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Colony-stimulating factor (CSF) radioimmunoassay: Detection of a CSF subclass stimulating macrophage production

(hemopoiesis/mononuclear phagocytes/macrophage growth factor/monopoietin/differentiation)

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ABSTRACT **Colony-stimulating factors (CSFs) stimulate the differentiation of immature precursor cells to mature granulocytes and macrophages. Purified ^{125}I-labeled murine L cell CSF has been used to develop a radioimmunoassay (RIA) that detects a subclass of CSFs that stimulates macrophage production. Murine CSF preparations that contain this subclass of CSF compete for all of the CSF binding sites on anti-L cell CSF antibody. With the exception of mouse serum, which can contain inhibitors of the bioassay, there is complete correspondence between activities determined by RIA and those determined by bioassay. The RIA is slightly more sensitive than the biosassay, detecting approximately 0.3 fmol of purified L cell CSF. It can also detect this subclass of CSF in chickens, rats, and humans. In the mouse, the subclass is distinguished from other CSFs by a murine cell bioassay dose–response curve in which 90% of the response occurs over a 10-fold (rather than a 100-fold) increase in concentration, by stimulating the formation of colonies containing a high proportion of mononuclear (rather than granulocytic) cells, and by certain physical characteristics.**

The production of mature granulocytes and macrophages from primary cultures of hemopoietic cells is dependent on the presence of nanomolar concentrations of specific regulatory molecules (1–4). These molecules have been termed colony-stimulating factors (CSFs) due to their ability to stimulate immature precursor cells to form clones of mature progeny cells (5–7). CSFs are found in body fluids, certain tissues, and the media conditioned by certain cells in culture (conditioned medium, CM) (6, 7). Whereas some CSFs appear to be glycoproteins of similar physicochemical properties (though of somewhat variable M_r) (6, 8–12), others exhibit different properties and, generally, a lower M_r (13–17). For example, purified CSFs from two murine sources, L cell CM (3) and endotoxin lung CM (4) are quite dissimilar. L cell CSF has a M_r of 70,000 and has chemical properties, a dose–response curve, and biological activity that differ from the lower M_r (23,000) CSF of endotoxin lung CM. In addition, significant differences in the size and density distributions of target cells have been observed between crude CSF preparations from different sources (18–21). These findings have indicated the existence of at least two types of CSF—one that primarily stimulates macrophage production (8, 22) and another (or others) that primarily stimulates granulocyte production. In this paper we describe the development of a CSF radioimmunoassay (RIA) and its use, in conjunction with the bioassay, in delineating a distinct subclass of CSF that stimulates macrophage production.

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MATERIALS AND METHODS

CFS Preparations. Stage 4 L cell CSF (3), L cell CM (23), C57BL mouse serum, C57BL mouse endotoxin serum (24), C57BL mouse endotoxin lung CM (13), and stage VI human urinary CSF (6, 25) were all prepared as described earlier. Wistar rat endotoxin serum was prepared in the same way as mouse endotoxin serum. Pregnant BALB/c mouse uterus extract (9), BALB/c mouse embryo cell CM (10), BALB/c mouse embryo extract (11), rat liver fibroblast CM, human fetal fibroblast CM, and human bone marrow fibroblast CM were gifts from T. R. Bradley. Purified 23,000 M_r C57BL mouse endotoxin lung CM CSF (4), mouse yolk sac CM (12), C57BL pokeweed mitogen mouse spleen CM (15), and human placental CM (26) were gifts from A. W. Burgess. The human embryo kidney CSF preparation (National Cancer Institute contract, CSF_{HEKS}), the human GCT-C cell CM (27), the mouse WEHI-3 cell CM (14), and the chicken endotoxin serum (28) were gifts received from J. Bull, J. DiPersio, N. Williams, and W. Dodge, respectively.

