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The Role of Colony-Stimulating Factor in Granulopoiesis

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The proliferation and maturation of granulocytic-monocytic stem cells appears to be controlled by a series of closely related glycoproteins termed "colony-stimulating factors" (CSFs). Recently, we devised a 6-step scheme for the purification of murine fibroblast (L-cell)-derived CSF. Ten liter pools of conditioned media were concentrated by ultrafiltration, precipitated by ethanol, and separated on DEAE cellulose, Con-A Sepharose, and Sephadex G150. The CSF was separated from trace contaminants, including endotoxin, by density gradient centrifugation. The purified material was radioiodinated and used to define the serum half-life and in vivo distribution. Following IV injection there was a biphasic serum clearance with a $t_{1/2}$ of 24–40 min and 2–2½ hours in the first and second phases. Approximately 25% of the tracer was excreted in the urine at 6 h; however, urinary radioactivity was due to low molecular weight peptides. Simultaneous studies by radioimmunoassay showed a similar rapid serum clearance of unlabeled CSF but virtually no urinary CSF activity. Thus, assays for urinary CSF may not provide useful measures of in vivo CSF activity. Further in vitro studies have defined the interaction of CSF with responsive cells in the marrow. Varying doses of CSF were incubated with 10^7 marrow cells for intervals of 24–48 h. The major increment in cell-associated radioactivity occurred between 6 and 16 h. The reaction was saturable with 1–2 ng/ml CSF. Binding was prevented by cold CSF, but not by other proteins. Irradiation yielded only a minimal reduction in CSF binding. The interaction of CSF with marrow cells appeared to require new protein synthesis, as binding was completely inhibited by cycloheximide and puromycin. Irradiated mice injected with antibodies to CSF showed an inhibition of granulopoiesis by marrow cells in peritoneal diffusion chambers; however, granulopoiesis in the intact bone marrow was unaffected. Granulpoiesis in long-term marrow cultures was also unaffected by anti-CSF. These different responses may be due to accelerated clearance of injected CSF in nonirradiated mice or to extensive stromal interactions that modulate and perhaps control granulocytic differentiation in the intact bone marrow microenvironment.

Key words: granulopoiesis, colony stimulating factor, diffusion chamber granulopoiesis, radioimmunoassay for colony stimulating factor, long-term marrow cultures, purification of colony stimulating factor, binding of colony stimulating factor

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During the past decade there has been a progressive accumulation of data suggesting that granulopoiesis is controlled by a humoral mechanism. In the preceding 10 years granulocyte differentiation was extensively characterized with definition of pool sizes, proliferative capacity, and transit times [1, 2]. The advent of techniques for the clonal growth of granulocytes and macrophages in vitro provided a further dimension for exploration of potential regulatory factors [3, 4].

Incubation of bone marrow cells in semi-solid medium in the presence of a stimulatory material termed colony-stimulating factor (CSF) leads to the development of discrete colonies of granulocytes and macrophages. Studies with the cell cycle active agent hydroxyurea showed that the colony-forming cells had a high rate of replication [5], which served to distinguish these progenitor cells from the spleen colony-forming units or pluripotential stem cells; the latter generally manifested a slow rate of cellular turnover. Based on the high rate of DNA synthesis and the apparent restriction to a single common (granulocyte and macrophage) line of differentiation, most investigators believe that the agar gel assay measures granulocyte, and perhaps monocyte, committed stem cells. Since colony formation results from the growth of individual granulocytic stem cells in response to CSF in vitro, it seemed reasonable to postulate that similar events might be occurring in vivo.

A series of correlative studies have shown an inverse relationship between the number of circulating neutrophils and serum levels of CSF [6–9]. Sera or urine from animals rendered neutropenic by X-irradiation, administration of an antineutrophil antibody, or injection of cytotoxic drugs show substantial increments in CSF activity. Moreover, serum samples from animals with cyclic neutropenia [10, 11] or from patients [12] with similar oscillations in their neutrophil counts also show periodic fluctuations in CSF activity. Higher levels of CSF are found during the neutropenic phase, with depression of CSF in conjunction with peak neutrophil values.

The use of a further experimental model – namely, the growth of granulocytic cells in peritoneal diffusion chambers [13] – has also provided data suggestive of a CSF effect in vivo. Cell growth is modest in control animals, whereas pretreatment with radiation [14] or cyclophosphamide [15] markedly enhances cellular proliferation. Since these treatments increase serum CSF activity, it is tempting to speculate that the accelerated granulopoiesis is due to this factor. Indeed, treatment of animals with an antibody to CSF markedly restricts the degree of diffusion chamber granulopoiesis [16].

Further studies with bacterial vaccines or lipopolysaccharides are also indicative of a CSF effect in vivo. For instance, a single injection of pertussis vaccine leads to a 48–72 h increment in circulating CSF, which in turn is followed by a heightened wave of granulocytic differentiation [17]. Various endotoxin preparations also stimulate an acute increase in CSF activity [18]. Following repetitive injection there is rapid tolerance to the CSF-inducing effect [19]. Nonetheless, animals treated in this fashion develop striking granulocytic hyperplasia of the bone marrow [19].

The rapid turnover rate and the marked lability of the granulocyte system have thus far impeded efforts to document the effect of CSF in vivo. Endotoxins and various foreign proteins, in addition to increasing CSF activity, cause an immediate release of mature neutrophils from the marrow [2]. This effect that is mediated by a neutrophil-releasing factor [20], might also be expected to stimulate cell production by reduction in a possible cell-contact negative feedback mechanism. Investigators have described numerous inhibitors of in vitro colony formation; however, it remains to be shown whether these materials are

active in vivo. High molecular weight inhibitors are readily demonstrated in the serum [21], yet the levels of these materials do not change in neutropenic or neutrophilic states [22]. Low molecular weight substances have been isolated from mature neutrophils [23]; however, such inhibitors are also derived from lymphocytes and various somatic tissues, which suggests a lack of specificity [24]. Recent data do indicate that neutrophil derived lactoferrin may serve a regulatory function by limiting the response of granulocytic progenitor cells both in vitro and in vivo [25].

