Effects of Bacterial Infection and Irradiation on Serum Colony-stimulating Factor Levels in Tolerant and Nontolerant CF, Mice

By P. Quesenberry, H. Cohen, J. Levin, R. Sullivan, P. Bealmear, and M. Ryan

Previous studies of germ-free and conventional irradiated mice led to the hypothesis that the increased levels of serum colony-stimulating factor (CSF) observed after whole-body irradiation (WBI) might be due to endotoxemia. Other investigations showed that CF1 mice preinjected with Salmonella typhosa endotoxin became tolerant or resistant to the CSF-elevating effect of endotoxin. In the current studies, tolerant CF1 mice were subjected to 1000 rads WBI; they developed serum CSF elevations comparable to those seen in control irradiated mice. Tolerance was not abolished by 1000 rads WBI or reversed by infection with gram-negative or gram-positive bacteria. Changes in inhibitor levels did not explain these results. These data indicated that serum CSF elevations observed after WBI may not be due solely to endotoxemia, despite the observation that limulus-reactive material, presumably endotoxin, was detected in 61.5% of CF1 mice 1–12 days after 1000 rads. In these studies, 29.4% of normal pooled plasma samples also were limulus positive, suggesting that transient endotoxemia may occur in some strains of normal mice. Infection of CF1 mice with gram-negative or gram-positive bacteria caused serum CSF elevations 6–24 hr after infection; levels returned to baseline by 48–144 hr in all groups. The return to normal in serum CSF levels in mice infected with gram-negative or gram-positive bacteria coincided with the development of tolerance to the CSF-elevating effect of endotoxin. Infected mice developed marked increases in marrow granulopoiesis and continued to show evidence of accelerated marrow granulocyte release 6–144 hr after infection.

COLONY-STIMULATING FACTOR (CSF) is defined by its unique action in inducing myeloid progenitor cells to form granulocyte–monocyte–macrophage colonies in culture system in vitro and may be a humoral regulator of granulopoiesis.1,2 We have previously observed that conventional but not germ-free CFW mice, subjected to lethal whole-body irradiation (WBI), develop elevations of serum CSF despite the presence of profound leukopenia in both groups.3 Germ-free and conventional mice, previously exposed to WBI, both respond to intraperitoneal (i.p.) injections of endotoxin with marked increases in serum CSF levels, indicating that the ability of germ-free mice to elaborate CSF has not been impaired. Furthermore, changes in the levels of...

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serum inhibitors of colony growth do not appear to account for these results. Chang and Pollard have confirmed the above observations on serum CSF levels in irradiated germ-free CFW mice, and in addition, have shown that germ-free CFW mice monocontaminated with gram-negative Escherichia coli develop serum CSF elevations, while those monocontaminated with gram-positive Streptococcus faecalis do not. These data suggest that serum CSF elevations after WBI might be due to gastrointestinal injury with ensuing bacteremia and endotoxemia.

The administration of endotoxin to man or experimental animals has been observed to produce a variety of biologic effects, including fever, antibody formation, intravascular coagulation, complement activation, marrow granulocyte release, changes in the number of proliferative status of hemopoietic stem cells, and, in sufficient doses, death. When endotoxin is administered repeatedly, a resistance or tolerance to many of these effects develops. The mechanisms underlying this tolerance have not been defined clearly, but it appears that the type of tolerance induced may depend upon the biologic effect and species under investigation. Different authors have proposed that tolerance may be secondary to neutralization of endotoxin with antibody, inactivation of endotoxin by a serum or tissue factor, or rapid clearance of endotoxin by the reticuloendothelial system. Greisman et al. have presented evidence that there may be two types of endotoxin tolerance: an early tolerance occurring shortly after endotoxin administration which is not dependent upon a humoral factor, and a late form of tolerance which is dependent upon a humoral factor specific for the endotoxin utilized in tolerance induction.

We have observed that repeated injections of endotoxin to CF1 or C57BL/6J mice also lead to a resistance or tolerance to the serum CSF-elevating effects of endotoxin (endotoxin-CSF) which persists for up to 1 yr after 1 wk of daily injections. The mechanisms underlying this form of tolerance also are unclear, although studies from our laboratory indicate that it may be mediated by a long-lived radioresistant tissue cell not resident in the bone marrow or spleen. Studies of the response of CF1 mice preinjected with a Salmonella typhosa endotoxin and then challenged with a heterologous endotoxin (E. coli) show a complete cross-tolerance immediately after cessation of preinjections, but only a partial cross-tolerance at later time intervals, suggesting that there might also be an early and late form of tolerance to the CSF-elevating effects of endotoxin.