Other Preparations. Purified preparations of nerve growth factor (mouse submaxillary glands), epidermal growth factor (mouse submaxillary glands), fibroblast growth factor (bovine pituitary glands), and multiplication-stimulating activity (Buffalo rat liver cell CM) were gifts from Collaborative Research, Inc. (Waltham, MA). Purified human urinary erythropoietin (29) was a gift from E. Goldwasser. Hormone preparations used were sheep pituitary luteotropic hormone (26 international units/mg, Sigma), porcine pituitary somatotropin (0.4 unit/mg, Sigma), crystalline bovine and porcine pancreatic glucagon (Sigma), porcine follicle-stimulating hormone (F-8001, Sigma), human chorionic gonadotropin (CG-2, Sigma), and recrystallized bovine pancreatic insulin (Calbiochem).

Bioassays. Murine cell bioassays were carried out as described (8, 30). All preparations (often concentrated) were serially diluted 1:2 for bioassay. The concentration at which colony numbers were half maximal was used to determine activity in colonies per ml. This figure was converted to units/ml by dividing by the number of colonies/unit determined from a standard, calibrated against a stable standard human urinary preparation (31, 32) included in each bioassay (1 unit $\approx$ 2.5 colonies under the culture conditions used). Human bioassays were carried out by using nonadherent human bone marrow cells (33) by the method of Pike and Robinson (34). Cell morphology was examined in cultures at CSF concentrations yielding 70–90% of the maximal response by staining individually picked colonies as described (25).

Iodinated CSF. Stage 4 L cell CSF (3) was iodinated by using

Abbreviations: CSF, colony-stimulating factor; RIA, radioimmunoassay; CSF_{RIA}, CSF detected by the RIA; CM, conditioned medium.

the method of Greenwood *et al.* (35) modified by including dimethyl sulfoxide to protect from loss of biological activity during oxidation (6, 36) and polyethylene glycol 6000 to preserve stability of CSF (6). Incubation mixtures contained 0.9 μg of stage 4 L cell CSF (approximately 13.0 pmol), 1.64 mCi (approximately 0.9 nmol; 1 Ci = 3.7×10^{10} becquerels) of carrier-free ^{125}I (Amersham), 28.0 μmol of dimethyl sulfoxide, 3.3 μg of polyethylene glycol 6000, and 2.0 nmol of chloramine-T in a total volume of 36.0 μl of 50 mM sodium phosphate, pH 6.5. Incubation (30 min at 0°C) was stopped by addition of 1 μl (2.4 nmol) of $K_2S_2O_5$. KI (2 μl, 0.2 μmol) was added and the protein-bound ^{125}I was separated from free ^{125}I on a Sephadex G-25 column (5.0 cm × 0.5 cm) in 0.3 mM Tris·HCl, pH 7.4, containing 0.01 g of polyethylene glycol 6000 per liter. Iodination efficiency was 8–11%. The fractions composing the protein-bound ^{125}I peak were pooled (total volume approximately 0.3 ml), lyophilized, resuspended to 100 μl, and gradient gel electrophoresed as described for stage 5 of the purification (3). Gel segments (0.4 mm) were eluted (overnight, 4°C, 0.5 ml of RIA buffer) eluate samples were (2 μl) assayed for ^{125}I, eluates from fractions included in the major peak were pooled as ^{125}I-labeled CSF (^{125}I-CSF) (3), made to 4.0 ml, sterilized by filtration, and stored at −20°C in 1.0-ml aliquots. Purity of at least 90% was established by each of four independent criteria, as described elsewhere (3) as well as by quantitative binding to target cells (25). The ^{125}I-CSF contained approximately six atoms of ^{125}I per CSF molecule (approximately 50,000 cpm/ng) and possessed full biological activity, and 90% of it combined with rabbit antibody to stage 4 L cell CSF (Fig. 1). Iodination of CSF under the conditions used rendered the molecule unstable and led to a gradual loss of biological and antibody-binding activity (half-life approximately 1 month).

