SPECIFIC INTERACTION OF MURINE COLONY-STIMULATING FACTOR WITH MONONUCLEAR PHAGOCYTIC CELLS

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ABSTRACT

L-cell colony-stimulating factor (CSF) is identical to macrophage growth factor and stimulates macrophage proliferation (Stanley et al., 1976, *J. Exp. Med.* **143:** 631–647). The nature of the interaction of iodinated L-cell CSF (^{125}I-CSF) with murine peritoneal exudate macrophages was studied. On incubation with 10 pM ^{125}I-CSF at 0°C, cellular binding of ^{125}I-CSF reaches a stable maximum within 15 h. This is in contrast to the association behavior at higher temperatures. At 37°C, cell-associated ^{125}I-CSF levels reach, within 45 min, an unstable maximum which is up to 10-fold less than that occurring under the same conditions at 0°C. At 0°C, binding is saturated ($\sim 5 \times 10^4$ sites/cell) at CSF concentrations of 1 nM. A comparison of binding and competition experiments indicates that iodinated L-cell CSF binds as effectively as L-cell CSF and that human urinary CSF and L-cell CSF equipotently compete for ^{125}I-CSF binding. Specificity of the CSF-binding site is demonstrated by the failure of other known growth factors and hormones to compete for ^{125}I-CSF binding. These studies and other findings suggest that ^{125}I-CSF binding is restricted to macrophages and their precursors and to macrophage cell lines and that the binding site(s) is the receptor mediating the biological action of this CSF.

Colony-stimulating factors (CSFs) are growth factors that are required for granulocyte and macrophage production from undifferentiated hemopoietic precursor cells in culture (2, 17, 26). One of these, a sialoglycoprotein of mol wt 70,000, has been purified from mouse L-cell conditioned medium (27) and is identical to macrophage growth factor (25). This particular factor belongs to the CSF subclass stimulating only macrophage production (23, 28) and will be, for convenience, hence-forth referred to as CSF. As CSF is absolutely required for the proliferation of both peripheral macrophages and their undifferentiated precursor cells (24), the CSF-target cell system is a potentially powerful one for studying the biochemical effects of a growth factor on cellular proliferation and differentiation.

The first step in the series of events leading to CSF-induced macrophage proliferation should involve the interaction of CSF with its target cells. We here report the existence of a specific and saturable CSF-binding site(s) on a relatively homogeneous population of peritoneal exudate macrophages. In addition, we present preliminary evidence that the occurrence of this binding site(s) is restricted to macrophages, their precursors, and macrophage cell lines.

MATERIALS AND METHODS

Reagents

L-cell CSF was purified as previously described (27). The purity was checked by polyacrylamide gel electrophoresis, with and without SDS, under reducing and nonreducing conditions, and by complexing with rabbit anti-CSF antibody (27). The CSF preparation was iodinated (one to two atoms ^{125}I per molecule, 59,000 cpm/ng) with full retention of biological and antibody-binding activities (23, 26, 27). ^{125}I-CSF was used within 3 wk of iodination as its biological activity decreases with time (half-life ~6 wk). There is a close correlation between biological and antibody-binding activities (23, 28), and the biologically active ^{125}I-CSF fraction can be rapidly determined as the fraction of

^{125}I combining with excess rabbit anti-CSF antibody (23). Molarities of biologically active ^{125}I-CSF were calculated from this fraction and the protein concentration (11) determined using a bovine serum albumin standard, assuming a mol wt of 70,000 (27).