Although the injection of crude sources of CSF produces a modest neutrophilia in experimental animals, the aforementioned concerns about endotoxin and foreign proteins clearly indicate the need for pure material. Recently, 3 groups have succeeded in purifying several sources of murine CSF [26–28]. We have used purified L-cell CSF for production of antibodies [29], for development of a radioimmunoassay [30], and for in vivo studies of plasma clearance and tissue distribution [31]. Both purified CSF and anti-CSF have been used for studies of granulopoietic control mechanisms using both diffusion chamber [16] and long-term marrow culture techniques [32].

METHODS AND RESULTS

Purification of CSF

CSF was produced by growth of L-cells in serum-free CMRL 1066 medium [28]. Each 10 l pool of conditioned medium was concentrated 100–250-fold by ultrafiltration and subjected to precipitation in 40% and 50% cold ethanol. The supernatant, which contained all of the active CSF, was applied to DEAE cellulose and eluted with a linear sodium chloride gradient (Fig. 1A). The active fractions were concentrated and applied to concanavalin-A Sepharose, wherein approximately 1/3 of the CSF was nonadherent; the remainder was bound and specifically eluted with alpha-methyl glucoside (Fig. 1B). Each active peak was separately concentrated and applied to coupled columns of Sephadex G-150. The CSF eluted in a volume 1.2 to 1.3 times the void volume of the column. With a series of protein markers, each peak eluted with an apparent but anomalous molecular weight of approximately 190,000 (Fig. 2). In the final purification step, CSF was separated from trace contaminants by sucrose density gradient centrifugation (Fig. 3). This proved successful as the high molecular weight contaminants, which eluted with CSF on Sephadex gels, were effectively sedimented to the bottom of the gradient, whereas CSF was retained in the upper portion. After ultracentrifugation, the resultant materials from both the Con-A adherent and nonadherent fractions appeared homogeneous after electrophoresis in varying concentrations of acrylamide, in SDS acrylamide, and by Ouchterlony gel diffusion [28]. The final materials were purified greater than 1,000-fold and showed a specific activity of $2–5 \times 10^7$ units/mg of protein. Maximum colony formation was detected using approximately 3 ng/ml in agar gel cultures.

Based on the migration in SDS gels with marker proteins (Fig. 4, top panel) and calculations using the sedimentation coefficient (3.0S) and Stoke's radius (50 Å), both CSF fractions had a molecular weight of 60,000–70,000. Both peaks contain substantial quantities of carbohydrates as judged by staining with PAS, by inactivation with periodate, and by altered electrophoretic behavior after incubation with neuraminidase. Reduction with 2-mercaptoethanol in the presence of SDS yielded two subunits of approximately 35,000 daltons (Fig. 4, bottom panel), however, these products were devoid of biologic activity.

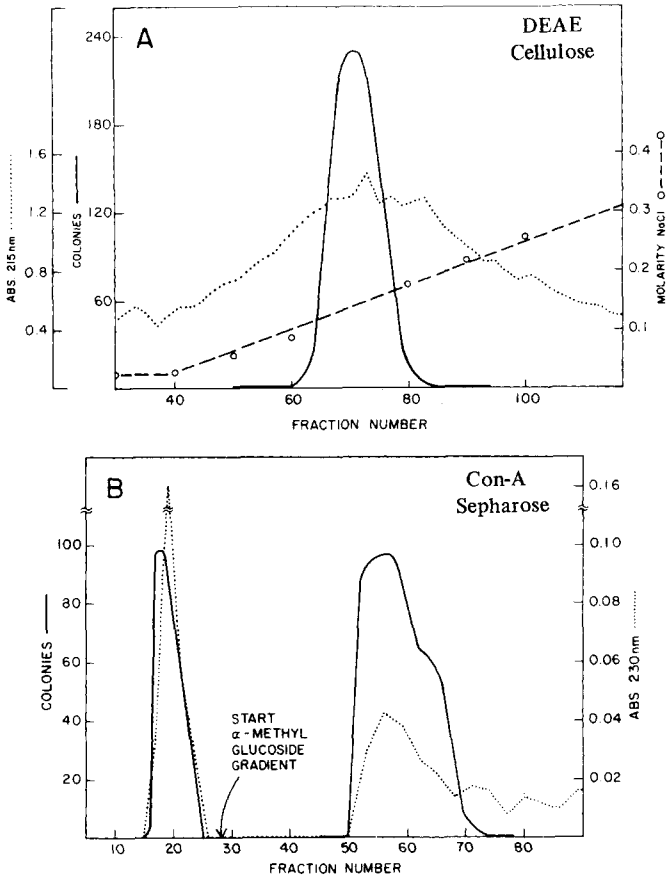

Fig. 1. A. Chromatographic separation of L-cell CSF with DEAE-cellulose. Biologic activity was detected in the 0.10–0.18 M NaCl fractions. B. Affinity chromatography of concentrated CSF from the DEAE-cellulose fraction on Con-A-Sepharose. Two peaks of activity were detected; nonadherent CSF (peak 1) and adherent (peak 2). Reproduced from Waheed and Shadduck: Purification and properties of L-cell derived colony-stimulating factor. J Lab Clin Med 94:180–194, 1979, with permission.

Clearance and Distribution Studies

Purified CSF has been radioiodinated using both the lactoperoxidase [30] and modified chloramine-T techniques [31]. With the inclusion of 0.06 M DMSO it has been possible to preserve 75–100% of the biologic activity with stability of the tracer for a minimum of 3 weeks after iodination. These various tracers have been used to define the clearance of CSF in vivo. After IV injection there was a rapid initial plasma clearance with a $t_{1/2}$ of 24–40 min through the first hour (Fig. 5). Thereafter, the disappearance rate was essentially linear, with a $t_{1/2}$ of 2–2½ hours. Plasma levels of radioactivity were lower after IP administration, with peak values at 1 h and linear clearance thereafter, with a half-life of 3½ hours.

