Macrophage Production during Murine Listeriosis: Colony-Stimulating Factor 1 (CSF-1) and CSF-1-Binding Cells in Genetically Resistant and Susceptible Mice

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The concentration of the macrophage-specific colony-stimulating factor (CSF-1) and the numbers of bone marrow and spleen cells with specific receptors for that factor have been investigated in a number of mouse strains under normal conditions and after infection with the facultative intracellular bacterium Listeria monocytogenes. The CSF-1 concentration in serum and tissue was markedly elevated in infected mice, the degree of stimulation reflecting the dose of L. monocytogenes. The CSF-1 titer did not correlate with genetic resistance or susceptibility of the mice to L. monocytogenes. In contrast to the effect of lipopolysaccharide, Listeria infection was able to increase the level of CSF-1 in the lipopolysaccharide nonresponder strain C3H/HeJ. In line with earlier findings on colony-forming cells, cells bearing receptors for CSF-1 in uninfected susceptible BALB/cJ mice were only half those in resistant C57BL/6J mice. After infection the majority of these cells disappeared from the bone marrow and spleen cells of both resistant and susceptible mice. The number of CSF-1 receptor-bearing cells in the normal bone marrow may determine the degree of resistance to L. monocytogenes.

Listeria monocytogenes is a facultative intracellular bacterium, pathogenic for humans, and widely studied as a model of cell-mediated immunity in mice. When infected into mice, it is rapidly engulfed by resident macrophages and can survive and multiply within those cells (11). Mouse strains differ in their innate resistance to L. monocytogenes, showing 100-fold differences in their 50% lethal dose controlled by a single major gene, Lsr (7, 18).

An early event in natural resistance, and a major determinant of genetic resistance or susceptibility, is the inflammatory response at the site of infection (12, 16). The response in both resistant (Lsr') and susceptible (Lsr) strains is an early influx of polymorphonuclear granulocytes to the site of infection (12), but resistant mice (C57BL and related strains) show an earlier monocyte response in the bloodstream (12, 16) and possibly also at the site of infection (23). These newly formed monocytes are highly bactericidal (23). Their earlier appearance is probably a consequence of higher numbers of hemopoietic precursor cells (colony-forming cells [CFCs]) in the bone marrow and spleen of the resistant mice (25).

It was therefore of interest to study during L. monocytogenes infection of mice the growth factor governing the development of macrophages, namely, colony-stimulating factor 1 (CSF-1), and the cells of the macrophage lineage which bear receptors for CSF-1. Because CSF-1 can be measured by radioimmunoassay (19, 20), it can be detected in tissue homogenates which might well be toxic in biological assays involving cell proliferation in vitro (13). Furthermore, the binding of 125I-CSF-1 to the cell, combined with autoradiography, provides a measure of the number of cells in the mononuclear phagocyte lineage (5, 20).

MATERIALS AND METHODS

Mice. C57BL/6J, BALB/cJ, CBA/J, C3H/HeJ, and C3HeB/FeJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. C3H/HeJ mice used in CSF-1-binding studies were the kind gift of R. Bradley, Cancer Research Institute, Melbourne, Australia. Sex-matched mice were infected intravenously at 6 to 8 weeks of age with L. monocytogenes as previously described (25). The 50% lethal dose of L. monocytogenes in the C57BL/6J mice was found to be \( 3 \times 10^7 \) organisms, while that for the other strains varied between \( 2 \times 10^3 \) and \( 5 \times 10^3 \) organisms.

Preparation and iodination of CSF-1. Two preparations of CSF-1 of different degrees of purity were made from mouse L-cell conditioned medium and used for different purposes. Their preparation is described in detail by Stanley (20). Stage 1 L-cell CSF-1 was made from L-cell conditioned medium by batch calcium phosphate gel chromatography, while highly purified stage 4 L-cell CSF-1 was prepared by subjecting stage 1 material to DEAE-cellulose chromatography, followed by gel filtration and affinity chromatography on concanavalin A-Sepharose. The purity of the stage 4 CSF-1 preparation was checked by polyacrylamide gel electrophoresis, and it was radioiodinated (300,000 cpm/ng of CSF-1 protein) as described previously (20) for use in the radioimmunoassay and cell-binding studies.

