Distribution of Cells Bearing Receptors for a Colony-stimulating Factor (CSF-1) in Murine Tissues

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ABSTRACT CSF-1 is a subclass of the colony-stimulating factors that specifically stimulates the growth of mononuclear phagocytes. We used the binding of 125I-CSF-1 at 0°C by single cell suspensions from various murine tissues, in conjunction with radioautography, to determine the frequency of binding cells, their identity, and the number of binding sites per binding cell. For all tissues examined, saturation of binding sites was achieved within 2 h at 2–3 × 10^-10 M 125I-CSF-1. The binding was irreversible and almost completely blocked by a 2 h preincubation with 5 × 10^-10 M CSF-1. 125I-CSF-1 binding was exhibited by 4.3% of bone marrow cells, 7.5% of blood mononuclear cells, 2.4% of spleen cells, 20.5% of peritoneal cells, 11.8% of pulmonary alveolar cells and 0.4% of lymph node cells. Four morphologically distinguishable cell types bound 125I-CSF-1: blast cells; mononuclear cells with a ratio of nuclear to cytoplasmic area (N/C) >1; cells with indented nuclei; and mononuclear cells with N/C ≤ 1. No CSF-1 binding cells were detected among blood granulocytes or thymus cells. Bone marrow promyelocytes, myelocytes, neutrophilic granulocytes, eosinophilic granulocytes, nucleated erythroid cells, enucleated erythroid cells, and megakaryocytes also failed to bind. The frequency distribution of grain counts per cell for blood mononuclear cells was homogenous. In contrast, those for bone marrow, spleen, alveolar, and peritoneal cells were heterogeneous. The monocytes in blood or bone marrow (small cells, with either indented nuclei or with N/C >1) were relatively uniformly labeled, possessing ~3,000 binding sites per cell. Larger binding cells (e.g., alveolar cells) may possess higher numbers of receptors. It is concluded that CSF-1 binding is restricted to mononuclear phagocytic cells and their precursors and that it can be used to identify both mature and immature cells of this series.

Colony-stimulating factors (CSFs) are growth factors which stimulate the formation of colonies of granulocytes and/or macrophages (3, 14) by precursor cells in hemopoietic tissues (reviewed in reference 13). Four subclasses may be discerned by their preferential effects on neutrophil, eosinophil, neutrophil-macrophage or macrophage colony formation (reviewed in reference 17). CSF-1 is a subclass discriminated from the other subclasses by its detection in subclass-specific radioimmuno- and radioreceptor assays (5, 16, 17). L-cell CSF-1 is identical to L-cell macrophage growth factor (19) and specifically stimulates the growth of mononuclear phagocytes and their precursors (17, 18, 20). It is a heavily glycosylated, sialic acid-containing glycoprotein (molecular weight 40,000–86,000) composed of two disulfide-bonded subunits (21, 22). The specific binding of radiolabeled CSF-1 to mononuclear phagocytes and to cell lines derived from mononuclear phagocytic cells has recently been demonstrated (8). Several independent findings indicate that the high affinity CSF-1 binding site described in these studies is the receptor through which the biological effects of CSF-1 are mediated (8, 21).

In this study we examine the distribution, frequency, and morphology of CSF-1 receptor-bearing cells in adult mouse tissues. Binding conditions were chosen to ensure saturation of the cell-surface receptors by 125I-CSF-1 and radioautography was used to identify the binding cells and to determine the number of CSF-1 receptors per cell. We report the existence of a CSF-1 receptor, with common properties, on cells in a variety of tissues and indicate its usefulness as a marker of both mature and immature cells of the mononuclear phagocytic series.

