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Colony Stimulating Factor (CSF) from Human Leukemic Urine: Affinity Chromatography and Isoelectric Focusing[1] (39122)

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Colony-stimulating factor (CSF)[3] is a term applied to proteinaceous materials that have been prepared from many sources including serum, urine, tissue extracts, and medium conditioned by a variety of tissues and cell types (1). The biological activity of CSF is generally measured by its ability to promote the formation of granulocyte and/or macrophage colonies in cultures of bone marrow cells suspended in either soft agar or methylcellulose (2–4). In addition, CSF has been shown to stimulate the *in vitro* incorporation of labeled precursors into DNA (5–7).

Several lines of indirect evidence have suggested that CSF from a number of sources is a glycoprotein. Thus colony-stimulating factors isolated from human urine (8), and from L-cell (9) and mouse embryo-conditioned media (10) were completely inactivated by mild periodate oxidation. Moreover, these same factors were comparatively resistant to inactivation by a variety of proteolytic enzymes (11). Molecular weights of several colony-stimulating factors as determined by gel filtration on Sephadex G-150 were considerably larger than values determined by velocity sedimentation in sucrose gradients (9, 12). Following treatment with neuraminidase, slight decreases in anodal electrophoretic mobilities were observed for the colony-stimulating activity associated with mouse embryo-conditioned medium (10) as well as a more purified preparation from human urine (13). Addition of Con A to a solution of human urinary CSF followed by centrifugation resulted in a supernatant having little or no colony-forming (14) or thymidine-incorporation-stimulating activity (15).

In this paper we present data concerning the affinity chromatography of CSF on Con A–Sepharose. This system provides further evidence for the glycoprotein nature of CSF and is a useful step in the partial purification of this substance. We also report the isoelectric point of CSF from human leukemic urine, both before and following treatment with neuraminidase.

Materials and methods. Assays. Bone marrow was obtained from both male and female C57Bl/6J mice supplied from our own breeding colony. Colony-forming activity of CSF was assayed in agar containing medium using a double layer technique (16). Thymidine-incorporation-stimulating activity of CSF was performed as described previously (7). A computer program was used to analyze the dose response curves (7). Specific activities are reported in this paper as the number of colonies per mg of protein relative to that given by the standard reference preparation of starting material (see below). The specific activity of the standard reference material was assayed in each experiment.

CSF preparation. CSF was obtained from the urine of leukemic patients admitted to Emory University Hospital. Twenty-four hour urines were collected in iced containers, liquid phenol (1 ml/liter of urine) was added, and the samples were frozen. Urines from several patients were pooled and processed at 4° using a modification of a previously reported method (7). Briefly, urine was

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[3] Abbreviations used are: CSF, colony stimulating factor; Con A, Concanavalin A; HSA, human serum albumin; αMM, α-Methyl-D-mannopyranoside; αMG, α-methyl-D-glucopyranoside; and TEMED, N:N:N′:N′-tetramethylethylenediamine.

precipitated with an equal volume of acetone. The precipitate was reconstituted in distilled water, dialyzed, and centrifuged at 43,000*g* for 20 min. The supernatant was subjected to gel filtration on Sephadex G-75 equilibrated in distilled water followed by ammonium sulfate precipitation. Precipitation was performed on material buffered in 0.1 *M* sodium phosphate, pH 6.5, and biological activity precipitated between 40–75% saturation. The 75% precipitate was dialyzed against 0.02 *M* phosphate buffer, pH 7.0, and then against distilled water. This material served as the standard reference preparation of starting material and had an average specific activity of 1047 colonies per mg protein. All specific activities reported in this paper are relative to this standard reference preparation.

