Purification and Some Properties of Colony-stimulating Factor From Normal Human Urine

By Kazuo Motoyoshi, Fumimaro Takaku, Hideaki Mizoguchi, and Yasusada Miura

A colony-stimulating factor (CSF) that stimulated human and mouse bone marrow cells to proliferate in vitro and form pure granuloid colonies was purified about 4000-fold from normal human urine. Purification procedures included concentration with polyethyleneglycol, ammonium sulfate precipitation, two chromatographic separations on DEAE-cellulose columns, gel filtration, and polyacrylamide gel electrophoresis. The molecular weight of the purified factor was estimated to be about 85,000 daltons by gel filtration, and the specific activity was found to be $10^6$ or $6.7 \times 10^4$ colonies/mg protein using mouse or human bone marrow cells, respectively. A urinary colony-inhibiting factor was separated from the CSF on the first DEAE-cellulose column. This inhibitor suppressed the formation of pure granuloid colonies of human and mouse bone marrow cells when employed in conjunction with the purified urinary CSF.

Certain progenitor cells in hemopoietic tissues have been shown to divide and differentiate in semisolid culture media to form colonies of granulocytes and/or macrophages. The formation in vitro of granulocyte macrophage colonies requires the presence of colony-stimulating factors (CSF), which can be provided indirectly via "feeder" cells or directly from cell-free preparations. CSF are found in serum, urine, certain tissues, and medium conditioned by certain cells. They have been highly purified by various groups.

Purification of CSF from human urine was first performed by Stanley et al., who showed that a 100,000-fold purified product was active on mouse bone marrow cells at $10^{-11} M$. The product was shown to be a sialic acid-containing glycoprotein of molecular weight 45,000–60,000 daltons that stimulated the early appearance of macrophages in murine colonies.

Recently we purified a CSF from normal human urine by procedures in which urine was concentrated with polyethyleneglycol (PEG), subjected to ammonium sulfate precipitation, chromatography on two successive DEAE-cellulose columns, gel filtration on Sephadex G-150, and polyacrylamide gel electrophoresis (PAGE). We found that CSF and its inhibitor, which were mixed in the crude fractions, were separated on the first DEAE-cellulose column and that highly purified CSF can induce the formation of pure granuloid colonies from human as well as mouse bone marrow cells.

In this report, the purification procedures, some properties of CSF and colony-inhibiting factor (CIF) from normal human urine, and the interaction between these two antagonistic factors are described.
MATERIALS AND METHODS

Concentration of normal human urine (CSFHU-1). As starting material for purification of CSF
4 liters of human urine from normal human adults were collected and concentrated to about 40
ml in cellulose tubing with PEG 6000. After dialysis against 1 liter of 0.1 M Tris-HCl buffer
(pH 7) for 6 hr, the proteins contained in the concentrated urine were precipitated by adding
21 g of solid ammonium sulfate (80% saturation). The precipitate collected by centrifugation
was dissolved in a small amount of 0.1 M Tris-HCl buffer (pH 7), and the solution was dialyzed
overnight against the same buffer. The dialyzed material (20 mg protein/ml, 6.65 ml) was used
as the starting material for CSF purification (CSFHU-1). The purification procedure described
below was performed at 0°-3°C, and the extent of purification at each step was assayed using
mouse bone marrow cells.

First DEAE-cellulose column chromatography (CSFHU-2). CSFHU-1 was applied to a column
of DEAE-cellulose (10 x 300 mm, 23.6 ml) that had been equilibrated with 0.1 M Tris-HCl
buffer (pH 7), and the column was washed with 120 ml of the same buffer. The flow rate was
adjusted to 6 ml/hr, and 3-ml fractions were collected. After washing the column, proteins were
eluted in two steps, first with 36 ml 0.1 M Tris-HCl buffer (pH 7) containing 0.2 M NaCl and
then with 40 ml 0.1 M Tris-HCl buffer (pH 7) containing 0.4 M NaCl. The active fractions were
pooled, concentrated under reduced pressure, and dialyzed overnight against 0.1 M Tris-HCl
buffer (pH 7) (CSFHU-2).

Second DEAE cellulose column chromatography (CSFHU-3). CSFHU-2 eluted from the
first DEAE-cellulose column was applied to a second column of DEAE-cellulose (10 x 382 mm,
30 ml) that had been equilibrated with 0.1 M Tris-HCl buffer (pH 7). The flow rate was
adjusted to 6 ml/hr, and 3-ml fractions were collected. After washing the column with 60 ml 0.1 M
Tris-HCl buffer (pH 7), elution was carried out with a linear concentration gradient of NaCl, using
150 ml 0.1 M Tris-HCl buffer (pH 7) in the mixing chamber and the same volume of 0.1 M Tris-
HCl buffer (pH 7) containing 0.2 M NaCl in the reservoir. The active fractions were pooled,
and the proteins were precipitated by adding solid ammonium sulfate (80% saturation) and were
dialyzed overnight against 0.1 M Tris-HCl buffer (pH 7) (CSFHU-3).

