Demonstration of a Blood–Bone Marrow Barrier to Macrophage Colony–Stimulating Factor

By Richard K. Shadduck, Abdul Waheed, and Edward J. Wing

Several previous studies suggested that murine macrophage colony–stimulating factor (CSF-1) might have impaired access to hematopoietic cells in the marrow. The apparent lack of hematopoietic responses to exogenous CSF and the finding of available or unoccupied CSF receptors despite saturating CSF levels in the serum led to studies of a potential blood–bone marrow barrier for this factor. Groups of mice were injected with pure unlabeled CSF-1 by either intravenous (IV) or intraperitoneal (IP) routes. Marrow and spleen cells were obtained at intervals after injection, held at 0°C, and assessed for changes in binding of 125I-CSF. Saturation of all available CSF receptors is achieved in vitro with 100 to 150 U CSF/mL. Despite achieving serum levels of 5,000 to 7,000 U/mL after IV injection of 25,000 units of CSF, less than 50% of the marrow receptors and less than 85% of the splenic receptors were saturated or downregulated. The decline in receptor availability was transient, with return of receptor sites in two to four hours. Increasing the IV dose to 125,000 units increased serum CSF values to ~20,000 U/mL and led to a virtual disappearance of available receptors for two to three hours. When administered IP, only ~40% of marrow and 80% of splenic receptors were affected for two hours. It was necessary to increase the dose of CSF to 250,000 units IP to saturate or downregulate receptors for three to four hours after injection. These observations indicate a marked blood–bone marrow barrier and lesser blood–spleen barrier for the transfer of serum CSF to responsive hematopoietic cells in vivo.

In contrast to these results, the systemic administration of M-CSF, or CSF-1, had virtually no effect on neutrophil or monocyte production in mice. This apparent lack of effect was unexpected because of the extensive cellular proliferation that M-CSF induces in vitro. Potential explanations for such ineffectiveness include a true lack of in vivo activity, administration of insufficient material, secondary release of inhibitors by accessory cells, or impaired access of the circulating CSF to the responsive progenitor cells.

The present studies were undertaken with a unique method we devised to examine the in vivo access of purified CSF-1 to responsive marrow and splenic cells. Since binding of CSF-1 is an irreversible process, experiments were conducted to measure the in vitro binding of 125I-CSF to marrow and spleen cells at intervals after the in vivo injection of pure unlabeled CSF-1. Any reduction in the ability of iodinated CSF to subsequently bind in vitro would then result from the receptor occupancy or downregulation that occurred in vivo by the injected unlabeled material. Since there is a rapid receptor turnover at 37°C, serial observations at intervals after a single injection of CSF-1 provided information on the duration of this receptor saturation or downregulation after a single dose of CSF-1. The reduction in subsequent receptor binding in vitro was compared with serum levels of CSF at each time interval following injection.

MATERIALS AND METHODS

L cell CSF, or CSF-1, was prepared by the growth of murine fibroblasts in serum-free medium as described previously. Ten-liter pools were concentrated 250-fold by ultrafiltration using an Amicon PM10 membrane (Amicon Corp, Danvers, MA). The CSF was purified by an affinity chromatography technique wherein the CSF was bound to a purified CSF antibody and eluted under low- mM protein. The purified material was devoid of contaminants as judged by migration in sodium dodecyl sulfate–acylamide gels and by finding an unambiguous N-terminal peptide sequence of this material.
BLOOD-BONE MARROW BARRIER TO CSF-1

CF1 female mice 12 to 16 weeks of age were used in all studies. Animals received 25,000 to 250,000 units (0.5 to 5.0 μg) of CSF in 0.1 ml/L Tris-HCl buffer, pH 7.5, containing 0.3% polyethylene glycol (molecular weight, 4,000) as a stabilizing agent. A group of five animals in each experiment served as controls after infection of the buffer. CSF preparations were rendered essentially free of endotoxin as described for in vivo studies. The CSF was injected in a 0.1- to 0.25-mL vol by either the intravenous (IV) or intraperitoneal (IP) route.

The animals were anesthetized by ether and killed at intervals extending from five minutes to six hours after injection. They were bled by cardiac puncture. Both femurs and the spleen were removed rapidly and placed in ice-cold McCoy's medium supplemented with 15% fetal calf serum. In one study peritoneal cells were harvested by injection of 10 mL of ice-cold Hank's medium containing 10 U heparin/mL before removal of the spleen. Five animals were used for each treatment group. Individual sera were stored at -20°C before assay for CSF. Bone marrow cells were flushed from the contents of femurs with fetal calf serum (molecular weight, 4,000) as a stabilizing agent. A group of five animals in each experiment served as controls after injection of the buffer. Cultures were incubated in I 2 x 75-mm test tubes at 0°C with 400,000 with fetal calf serum-free McCoy's medium before binding assays. Each spleen was pressed through a wire mesh. Five animals were used for in vivo studies. 