By use of a radioimmunoassay [30] it was also possible to monitor the serum clearance of unlabeled CSF. In these studies CSF disappeared from the plasma in a near linear fashion, with a $t_{1/2}$ of 1½ hours. The differences in behavior between radiolabeled

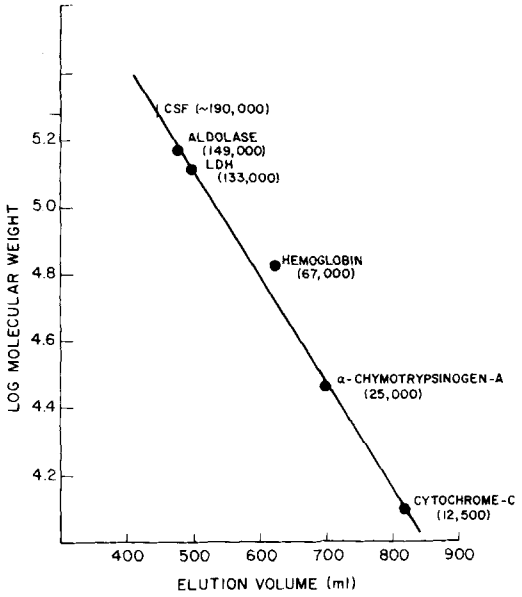

Fig. 2. The apparent molecular weight of CSF on Sephadex G150. The elution of CSF was compared to a series of marker proteins and plotted as a log of the molecular weight vs elution volume.

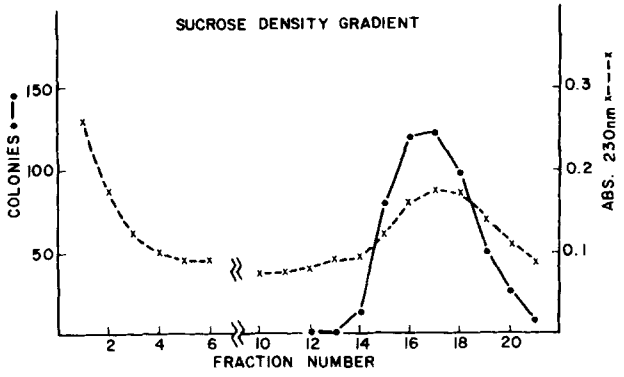

Fig. 3. Final separation of a peak 2 CSF fraction by sucrose density gradient centrifugation. High molecular weight contaminants were removed in fractions 1–4, which were obtained from the bottom of the centrifuge tube. CSF was detected in fractions 14–20, which represented the upper portion of the gradient. Reproduced from Waheed and Shadduck: Purification and properties of L-cell derived colony-stimulating factor. J Lab Clin Med 94:180–194, 1979, with permission.

and unlabeled material may be more apparent than real, as tracer studies only utilized 10–20 units of CSF whereas 125,000 units of cold material were employed.

Although in these experiments 15–30% of the radioactivity was excreted in the urine over 6 h, only 0.3–0.6% of intact CSF could be detected by immunoassay (Table I). Separation of the urinary radioactivity on Sephadex gel showed that virtually all of the tracer was degraded into low molecular weight peptides.

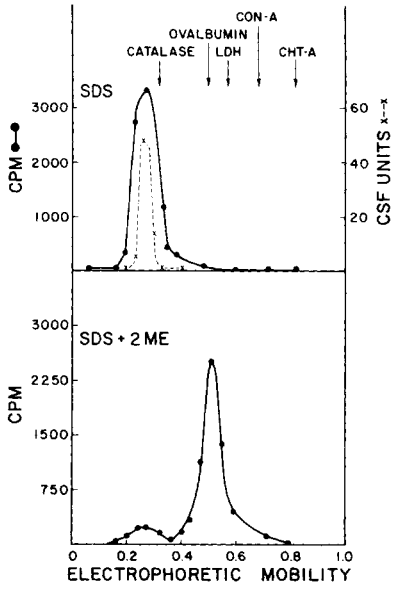

Fig. 4. A. Electrophoretic mobility of step VI, peak 2 CSF in SDS-acrylamide. B. Mobility of step VI, peak 2 CSF after reduction with 2-mercaptoethanol. Abbreviations refer to lactate dehydrogenase (LDH), concanavalin A (Con-A), and alpha chymotrypsinogen A (CHT-A). Reproduced from Waheed and Shadduck: Purification and properties of L-cell derived colony-stimulating factor. J Lab Clin Med 94:180–194, 1979, with permission.

TABLE I. Serum and Urinary CSF Values 6 Hours After Injection*

Injection	Measurement	Serum	Urine
^{125}I-CSF + 31,000 U cold CSF	Radioactivity	8.6% ± 0.4%	28.4% ± 4.2%
^{125}I-CSF + 125,000 U cold CSF	Radioactivity	6.4% ± 0.3%	14.3% ± 2.4%
31,000 units cold CSF	RIA	4.6% ± 0.5%	0.6% ± 0.05%
125,000 units cold CSF	RIA	2.9% ± 0.2%	0.3% ± 0.03%

*Groups of 5 mice each were injected with unlabeled cold CSF alone or mixed with ^{125}I-labeled CSF. Six-hour serum and urinary CSF was measured by either radioactive counting or radioimmunoassay (RIA). Values are means ± 1SE. Reproduced from Shadduck, Waheed, Porcellini, Rizzoli and Pigoli: Physiologic distribution of colony-stimulating factor in vivo. Blood 54:894–905, 1979, with permission.

In these studies the great majority of CSF was rapidly removed from the circulation, and within 5 min approximately 40% of the injected dose was detected in the liver (Fig. 6). Lesser quantities were found in the kidneys, lungs, and spleen, with clearance patterns that approximated those of the plasma. It is intriguing to note that only extremely small quantities of the isotope accumulated in the marrow. This could be due to the relatively low quantity of tracer injected, to a delay in cell binding, or perhaps to sequestration of the responsive cells in ecologic niches created by marrow stromal cells.