Partially purified (stage VI) human urinary CSF was prepared as previously described (26, 28). Purified preparations of nerve growth factor (NGF) and epidermal growth factor (EGF) (mouse submaxillary glands), fibroblast growth factor (FGF) (bovine pituitary glands), and multiplication stimulating activity (MSA) (Buffalo rat liver cell conditioned medium) were gifts from Collaborative Research Inc. (Waltham, Mass.). Purified human urinary erythropoietin (13) was a gift of Dr. E. Goldwasser. Hormone preparations used were recrystallized bovine pancreatic insulin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), crystalline bovine and porcine pancreatic glucagon (Sigma Chemical Co., St. Louis, Mo.), sheep pituitary luteotropic hormone (LH) (26 IU/mg, Sigma Chemical Co.), porcine pituitary somatotropin (0.4 U/mg, Sigma Chemical Co.), porcine follicle-stimulating hormone (FSH) (2 U/mg, Sigma Chemical Co.), and synthetic adrenocorticotropic hormone (ACTH) (Cortrosyn, Organon Inc., West Orange, N. J.).

Bioassay

CSF bioassays were carried out using murine bone marrow cells as described previously (12, 25).

Target Cells

Peritoneal exudate macrophages were prepared from 3 mo-old C3H/Anf mice that had received 3 d previously an intraperitoneal injection of 1.5 ml of 2% hydrolyzed starch (Connaught Laboratories, Toronto, Canada) in PBS (0.15 M NaCl, 0.005 M phosphate, pH 7.35). Cells were collected from the peritoneal cavity in 10 ml of α medium (Kansas City Biological, Kansas City, Mo.) containing 0.025 M HEPES (Grand Island Biological Co., Grand Island, N. Y.) in lieu of bicarbonate, pH 7.35, at 23°C (α-HEPES) made 10% (vol/vol) in fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.) (FCS-α-HEPES), and 5 U/ml in heparin (Upjohn Co., Agricultural Prods MKT, Kalamazoo, Mich.). They were washed once by centrifugation (800 *g*, 10 min) in 10 ml of α-HEPES, and 10^6 cells in 2 ml of FCS-α-HEPES was incubated in 35-mm tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) for 15–20 min at 37°C. The medium was swirled to loosen cells not firmly attached, and the supernatant fluid was aspirated. The adherent cells were washed once with α medium (2 ml/dish) and incubated for ≥15 h in 10% FCS (vol/vol) in α medium (FCS-α) at 37°C in a humidified, 10% CO_2 in air atmosphere before ^{125}I-CSF-binding studies. 100% of these cells are capable of Fc-mediated phagocytosis of antibody-coated sheep erythrocytes (5). Unstimulated or "resident" peritoneal macrophages were harvested from untreated C3H/Anf mice and plated as described above except that the adherent cells were allowed 2 h for attachment rather than 15 min. Bone marrow cells were obtained by gently aspirating the bone marrow plugs from trimmed femoral shafts of C3H/Anf mice through a 22-gauge needle into FCS-α-HEPES. Tissue clumps were allowed to settle, and the supernatant cell suspension was diluted to 10^7 nucleated cells/ml for ^{125}I-CSF-binding studies. The mouse myeloma cell line MOPC-21 (8) and the myeloma-macrophage hybrid line FC-1 (5) were supplied by Dr. B. Diamond; the mouse macrophage lines J774.2 and J774.16 (5, 20) were provided by Dr. B. Bloom; the mouse Friend erythroleukemic line DS19 (15) by Dr. C. Schildkraut; the L_6E_9 rat myoblast cells (14) by Dr. B. Nadal-Ginard; the Chinese hamster ovary cells (18) by Dr. P. Stanley; the M1 mouse myeloid leukemia line (9) by Dr. D. Housman; the mouse lines WEHI-3, P388D1, PU5-1.8, and RAW264 (19) by Dr. P. Ralph; and the mouse 3T3-L1 cells (7) by Dr. O Rosen.