Tissue preparation for CSF-1 assay. Mice were ether anesthetized and bled from the heart to collect the maximum amount of blood. The blood was allowed to clot for 1 h at room temperature, and the clots were retracted overnight at 4°C to collect the serum. The following organs were removed for assay: spleen and liver (the major sites of L. monocytogenes localization) (8) and lung and submaxillary salivary glands (tissues containing a high concentration of CSF-1) (2). The tissues were weighed and suspended in 20 times their weight of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered α medium (KC Biologicals, Le-
nexa, Kans.) with 0.2% bovine serum albumin and 0.02% sodium azide. They were homogenized on ice with an Eberbach homogenizer (7265; Fisher Scientific Co., Pittsburgh, Pa.) and centrifuged at 800 × g for 10 min at 4°C to remove debris. The supernatants were heated to 56°C for 30 min, and the preparations were centrifuged again to remove the precipitated proteins. The concentration of CSF-1, which is quantitatively recovered in the supernatant, was assayed by a specific radioimmunoassay, as described in detail elsewhere (19, 20). One unit of CSF-1 represents approximately 0.44 fmol, or 12 pg, of CSF-1 protein.

**Cell binding of 125I-CSF-1.** Infected or uninfected mice were killed by cervical dislocation, and femurs, tibias, and/or the spleen were removed. Bone marrow cells were prepared by cutting off the epiphyses and washing out the plug of marrow with a 1-ml tuberculin syringe with a 26-gauge needle. The cells were aspirated several times through the 26-gauge needle to ensure a single-cell suspension. Spleen cells were prepared by gently teasing the spleens with a syringe and needle and passed through an 80-gauge, 80-μm stainless steel sieve. The resulting cell suspension was layered was HEPES-buffered α medium with 0.2% bovine serum albumin and 0.02% NaN₃, and the cells were maintained at all times at 0 to 4°C, unless noted otherwise. Debris was removed from the cell suspension by settling on a fetal calf serum cushion, and when the removal of erythrocytes was necessary, they were lysed in Tris-buffered 0.83% ammonium chloride at 37°C for 5 min (3).

125I-CSF-1 binding was carried out in 400-μl microtiter plates (nos. 72700, Sansted Inc.) containing 50 μl of a suspension of 10⁶ nucleated cells per ml. Five microliters of stage 1 CSF-1 (30,000 U/ml) was added to control tubes, which were incubated for 1 h at 4°C to saturate all CSF-1-binding sites before the addition of 125I-CSF-1. Five microliters of medium of each was added to test groups, which were similarly incubated. 125I-CSF-1 was added to each tube (5 μl, approximately 10⁶ cpm), and the tubes were incubated at 4°C for at least 2 h. Binding of CSF-1 to its receptor on cells at 4°C is virtually irreversible, and maximum binding is observed within 0.5 to 1 h under the conditions used (5, 10). A 50-μl sample of the incubation mixture was layered onto 3 ml of filtered serum in a 4-ml tube (BSS8010; Bunzl, Hendon, South Australia), and the cells were centrifuged at 400 × g for 15 min at 4°C. The supernatant was removed, and the cells were transferred to another tube with 4 ml of phosphate-buffered saline and centrifuged. After the cells were suspended in 4 ml of phosphate-buffered saline, 40 μl was added to 200 μl of fetal calf serum in a cytocentrifuge and spun at 100 × g for 5 min. The slides were air dried and fixed in methanol for autoradiography. Remaining cells were centrifuged and the cell pellet was counted in a Packard Auto-gamma Scintillation Spectrometer.

