Tolerance to the Granulocyte-releasing and Colony-stimulating Factor Elevating Effects of Endotoxin

By P. Quesenberry, J. Halperin, M. Ryan, and F. Stohlman, Jr.

Injection of Salmonella typhosa endotoxin into either CF, or C57bl/6J mice leads to prompt increases in serum colony-stimulating factor (CSF). Repeated injections of endotoxin result in a dose-related hyporesponsiveness or tolerance to this effect. Tolerance is seen after either intravenous (i.v.) or intraperitoneal (i.p.) routes of administration or challenge and occurs after one to two preinjections. Cross-tolerance to heterologous endotoxin (Escherichia coli) was also shown. This cross-tolerance is complete immediately after cessation of preinjections, but partial at later time intervals. Levels of a serum inhibitor of colony growth were decreased in tolerant mice, although this decrease is not statistically significant. Tolerant mice injected with endotoxin release granulocytes from the bone marrow normally, in spite of the absence of a CSF response. This suggests that neutrophil releasing activity (NRA) and CSF are separate entities. A marked marrow granulocytic hyperplasia develops after 7 or 20 days of endotoxin injections, despite the tolerance to the CSF-elevating effect of endotoxin. This granulocytic hyperplasia could still be mediated by serum CSF increases. A negative medullary feedback secondary to the repetitive release of marrow granulocytes, however, is an equally plausible mechanism for the stimulation of granulocyte production. It is also possible that the decrease in serum inhibitors played a role in the sustained increase in granulopoiesis seen here.

Injection of experimental animals and man with bacterial endotoxin leads to a wide spectrum of pathophysiologic changes. These include fever, induction of antibody formation, complement activation, intravascular coagulation, the Schwartzman phenomenon, neutropenia followed by marrow granulocyte release, changes in hemopoietic stem cell numbers, and proliferative status and, in sufficient doses, death. Repeated injections of endotoxin result in a marked resistance to the development of many of these effects—a state termed by most as tolerance. This latter phenomena may depend on a number of variables, including dose and route of administration of endotoxin, the species of experimental animals and the parameter under investigation.

A variety of mechanisms have been proposed as underlying these states of tolerance, including reticuloendothelial stimulation with rapid clearance of endotoxin, neutralization of endotoxin with antibody, or inactivation of endotoxin by a serum or tissue factor separate from antibody. Recently evidence has been presented that at least two types of tolerance may occur; an early tolerance which is not associated with a humoral factor, and a late form dependent on a transferable humoral factor specific for the endotoxin employed.
We have observed that endotoxin injection into CF1 mice results in marked elevation of serum colony-stimulating factor (CSF).26 This latter entity is defined by its ability to induce myeloid progenitor cells to form granulocyte-monocyte-macrophage colonies in an in vitro culture system and may be a humoral regulator of granulopoiesis.27 Endotoxin injection also results in the release of marrow granulocytes from the marrow reserve pool.7'28-31 A serum factor termed neutrophil-releasing activity (NRA) is believed to moderate this release.32-33 There is a temporal relationship between serum NRA and CSF activity after endotoxin,34 but whether they are the same or separate entities has not been established. In the present communication, we present studies on the development of tolerance to the CSF-elevating and granulocyte-releasing effects of endotoxin.

MATERIALS AND METHODS

Tolerance Induction

S. typhosa endotoxin (Difco No. 3940) was dissolved in saline immediately prior to use and groups of four to nine CF1 mice (Carworth Farms) were injected intraperitoneally (i.p.) daily with either 10 μg of endotoxin or 0.1 ml of sterile saline over a period of 1-20 days. At varying time intervals (24 hr to 1 yr) after the last preinjection, the mice were injected with 5 μg endotoxin or 0.1 ml saline and bled from 1/2 to 40 hrs later. In other experiments, the preinjection doses were spaced at varying intervals, and in still others the challenge dose was varied from 5 to 200 μg of endotoxin given i.p. or intravenously (i.v.). Blood was collected under ether anesthesia by cardiac puncture and pooled. In most instances, sera were separated, frozen and later assayed for colony-stimulating or inhibiting factors. In several experiments, blood was anticoagulated with a preservative-free heparin (Panhepnin-Abbott) and plasma collected, frozen, and assayed at a later date. The results of assays were the same whether plasma or sera were assayed, and these results have been pooled. In several experiments, CSF were assessed for serum or plasma CSF levels: (1) endotoxin preinjected (tolerant) mice injected with endotoxin prior to sacrifice; (2) endotoxin preinjected (tolerant) mice injected with saline prior to sacrifice; (3) saline preinjected mice (control) injected with endotoxin; and (4) saline preinjected mice (control) injected with saline.