Since CF1 mice preinjected with endotoxin (tolerant mice) do not show serum CSF elevations after the administration of relatively large doses of endotoxin (5 μg), they provide an experimental model for assessing the possible role of endotoxin in irradiation-induced serum CSF elevations. If endotoxemia is the cause of serum CSF increases after WBI, than endotoxin-tolerant mice would not be expected to show serum CSF increases after WBI unless this tolerance is abolished by irradiation or influenced by irradiation-related bacterial infection. In the present communication, we have attempted to assess the role of endotoxemia in determining serum CSF levels of nontolerant and tolerant CF1 mice after WBI. We have also assessed the effect of WBI and bacterial infection on tolerance and have measured plasma endotoxin levels (as determined by the limulus lysate assay) in CF1 mice at various time intervals after irradiation. Finally, we have evaluated the effect of infection with gram-negative or gram-
positive bacterial organisms on serum CSF levels in normal and tolerant CF
mice, along with the granulopoietic response of normal CFmice to bacterial
infection.

MATERIALS AND METHODS

Virgin female CFmice (Carworth Farms), 12-16 wk old, were used throughout. S. typhosa
endotoxin (Difco No. 3940) was dissolved in saline immediately prior to use. Endotoxin-CSF
tolerance was induced as previously described. Daily intraperitoneal injections of endotoxin
(10 μg/day for 3-7 days) were administered to CFmice, which then were irradiated or infected
1-420 days later, dependent upon the particular experimental protocol. Mice were then sacrificed
at various time intervals after infection or irradiation, and serum was collected for CSF and
inhibitor assays. In some experiments, infected or irradiated control and tolerant mice were as-
essessed for their serum CSF or inhibitor response 3-4 hr after 5 μg S. typhosa endotoxin i.p.

Bacterial Infections

Virulent strains of bacteria were isolated from the blood or cerebrospinal fluid of patients at
Children's Hospital Medical Center, Boston, Mass. Trypticase soy broth and brain heart infusion
broth were obtained from Difco Laboratories. E. coli, Staphylococcus aureus, and Pseudomonas
aeruginosa were grown in trypticase soy broth; Hemophilus influenzae was grown in brain heart
infusion broth with DMP and defibrinated horse blood added, as described by Anderson et al., and
Diplococcus pneumoniae was grown in Todd-Hewitt medium. All organisms were grown at
37°C in a shaking water bath in a Nephloflask containing 10 ml of medium, with approximately
10⁶ organisms/ml. This concentration was estimated by a change of absorbance of 0.2 optical den-
sity (OD) at 490 nm. The cultures were then kept at 0-4°C and centrifuged at 14,000 g for 15
min in an RC 2B centrifuge. The supernatant was discarded and the bacteria were washed twice
with 10 ml of phosphate-buffered saline (PBS). PBS contained 8.1 g sodium chloride, 0.157 g
NaH₂PO₄·H₂O, and 1.3 g Na₂PO₄·H₂O per liter (pH 7.3 at 25°C). Various numbers of bac-
teria were suspended in PBS and were injected into the peritoneal cavity of the mice in a 0.1-ml
volume.

Bacterial quantification was performed by plating out the bacteria after diluting 10⁴ and
10³-fold and incubating at 37°C for 1 day. E. coli, P. aeruginosa, and Staph. aureus were
plated on trypticase soy agar, H. influenzae on brain heart infusate agar with DPN and defi-
brinated mouse blood, and D. pneumoniae on blood agar plates. Colonies were counted using a
colony counter from New Brunswick Scientific and the results given were the averages of 2-4
plates. Staph. aureus was heat killed by autoclaving at 20 lb steam pressure/sq inch for 12 min.

Irradiation

WBI mice were exposed to 1000 rads from a Cesium 137 irradiation unit with a dose rate of
110 rads/min, or to 850 rads from an x-ray source at a dose rate of 40 rads/min, as described
previously.

CSF Assay

We have utilized a modification of an in vitro double-layer agar culture technique. We cur-
rently employ a final concentration of fetal calf serum of 19%, but otherwise the method is as we
have reported previously. CSF dose-response curves were determined for serum samples by
assaying at various concentrations, and CSF activities were then compared on the linear part of
the dose-response curve. CSF activity was expressed as the number of colonies stimulated per
10⁵ murine cells ± 1 SEM. Colony morphology was determined by picking individual colonies,
staining them with acetoorcein, and then categorizing them as granulocyte (over 90% granulo-
cytes), macrophage (over 90% macrophages), or mixed (all others).

