Purification of Colony-Stimulating Factor by Affinity Chromatography

By Abdul Waheed and Richard K. Shadduck

Studies were undertaken to determine whether L-cell-derived colony-stimulating factor (CSF) could be purified by a single step affinity chromatographic technique. A quantity of 100 x 10^6 units of purified anti-CSF was coupled to cyanogen bromide activated Sepharose 4B; colony assays revealed complete binding of the antibodies to the gel. Three 10-liter pools of serum-free L-cell CSF were concentrated by ultrafiltration, applied to the gel, and eluted with a low pH, high molarity buffer. Recovery of CSF ranged from 68%–100% with greater than 1000-fold decreases in protein content. Specific activity of the purified CSF ranged from 2.8 to 5.9 x 10^9 U of CSF/mg protein. Following iodination, each purified pool of CSF revealed a major 63,000-dalton peak of radioactivity that comigrated with CSF activity in SDS-acrylamide gels. In addition, several smaller peaks of 50,000 and 40,000 molecular weights were also detected. Approximately two-thirds of the purified CSF was adherent to concanavalin-A with elution by a competing sugar. Electrophoretic mobility was retarded by incubation with neuraminidase. These chromatographic studies confirm the observation that CSF is a glycoprotein but also suggest variable degrees of glycosylation of the molecule. This chromatographic technique should prove useful in the rapid purification of large quantities of CSF for physiologic and biochemical characterization.

COLONY-STIMULATING FACTOR (CSF) is a glycoprotein material that is required for the in vitro growth of marrow cells into colonies of granulocytes and macrophages. Based on this finding and the belief that the responsive progenitor cells represent a class of hemopoietic stem cells committed to differentiation only in the granulocyte-macrophage pathway, many studies have attempted to show a granulo-poietic effect of CSF in vivo. Induction of neutropenia is associated with increased serum levels of CSF, and injection of impure sources of CSF has led to modest stimulation of granulopoiesis. Although these correlative studies suggest a regulatory role for CSF, bacterial endotoxins also increase CSF activity, and, on repetitive injection, induce granulocytic hyperplasia. These problems with endotoxin and other potential contaminants indicate the need for pure sources of CSF for in vivo studies. Several groups have described extensive techniques for the purification of murine types of CSF. These entail tedious and time-consuming steps that require a minimum of several months for completion. Recoveries of biologic activity have been variable and have ranged from 25% to 70%.

Preliminary characterization of the L-cell type of CSF has shown that it is a glycoprotein with a molecular weight of 65,000–70,000. There is heterogeneity with respect to carbohydrate content as only two-thirds of the material binds to concanavalin-A, whereas the remainder is nonadherent. Both fractions stain avidly with periodic acid Schiff (PAS) and are inactivated with periodate. Removal of sialic acid residues with neuraminidase alters electrophoretic mobility of both fractions but does not decrease biologic activity. The active molecule consists of two major subunits of 35,000 molecular weight bound by disulphide bonds. Further characterization has been hampered by a relative paucity of material for study.

Recently, we have described a technique for the selective purification of antibodies directed against L-cell CSF. In these studies, purified CSF was covalently bound to an immunoabsorbent gel; this gel was then used for the preferential adsorption and subsequent elution of presumed "monospecific" antibodies.

This report describes a similar approach to the purification of CSF. Large quantities of purified CSF antibodies were coupled to cyanogen bromide activated Sepharose; concentrated serum-free L-cell conditioned medium was passed through the gel and eluted with low pH, high molarity conditions. This simple one-step procedure yielded highly purified CSF that is comparable in specific activity to material produced by the previously described techniques.

MATERIALS AND METHODS

Murine CSF was obtained by the growth of L-cells in serum-free CMRL 1066 tissue culture medium as previously described. The supernatant conditioned medium, which contained 800–1200 U of CSF/ml, was concentrated 250-fold by ultrafiltration using an Amicon PM-10 membrane and subjected to a 6-step purification procedure as previously described. Using this technique, the specific activity of CSF increased greater than 1000-fold to approximately 5 x 10^9 U/mg of protein. Total recovery of CSF was approximately 70% of the starting material. The CSF appeared homogeneous as judged by mobility in various concentrations of acrylamide, by behavior in SDS acrylamide gel, and by development of a single precipitin line when reacted with anti-CSF serum.