Albumin, which is the largest single contaminant in the preparation of urinary CSF, was removed by chromatography on a column of Blue Dextran 2000-Sepharose 4B (Pharmacia) prepared by the method of Cuatrecasas (17) after the rationale of Travis and Pannell (18). CSF was applied to a column (1.8 × 20 cm) that had been equilibrated at 4° in 0.001 *M* sodium phosphate, 0.05 *M* ammonium sulfate, 0.01 *M* β-mercaptoethanol, pH 7.0. Biologically active material was eluted with the equilibration buffer, but albumin was retained on the support. This observation was confirmed by noting the absence of albumin in the eluted fraction when examined by polyacrylamide gel electrophoresis and Ouchertlony double diffusion using an anti-HSA IgG preparation. Furthermore, albumin could be recovered by eluting the column with 0.5 *M* NaCl and/or 6 *M* urea. Recovery of colony-forming activity ranged from 70–150% with a specific activity of 1183 to 2534 colonies/mg protein. This material was used for the particular studies reported in this paper.

Preparation of Con A–Sepharose 4B and affinity chromatography. Con A was attached to Sepharose 4B using the cyanogen bromide activation method (17). The resulting affinity adsorbent contained approximately 4 mg of Con A per ml of gravity-packed wet Sepharose and was stored in 0.1 *M* acetate buffer, pH 6.0, containing 1 *M* NaCl, 0.001 *M* each of $CaCl_2$, $MnCl_2$, $MgCl_2$, and 0.02% merthiolate. Chromatography was performed at 4° using columns equilibrated in 0.02 *M* sodium phosphate, 1 *M* NaCl, pH 7.0 (19). All samples were applied in the equilibration buffer. For preparing CSF for subsequent studies, 50 to 125 mg were applied ($\leq$8.5 ml) to columns measuring 0.9 × 60 cm. For studies involving rechromatography of control and neuraminidase-treated CSF (see below), 0.3 to 1.5 mg were loaded onto columns measuring 0.9 × 30 cm in a volume no greater than 2 ml. Unbound protein was removed by elution with 5–15 column volumes of equilibrating buffer. Subsequently, a linear gradient (total volume 250 ml) of 0.001 *M*–0.06 *M* α-methyl-D-mannopyranoside (αMM) in the buffer was employed to specifically elute bound glycoproteins. Similar results could be obtained using a gradient of α-methyl-D-glucopyranoside (αMG). Equilibration buffer containing 2% (w/v, approximately 0.1 *M*) αMM was used as a final column eluant and served to prepare the column for reequilibration with starting buffer. When run at 4° under these conditions of neutral pH, columns could be reused two to three times without loss of selectivity or resolution. However, a single run at 25° caused substantial removal of Con A, resulting in poor performance if the same column were reused. Selected effluent fractions were assayed to determine the region containing biologically active material. Appropriate fractions were then pooled, dialyzed, concentrated by lyophilization, and assayed to determine recovery of biological activity. Carbohydrate was determined using an anthrone–sulfuric acid procedure (20).

Neuraminidase treatment. Biologically active CSF eluted from Con A–Sepharose was treated with neuraminidase (*Cl. perfringens*, Type VI, Sigma, Lot #31C-8100-1) to remove sialic acid residues. The enzyme (1.1 units/mg) was incubated with CSF in 0.1 *M* sodium acetate buffer, pH 6.0, in the ratio of 3 ml CSF (1.1 mg per ml) to 0.2 ml enzyme (0.2 units). Incubation was at 37° with shaking for 16 hr. Following incubation, the reaction mixture was dialyzed at 4° against distilled water. Appropriate controls of CSF

incubated without enzyme and enzyme without CSF were performed. Under these incubation conditions, all sialic acid was removed as judged by similar quantitative values for sialic acid when the sample was hydrolyzed for 1 hr at 80° in the presence of 0.1 *N* sulfuric acid (21).