The effect of route of administration on tolerance was assessed by injecting mice i.v. (tail vein) with 10 μg S. typhosa endotoxin on 0.1 ml saline on days 1, 4, and 7, and then challenging these mice on day 8 with 5 μg of endotoxin i.p. or i.v. 6 hr prior to sacrifice.

To evaluate whether cross-tolerance to a heterologous endotoxin was demonstrable, mice preinjected with S. typhosa endotoxin were challenged with E. coli endotoxin (Difco—3923-10).

CSF Assay

We have utilized a modification of an in vitro double layer agar culture technique,35 which we have previously described.36 CSF dose response curves were determined for serum or plasma samples by assaying at multiple concentrations. CSF activities were then compared on the linear part of the dose-response curve. CSF activity is expressed as the number of colonies stimulated per 10^5 murine marrow cells ±1 SE of the mean.

Inhibitor Assay

Serum inhibitors of colony growth were measured in an in vitro single layer culture system.37 Serum samples or saline were mixed with an excess of CSF derived from either murine endotoxin serum38 or mouse lung conditioned medium (see below), and the number of colonies formed in plates containing CSF and test sera compared to the number formed in plates with saline and CSF. The results were then expressed as percent inhibition caused by test sera.

Lung-conditioned medium was prepared by mincing lungs from CF1 mice with scissors and incubating the fragments in Hank's balanced salt solution or single strength Eagle's medium for
3 days at 37°C in 10% CO₂. Fragments from one lung were incubated in 2 ml volumes in Falcon petri dishes. The incubates were centrifuged at 975 g for 10 min and the supernatant solution then dialyzed against sterile H₂O (6250 units of penicillin and 6250 mg streptomycin per liter) for 3 days with one change per day. The dialyzed conditioned media were then sterilized by pressure filtration through a 0.45 μ Millipore filter and stored at –20°C. Marrow and peripheral blood differentials were measured as previously reported. At least 100 cells per animal were counted for peripheral blood, and from 386-1000 cells per animal for bone marrow. Probability figures were calculated using Student’s T test.

RESULTS

Mice subjected to repeated endotoxin injections over 7–20 days did not show any apparent adverse effects. Weights were determined on endotoxin and saline preinjected mice before and after 7 days of injections; 60 control and 100 tolerant mice were evaluated. The mean weights ± 1 SE of mice immediately before saline injections were begun, and 24 hr after 7 days of injections were 27.2 ± 0.26 and 27.3 ± 0.29 g, respectively, while the mean weights of mice before and after a 7-day injection schedule of 10 μg endotoxin were 27.4 ± 0.19 and 28.2 ± 0.2 g, respectively.

There were no significant CSF elevations in mice preinjected with endotoxin for 7 days and challenged with endotoxin 5–6 hr prior to sacrifice on day 8 (Table 1). In two experiments, C57bl/6J mice were preinjected with 10 μg endotoxin i.p. every day for 7 and 14 days, and then challenged on day 8 and 15, respectively, with 5 μg endotoxin 4-5 hr prior to sacrifice. In these experiments, 5% sera from the endotoxin-injected controls stimulated from 68 to 110 CFC/10⁵ cells, while 5% sera from tolerant mice injected with endotoxin stimulated 0.7 to 6.7 CFC/10⁵ cells.

The serum CSF values from ½ to 4 hr after 5 μg of endotoxin in tolerant and control mice on day 8, after a 7 day preinjection schedule, are presented in Fig. 1. No significant elevations were noted in the tolerant mice injected with endotoxin. In one experiment, on day 8 after a 7 day preinjection schedule, mice were challenged with 10 μg of endotoxin, the same dose used for tolerance induction, and serum collected at 2, 4, 6, 8, 12, 16, 20, 24, and 40 hr after injection. When these sera were tested for CSF activity, at a 2.5% concentration, small suboptimal CSF increases were seen in the 2 and 4-hr samples, but not in the other samples.