MATERIALS AND METHODS

Preparation of 125I-CSF-1

L-cell CSF-1 was purified as previously described (21, 22). The purity was checked by PAGE, with and without SDS under reducing and nonreducing
conditions and by complexing with rabbit anti-CSF-1 antibody (21, 22). The purified CSF-1 was iodinated with carrier-free 125I (Amersham Corp., Arlington Heights, Ill.) with full retention of biological activity (16, 21). The biological activity of the 125I-CSF-1 preparation was assessed either by radioimmunoassay with 125I-CSF-1 and double label counting (16, 21) or by immunoprecipitation with excess rabbit anti-CSF-1 antiserum (21) on the basis of the close correlation between bioactivities and antibody-binding activities (16, 21). Three different preparations of 125I-CSF-1 were used in the experiments described here. 125I-CSF-1 preparations were stable when stored in HS-a-HEPES, alpha medium (KC Biological Inc., Kansas City, Mo.) containing 25 mM HEPES (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) in lieu of bicarbonate, pH 7.3, with 10% (vol/vol) horse serum (Flow Laboratories, Inc., Rockville, Md.), at -20°C (half-life of biologically active CSF-1 = 50 d). They were used no longer than 3 wk postiodination. Determinations of the specific radioactivities, as described elsewhere (21), were made at the time of each experiment. The mean value was ~106,000 cpm/ng of biologically active CSF-1 protein or 2,900 cpm/nmol, corresponding to an average of 1 atom of 125I per biologically active CSF molecule. Molarity conversions are based on an M, for the polypeptide moiety of CSF-1 of ~28,000 (S. K. Das and R. E. Stanley, manuscript in preparation) (21) and the specific activity of purified CSF-1 (~1.6 × 10^6 U/mg protein) (22).

**Preparation of Cells**

8- to 12-wk-old female C3H/An mice (Cumberland Farms, Nashville, Tenn.) were used exclusively. Unless otherwise stated, the animals were killed by cervical dislocation and the cells suspended in HS-a-HEPES containing 5 U/ml Heparin (Upjohn Co., Agricultural Prods. MKT, Kalamazoo, Mich.). When necessary, cell suspensions were cleared of large and small cellular debris as described elsewhere (15). Viable cell counts were determined by Trypan blue exclusion and cell suspensions with viabilities of >95% were used in binding experiments. Unless otherwise stated, all operations were carried out at 4°C.

Bone marrow cells were obtained from femora, cleared of surrounding tissue and opened by scalpel at both ends. The narrow plug was ejected into medium by means of a syringe connected to the femur via a piece of silicon tubing. The cells were aspirated several times through the femur to ensure a single-cell suspension. The cell yield was ~7 × 10^6 nucleated cells/femur.

Spleen cells were obtained by forcing the tissue through a metal screen into the collecting medium. The suspension was aspirated several times with a Pasteur pipette to obtain a single-cell suspension. Approximately 10^7 cells were obtained from each spleen.

Blood mononuclear cells and granulocytes were obtained by the method of Boyum (2). Mice were anesthetized with ether and blood collected from the axillary artery and mixed with 0.3 M EDTA (50:1) to prevent coagulation. It was diluted (1:4) with 0.15 M NaCl and layered over a mixture of 6.3% Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and 10% Hypaque (Winthrop Laboratories, New York, N. Y.). The tube was centrifuged at 400 g for 30 min at 22°C. The interface layer of mononuclear cells was collected and washed three times with phosphate buffered saline (PBS) and resuspended in medium. The cell yield was 2–3 × 10^7 cells per mouse. The pellet of erythrocytes and granulocytes was resuspended in mouse serum containing 40 μM Dextran T500 (Pharmacia Fine Chemicals). The cells were allowed to sediment (1 g, 4°C, 1 h) and the supernatant granulocytes were removed, washed twice with PBS (400 g, 4°C, 10 min), and resuspended in medium.

A single-cell suspension of thymus cells was obtained by teasing apart thymus tissue with a pair of #26 needles. The cells were washed twice with medium. Thymus gland yielded 5–8 × 10^7 cells.

Lymph node cells were collected from the inguinal, brachial, and mesenteric lymph nodes by gently teasing them apart with #26 needles until a single-cell suspension was obtained. The cells were washed twice; 2–4 × 10^7 cells were obtained per mouse.

Resident peritoneal cells were obtained by flushing the peritoneal cavity with 3 ml of medium in the range 1 × 10^6 to 1 × 10^7/ml (data not shown).