**Autoradiography.** Slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) that had been warmed to 42°C. The slides were air dried and enclosed in lighttight boxes containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) as the desiccant. They were exposed at 4°C for 4, 7, or 14 days. Slides were developed at 22°C for 2 min in Kodak K-19 developer, dipped in 0.2 M acetic acid for 30 s, fixed for 3 min in Kodak Rapid Fixer, washed in running tap water for 15 min, and rinsed in distilled water. They were stained for 10 min with 10% Giemsa in 3 ml sodium phosphate buffer (pH 6.8) and mounted using DePeX (Gurr, BDH, Poole, England). The grain threshold was determined by examining the control cells which had been preincubated with unlabeled CSF-1. At least 500 sequential cells were examined on each slide, with triplicate slides for each experimental group.

**In vitro up regulation of CSF-1 receptors.** Cells were prepared initially as above, but before exposure to CSF-1, they were incubated (2 × 10⁶ cells per ml; 3 ml) in HEPES-buffered α medium with 10% fetal calf serum in 5-ml polypropylene tubes at 37°C for 16 h (1). The cells were then cooled to 4°C, centrifuged at 400 × g for 10 min, suspended to a concentration of 10⁶/ml, and kept at 4°C before 125I-CSF-1 binding as described above.

**RESULTS**

**CSF-1 in the serum of L. monocytogenes-infected mice.** C57BL/6J and BALB/cJ mice were infected intravenously with various doses of L. monocytogenes, namely, 1 × 10⁶, 1 × 10⁷, and 5 × 10⁸ viable units, and the CSF-1 in their serum was assayed by radioimmunoassay 1, 2, and 3 days later. Sera from uninfected mice were assayed at the same time. Figure 1 shows that the response was extremely dose dependent and peaked at 2 to 3 days postinfection. The BALB/cJ mice, which rapidly develop higher numbers of L. monocytogenes than do the resistant C57BL/6J mice given the same dose (Fig. 2), showed higher concentrations of CSF-1 in the serum. At the highest dose, BALB/cJ mice were dead by day 3.

**CSF-1 in the tissue of L. monocytogenes-infected mice.** C57BL/6J and BALB/cJ mice were infected intravenously with 10⁶ L. monocytogenes organisms, a dose judged to be high enough to produce a response but low enough to allow the survival of BALB/cJ mice over 3 days. At daily intervals the mice were kill-bled and the tissues were removed for extraction of CSF-1 as described in Materials and Methods. The results are shown in Fig. 3. A pronounced (10- to 20-fold) increase in CSF-1 concentration in the spleen may be seen. Other organs also showed an increase: liver, 3-fold from a very low base level; lung and salivary gland, 3-fold. The concentration in the spleen was far in excess of the concentration in the serum. The lung and salivary gland also contained higher concentrations than did serum. It should be noted that the liver, because of its larger weight, contained by far the greatest total amount of CSF-1 after infection. The concentration, even in the liver, was higher than could be accounted for by residual serum. There was no consistent difference between the two strains in the various tissues.

**CSF-1 response in genetically resistant and susceptible mice.** Mice of the resistant C57BL/6J strain or of the susceptible BALB/cJ or CBA/J strain were infected with 5 × 10⁶ L. monocytogenes organisms, and 2 days later the concentrations of CSF-1 in their serum, spleen, liver, lung, and submaxillary salivary glands were assayed. Assays of serum and tissue extracts of normal mice were included for comparison (Fig. 4). The response in serum to this relatively low dose was slight. However, CSF-1 concentrations were elevated in all organs in both resistant and susceptible strains, and no correlation was observed between genetic resistance to L. monocytogenes and the CSF-1 concentration in an organ.

**CSF-1 response to L. monocytogenes or LPS in LPS responder and nonresponder strains of mice.** A comparison was made between C3HeB/FeJ mice, which are capable of responding to Escherichia coli lipopolysaccharide (LPS) and CSF-1, and C3H/HeJ mice, which are nonresponders. Mice were infected intravenously with 5 × 10⁶ L. monocytogenes organisms and their tissues were sampled 2 days later, or they were injected intraperitoneally with 5 μg of E. coli LPS
(lipopolysaccharide W E. coli O111-84; Difco Laboratories, Detroit, Mich.) and their tissues were sampled at the peak response of 7 h. Normal mice were also assayed. Although the distinction between C3H/HeJ and C3HeB/FeJ mice was clear in their response to E. coli LPS, both C3H/HeJ and C3HeB/FeJ mice were able to respond to L. monocytogenes infection (Fig. 5).