Tolerance is demonstrable after one preinjection of endotoxin and is quite marked after three preinjections (Fig. 2). These data have been confirmed in three additional experiments.

| Table 1. Serum CSF Values in Tolerant and Control CF1 Mice Injected with Saline or Endotoxin |
|-------------------------------|-------------------------------|----------------|
| Group                        | Challenge Injection | CFU/10⁵ ± SE |
| Control                      | Saline                  | 0.5 ± 0.2    |
| Control                      | Endotoxin               | 87 ± 15      |
| Tolerant                     | Saline                  | 0.4 ± 0.4    |
| Tolerant                     | Endotoxin               | 1.6 ± 0.3    |

Serum CSF values from mice preinjected i.p. for 7 days with saline or 10 μg S. typhosa endotoxin and challenged on day 8 with saline or 5 μg of S. typhosa endotoxin 5–6 hr prior to sacrifice. Pooled sera were collected from four to eight mice per experiment in four separate experiments.

*Number of colonies per 10⁵ murine marrow cells stimulated by 2.5% concentration of sera.
Fig. 1. Serum CSF values expressed as the number of CFC/10^5 murine marrow cells ± 1 SE of the mean stimulated by 5% serum from tolerant and control mice at varying time intervals after 5 μg endotoxin or 0.1 ml saline i.p. CSF values for the sera from saline injected mice at the various time points were pooled and are represented by the hatched bar. The values at each time point represent the mean CSF values of pooled sera collected in three to four separate experiments.

Fig. 2. CSF levels in CF1 mice expressed as CFC/10^5 marrow cells ± 1 SE of the mean in mice preinjected with 10 μg endotoxin i.p. for 1–3 days and then challenged 24 hr after the last preinjection with 5 μg endotoxin i.p. and bled 3 hr later. Serum was tested at a 2.5% concentration; 2.5% serum from saline injected control or tolerant mice did not stimulate over 0.9 ± 0.4 CFC/10^5 cells. Serum from mice which had received three preinjections of saline and were then challenged with 5 μg of endotoxin stimulated 124 ± 8 CFC/10^5 cells when tested at a 2.5% concentration.

Tolerance decreases when the intervals between the last preinjection and the challenge dose is lengthened. Serum CSF levels at different time intervals after either 7 or 20 days of preinjections are presented in Fig. 3. In these experiments, tolerant mice did not respond to endotoxin with serum CSF elevations 1 day after either the 7 or 20 day schedule. CSF responses became demonstrable, but remained suboptimal up to 30–32 days after the end of the injection schedules. In other experiments, tolerant CF1 mice were assessed for their serum CSF response to endotoxin at 8, 16, 291, and 333 days after a 7-day preinjection schedule.

Fig. 3. Serum CSF expressed as CFC/10^5 marrow cells ± 1 SE of the mean stimulated by 2.5% sera from mice bled at varying time intervals after a 7 or 20-day preinjection schedule. Consecutive daily injections of saline or 10 μg endotoxin over the 7-day schedule. The 20-day schedule consisted of 13 injections of 10 μg endotoxin or saline over 20 days; the last eight injections were given consecutively. Serum from tolerant or control mice injected with saline at these various time points did not stimulate over 2.6 ± 0.3 CFC/10^5 marrow cells when tested at a 2.5% concentration.
**Table 2. Induction of Tolerance Intravenously**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 8 Injection</th>
<th>Route Administration</th>
<th>CSF (CFC/10⁵ Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Endotoxin</td>
<td>i.v.</td>
<td>129 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Endotoxin</td>
<td>i.v.</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td>Saline</td>
<td>i.v.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>0</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Saline</td>
<td>i.v.</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>0</td>
</tr>
</tbody>
</table>

Control mice and tolerant mice were injected with either 0.1 ml saline or 10 μg endotoxin i.v. on days 1, 4 and 7 and challenged with 5 μg endotoxin on day 8 (6 hr prior to sacrifice). Sera from at least five mice tested at a final concentration of 2.5%, and the results expressed as the number of CFC/10⁵ cells ± 1 SE of the mean.

schedule, and at 7, 52, and 364 days after a 20-day preinjection schedule. In all instances, small increases in serum CSF were seen in the endotoxin-injected tolerant mice, but these increases were all significantly less than those seen in control mice injected with endotoxin. In one experiment, C57bl/6J mice were evaluated 7 mo after the induction of tolerance and found still to be tolerant.

Tolerance could be induced by either the i.p. or i.v. route. When tolerance is induced by i.v. injection, it can be demonstrated regardless of whether the challenge is given i.p. or i.v. (Table 2).