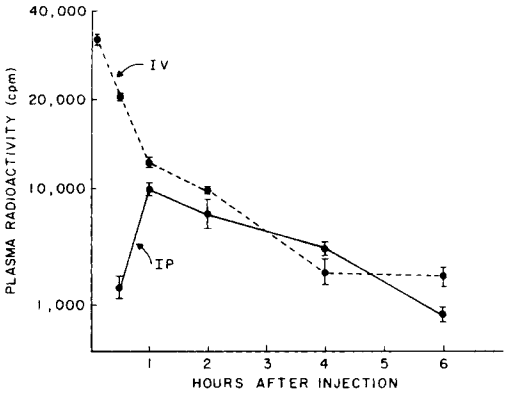

Fig. 5. Plasma clearance of radiolabeled CSF after IV or IP injection of the tracer. All mice received 100,000 cpm of tracer and were bled at intervals of 5 min to 6 h. Values represent total plasma radioactivity (means ± 1 SE). Reproduced from Shadduck, Waheed, Porcellini, Rizzoli and Pigoli: Physiologic distribution of colony-stimulating factor in vivo. Blood 54:894–905, 1979, with permission.

Binding of CSF to Bone Marrow Cells

In further in vitro experiments radioiodinated CSF was directly incubated with murine bone marrow cells for periods up to 48 h [31]. Control cultures contained an 80-fold excess of cold CSF to provide a measure of nonspecific binding. With 10^7 marrow cells, only minimal cell-associated radioactivity was detected over 1–3 h (Table II). The major increment in cell binding occurred between 6 and 16 h, with a near plateau thereafter. Saturation was achieved with concentrations of approximately 1 ng/ml, wherein 8% of the tracer was bound.

Cellular binding appeared specific as competition studies showed a prevention of binding with excess cold CSF but no inhibition with human serum, bovine albumin, ovalbumin, chorionic gonadotropin, porcine insulin, thyroxine, or dexamethasone. Furthermore, ^{125}I CSF did not bind to lymph node, thymic, hepatic, or renal cells, and showed markedly reduced binding with splenic cells, which are known to contain only small numbers of granulocyte-macrophage colony- forming cells. L-cell CSF is specific to murine progenitor cells; incubation of crude or purified material with human marrow does not generate colony formation. As a further measure of specificity ^{125}I CSF was incubated with 2 samples of normal human marrow. With 4×10^{-11} M CSF, no binding was observed at 24 h with the human samples, whereas approximately 9% of the tracer was bound to murine cells.

Although colony-forming cells show a high rate of DNA synthesis [5], only a modest decrease in cellular binding of radiolabeled CSF was seen with X-irradiation. As shown in Figure 7, binding was not inhibited by treatment of the cultures with 500 rads; a 24% reduction was observed with 1,000 rads. Similar findings were noted with cycle, active, phase-specific agents such as cytosine arabinoside or hydroxyurea. Preliminary autoradiographic studies done in collaboration with Dr. Lewis Schiffer of Allegheny General Hospital, Pittsburgh, PA, are in accord with these findings. As shown in Figure 8, large mononuclear or blast cells were labeled after 1–6 h exposure to the tracer. With increasing incubation time, virtually all cells of the granulocytic series, including mature neutrophils, were labeled.

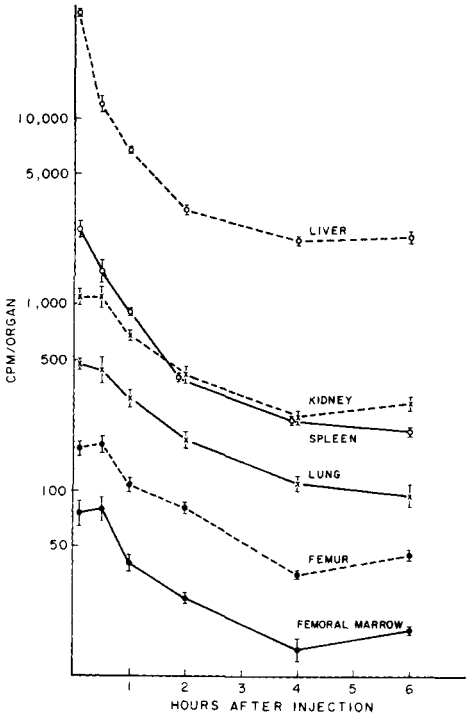

Fig. 6. Patterns of radioactivity in the tissues after IV injection of radiolabeled CSF. Values represent total tissue radioactivity and are means ± 1 SE for 5 animals/point. Reproduced from Shadduck, Waheed, Porcellini, Rizzoli and Pigoli: Physiologic distribution of colony-stimulating factor in vivo. Blood 54:894–895, 1979, with permission.

TABLE II. Bone Marrow Cell Uptake of ^{125}I-CSF*

		cpm/10^7 Marrow cells	
Time (h)	^{125}I-CSF	^{125}I-CSF plus cold CSF	Specific binding
1	1,882	642	1,240
3	2,532	728	1,804
6	5,663	1,009	4,654
10	13,934	1,619	12,315
16	31,501	2,840	28,661
24	40,003	7,550	32,453
48	46,480	10,510	35,970

*Marrow cells (10^7) were incubated for 1–48 h in 1 ml of supplemented McCoy's medium at 37°C, with 400,000 cpm of CSF. Control tubes for nonspecific binding contained an 80-fold excess of unlabeled CSF. Reproduced from Shadduck, Waheed, Porcellini, Rizzoli and Pigoli: Physiologic distribution of colony-stimulating factor in vivo. Blood 54:894–905, 1979, with permission.