CSF-1-binding cells in the bone marrow and spleen of infected mice. The binding of 125I-CSF-1 allows the identification of cells of the macrophage lineage, ranging from precursor macrophage-CFCs to mature macrophages. Therefore, cells from normal mice and from mice at the peak of their CSF-1 response were tested for their ability to bind CSF-1.

C57BL/6J and BALB/cJ mice were infected with 6 x 10^4 Listeria organisms. Two days later their bone marrow was removed and the cells were exposed to 125I-CSF-1 as described in Materials and Methods (Table 1). Cells from uninfected C57BL/6J mice bound twice as much 125I-CSF-1 as did cells from BALB/cJ mice (P < 0.001). This was due to a significantly higher proportion (1.4-fold, P < 0.01) of CSF-1-binding cells in the resistant C57BL/6J mice, combined with a slightly higher density of CSF-1 receptor sites on their CSF-1-binding cells. Of additional interest was the higher total number of cells in the bone marrow from C57BL/6J mice compared with susceptible BALB/cJ mice, leading to a greater-than-twofold difference in the total number of binding cells. Infection with L. monocytogenes resulted in dramatically decreased binding and an apparent decrease in the percentage of CSF-1-binding cells in the bone marrow of both strains, coupled with a marked loss of total bone marrow cells. The binding data for splenic cells from these

FIG. 1. CSF-1 concentrations in the serum of C57BL/6J (●) and BALB/cJ (○) mice infected with 1 x 10^6 (A), 1 x 10^4 (B), or 5 x 10^2 (C) Listeria organisms. Each point represents the mean and standard deviation for five mice kill-bled at different times after infection.

FIG. 2. Total numbers of Listeria organisms in the spleen and liver of C57BL/6J (●) or BALB/c (○) mice given 1 x 10^6 (A) 1 x 10^4 (B), 1 x 10^6 (C), or 5 x 10^2 (D) Listeria organisms intravenously. Each point represents the mean and standard deviation for a group of five mice.
two strains also showed that there was a twofold-higher binding to resistant C57BL/6J mice (2,310 ± 283 cpm) compared with susceptible BALB/cJ mice (1,134 ± 144 cpm, P < 0.01). Infection reduced binding to 465 ± 60 cpm for C57BL/6J spleen cells and 578 ± 17 cpm for BALB/c (P < 0.01 compared with uninfected mice).

Since CSF-1 down regulates and LPS down modulates CSF-1 receptors (9, 10), it was possible that the decreased density of CSF-1 receptor sites on binding cells from infected mice could have been due to exposure of the cells to CSF-1 or to LPS-like molecules from the L. monocytogenes or both. C3H/HeJ mice, which do not respond to E. coli LPS but do respond to L. monocytogenes (Fig. 5), were infected with 5 × 10⁴ Listeria organisms. Two days later bone marrow cells were recovered from them and from control mice. Half the bone marrow cells were immediately subjected to [¹²⁵I]-CSF-1 binding. CSF-1 receptors on the other half were up regulated by incubation for 16 h at 37°C without added CSF-1 before [¹²⁵I]-CSF-1 binding. The results (Table 2) indicate that up regulation led to an increase in the total [¹²⁵I]-CSF-1 bound of approximately threefold among control cells and of approximately eightfold among cells from infected mice, while the percentage of binding cells increased five- and ninefold, respectively. Nevertheless, there remained in the infected mice a lower percentage of binding cells. This was compounded by the loss of total cells from the bone marrow after infection, so that the infected mice had only half the number of binding cells per tibia, compared with normal mice.