The CSF was injected in 10 mL of ice-cold Hank's medium containing 10 U heparin/mL before removal of the spleen. Five animals were used for each treatment group. Individual sera were stored at -20°C before assay for CSF. Bone marrow cells were flushed from the contents of both femurs into supplemented McCoy's medium as outlined earlier. Cells were dispersed thoroughly by vigorous pipetting and held at 0°C before binding assays. Each spleen was washed with ice-cold serum-free McCoy's medium and placed in ice-cold McCoy's medium supplemented with 10 U heparin/mL before

Table 1. Serum CSF Levels and Receptor Binding After IV Injection of 25,000 Units of CSF

<table>
<thead>
<tr>
<th>Minutes</th>
<th>Serum CSF (U/mL)</th>
<th>cpm Bound</th>
<th>Receptor Binding (%)</th>
<th>cpm Bound</th>
<th>Receptor Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1,402 ± 145</td>
<td>8,943 ± 645</td>
<td>100</td>
<td>31,221 ± 2,997</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>7,415 ± 356</td>
<td>9,319 ± 646</td>
<td>104</td>
<td>19,864 ± 1,998</td>
<td>63</td>
</tr>
<tr>
<td>15</td>
<td>3,913 ± 431</td>
<td>7,341 ± 1,066</td>
<td>82</td>
<td>11,986 ± 3,657</td>
<td>38</td>
</tr>
<tr>
<td>30</td>
<td>2,646 ± 308</td>
<td>6,241 ± 1,468</td>
<td>69</td>
<td>5,076 ± 3,831</td>
<td>16</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>949 ± 50</td>
<td>16,044 ± 1,372</td>
<td>100</td>
<td>51,724 ± 12,052</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>4,618 ± 696</td>
<td>13,292 ± 728</td>
<td>86</td>
<td>32,841 ± 4,015</td>
<td>69</td>
</tr>
<tr>
<td>30</td>
<td>3,888 ± 424</td>
<td>11,390 ± 574</td>
<td>71</td>
<td>9,875 ± 3,167</td>
<td>19</td>
</tr>
<tr>
<td>60</td>
<td>1,382 ± 93</td>
<td>13,607 ± 1,256</td>
<td>85</td>
<td>25,416 ± 6,647</td>
<td>49</td>
</tr>
</tbody>
</table>

Serum CSF levels and marrow and splenic 125I-CSF binding were evaluated at times up to 60 minutes after IV injection of CSF-1. Serum CSF values were determined by RIA. Binding values represent specific counts per minute bound after 30 minutes' incubation with 400,000 cpm of 125I-CSF at 0°C. Studies used 5 × 10⁶ marrow and 25 × 10⁶ spleen cells per tube. Values are means ± 1 SE.

RESULTS

In the initial studies, 25,000 U of CSF-1 was administered IV, and parameters were evaluated five to 60 minutes after injection. The results of two studies are shown in Table 1. Serum CSF levels increased to 4,618 to 7,415 U/mL at the earliest time point evaluated, ie, five minutes after injection. This was associated with a decrease in splenic binding capacity for 125I-CSF to 63% of control but little immediate reduction in marrow cell binding. Serum CSF declined towards baseline values over the 30- to 60-minute time intervals. Splenic receptor availability decreased to 16% to

Cell-associated radioactivity was determined by repeat counting. In each experiment, control tubes contained a 60- to 100-fold excess of unlabelled CSF as a measure of nonspecific binding. In general, this ranged from 6% to 8% of the total binding. To determine the specific counts per minute bound, the values obtained in tubes containing the unlabelled CSF were subtracted from those containing the radiolabelled CSF alone.

CSF receptor availability was determined by the degree of subsequent binding of 125I-CSF in vitro after injection of the unlabelled material. Any decrease in receptor availability might be due to a variety of events that would reduce binding such as receptor downregulation, internalization, or occupancy at the time of in vitro study.

