Demonstration of a Circulating Endotoxin Hapten in Burned Mice (35626)

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Previous evidence that *E. coli* endotoxin plays an important role in mortality after burns includes the following experimental findings: (a) *E. coli* and other gram-negative microorganisms were isolated from over 50% of blood cultures of conventional mice after a severe scald (1); (b) *E. coli* added to the environment of specific pathogen-free mice reared in the absence of *E. coli* doubled shock mortality after severe burns (2); (c) burned conventional mice were up to 1000 times more sensitive to *E. coli* endotoxin than were nonburned mice (3); (d) conventional mice made tolerant to a lethal dose of *E. coli* endotoxin were significantly more resistant to burn trauma (3).

Demonstration of biologically active circulating endotoxin in burned animals would furnish direct evidence to support the endotoxin theory of shock (4). The present experiments were designed to test for such evidence by using an immunological test for circulating endotoxin[1] and two separate tests (5, 6) for biological activity of circulating endotoxin.

Materials and Methods. Animals. Conventional, 18- to 25-g female albino Swiss Webster mice of the NIH stock colony were used.

Types of burn trauma studied. Mice were anesthetized with diethyl ether prior to burning. A mild hip burn was produced by immersion to the level of the pelvic crests in water at 70° for 5 sec. No fluid therapy was administered postburn. This burn produced no mortality in 21 days. Other mice were given a severe burn of ⅔ body surface area by immersing to the axilla in water at the same temperature for 8 sec. Immediately postburn these mice received an amount of 0.85% NaCl subcutaneously equivalent to 15% of body weight. With this amount of fluid therapy, only 15% of the mice died in 48 hr postburn; without fluid therapy, 100% of the mice died during the same time period.

Collection of serum samples. At various time intervals after burn trauma, blood was collected by either of two methods: (a) nonsterile technique: mice were decapitated and bled into sterile centrifuge tubes or (b) sterile technique: mice were bled from underneath the right foreleg using alcohol-flamed scissors to cut away the skin and to cut the brachial artery. The blood was collected in sterile centrifuge tubes, allowed to stand for several hours at room temperature, and centrifuged at 1800 rpm for 30 min. The serum was separated and stored at −10°. Routine bacteriological cultures were performed on all sera. The sera collected by the sterile technique were always sterile. Those sera collected by the nonsterile technique which yielded *E. coli* were discarded.

Serum treatment. All sera were heated in a water bath at 56° for 30 min before using in the hemagglutination tests. In the epinephrine test some sera were heated, while others were not. In the pyrogenicity studies the sera were not heated.

Preparation of sensitized erythrocytes. Normal human O Rh-negative blood was diluted with an equal volume of Alsever's solution and allowed to stand for several days before use. At the time of the experiment the erythrocytes were washed three times with 3 vol of 10% triethanolamine-buffered saline containing 0.1% bovine albumin (TBS) and sedimented by centrifuging for 5 min at 1800 rpm. A 10% suspension of the packed cells in

[1] Preliminary results were presented previously (7).

TBS was sensitized with *E. coli* lipopolysaccharide (Difco) by incubating equal volumes of erythrocytes and antigen (1–5 mg) in TBS in a water bath for 2 hr at 37° with occasional shaking. The resulting antigen-sensitized erythrocytes were washed three times in TBS and resuspended to make a 1% solution. These cells were used for 2 days only, after which a new preparation was made.

Standardization of sensitized erythrocytes. Serial dilutions of 0.2 ml rabbit serum (Difco) hyperimmunized to the antigen used in sensitizing the erythrocytes were made in 0.2 ml TBS in a series of Wasserman tubes. Two-fold serial dilutions were carried out to a titer of 1:32,768. Then 0.1 ml of each suspension of sensitized erythrocytes was added to each row of serially diluted antiserum, and the mixture was incubated at 37° for 1 hr and allowed to stand overnight at 4°. The hemagglutination titer was read by pattern as the reciprocal of the highest dilution giving complete agglutination of erythrocytes. The suspension of endotoxin-sensitized erythrocytes which gave the highest titer using the smallest quantity of endotoxin was the one used in the hemagglutination and hemagglutination-inhibition tests.