Sephadex G-150 column chromatography (CSFHU-4). CSFHU-3 was applied on a column of
Sephadex G-150 (10 x 226 mm, 19 ml) that had been equilibrated with 0.1 M Tris-HCl buffer
(pH 7) and eluted with the same buffer. The flow rate was adjusted to 3 ml/hr, and 0.9-ml frac-
tions were collected. V₀ and Vₑ values of this column were determined by the elution volumes
of blue dextran and ATP. The active fractions were collected, concentrated under reduced
pressure, and dialyzed overnight against 0.1 M Tris-HCl buffer (pH 7) (CSFHU-4).

Polyacrylamide gel electrophoresis (PAGE) (CSFHU-5). After concentration of the CSFHU-4,
several samples were analyzed by PAGE according to the method of Davis¹² using a 10% poly-
acrylamide gel at pH 8.9. Electrophoretic mobilities were expressed relative to bromphenol blue.
The gels were sliced longitudinally, and half of each gel was stained for proteins with Coomasie
Brilliant Blue (2.5% in methanol:acetic acid:water 2:1:5) and destained with acetic acid (7.5% v/v); the other half was sliced into 2.5-mm sections that were crushed and eluted with 1.5 ml
0.1 M Tris-HCl buffer (pH 7) and assayed for colony-stimulating activities after sterilization
by Millipore filtration.

Protein estimation. Protein was estimated quantitatively by the method of Lowry et al.¹³
using bovine serum albumin as the standard. Optical density at 280 nm was used to monitor the
protein concentration of column effluents. Densitometric tracing of Coomasie Brilliant Blue-
stained polyacrylamide gel was performed using a Gilford spectrophotometer model 2400-2.

Assay for colony-stimulating activity (CSA). CSA was assayed in 1.0-ml cultures in 35-mm
plastic dishes (Falcon Plastics, Los Angeles, Calif.) containing mouse or human bone marrow
cells. Mouse bone marrow cells were obtained from the femurs of 6-8-wk-old C3H/He mice.
Human bone marrow cells were obtained from patients with iron-deficiency anemia by sternal
puncture with heparinized syringes. They were used after removal of erythrocytes by centrifugation
and washing with McCoy’s 5A medium. A constant number of nucleated cells (7.5 x 10⁶ mouse or
25 x 10⁶ human cells) were cultured in a single layer in 1.0 ml of the supplemented McCoy’s
5A medium containing 0.3% agar, 20% fetal calf serum, and 10% (0.1 ml) CSF sample. CSF
samples were previously dialyzed against 0.1 M Tris-HCl buffer (pH 7) and Millipore filtered
for sterility. After 7-9 days of incubation in a humidified 5% CO₂ atmosphere, discrete colonies
suspended in 0.1 M Tris-HCl buffer (pH 7) for 6 hr. the proteins contained in the concentrated urine were precipitated by adding
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samples were previously dialyzed against 0.1 M Tris-HCl buffer (pH 7) and Millipore filtered
for sterility. After 7-9 days of incubation in a humidified 5% CO₂ atmosphere, discrete colonies

containing more than 50 cells (for mouse) or more than 20 cells (for human) were counted with an inverted microscope. For morphologic analysis of colonies, 30 of them were picked up with microhematocrit tubes and stained with 0.6% orcein in 40% acetic acid.

RESULTS

First DEAE-cellulose column chromatography (CSF<sub>HU</sub>-2). CSF<sub>HU</sub>-1 (20 mg protein/ml, 6.65 ml) was applied to a column of DEAE-cellulose and the column was washed with 0.1 M Tris-HCl buffer (pH 7). CSF activities were not eluted with this buffer. After complete elution of nonabsorbed proteins, washing of the column was continued with three or four column volumes of the same buffer. In this way, CIF (peak A, Fig. 1) was completely removed from the CSF fraction (peak B, Fig. 1), as will be described below. The column was then treated with 0.1 M Tris-HCl buffer (pH 7) containing 0.2 M NaCl and 0.1 M Tris-HCl buffer (pH 7) containing 0.4 M NaCl. CSF activity was found only in the fractions that eluted with 0.1 M Tris-HCl buffer (pH 7) containing 0.2 M NaCl (peak B, Fig. 1). None of protein was eluted with 0.1 M Tris-HCl buffer (pH 7) containing 0.4 M NaCl. The active fractions (Fr. 42 to Fr. 46)
were combined (15 ml) and dialyzed overnight against 1 liter of 0.1 M Tris-HCl buffer (pH 7). After dialysis, 20 ml of solution containing 1.5 mg/ml of protein was obtained (CSFU-2). The dose-response relationship between CSFU-2 and colony numbers is shown in Fig. 2.