**DISCUSSION**

CSFs are a group of growth factors which regulate leukocyte proliferation and differentiation in vitro (13) and in vivo (4, 14, 17). Since the rapidity with which newly formed monocytes are mobilized to the site of infection is the major determinant of innate resistance of mice to L. monocytogenes, as well as the basis of marked differences in their genetic resistance (12, 16, 21, 23), it has been of interest to study CSFs and CFCs during that infection. We have previously found that after infection the concentration of total colony-stimulating activity (CSA) in serum increased with higher numbers of bacteria in the mice, whether the bacterial numbers reflected the infecting dose or the relative susceptibilities of the strain of mouse (25). Thus, as the CSA concentration in serum is higher in infected susceptible mice than in infected resistant mice, an increased CSA concentration in serum after infection cannot explain resistance. On the other hand, the number of CFCs in resistant mice before infection is higher than that in susceptible mice, providing a possible explanation for resistance.

Since it is the monocyte-macrophage series of cells which appears to be particularly important in resistance to L. monocytogenes, the present paper focuses on the CSF (CSF-1) which selectively regulates mononuclear phagocytes from the CFC precursor to the mature nondividing macrophage. All of these cells selectively express the CSF-1 receptor; larger numbers of receptors are expressed on more mature cells of the series (1, 5).

As with total serum CSA, the CSF-1 concentration in serum depended on the infecting dose of bacteria and was higher in susceptible than in resistant mice given the same dose. A marked increase in serum CSF-1 was only achieved by very high doses of L. monocytogenes. When levels of CSF-1 in tissue were examined, lower infecting doses were used (5 × 10⁴ to 10 × 10⁴ bacteria) for two reasons: first, to allow the survival of the mice over the period of observation,
and second, to minimize the contribution of blood CSF-1 to the CSF-1 concentration in tissue.

It will be noted that in all experiments the spleen, which together with the liver, is the major site of infection, contained the highest concentration of CSF-1 of all the organs examined in infected mice. It is relevant that splenectomy of mice before infection abrogates the increase in serum CSA (24), so the spleen is clearly a major source of CSF. The liver, which normally has a very low CSF-1 concentration, after infection exhibited the greatest fold increase in CSF-1 concentration and, because of its size, the greatest total CSF-1 of any organ. Significant increases were also observed in the lung and salivary gland. In each case, concentrations were too high to be significantly augmented by blood in the tissue. There was no correlation between genetic resistance of C57BL/6J mice or susceptibility of BALB/cJ, CBA/J, C3H/HeJ, or C3HeB/FeJ mice and their CSF-1 concentration before or 2 days after infection with 5 x 10^4 Listeria organisms. In this respect, a direct comparison can be made between Fig. 3 and 4 since they actually represent the same experiment. It was of interest that the LPS nonresponder C3H/HeJ mice were able to respond to L. monocytogenes as strongly as the C3HeB/FeJ LPS responder mice. This indicates that L. monocytogenes may stimulate the production of CSF-1 by a mechanism that differs from the one utilized by LPS or that the LPS-like molecule expressed in the Listeria cell wall (22) is recognized by the C3H/HeJ mice as different from E. coli LPS.

When the number of CSF-1-binding cells was examined, it was found, in line with our previous studies on CFCs, that the uninfected resistant C57BL/6J mice had a higher percentage of CSF-1-binding cells in their bone marrow and spleen than did uninfected susceptible mice. Furthermore, more cells were consistently recovered from the bone marrow (the major source of hemopoietic cells) of C57BL/6J mice than from BALB/cJ, CBA/J, or C3H mice. Thus, it seems that the resistant mice, as well as having intrinsically high numbers of total CFCs (25), also have higher numbers of cells which specifically give rise to monocytes and macrophages.

<table>
<thead>
<tr>
<th>Mice</th>
<th>^125I-CSF-1 bound (cpm/5 x 10^6 cells)</th>
<th>CSF-1-binding cells (% of total)</th>
<th>Total CFCs (10^6) per tibia</th>
<th>Total CSF-1-binding cells (10^6) per tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57BL/6J</td>
<td>41,028 ± 1,321</td>
<td>5.4 ± 0.2</td>
<td>11.0</td>
<td>59.4</td>
</tr>
<tr>
<td>Normal BALB/cJ</td>
<td>23,048 ± 1,155</td>
<td>3.8 ± 0.4</td>
<td>7.5</td>
<td>28.5</td>
</tr>
<tr>
<td>Infected* C57BL/6J</td>
<td>6,848 ± 534</td>
<td>1.5 ± 0.5</td>
<td>6.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Infected* BALB/cJ</td>
<td>4,835 ± 752</td>
<td>1.9 ± 0.7</td>
<td>4.0</td>
<td>7.6</td>
</tr>
</tbody>
</table>