Isoelectric focusing. Isoelectric points were determined using an LKB 8100 column (110 ml) containing a 20–40% (w/v) sucrose gradient and a 1.6% (v/v) concentration of pH 3–10 ampholine solution (LKB). The cathode and anode solutions were 2.9% (v/v) ethylenediamine and 1% (v/v) phosphoric acid, respectively. The column (4°) was prefocused at 300 V (5 mA) prior to introduction of the sample, and the run itself was performed at 500 V (1.5 mA) for 72–96 hr. Polyacrylamide gels for isoelectric focusing were prepared using a modification of the method of Dale and Latner (22). For an 8 ml gel solution, the following were combined: 1 ml of 0.8% TEMED, 2 ml monomer solution [28% (w/v) acrylamide, 0.735% (w/v) bis-acrylamide], 0.3 ml 40% ampholine (LKB, pH 3.5–10), 3.7 ml distilled water, and 1 ml 0.004% (w/v) riboflavin. The samples were added to this mixture in amounts necessary to give a concentration of 50–150 μg/ml. The gels were photopolymerized for 1.5 hr and then equilibrated to 4°. The upper, cathodal chamber contained 2.9% ethylenediamine and the lower, anodal chamber contained 1% phosphoric acid. Gels were run at 100 V (1.5 mA) for 16–18 hr, and sliced with the aid of a Gilson automatic gel slicer.

Results. A typical elution profile of partially purified CSF on Con A–Sepharose is shown in Fig. 1. It was observed that no statistically significant biological activity was eluted by the equilibration buffer. However, this unretarded fraction accounted for about 55% of the total protein recovered from the column. Application of a linear gradient of αMM resulted in elution of colony-forming activity and approximately 33% of the recovered protein (9% of the total protein applied to the column). Thymidine-incorporation-stimulating activity was also eluted under these same conditions. Valid dose response curves were obtained for only selected effluent fractions because of the limited amount of recovered material. As can be seen in Fig. 1, specific activities (colony formation) of individual fractions were increased five- to 15-fold over that of the standard reference material. Fractions from appropriate regions of the elution pattern were pooled and assayed for recovery of protein and biological activity. Protein recovery was generally about 30% of that applied to the column while recovery of biological activity (colony formation) was usually about 50%. Additional protein (less than 5% of that applied to the column) could be eluted by applying a 2% αMM buffer; however, this material had no significant biological activity. Colony-forming activity of the active pool exhibited a six- to ninefold increase in specific activity over the standard reference material, while in one experiment thymidine-incorporation-stimulating activity increased by as much as 25-fold.[4]

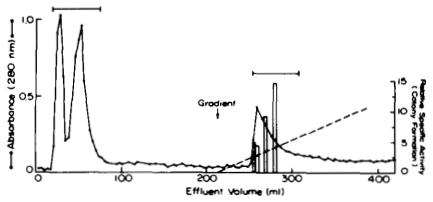

FIG. 1. Affinity chromatography of CSF on Con A–Sepharose 4B. The sample (123 mg, 8.25 ml, sp act 2429 colonies/mg protein) was applied to a column (0.9 × 60 cm) equilibrated at 4° in 0.02 *M* sodium phosphate, 1 *M* NaCl, pH 7.0. A flow rate of 17 ml per hr was maintained. Following elution of unbound protein with the equilibrating buffer, a linear gradient of αMM (1–60 m*M*) was applied. Selected fractions were assayed for protein and colony-forming activity, and the darkeed bars indicate specific activities relative to the standard reference preparation described in Materials and Methods. Pooled fractions are indicated by the horizontal lines.

Not all glycoproteins were selectively retained on the column under the initial elution conditions. Thus, both the unadsorbed and adsorbed fractions contained anthrone-positive carbohydrate when analyzed following dialysis. In addition, further evidence for the selective retention of CSF activity

[4] Such differences in these two specific activities of CSF will be the subject of a separate paper.

was provided by the finding that pretreatment of the affinity matrix with αMM (0.06 *M*) caused the material responsible for biological activity to pass unretarded through the column.