When the 10 μg dose of endotoxin used to induce tolerance was employed as a challenge 24 hr after the cessation of seven daily preinjections, increased CSF levels were noted in the endotoxin-treated tolerant mice (Fig. 4 and above). Increasing the challenge dose given 24 hr after tolerance induction to from 50 to 100 μg of endotoxin resulted in further increases in serum CSF levels, but these levels were still suboptimal when compared to the control animals injected with 5 μg of endotoxin (Fig. 4 and Table 3). These results were confirmed in two additional experiments. When similar groups of tolerant mice were challenged with 100 μg of S. typhosa endotoxin i.p. 7 and 16 days after a 7-day preinjection schedule, serum CSF increases were equivalent to control mice injected with 5 μg of endotoxin i.p. These animals could be shown to be tolerant by utilizing a 5 μg challenge dose.

**Fig. 4.** Serum CSF levels expressed as the number of CFC/10⁵ marrow cells ± 1 SE of the mean. Tolerant or control mice were given different doses of endotoxin and then bled at varying time intervals and sera from these mice were assayed at a 5% concentration. These data are from one experiment with five mice per experimental group.
Table 3. Serum CSF in Tolerant and Control Mice Injected with Saline or Endotoxin (CFC/10^5)

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge Injection</th>
<th>S. typhosa Endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline 0.1 ml</td>
<td>E. coli Endotoxin</td>
</tr>
<tr>
<td></td>
<td>5 μg</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td>100 μg</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.7 ± .3</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0</td>
<td>0.3 ± .3</td>
</tr>
</tbody>
</table>

Tolerant and control mice were injected i.p. for 4 days with either 10 μg S. typhosa endotoxin or 0.1 ml saline and on day 5 challenge injections were administered and the animals bled 5–6 hr later. Pooled sera tested at 2.5% concentrations and results expressed as the number of CFC/10^5 marrow cells ± 1 SE of the mean.

Cross-tolerance to E. coli endotoxin was demonstrable in CF1 mice 24 hr after the cessation of preinjections with S. typhosa endotoxin (Table 3). These results were confirmed in two other experiments in which mice were rendered tolerant by either 4 or 7 days of preinjections. The data presented in Table 3 show that complete cross-tolerance to E. coli endotoxin was also demonstrable with higher challenge doses. These data were confirmed in one additional experiment. Cross-tolerance was also assessed in four experiments at from 12 to 100 days after the end of preinjection schedules. Cross-tolerance to E. coli endotoxin was only partial at these later time intervals after tolerance induction. In these experiments, control mice developed comparable CSF elevations in response to 5 μg of E. coli or S. typhosa endotoxin. S. typhosa preinjected mice showed a diminished CSF response to both endotoxins, but the tolerant mice injected with 5 μg of E. coli endotoxin evolved more CSF activity than those injected with 5 μg of S. typhosa endotoxin. The CSF levels of serum (tested at 5%) from the tolerant mice injected with E. coli endotoxin were 308 ± 173% of the levels which were seen in comparable tolerant mice challenged with S. typhosa endotoxin.

A number of different endotoxin preinjection schedules were studied with regard to the induction of tolerance. Table 4 shows that mice preinjected with 10 μg of endotoxin on days 1 and 9 and challenged on day 14 no longer show CSF increases in response to 5 μg of endotoxin.

Serum inhibitor levels in tolerant and control mice are presented in Table 5. Sera from tolerant and control mice at from 1/2 to 6 hr after saline injection (24± to 30 hrs after the last preinjection) were assessed for inhibition of colony growth. Inhibitor levels were generally lower in the sera from tolerant mice, but none of these differences were statistically significant. In three experiments,

Table 4. Serum CSF after Spaced Injections of Endotoxin

<table>
<thead>
<tr>
<th>Group</th>
<th>Injection</th>
<th>CSF (CFC/10^5)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>1 ± 0.7</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Saline</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>Endotoxin</td>
<td>117 ± 7</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Endotoxin</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

*5.0 μg of endotoxin or 0.1 ml of saline injected 6 hr prior to sacrifice.
†The mean number of CFC/10^5 cells ± 1 SE of the mean stimulated by 2.5% of pooled test sera. Preinjections of either 10 μg of S. typhosa endotoxin or 0.1 ml of saline i.p. were given on days 1 and 9 and the mice injected and bled on day 14.
serum inhibitor levels from mice preinjected for 7 days and challenged on day 8 were determined at 1/2, 1, 2, 3 and 4 hrs after 5 μg of endotoxin and compared to inhibitor levels in saline injected mice. In two experiments, inhibitor levels were also determined 6 hr after injection. There were no significant differences between inhibitor levels in the serum of tolerant and control mice injected with endotoxin. There was a wide scatter of inhibitor levels in both tolerant and control mice after endotoxin, but in the majority of instances sera from endotoxin-injected tolerant and control mice showed either similar or lower inhibitor levels when compared to their saline-injected counterparts.

