Bacterial Endotoxin Both Enhances and Inhibits the Toxicity of Shiga-Like Toxin II in Rabbits and Mice

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The ability of bacterial lipopolysaccharide (LPS) to enhance the toxicity of Shiga-like toxin II (SLT-II) was investigated in rabbits and mice. Rabbits were continuously infused with 0.5 50% lethal dose (LD₅₀) of SLT-II per day. Rabbits that received a 30-µg/kg dose of LPS (0.02 LD₅₀) on day 3 of infusion were significantly more likely to die than were rabbits receiving SLT-II only. Rabbits receiving SLT-II and a lower dose of LPS (3 µg/kg) did not die but lost an average 3.3% ± 1.0% of initial body weight during the first 5 days of infusion, compared with weight gains of 4.2% ± 0.6% and 17.1% ± 0.9% for rabbits receiving only SLT-II or LPS, respectively. Rabbits that were pretreated with LPS 20 h before challenge with a single dose of SLT-II showed highly significant protection from both the diarrheagenic and lethal effects of SLT-II. Pretreatment of endotoxin-responsive C3H/HeN mice protected the animals from challenge with an LD₅₀ but not an LD₁₀₀ of SLT-II. LPS enhanced the lethal toxicity of SLT-II for C3H/HeN mice when it was given at 8 or 24 h but not 0 or 72 h after SLT-II challenge. LPS did not affect the lethal toxicity of SLT-II for endotoxin-resistant C3H/HeJ mice. These results suggest that LPS enhances the effects of SLT-II in vivo. Since cecal changes that increase mucosal permeability occur in response to SLT in rabbits, this synergy may be directly relevant to disease processes.

Strains of Escherichia coli belonging to serogroup O157:H7 were first reported to be associated with outbreaks of hemorrhagic colitis in 1983 (19, 21). Later that year, it was reported that the strains responsible for these outbreaks produced a Shiga-like toxin (SLT) (A. D. O'Brien, T. A. Lively, M. A. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702, 1983). On further study, it became clear that these organisms produced two antigenically distinct toxins with similar biological activities, termed SLT-I and -II (20). These toxins are also referred to as verotoxins 1 and 2 (S. M. Scotland, H. R. Smith, and B. Rowe, Letter, Lancet ii:885-886, 1985), in observance of the original description of SLT-I (verotoxin 1) as a Vero cell cytotoxin (12). Strains of SLT-producing E. coli were later associated with hemolytic uremic syndrome (10) and thrombotic thrombocytopenic purpura (4).

In addition to being cytotoxic for selected cell cultures, SLT-I and -II are paralytic-lethal for mice and cause fluid accumulation in the ligated rabbit ileal loop (20). Both toxins have been shown to inhibit protein synthesis by interfering with elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes (8, 15, 16). The relationship of this activity to the biological effects of SLT remains to be demonstrated.

We recently reported attempts to develop an animal model for SLT-II-induced diseases (1). Using highly purified SLT-II (7) administered by continuous infusion to rabbits, we were able to cause diarrhea accompanied by cecal lesions that resembled those seen in the colons of humans with hemorrhagic colitis (11, 19). Although some animals developed renal failure in response to SLT-II, the pathologic changes did not resemble those seen in the kidneys of humans with hemolytic uremic syndrome (18). Large doses of toxin caused death from vascular damage to the central nervous system, as was reported for Shiga toxin more than 30 years ago (2).

Because of the extensive cecal lesions and occasional complete interruption of the mucosal integrity, we wondered whether lipopolysaccharide (LPS) from the bacterial flora of the gut might contribute to the pathologic changes. Such a speculative role for LPS in the pathogenesis of hemolytic uremic syndrome was recently suggested by Cleary (5). In this study, we examined the ability of LPS to act synergistically with SLT-II in causing disease and death in both rabbits and mice. We also found that LPS administered before SLT-II could protect animals from SLT-II challenge.

MATERIALS AND METHODS

Rabbits. Male New Zealand White rabbits weighing 1 to 2 kg were obtained from the Centers for Disease Control breeding facility. The animals were caged individually and provided free access to food and water.

Mice. C3H/HeN mice were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. C3H/HeJ mice were purchased from Jackson Laboratory, Bar Harbor, Maine. All mice were male, were 4 to 5 weeks of age, and were used within 5 days of arrival. The 50% lethal dose (LD₅₀) of SLT-II was determined by the method of Reed and Muench (17). Groups of seven mice were given twofold dilutions of SLT-II in pyrogen-free saline, with doses ranging from 100 to 3,200 pg per mouse.