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Purified CSF (473 μg) was coupled to 1.5 g of cyanogen bromide activated Sepharose and poured into a 1 x 5 cm column. Thirty milliliters of antiserum11 from each of two rabbits with high titer antibodies were dialyzed against 0.1 M Tris-0.1 M NaCl-0.1 M glycine buffer, pH 7.5, containing 0.3% polyethylene glycol 4000 (PEG). Three milliliters of antiserum was applied to the column, rinsed with 50 ml of buffer, and eluted with 10 ml of 2 M guanidine in starting buffer adjusted to pH 4.0. The eluted anti-CSF from 10 cm columns for each antibody was pooled and dialyzed 10 times to remove the guanidine using an Amicon PM-10 membrane. Anti-CSF assays indicated recovery of approximately 50% of the starting material or 100 x 10^6 U of anti-CSF. Total protein was reduced to 1.86 mg, which represented approximately 0.7% of the starting IgG or 0.07% of the starting serum concentration. The eluted anti-CSF appeared free of contaminating proteins, as only a single line was noted in Ouchterlony gel diffusion using goat anti-rabbit serum and goat anti-rabbit IgG serum.

The purified CSF was dialyzed against 0.1 M NaHCO₃-0.5 M NaCl containing 0.3% PEG, pH 8.0. The material was coupled to 3 g of cyanogen bromide activated Sepharose as previously described for CSF12 and poured into a 1 x 13 cm glass column. All coupling, blocking, and washing reagents were dialyzed against buffer and subsequently assayed for anti-CSF to determine the extent of binding of antibody to the gel. The capacity of the anti-CSF column to bind L-cell CSF was determined using incremental quantities of serum-free conditioned media.

Initially, a 5-liter pool of L-cell CSF was concentrated to 25 ml and dialyzed against 0.1 M NaCl-0.3% PEG buffer, pH 7.5. Five-milliliter aliquots, each of which represented the CSF from 1 liter of starting material, were passed through the column and rinsed with 50 ml of buffer to remove nonadsorbed proteins. This process was repeated 5 times until the CSF from 5 liters of starting material had been applied to the gel. The unadsorbed conditioned media as well as the buffer rinses were saved for CSF determinations. Attempts were made to elute the adsorbed CSF by sequential passage of 10-ml aliquots of 0.5, 1.0, 1.5, and 2 M guanidine dissolved in starting buffer adjusted to pH 6.5. To minimize denaturation of CSF by guanidine, the eluates were collected with continuous stirring into 10 ml of starting buffer maintained at 4°C. All binding, washing, and other elution studies were carried out at room temperature. Prior to assay for CSF, the resultant eluates were concentrated and dialyzed using Amicon PM-10 membranes.

Once it had been established that the anti-CSF column was capable of binding CSF from 5 liters of starting material, a second 10-liter pool was concentrated to 40 ml and divided into aliquots. Twenty milliliters of the CSF concentrate was applied to the column and washed with 50 ml of buffer. Two additional 10-ml aliquots were added to the column as above and the unadsorbed samples saved for determination of CSF. The column was subsequently eluted with 10-ml aliquots of 0.5, 1.0, 1.5, and 2 M guanidine that had been adjusted to pH 4.0 in order to improve CSF elution. Each individual aliquot was dialyzed 10 times against starting buffer to remove the guanidine and subsequently tested for CSF bioactivity.

The next two 10-liter pools of serum-free L-cell conditioned media (pool 11 and 12) were concentrated to 50 ml using an Amicon PM-10 membrane and equilibrated against 0.1 M Tris-0.1 M NaCl-0.1 M Tris-0.1 M NaCl containing 0.3% PEG buffer, pH 7.5. These conditioned media, each of which represented the CSF from 10 liters of starting material, were each separately passed through the anti-CSF column and unadsorbed contaminating proteins removed by washing the column with 300 ml of buffer. The CSF was eluted by a single passage of 25 ml of 2 M guanidine, pH 4.0, in starting buffer; eluates were collected with constant stirring into 25 ml of starting buffer held at 4°C. The guanidine eluates were subsequently concentrated to approximately 2 ml and dialyzed extensively to remove any residual guanidine.

For CSF bioassays, 10^1 CFU, mouse marrow cells were mixed in 1 ml of 0.3% McCoy’s agar2 in the presence of 0.1 ml of serum-free L-cell conditioned medium or 0.1 ml of appropriate dilutions of purified fractions. All samples were sterile-filtered by passage through autoclaved 0.45 μm Millipore filters prior to assay. CSF activity was calculated from the linear portion of the dilution curve wherein formation of one colony was defined as 1 U of CSF activity. Anti-CSF activity was determined by addition of 0.1 ml of antiserum or purified fractions to agar gel cultures that were stimulated by 0.05 ml of standard CSF. Inhibition was determined by reduction in colony formation as compared to control plates. The titer of anti-CSF activity was defined as the dilution of antibody that caused 50% inhibition of colony formation. This value was determined by interpolation using a standard dilution curve. Units of anti-CSF were calculated by multiplying the reciprocal of the 50% antibody neutralization titer by the number of colonies stimulated with the standard CSF. Thus, anti-CSF units exceeded CSF units by at least twofold.