The CIF fractions (Fr. 4 to Fr. 41) were also pooled, concentrated with ammonium sulfate precipitation (80% saturation), dialyzed against 0.1 M Tris-HCl buffer (pH 7), and used as an inhibitor in the colony inhibition assay, which will be described below.

Second DEAE-cellulose column chromatography (CSFU-3). CSFU-2 obtained as described above was applied to a second column of DEAE-cellulose that had been equilibrated with 0.1 M Tris-HCl buffer (pH 7) (details in Materials and Methods). After washing the column with the same buffer, it was eluted with a linear concentration gradient of Cl\(^-\) ions (0.1–0.3 M).

As shown in Fig. 3, CSF activity was eluted from the column as a single peak at about 0.15 M Cl\(^-\) ion. The peak fractions were combined (Fr. 43 to Fr. 58, 37 ml) and the proteins were precipitated by adding 19.1 g solid ammonium sulfate (80% saturation). The precipitate was collected by centrifugation, dissolved in a small amount of 0.1 M Tris-HCl buffer (pH 7), and dialyzed overnight against 500 ml of the same buffer. After dialysis, 0.5 ml of the material containing 23.6 mg/ml of proteins was obtained (CSFU-3).

Sephadex G-150 chromatography (CSFU-4). CSFU-3 was chromatographed on Sephadex G-150. As shown in Fig. 4, CSF activity was eluted as a single and almost symmetrical peak. The elution position was slightly ahead of bovine
serum albumin (BSA). From comparison of $K_d$ values between CSF and BSA (molecular weight of 68,000 daltons), the molecular weight of CSF was assumed to be about 85,000 daltons. Active fractions (Fr. 10 to Fr. 13) were pooled, concentrated under reduced pressure, and dialyzed overnight against 1 liter of 0.1 M Tris-HCl buffer (pH 7). After dialysis, 1.8 ml of the solution containing 1 mg/ml of protein was obtained (CSFHU-4).

PAGE of CSFHU-4 was carried out on a 10% polyacrylamide gel (Fig. 5). CSFHU-5 electrophoresed as a single peak with a mobility comparable to that of one of the five protein bands. From the results of assays using mouse or human bone marrow cells, the specific activity of CSFHU-5 was estimated to be about $10^6$ or $6.7 \times 10^5$ colonies/mg protein, respectively.

Summary of purification of CSFHU. A summary of the data obtained during the purification procedures is presented in Table 1. The procedures used resulted in an approximately 4000-fold increase in specific activity in both mouse and human bone marrow assays. The increase in specific activity at each step was nearly parallel for the mouse and human assays. The fact that the total activity

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<th>Table 1. Purification of CSFHU From Normal Human Urine</th>
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<td><strong>Mouse Bone Marrow Assay</strong></td>
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*Estimated from Coomassie Brilliant Blue-stained acrylamide gels.
Purification and Properties of CSF

Fig. 6. Colony-inhibiting assay. A, 30 μg CSF_HU-2 and indicated amount of CIF used in the assay with 7.5 x 10^6 mouse bone marrow cells. B, 9 μg CSF_HU-3 and indicated amount of CIF used in assay with 2.5 x 10^5 unfractionated human bone marrow cells. Vertical bars, SD.

Effects of CSF_HU on the colony formation by nonadherent human bone marrow cells. Experiments using nonadherent human bone marrow cells were carried out in order to clarify whether our CSF_HU preparations act directly on human granulocyte progenitor cells or whether they act indirectly by the stimulation

Colony inhibition assay was carried out using peak A in Fig. 1 as an inhibitor and CSF_HU-2 or CSF_HU-3 as a stimulator; results obtained are shown in Fig. 6. As shown in Fig. 6A, the reduction in colony numbers obtained by adding the inhibitor was nearly proportional to the amount of inhibitor added to the culture medium containing mouse bone marrow cells and CSF_HU-2 as a stimulator. Figure 6B shows that the same phenomenon was observed in the colony inhibition assay using unfractionated human bone marrow cells and CSF_HU-3 as a stimulator.

Fig. 7. Effects of CSF_HU on colony formation by nonadherent human bone marrow cells; 1.2 x 10^5 nonadherent cells were used. Vertical bars, SD.
of the production of macrophages and/or monocytes, which are considered to be the CSF producing cells. Preparation of nonadherent cells were carried out according to the method of Messner et al. As shown in Figs. 7B-7D, CSF$_{HU}$-2, 3, and 4 stimulated the colony formation by nonadherent cells with a dose-response relationship to the amount of added samples. The stimulation by CSF$_{HU}$-1 reached a plateau at 500 µg probably because of the existence of CIF in this fraction (Fig. 7A).