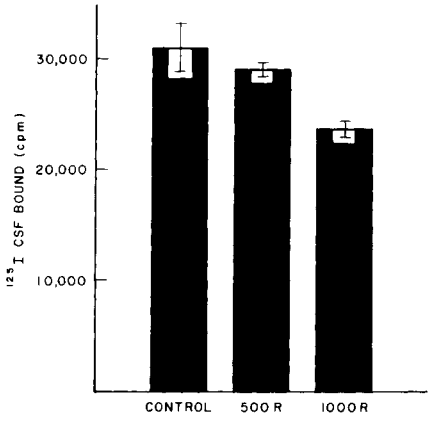

Fig. 7. The effect of irradiation on binding of radiolabeled CSF to murine bone marrow cells. Each culture contained 10^7 marrow cells and 400,000 cpm of tracer. Cultures were irradiated immediately after preparation; values represent specific cell-associated radioactivity following 24 h incubation.

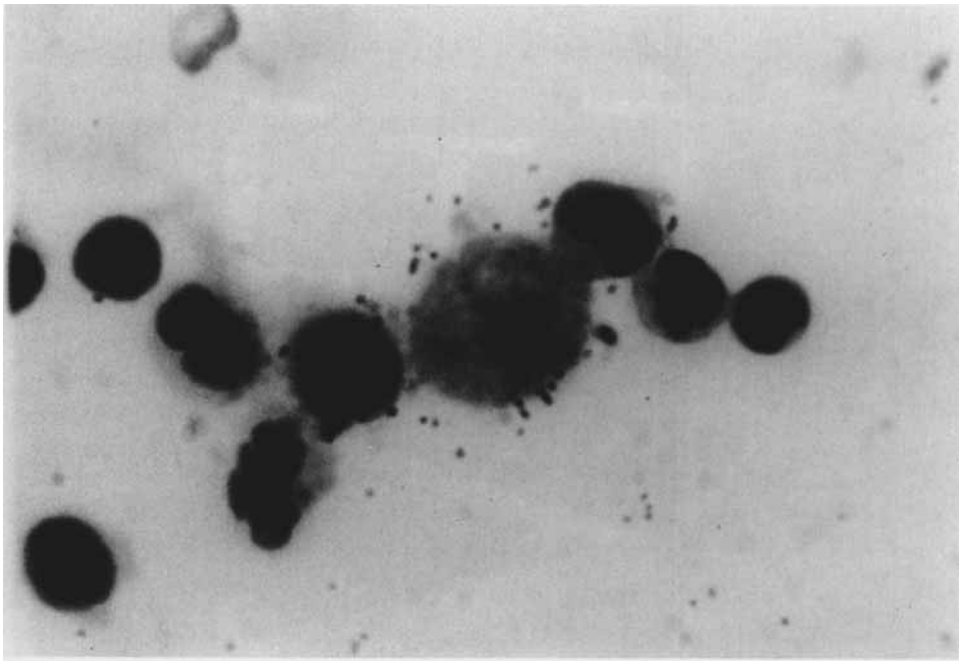

Fig. 8. Autoradiograph obtained after 6 h exposure of the radioiodinated CSF to murine bone marrow cells. A large mononuclear or blast cell is labeled, whereas lymphocytes and erythroid cells show no cell-associated radioactivity.

The delay in cellular binding of CSF appears to require synthesis of new protein as both cycloheximide (1 μg/ml) and puromycin (10 μg/ml) inhibited cellular uptake of radioactivity.

In Vivo Studies With Anti-CSF

Although foreign proteins may stimulate granulopoiesis by accelerating release of neutrophils from the marrow or by other nonspecific mechanisms, such materials are not known to depress granulocyte production. It was therefore reasonable to postulate that neutralization of all circulating CSF by antiserum should diminish or perhaps abolish cellular production. Initially, it was necessary to document the ability of rabbit antiserum directed against L-cell CSF to neutralize completely serum colony-stimulating activity. Colony formation by both L-cell and murine serum CSF was completely inhibited with a 1:512 dilution of antiserum. The in vivo serum disappearance of anti-CSF was studied using a highly purified antibody fraction, which had been obtained by an immunoadsorbent technique [33]. In addition this material was repeatedly adsorbed with limulus lysate by Dr. Jack Levin of Johns Hopkins University School of Medicine to remove detectable levels of endotoxin. A total of 25,000 units of this purified anti-CSF was injected IP into each of 9 mice, and serum was obtained 2, 7, and 12 h thereafter. Circulating antibody activity was measured by determining the ability of the various serum samples to precipitate ^{125}I-labeled CSF. When compared to a standard curve, the 2 h serum value reflected a 10.4% recovery of the injected dose (Fig. 9). Clearance was essentially linear through 12 h, with a $t_{1/2}$ of approximately 2 h. This compares quite favorably with the clearance of CSF, thereby suggesting that antibody clearance is due to interaction with newly synthesized CSF. Bioassay of serum samples from 2 and 7 h showed no CSF; only 80 units/ml were detected at 12 h. This study and the in vitro incubation of anti-CSF with mouse serum clearly indicate that this antibody completely neutralizes circulating CSF activity.

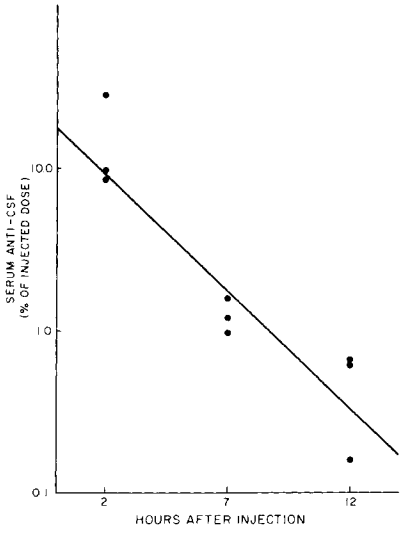

Fig. 9. Serum half-life of purified anti-CSF. Animals were injected with 25,000 units of purified anti-CSF and circulating antibody determined at intervals thereafter. Values represent the % of injected antibody activity recovered from serum at the indicated time intervals.