**Radioautographs**

Kodak NTB2 (Eastman Kodak Co., Rochester, N. Y.) was heated to 42°C and a thin layer was applied to each slide by dipping. They were enclosed in light-tight slide boxes, containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) as a desiccant, and exposed to 4°C for 4, 7, or 14 d. Slides were developed at 22°C for 2 min in Kodak D-19 developer, dipped in 0.2 M acetic acid for 30 s and fixed for 3 min in Kodak Rapid Fixer. They were washed in running tap water for 15 min and rinsed in distilled water. The cells were stained in 0.3% (wt/vol) May-Grünwald in methanol for 15 min followed by 0.02% (wt/vol) Giemsa in 3 mM sodium phosphate buffer pH 6.8 for 30 min. The emulsion was destained in 20 mM acetic acid in ethanol, rinsed in buffer, and air-dried. Cells were examined with a Zeiss Standard WL Microscope (Carl Zeiss, Inc., N. Y.). The grain threshold was determined with the control cells that had been preincubated with unlabelled CSF-1. At least 1,000 sequential cells were examined in each preparation.

**RESULTS**

The binding conditions were determined using the macrophage cell line J-774.2 as the target cell population. Their ability to bind 125I-CSF-1 at 0°C has been previously demonstrated (8). Saturation of cellular binding was achieved, at concentrations of 125I-CSF-1 >2 × 10^-10 M, within 2 h of incubation at 0°C (Fig. 1a). There was a linear relationship between cpm bound and cell concentration in the range 1 × 10^6 to 1 × 10^7 cells/ml (Fig. 1b). Binding was essentially irreversible as 125I counts remained cell bound when loaded cells were resuspended in CSF-1-free medium for 24 h at 0°C (data not shown). Binding could be inhibited either by incubation in the presence of a 50-fold excess of "cold" unlabelled CSF-1 or by a 2 h preincubation with a saturating concentration of unlabelled CSF-1 (5 × 10^-10 M). This latter procedure was routinely adopted to minimize the use of unlabelled CSF-1. Complete inhibition of 125I binding could not be achieved and the "net binding" refers to that which could be inhibited. Net binding is the difference between the two lines of Fig. 1b.

Tissue cells were examined to see if the same binding conditions applied. Bone marrow and spleen cells were incubated with different concentrations of 125I-CSF-1. Because of the lower incidence of binding cells in these suspensions, a higher cell concentration of 5 × 10^6 cells/ml was necessary. A linear relationship was found between cpm bound and cell concentration in the range 1 × 10^6 to 1 × 10^7/ml (data not shown).
Fig. 2 shows saturation binding curves for bone marrow and spleen cells. Binding sites were saturated at concentrations between 2 and $3 \times 10^{-10}$ M. Saturation was achieved within 2 h at 0°C. The binding was essentially irreversible for all the tissues studied as further incubation of the loaded cells for 24 h at 0°C in CSF-1-free medium resulted in no loss of $^{125}$I.

Table I presents data on net binding for each of the tissues studied. The percentage of labeled cells in each tissue was determined from radioautographs which had been exposed for 4, 7, or 14 d. This percentage did not vary with exposure time, although the number of grains per cell increased. Two cell populations, thymic cells and blood granulocytes, showed no CSF-1 binding. CSF-1 binding cells constituted a minor fraction of the cells in the remaining tissues. The average number of $^{125}$I-CSF-1 molecules bound per binding cell in these tissues provides an estimate of the average number of receptors per cell assuming that a receptor binds only one molecule of CSF-1. Values ranged from ~3,000 for blood mononuclear cells to ~16,000 for pulmonary alveolar cells. A higher value of ~19,000 was found with the macrophage cell line J-774.2.