Neuraminidase treatment of CSF eluted from Con A–Sepharose. Neuraminidase treatment of CSF was found to have no adverse effect on its *in vitro* biological activity. In fact, an approximate two- to threefold increase in colony-forming activity was consistently seen in both the control and enzyme-treated samples. At the present time we cannot account for the increase in activity of the incubated control, although inactivation of inhibitory material by neuraminidase or by contaminating protease (0.01 units/mg protein, personal communication, Sigma) might explain the elevated activity of the enzyme-treated preparation. When neuraminidase-treated CSF was rechromatographed on the affinity column, no activity was eluted in the absence of the sugar, while application of the αMM gradient resulted in elution of colony-forming activity in both control (Fig. 2A) and treated (Fig. 2B) preparations over the same effluent volume. The specific activities (colony formation) of the pooled fractions

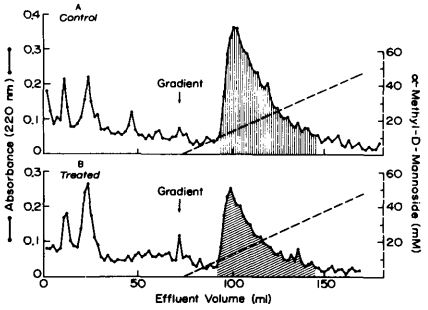

FIG. 2. Rechromatography of untreated (A) and neuraminidase-treated (B) CSF on Con A–Sepharose 4B. Starting material was obtained by chromatography as described in the legend to Fig. 1 and had an average sp act of 21,000 colonies/mg protein. Control preparations were incubated under identical conditions except that no enzyme was present. Conditions for rechromatography were identical except for size of the column (0.9 × 30 cm) and sample (1.5 mg protein, 1.8 ml). Recovery of protein and activity were essentially complete so that the specific activities (colony formation) for the pools of material represented by the shaded areas of the profiles were the same as those of the respective samples applied to each column.

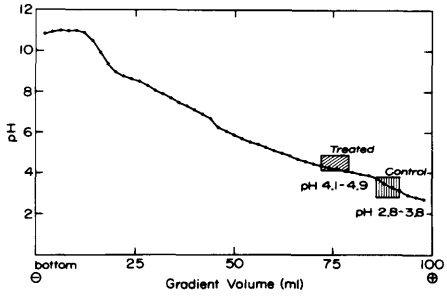

FIG. 3. Isoelectric focusing of CSF in a sucrose stabilized gradient (pH 3–10) before (control) and after (treated) treatment with neuraminidase. Control preparations were incubated under identical conditions except that no enzyme was present, and the specific activity of the sample was approximately 21,000 colonies/mg protein. Treated and control preparations (270 μg, 1 ml) were focused in separate experiments. The pH gradient is an average of three separate experiments. Because of the small amount of material, it was impossible to obtain valid dose response curves and recovery values. Thus, shaded areas represent regions of biological activity (about 40 colonies). When a sample (2.82 mg) of biologically active material obtained as described in the legend to Fig. 1 was focused under identical conditions, 34% of the colony-forming activity was recovered.

from both treated and control samples were not significantly different from those of the respective samples applied to the column. Similar results were observed when thymidine-incorporation-stimulating activity was measured.

Isoelectric point of CSF before and after treatment with neuraminidase. The isoelectric point of CSF which had been eluted from a Con A–Sepharose column was determined, and this value was compared with that obtained after the sample had been treated with neuraminidase. It was found (Fig. 3) that biological activity of the untreated control was associated with material having an average isoelectric point of pH 3.3. Treatment with neuraminidase caused the isoelectric point to increase to pH 4.5, which was consistent with the removal of sialic acid residues. Of even greater interest was the observation that both treated and control preparations exhibited isoelectric points over a strikingly broad range of pH. In order to ascertain whether or not such an observation was due to the method of analysis used, we carried out similar experiments using an