RIA. (See refs. 37 and 38.) To 200 μl of test sample diluted in RIA buffer [50 mM sodium phosphate containing 2.0 g of crystalline bovine serum albumin per liter (Sigma), 3 mM NaN_3, and 0.02 g of polyethylene glycol 6000 per liter, pH 6.5] in a plastic tube (Falcon 2052), 50 μl of ^{125}I-CSF (approximately 0.2 ng, 24 units, 10,000 cpm) in RIA buffer was added, followed by 250 μl of rabbit antiserum to stage 4 L cell CSF (8) diluted 1/1024 in a mixture of 1 part normal rabbit serum to 7 parts RIA buffer (the normal rabbit serum was a pretested batch that precipitated <6% of the ^{125}I in tubes containing ^{125}I-CSF without antiserum). All dilutions were set up in duplicate and each RIA included "total counts" tubes containing 50 μl of ^{125}I-CSF alone, tubes in which 1:8 normal rabbit serum replaced 1:1024 antiserum and tubes in which RIA buffer replaced the test sample. Incubation was for 20 hr at room temperature or 48 hr at 4°C, conditions under which binding was 95–100% of equilibrium values. This "one-step" procedure could be altered to a more sensitive but less rapid "two-step" procedure by incubating antiserum and test sample for 20 hr at room temperature, prior to addition of ^{125}I-CSF, and further incubating for 20 hr at room temperature. After both procedures, $(NH_4)_2SO_4$ (4.0 ml, 2.18 M, 4°C) was added to precipitate complexes in all tubes except "total counts" tubes. After incubation (4°C, 30 min), these tubes were centrifuged (1800 × *g*, 4°C, 30 min), supernates were decanted, and the radioactivities of precipitates were measured along with "total counts" tubes. The antiserum concentration was chosen to yield a bound-to-free ratio (*B/F*) of approximately 1.0. Due to the instability of ^{125}I-CSF, this ratio decreased with time after preparation of ^{125}I-CSF, and, in order to graph data from different experiments, *B/F* values obtained for ^{125}I-CSF and antiserum alone in each RIA were normalized to yield a value of 1.0. Satisfactory RIA results could be obtained only up to 3 months after iodination.

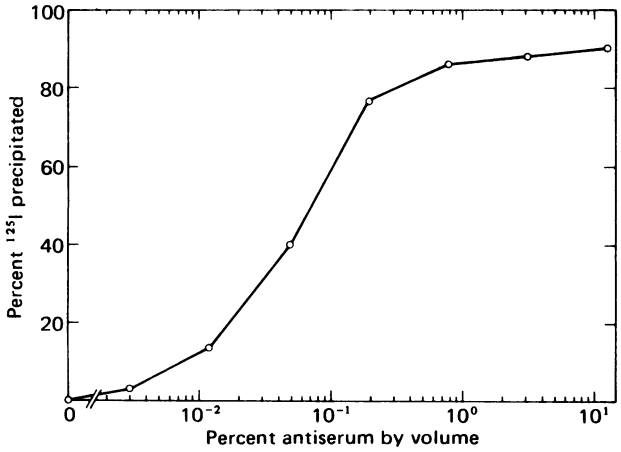

FIG. 1. Reaction of purified ^{125}I-CSF with rabbit antiserum to stage 4 L cell CSF. Incubation mixtures contained 50 μl of ^{125}I-CSF (approximately 0.2 ng, 24 units, 10,000 cpm) in RIA buffer, 250 μl of rabbit antiserum diluted in 1:8 normal rabbit serum, and 200 μl of RIA buffer. Tubes were incubated and processed as for RIA. Figures for percent ^{125}I precipitated (ordinate) include a subtraction for precipitation occurring in the presence of 1:8 normal rabbit serum alone (5.2%).

RESULTS

Murine CSF Sources. The bioactivity of certain sources, including conditioned media, tissue extracts, and body fluids, has been shown to be completely neutralized by antiserum to L cell CSF (6, 8). Results of both RIA and murine bioassays on several of these sources and on two biologically inactive conditioned media (M-1 murine myeloid leukemic CM and a murine lymphoma CM) are shown in Fig. 2. The latter two