The peripheral blood band and polymorphonuclear granulocyte (PMN) counts in tolerant and control mice injected with endotoxin or saline i.p. 5-6 hr prior to sacrifice are presented in Fig. 5. Both the tolerant and control mice show increases in peripheral blood granulocytes in response to endotoxin. The absolute tibial proliferative granulocytes (myeloblasts, promyelocytes, and myelocytes), metamyelocytes, bands, and PMN’s from the same groups presented in Fig. 5 are shown in Table 6. An absolute increase in marrow granulopoiesis in the tolerant mice is apparent (194% of control), and both the tolerant

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**Table 5. Serum Inhibitor in Tolerant and Control Mice**

<table>
<thead>
<tr>
<th>Hours After</th>
<th>Per Cent Inhibition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Injection</td>
<td>Control</td>
<td>Tolerant</td>
</tr>
<tr>
<td>1/2</td>
<td>86 ± 5</td>
<td>61 ± 21</td>
</tr>
<tr>
<td>1</td>
<td>95 ± 1</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>3</td>
<td>76 ± 9</td>
<td>73 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>66 ± 8</td>
<td>74 ± 22</td>
</tr>
<tr>
<td>6</td>
<td>61 ± 12</td>
<td>38 ± 15</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>28</td>
</tr>
</tbody>
</table>

*Number of experiments per time point. Pooled sera from at least four mice were collected for each time point in each experiment. The results are expressed as the per cent inhibition ± 1 SE of the mean.
and control mice show a marked and comparable decrease in marrow bands and PMNs 5–6 hrs after 5 μg of endotoxin. Serum CSF levels were determined in tolerant mice 5–6 hr after 5 μg of endotoxin or 0.1 ml of saline in each of these three experiments. No serum CSF increases were demonstrable in the endotoxin-injected tolerant mice. Table 7 shows absolute tibial proliferative granulocytes, metamyelocytes, bands and PMNs from tolerant and control mice preinjected for 20 days. There is also a marked increase in marrow granulopoiesis in the tolerant mice (236\% of control), and comparable decreases in marrow granulocytes 5–6 hrs after endotoxin. In the tolerant mice injected with endotoxin, there was a decrease of 3.01 × 10^6 marrow bands and PMN’s, while in the control mice injected with endotoxin this figure was 2.68 × 10^6.

**DISCUSSION**

Repeated injections of *S. typhosa* endotoxin administered to CF1 or C57bl/6J mice leads quite rapidly to a hyporesponsiveness or tolerance to the serum-CSF elevating effects of endotoxin. This CSF-tolerance may be induced via i.p. or i.v. injections, is demonstrable by either route and may be partially overcome by increasing the challenge dose of endotoxin. CSF-tolerance wanes but is still demonstrable for 1 yr after the cessation of preinjections. Mice preinjected with *S. typhosa* endotoxin show a cross-tolerance to *E. coli* endotoxin. Cross-tolerance to heterologous endotoxin was complete 24 hr after the last preinjection (early tolerance stage), but at later time intervals (late tolerance stage) tolerance to heterologous *E. coli* endotoxin was less than that seen with the homologous *S. typhosa* endotoxin. These data indicate that tolerance is non-specific during the early tolerant stage, but in the late tolerant stage there is evidence of partial specificity.

The rapidity with which tolerance occurs and the lack of specificity in the early tolerance period suggests that the tolerance seen immediately upon ces-
oration of preinjections may be on a nonimmune basis. However, the prolonged decreased responsiveness and the apparent partial specificity seen in the late tolerance period suggest that immune mechanisms may be involved at the longer time intervals after tolerance induction. This interpretation would be consistent with the work of Greisman et al., suggesting that separate mechanisms exist for early and late tolerance.

Inhibitor levels appeared decreased in tolerant mice, but these decreases were not statistically significant. The meaning of these inhibitors is not yet clear, and it is possible that they represent only in vitro artifacts of the culture system. The level of serum inhibitors, however, may influence CSF assays and, thus, regardless of their possible physiologic significance, it is important that inhibitor levels be determined. In these experiments, it appeared that changes in inhibitor levels did not significantly influence the CSF assays.