Hemagglutination test (HA). A preliminary HA test was carried out on all experimental sera for the presence of antibodies to endotoxin. Two-tenths of the experimental serum was serially diluted 2-fold in 10% TBS. Then 0.1 ml of the 1% endotoxin-sensitized erythrocytes was added to all tubes and incubated for 1 hr at 37°. The reciprocal of the highest dilution containing agglutination of the erythrocytes on overnight standing at 4° was taken as the titer of antibodies to the specific endotoxin used.

Hemagglutination-inhibition test (HI). The test developed here was based on modifications of the techniques of Noyes *et al.* (8) and Young *et al.* (9), and has the advantage of quantitating endotoxin antigen in serum.

A series of 15 Wasserman tubes is set up. Tube 1 contains 0.4 ml 10% TBS. Tube 2 contains 0.2 ml antiserum and 0.2 ml 10% TBS. Tube 3 contains 0.2 ml experimental serum. Tubes 4–15 contain 0.2 ml 10% TBS. Next, 0.2 ml of experimental serum is added to Tube 4 and serially diluted to Tube 15. Three sets of each experimental serum are prepared. Then, 0.2 ml of the highest dilution of hyperimmune serum giving agglutination of sensitized erythrocytes (as described under standardization of sensitized erythrocytes) is added to one set of the experimental serum starting from tube 15 through tube 3. The same procedure is repeated with the other two sets of experimental sera using the second and third highest dilutions, respectively, of hyperimmune serum giving agglutination of sensitized erythrocytes. All tubes are covered with aluminum foil and incubated at 37° for 2 hr. Lastly, 0.1 ml of endotoxin-sensitized erythrocytes is added to all tubes except 2, which are then reincubated for 1 hr at 37°. The pattern of agglutination is read on standing overnight at 4°. The hemagglutination-inhibition titer is taken as the most dilute tube with no agglutination.

Tests for biological activity. In the Thomas epinephrine test (5) normal female albino rabbits were given a 1-ml intravenous injection of either 0.85% NaCl, 5–10 μg *E. coli* 026:B6 lipopolysaccharide, normal or burned mouse sera. One-half hour later, 0.1 ml of epinephrine (adrenalin chloride solution, 1:1000, Parke-Davis) was injected intradermally on both shaved flanks of the rabbit. Five to six hours later, the amount of reaction at the intradermal injection site was recorded.

The second test used for biological activity was based on the pyrogenic response of rabbits to endotoxin (6). One to two milliliters of burned mouse sera containing elevated titers of *E. coli* endotoxin hapten, determined by immunological means, were injected intravenously into trained rabbits, and a continuous recording of rectal temperature was measured for 5–7 hr postinjection. Quantitative evidence for endotoxin was obtained by measuring the area under the fever curve. The following controls were injected in the same manner: (a) normal mouse sera; (b) normal mouse serum to which were added 1 or 8 μg/ml of *E. coli* 026:B6 lipopolysaccharide; and (c) mouse sera obtained at 2, 5, and 21 hr after intraperitoneal injection of 0.05 mg *E. coli* 026:B6 lipopolysaccharide/mouse.

Results. Validity of the hemagglutination-inhibition test for endotoxin. Figure 1 shows the titers obtained in the HI test when increasing amounts of *E. coli* endotoxin were added to normal mouse serum and incubated with two different dilutions of antiserum. A linear relationship was observed between micrograms of *E. coli* lipopolysaccharide (endotoxin) and hemagglutination-inhibition titers for both dilutions of specific antiserum.

Table I demonstrates the specificity of the HI test. In the case illustrated, the erythrocytes were sensitized with *E. coli* 026:B6 lipopolysaccharide. When a specific antiserum and antigen were added, high titers up to 512 were found. When nonspecific antigens were added, including various strains of *E. coli, Salmonella, Pseudomonas,* or lipopolysaccharide extracted from a tissue culture of Burkitt's tumor, no significant titers were obtained, even with 50-μg quantities. Thus, the test appears specific.

