Molecular Properties of a Granulocyte/Macrophage Colony-Stimulating Factor Obtained from the Serum-Free Culture of Yoshida Sarcoma Cell Line YSSF-212T

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ABSTRACT. A granulocyte/macrophage colony-stimulating factor (Peak-1 CSF) was partially purified from the medium of a serum-free culture of Yoshida sarcoma cells (Line YSSF-212T). Its elution position in gel-filtration chromatography corresponded to a molecular weight of about 22,000. The factor had an isoelectric point at pH 4.5 and a sedimentation coefficient of 2.3 S. The major part of its activity was not bound by Concanavalin A-Sepharose. Although CSF activity behaved as a single component in the gel-filtration and isoelectrofocussing procedures, subsequently it was resolved into two species by preparative discontinuous polyacrylamide gel-electrophoresis. This resolution indicates microheterogeneity of the CSF molecule. Oxidation with periodate readily inactivated L·P3-cell CSF, but the YSSF-cell CSF was fairly resistant. Moreover, titration with anti-L cell CSF serum showed a definite difference between L·P3-cell CSF and YSSF-cell CSF.

The genealogy of blood cells involves bipotential myeloid stem cells that can develop into a lineage of neutrophilic granulocytes or a lineage of monocyte/macrophages (11, 19). This development is controlled by three different classes of humoral regulators, probably all of which are sialoglycoproteins. The first is the granulocyte-macrophage colony-stimulating factor (GM-CSF) (3, 5, 6, 8, 9, 17, 22, 30, 33) that produces both granulocytes and macrophages from bipotential stem cells. The two other classes of CSF are the granulocyte colony-stimulating factor (G-CSF) (4, 23, 34, 35) and the macrophage colony-stimulating factor (M-CSF or CSF-1) (1, 13, 20, 28, 29). The former acts on stem cells to produce mainly granulocytes, whereas the latter produces mainly macrophages from the stem cells.

These regulators appear to be produced locally in hematopoietic tissues (10, 15) and to perform their functions in the area in which they are produced (24). In addition to hematopoietic tissues, many other tissues contain cells (presumably macrophages and lymphocytes) capable of producing myelopoietic regulators such as those described above (18, 21, 25). The entire mechanism of humoral regulation of myelopoiesis has yet to be determined, and detailed studies are needed.

Abbreviations used: GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; CM, conditioned medium; Con A, Concanavalin A; Peak-1 CSF, the first peak CSF from DEAE-cellulose column chromatography.

Previously, we reported (22) that Yoshida sarcoma cells (Line YSSF-212T) derived from a rat produce a CSF that can promote formation of granulocyte colonies in both human and murine bone marrow cell cultures. The YSSF-cell CSF attracted our attention because of its activity beyond species specificity. The molecular properties of the YSSF-cell CSF reported here resemble those of RSP-2·P3 cell CSF (30) and markedly differ from those of L·P3-cell CSF (29, 31).

MATERIALS AND METHODS

Cells. Yoshida sarcoma cells (Line YSSF-212T) basically were cultured as reported previously (22). Cells were suspended in a serum-free medium (RITC-1000PA) (36) at an initial density of 5×10^4 /ml, then 200-ml samples were cultured for 9 days in 2-liter roller bottles at 37°C.

Purification of Peak-1 CSF. About 8,600 ml of the cell-conditioned medium was centrifuged at $1000 \times g$ for 15 min, then the supernatant was brought to 90% saturation with ammonium sulphate. The resulting precipitate (about 10 g protein) was taken by centrifugation and dialyzed against 2 mM phosphate buffer of pH 6.5. The dialyzate was chromatographed on a DEAE-cellulose column (6×23 cm) in two portions. After washing the column with 2.5 liters of 10 mM Na phosphate/25 mM NaCl (pH 6.5) then with 350 ml of 10 mM Na phosphate/50 mM NaCl, it was washed again with one liter of the latter phosphate-saline solution. The eluate (Peak-1 CSF) was concentrated to 7.3 ml by a PM-10 membrane (Amicon), then 6.0 ml of the 7.3 ml was applied to a column of Ultro-gel AcA44 (2.5×100 cm) that had been equilibrated with 10 mM Na-phosphate (pH 7.4)/150 mM NaCl (phosphate buffered saline) containing 0.01% Tween 20 and 0.02% Na azide. The first 350-ml eluate was discarded, and the second 80-ml eluate kept. This CSF solution was concentrated to about 10 ml by ultrafiltration and stored in small portions at -20°C. The final yield of CSF was 8.2% of the starting activity. Other conditions for the purification procedures were similar to those reported previously (22).

