat stable and is relatively resistant to trypsin digestion, but is very sensitive to chymotrypsin. Type II is heat labile and sensitive to both trypsin and chymotrypsin. Neither type is affected by mercaptoethanol or neuraminidase. This is in contrast to purified mouse L-cell CSF, which is sensitive to mercaptoethanol (Stanley & Heard, 1977).

The finding of two distinct types of CSF is a common pattern for CSFs from human sources (Wu & Yunis, 1980). This pattern had not been previously observed in our work with human lung CSF (Fojo et al., 1978). However, isoelectrofocusing was not employed at the same stage of purification. When human lung conditioned medium was subjected to the same procedures as described here, a similar pattern of two

distinct types of CSF was also observed (Wu & Yunis, 1980). The CSFs from human lung and placenta share all the properties we have described above.

Perhaps the most interesting aspect of the two types of CSF are their species specificities and the type of cell differentiation they promote. It is not clear at present why a given CSF from a human source should exhibit activity in mouse but not in human marrow. Neither is the biochemical basis for stimulating the formation of granulocyte or macrophage colonies apparent. Detailed biochemical and functional studies on homogeneous preparations of these two types of CSF should help provide some answers to these important questions.

Acknowledgments

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p-Isothiocyanatophenyl 6-Phospho-α-D-mannopyranoside Coupled to Albumin. A Model Compound Recognized by the Fibroblast Lysosomal Enzyme Uptake System. 1. Chemical Synthesis and Characterization†

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ABSTRACT: We have developed a simple synthesis for a conjugate of albumin and *p*-aminophenyl 6-phospho-α-D-mannopyranoside to study the requirements of the fibroblast lysosomal enzyme recognition system. *p*-Aminophenyl 6-phospho-α-D-mannopyranoside was prepared in two ways: (1) phosphorylation of *p*-nitrophenyl α-D-mannopyranoside and subsequent reduction of the nitro group by catalytic hydrogenation and (2) direct phosphorylation of *p*-aminophenyl α-D-mannopyranoside. Mannosides were phosphorylated in a reaction with phosphoryl chloride, pyridine, and water at 0 °C for 1 h, by a procedure selective for primary hydroxyl groups. Purified *p*-aminophenyl 6-phospho-α-D-mannopyranoside was characterized by chromatographic, enzymatic, and ^{13}C nuclear magnetic resonance spectroscopic methods. *p*-Isothiocyanatophenyl 6-phospho-α-D-mannopyranoside and the *p*-isothiocyanatophenyl glycosides of α-mannose, α-glucose, α- and β-galactose, and α-L-fucose were formed by reaction of the respective *p*-aminophenyl glycosides with thiophosgene. Incubation of the *p*-isothiocyanatophenyl glycosides with bovine serum albumin at pH 8.5, 25 °C, for 18 h generally resulted in the coupling, primarily through lysine residues, of up to 20-30 mol of glycoside per mol of protein. Biological properties of the conjugates in the fibroblast lysosomal enzyme recognition system are described in the accompanying paper.

The potential of synthetic sugar-protein conjugates (neoglycoproteins) as mechanistic probes and as models for the design of specific cell-directed substances has been demonstrated in studies of carbohydrate recognition systems for receptor-mediated endocytosis. Following the discovery by Morell et al. (1971) that mammalian hepatocytes recognized and removed from the circulation a large series of galactose-terminated glycoproteins, Rogers & Kornfeld (1971) showed that the uptake of certain nonglycosylated proteins by the liver was enhanced when they were chemically coupled to fetuin asialoglycopeptides. More recently, chemical coupling

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