There is a marked increase in marrow granulopoiesis after either 7 or 20 consecutive IP injections of 10 μg of S. typhosa endotoxin. Chervenick and Boggs noted similar increases in marrow granulopoiesis from 7 to 101 days after the start of daily i.p. injections of 0.5 μg endotoxin in C57bl female × DBA male F1 mice, but they observed that if the daily dose of endotoxin was raised to 2 μg, the increase in marrow granulopoiesis was only transient and had returned to control levels by 4 wk. The increases which we observed in marrow granulopoiesis developed in the face of a markedly diminished CSF response of endotoxin; this latter occurring within 1–2 days of the initiation of preinjections. An understanding of the details of the experimental protocols is crucial in interpreting these data. In the experiments in which granulocyte release occurred in the face of no demonstrable CSF elevations, the mice were preinjected with 10 μg of endotoxin and challenged with 5 μg (see below). In analyzing the relationship of serum CSF levels to the development of granulocyte hyperplasia, the critical question is whether or not serum CSF levels were increased after repetitive 10 μg injections. When serum CSF levels in tolerant mice were evaluated after a 10 μg rather than a 5 μg endotoxin challenge, small but significant CSF elevations were noted for at least 8 days of injections. It can be inferred from the above that mice injected repetitively with 10 μg of endotoxin have marked increases in serum CSF over the first 24 to 48 hr of injections and thereafter, at least up to 8 days, have small but demonstrable increases in serum CSF. Although these data offer no compelling evidence for or against CSF as a hormonal regulator of granulopoiesis, the pattern of CSF response to a sustained stimulus (repetitive endotoxin injection) is not too dissimilar from the pattern of serum erythropoietin elevations seen in response to chronic hypoxia. There is a significant difference in these responses, however, in that mice previously subjected to chronic hypoxia do not show a diminished erythropoietin response on reexposure to hypoxia. We, as yet, have no data on the marrow granulocyte response of tolerant mice given a series of endotoxin injections at prolonged time intervals after the first series of preinjections. Considering the observations of Chervenick and Boggs of an eventual decrease in marrow granulopoiesis in mice given 2 μg endotoxin daily, one might expect that mice in the late tolerant phase would show a diminished granulopoietic response to repetitive endotoxin injections when compared to their appropriate endotoxin injected controls.
There are a number of possible mechanisms for the increased granulopoiesis noted in tolerant animals. Serum CSF elevations remain one possibility, and the decrease in the extent of CSF elevations noted after 1–2 days of injections does not weigh too heavily against this possibility. Marrow cells could become more sensitive to the action of CSF, CSF turnover, and utilization could be increased or the initial CSF elevations may have been far in excess of that needed to stimulate granulopoiesis. Alternatively, it is possible, as Metcalf has suggested, that the local production of CSF by bone is a critical means of stimulating granulopoiesis. Preliminary studies evaluating the production of CSF in short-term organ cultures of bone from tolerant and control mice show decreased production of CSF from tolerant bone, suggesting that local production is not the mechanism for the increased granulopoiesis seen under these experimental conditions. It is equally plausible that the repetitive release of marrow granulocytes, induced presumably by NRA, alone or in concert with CSF elevations, may serve as the signal for increasing the rate of granulocyte production. It is also possible that decreases in serum inhibitors may have facilitated the increased granulopoiesis seen in these studies, although the decreases which we observed were not statistically significant. Finally, the increased granulopoiesis noted in tolerant animals may be secondary to a direct action of endotoxin or an endotoxin-induced elevation of a humoral factor separate from CSF or NRA, such as the diffusible granulocytopoietic stimulator (DGS) described by Rothstein et al.

Both NRA and CSF are detectable in animals given cytotoxic drugs or endotoxin, and, at least after endotoxin, the increases in the two factors follow a similar time course. These observations led to the suggestion that these two factors might, in fact, represent different activities of a single molecule. The present observations, however, of a continued release of marrow granulocytes in the face of an absent CSF response to 5 μg of endotoxin suggest otherwise. The temporal discordance in the onset of tolerance to the CSF-elevating and granulocyte-releasing effects of endotoxin implies that CSF is not the mediator for granulocyte release. A corollary to this is that CSF and NRA are probably separate factors. Support for this contention is provided by the recent observations of Broxmeyer et al., who utilized two direct assays for NRA in the rat, and who were able to show that some sera without CSF activity had NRA activity.

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