Determination of Cell-associated ^{125}I

After incubation for 15 h in FCS-α without CSF, adherent cells on 35-mm tissue culture dishes were washed once with PBS and 0.9 ml of FCS-α-HEPES, or, in some cases, 10% dialysed FCS in PBS was added. The cells were brought to the appropriate temperature before addition of 0.1 ml of the same medium containing ^{125}I-CSF or ^{125}I-CSF plus competing unlabeled CSF, hormones, or growth factors. Incubations were carried out in a humidified atmosphere at the appropriate temperature. The reaction was stopped by 4 × 2.5 ml washes of ice-cold PBS and the cells were either instantly fixed *in situ* with cold methanol (for staining or autoradiography) or solubilized from the dishes with 3 × 0.5 ml washes of 0.05 M Tris-HCl, 0.5% SDS, pH 7.4[1] (for gamma counting). Cell numbers were determined from counts of 20 randomly selected fields on methanol-fixed, Giemsa-stained dishes. For ^{125}I-CSF-binding studies with nonadherent cells, the cells were incubated for at least 4 h in FCS-α (without CSF at 37°C), washed once with PBS (800 *g*, 10 min, 2°C), resuspended to 1.1×10^6 cells/ml in FCS-α-HEPES, cooled to 0°C, and 0.9 ml was aliquoted into cold 35-mm plastic petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). Incubations with ^{125}I-CSF were carried out as for the adherent cells. The reaction was stopped by layering the cell suspension over 3 ml of ice-cold FCS in a 5-ml culture tube (Falcon No. 2054) and centrifuging (800 *g*, 10 min, 2°C). While the pellet was kept at 4°C, the supernatant fluid was aspirated away. The cell pellets were either counted in a gamma counter or resuspended in ice-cold FCS, spun onto ice-cold slides in a cooled cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.), and fixed in cold methanol for autoradiography (25). After development, the emulsion-covered cells were stained with Giemsa or Wright's stain in 0.1 M citrate, pH 5.75.

RESULTS

The time and temperature dependence of ^{125}I binding[2] by peritoneal exudate macrophages in the presence of 10 pM ^{125}I-CSF is shown in Fig. 1. Maximal ^{125}I bound by the cells is some 10 times greater at 0° than at 37°C. At 0°C, a stable maximum level of bound ^{125}I is achieved. In contrast, a stable maximal equilibrium is not reached at 22° or 37°C. The decrease in cell-associated ^{125}I at 37°C after maximal levels have been reached cannot be explained by the much slower decrease in the supernatant concentration of biologically active ^{125}I-CSF because of binding site mediated ^{125}I-CSF destruction (28). Preliminary experiments

[1] This treatment removes all ^{125}I counts from the dishes.
[2] ^{125}I binding refers to total cell-associated ^{125}I after incubation with ^{125}I-CSF as described in Materials and Methods and doesn't distinguish between surface-bound and internalized ^{125}I.

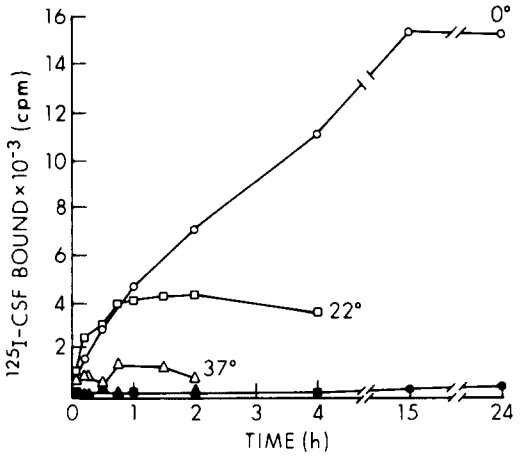

FIGURE 1 Kinetics of binding of ^{125}I-CSF (10 pM) by murine peritoneal exudate macrophages at 0° (○), 22° (□), and 37°C (△). Data are normalized to present ^{125}I binding in cpm/10^5 cells. Open symbols represent total binding of ^{125}I and filled symbols the binding in the presence of 1 nM unlabeled CSF. Details are given in the text.