* Results represent the mean and standard deviation of groups of three. Non-specific binding in the presence of unlabeled CSF-1 has been subtracted.  
* Infected mice received 6 x 10^6 Listeria organisms intravenously.
TABLE 2. Up regulation of CSF-1-binding cells from C3H/HeJ bone marrow of normal or 2-day-infected mice

<table>
<thead>
<tr>
<th>Conditions of testing</th>
<th>Mice</th>
<th>125I-CSF-1 bound (cpm/5 x 10^6 cells)</th>
<th>CSF-1-binding cells (%)</th>
<th>Total cells (10^6/tibia)</th>
<th>Total CSF-1-binding cells (10^6/tibia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Uninfected</td>
<td>5,437 ± 1,157a</td>
<td>1.9 ± 0.5a</td>
<td>12 x 10^6</td>
<td>22.8</td>
</tr>
<tr>
<td>Up regulated</td>
<td>Infected</td>
<td>1,461 ± 590</td>
<td>0.7 ± 0.1</td>
<td>6.5 x 10^6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

a Normal conditions of testing involved placing the cells at 4°C immediately upon removal from the mice and throughout CSF-1 binding. Up regulation of the receptors was achieved by incubating the cells at 37°C for 16 h without CSF-1 and then testing CSF-1 binding at 4°C.

Bone marrow cells from *L. monocytogenes*-infected mice exhibited a lower number of CSF-1-binding cells and a lower density of binding sites than did cells from normal mice. The possibility that this was simply due to mass migration of high-receptor-density cells (mature macrophages) to the site of infection was ruled out by the low binding of CSF-1 to spleen cells of infected mice. Another possibility was the down modulation-regulation of receptors by higher CSF-1 or LPS concentrations in the infected mice (9, 10). It was found that after up regulation in vitro in the absence of CSF-1, the total CSF-1 bound by 5 x 10^6 bone marrow cells from infected or uninfected mice reached approximately the same high level, reflecting an increase in the percentage of binding cells. The increase was almost fivefold for normal cells and ninefold for infected cells. Thus, cells from normal mice were down modulated-regulated to some extent, but the effect was far greater in infected mice. The percentage of binding cells remained somewhat lower in infected mice, while total bone marrow cells were almost halved. This suggests that there was some migration of cells of the macrophage lineage from the bone marrow to the site of infection. It was necessary to perform these experiments with the LPS nonresponder strain of mice (C3H/HeJ) since no up regulation was achieved with either C57BL/6J or BALB/cJ mice under the same conditions (data not shown). This was probably due to the sensitivity of cells from LPS responder strains to down modulation of their CSF-1 receptors to even extremely low concentrations of LPS in vitro (9). No direct comparison with respect to the amount of CSF-1 bound can be made between Tables 1 and 2 because they involved different batches of CSF-1 and different sources of mice.

The down regulation of receptors during infection raises questions concerning its mechanism. It is known that incubation of cells in CSF-1 down regulates the CSF-1 receptor. Furthermore, several other CSFs are known to down modulate the CSF-1 receptor (15). Granulocyte-CSF appears at a high concentration in the serum after infection (6), and other CSFs are probably produced in the tissues. On the other hand, it is also known that incubation of cells in vitro with LPS down modulates the CSF-1 receptor (9). Both mechanisms may be operating. The observed down regulation in the infected LPS nonresponder C3H/HeJ mice could have been due to an increased local CSF-1 concentration in the bone marrow, since even cells from normal uninfected control mice appeared to have significantly down regulated their CSF-1 receptors, perhaps reflecting the effect of basal levels of CSF. Experiments are in progress to determine whether products of *L. monocytogenes* are also involved in down modulating. In addition, the role of CSF-1 receptor down modulation-regulation in the control of mononuclear phagocyte production during infection is being examined.

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LITERATURE CITED