Following these experiments, groups of mice were injected with 35,000 units of anti-CSF IP every 12 h for 7 days. Peripheral blood granulocytes were reduced from 815 to 510/μl on day 7; however, bone marrow cellularity was essentially unchanged. Further studies using 175,000 units of purified anti-CSF showed essentially no decrease in circulating neutrophils or marrow cellularity. However, a modest decrease in bone marrow granulocyte-macrophage colony-forming cells from 18,000 to 8,000 per femur was observed.

One explanation for this relatively limited inhibition of granulopoiesis may relate to the rapid development of antibodies against the injected anti-CSF. Although high levels of circulating anti-CSF were detected on days 1 and 3 of this study, virtually none could be detected on days 5 and 7, despite continued administration every 12 h. Modified radioimmunoassays showed that the recipient mice had developed precipitating antibody against the anti-CSF.

If further experiments, mice were treated with 2.5 mg of cortisone acetate on day -1, day 2, and day 5 in an attempt to limit their development of antibodies directed against anti-CSF. Groups of mice received high titer anti-CSF serum (230,000 units of anti-CSF) every 12 h and were evaluated on days 1–7 of treatment. Although circulating anti-CSF could be detected for up to 5 days, no decrease in circulating granulocytes or in the proliferative or nonproliferative compartments in the marrow was noted (Fig. 10). Thus, these studies show that the in vivo administration of anti-CSF has only minimal effects in intact mice.

Diffusion Chamber Studies With Anti-CSF

As noted above, increased proliferation of granulocytic cells occurs in diffusion chambers implanted in neutropenic as compared to normal hosts. Studies were undertaken in collaboration with investigators at Brookhaven National Laboratories to determine whether CSF might be responsible for this effect. Groups of mice received 700 R total body irradiation and were each implanted with 2 intraperitoneal diffusion chambers containing 5×10^5 nonadherent marrow cells. Each group was injected with 0.5 ml of saline, control serum, or CSF antiserum (titer, 1:128) every 12 h for 4 days. Diffusion chambers were harvested at 2, 4, and 7 days for evaluation of cell counts, differentials, and colony-forming assays [16]. The results showed marked inhibition of granulocytic differentiation in chambers from antibody recipients (Fig. 11). Both control serum and saline groups had a 12- to 18-fold increase in granulocyte production. In addition, chamber CFU-C content was markedly depressed in antibody recipients (Fig. 12). These findings, as well as the continued requirement for CSF in agar cultures, suggest that CSF is necessary both for the growth and maturation of differentiated cells and for proliferation of the granulocytic stem cell compartment. Thus, these observations are strongly suggestive that CSF may function as an in vivo as well as an in vitro granulopoietin.

Effect of CSF and Anti-CSF on Long-Term Bone Marrow Cultures

As noted above the stromal microenvironment may represent an important determinant of stem cell proliferation and differentiation. Recently, a system was devised in which a bone marrow-derived adherent cell layer provides the necessary environment for sustained hemopoiesis [34]. Murine bone marrow cells are suspended in culture medium supplemented

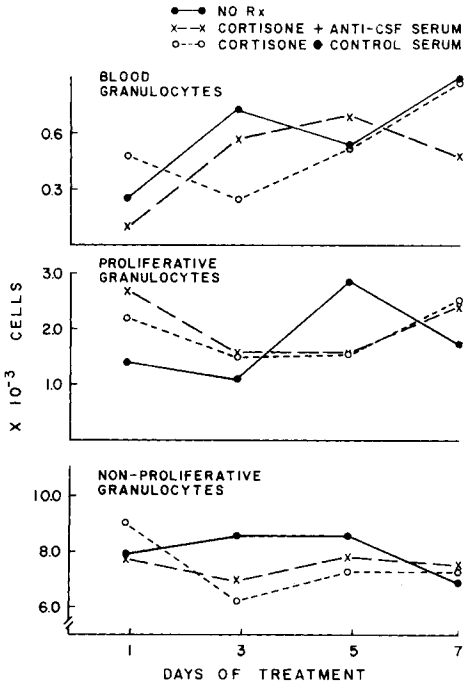

Fig. 10. Effect of anti-CSF on in vivo granulopoiesis. Values represent absolute peripheral blood neutrophil counts or quantitative determinations of bone marrow granulocytic cells at the times indicated. Test animals received 0.5 ml of antiserum or control serum every 12 h throughout the experiment. Values are means from 5 animals/point.

with 20% horse serum and incubated at 33°C. With half depopulation and refeeding on a weekly basis, the nonadherent cells show continual production of granulocytes, macrophages, and maintenance of granulocytic and pluripotential colony-forming cell populations.

Studies were undertaken in collaboration with Dr. Michael Dexter of Manchester, England, to determine whether exogenous CSF or anti-CSF influenced these long-term cultures. Previous experiments had shown virtually no CSF in culture supernatants [34, 35] and a remarkable decrease in cellular differentiation following addition of crude sources of CSF. In our recent studies impure CSF derived from heart conditioned medium or standard L-cell CSF caused a progressive decline in culture cellularity and granulocytic differentiation [32]. In contrast, purified L-cell CSF had no such effect. This suggests that the decline in cellular differentiation was probably due to exogenous materials in the impure CSF preparations. In the opposite type of experiment, CSF antiserum had essentially no effect on granulocyte differentiation [32]. Despite addition of 10% antiserum to these cultures, there was no decline in cellularity, granulocytic growth, or proliferation of CFU-C or CFU-S compartments.