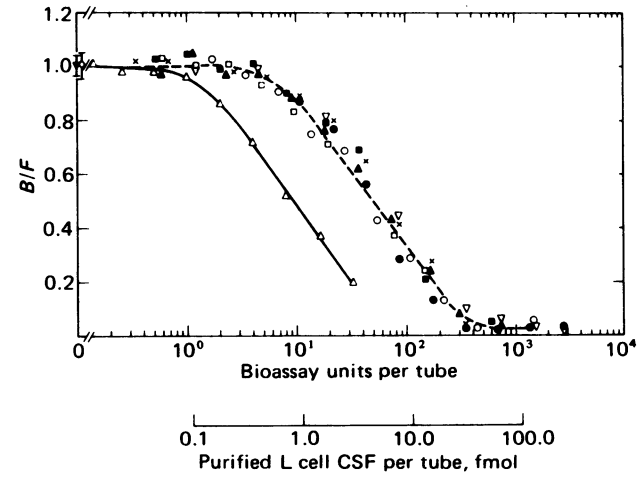

FIG. 2. Displacement curves for murine sources of CSF for which murine bioactivity is completely neutralized by anti-L-cell CSF antiserum (one-step assay). Ordinate, ratio bound ^{125}I-CSF to free ^{125}I-CSF; abscissa, murine bioactivity in units per tube or approximate molar concentration of purified L cell CSF per tube (1 fmol equals approximately 70 pg). Control (biologically inactive) sources: M-1 murine myeloid leukemic cell CM (39) (▼) and a murine lymphoma cell CM (◇). Other sources: purified stage 5 L cell CSF (×), L cell CM (○), pregnant BALB/c mouse uterus extract (●), BALB/c mouse embryo cell CM (□), mouse yolk sac CM (■), BALB/c mouse embryo extract (▽), C57BL mouse endotoxin serum (▲), and C57BL mouse serum (△).

sources were not detected in the RIA. Each of the other sources competed for all the CSF binding sites in the antiserum to L cell CSF and exhibited slopes that were the same as the slope obtained for purified L cell CSF. Furthermore, for each of these sources (with the exception of mouse serum) there was complete correspondence between RIA and bioassay measurements of CSF concentration. Greater displacement was observed with normal mouse serum than expected from the bioassay results. The close correlation between bioassay and RIA results observed for the majority of murine sources examined (Fig. 2) is also observed for L cell CSF undergoing various treatments, including heat inactivation at 90°C and chromatographic fractionation (data not shown).

Murine sources of CSF for which the murine bioactivity is only partially neutralized or completely unaffected by antiserum to L cell CSF were investigated by using the more sensitive "two-step" RIA (Fig. 3). All the unfractionated sources in this group appear to contain at least two chromatographically separable subclasses of CSF, one of which is the CSF detected by the RIA (henceforth referred to as CSF_{RIA}) (refs. 14, 15, 17, 40; unpublished observations). For each of these sources, there was little correlation between results of the bioassay (which detects all CSF subclasses) and results of the RIA (which detects only one CSF subclass). Thus addition of considerably more bioassay units of CSF was necessary than in the case of L cell CSF in order to obtain equivalent displacement in the RIA. In the extreme case of the purified 23,000 M_r mouse endotoxin lung CM CSF, no displacement was observed even with 1000 times the number of units required for the detection of L cell CSF in the RIA.

Specificity of RIA. Preparations of a number of different growth factors and hormones were tested and found not to be detected in the RIA (data not shown). They included purified nerve growth factor (20 pmol), epidermal growth factor (200 pmol), fibroblast growth factor (20 pmol), multiplication-stimulating activity (20 pmol), erythropoietin (6 units) as well as luteotropic hormone (20 units), somatotropin (0.4 milliunit), glucagon (200 pmol), follicle-stimulating hormone (1 unit), human chorionic gonadotropin (200 units), and insulin (200 pmol).

Relationship between Detection in the RIA and Other Characteristics of the Murine Sources Tested. All the murine sources exclusively containing CSF_{RIA} had bioassay dose–response curves in which 90% of the response occurred over a 10-fold increase in CSF concentration. These sources were purified L cell CSF, L cell CM, pregnant mouse uterus extract, mouse embryo cell CM, mouse yolk sac CM, mouse endotoxin serum, and mouse serum. By contrast, those murine sources containing CSF(s) other than CSF_{RIA} had bioassay dose–response curves in which 90% of the response occurred over a 100-fold increase in CSF concentration. These sources were purified 23,000 M_r mouse endotoxin lung CM CSF, mouse endotoxin lung CM, mouse WEHI-3 cell CM, and mouse pokeweed mitogen spleen CM (for examples of dose–response curves see ref. 25).