SLT-II preparation. SLT-II was purified from E. coli NM522(pEBI) as previously described (7). Endotoxin contamination was determined by the Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.). Bacterial endotoxin was removed by repeated passage through a Detoxigel column (Pierce Chemical Co., Rockford, Ill.). After passage, endotoxin contamination was 6 pg/µg of SLT-II.

Protein determination. The protein content of SLT-II preparations was determined by using the Coomassie blue G-250 reagent (Pierce).

Endotoxin preparation. E. coli serotype O26 LPS (Sigma Chemical Co., St. Louis, Mo.) was diluted in sterile saline...
prepared with pyrogen-free distilled water immediately before use.

Continuous infusion of SLT-II. Rabbits were infused with SLT-II as previously described (1). Miniosmotic pumps (model 2002; Alza Corp., Palo Alto, Calif.) were loaded with sufficient SLT-II to deliver 100 ng of toxin per kg of body weight per day for 14 days. Rabbits were anesthetized with 0.4 ml of ketamine sulfate (Ketaset; Bristol Laboratories, Syracuse, N.Y.) containing 4 mg of xylazine (Rompun; Miles Laboratories, Inc., Shawnee, Kans.) per ml per kg of body weight. A midline incision was made, the pumps were placed in the peritoneal cavity, and the incision was closed.

SLT-II-LPS synergy in rabbits. The ability of LPS to enhance the diarrheagenic and lethal activities of SLT-II in rabbits was investigated by intravenous (i.v.) injection of LPS into animals on day 3 of SLT-II infusion as described above. In the first experiment, 18 rabbits received SLT-II-containing pumps. Of these 18 animals, 12 received LPS (30 μg/kg) on day 3 of infusion while the other 6 received a saline placebo. Six rabbits received only the LPS injection. In the second experiment, 16 rabbits were implanted with SLT-II pumps. Eight rabbits were injected with a much smaller dose of LPS (3 μg/kg) on day 3, while eight received saline. Eight additional rabbits received only the LPS injection. In this experiment, all animals were weighed on days 0, 5, and 10 post-surgery. In all rabbit experiments, animals that were determined by two investigators to be moribund were humanely killed.

Pretreatment of rabbits with LPS. Nine rabbits were given 60 μg of LPS per kg of body weight by i.v. injection 20 h before i.v. injection with 200 ng of SLT-II. Fifteen rabbits received the same dose of SLT-II with no LPS pretreatment.

SLT-II-LPS synergy in mice. Seven groups of six C3H/HeN mice were given an LD₅₀ of SLT-II (400 pg) by intraperitoneal injection. Each group also received an intraperitoneal injection of 20 μg of LPS (0.05 LD₅₀; unpublished observations) at specified intervals before and after SLT-II injection (see Table 3). This experiment was later repeated exactly, and the data from the two experiments were pooled. In another experiment, groups of six C3H/HeN or C3H/HeJ mice were given the 100% lethal injection of SLT-II (approximately 2 LD₅₀) and an intraperitoneal injection of 20 μg of LPS at the following times before or after SLT-II: –48, –18, 0, +8, +24, +48, and +72 h.

RESULTS

In the first SLT-II-LPS synergy experiment, the number of deaths among rabbits receiving both SLT-II (100 ng/kg per day) and LPS (30 μg/kg) was significantly greater than among those receiving either SLT-II or LPS alone (P = 0.002, Fisher's exact test) (Table 1). The number of rabbits developing diarrhea in the SLT-II-LPS group was significantly greater than in the LPS-only group (P = 0.009, Fisher's exact test). More rabbits developed diarrhea when both SLT-II and LPS were given than when SLT-II alone, but the difference was not significant (P = 0.344, Fisher's exact test).

A much smaller dose of LPS (3 μg/kg) was used in the second experiment, and no deaths occurred in any treatment group (Table 2). During the first 5 days of infusion, rabbits receiving SLT-II or LPS alone increased initial body weight by means of 4.2 and 17.1%, respectively, while the mean weight of rabbits receiving both toxins decreased by 3.3%. The one-way analysis of variance performed to investigate the relationship between treatment group and percent change in body weight was significant (overall F = 51.59; df = 2, 21; P < 0.0001). Duncan's multiple comparison procedure revealed that all three groups were significantly different from each other. The weight change for an untreated control group was +17.6% ± 2.4%. The same number of rabbits developed diarrhea when challenged with SLT-II alone as when receiving both SLT-II and LPS.