These findings as well, as the previous inability to detect CSF in the long-term bone marrow cultures, call into question the involvement of CSF in this type of granulocytic differentiation. To examine this issue further, supernatant material from long-term cultures

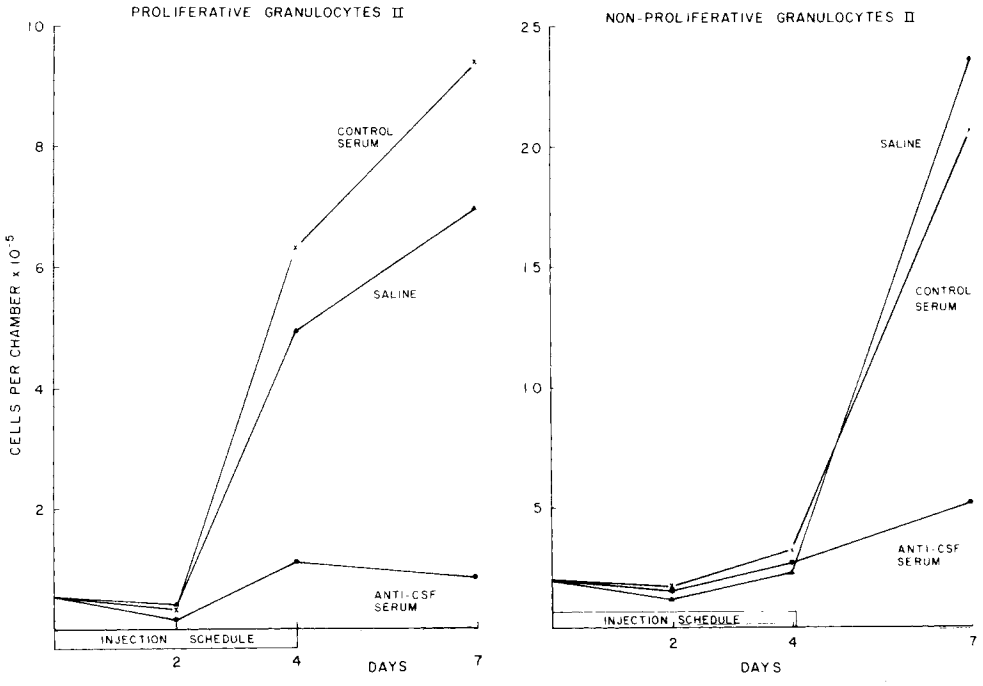

Fig. 11. Effect of anti-CSF serum on diffusion chamber granulopoiesis. After 700 R irradiation, groups of mice were injected with 0.5 ml of saline, control serum, or antiserum every 12 h through 4 days. Shown are the total number of proliferative and nonproliferative granulocytes in each treatment group on the 2nd, 4th, and 7th days of study. Reproduced from Shadduck, Carsten, Chikkappa, Cronkite and Gerard: Inhibition of diffusion chamber (DC) granulopoiesis by anti-CSF serum. Proc Soc Exp Biol Med 158:542–549, 1978, with permission.

was kindly provided by Dr. Michael Dexter and Dr. Joel Greenberger (Harvard Medical School). The results of CSF assays are shown in Table III. Although no CSF activity could be determined by agar gel bioassay, substantial quantities were found in all samples by a sensitive radioimmunoassay. Preliminary experiments suggest that the inability to detect CSF in these cultures may result from high levels of inhibitory substances that prevent colony formation. Clearly, the further delineation of granulopoietic control mechanisms in these long-term cultures may have important implications concerning regulatory mechanisms that may be operative in vivo.

DISCUSSION

As shown in these and other recent studies [26, 27], murine fibroblast or L-cell CSF has been purified to homogeneity. This factor has a molecular weight of 60,000–70,000 daltons and contains substantial quantities of carbohydrate as evidenced by PAS staining, inactivation by periodate, and altered electrophoretic mobility following incubation with neuraminidase. Based on the variable binding to concanavalin-A, the L-cell CSF appears to have some heterogeneity in carbohydrate residues; however, this difference

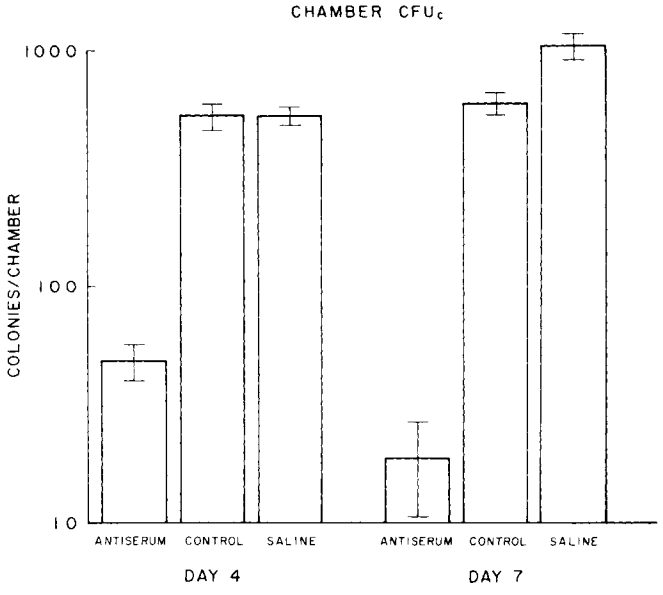

Fig. 12. Recovery of granulocyte-macrophage colony-forming cells or CFU-C from diffusion chambers following treatment with CSF antiserum. Hosts were treated as in Figure 11. Values are means ± 1 SE. Modified from Shadduck, Carsten, Chikkappa, Cronkite and Gerard: Inhibition of diffusion chamber (DC) granulopoiesis by anti-CSF serum. Proc Soc Exp Biol Med 158:542–549, 1978, with permission.

does not influence specific activity [28] or colony morphology [33]. The purified CSF is active in concentrations of 5×10^{-12} M and yields maximal colony formation with 5×10^{-11} M, or approximately 3 ng/ml.

Although in our studies [28] 50–70% of the biologic activity is retained after purification, the final yields of material only range from 200 to 300 μg/10 l pool. This problem and the marked time delay inherent in the separative procedures have substantially hampered large scale production and purification of this factor. Recently, using "monospecific" antibodies to CSF [33], we have found that the crude L-cell material can be purified by a single-step immunoadsorbent technique [36]. Yields have ranged from 65% to 100%, and specific activities are essentially identical to those obtained by the 6-step procedure. This improved technique may now provide the relatively large quantities of material required for in vivo studies and for further characterization of subunit structure.