The sources exclusively containing murine CSF_{RIA} exhibited a common colony morphology. At ×20 magnifications, colonies were seen to be loose to fairly dense aggregates of large cells and cultures were characterized by an absence of both colonies with very dense centers and colonies composed of small cells. The cell morphology was mononuclear. By contrast, 30–90% of all colonies stimulated by murine sources containing CSF(s) other than CSF_{RIA} exhibited a high proportion of granulocytic cells. In addition, significant proportions of colonies with very dense centers and of small-cell colonies were observed. The exception to these correlations was mouse endotoxin serum (31% of colonies composed of small polymorphonuclear granulocytic cells, 31% both immature granulocytes and mononuclear cells, and 38% mononuclear cells).

Neutralization Studies. Bioassay neutralization studies with excess antiserum to CSF_{RIA} were carried out on murine sources containing both CSF_{RIA} and other CSFs to determine whether the calculated proportion of CSF_{RIA} (from Fig. 3) corresponded to the proportion of their bioactivity that could be neutralized. The degree of neutralization was much greater than the calculated proportion of CSF_{RIA} for WEHI-3 cell CM (Table 1), raising the possibility of synergism between CSF_{RIA} and the other CSFs during colony formation stimulated by WEHI-3 cell CM.

CSF Sources from Other Species. A number of nonmurine CSF sources were examined to determine the sensitivity of the murine RIA across species (Fig. 4). Approximately 80 times the number of murine bioassay units required with L cell CM were required to obtain an equivalent RIA displacement by stage VI human urinary CSF or rat liver fibroblast CM. However, displacement curves for these sources were parallel and the latter two sources competed for at least 80% of the CSF binding sites in the antiserum to L cell CSF. The rat endotoxin serum tested

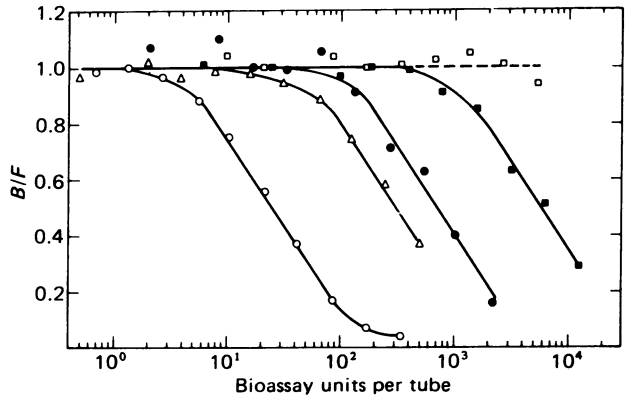

FIG. 3. Displacement curves for murine sources of CSF for which the murine bioactivity is partially neutralized or unaffected by antiserum to L cell CSF (two-step assay). Sources: control L cell CM (○), WEHI-3 cell CM (△), C57BL pokeweed mitogen mouse spleen CM (●), C57BL mouse endotoxin lung CM (■), and purified 23,000 M_r C57BL mouse endotoxin lung CM CSF (□).

Table 1. Degree of neutralization of colony-stimulating activity of various murine preparations by antiserum to L cell CSF

Source	Colony-stimulating activity detectable by RIA (calculated*), %	Neutralization by anti-L cell CSF antiserum, %
L cell CM	100	100
WEHI-3 cell CM	8	66
Pokeweed mitogen spleen CM	4	3 (NS)
Endotoxin lung CM	0.4	1 (NS)

NS, not significant (<10%).

* Calculated with reference to the RIA-bioassay curves in Fig. 3. For each preparation, the number of bioassay units required to obtain a degree of displacement in the RIA equivalent to that of 20 units of L cell CSF was determined. The latter figure divided by the former was assumed to represent the fraction of colony-stimulating activity detectable by RIA. Calculations do not take into account differences in the murine bioassay dose–response curves between L cell CSF and the other preparations examined, making the calculations approximate.