Pretreatment of rabbits with 60 μg of LPS per kg of body weight 20 h before challenge with an i.v. injection of 200 ng of SLT-II per kg resulted in significant protection from the diarrheagenic effect of SLT-II. Of 9 pretreated rabbits, 3 developed diarrhea, compared with all 15 rabbits which were not pretreated (P = 0.0006, Fisher's exact test). LPS pretreatment provided complete protection from the lethal effect of the SLT-II challenge. Of 15 rabbits not receiving pretreatment, 10 died, compared with none of 9 LPS-treated animals (P = 0.002, Fisher's exact test). Therefore, LPS had either a synergistic or protective effect, depending on the time of administration. This time-dependent effect was further investigated with endotoxin-normoreponsive (C3H/HeN) and endotoxin-hyporesponsive (C3H/HeJ) mice.

Pretreatment of C3H/HeN mice with LPS 18 h before injection with 400 pg of SLT-II (1 LD₅₀) provided complete protection from the lethal effects of SLT-II (Table 3). Treatment with LPS after exposure to SLT-II had a demonstrable effect only when LPS was given 8 or 24 h following SLT-II. While LPS treatment did not result in a significantly greater number of deaths compared with SLT-II treatment only (about 50% died in each group), the mean time to death was significantly reduced by LPS treatment at 8 or 24 (but not 48) h post-SLT-II. There were no significant differences between groups 5 days after the injection of SLT-II. The number of deaths among rabbits receiving both SLT-II (100 ng/kg per day) and LPS (30 μg/kg) was significantly greater than among those receiving either SLT-II or LPS alone (P = 0.002, Fisher's exact test) (Table 1). The number of rabbits developing diarrhea in the SLT-II-LPS group was significantly greater than in the LPS-only group (P = 0.009, Fisher's exact test). More rabbits developed diarrhea when both SLT-II and LPS were given than when SLT-II alone, but the difference was not significant (P = 0.344, Fisher's exact test).

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### TABLE 1. Outcome of treatment of rabbits with SLT-II or LPS or both

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>With diarrhea</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLT-II (100 ng/kg per day)*</td>
<td>6</td>
<td>3</td>
<td>0*</td>
</tr>
<tr>
<td>LPS (30 μg/kg)</td>
<td>6</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>SLT-II and LPS</td>
<td>12</td>
<td>9*</td>
<td>10*</td>
</tr>
</tbody>
</table>

* 0.5 LD₅₀/day by continuous infusion.

### TABLE 2. Outcome of treatment of groups of eight rabbits with SLT-II or a low dose of LPS or both

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. with diarrhea</th>
<th>Mean wt change (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLT-II (100 ng/kg per day)</td>
<td>6d</td>
<td>+4.2 ± 0.6</td>
</tr>
<tr>
<td>LPS (3 μg/kg)</td>
<td>0df</td>
<td>+17.1 ± 0.9</td>
</tr>
<tr>
<td>SLT-II and LPS</td>
<td>6f</td>
<td>−3.3 ± 1.0</td>
</tr>
</tbody>
</table>

* There were no deaths in any of the groups.

b Mean weight change 5 days after the beginning of the experiment as percentage of initial body weight. Differences were significant by one-way analysis of variance (overall F = 51.59; df = 2, 21; P < 0.0001). All groups differ from each other by Duncan's multiple comparison procedure. The weight change for untreated rabbits was +17.6% ± 2.4%.

c 0.5 LD₅₀/day by continuous infusion.

d P = 0.007, Fisher's exact test (two-tailed).

f 0.002 LD₅₀ injected i.v. 72 h after SLT-II was begun.

/ P = 0.007, Fisher's exact test (two-tailed).

Same doses as indicated above.
TABLE 3. Time-dependent effect of LPS treatment on mean time to death of C3H/HeN mice given an LD₅₀ of SLT-II

<table>
<thead>
<tr>
<th>Time of LPS dose (h)</th>
<th>Time to death (days; mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−18</td>
<td>—</td>
</tr>
<tr>
<td>0</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>+2</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>+8</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>+24</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>+48</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>No dose</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>

a LPS (20 μg; 0.05 LD₅₀) was given by intraperitoneal injection at the stated number of hours before or after SLT-II injection.
b — No deaths.