Inhibitor Assay

Serum inhibitors of colony growth were measured in an in vitro single-layer culture system. Serum samples or saline were mixed with an excess of CSF derived from either mouse lung-conditioned medium or serum obtained from mice 2-4 hr after intraperitoneal injection with...
endotoxin. The number of colonies formed in plates containing CSF and test sera was compared to the number formed in control plates with CSF and saline. The results were expressed as a percentage of control.

Differential Counts

Marrow and peripheral blood differential counts were determined as previously reported. An average of 98 cells/animal were counted for peripheral blood and 630 cells/animal for bone marrow differentials.

Limulus Lysate Assay

Heparinized samples of plasma were collected from irradiated (1000 rads) or control CF₁ mice by cardiac puncture under ether anesthesia. The chest wall of the mouse was exposed by surgical procedures and cardiac puncture performed. One aspiration was allowed per mouse. The plasma was separated by centrifugation and frozen at −20°C. These samples were tested for limulus-reactive materials using the limulus assay for endotoxin, as described by Levin et al.

Empty glass tubes, heparin, and syringes also were tested for limulus-reactive material, and if any were positive the data from that assay were discarded.

Statistical significance was determined utilizing the fourfold contingency table $\chi^2$ square with Yates' correction. When individual experiments are presented the SEM refers to the mean of at least triplicate culture plates; when results from two experiments are presented the SEM refers to the combined mean of these experiments representing six or more culture plates.

RESULTS

Effect of Tolerance on CSF Levels After Irradiation

Serum CSF levels in irradiated (1000 rads) and unirradiated (0 rad) CF₁ mice at various time intervals after induction of tolerance are presented in Fig. 1. Comparable serum CSF elevations were seen in both nontolerant and tolerant irradiated mice. The morphology of colonies stimulated by 4.8% serum obtained from tolerant or control mice 7 days after 1000 rads was similar. At least 100 colonies were analyzed for each group. Colonies stimulated by serum from tolerant irradiated mice were 52.7% macrophage, 25.5% granulocyte, and 21.8% mixed, while those stimulated by serum from control irradiated mice were 53.5% macrophage, 16.8% granulocyte, and 29.7% mixed.

Serum inhibitor levels were determined in the same experiments presented in Fig. 1 A and D. The inhibitor levels showed a marked scatter and a lack of correlation with serum CSF levels. Inhibitor levels also were determined in the experiment presented in Fig. 1 C, but in this instance, the sera, tested at a final concentration of 4.5% with a standard source of CSF (4.5% murine endotoxin serum), failed to show any inhibition.

Effect of Irradiation on Tolerance

The effect of 1000 rads WBI on endotoxin-CSF tolerance is demonstrated in Fig. 2. In these experiments, CF₁ mice were injected with either saline or endotoxin for 7 consecutive days. One day later, they were exposed to 1000 rads WBI or left unirradiated, and 8 days after irradiation they were challenged with either 5 μg endotoxin or saline and bled 4 hr later. The irradiated mice remained tolerant, showing a markedly subnormal serum CSF response to challenge with endotoxin. These findings were confirmed in two additional experiments, in which mice were bled at 8 and 12 days after 1000 rads WBI. In three other experiments, CF₁ mice were irradiated (1000 rads) at 21 days, 4 mo,
CSF LEVELS: BACTERIA AND IRRADIATION

Fig. 1. Serum CSF values: mean number of colonies/10^4 cells ± 1 SEM, stimulated by 4.8% serum from unirradiated or irradiated tolerant and control CF1 mice at various time intervals after 0 or 1000 rads WBI. Control and tolerant mice were injected daily for 7 days with saline or endotoxin, respectively, and 1 day after the last injection they were subjected to 1000 R WBI or no irradiation. (A) CSF values in serum from unirradiated mice were determined on day 1 and after the conclusion of the injection schedule; a mean control value ± 1 SEM was calculated. (B)–(D) Serum from unirradiated tolerant and nontolerant mice were collected daily and CSF levels were determined. Data at each time point were derived from pooled serum samples from 4–6 CF1 mice. Day 0 represents the time of irradiation.

and 14 mo after tolerance induction and tolerance was assessed 8 days after irradiation. In each instance, tolerance persisted despite irradiation.