From the observation of 30 specimens in which the cells picked up from colonies were stained with acetoorcein, it was clear that almost all of the colonies stimulated by the factor consisted of 20-100 granulocytes. One of these specimens is shown in Fig. 8. From the results described above, it is probable that the CSF$_{HU}$ obtained by us directly stimulated the granulocyte progenitor cells to proliferate and differentiate into the mature granuloid cells.

**DISCUSSION**

The most important step in our purification procedures of CSF from normal human urine is the elimination of the CIF from the crude CSF fraction in the first DEAE-cellulose column (Fig. 1). Since CSF can bind to the DEAE-cellulose column in the presence of 0.1 M Cl$^-$ ion, while CIF cannot do so under the same conditions, we were able to separate CIF from CSF by elution of the column with a sufficient volume of 0.1 M Tris-HCl buffer (pH 7). The marked increase of total activity at the first DEAE-cellulose column (CSF$_{HU}$-2) probably reflects the exclusion of CIF from the crude CSF sample (CSF$_{HU}$-1). Coexistence of CSF and CIF in crude sample has been reported by many previous authors.  

Stanley and co-workers reported that CSF in unpurified human urine had a molecular weight of about 190,000 daltons on gel filtration while highly purified CSF had a molecular weight of 60,000 daltons. The difference in molecular
weight was suggested to be due to an interaction with other components (or polymerization) under the condition of gel filtration. We also observed that the crude CSF (CSF_{HU-1}) had a larger molecular weight (about 200,000 daltons; data not shown) than that of a more purified preparation (CSF_{HU-4}), which was about 85,000 daltons (Fig. 4). The difference between the molecular weight of CSF purified by us and the molecular weight of CSF reported by Stanley and Metcalf\textsuperscript{18} seemed probably due to the different conditions used for gel filtration. However, it is also possible that because of the unique mode of action of our preparation (Figs. 7 and 8, Table 1), we were dealing with a different protein species. CSF purified by us stimulated the granulocyte progenitor cells from human and mouse bone marrow to proliferate into the mature granulocytes, while the CSF reported by previous authors\textsuperscript{11} was mainly effective on the formation of macrophage colonies from mouse bone marrow cells. CSF effective in the formation of granuloid colonies by human bone marrow cells was previously purified by Price et al.\textsuperscript{17} and Burgess et al.\textsuperscript{20} Price et al. reported that the medium from cultures of nonleukemic cells contained three molecular species of CSA with approximate molecular weight 90,000 (fraction E\textsubscript{1}), 36,000 (fraction E\textsubscript{2}), and 15,000 daltons (fraction E\textsubscript{3}). Burgess et al., on the other hand, purified CSF\textsubscript{HPCM} with a molecular weight of about 30,000 daltons from human placental conditioned medium. With respect to molecular weight, CSF\textsubscript{HU} resembles fraction E\textsubscript{1} reported by Price et al. Fraction E\textsubscript{1} purified by four purification steps had a specific activity of $6.4 \times 10^5$ colonies/mg protein in human bone marrow assay, and CSF\textsubscript{HPCM} purified by five steps had a specific activity of $2.5 \times 10^5$ colonies/mg protein. Because CSF\textsubscript{HU}-5 had a specific activity of $6.7 \times 10^5$ colonies/mg protein in the human bone marrow assay, its purity would appear to be the same as that of fraction E\textsubscript{1} and CSF\textsubscript{HPCM}, considering the differences in assay conditions among these three laboratories. Physico-chemical studies of our CSF are now in progress to clarify its properties and to compare them with those of the CSF reported previously.\textsuperscript{10,11,16 19}

From the data shown in Figs. 7 and 8, it is suggested that CSF\textsubscript{HU} directly stimulates the formation of granuloid colonies from unfractionated as well as nonadherent human bone marrow cells. The plateau of stimulation obtained with CSF\textsubscript{HU}-1 (Fig. 7A) is probably the result of the inhibiting activity by CIF that contaminates CSF\textsubscript{HU}-1. CIF (peak A, Fig. 1) inhibits the formation of pure granuloid colonies from mouse bone marrow cells in the presence of CSF\textsubscript{HU}-2 (Fig. 6A) and from unfractionated human bone marrow cells in the presence of CSF\textsubscript{HU}-3 (Fig. 6B). Since CIF is a nondialyzable macromolecule, it is apparently different from the dialyzable inhibitor reported by Chan et al.\textsuperscript{21} and Vogler et al.\textsuperscript{21} Further purification of CIF and studies of interaction between CSF and CIF are now proceeding.

ACKNOWLEDGMENT

We thank Dr. I. Deitch of Columbia University for her advice in preparation for this manuscript.

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