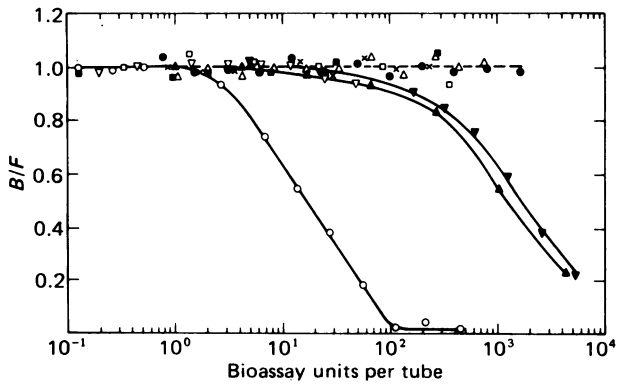

FIG. 4. Displacement curves for human and rat CSF sources (two-step assay). Sources: control L cell CM (○), partially purified human urinary CSF (▲), rat liver fibroblast CM (▼), rat endotoxin serum (▽), human placental CM (●), human embryo kidney CSF preparation (□), GCT-C cell CM (■), human fetal fibroblast CM (△), and human bone marrow fibroblast CM (×).

did not possess enough activity to cause a very marked displacement but behaved similarly to the rat liver fibroblast CM. On a plot of B/F against dilution, preparations of chicken endotoxin serum and L cell CM had identical slopes. This data could not be included in Fig. 4 because the chicken endotoxin serum tested was inactive in the murine bioassay [the general case for chicken sources of CSF of this type (W. Dodge, personal communication)]. All the human sources of colony-stimulating activity tested were active in the human bioassay as well as in the murine bioassay but, with the exception of human urinary CSF, failed to be detected in the RIA.

DISCUSSION

RIA. Purified L cell ^{125}I-CSF and anti-L cell CSF antibody have been used to develop a rapid, sensitive, and reliable RIA for a particular subclass of CSFs. The close correlation between bioassay and RIA results indicates that the RIA may be used to replace the bioassay for this subclass. In many respects the RIA has definite advantages over the bioassay: It is more specific in that it detects one CSF subclass; it eliminates the general problems associated with tissue culture; it is faster and less laborious than the bioassay and, for murine sources, more sensitive than the bioassay. However, for CSFs from species other than the mouse, the murine RIA is less sensitive than the murine bioassay. In addition, caution should be exercised in equating the RIA with the bioassay for tissue culture sources, because the ordinarily small percentage (approximately 0.4%) of L cell CM colony-stimulating activity that is of low M_r and not recognized by antibody raised to the 70,000 M_r species (8) may be increased (up to 10%) when poor L cell culture conditions lead to significant cell death (25).

The specificity of the RIA is exemplified by the close correlation between CSF levels determined by RIA and bioassay in a number of disparate sources and during the fractionation of L cell CM, the coordinate loss of CSF by RIA and bioassay during heat inactivation, and the failure of the RIA to detect CSF in purified preparations of other growth factors and hormones. The exceptions to this correlation between RIA and bioassay results are those sources that contain different types of CSF not detected by RIA (Fig. 3) and mouse serum. In the former case, as one would expect, higher CSF levels were detected by the bioassay, which does not discriminate between different CSF types. In the case of mouse serum, higher CSF levels were detected by RIA (0.15 nM) than by bioassay (0.03 nM, curve shifted to the left; Fig. 2). This result is probably explained by the presence of inhibitors of the tissue culture bioassay that are known to be present in mouse serum (41) but that apparently have no effect on the determination of CSF by RIA. The results with mouse endotoxin serum (which contained 0.6 nM CSF by both bioassay and RIA and for which the effect was not observed) support this explanation. Due to the elevated CSF levels in mouse endotoxin serum (24), the bioassay figures were calculated from colony counts of cultures in which the serum (and, therefore, any inhibitory material) was approximately 1/16th as concentrated as in the case of normal mouse serum.