Endotoxin–CSF tolerance also was inducible in mice which had been subjected to 1000 rads WBI. Table 1 presents a representative experiment in which CF1 mice were subjected to 1000 rads WBI and then, starting 5 days later, received three consecutive daily i.p. injections of 10 μg endotoxin (total of 30 μg). Twenty-four hours after the end of the injection schedule (9 days after irradiation), they were challenged with 5 μg endotoxin or 0.1 ml saline i.p. and were sacrificed 4 hr later. It was apparent that mice became tolerant despite having previously been subjected to 1000 rads WBI. These results were confirmed in
Fig. 2. CF₁ mice were injected with 10 µg S. typhosa endotoxin (tolerant) or 0.1 ml saline (control) daily for 7 days; 1 day later they were subjected to 1000 rads WBI or no irradiation; 8 days later they were challenged with 5 µg endotoxin or 0.1 ml saline i.p. 4 hr prior to sacrifice. Serum CSF levels: mean number of colonies/10⁵ cells ± 1 SEM, stimulated by 2.4% sera. Data are mean values from two experiments; each pooled serum sample in each experiment was derived from 4 or 5 mice.

Effects of Gram-negative and Gram-positive Infections on CSF Levels and Tolerance

The above experiments indicated that tolerant mice developed true serum CSF elevations after 1000 rads WBI and that tolerance was not abolished by exposure to 1000 rads WBI. In the next series of experiments, we evaluated the effects of gram-negative or gram-positive bacterial infections on serum CSF levels, endotoxin responsiveness, and tolerance. The serum CSF levels of CF₁ mice at various times after i.p. infection with either gram-negative or gram-positive bacteria are shown in Fig. 3. The number of bacteria injected i.p. and the mortality produced by these infections are presented in Table 2. Both gram-negative and gram-positive infections induced serum CSF increases in CF₁ mice, but gram-negative infections usually produced higher levels of CSF. In general, increasing numbers of bacteria led to higher serum CSF levels. Lethality also increased with increasing numbers of bacteria and tended to parallel serum CSF levels, although marked CSF elevations were seen with H. influenzae infections which resulted in no mortality. It was of interest that in one experiment in which tolerant and control mice were infected with 1 x 10⁶ E. coli (Table 2, part C), the tolerant state appeared to provide significant protection against the lethal effects of the infection. These data also show that four other separate experiments: one identical to the above, two in which mice were bled 2 hr after challenge rather than 4 hr, and one in which they received endotoxin or saline injections for 4 days rather than 3. In each experiment, the irradiated mice developed tolerance to the serum CSF-elevating effects of endotoxin.

### Table 1. Induction of Tolerance in Irradiated Mice

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Preinjections (× 3)</th>
<th>Challenge</th>
<th>CSF (CFC/10⁵)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No irradiation</td>
<td>Saline</td>
<td>Saline</td>
<td>0.3 ± 0.3</td>
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<tr>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
<td>43 ± 6</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Saline</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Endotoxin</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>1000 rads WBI</td>
<td>Saline</td>
<td>Saline</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
<td>50 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Saline</td>
<td>3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Endotoxin</td>
<td>2 ± 0.9</td>
</tr>
</tbody>
</table>

*Mean number of colonies per 10⁵ marrow cells ± 1 SEM, stimulated by 2.4% sera.
Fig. 3. Serum CSF levels in CF1 mice at various time intervals after i.p. bacterial infection. CF1 mice were injected with various numbers of gram-negative or gram-positive bacteria or with PBS. Sera at each time point in each experiment were pooled from 3-5 mice. Mean number of colonies/10^5 cells ± 1 SEM, stimulated by 2.4%-4.8% sera.

Serum CSF levels returned to normal by 48 hr in all infected groups, regardless of the severity (as determined by lethality) of the infections. Inhibitor assays were carried out on the samples included in Fig. 3A; the sera were assayed at 4.8% with 9% lung conditioned medium as a source of CSF. No inhibition was demonstrable. The absence of any demonstrable inhibition at a concentration of serum which was twice that utilized for the CSF assay suggests that changes in inhibitor levels did not influence the results of the CSF assay.

We evaluated whether endotoxin-tolerant mice would respond to bactericidal infection with increases in serum CSF levels, and what effect bacterial infection would have on endotoxin tolerance (Table 3). Mice received three consecutive daily injections of endotoxin (tolerant) or saline (nontolerant), and then 1-2 days later these mice were injected i.p. with PBS, E. coli, H. influenzae, Staph. aureus, or heat-killed (HK) Staph. aureus. At various times after infection, these mice were challenged with 5 μg endotoxin or 0.1 ml saline i.p., and their serum CSF levels were determined 3-3½ hr after challenge. Serum CSF levels in saline-injected nontolerant and tolerant infected mice are presented in columns 3 and 4 of Table 3. Serum CSF levels at 9 and 26-27 hr after infection were lower in the tolerant mice infected with E. coli or Staph. aureus as compared to levels in control mice infected with the same organisms.