CSF assay. Male C3H/He mice about 5 months old were used for all the experiments. One hundred thousand cells of femoral marrow were plated in separate 35-mm plastic petri dishes (Lux) as a single-cell suspension in 1 ml of McCoy's 5A medium containing 0.32% agar, 20% horse serum (Gibco) and an appropriate amount of CSF. The inoculated dishes were incubated for 7 days at 37°C under humidified air containing 5% carbon dioxide. The number of colonies (50 cells or more) was counted microscopically at the end of incubation. One unit of CSF is the activity that produces one such colony in the culture.

CSF activity also could be detected by the cellular uptake of radioactive thymidine. Bone marrow cells together with an appropriate amount of the CSF solution were suspended at a density of 2×10^5 /ml in McCoy's 5A medium containing 20% horse serum, then 0.5-ml portions were placed in plastic culture tubes (Nunc N-1409) and the tubes incubated at 37°C for 5 days. Tritiated thymidine (Amersham, 5 μ Ci/nmol/250 μ l) was added at 0.4 μ Ci/tube. Six hours later, the cells were suspended in the phosphate buffered saline containing 25 mM EDTA and transferred to glass fibre filter paper (Whatman GF/F, 2.4 cm diameter) then washed with 5% trichloroacetic acid followed by methanol. The radioactivity retained on the filter was measured with a liquid scintillation spectrometer and corrected for the zero-time controls (64 cpm/tube). The incorporation of radioactivity in the acid-insoluble materials increased linearly in the range of 0–15,000 cpm/tube with the increase of the added CSF activity.

Chromatography and electrophoresis of CSF. A column, 2.5×70 cm, was used for

Sephadex G-150 chromatography. Phosphate buffered saline was used for the elution of CSF at a flow rate of 27 ml/h.

Isoelectrofocussing was done as described previously (29). The CSF solution was dialyzed against 0.02% Tween and placed on 110 ml of a 5–50% sucrose concentration gradient containing 0.1% Ampholine (LKB). Electrophoresis was continued for 48 h at 500 volt at 5°C.

Concanavalin A (Con A) Sepharose was obtained from Pharmacia and packed in a column, 1 × 5 cm, after equilibration with 10 mM Na-acetate buffer at pH 6.0 containing 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.01% Tween 20 and 0.02% Na-azide. This acetate-buffered cocktail containing 50 mM α -methyl-D-glucoside was used for the elution of bound glycoproteins.

Preparative discontinuous polyacrylamide gel-electrophoresis was carried out in Canaco Prep-Disc equipped with a 2/150 column. The stacking and separating gels were both 4 ml in volume and contained 7% acrylamide and 0.184% bisacrylamide. Electrophoresis buffer was prepared according to Williams and Reisfeld (32). Tween 20 was added at a final concentration of 0.01%. Before the application of the CSF solution, the gel was electrophoresed for 1 h to remove soluble materials. The CSF solution was dialyzed against 0.05 M Tris-HCl (pH 5.5) containing 0.01% Tween 20, 3% sucrose and a trace of brom phenol blue, then a 2.25-ml portion was applied to the column. The eluate was collected for 7 h in 12.6 ml portions during electrophoresis at 8°C with a constant current of 10 mA and 1,100 volts.

Other methods. The sedimentation coefficient of the CSF was determined by the measurement of the distance of migration of the peak of CSF activity through a 5–50% sucrose concentration gradient, as reported previously (29, 31). After 0.2 ml of the CSF solution had been layered on the top of 5 ml of the gradient, it was centrifuged for 17 hr at 5°C. Fractions of 0.23 ml each were collected from the bottom of the tube and used to locate the peak CSF activity on the gradient.

Sodium periodate (18 mM) was dissolved in 0.2 M Na-acetate buffer at pH 5.0. One tenth milliliter of this solution was added to 0.8 ml of the CSF solution. The reaction was allowed to proceed at 4°C in the dark for 0–18 h and was stopped by the addition of 0.1 ml of 50% sucrose solution.

