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A Method for the Removal of Endotoxin from Purified Colony-Stimulating Factor (40821)[1]

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Bacterial endotoxins have a number of diverse effects on hemopoiesis. These include changes in peripheral blood and bone marrow neutrophil populations (1) and profound influences on various classes of marrow and splenic stem cell populations (2–5). For these reasons, studies of hemopoiesis, which require injection of biologic materials, should be carefully controlled for contamination by endotoxin.

Recent studies have shown that many sources of erythropoietin are heavily contaminated with endotoxin (6, 7). Some of the effects of erythropoietin preparations on erythropoiesis, may be due to bacterial lipopolysaccharides (5, 7–9). These considerations are of greater importance when evaluating changes in granulopoiesis. Endotoxin induces changes in peripheral blood neutrophil counts (1), marked stimulation of marrow granulopoiesis (10), and increased release of colony-stimulating factor (CSF), (11, 12) a candidate granulopoietin.

In preparation for *in vivo* studies with purified murine CSF (13) we have devised techniques for removing substantial quantities of endotoxin from these preparations. In addition, studies were conducted in mice using low doses of endotoxin to determine levels of this contaminant which would not influence parameters of bone marrow or splenic hemopoiesis.

Materials and methods. Murine CSF was prepared by the growth of L-cells (murine fibroblasts) in serum-free medium (13). The resultant conditioned medium was concentrated by ultrafiltration, precipitated by ice-cold ethanol, and applied to a column of concanavalin-A Sepharose. Adherent CSF (which represented two-thirds of the total activity) was eluted with α-methylglucoside and subsequently passed through Sephadex G-150. In the final purification step, CSF was separated from trace contaminants by ultracentrifugation using a 5 to 15% sucrose gradient (13). Individual fractions were obtained by puncturing the bottom of the separation tube and collecting samples by gravity. The CSF appeared homogeneous following electrophoresis in various concentrations of acrylamide or sodium dodecyl sulfate–acrylamide gel and after Ouchterlony gel diffusion. The specific activity of the pure CSF was increased 1000-fold to 5×10^7 units per milligram of protein (13). The final material was diluted in 0.1 *M* Tris HCl, pH 7.4, containing 0.3% polyethylene glycol 4000 as a stabilizing agent.

All glassware used in these studies was thoroughly cleansed with detergent (E-Toxa-Clean, Sigma Chemical Co.) and rinsed 10 times with Water for Injection (Abbott Laboratories). It was sterilized by autoclaving at 132°C, 15 psi, for 30 min and rendered free of pyrogens by exposure to 180°C dry heat for 2 hr. Plastic Millipore units (Swinnex-Millipore Corp.) and centrifuge tubes (Beckman Corp.) were soaked in polymyxin solution (Burroughs–Wellcome), 200 units/ml, for 1 hr and then washed and rinsed as above. After rapid drying at 60°C, they were sterilized by autoclaving or by exposure to ethylene oxide (14). Samples of glassware and centrifuge tubes were rinsed with Water for Injection and tested for endotoxin contamination to assure the validity of these washing and sterilization techniques. Buffers

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were prepared by addition of the appropriate materials to Water for Injection. After filtration through 0.45-μm Millipore filters, they were autoclaved for 30 min.

Endotoxin activity was detected by the use of the limulus lysate assay (15, 16). Limulus lysate, prepared in the laboratory of one of the authors (J.L.), was mixed in equal volumes with dilutions of the buffers or sources of CSF. The mixtures were incubated at 37°C for 4 hr, and then at room temperature for an additional 20 hr. Gelation, increased viscosity, or marked flocculation was interpreted as a positive result. *Escherichia coli* endotoxin (Lipopolysaccharide B, 055:B5, Difco) was used as a standard. In most experiments, gelation was produced with 0.001 μg/ml and flocculation with 0.0001 μg/ml of the reference endotoxin. Endotoxin concentrations were derived from observations which included both the rate and extent of gelation of the lysate (15, 16).