Serum CSF levels in the endotoxin-injected nontolerant and tolerant infected mice are presented in the last two columns of Table 3. It is apparent that endotoxin-preinjected mice remained tolerant at each time point evaluated after infection (9-75 hr) as shown by their lower serum CSF levels in response to endotoxin as compared to nontolerant infected mice. Furthermore, serum
Table 2. Mortality of CF1 Mice Infected With Bacteria

<table>
<thead>
<tr>
<th>i.p. Injection*</th>
<th>No. of Bacteria Injected</th>
<th>No. D/dead/No. at Risk (%): 1 hr</th>
<th>No. D/dead/No. at Risk (%): 6 hr</th>
<th>No. D/dead/No. at Risk (%): 24 hr</th>
<th>No. D/dead/No. at Risk (%): 48 hr</th>
<th>No. D/dead/No. at Risk (%): 72 hr</th>
<th>No. D/dead/No. at Risk (%): 144 hr</th>
<th>Total No. Dead</th>
</tr>
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<td>A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>PBS</td>
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<td>E. coli</td>
<td>$1.6 \times 10^5$</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>18</td>
<td>0</td>
<td>0</td>
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<td>H. influenzae</td>
<td>$5.4 \times 10^6$</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>P. aeruginosa</td>
<td>$6.3 \times 10^5$</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>9</td>
<td>67</td>
<td>0</td>
<td>7</td>
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<tr>
<td>Staph. aureus</td>
<td>$2.2 \times 10^6$</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>D. pneumoniae</td>
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<td>E. coli</td>
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<td>0</td>
<td>0</td>
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<td>H. influenzae</td>
<td>$4.6 \times 10^7$</td>
<td>0</td>
<td>0</td>
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<td>Staph. aureus</td>
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<td>8</td>
<td>11</td>
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<td>D. pneumoniae</td>
<td>$1.2 \times 10^7$</td>
<td>0</td>
<td>10</td>
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<td>25</td>
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<td>94</td>
<td>0</td>
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<td>0</td>
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<td>20</td>
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<tr>
<td>E. coli†</td>
<td>$1 \times 10^6$</td>
<td>0</td>
<td>13</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Staph. aureus</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*In parts A and B (Fig. 5) each bacteria (or PBS) was injected into a different group of 30 mice, and in part C, each group had 24–25 mice; 5 mice were sacrificed at each time point to determine CSF levels.

†Tolerant CF1 host mice; all other groups were normal CF1 mice.

Table 3. Serum CSF Levels in Nontolerant and Tolerant CF1 Mice Infected With Gram-negative or Gram-positive Bacteria and Challenged 3–34 hr Prior to Sacrifice With i.p. Endotoxin or Saline

<table>
<thead>
<tr>
<th>i.p. Injection*</th>
<th>Hours After i.p. Injection</th>
<th>CSF Levels (CFC/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline 0.1 ml Endotoxin 5 μg</td>
<td>Nontolerant</td>
</tr>
<tr>
<td>PBS</td>
<td>9</td>
<td>1.5 ± 1.5 1 ± 1</td>
</tr>
<tr>
<td>E. coli</td>
<td>121 ± 3 3 ± 2</td>
<td>159 ± 261 31 ± 0</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>31 ± 7 1 ± 0</td>
<td>140 ± 45 20 ± 9</td>
</tr>
<tr>
<td>PBS</td>
<td>26–27</td>
<td>4.5 ± 4.5 1.5 ± 1.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>91 ± 65 1.9 ± 1.1</td>
<td>21 ± 2 3 ± 2</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>1 ± 0.7 0.4 ± 0.4</td>
<td>70 ± 36 8 ± 6</td>
</tr>
<tr>
<td>H. influenzae†</td>
<td>2 ± 2 1.7 ± 0.3</td>
<td>55 ± 15 1 ± 1</td>
</tr>
<tr>
<td>PBS</td>
<td>74–75</td>
<td>0.4 ± 0.4 0.7 ± 0.7</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.3 ± 0.4 0</td>
<td>41 ± 31 6 ± 6</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>1.5 ± 1.5 0.85 ± 0.85</td>
<td>75 ± 30 1.5 ± 1.5</td>
</tr>
<tr>
<td>H. influenzae†</td>
<td>2 ± 2 1.7 ± 0.3</td>
<td>54 ± 13 7 ± 1.5</td>
</tr>
<tr>
<td>HK Staph. aureus†</td>
<td>1 ± 1 0</td>
<td>71 ± 4 2.3 ± 0.3</td>
</tr>
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</table>

Sera were collected from 4 or 5 mice for each time point in each experiment.