Normal conventional mouse serum did not contain endotoxin of *E. coli* 026:B6, regardless of the sterility of the technique used to obtain blood. On the other hand, titers of 128 to the 055:B5 and 0111:B4 strains of *E. coli* were obtained from serum of normal conventional mice; consequently all HI experiments, unless otherwise mentioned, were performed with erythrocytes sensitized to *E. coli*

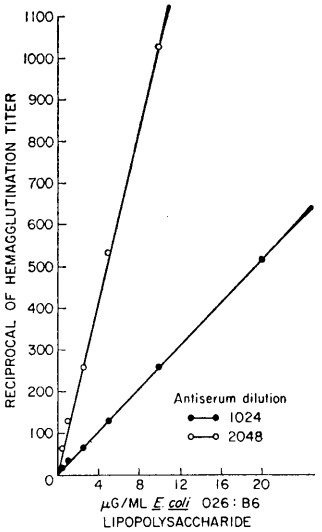

FIG. 1. Antigen titers obtained with increasing amounts of *E. coli* 026:B6 lipopolysaccharide in the HI test. Various amounts of *E. coli* 026:B6 lipopolysaccharide were incubated at 37° for 2 hr with 1:1024 and 1:2048 dilutions of specific antisera. Then equal amounts of erythrocytes sensitized with 4 mg/ml *E. coli* 026:B6 lipopolysaccharide were added to each tube and reincubated at 37° for 1 hr. Hemagglutination of erythrocytes was recorded on standing overnight at 4°.

TABLE I. Specificity of Hemagglutination Inhibition.

Erythrocytes sensitized with	Antiserum to	Antigen added	Quantity (μg)	Inhibition titer
E. coli 026:B6	*E. coli* 026:B6	*E. coli* 026:B6	0.5	16
			10	512
E. coli 026:B6	*E. coli* 026:B6	*E. coli* 055:B5	0.25	0
			50	16
E. coli 026:B6	*E. coli* 026:B6	*E. coli* 0111:B4	0.25	0
			50	2
E. coli 026:B6	*E. coli* 026:B6	*E. coli* 0127:B8	5	0
			50	0
E. coli 026:B6	*E. coli* 026:B6	*Salmonella typhosa* 0901	2.5	0
			50	0
E. coli 026:B6	*E. coli* 026:B6	*Pseudomonas aeruginosa*	Undil.	0
E. coli 026:B6	*E. coli* 026:B6	Burkitt cell tumor lipopolysaccharide[a]	Undil.	0

[a] Gift of Dr. R. B. Herberman, Cancer Institute, NIH, Bethesda, Maryland (5 mg protein/ml).

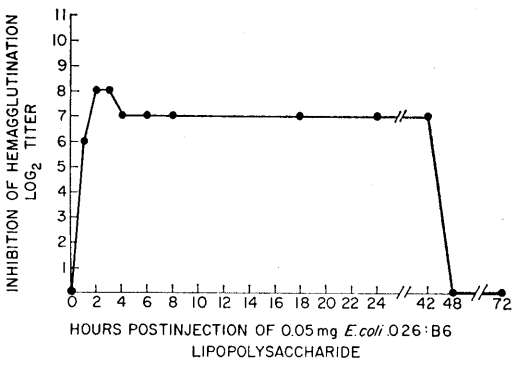

FIG. 2. The titer of *E. coli* endotoxin obtained in the serum of normal mice after injection of 0.05 mg *E. coli* 026:B6 lipopolysaccharide. In the HI test the erythrocytes were sensitized with 4 mg/ml *E. coli* 026:B6 lipopolysaccharide, and a 1:2048 dilution of specific antiserum was used.

026:B6 lipopolysaccharide to obtain control titers of zero. In order to determine whether there was any correlation between the serological type of endotoxin found in serum and microorganisms present in the gut, all strains of *E. coli* which were isolated from the feces were tested serologically. None of the previously mentioned strains, 026:B6, 055:B5, or 0111:B4, were found in the feces.