Anti-L cell CSF rabbit serum was a gift from Dr. E.R. Stanley of the Albert Einstein College of Medicine, New York City. It was used after it had been heated at 56°C for 30 min and diluted 120-fold with McCoy's 5A medium.

L·P3 cell CSF was partially purified by precipitation by ammonium sulphate. The specific activity of this preparation was about 2.5×10^4 units/mg protein; nearly comparable to that of the Peak-1 CSF of the YSSF cells.

RESULTS

Chromatographic behaviour of the YSSF-cell CSF. In a separate experiment, all CSF activity was precipitated by ammonium sulphate from the serum-free conditioned medium, and a sample of it (216 mg protein) was chromatographed on Sephadex G-150. A major part of the CSF activity was eluted as a peak with the V/V_0 value of 2.20 (Fig. 1). The position of the peak was nearly the same for the agar colony assay and the [³H]thymidine uptake assay. Chymotrypsinogen (molecular weight—22,500) was eluted from this gel-filtration column with the V/V_0 value of 2.22, indicating that the molecular weight of this major peak CSF is similar to that of chymotrypsinogen. In addition to the major peak, a few minor peaks were detected

Fig. 1. Sephadex G-150 column chromatography of YSSF-cell CSF. See the text for details of the chromatography. Both the agar-colony and [^3H]thymidine-uptake assays were done in duplicate with 0.2-ml and 0.1-ml portions of the eluate, respectively. Arrows V_0 and Ch show the positions of the peaks of blue dextran and chymotrypsinogen in separate runs. The total CSF activity recovered was about 70% based on the agar-colony assay.

by the [^3H]thymidine uptake assay. The second largest peak was eluted with the V/V_0 value of 2.76; this value did not match that of the small peak of CSF activity detected by the agar colony assay method.

To prepare Peak-1 CSF, another large batch of ammonium sulphate precipitate was chromatographed through DEAE-cellulose as described in MATERIALS AND METHODS. The first CSF peak represented about one tenth of the total CSF activity. This material was chromatographed on Ultro-gel AcA44 chromatography and was recovered from the fraction that had an average V/V_0 value of 1.43, which value was

Fig. 2. Isoelectrofocussing of Peak-1 CSF from the YSSF-cell. CSF activity was assayed in duplicate with 0.1-ml portions of each fraction after dialysis. The total CSF activity recovered was about 85%.

Fig. 3. Preparative polyacrylamide gel-electrophoresis of the Con A Sepharose unbound fraction of Peak-1 CSF. A 0.8-ml sample of each fraction was mixed with 0.2 ml of bovine serum albumin solution (2.5 mg/ml) then dialyzed overnight against excess saline. A 0.2-ml portions of the dialyzate was used for the duplicate CSF assays. See Materials and Methods for details of electrophoresis.

consistent with a previous result (22). This elution position from the Ultro-gel column corresponded to a molecular weight of 22,000, determined with bovine serum albumin, lactoglobulin, and cytochrome c as standards. This two-step-purified Peak-1 CSF was stored and used for the experiments reported below.

A sample (0.31 mg protein) of the purified stock was used to determine the sedimentation coefficient of the CSF. Upon centrifugation in the sucrose density gradient, the CSF activity sedimented at a distance of 10.5 mm in 17 h as a single peak. This peak corresponded to a coefficient of 2.3 S, or to a molecular weight of 21,000.

Another sample (0.31 mg protein) of the stock was put through isoelectric focussing with the carrier Ampholine. The CSF activity was focussed at pH 4.5 as a single peak (Fig. 2).

The major part (12.4 mg protein) of the stock was applied to a Con A Sepharose column. A total of 2.8 mg or 22.5% of the applied protein was recovered in the

TABLE 1. HEAT STABILITY OF YSSF-CELL CSF AND L-P3-CELL CSF

Temperature	CSF activity (colonies/dish) ^a		
	YSSF-cell-conditioned medium	90% (NH ₄) ₂ SO ₄ precipitate of YSSF-cell CM	90% (NH ₄) ₂ SO ₄ precipitate of L-P3-cell CM
Control (5°C)	61 (100) ^b	95 (100)	98 (100)
60°C, 30 min	46 (75)	93 (98)	90 (92)
60°C, 60 min	n.d.	104 (109)	86 (88)
100°C, 5 min	26 (43)	71 (75)	23 (23)

^a CSF solutions were dialyzed against phosphate buffered saline, then appropriately diluted and heated in 2-ml portions as above. Samples of these solutions (0.1 ml) were assayed for CSF activity in each 1-ml bone marrow cell culture. Figures in the table are the averages of three dishes.