* $1 \times 10^6$ E. coli, $1 \times 10^7$ Staph. aureus, $1 \times 10^7$ H. influenzae, or 0.1 ml PBS.
†Mean number of colonies per 10^5 marrow cells ± 1 SEM, stimulated by 2.4% serum.
‡Data from one experiment; all other data were derived from two experiments.
Table 4. Serum CSF Levels in CF1 Mice Infected at Prolonged Time Intervals After Tolerance Induction and Then Challenged With either Saline or Endotoxin 3–34 hr Prior to Sacrifice

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Injection</th>
<th>Nontolerant CSF Levels (CFC/10^5)</th>
<th>Tolerant CSF Levels (CFC/10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>0.7 ± 0.3</td>
<td>169 ± 10</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>35 ± 4</td>
<td>39 ± 1</td>
</tr>
<tr>
<td></td>
<td>Staph. aureus</td>
<td>12 ± 2</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>PBS</td>
<td>1.0 ± 0</td>
<td>97 ± 9</td>
</tr>
<tr>
<td></td>
<td>H. influenzae</td>
<td>1.3 ± 0.9</td>
<td>40 ± 8</td>
</tr>
</tbody>
</table>

Mice were injected with PBS, E. coli (1 x 10^6 organisms), H. influenzae (1 x 10^6 organisms), or Staph. aureus (1 x 10^7 organisms) 26–27 hr prior to sacrifice; 3–34 hr prior to sacrifice these mice were injected with either 0.1 ml saline or 5 μg endotoxin i.p. Data are expressed as the mean number of colonies per 10^5 cells ± 1 SEM, stimulated by 2.4% sera. Nontolerant and tolerant mice were preinjected with 0.1 ml saline or 10 μg endotoxin, respectively, i.p. daily for 7 days, 2–4 mo prior to sacrifice.

CSF response to endotoxin was actually decreased in both the nontolerant and tolerant mice at 26–27 and 74–75 hr after i.p. injection of E. coli, Staph. aureus, H. influenzae, or HK Staph. aureus as compared to the CSF response to endotoxin of noninfected, nontolerant and tolerant mice injected i.p. with PBS. This finding suggests that infection with gram-negative or gram-positive bacteria, rather than reversing endotoxin tolerance, may actually induce it.

As noted above, evidence has been provided that the tolerance observed immediately after a series of endotoxin injections (immediate tolerance) may be different from that seen at longer time intervals after tolerance induction (delayed tolerance). We thus evaluated the endotoxin–CSF responsiveness of delayed tolerant mice which had been infected 2–4 mo after tolerance induction with either gram-negative or gram-positive bacteria (Table 4). Again, endotoxin–CSF tolerance appeared to be induced by infection of the nontolerant control mice. Infection of the delayed tolerant mice either did not affect the CSF response to endotoxin or appeared to decrease it as compared to that seen with delayed tolerant mice injected with PBS. In one additional experiment, delayed tolerant (6 mo after tolerance induction) and nontolerant mice were injected i.p. with PBS, or 1 x 10^7 or 5 x 10^7 Staph. aureus, and the serum CSF levels were determined 6 hrs after i.p. injection. Delayed tolerant mice appeared to be hyporesponsive to the CSF-elevating effect of infection with Staph. aureus (Table 5).

Table 5. Serum CSF Levels in CF1 Mice Infected With Staph. aureus 6 mo After Tolerance Induction

<table>
<thead>
<tr>
<th>Injection</th>
<th>CSF Levels (CFC/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nontolerant</td>
</tr>
<tr>
<td>PBS</td>
<td>9.3 ± 1.9</td>
</tr>
<tr>
<td>Staph. aureus, 1 x 10^7</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Staph. aureus, 5 x 10^7</td>
<td>35 ± 2</td>
</tr>
</tbody>
</table>

Control and tolerant mice were preinjected i.p. for 5 days with either saline or 10 μg endotoxin; 6 mo later they were injected i.p. with PBS or Staph. aureus and then sacrificed 6 hr later. Results are expressed as the mean number of colonies per 10^5 cells ± 1 SEM, stimulated by 4.8% sera.
We also evaluated normal (nontolerant) mice at various times after lethal WBI for the presence of endotoxin in their plasma, as determined by the limulus lysate assay (Table 6). Limulus-reactive material was detected in the plasma of 61.5% of the irradiated mice and in 29.4% of the normal unirradiated controls. This difference was significant at $p < 0.001$.