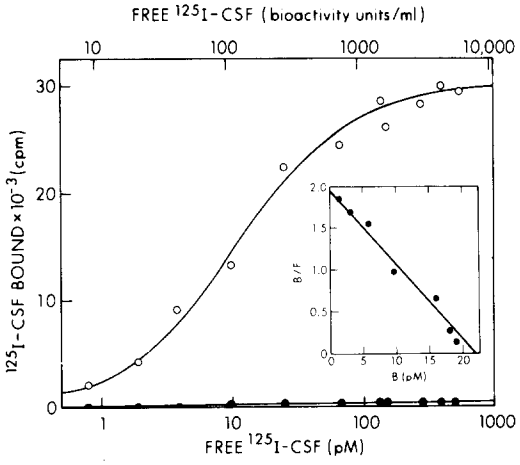

FIGURE 2 ^{125}I-CSF bound to peritoneal exudate macrophages at 0°C as a function of the concentration of free ^{125}I-CSF at equilibrium. Both total binding (○) and binding in the presence of a 50× excess of unlabeled CSF (●) has been normalized to cpm per 10^5 cells. (Experimental cell concentration was 2.55 × 10^5 cells/ml.) *Inset:* Data plotted according to the method of Scatchard (21). Abscissa: Bound ^{125} I-CSF concentration in picomoles per liter. Ordinate: Ratio of bound to free ^{125}I-CSF (unitless).

indicate that the decrease from maximal levels of binding with time at higher temperatures is caused by changes in the ability of the cells to bind ^{125}I-CSF combined with a loss of cell-associated ^{125}I. Immediately after exposure of ^{125}I-CSF to cells at 37°C, their ability to continue to bind ^{125}I-CSF is rapidly lost and remains absent for the next few hours. Concomitantly the ^{125}I-CSF which has bound is degraded to TCA-soluble ^{125}I and released causing an observed net loss (data not shown). Similar behavior is observed in the interaction of ^{125}I-EGF with human fibroblasts (4). The proportion of the ^{125}I binding which is competed for by a 50-fold excess of unlabeled CSF (specific binding) is larger at 0° (98%) than at 37°C (89%).

Because ^{125}I binding at 0°C reaches a stable maximum with the highest proportion of competable to total binding, studies on saturability and specificity were carried out at 0°C. Under these conditions, stable equilibrium levels are attained in ≤20 h (at ≥2 pM ^{125}I-CSF), at which time >95% of the cells were viable as assessed by trypan blue exclusion. ^{125}I binding by peritoneal exudate macrophages at 0°C saturates at $<10^{-9}$ M (Fig. 2). A Scatchard plot (21) of these binding data (Fig. 2, *inset*) is linear over the free ^{125}I-CSF concentration range 8×10^{-13} to 2×10^{-10} M. From this plot the calculated equilibrium constant for ^{125}I-CSF-binding site dissociation ($K_{^{125}\text{I-CSF}}$ = 1/slope) is 1.1×10^{-11} M, and the number of sites per cell, calculated from the abscissa intercept, is 52,000. The values of $K_{^{125}\text{I-CSF}}$ calculated from the slope of the Scatchard plot and from the ratio of the initial dissociation rate constant to the initial association rate constant agree; however, only 10–20% of the bound ^{125}I-CSF dissociates at 0°.[3] We have no explanation at present for the partial reversible nature of the interaction or its effect on the interpretation of the Scatchard plot.

The specificity of the ^{125}I-CSF-binding site(s) on macrophages for CSF was established by testing the ability of known hormones and growth factors to compete with ^{125}I-CSF for binding at 0°C (Fig. 3). In the concentration range 10^{-12} to 10^{-8} M, only purified mouse L-cell CSF and partially purified human urinary CSF (26, 28) compete with ^{125}I-CSF (2 pM) for binding. Equivalent concentrations (in bioactivity units/ml) of human urinary CSF and mouse L-cell CSF compete equivalently for mouse ^{125}I-CSF binding. The relationship between the unlabeled CSF concentration at 50% inhibition (~1.8×10^{-11} M, Fig. 3) and the apparent dissociation constants for ^{125}I-CSF ($K_{^{125}\text{I-CSF}}$ =

[3] Guilbert, L. J., and E. R. Stanley. The nature of the interaction of ^{125}I-CSF with macrophages. Manuscript in preparation.