^b Figures in parentheses represent CSF activity as a percentage of the respective control.

Fig. 4. Loss of CSF activity by treatment with periodate. See MATERIALS AND METHODS for details. After the reaction, the CSF solution was dialyzed against phosphate-buffered saline, and 0.1-ml portions were used for the colony assay. Data shown in the figure are averages of two dishes. Controls: YSSF-cell CSF—105 colonies/dish; L-P3 cell CSF—214 colonies/dish.

fraction not bound to the column. This break-through fraction contained more than 84% of the applied CSF activity. The other 16% of the CSF activity was found in the fraction eluted from the column with α -methyl-D-glucoside. A large sample (2.70 mg protein) of the unbound fraction was put through preparative polyacrylamide gel electrophoresis. The CSF was resolved into two peaks of similar size as shown in Fig. 3.

Other properties of the YSSF-cell CSF. The heat-stability of the YSSF-cell CSF in the crude preparations is shown in Table 1. The CSF activity precipitated with

Fig. 5. Inhibition of CSF activity by the addition of anti-L cell CSF serum (120-fold dilution) to the bone marrow cell culture. Controls: YSSF-cell CSF—62 colonies/dish; L-P3-cell CSF—56 colonies/dish.

ammonium sulphate seems to be more stable than that in the original cell-conditioned medium. Exposure of the crude CSF to acidic (pH 2.0 by HCl) and basic (pH 11.5 by NaOH) environments resulted in 48% or 78% loss of activity within 2 h at room temperature. Peak-1 CSF also was fairly stable; nearly 100% of the activity was recovered after the crude preparation was filtered through an Ultro-gel AcA44 column as well as when it was chromatographed on Con A Sepharose. The L-P3-cell CSF was slightly less stable than the YSSF-cell CSF (Table 1).

Oxidation with periodate showed a significant difference in the stability of the L-P3-cell CSF and the Peak-1 CSF of the YSSF cells (Fig. 4). Nearly all the activity of the L-P3-cell CSF was lost by an 18-h treatment with 2 mM periodate, whereas loss of the YSSF-cell CSF activity during the same treatment was negligible.

An addition of anti-L cell CSF serum to a soft-agar culture of bone marrow

TABLE 2. MOLECULAR WEIGHT OF THREE CLASSES OF CSF ESTIMATED BY GEL-FILTRATION CHROMATOGRAPHY

Sources	Molecular weight estimate ^a			Rf.
	G-CSF	GM-CSF	M-CSF	
Human placenta CM	30,000 (G-150) ^{b,c}			4
	27,000 (AcA44)		41,000 (AcA44)	34
MIA PaCa cell CM	27,000 (AcA44)		50,000 (AcA44)	35
T3M-1 cell CM	30,000 (G-75)			23
RSP-2-PC cell CM		20,000 (HW55F/G) ^d		30
YSSF-212T cell CM		22,000 (AcA44)		22
Post-LPS tissue extracts		23,000 (CL6B/G) ^d		21
T-cell hybridoma CM		24,000 (AcA44)		6
		35,000 (S-200)		12
Mouse lung CM		29,000 (AcA44)		3
Mo cell CM		34,000 (AcA44)		17
GCT cell CM		35,000 (S-200)		8
PWM-spleen cell CM		37,000 (G-150)		5
		24,000 (CL6B/G) ^d		5
Human T-cell CM		40,000 (G-150)		33
Human lung CM		41,000 (AcA44)		9
Mouse yolk sac CM			60,000 (G-100)	13
L-cell CM			73,000 (G-150)	1
Human urine			75,000 (G-100)	16
			85,000 (G-150)	20
L-P3 cell CM			80,000 (AcA34)	29
			47,000 (HW55F/G)	30

^a In much of the literature cited, the molecular weight estimate is given as a range. Median values have been used in this table for simplicity.