The detection of CSF by RIA in chicken, mouse, and rat endotoxin sera and human urine indicate that it may be possible to carry out significant phylogenetic studies by murine RIA that otherwise would be difficult. In this regard, it should be noted that detection of CSF in the murine bioassay was neither a necessary nor sufficient criterion for detection in the RIA. CSF of chicken endotoxin serum, although active in a chicken bioassay (28), was inactive in the murine bioassay but detected in the RIA. Also, sources of granulocytic CSFs were active in the bioassay and not detected by the RIA. On the basis of neutralization data with an anti-human CSF antiserum that recognizes human urinary CSF (42), CSF in at least one human source (placental CM) should have been detected by murine RIA. It may be possible to characterize the human sources further with a recently developed RIA utilizing human urinary ^{125}I-CSF.

Relationship between CSF_{RIA} and Other CSFs. Earlier neutralization studies with antisera to both L cell and human urinary CSF had suggested the existence of a distinct subclass of CSF (refs. 6, 8; unpublished observations). In the present study, a subclass of murine CSFs detected by the RIA, stimulating the formation of colonies of mononuclear cells, and possessing a distinctive bioassay dose–response curve has been defined. It appears that CSFs belonging to this subclass share physical characteristics.

In our murine bioassay, 90% of the response to murine CSF_{RIA} occurs over approximately a 10-fold change in concentration. This confirms earlier findings for most of the sources examined here, with the exception of mouse endotoxin serum, which has been reported to exhibit a dose–response curve in which 90% of the response occurs over approximately a 100-fold change in concentration (43). A possible explanation of this discrepancy is the fortuitous absence from our preparation of a separate augmenting activity that appears to affect the CSF dose–response curve (43). Sources exclusively containing murine CSF_{RIA} generally stimulate formation of relatively loose colonies of large mononuclear cells. The exception is mouse endotoxin serum, for which, in addition, significant numbers of granulocytic and mixed granulocyte/mononuclear cell colonies are observed. Because colonies stimulated by highly purified mouse L cell CSF are characterized by an absence of granulocytic cells (25), it will be of interest to examine purified CSF from mouse endotoxin serum when it becomes available. If made by unstimulated cells in culture, murine $CSFs_{RIA}$ are sialic acid-containing glycoproteins with M_rs of approximately 70,000 (3, 12, 44). Apparent M_r may be smaller for CSF_s from other sources, including murine tissue extracts (9, 45, 46). The distinguishing features of this subclass, in the murine sources that have been examined, appear to be a relative resistance to inactivation by proteases (8, 23, 44, 46), a sensitivity to inactivation by 2-mercaptoethanol (3, 6, 46), and antigenicity in rabbits (8). These characteristics are shared by human urinary CFS (6, 25, 47–49), which is also detected in the RIA.

It is somewhat premature to tell whether the CSFs other than CSF_{RIA} have common physical characteristics. For example,

the separation of eosinophil colony-stimulating activity from neutrophil/macrophage colony-stimulating activity in mouse pokeweed mitogen spleen CM (15) suggests that there is more than one type of CSF that is not detected by the RIA. Those CSFs examined that are not detected by the RIA appear to be of lower M_r than CSF_{RIA} (13, 15, 16), resistant to inactivation by 2-mercaptoethanol (48), and relatively sensitive to inactivation by proteases (16, 48). Attempts to produce significant antibody to this CSF subclass(es) in rabbits have not been successful. These CSFs all exhibit murine bioassay dose–response curves in which 90% of the response occurs over approximately a 100-fold change in concentration. In addition, they stimulate colony formation in which colonies containing granulocytic cells are always observed.

Sources used in the neutralization studies contained a small proportion of the CSF_{RIA} and a much larger proportion of other CSF(s). The marked effect of antibody to CSF_{RIA} on the colony-stimulating activity of one of these sources, WEHI-3 cell CM, could suggest a synergism between the CSF_{RIA} and other CSF(s). For example, this may be reflected in the action of the CSF_{RIA} on the target cells of the other CSF(s), or their progeny or precursors. The question of possible synergism can now be tested by mixing experiments with purified preparations and by direct target cell binding assays (25).

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