Protein determinations were performed using the Lowry technique after precipitation of protein by trichloroacetic acid. This step was necessary, as the initial conditioned media contained phenol red as an indicator that interfered with the colorimetric determination of protein. In purified samples with extremely low protein values, a dye binding technique with Coomassie brilliant blue G-250 was utilized.

The purified CSF was radioiodinated using a modified chloramine-T technique as previously described. To determine the apparent purity of CSF, 50,000-100,000 cpm of labeled material or 0.5-1.1 μg of unlabeled CSF were subjected to electrophoresis in acrylamide gels both alone or were coupled to acrylamide gels, as the initial conditioned media contained phenol red as an indicator that interfered with the colorimetric determination of protein. In purified samples with extremely low protein values, a dye binding technique with Coomassie brilliant blue G-250 was utilized.

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The apparent molecular weight of CSF was determined with SDS acrylamide gels. The unlabeled or radioiodinated CSF was incubated with SDS (1% final concentration) for 3 hr at room temperature. A series of marker proteins including beef liver catalase (mol wt 58,000), ovalbumin, grade VI (mol wt 43,000), lactate dehydrogenase (mol wt 36,000), concanavalin A, Grade IV, (subunit mol wt 27,000), and alpha-chymotrypsinogen (mol wt 25,000) (Sigma, St. Louis, Mo.) were similarly treated. These proteins were subjected to electrophoresis in SDS, 7.5%–10% acrylamide gels and their degree of migration relative to a bromophenol blue dye marker was determined. The mobility of the CSF was monitored by both radioactivity and radioimmunoassay. The molecular weight of CSF was calculated by plotting the log of the molecular weight against the relative degree of migration and comparing this to the behavior of marker proteins.

Further studies examined the effect of neuraminidase on carbohydrate residues of CSF. Fifty microliters of radiiodinated CSF (100,000 cpm) and unlabeled CSF (1.1 μg) were adjusted to pH 5.0 with 40 μl of 0.4 M acetate, 0.3% PEG buffer. These samples were reacted with 1.1 μg of purified neuraminidase (147 U/mg, Worthington Biochemical Corp. Freehold, N.J.) at 37°C for 20 hr. Samples were run on 7.5%–10% acrylamide gels and their degree of migration relative to a bromophenol blue dye marker was determined. The mobility of the CSF was monitored by both radioactivity and radioimmunoassay. The molecular weight of CSF was calculated by plotting the log of the molecular weight against the relative degree of migration and comparing this to the behavior of marker proteins.
herent to concanavalin-A Sepharose, whereas the remaining two-thirds was bound and subsequently eluted with a competing sugar. In order to compare the behavior of CSF purified by the present affinity techniques, either 1.4 x 10^6 U of unlabeled CSF or 7.4 x 10^6 cpm of 125I-labeled CSF were applied to a 1.5 x 25 cm column of concanavalin-A-Sepharose. The gel was rinsed with 50 ml of 0.1 M sodium acetate buffer containing 1 M NaCl with 10^-3 M MnCl2, MgCl2 and CaCl2, pH 6.0. The adherent CSF was eluted with 50 ml of this buffer, which contained 0.1 M alpha-methyl glucoside.

RESULTS

A quantity of 100 x 10^6 U of purified anti-CSF was coupled to 3 g of cyanogen bromide activated Sepharose and poured into a 1 x 13 cm glass column. Coupling, blocking, and washing agents were dialyzed against buffer and assayed for anti-CSF by inhibition of colony formation. None of the samples decreased colony growth, thus indicating that virtually all anti-CSF was bound to the Sepharose gel.

Attempts were made to determine the maximum capacity of this gel for CSF. Five liters of serum-free L-cell conditioned media (pool 9) were dialyzed and concentrated to 25 ml. Five-milliliter aliquots were each separately applied to the column; each application was followed by a 50-ml buffer wash. No CSF could be detected in any of these washes; this suggested complete adsorption of CSF to the column. In order to elute the adsorbed material, 10 ml of 0.5, 1.0, 1.5, and 2 M guanidine, pH 6.5, were applied. As shown in Table 1, CSF was only eluted with the 2 M guanidine. Once the results of this assay were available, the column was further treated with an additional 10 ml of 2 M guanidine that had been adjusted to pH 4.0. This led to the additional recovery of 0.45 x 10^6 U of CSF. The total recovery of CSF (1.89 x 10^6 U) represented 30% of the applied CSF activity. This low recovery may have resulted, in part, from the 10-day time delay that occurred between the two elution steps.