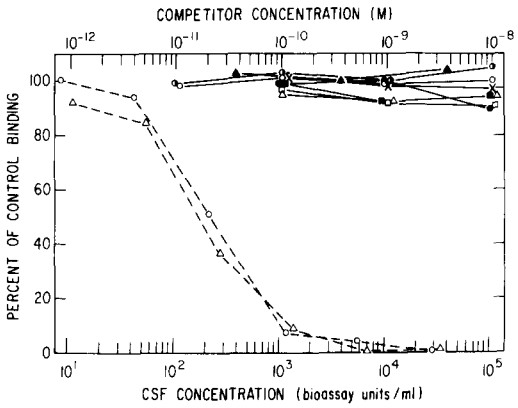

FIGURE 3 Competition for ^{125}I-CSF binding to peritoneal exudate macrophages by unlabeled CSFs and other growth factors and hormones. Incubations were carried out for 20 h at 0°C in the presence of 2 pM ^{125}I-CSF and 5.9×10^4 macrophages/ml. Abscissa: Upper scale (molar concentration) applies to all competing agents except human urinary CSF, and lower scale (bioactivity units/ml) applies only to mouse L-cell and human urinary CSF. Ordinate: Percent of control binding = 100 × cpm bound in presence of competing agent ÷ cpm bound in absence of competing agent. Competing agents are: (solid lines) EGF (△), erythropoietin (▲), insulin (○), glucagon (◑), NGF (●), MSA (□), ACTH (■), FGF (×); (broken lines) mouse L-cell CSF (○), and human urinary CSF (△).

1.1×10^{-11} M) and CSF (K_{CSF}, unknown) is given by Eq. 1 (10):

$$[CSF]_{50\%} = K_{CSF} + (K_{CSF}/K_{^{125}I\text{-}CSF}) \cdot ([^{125}I\text{-}CSF]_0 + R_0 - 3/2\ B). \quad (1)$$

For the competition curve shown in Fig. 3, $[^{125}I\text{-}CSF]_0$ (total ^{125}I-CSF) = 2×10^{-12} M, R_0 (total binding site concentration, calculated from the No. of cells/ml) = 5.1×10^{-12} M, and B (concentration of bound ^{125}I-CSF in the absence of competing CSF) = 5.8×10^{-13} M (data not shown). Solving Eq. 1 for K_{CSF} gives 1.2×10^{-11} M. With the reasonable assumption that the partial reversibility of the CSF-macrophage interaction affects the outcome of $K_{^{125}I\text{-}CSF}$ and K_{CSF} calculations equally, it appears that ^{125}I-CSF and unlabeled CSF have the same affinity for a common binding site(s). This observation is in accord with the observed complete retention of biological activity after iodination of CSF.

The other hormones and growth factors tested (Fig. 3), including somatotropin (10^{-2} U/ml), LH (100 IU/ml), and FSH (5 U/ml) (data not shown), failed to compete. In addition, as there was no difference in the degree of specific ^{125}I-CSF binding for incubations in α-HEPES, FCS-α-HEPES, or PBS containing EDTA (3 mM) and dialysed FCS[4] (10% vol/vol) (data not shown), binding is not competed for by serum components, e.g., glycoproteins (1, 22) or lipoproteins (6), or influenced by Ca^{++}. Binding of CSF (a glycoprotein) is not mediated "nonspecifically" by the mannosyl-glucosyl-binding site found on some macrophage populations (22) because ^{125}I-CSF binding is not affected by yeast mannan (1 mg/ml) (data not shown). Furthermore, the mannosyl-glucosyl-binding site and the galactose-specific recognition site for glycoprotein uptake have been reported to be absent from peritoneal exudate macrophages[5] (1).