Further studies were done to determine whether minute quantities of endotoxin had an effect on *in vivo* hemopoiesis. Groups of CF_1 mice were injected IP with 0.01 or 0.1 μg of *E. coli* endotoxin every 6 hr for intervals up to 8 days. Serum was obtained by cardiac puncture and assayed for CSF using a sensitive radioimmunoassay (17). Bone marrow and splenic cell suspensions were prepared as previously described (18). They were assayed for granulocytic (CFU-C) (19) and erythroid (CFU-E) (20) colony-forming cells by standard techniques. All values were obtained using five animals per point.

Results. The initial limulus assays performed on two pools of purified CSF are shown in Table I. Both CSF preparations were markedly contaminated with endotoxin whereas the Water for Injection and Tris buffer showed low levels of activity.

Since most endotoxins have a molecular weight in excess of 10^6 daltons (21) and CSF has an apparent molecular weight of 65,000–70,000 daltons (13), further studies were undertaken to separate these substances by density gradient centrifugation. Following appropriate cleansing and sterilization, gradients were loaded with 10 μg of *E. coli* endotoxin, 200 μg of CSF, or Tris buffer. The tubes were centrifuged at 100,000*g* for 18 hr at 4°C. In these experiments, the recovery technique was modified to avoid recontamination of the CSF by the high molecular weight endotoxin; gradients were aspirated from the top of the tube rather than by downward displacement.

TABLE I. ENDOTOXIN CONTENT OF PURIFIED CSF[a]

Material	μg/ml
Pool 4 CSF	>10
Pool 5 CSF	>1
Water for Injection	0.0001
Tris buffer	0.0005

[a] Values were derived from limulus assays using 10- to 10,000-fold dilutions of the test materials.

Results of limulus assays of the top half and bottom half of the gradients are shown in Table II. Greater than 99% of the detectable *E. coli* endotoxin sedimented to the lower half of the gradient. Only 52% of the endotoxin was recovered, a discrepancy probably due to the marked dilution necessary for assay. Importantly, the pure CSF activity was recovered in the top half of the gradient whereas 95% of the endotoxin was detected in the bottom half of the tube. Samples from the buffer gradient had only minimal endotoxin activity in either fraction. To verify these findings, three additional pools of purified CSF were subjected to density gradient centrifugation and assayed for CSF and endotoxin. As before, all CSF was found in the upper half of the gradients. Endotoxin activity was markedly reduced; concentrations in the upper half ranged between 0.001 and 0.0001 μg/ml.

Further studies explored the effect of trace levels of endotoxin on hemopoiesis. Repetitive injections of 0.01 μg of endotoxin had only minimal effects on splenic CFU-C or CFU-E (Figs. 1 and 2). In contrast, 0.1 μg of endotoxin caused a significant early increase in these progenitor cells. Despite repeated administration of endotoxin, both CFU-C and CFU-E returned to control levels by Day 8. The effect on bone marrow colony-forming cells is shown in Table III. There was considerable variation in marrow CFU-E in all three treatment groups but no discernable dose-related ef-

TABLE II. THE EFFECT OF DENSITY GRADIENT CENTRIFUGATION ON DISTRIBUTION OF ENDOTOXIN ACTIVITY[a]

Material	Portion of gradient	μg/ml	Volume (ml)	Total endotoxin (μg)
Endotoxin	Upper half	0.00075	30	0.02
Endotoxin	Lower half	0.75	7	5.25
CSF	Upper half	0.05	30	1.5
CSF	Lower half	1.0	30	30.0
Tris buffer	Upper half	0.001	30	0.03
Tris buffer	Lower half	0.001	30	0.03

[a] Values were derived from limulus assays using 10- to 10,000-fold dilutions of the test materials.

fect from endotoxin. A modest increase in marrow CFU-C was seen on Days 6 and 8 in those animals treated with 0.1 μg but not with 0.01 μg of endotoxin.

Serum CSF levels were obtained on pooled sera from each treatment group. Two uninjected control groups on Days 1 and 8 yielded 840 and 790 units of CSF/milliliter, respectively. As shown in Table IV, sera from buffer injected animals contained from 480 to 1240 units/ml of CSF. A similar range in activity was seen in animals that received 0.01 μg of endotoxin. High dose (0.1 μg) endotoxin recipients had an acute rise in serum CSF at 6 hr (1720 units/ml) but no further increase in activity during the course of the experiment.