Figure 2 illustrates the titers of endotoxin obtained in the HI test when mice were given a nonlethal dose of 0.05 mg of specific *E. coli* 026:B6 lipopolysaccharide intraperitoneally. High titers of endotoxin in mouse serum were obtained as early as 1 hr postinjection. The high titers persisted for 42 hr, after which there was a precipitous drop to 0 at 48 hr postinjection.

Serum antibody levels to endotoxin. Routine examination of all normal or burn sera for antibodies against the 026:B6 strain of *E. coli* showed zero titers in the hemagglutination test; however, normal mouse serum had an antibody titer of 32 against *E. coli* 055:B5 antigen and a titer of 4 against *E. coli* 0111:B4 antigen.

Serum endotoxin of burned mice. Figure 3A shows a scatter diagram of serum endotoxin titers obtained at various time intervals after mice had been given a minimal hip burn. No parenteral fluid therapy was given. Although there were a few negative titers during the first 6 hr after trauma, the majority of the titers were elevated to 256 from 1–48 hr postburn.

Figure 3B illustrates the serum endotoxin titers after a more severe burn when mice were given a ⅔ body surface area burn and subcutaneous saline therapy immediately postburn. As in the case of the minimal burn, there were a few titers at the zero level during the first 5 hr after burning, but the majority showed high titers of 256 for 48 hr postburn.

In order to rule out the possibility that injection of saline might produce an endotoxemia, normal mice were given 3 ml of sterile 0.85% NaCl subcutaneously and bled by the sterile technique at 1, 4, and 6 hr postinjection. The results of the HI test showed no evidence for circulating endotoxin in the sera of these mice.

In order to determine whether the circulating endotoxin found in burned mice was limited to the *E. coli* 026:B6 subgroup, the HI test was also performed using *E. coli* 055:B5

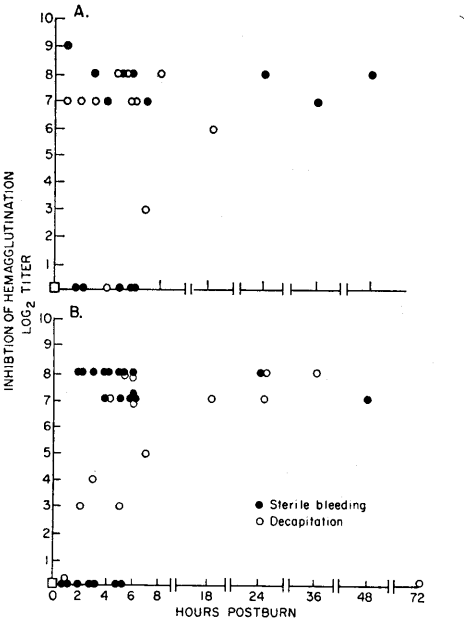

FIG. 3. Endotoxin titers in mouse serum obtained after burns of varying severity. In A, the mice were given a minimal hip burn, and in B, a severe 2/3 body surface area burn. In the HI test, the erythrocytes were sensitized with 4 mg/ml *E. coli* 026:B6 lipopolysaccharide, and a 1:4096 dilution of specific antiserum was used.

TABLE II. Test for Biological Activity of Endotoxin.

Material injected iv	No. of tests	Reaction to intradermal epinephrine 5–6 hr later[a]			
		0	+	++	+++
5–10 μg *E. coli* 026:B6 lipopolysaccharide	16	4	1	2	9
1 ml 0.85% NaCl	14	8	0	0	6
1 ml normal mouse serum	20	18	1	0	1
1 ml burned mouse serum					
2 hr postburn	2	1	0	0	1
3 hr postburn	2	0	0	0	2
4 hr postburn	2	1	0	0	1
5 hr postburn	6	1	2	1	1
6 hr postburn	6	1	2	1	1
24 hr postburn	2	0	1	0	1
Total	20	4	5	3	8

[a] 0, no reaction; +, pink color; ++, redness and swelling; +++, hemorrhagic areas.

lipopolysaccharide antigen and antiserum. In a small number of sera collected 5 or 6 hr after a hip burn, titers of 1024 were found compared to the normal control titer of 128.