^b Column matrices used for gel-filtration chromatography are shown in parentheses; G-75, G-100, G-150—Sephadex; AcA34, AcA44—Ultro-gel; S-200—Sephacryl; HW55F/G—Toyo-Pearl HW55F run in the presence of 6M guanidine; CL6B/G—Sepharose CL-6B run in the presence of 6M guanidine.

^c Underlined estimates are those of CSFs known to be effective toward human stem cells.

^d Analysis after neuraminidase treatment.

cells significantly decreased the number of cell colonies when L-P3-cell CSF was used to stimulate cells of monocyte/macrophage lineage.

Granulocyte/macrophage colony formation by Peak-1 CSF of the YSSF cells, however, was not much affected by the anti-L cell CSF serum (Fig. 5). Microscopic examination of the cell colonies showed that there was no selective change in cell morphology in the presence of the antiserum.

DISCUSSION

Results of gel-filtration chromatography, Sephadex G-150 and Ultro-gel AcA44, and the measurement of sedimentation coefficient suggested that the YSSF-cell Peak-1 CSF has a molecular weight of about 22,000. Molecular weight estimates of three classes of CSF from various sources are given in Table 2. The YSSF-cell CSF is one of the smallest of these CSFs. Since all these CSFs presumably are sialoglycoproteins, their behaviour in gel-filtration chromatography does not precisely reflect their molecular weight. Nevertheless, GM-CSF seems to be generally smaller than M-CSF, the difference between GM-CSF and M-CSF shown in Table 2 being statistically significant ($P < 1\%$). In contrast, the difference between G-CSF and GM-CSF is not as great as the difference between M-CSF and GM-CSF.

Molecular heterogeneity has been found for many kinds of CSF. This also was true for the YSSF CSF. Peak-1 CSF was contained in the fraction that bound to the DEAE-cellulose most loosely. As reported previously (22), a large part of the total CSF remained bound on the column under the conditions that eluted Peak-1 CSF. Therefore, Peak-1 CSF seems to be least heavily sialylated of the several homologous CSFs of the YSSF cells. Although Peak-1 CSF behaved as a single peak both in gel-filtration chromatography and in isoelectrofocussing, it was resolved into two components by subsequent polyacrylamide gel-electrophoresis. Probably, a slight

TABLE 3. ISOELECTRIC POINTS OF THREE CLASSES OF CSF

Sources	Isoelectric point ^a			Rf.
	G-CSF	GM-CSF	M-CSF	
Human placenta CM	<u>5.7</u> ^b		4.2	34
MIA PaCa cell CM	<u>5.7</u>		4.2	35
Human lung CM		3.8(4.3) ^c		9
Mo cell CM		4.0(4.6)		17
T-cell hybridoma CM		4.3		6
RSP-2-P3 cell CM		4.4		30
YSSF-212T cell CM		4.5		^d
PWM-spleen cell CM		4.8		5
Human urine			3.3(4.5)	14
L-P3 cell CM			3.5(5.4)	29
L60T cell CM			4.3	27

^a Median values of the pI ranges reported in the literature.

^b Underlined values are those of CSFs known to be effective toward human stem cells.

^c Figures in parentheses are isoelectric points of the CSFs after treatment with neuraminidase. Information on the pI of neuraminidase-treated CSFs was not available for all the CSFs listed.

^d Present paper.

difference in the molecular properties of the two components resulted in their separation by the most sensitive method. A similar microheterogeneity recently was reported by Burgess *et al.* (7) for the epidermal growth factor from mouse submaxillary gland. In our study, however, the possibility can not be excluded that the microheterogeneity may have been artificially induced during electrophoresis.

The heterogeneity of our CSF seems mainly to be due to the molecule being a sialoglycoprotein (2, 26, 31). Variations in the isoelectric points of the various CSFs are shown in Table 3. Probably the diversity of molecular charges of the CSFs from different sources also is due to the degree of sialylation. Although our information is limited, M-CSF seems to be more heavily sialylated than GM-CSF.

Desialylation of human urine and L-P3-cell M-CSF increased their isoelectric points by more than one pH unit, whereas the shift in pI was only a one-half pH unit in the GM-CSFs from human lung and Mo-cell CM.

Tables 2 and 3 also show which CSFs are active on human cells. Generally speaking, the CSFs that are not specific for animal species are small and less acidic than species-specific CSF. Alternatively, it can be said that the granulopoietic pathway is less dependent on species difference than the monocyte/macrophage pathway.

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