Table 2. The Binding of L-Cell CSF to an Affinity Column of Anti-CSF

<table>
<thead>
<tr>
<th>Application</th>
<th>Elution</th>
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<tbody>
<tr>
<td>5 liters</td>
<td>Guanidine</td>
</tr>
<tr>
<td>2.5 liters</td>
<td>0.5 M</td>
</tr>
<tr>
<td>2.5 liters</td>
<td>1.0 M</td>
</tr>
<tr>
<td>2.5 liters</td>
<td>1.5 M</td>
</tr>
<tr>
<td>2.5 liters</td>
<td>2.0 M</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>0.5 M</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>1.0 M</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>1.5 M</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>2.0 M</td>
</tr>
</tbody>
</table>

Each pool showed a major peak of radioactivity with a relative mobility of 0.42–0.44 with respect to a bromophenol blue marker.

The binding and elution of L-cell CSF using a 5-liter pool of conditioned media:

<table>
<thead>
<tr>
<th>Application</th>
<th>Guanidine elutions</th>
<th>Total recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 1 liter</td>
<td>6.2 x 10^6</td>
<td>1.85 x 10^6 (30.5%)</td>
</tr>
<tr>
<td>Buffer wash</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total bound</td>
<td>6.2 x 10^6</td>
<td></td>
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</tbody>
</table>

The CSF from 5 liters of conditioned medium was concentrated to 25 ml. Aliquots of 5 ml each were passed through the anti-CSF column and rinsed with 50 ml of buffer. Ten milliliters of varying concentrations of guanidine were used to sequentially elute the gel. The pH 4.0 guanidine was added 10 days later after colony assays showed low recovery of the bound CSF. Units of CSF were determined by bioassay.
Three separate 10-liter pools of CSF were concentrated 200–250-fold and applied to the anti-CSF column. Purified CSF was eluted with stepwise increments in guanidine (pool 10) or 10 ml of 2 M guanidine, pH 4.0 (pools 11 and 12). CSF activity was determined by bioassay.

Secondary peaks of radioactivity with relative mobilities of 0.56–0.60 were observed in all 3 pools. In addition, small tertiary peaks of radioactivity were observed with pools 11 and 12.

Further studies were done with pool 11 and pool 12 to determine whether the peaks of radioactivity corresponded to the CSF activity. Both radioiodinated and “cold” CSF were subjected to SDS acrylamide gel electrophoresis. CSF activity from the gel containing “cold” CSF was determined by radioimmunoassay after exposure to a mixed bed resin for removal of SDS. As shown in Fig. 2, the major band of radioactivity corresponded to a similar band of CSF activity. In addition, the small secondary and tertiary peaks of radioactivity were also active as judged by radioimmunoassay. Pool 11 had 84% of the radioactivity in the major peak, with an additional 11% and 5% in the secondary and tertiary peaks, respectively.

The biologic activities of the 2 peaks of CSF from pool 12 were studied in further experiments. The migration of unlabeled CSF in SDS acrylamide gel slices was monitored by radioimmunoassay after 3-day exposure to a mixed bed resin to remove SDS. By RIA,
50–60 U of CSF were found in each peak; however, little activity could be detected in the bioassay. The 2 peaks were further dialyzed, concentrated, and reasayed. Small numbers of clusters (3–5/plate) were obtained on day 4 with development of 1–3 colonies on day 7. Individual clusters from the high molecular weight peak were 50% macrophage, 11% granulocytic, and 39% mixed type. The second peak generated 40% macrophage, 20% granulocytic, and 40% mixed clusters. All 7-day colonies were composed entirely of macrophages. Thus, there did not appear to be major differences in the biologic activities of the two forms of CSF.

The molecular weights of the various peaks of CSF from pool 12 were determined by electrophoresis in SDS acrylamide gels. The results are shown in Fig. 3. The major peak of CSF activity corresponded to a molecular weight of approximately 63,000 with two smaller peaks of approximately 50,000 and 40,000 daltons, respectively. Similar results were noted with pools 10 and 11 in which the major peaks of radioactivity migrated with apparent molecular weights of 59,000 and 62,000, respectively.