To determine whether the occurrence of CSF-binding sites is restricted to particular cell types, CSF binding by various primary cells and continuous cell lines was studied. In the case of the primary cell populations examined, binding by "resident" peritoneal macrophages was ⅓, and binding by bone marrow cells was 1/500, of the amount bound by peritoneal exudate macrophages (Table I). To determine whether the lower degree of binding exhibited by both resident peritoneal macrophages and bone marrow cells was caused by a decrease in the average amount of ^{125}I-CSF bound per cell or to a lower frequency of binding cells, all primary cell populations examined were subjected to autoradiography. In autoradiographs, >98% of peritoneal exudate macrophages incubated with 15 pM ^{125}I-CSF at 0°C for 15 h had >20 grains associated with them (Fig. 4 *a*) in contrast to cells incubated under the same conditions with ^{125}I-CSF plus a 50-fold excess of unlabeled CSF, 100% of which had less than five grains per cell. Autoradiographs show that the lower binding by resident peritoneal macrophages is caused by a lower average amount of ^{125}I-CSF bound per cell rather than by a lowered frequency of binding cells (data not shown). In contrast, the very low binding of ^{125}I-CSF by bone marrow cells (on an average per cell basis) is explained by the autora-

[4] Even experiments carried out in the presence of 50% (vol/vol) FCS showed little loss in specific ^{125}I-CSF binding (data not shown). Although FCS likely has some bovine CSF activity, the lot used here was selected for the absence of activity in the murine bioassay.

[5] Stahl, P., Washington University School of Medicine, St. Louis. Personal communication.

TABLE I

^{125}I-CSF Binding by Various Rodent Cell Types at 0°C

Cell Type*	cpm Specifically bound/ 10^5 cells‡
Peritoneal exudate macrophages	23,500
Resident peritoneal macrophages	7,900
Nucleated bone marrow cells§	44
P388D1 macrophage§	3,500
J774.2 macrophage	2,900
J774.16 macrophage	2,300
FC-1 macrophage-myeloma hybrid	1,600
WEHI-3 monocytic§	650
PU5-1.8 macrophage§	540
RAW264 macrophage§	0
M1 myeloid leukemic§	40
MOPC-21 myeloma§	10
DS19 erythroleukemic§	0
L_6E_9 myoblast (rat)	42
CHO (hamster)	6
3T3-L1 fibroblast§	0
L 929 fibroblast	0

* Unless otherwise indicated, cells are of murine origin.
‡ The binding experiments were carried out at 15 pM ^{125}I-CSF. Specifically bound ^{125}I-CSF represents the binding that is competed for by 1 nM unlabeled CSF.
§ Incubations as described in text for nonadherent cells.

diographic observation that only 6% of the cell population binds ^{125}I-CSF and that the average amount bound by this binding fraction is much lower (<10%) than the average amount bound by peritoneal exudate macrophages. Greater than 90% of these CSF-binding cells were mononuclear and appeared to be macrophages, monocytes, or undifferentiated cells (monoblasts) (Fig. 4*b*). Fewer than 10% of the CSF-binding cells had the typical ring-shaped nucleus of an immature mouse granulocyte (Fig. 4*b*). However, most cells with ring-shaped nuclei were unlabeled, raising the question of whether the labeled fraction is indeed comprised of granulocyte (rather than macrophage) precursors. In this respect, recent reports of the existence of mononuclear phagocytic cells with ring-shaped nuclei are relevant (3, 16). ^{125}I binding by erythrocytes and recognizable erythroid cells, megakaryocytes, and mature granulocytes could not be detected.

A number of rodent cell lines were tested for ^{125}I-CSF binding at 0°C (Table I). Of the 14 lines examined, six were positive: J774.2 and J774.16, FC-1, P388D1, PU5-1.8, and WEHI-3. All are macrophage or monocytic lines (19). However, the macrophage line RAW264 and the myeloid leukemic line M1 did not bind significant amounts of ^{125}I-CSF.