LPS enhanced the toxicity of SLT-II for the endotoxin-responsive C3H/HeN mice but only when given 8 or 24 h after SLT-II. The effect of LPS must be on a specific pathway and not simply an additional insult to host integrity, since LPS had no effect on mortality when given at 0 or 48 h post-SLT-II. Protection of mice by LPS pretreatment was seen only with an LD₅₀ of SLT-II. It thus appears that the protective effect of pretreatment may be overcome by larger doses of toxin. Since mice are far less susceptible to endotoxin than are rabbits, it is not surprising that the effects of LPS were more obvious in rabbits.

In summary, we have shown that bacterial LPS enhances both the diarrheagenic and lethal effects of SLT-II when given to rabbits after SLT-II exposure. LPS protected rabbits from SLT-II toxicity when given before SLT-II challenge. The lethal effects of SLT-II were also enhanced by LPS in C3H/HeN but not C3H/HeJ mice. These results suggest that LPS acts in a macrophage-mediated synergistic manner to enhance SLT-II-induced injury.

LITERATURE CITED

13. Morrison, D. C., and J. L. Ryan. 1987. Endotoxins and disease effects on the normal course of illness and that pretreatment with LPS rendered the test rabbits hyporesponsive to endotoxin absorbed from the gut after initial response to SLT-II. We were unable to detect significant levels of circulating endotoxin in the blood of another group of SLT-II-treated rabbits (unpublished observations); the failure to detect LPS could be due to the difficulties in measuring endotoxin in blood (6), inappropriate collection of specimens, or a localized rather than systemic effect.

The protective effect of LPS pretreatment may also be due to endotoxin interference with the effect of SLT-II on targets or mediators (14). The macrophage plays a central role in promoting the lethal effects of endotoxin through production of inflammatory mediators, such as tumor necrosis factor (reviewed in reference 13). It is possible that LPS and SLT-II either enhance or antagonize each other’s activity, depending on the timing of administration, through action upon the macrophage.

In a previous study, we reported that rabbits infused with SLT-II developed diarrhea and cecal lesions on day 3 of infusion (1). If LPS from the gut flora contributes to the pathologic changes, this seemed to be the most likely time for such synergy to occur. Day 3 was thus chosen for LPS challenge. As shown in Table 1, a 30-μg/kg dose of LPS was clearly able to increase the lethal effect of SLT-II. The effect appeared to be synergistic rather than additive, since 30 μg of LPS per kg is approximately 0.02 LD₅₀ (3; unpublished observations). A 10-fold-lower dose of LPS did not increase the lethality of SLT-II but did result in a significant weight loss compared with that in rabbits receiving only SLT-II or LPS. It thus appears that even small doses of LPS may cause increased morbidity in animals challenged with SLT-II.

The protection of rabbits from SLT-II-induced diarrhea and death by pretreatment with LPS has several possible explanations. Animals treated with sublethal doses of endotoxin become refractory to LPS challenge 3 to 4 days later (9). It is possible that LPS does in fact participate in the normal course of illness and that pretreatment with LPS rendered the test rabbits hyporesponsive to endotoxin absorbed from the gut after initial response to SLT-II. We were unable to detect significant levels of circulating endotoxin in the blood of another group of SLT-II-treated rabbits (unpublished observations); the failure to detect LPS could be due to the difficulties in measuring endotoxin in blood (6), inappropriate collection of specimens, or a localized rather than systemic effect.

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DISCUSSION

In between identical groups in the duplicate runs of this experiment in either number of deaths or mean time to death. When a similar experiment was performed with 2 LD₅₀ of SLT-II, all of the experimental animals died and the differences between groups receiving SLT-II only and SLT-II-LPS were reduced. The mean time to death were reduced to 3.9 ± 0.2 days for mice receiving SLT-II only, 2.8 ± 0.3 days for mice receiving LPS at +8 h, and 2.9 ± 0.2 days for mice injected with LPS 24 h after SLT-II. LPS treatment had no effect on the survival of endotoxin-resistant C3H/HeJ mice (data not shown).

LPS enhanced the toxicity of SLT-II for the endotoxin-responsive C3H/HeN mice but only when given 8 or 24 h after SLT-II. The effect of LPS must be on a specific pathway and not simply an additional insult to host integrity, since LPS had no effect on mortality when given at 0 or 48 h post-SLT-II. Protection of mice by LPS pretreatment was seen only with an LD₅₀ of SLT-II. It thus appears that the protective effect of pretreatment may be overcome by larger doses of toxin. Since mice are far less susceptible to endotoxin than are rabbits, it is not surprising that the effects of LPS were more obvious in rabbits.

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