To determine whether the secondary peak of radioactivity was due to variation in carbohydrate content, purified pool 12 CSF was incubated with or without neuraminidase for 30 hr at 37°C. The samples were then subjected to electrophoresis in 7.5% acrylamide gel. Both radioiodinated and unlabeled pool 12 CSF were incubated with neuraminidase as above and subsequently treated with 1% SDS for 3 hr. All samples were electrophoresed in SDS 7.5% acrylamide gels. Both neuraminidase-treated and control CSF showed identical patterns of migration; the latter is not shown.

Neuraminidase treatment did not affect CSF activity nor did it change the migration of the small secondary peak of bioactivity and radioactivity.

In previous studies, approximately two-thirds of the

<table>
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<th>Peak I (Nonadherent)</th>
<th>Peak II (Adherent)</th>
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<tr>
<td>&quot;Cold&quot; CSF</td>
<td>32.1%</td>
<td>67.8%</td>
</tr>
<tr>
<td>125I-CSF</td>
<td>58.5%</td>
<td>41.4%</td>
</tr>
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</table>

Two sources of purified pool 12 CSF (1.4 x 10^6 U of unlabeled material or 7.4 x 10^4 cpm of labeled material) were passed through a concanavalin-A-Sepharose gel. Peak I passed through the gel whereas peak II was retained and eluted with alpha-methyl glucoside.
CSF was selectively bound to concanavalin-A-Sepharose, whereas the remaining one-third was nonadherent. Material purified by the affinity column was passed through a column of concanavalin-A-Sepharose and the adherent variety eluted with 0.1 M alpha-methyl glucoside. As shown in Table 4, 32% of the CSF was recovered in peak I, which was nonadherent to concanavalin-A. The remaining two-thirds of the CSF was eluted with the competing sugar. After iodination, a greater proportion of CSF (58.5%) passed through the concanavalin-A gel. This change in behavior may be due to oxidation of some of the carbohydrate residues without affecting bioactivity. After separation on concanavalin-A, each peak was applied separately to SDS acrylamide gels. The results are shown in Fig. 5. The first peak, which was nonadherent to concanavalin-A, was relatively symmetrical with essentially no secondary peaks of radioactivity. In contrast, peak 2, which was eluted with alpha-methyl glucoside, showed a prominent secondary peak of radioactivity. This suggests that the lower molecular weight species of CSF may have variations in the carbohydrate residues, which confer greater affinity to concanavalin-A.

**DISCUSSION**

These studies show that CSF can be purified by a one-step affinity chromatographic technique using a column of purified anti-CSF. This purification scheme is relatively simple and can be completed within several days as compared to 2–3 mo for the previous 6-step purification procedure. The specific activity of the CSF varied from 2.8 to $5.9 \times 10^7$ U/mg of protein. These specific activities compare favorably with those obtained by the more lengthy purification schedule. Moreover, the yield of active material ranged from 68% to 100% of starting material. This is somewhat better than the previous 50%–60% yields obtained with the 6-step procedure.

Each pool of purified CSF showed a major peak of radioactivity that comprised 80%–85% of the total radioactivity. The molecular weight of the main peak was approximately 59,000–63,000 as compared to 65,000–70,000 in previous studies. Several smaller peaks were also identified that had molecular weights of 50,000 and 40,000 daltons, respectively. Such lower molecular weight species had not been found in previous studies. It is likely that the lower molecular weight species had been separated from the major peak of activity and thus were lost in the numerous purification steps. In preliminary studies, we have also prepared serum-free L-cell CSF in the presence of tunicamycin, which is an inhibitor of glycosylation. After purification on the affinity column, this CSF revealed a number of molecular weight species with a major peak of activity in the range of 26,000. Thus, it appears that a substantial proportion of the CSF molecule is composed of carbohydrate residues that may not be essential for biologic activity.

The purified CSF obtained in these studies appears to be a glycoprotein with neuraminic acid residues. Incubation with neuraminidase reduced the electrophoretic mobility but did not affect the biologic activity. Approximately two-thirds of the CSF was adherent to concanavalin-A and was eluted with a specific competing sugar. As in previous studies, approximately one-third was nonadherent, which suggests some heterogeneity in the carbohydrate residues. After iodination there was a change in the behavior on concanavalin-A that was probably due to oxidation of carbohydrates.

In summary, the modified purification technique described here is rapid and simple and produces large quantities of purified material. This affinity technique should be useful in the rapid purification of a number of pure glycoprotein samples.
of forms of murine CSF. As fewer steps are involved, it may be possible to obtain high yields as compared to earlier techniques. In addition, use of this technique should provide the large quantities of purified CSF that will be required for extensive in vivo studies and for detailed biochemical analysis of the CSF molecule.

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