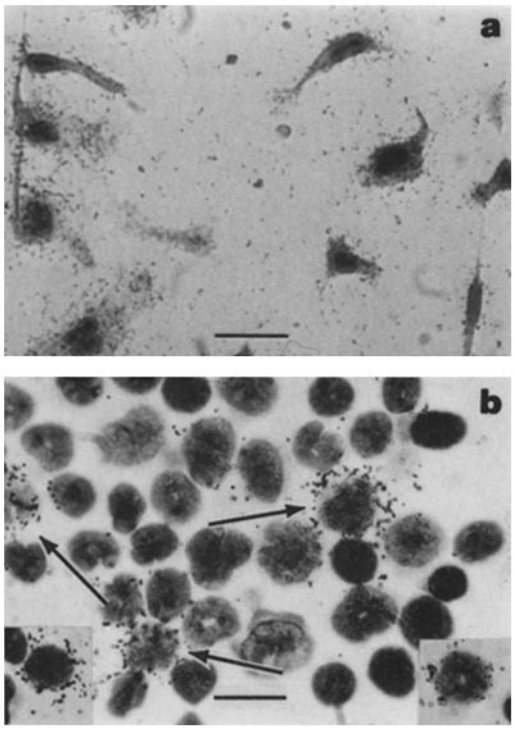

FIGURE 4 Autoradiographs of mouse peritoneal exudate macrophages and bone marrow cells which have been incubated with 15 pM ^{125}I-CSF for 15 h at 0°C. *(a)* Peritoneal exudate macrophages. Exposure time 5 d, stained with Giemsa. Bar, 25 μm. *(b)* Bone marrow cells. Exposure time 22 d, stained with Wright-Giemsa. *Right inset:* Labeled cell with ring-shaped nucleus. *Left inset:* Labeled immature mononuclear cell. Arrows point to labeled mature macrophages. Note unlabeled immature granulocytes and erythroid cells. Bar, 10 μm.

DISCUSSION

The data indicate a highly specific and saturable binding site for ^{125}I-CSF on macrophages. We are currently determining the nature (mononuclear phagocytic or granulocytic) of the ^{125}I-CSF-binding cells with ring-shaped nuclei. However, the possibility that the CSF-binding site exists exclusively on cells of the mononuclear phagocytic system (29) is in accord with the observation that this CSF subclass stimulates the formation of colonies containing only macrophages (28). The presence of CSF-binding sites may well be a specific marker for cells of this system.

Several characteristics of ^{125}I-CSF binding to

target cells suggest that the binding site is the receptor through which the biological effects of this CSF subclass are mediated. *(a)* Among the cells examined, all those known to biologically respond to this CSF possess the binding site. *(b)* All the cells and cell lines which bind this CSF belong to, or are derived from, the mononuclear phagocytic line progression on which it specifically (23) acts. *(c)* Competition for binding is exhibited by this CSF subclass (human or murine), and the degree of competition is directly related to the biological activity of the competing preparation on murine target cells. *(d)* Other CSF subclasses that affect granulocyte production fail to compete for binding.[6] *(e)* The CSF concentration necessary for maximal stimulation of peritoneal exudate macrophage colony formation is 100 pM (24) and the serum CSF concentration in normal C3H/Anf mice is 50 pM[7] (see footnote 4). Both these concentrations fall on the ascending portion of the 0°C binding curve (Fig. 2).

It is apparent that a variety of functionally distinct cell types of mononuclear phagocytic origin are capable of specifically binding CSF. These range from immature to mature mononuclear phagocytes and include continuous cell lines. They exhibit a spectrum of responses to CSF: some (immature bone marrow cells and peritoneal exudate cells) have an absolute requirement for CSF in order to proliferate (24); others (resident peritoneal macrophages) fail to proliferate in its presence (24) and the continuous cell lines proliferate in the absence of added CSF. Because of the variety of CSF binding cell types available, the CSF-target cell system is an attractive model for studying the effects of a growth factor on proliferation and differentiation.

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[6] Das, S. K., E. R. Stanley, L. J. Guilbert, and L. W. Forman. Discrimination of colony stimulating factor subclass by a specific receptor on a macrophage cell line. Manuscript submitted for publication.

[7] Zak, D., and E. R. Stanley. Unpublished observations.

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