The effect of various doses of endotoxin on CSF activity was evaluated in a repeat study. Groups of five mice each were injected with buffer, 0.001, 0.01, or 0.1 μg of endotoxin and bled 6 hr later. Each serum was individually assayed for CSF. Although values were higher than in the previous study, the same trend was apparent. Recipients of 0.001 and 0.01 μg of endotoxin yielded 1240 ± 63 (±1 SE) and 1400 ± 98 units/ml which did not vary from buffer-treated mice (1236 ± 51 units/ml). In contrast, mice injected with 0.1 μg of endotoxin showed a significant increase in activity 1828 ± 163 units/ml).

Discussion. These studies show that samples of purified L-cell CSF can contain

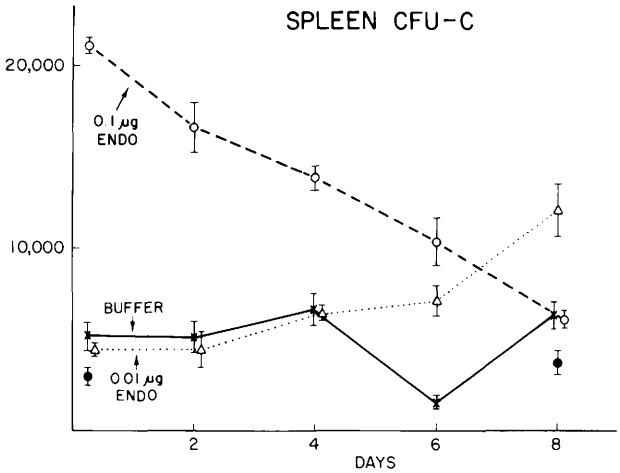

FIG. 1. The splenic CFU-C response to repeated injections of endotoxin. Groups of five mice each were injected ip with endotoxin or endotoxin-poor buffer every 6 hr for intervals up to 8 days. Pooled spleen cell suspensions were assayed for CFU-C using 10^6 cells per culture plate. Five plates each were used for each datum point. Values are the means ± one standard error for total splenic CFU-C content. Uninjected controls also were evaluated on Day 0 and Day 8 and are shown in solid circles.

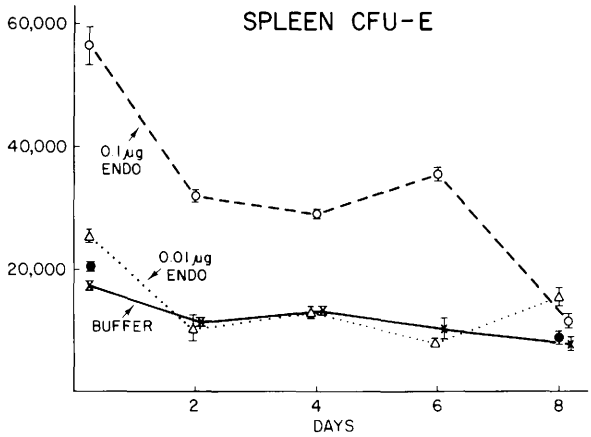

FIG. 2. The splenic CFU-E response to repeated injections of endotoxin. Groups of five mice each were injected ip with endotoxin or endotoxin-poor buffer every 6 hr for intervals up to 8 days. Pooled spleen cell suspensions were assayed for CFU-E using 10^6 cells per culture plate. Five plates each were used for each datum point. Values are the means ± one standard error for total splenic CFU-E content. Uninjected controls also were evaluated on Day 0 and Day 8 and are shown in solid circles.

substantial quantities of endotoxin. Since the molecular weight of bacterial endotoxin is in the range of 10^6 daltons, a variety of procedures such as column chromatography, gel electrophoresis, or density gradient centrifugation would be expected to separate this contaminant from CSF. However, as shown in these studies, care must be taken in the collection of fractions. Although CSF is readily separated from endotoxin by density gradient centrifugation, previous purification schemes which collected samples by gravity (13) led to recontamination of the CSF fractions. As shown in the present studies, simple aspiration of the top half of the gradient obviated this problem.