The labeled cells were morphologically classified into four types: blast cells (Fig. 3a); mononuclear cells with N/C >1 (Fig. 3b); cells with indented nuclei (including large and small indentations) (Fig. 3c); and mononuclear cells with N/C ≤1 (Fig. 3d). Blast cells were distinguished by finely divided chromatin and sparse, often basophilic, cytoplasm. In the case of the peritoneal and pulmonary alveolar cells, a large proportion of the labeled cells that were classified as blast cells on the basis of their sparse peripheral cytoplasm, could have been multinucleated giant cells (macrophages), as their nuclear morphology was obscured by the large number of overlying grains (Fig. 3e). The greatest proportion of labeled cells in each tissue consisted of mononuclear cells with N/C >1. Labeled mononuclear cells with N/C ≤1 were found among lymph node, peritoneal and alveolar cells. Labeled cells with indented nuclei were found in all tissues except among alveolar cells.

Table II illustrates the extent to which a cell of a given morphology possesses receptors for CSF-1. In most cases both labeled and unlabeled cells of a particular morphology were found. However, in the case of blood mononuclear cells, all monocytes with the distinctive indented or horseshoe-shaped nuclei were found to be labeled. Granulocytic cells, nucleated erythroid cells, and mature erythrocytes were always found to be unlabeled. The number of grains per cell is a function of the number of $^{125}$I-CSF-1 molecules bound. The distributions of grains per cell among binding cells were heterogeneous in most populations examined, including bone marrow (Fig. 4a), alveolar (Fig. 4c) and spleen and peritoneal cells (not shown). However, as seen in Fig. 4b, blood mononuclear cells were relatively homogeneous in terms of the number of grains per cell (10-45 grains/cell, ~3,000 binding sites/cell). They consisted of mononuclear cells with N/C >1 (68%) and cells with accentuated nuclear indentations (32%) (Table II), both of which were characterized as monocytes (see Discussion). Analysis of all three tissues represented in Fig. 4 failed to reveal any other striking correlation between cellular morphology and grain counts per cell. However, the binding cells in the pulmonary alveolar
preparation were larger and bound more $^{125}$I-CSF-1 per cell than the blood mononuclear binding cells.

DISCUSSION

A prior report demonstrated stable binding of $^{125}$I-CSF-1 to mouse peritoneal exudate cells at $0^\circ$C (8). At $37^\circ$C, $^{125}$I-CSF-1 is internalized and destroyed (21). Binding at $0^\circ$C was therefore used to determine the frequency of binding cells in various tissues and to quantitate the number of unoccupied cell surface CSF-1 binding sites per cell in these tissues. Despite variations in the number of sites per cell between and often within the tissue examined, the CSF-1 receptor exhibited similar characteristics in all cell populations; the saturation binding conditions were found to be similar and binding was essentially irreversible.

In all tissues examined, CSF-1 binding was restricted to four cell types: blast cells; mononuclear cells with N/C >1; cells with indented nuclei; and mononuclear cells with N/C $\leq 1$. CSF-1 binding blast cells were found in bone marrow, and in a higher proportion among peritoneal and alveolar cells (in the latter populations many "blast" cells were possibly multinucleated giant cells). Mononuclear cells (N/C $>1$) binding CSF-1 (cells with lymphocytelike morphology) were found in all tissues. Binding cells with indented nuclei included promonocytes (7) and monocytes. Mononuclear cells (N/C $\leq 1$) (cells with a macrophagelike morphology) that bound CSF-1 were found among peritoneal, alveolar, and lymph node cells. In all tissues studied, granulocytes (promyelocytes to mature neutrophils and eosinophils), nucleated and enucleated erythroid cells, and megakaryocytes failed to bind CSF-1. Furthermore, the low frequency of binding cells among lymphocytelike cells (N/C $>1$) in the blood mononuclear, thymic, and lymph node cell populations makes it very unlikely that B or T lymphocytes possess CSF-1 receptors. Thus the morphology of the binding cells is consistent with the conclusion that they are either mononuclear phagocytes or undifferentiated mononuclear phagocytic precursor cells. Other kinds of evidence support this conclusion. (a) Although many of the binding cells cannot be identified (especially the blasts and mononuclear cells with N/C $>1$), all of the identifiable mononuclear phagocytic cells in most populations bind $^{125}$I-CSF-1. All blood mononuclear cells with an accentuated nuclear indentation (classical blood monocytes) bound $^{125}$I-CSF-1. It was reported (8) that $>95\%$ of adherent peritoneal cells (peritoneal macrophages) bound $^{125}$I-CSF-1 and that $>98\%$ of a population of adherent peritoneal exudate cells ($>99\%$ of which displayed Fc-mediated sheep erythrocyte phagocytosis) bound $^{125}$I-CSF-1. (b) In this study, CSF-1 binding cells were preferentially distributed among tissues known to contain mononuclear phagocytic cells (25). (c) The frequencies of binding cells (Table I) in the tissues examined are in general agreement with the previously reported frequencies of mononuclear phagocytic cells in these tissues (bone marrow [27], blood monocytes from Ficoll-Hypaque sedimentation [4, 10], splenic monocytes or macrophages [12], and peritoneal macrophages [23, 26]). An exception to this