CSF activity was measured by a sensitive double-antibody RIA as described previously. In this assay, rabbit anti-CSF serum was reacted with purified radiolabeled CSF and precipitated with sheep antirabbit IgG serum. A standard curve was defined by using 0 to 150 units of CSF. Activity was measured by the displacement of radiolabeled CSF by the unlabelled material. To determine serum CSF levels, 20 μL from individual mice was substituted for the standards in the RIA. Duplicate tubes were prepared for each sample. The activity of the sera was calculated by using a computer program as described previously. Units of CSF activity were determined originally by bioassay. A total of 10⁴ CFU, mouse bone marrow cells was mixed in 1 mL of 0.3% agar in McCoy's medium supplemented with 15% fetal calf serum. Assays contained 0.1 mL of the unknown CSF sample in appropriate dilutions. Total CSF activity was determined from the linear portion of the dilution curve wherein 1 unit of CSF activity was defined as that amount required to produce one colony.

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23%, whereas ~70% of marrow receptors were unsaturated during this time interval.

The effects of CSF injection on receptor binding were relatively transitory. The results of a four-hour study are shown in Fig 1. Serum CSF levels peaked at ~5,600 U/mL at 15 minutes and decreased to near baseline values within one hour. In this experiment there was a 50% decline in available marrow receptors and a 76% decline in splenic receptor binding at the earliest time point. There was a rapid return in available CSF receptors as serum CSF values declined at one to two hours.

The serum clearance of exogenous CSF was relatively rapid after IV injection of 25,000 units of CSF (Fig 2). Peak values were estimated by extrapolation to time 0 as approximately 6,500 U/mL immediately after injection. Since the plasma volume of such mice is 1 to 1.2 mL, this suggests either a greater volume of distribution, extensive receptor binding, rapid degradation, or very rapid excretion of the CSF. The estimated initial plasma clearance (t1/2α) was approximately 20 minutes. Since these mice had appreciable endogenous levels of CSF and the injected material was unlabeled, it was not possible to calculate a secondary clearance pattern (t1/2β).

These preliminary observations indicated a substantial barrier between the circulating CSF and the receptive cells in the marrow and spleen. Even though serum levels were increased 20- to 50-fold greater than that required for receptor saturation in vitro, many available receptor sites were unaffected. To see whether this barrier could be overcome, further groups of mice were injected with 25,000, 125,000 and 250,000 units of CSF IV and evaluated 15 minutes after the injection (Fig 3). As in previous studies, the CSF had greater access to splenic progenitor cells at the low dose level, with a greater than 70% reduction in available receptors as opposed to only a 28% reduction in marrow receptors. With the higher dose levels of CSF, serum values rose to 21,400 and 41,200 U/mL after the 125,000- and 250,000-unit doses respectively. This led to a near total loss of available receptors at the 15-minute time point.

The duration of receptor saturation or downregulation was evaluated in a further study at intervals up to three hours after the IV injection of 125,000 units of CSF (Fig 4). Serum CSF values were ~21,700 U/mL at 30 minutes and declined ~11,000 U/mL/h with a serum t1/2α of approximately 45 minutes. Virtually no marrow and splenic binding sites could be detected through two hours, but available receptors began to reappear by three hours as serum CSF values declined below 4,000 U/mL.

Although these studies indicated that high doses of CSF were capable of reaching responsive hematopoietic cells, repeated IV doses of CSF are impractical in the murine model. For this reason, 125,000 units of CSF was administered IP, and observations were made at the same time intervals as in the IV study. Serum CSF values were lower at all time points after IP as opposed to IV injection; peak values of 5,920 U/mL were noted 30 minutes after injection. This caused an 80% decline in splenic and a 40% decline in available marrow receptors for at least two hours after the injection.

In a final study, 250,000 units of CSF were administered IP, and observations were extended over a period of six hours; binding was also measured by using peritoneal exudate cells (Fig 5). Serum CSF values peaked at one hour. There was a near complete disappearance of marrow and splenic CSF receptors at one and two hours. This dose of CSF was sufficient to maintain an ~50% decrease in marrow and splenic receptor availability through six hours after injection. In contrast to the return in available receptors for these cells, no peritoneal cell receptors could be detected through the six-hour time point.
**DISCUSSION**

Initially the CSFs were described as activities that induced the in vitro growth of granulocyte and macrophage colonies. With purification and molecular cloning it has been possible to produce relatively large quantities of recombinant murine and human hematopoietic growth factors for testing in vivo. It was anticipated that such factors should act in vivo as they do in vitro, ie, to cause extensive cellular proliferation and differentiation. In part this has been the case. G-CSF\(^\text{15}\) and, to a lesser extent, GM-CSF\(^\text{16}\) and IL-3\(^\text{17,18}\) stimulate hematopoiesis in the murine model, while M-CSF, or CSF-1, has been essentially devoid of activity.\(^\text{19}\) All have been used in nanogram dose ranges; CSF-1 has been inactive with injection of 25,000 units (~500 ng) every six hours.