**Effects of Gram-negative and Gram-positive Infections on Peripheral and Bone Marrow Differential Cell Values**

The absolute peripheral blood and bone marrow differential cell values at 6, 48, 72, and 144 hr after infection with gram-positive or gram-negative bacteria were determined in two experiments. Peripheral blood polymorphonuclear leukocytes (PMN) and bands were markedly increased at 6 hr and remained variably elevated in most groups for 144 hr, while peripheral blood lymphocyte values were depressed at 6 hr in all groups and generally returned to or above control values by 72–144 hr. The marrow values from the experiment presented in Fig. 3B are presented in Fig. 4. Marrow mature granulocytes (PMN) were markedly depressed at 6 hr and variably decreased thereafter, indicating a release of marrow granulocytes, while marrow granulopoiesis was stimulated at 48–144 hr after infection.

**DISCUSSION**

Previous studies, referred to above, have shown that conventional but not germ-free mice develop serum CSF elevations after lethal WBI. These data,
along with the observations that both irradiated and unirradiated germ-free and conventional mice responded to endotoxin with marked and comparable elevations of serum CSF, suggested that the serum CSF elevations seen after irradiation might be related to irradiation-induced gastrointestinal injury with ensuing endotoxemia and/or bacteremia.\textsuperscript{3,4} The present data, which demonstrate that both gram-negative and gram-positive infections caused serum CSF elevations in CF\textsubscript{1} mice (Fig. 3), also are consistent with this hypothesis, as are the observations that a significant percentage (61.5\%) of irradiated CF\textsubscript{1} mice had detectable limulus-reactive material, presumably endotoxin, in their plasma (Table 6).

However, the findings that tolerant CF\textsubscript{1} mice, which are resistant to the CSF-elevating effects of relatively large amounts of endotoxin (5 \( \mu \)g), developed serum CSF elevations after irradiation comparable to those seen in irradiated nontolerant CF\textsubscript{1} mice (Fig. 1) suggest that endotoxemia may not be a prerequisite for the serum CSF elevations seen in irradiated mice. The administration of 5 \( \mu \)g of endotoxin to normal or irradiated CF\textsubscript{1} mice reproducibly resulted in increases in serum CSF levels far in excess of those seen after irradiation alone. If the sole cause of serum CSF elevations in irradiated mice were endotoxemia, it would follow that irradiated mice probably develop lower serum levels of endotoxin than do irradiated or normal mice injected i.p. with
5 μg endotoxin. Accordingly, irradiated mice in which tolerance to the CSF-elevating effect of 5 μg endotoxin i.p. has been produced would not be expected to respond with serum CSF elevations to the presumably lower levels of endogenous endotoxemia seen after irradiation. Thus, the observation of comparable serum CSF increases in tolerant and control irradiated mice suggests that some factor other than endotoxin may have produced these serum CSF increases, at least in the irradiated tolerant mice.

It remains possible, of course, that although following irradiation endotoxin levels are lower, endotoxemia could be more prolonged, thereby maximizing biologic effect. Other possible explanations of these results include alterations in serum inhibitor levels or reversal of tolerance by irradiation. These have been effectively ruled out by our data (in text and Fig. 2), and, in fact, tolerance could be induced after irradiation (Table 1).

Another possible explanation of these data, consistent with endotoxemia as the proximate cause of serum CSF elevations in irradiated mice, is that bacterial infection may have either reversed tolerance or induced a relative hypersensitivity to the CSF-elevating effects of endotoxin. Studies of humans rendered tolerant to the pyrogenic effects of endotoxin have shown that experimentally induced bacterial infection results in hyperreactivity to the pyrogenic effects of endotoxin, although tolerance (relative to infected nontolerant humans) persists.34,35 When these subjects are treated with appropriate antibiotics, the infections clear and hyperreactivity to the pyrogenic effects of endotoxin is no longer present. Our studies in nonirradiated CF1 mice show that bacterial infection, rather than inducing hyperreactivity or reversing tolerance, is actually capable of inducing endotoxin–CSF tolerance (Tables 3–5). The latter studies on the effect of infection have been carried out with unirradiated tolerant mice, and conceivably, different results might have been obtained if irradiated tolerant mice had been evaluated. However, in toto, the above data suggest that endotoxemia is probably not the sole cause of irradiation-induced serum CSF elevations. Considering the established relationship, in irradiated mice, between host bacterial flora and serum CSF levels,33 it seems most likely that endotoxin is one of a number of bacterial substances which determine serum CSF levels in irradiated mice.