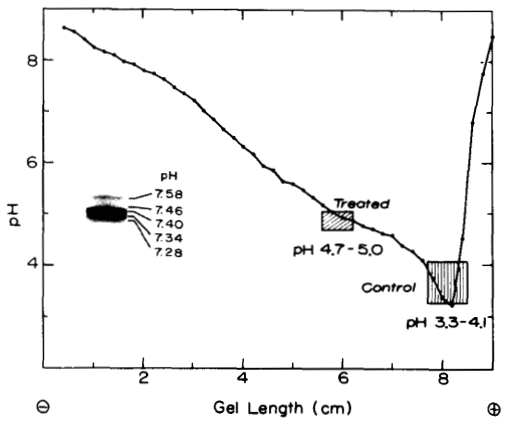

FIG. 4. Isoelectric focusing of CSF in a polyacrylamide gel stabilized gradient (pH 3–8) before (control) and after (treated) treatment with neuraminidase. The pH curve is a composite of three separate gel gradients. Both control (312 μg) and treated (406 μg) preparations were focused in separate gels. The sp act of the samples was about 21,000 colonies/mg protein. Owing to poor recovery from the gel and loss of activity, it was not possible to obtain valid dose response curves. Thus, biological activity (20–30 colonies) is given by the shaded regions. The sharp rise in pH at the anode is an artifact resulting from drainage of a small amount of basic cathode solution along the glass tube prior to slicing the gels. The insert is a photograph of mouse hemoglobin (unstained) which was used as a marker to assess the reliability of each experiment. Other details are given in the text.

alternative procedure. Fig. 4 presents the results obtained when the isoelectric points of both treated and control CSF preparations were determined in pH gradients stabilized by a polyacrylamide gel. In this system, the untreated control CSF had an average isoelectric point of pH 3.7, while the treated material had an isoelectric point of about pH 4.8.

As an additional check to be sure that the spreading of biological activity over the rather wide pH range was not an artifact of this system, we included a sample of mouse hemoglobin as a marker. Rodent hemoglobin is known to contain at least five species, each having a distinct and different isoelectric point in the range of pH 7–8 (23). Prior to switching off the current, we observed five major bands (Fig. 4, insert) within a range of less than 0.5 pH units. Gels were sectioned as soon as the focusing run was terminated. Also, there was negligible diffusion of the hemoglobin bands for at least 2 hr following termination. Thus, we have concluded that the apparent heterogeneity of CSF, as indicated by such broad bands of activity, is a real phenomenon.

Discussion. Previous work suggested that the colony-forming activity of CSF was associated with a material which was glycoprotein in nature (9–13). Our observation that CSF activity was retained on a Con A–Sepharose matrix was consistent with this idea. However, since a protein may interact in a number of ways with an affinity support and thereby be retained, it was necessary to demonstrate the selectivity of such interaction. It was observed that the biological activity of CSF was retained on the column in the presence of 1 *M* NaCl over a period when an effluent volume of at least 15 column volumes was collected (Fig. 1). This finding supported the idea that ionic interactions were not responsible for retention of CSF. Secondly, it was found that not all glycoproteins in the preparation of CSF were bound to the support under conditions where all biological activity of CSF was retained. Thirdly, it was observed that low concentrations of either αMM or αMG, both of which specifically interact with Con A (24), were capable of eluting the biological activity from the support. Pretreatment of the affinity column with αMM completely inhibited binding of any CSF to the support. Finally, rechromatography of the biologically active material under identical conditions, either before or after treatment with neuraminidase, resulted in elution of the activities over the same effluent volume (Fig. 2); specific activities of the pooled fractions from both treated and control were the same. Taken together, these data strongly supported the conclusion that the biological activity associated with CSF was a glycoprotein that selectively interacted with the Con A–Sepharose adsorbent. It should be noted that affinity chromatography of CSF under these conditions often resulted in an apparent instability of the biological activity as reflected by a significant loss of activity as a function of time (as much as 90%). This problem has been reported by others (29),

and until it is reproducibly eliminated, limits the usefulness of the procedure as a step in the purification of CSF.