A variety of observations suggested that CSF-1 should induce changes in either neutrophil or monocyte production. These include the well-known activity in vitro, the increased cycling of various hematopoietic progenitor cells seen after IV injection of CSF-1,\(^\text{26,37}\) and the inhibition of in vivo diffusion chamber granulopoiesis by anti-CSF-1 serum.\(^\text{18,29}\)

The latter may be explained by the secondary production of G-CSF by macrophages in response to CSF-1.\(^\text{30}\)

One observation suggested to us that the CSF-1 might have impaired access to the responsive progenitor cells in vitro. Plateau colony formation occurs with 100 to 150 U/mL of CSF. A similar plateau in CSF binding occurs with this concentration of either native or radiolabeled CSF.\(^\text{26}\) Such binding is irreversible and cannot be displaced by a 100- to 1,000-fold excess of unlabeled material. Since serum levels of CSF-1 are ~500 to 1,500 U CSF/mL or at least ten times those that induce plateau binding, one would anticipate...
saturation or downregulation of all receptors in vivo. Such is not the case. Indeed, virtually all receptors are unsaturated to a high degree in vitro binding of $^{125}$I-CSF is observed with fresh marrow or spleen cells. Further, when using a technique with low pH exposure to dissociate CSF from the receptor, we have found only a marginal increase in the number of available receptors with fresh marrow cells. Serum CSF does indeed bind avidly to the CSF-1 receptor because a 15-minute exposure of marrow cells to murine serum in vitro prevents the subsequent binding of $^{125}$I-CSF.

These observations suggested to us a significant blood-marrow barrier for CSF-1 and provided the basis for the present studies. The present findings indicate that after an IV injection the $t_{1/2}$ of CSF is on the order of 20 to 45 minutes, depending on the total dose of CSF used. Peak serum values of 5,000 to 7,000 U/mL were seen 15 minutes after an IV injection of 25,000 units CSF-1. This caused a 75% to 85% loss of available splenic receptors but less than a 50% decline in available CSF receptors. The effect was transient, with a return to normal receptor status within two to four hours. A fivefold increase in the dose of CSF-1 to 125,000 units IV raised the serum CSF-1 value to 21,700 U/mL. This caused an essentially complete loss of spleen and marrow receptors; however, available receptors returned within three hours after the CSF injection. CSF-1, 125,000 units IP, yielded peak serum values of ~6,000 U/mL with only a 40% decline in marrow receptors. An increase in the dose of IP CSF-1 to 250,000 units achieved peak serum CSF-1 values of ~22,200 U/mL. This saturated or downregulated a high proportion of spleen and marrow receptors for two to four hours after the injection and all peritoneal cell receptors throughout the six-hour study.

On the basis of these findings it would appear that serum CSF-1 values must be increased from baseline levels of ~1,000 U/mL to 5,000 to 20,000 U/mL to reach and bind to available CSF receptors. The effect is relatively transient, with apparent synthesis of new CSF-1 receptors within one to four hours postinjection. Since saturation of binding occurs with 100 to 150 U CSF-1/mL in vitro, these findings indicate a substantial blood–bone marrow barrier for this factor. These observations suggest that the previous ineffectiveness of CSF-1 in vivo may have been due to this barrier and may require larger quantities of CSF-1 for further in vivo studies.

Although the present explanations for the ineffectiveness of CSF-1 in vivo are appealing, it is not known how many receptors need to be occupied for an in vivo effect. It will be interesting to explore, with this technique, the in vivo receptor saturation or downregulation with other murine CSFs that are known to be active in vivo. Another recent observation suggests that CSF-1 may induce a secondary release of certain inhibitors following repeated injections. A single or double injection of CSF-1 causes a marked increase in the number of colony-forming units in cycle 24 to 36 hours after the injection. However, if a third injection of CSF is administered during this 24-hour cycle, there is a near complete inhibition of cycling of the CFU-GM as determined by thymidine suicide studies. This suggests that repeated injections of CSF may induce the release of inhibitory molecules such as prostaglandins, lactoferrins, acidic isoferritins, or other interactive molecules. It will be important to explore the effects of such inhibitors in further in vivo studies with large doses of CSF-1 as indicated by the present studies.

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REFERENCES


Demonstration of a blood-bone marrow barrier to macrophage colony-stimulating factor

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