The present data show that limulus-reactive material, presumably endotoxin, was demonstrable in the plasma of lethally irradiated mice (Table 6). However, positive limulus tests also were found in a small proportion of samples from normal control mice. Although significantly more samples from irradiated CF1 mice were positive, as compared to samples from the normal controls, the positive samples in the control groups require explanation. One possibility is that there was extraneous contamination of some samples with endotoxin. Our methods were structured to minimize this possibility, and results were utilized only for those assays in which all glassware and heparin samples were limulus-negative. Alternatively, these results could be interpreted as indicating that occasional normal CF1 mice have transient endotoxemia. Our samples consisted of pooled plasma from 6–10 mice, and therefore only 1 endotoxemic animal per pool could have produced a positive sample. As a result, a relatively small number of endotoxemic mice theoretically could have accounted for the positive control samples.
As noted above, infection with gram-negative or gram-positive bacteria induced serum CSF elevations (Fig. 3). The serum CSF levels returned toward baseline values by 48 hr in infected mice, regardless of the severity of the infection. The pattern of CSF elevations was similar to that observed when CF<sub>1</sub> mice are repeatedly injected with endotoxin. In the latter situation, there is a progressive decrease in the peak serum CSF levels after consecutive injections of endotoxin secondary to the development of tolerance; this results in a pattern of serum CSF elevations in which levels are quite high for the first several days and thereafter are only slightly elevated.

The present data indicate that a similar type of endotoxin-CSF tolerance develops in response to infection with gram-negative bacteria (Tables 3 and 4) and this possibly explains the eventual decrease in serum CSF levels observed in the infected mice. The development of endotoxin tolerance is not surprising since infection with gram-negative bacterial organisms represents a continued exposure to endotoxin. It is, however, puzzling that infection with a gram-positive organism (*Staph. aureus*) resulted in the development of a resistance to the serum CSF-elevating properties of endotoxin, similar to that seen in endotoxin preinjected animals. The question of whether or not the hyporesponsiveness to the CSF-elevating effects of endotoxin seen in *Staph. aureus* infected mice is the same phenomenon as that seen in mice preinjected with endotoxin is not answered by the present data. The observation that endotoxin-tolerant mice (delayed or immediate) are also hyporesponsive to the CSF-elevating effects of infection with *Staph. aureus* suggests that "*Staph. aureus*-CSF tolerance" may be closely related to endotoxin-CSF tolerance, if not the same phenomenon. These data can be interpreted as suggesting that "tolerance" to the CSF effects of endotoxin may not be specific for endotoxin but may extend to a variety of bacterial and antigenic substances.

These findings also could be explain by contamination of the *Staph. aureus* isolates with endotoxin. In the experiment presented in Table 5 the *Staph. aureus* isolate was tested for endotoxin by the limulus lysate assay. Reports from many laboratories have confirmed that fungi and gram-positive organisms, including *Staph. aureus* at concentrations of 10<sup>8</sup>–10<sup>9</sup>/ml, do not react with limulus lysate. However, the isolate had low levels of detectable limulus-reactive material equivalent to approximately 0.1 µg/ml of *E. coli* endotoxin. Thus, mice utilized in the experiment presented in Table 5 would have received a total of 0.01 µg of endotoxin when injected i.p. with 0.1 ml of the *Staph. aureus* suspension. Therefore, in separate experiments, it was determined that i.p. injection of CF<sub>1</sub> mice with 0.01 or 0.1 µg *S. typhosa* endotoxin neither increased CSF levels nor induced CSF tolerance 24 hr after injection, so that endotoxin contamination does not appear to provide a sufficient explanation for these results. One other possible explanation for the apparent capacity of i.p. infection with *Staph. aureus* to induce refractoriness to endotoxin is that gram-positive infection resulted in gastrointestinal tract damage with subsequent endogenous endotoxemia and the development of endotoxin-CSF tolerance. We have no data bearing directly on this point.

Alterations of peripheral blood and marrow differential cell values clearly show that marrow granulopoiesis was stimulated by infection with either gram-positive or gram-negative bacteria, and that a continuing release of marrow
granulocytes occurred with both types of infections. (Fig. 4). This observation is consistent with previous reports that endotoxin and gram-positive bacterial infection release marrow granulocytes.

The present studies do not deal directly with the mechanisms of endotoxin-CSF tolerance. However, studies of the effect of 1000 rads WBI on tolerance (Fig. 2) show that the phenomenon is radioresistant, and, as noted above, other studies have suggested that this type of tolerance may be mediated by a long-lived radioresistant tissue cell. Furthermore, the observation that serum CSF levels were elevated to a comparable degree in both nontolerant and tolerant irradiated mice indicates that endotoxin-CSF tolerance does not represent a nonspecific exhaustion of body CSF reserves, but rather relates to a specific refractoriness to the effects of endotoxin and possibly other bacterial products.

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Effects of bacterial infection and irradiation on serum colony-stimulating factor levels in tolerant and nontolerant CF1 mice

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