Tests for biological activity. Table II shows the results of the epinephrine test for biological activity. When 5–10 μg *E. coli* 026:B6 lipopolysaccharide were injected intravenously, a positive skin reaction was found in 75% of the tests; however, the saline control also produced a positive reaction in 43% of the tests. On the other hand, normal mouse serum produced a positive reaction in only 10% of the tests ($p<.001$ compared with the endotoxin control), while burned mouse serum produced a positive reaction in 80% of the tests ($p>.9$ compared with the endotoxin control). These results suggest that the endotoxin hapten in burn serum is biologically active.

In the pyrogenic response assay for endotoxin, however, no significant fever was produced by three samples of normal mouse sera containing no endotoxin hapten or by four samples of 5- to 6-hr postburn mouse sera containing titers of 256 or 512 of *E. coli* 026:B6 hapten. The test appears valid, since a high fever was produced in rabbits injected with 1 ml normal mouse serum to which had been added 1 or 8 μg/ml of *E. coli* 026:B6 lipopolysaccharide. To rule out the possibility that circulating endotoxin could be detoxified and thereby lose biological activity by this test, serum was collected from mice that had been injected intraperitoneally 2, 5, or 21 hr previously. The three samples had hemagglutination-inhibition titers for endotoxin hapten of 256, 512, and 512 respectively. In the pyrogenicity test, the serum collected at 2 and 5 hr after endotoxin injected produced a single hump of moderate fever, while the 21-hr sample produced no fever at all.

This test, therefore, showed no evidence of circulating endotoxin that was biologically active in burned animals.

Discussion. The results of these experiments furnish partial direct evidence to support the endotoxin theory of shock (4). The positive evidence is based on the demonstration of circulating endotoxin hapten in the sera of burned mice. The hemagglutination inhibition test devised for this purpose appears valid, since it: (a) is specific for all of the antigens tested; (b) detects specific circulating antigen injected *in vivo*; and (c) responds in a linear fashion to increasing amounts of antigen used in the test.

On the other hand, some of the data obtained may raise doubts as to the validity of the endotoxin theory; for example: (a) no adequate explanation has been found as yet to account for *E. coli* 026:B6 endotoxin hapten observed in the serum of nonburned, germ-free mice (7); (b) the presence of some zero titers of endotoxin in the pooled sera of burned conventional mice during the

first 6 hr postburn seems contradictory, but could be explained by the existence of a saturation point in the ability of the reticuloendothelial system in clearing circulating endotoxin; and (c) the absence of fecal strains of *E. coli* corresponding to the serotype of endotoxin found in the serum of burned mice appears conflicting, unless the circulating endotoxin did not come from the gut or that these strains disappeared proximal to the ileum with overgrowth of other strains in the feces.

The second criterion for direct proof of the endotoxin theory of shock, however, was not met. The epinephrine test suggested that the circulating endotoxin was biologically active, but the evidence was not convincing, since 43% of the saline controls also gave a positive reaction. This finding indicated nonspecificity of the test, unless the saline was contaminated with endotoxin or the skin at the test site was contaminated by nicks during the clipping of fur. In the more sensitive rabbit pyrogenicity test, there was no evidence of a fever response by four mouse sera containing endotoxin hapten. Unless the endotoxin was altered on standing at $-10°$ for 1 week prior to testing (which seems doubtful), these results show no evidence for biologically active endotoxin.

The finding of a circulating endotoxin hapten that is not biologically active both in burned animals and in animals injected with endotoxin suggests a mechanism by which an animal can protect itself against endotoxin. It is possible that the phagocytic cells of the reticuloendothelial system hydrolyze endotoxin, secreting the polysaccharide hapten into the circulation and destroying the toxic lipid portion of the molecule. In the burned animal the phagocytes may be able to handle the quantities of endotoxin present, and for that reason only the nonbiologically active hapten was found in the serum.

Summary. A quantitative hemagglutination-inhibition test was developed for the determination of endotoxin (hapten) in serum of mice. When the blood sera of conventional mice given a minimal or severe burn were tested for circulating endotoxin, significantly elevated titers of *E. coli* endotoxin (or hapten) were found in the sera. There is as yet no conclusive evidence, however, that the circulating endotoxin is biologically active.

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