**FIGURE 3** Radioautographs of $^{125}$I-CSF-1 labeled cells at 4 d of exposure. Bar, 20 pm. (a) Labeled blast cell (bone marrow). (b) Labeled mononuclear cell (N/C $>1$) (bone marrow). (c) Labeled cell with indented nucleus (blood mononuclear cell). (d) Labeled mononuclear cells (N/C $\leq 1$) (pulmonary alveolar cells). (e) Large labeled "blast" cell (pulmonary alveolar cells).
observation was that the frequency of alveolar cells binding CSF-1 was much lower than the reported frequencies of alveolar macrophages in such preparations (1, 10). Indeed, many alveolar cells, which by classical morphological criteria are macrophages (N/C \(_{<1}\), Fig. 3d), did not bind CSF-1. (d) In all tissues examined the frequency of cells which respond to CSF-1 by forming colonies of macrophages (18) was equal to or less than the frequency of binding cells. Taken together, the above evidence strongly suggests that the CSF-1 receptor is restricted to undifferentiated and differentiated members of the mononuclear phagocytic cell lineage. The reason why some macrophages, i.e., the alveolar subset mentioned above, do not bind CSF-1 is unclear.

Grain counts in radioautographs indicated that the number of CSF-1 binding sites per cell varied among binding cells (Fig. 4). With the exception of the relatively homogeneous distribution for blood mononuclear cells, all tissues examined exhibited heterogeneous distributions of grain counts per cell. CSF-1 binding alveolar cells, of larger average size than binding cells from other tissues, also possessed a higher average number of receptors than cells from other tissues (Table I). In agreement with this, the frequency distribution of grain counts associated with these cells was shifted towards higher grain counts per cell compared with the distributions of CSF-1 binding cells in bone marrow and blood (Fig. 4). The observed heterogeneity among CSF-1 binding cells in tissues other than blood could be explained in a variety of ways, e.g., microenvironmnetal variations in CSF-1 concentration, cell cycle state, or state of differentiation.

An excellent correlation exists between the reported tissue distribution of monocytes and the tissue distribution of cells with grain counts of between 10 and 45 grains. Virtually all of the mononuclear phagocytic cells in the peripheral blood