The CSF samples used in these studies were derived from 10-liter pools of serum free conditioned media; each contained approximately 10^7 units of CSF activity. Using the technique outlined herein, endotoxin activity in these samples was reduced to 0.0001 to 0.05 μg/ml. Although this still represents low-level contamination, the large quantity of CSF in each pool

TABLE III. BONE MARROW PROGENITOR CELL RESPONSE TO REPETITIVE INJECTIONS OF ENDOTOXIN[a]

Treatment	6 hr	Day 2	Day 4	Day 6	Day 8
			CFU-E/Femur		
Buffer	18,445 ± 1,356	24,664 ± 818	37,490 ± 3,450	37,268 ± 1,635	25,108 ± 2,509
Endo 0.01 μg	19,059 ± 2,458	24,016 ± 1,017	20,818 ± 1,420	16,974 ± 706	32,872 ± 4,508
Endo 0.1 μg	10,569 ± 536	22,651 ± 518	24,346 ± 1,382	43,963 ± 1,134	28,811 ± 1,132
Untreated controls	15,944 ± 203	nd	nd	nd	18,017 ± 1,957
			CFU-C/Femur		
Buffer	29,917 ± 2,810	35,736 ± 2,157	41,216 ± 5,002	38,236 ± 5,611	44,237 ± 2,946
Endo 0.01 μg	35,230 ± 2,505	44,161 ± 3,426	46,216 ± 2,279	36,147 ± 1,058	47,824 ± 5,880
Endo 0.1 μg	25,584 ± 3,471	43,850 ± 3,866	43,469 ± 7,795	55,742 ± 5,101	60,098 ± 1,721
Untreated control	23,955 ± 7,082	nd	nd	nd	40,248 ± 3,117

[a] Values represent mean total femoral CFU-E or CFU-C ± 1 S.E. on the indicated days of treatment. Five animals per point were injected I.P. every 6 hr for the duration of the experiment, with Tris–buffer, 0.01 μg or 0.1 μg of *E. coli* endotoxin (Endo). Untreated controls were evaluated only on Days 0 and 8.

TABLE IV. SERUM CSF RESPONSE TO REPETITIVE INJECTIONS OF ENDOTOXIN[a]

Injection	6 hr	Day 2	Day 4	Day 6	Day 8
Buffer	1240	800	720	480	600
0.01 μg endotoxin	780	790	750	620	1040
0.1 μg endotoxin	1720	920	840	640	400
Uninjected control	840	nd	nd	nd	790

[a] Values represent CSF assays on pooled serum samples. Each group contained five animals. All mice received the indicated test material ip every 6 hours for intervals up to 8 days.

can be diluted at least 100-fold in pyrogen-free buffer for *in vivo* studies. Thus, the endotoxin concentration can readily be reduced to less than 0.001 μg/ml.

The *in vivo* studies with known quantities of *E. coli* endotoxin showed marked changes in splenic progenitor cell content with repetitive doses of 0.1 μg of this bacterial lipopolysaccharide. Only minimal changes were observed following administration of 0.01 μg. Serum CSF assays indicated a similar level of sensitivity. Mice injected with 0.1 μg of endotoxin had an acute rise in serum CSF whereas no changes were observed with a dose of 0.01 μg. Collectively, these observations show that CSF can be rendered free of biologically significant levels of endotoxin. Future *in vivo* studies with CSF should be carefully controlled for this important contaminant.

Summary. Limulus lysate assays showed that several samples of purified L-cell CSF were markedly contaminated with endotoxin. Several pools of material contained 1 to 10 μg/ml of this bacterial lipopolysaccharide. Ultracentrifugation in sucrose density gradients led to a 95% separation of CSF and endotoxin activities. In four studies, endotoxin content of the CSF preparations was reduced to levels varying from 0.05 to 0.0001 μg/ml.

In vivo studies with known quantities of *E. coli* endotoxin were conducted in mice. Acute and repeated injections of 0.1 μg of endotoxin led to increased serum CSF activity and increased number of splenic CFU-C and CFU-E. In contrast, similar injections of 0.01 μg of endotoxin produced virtually no effect on these parameters of hemopoiesis. Thus, the removal of endotoxin from CSF as outlined herein will permit *in vivo* studies of the effects of CSF on hemopoiesis, without concern for the complicating effects of endotoxin.

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