One of the major problems in the study of in vivo granulopoiesis has been the invariable contamination of test solutions with endotoxin. Since such lipopolysaccharides cause both the production of CSF in vivo [18] and stimulation of granulopoiesis [19], purified CSF must be rendered virtually free of biologically active levels of such contaminants. By virtue of the widely differing molecular weights, it has now been possible to markedly reduce the level of endotoxin in purified CSF preparations [37]. Density gradient centrifugation sediments greater than 95% of endotoxin activity to the bottom of the tube, whereas CSF is retained in the upper portion of the gradient. Levels of endotoxin are generally reduced to < 0.01 μg/ml – a concentration that is essentially devoid of effects on hemopoiesis [37].

TABLE III. CSF Activity in Long-Term Marrow Cultures*

Duration of culture	Bioassay (units/ml)	Radioimmunoassay (units/ml)
1–3 weeks (Dexter)	4.4 (2–6)	79.2 (44–145)
4–13 weeks (Greenberger)	3.0 (0–8)	69.6 (47–135)

*Values represent units/ml of CSF in supernatants harvested from long-term marrow cultures at the indicated times. One unit of CSF is defined as that activity which stimulates 1 colony in the agar-gel bioassay. Units were calculated, when feasible, from the linear portion of the dose-response curve.

With radiolabeled CSF, it has been possible to define the plasma disappearance rate and partially characterize the degradative pathways. The major plasma clearance has a $t_{1/2}$ of approximately 2 h. Only minimal activity is detected in target marrow cells, whereas > 40% of the tracer rapidly accumulates in the liver. Within 6 h as much as 30% of the tracer is excreted in the urine. However, this is composed entirely of low molecular weight peptides. Parallel studies with unlabeled CSF show a similar plasma clearance; however, less than 1% of the intact molecule is recovered in the urine. It appears clear from these studies that urinary CSF levels may not reflect serum levels of this factor. Despite the fact that human urine is a good source of CSF for murine cultures, these findings, as well as the inability of human urinary CSF to stimulate human cultures, cast serious doubt on the value of such determinations.

In vitro incubation of iodinated CSF with murine marrow cells leads to delayed but extensive binding. The kinetics differ markedly from most other hormone–receptor interactions by virture of a 6–16 h time lag. The binding is specific and saturable, and it is not influenced by addition of various exogenous proteins. Preliminary results indicate that once CSF is bound to the cells it cannot be displaced by a 100- to 1,000-fold excess of cold material. Perhaps the delay in binding results from irreversible receptor occupancy in vivo such that in vitro binding requires genesis of new cellular receptors.

Recently, increasing attention has been directed at colony morphology. Various sources of CSF yield colonies of granulocytes (G), macrophages (M), or a mixture thereof (GM) when scored after 7 days of incubation. Based on this terminology, L-cell CSF is believed to represent a pure M type CSF [38]. However, when colonies are examined on the 3rd or 4th day of growth in agar gels [33] or in plasma clots [39], substantial numbers of granulocytic cells are observed.

Our preliminary autoradiographic studies also indicate that CSF binds to cells of the granulocytic series. Between 35% and 60% of large mononuclear cells are labeled after 6–24 h incubation. Fifty percent of myeloblasts and promyelocytes are labeled at 6 h, with an increase to 95% at 24 h. Between 30% and 60% of later cells in the myeloid series are labeled after 24 h incubation [40]. Thus, it would seem clear that this variety of CSF binds to and causes limited proliferation of cells in the granulocytic as well as monocyte-macrophage lineages.

Initially, it was believed that if CSF is a biologically important regulator of granulopoiesis, simple in vivo neutralization by antibodies should lead to granulocytic aplasia. Although anti-CSF completely neutralizes circulating CSF, studies with crude antiserum

indicated no depression of in vivo granulopoiesis [16]. As shown in this report, highly purified anti-CSF is also devoid of activity. In part this may result from second antibody formation, with rapid clearance of anti-CSF within 5 days of repetitive injection. Animals treated with anti-CSF and cortisone to prevent second antibody formation still manifest active granulocytic differentiation. Thus, it must be concluded that CSF in the serum may not be a controlling factor in granulopoiesis.

Although anti-CSF has not suppressed bone marrow granulopoiesis, such treatment is markedly inhibitory to diffusion chamber granulopoiesis. Either crude antiserum [16] or, in recent experiments with Drs. Cronkite and Carsten, the highly purified antibody reduced both the number and size of diffusion chamber granulocytic colonies. This striking difference from the effect in intact animals may be due to altered clearance of antibody in irradiated mice – a step necessary to induce heightened diffusion chamber granulopoiesis. Alternatively, there may be extensive local stromal production of CSF in the intact marrow, which is difficult to neutralize by systemic administration of antibody. Diffusion chamber studies obviate this problem by selectively removing marrow cells from their intact microenvironment.

To examine further the question of stromal interaction, the effect of anti-CSF was determined in long-term marrow cultures. In this system adherent "stromal" layers are established after 3–4 weeks of marrow cell culture [34]. With refeeding on a weekly basis, granulopoiesis continues for several months without addition of CSF. Early studies showed no CSF production in these cultures [34, 35]. However, the results reported herein indicate this is an artifact caused by coexistent inhibitors. Virtually no CSF is detected in supernatants by agar gel bioassay, whereas substantial quantities of CSF are found by radioimmunoassay.

Although CSF is present in these cultures, addition of anti-CSF does not prevent continuing granulocytic differentiation [32]. Thus, it appears clear both from the in vivo studies and from the long-term "Dexter-type" cultures that granulopoiesis cannot be inhibited by anti-CSF when the responsive colony-forming cells are in contact with stromal elements of the marrow. When removed from the microenvironment either in the agar gel [29] or in the diffusion chamber system [16], marrow cells are markedly suppressed by this antiserum. This suggests that granulopoiesis is normally CSF mediated by a short-range cell–cell contact within the intact marrow microenvironment.

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