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Table II

| Cell population          | Blast\(^*\) | Mononuclear cells (N/C >1)\(\ddagger\) | Cells with indented nuclei | Mononuclear cells (N/C \(\leq 1\)) | Granulocytic cells\(\S\) | Nucleated erythroid cells | Others\(\|$ |
|-------------------------|------------|-----------------------------------|----------------------------|-----------------------------------|--------------------------|---------------------------|-------------------------|
| % Total cells           |            |                                   |                            |                                   |                          |                           |                         |
| Bone marrow             | 3.9        | 24.9                              | 1.3                        | 0                                 | 50.6                     | 17.4                      | 1.9                     |
| Spleen                  | 0.7        | 88.2                              | 4.7                        | 0                                 | 4.4                      | 1.6                       | 0.4                     |
| Blood mononuclear       | 0          | 95.1                              | 4.2\(\ddagger\)           | 0                                 | 0.7                      | 0                         | 0                       |
| Pulmonary alveolar      | 1.4        | 33.5                              | 1.0                        | 63.7                              | 0.3                      | 0                         | 0                       |
| % Total cells labeled   |            |                                   |                            |                                   |                          |                           |                         |
| Bone marrow             | 0.1        | 3.1                               | 1.1                        | 0                                 | 0                        | 0                         | 0                       |
| Spleen                  | 0          | 1.6                               | 0.8                        | 0                                 | 0                        | 0                         | 0                       |
| Blood mononuclear       | 0          | 5.1                               | 2.4\(\ddagger\)           | 0                                 | 0                        | 0                         | 0                       |
| Pulmonary alveolar      | 1.3        | 8.1                               | 0                          | 2.4                               | 0                        | 0                         | 0                       |
| Peritoneal              | 0.4        | 17.0                              | 2.7                        | 0.4                               | 0                        | 0                         | 0                       |
| Lymph node              | 0          | 0.2                               | 0.1                        | 0.1                               | 0                        | 0                         | 0                       |

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\(^*\) Includes pulmonary alveolar and peritoneal cells which might be characterized as multinucleated giant cells.

\(\ddagger\) Includes small, medium, and large lymphocytes and lymphocytelike cells.

\(\S\) Includes the series from promyelocyte to neutrophil and eosinophil.

\(\|$\) Includes megakaryocytes, reticulum cells, and unidentified cells.

\(\ddagger\) 2.4% of the blood mononuclear cells possessed the accentuated nuclear indentation of typical monocytes and all of these cells were labeled.

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Figure 4: Distribution of grains per cell in 4-d radioautographs of (a) bone marrow, (b) blood mononuclear, and (c) pulmonary alveolar cells. 200 labeled cells were examined in each case. Since the alveolar cells were labeled with a \(^{125}\)I-CSF-1 preparation of lower specific radioactivity (2,400 cpm/fmol) than the preparation used for the bone marrow and blood mononuclear cells (3,700 cpm/fmol), the data in histogram c were normalized. The average grain count per labeled bone marrow cell was divided by the average number of receptors per labeled bone marrow cell (Table I) and the resulting ratio multiplied by the average number of receptors per labeled alveolar cell (Table I) to obtain a normalized average grain count per labeled alveolar cell (~1.3 times the actual average grain count). The distribution of grain counts per labeled alveolar cell was then moved to the right by an amount equal to the difference between the normalized and actual average grain count per labeled alveolar cell.

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1 In this regard, it would appear that the presence of the receptor is necessary but is not sufficient for the CSF-1 proliferative response. However, it has recently become clear that although not all CSF-1 receptor bearing cells have the capacity to proliferate, those that do not may respond to CSF-1 in other ways (9, 10).
mononuclear cell population are monocytes and 70–80% of those in bone marrow are monocytes (7, 24). CSF-1 binding blood mononuclear cells are tightly distributed (98% of binding cells have between 10 and 45 grains per cell). The major peak (71%) of the more heterogeneously distributed binding cells in bone marrow fall within this “monocyte” range. Thus it appears that the major binding cell class in bone and bone marrow is the monocyte, of which approximately two-thirds are mononuclear cells with N/C >1 and one-third cells with deeply indented nuclei (Table II) and which are relatively homogeneous in their expression of the CSF-1 receptor (~3,000 receptors per cell).

By contrast, there are very few CSF-1 binding pulmonary alveolar cells with the morphology of the CSF-1 binding blood mononuclear cells (cells with indented nuclei or small (5–10 μM) cells with N/C >1, Table II). It is significant that <5% of the CSF-1 binding cells in this population have <45 grains per cell; i.e., the alveolar cell distribution (Fig. 4c) lacks the “monocyte” peak.

The distribution, frequencies and morphologies of CSF-1 binding cells in murine tissues suggest that the CSF-1 receptor is a specific marker of mononuclear phagocytic cells and their precursors. The results demonstrate that binding by specific growth factors may be used as a means of identifying both mature and immature cells of a differentiating cell lineage.

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