The results of our studies on the isoelectric point of CSF were essentially in agreement with those of Ecsenyi (25). Moreover, neuraminidase treatment was found to have no effect on the *in vitro* biological activity of CSF, thus confirming the work of Stanley and Metcalf (11). A similar observation has been made for erythropoietin, a more fully characterized glycoprotein that is similar in many respects to CSF; however, such treatment did abolish its biological activity *in vivo* (26). Our data concerning the effect of neuraminidase treatment on the isoelectric point of CSF directly supports the glycoprotein nature of this molecule(s). When all sialic acid residues were removed enzymatically, as judged by comparison with the amount of sialic acid released by acid hydrolysis, the isoelectric point of the biological activities associated with CSF showed a dramatic increase from about pH 3.5 to about 4.7. This observation was made using either a sucrose (Fig. 3) or polyacrylamide gel (Fig. 4) stabilized pH gradient. Recently, it was reported that a CSF preparation obtained from EI-cell-conditioned medium (27) contained no carbohydrate. Although this preparation has not been subjected to chromatography on Con A–Sepharose, it is interesting that the isoelectric point of the protein (pH 4.5–5.5) (28) is similar to that obtained from our human leukemic urinary CSF (pH 47) after treatment with neuraminidase.

Previous work (19) showed that under the conditions employed in this study, albumin was not retained by the Con A–Sepharose support. We found that a more effective means of removing the contaminating albumin was passage of the sample through an affinity matrix of Blue Dextran–Sepharose. This method would appear to have advantages over relatively nonspecific digestion with proteases or use of an anti-HSA IgG (13, 29), judging from the simplicity, absence of exogeneous agents, and ease of scaling up the affinity procedure.

The most interesting observation resulting from the present work concerns the apparent heterogeneity of the molecules associated with biological activity of CSF. Such heterogeneity has previously been observed in a number of systems such as starch gel electrophoresis and chromatography on DEAE, hydroxyapatite, and various Sephadexes (9, 10). In studies on mouse embryo-conditioned medium (10), it was speculated that differing amounts of sialic acid might be responsible for the observed heterogeneity. Our results indicated that despite removal of all sialic acid residues, the biological activities of CSF appeared to be associated with a population of molecular species having different isoelectric points. The fact that the biological activities of CSF, both before and after neuraminidase treatment, eluted over a rather wide range of effluent volume might also be taken as supporting evidence for this heterogeneity. This same phenomenon has been reported for erythropoietin (30). Possible explanations might include differences in amide content, microheterogeneity of the carbohydrate moiety, or genetic polymorphism arising from the multipatient source of urine (30). The major question, of course, is whether this apparent heterogeneity has any physiological significance. In this regard, it should be noted that erythropoietin has been postulated to contain a microheterogeneous population of molecules associated with a number of differing biological activities (31, 32). Our own results (33) indicate that the same may be true for CSF. Nevertheless, the affinity procedures developed in the present study have proved useful in the partial purification of CSF (six- to ninefold), and they provide a further tool for the comparative study of glycoprotein colony-stimulating factors from other sources.

Summary. Biological activities associated with colony-stimulating factor (CSF) from human leukemic urine were found to be selectively retained on an affinity adsorbent of Con A–Sepharose. Elution of activity was achieved using a linear gradient of either α-methyl-D-mannopyranoside (αMM) or α-methyl-D-glucopyranoside (αMG), and resulted in significant increases in specific biological activity. Rechromatography of appropriate fractions indicated that retention of CSF activities was not artifactual. Pre-

treatment of the affinity matrix with αMM completely inhibited binding of CSF. Affinity chromatography of CSF on a Blue Dextran–Sepharose adsorbent was found to be an effective method for removing albumin, a major protein contaminant in urinary preparations. Treatment of CSF with neuraminidase had no effect on its *in vitro* activity; however, such treatment resulted in an increase in the isoelectric point of CSF from pH 3.5 to pH 4.7, as determined using both sucrose and polyacrylamide gel stabilized pH gradients. Relatively broad regions of biological activity were observed following isoelectric focusing of both neuraminidase-treated and untreated CSF, suggesting that activity was associated with a heterogeneous/polydisperse population of molecular species.

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