Physiology of the Potentiation of Lethal Endotoxin Shock by Streptococcal Pyrogenic Exotoxin in Rabbits

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Streptococcal pyrogenic exotoxin (SPE) dramatically potentiates the lethal shock induced by gram-negative bacterial endotoxin. To provide further understanding of the mechanism underlying the potentiating effect, the physiological basis for the toxic synergism of the two toxins was investigated. Pretreatment of rabbits with an intravenous (i.v.) dose (10 μg/kg of body weight) of SPE greatly enhanced the endotoxin lethality and reduced the 50% lethal dose to less than 5 μg of endotoxin per kg. The SPE pretreatment dose caused severe pathophysiological changes in combination with a small i.v. dose of endotoxin (1 μg/kg). These changes included transient hyperglycemia followed by profound hypoglycemia, elevation of the blood lipoperoxide level, and an acute increase in plasma β-glucuronidase activity. These changes were comparable with those in animals given a large i.v. dose of endotoxin (100 μg/kg) alone. An injection of SPE alone did not alter any of the parameters described above. These results suggest that SPE renders rabbits more sensitive to extensive pathophysiological effects of endotoxin, and the potentiating effect on endotoxin lethality may thus involve a general potentiation of physiologic failures. The SPE pretreatment depressed the vascular clearance of a large dose of endotoxin (100 μg/kg) but failed to affect that of a small dose of endotoxin (1 μg/kg). The data suggest that the potentiating effect is not readily explained solely on the basis of the decreased clearance of endotoxin.

Group A streptococcal pyrogenic exotoxin (SPE) possesses a wide variety of biological properties, including pyrogenicity (24, 35), T-cell mitogenicity (1), and capacities to alter the blood-brain barrier (31) and antibody response to a second antigen (7, 18, 19). The most striking property of SPE is its capacity to potentiate lethal shock induced by gram-negative bacterial endotoxin (24, 31, 32, 35). The clinical implications of the potentiating effect for human disease have been pointed out since the ubiquity of endotoxins and their potential for contaminating parenteral drugs could result in fatal accidents when the drugs are administered to patients with clinical or subclinical group A streptococcal infections (24).

The capacity of SPE to potentiate endotoxin lethality is independent of its ability to induce fever and not dependent on an effect on the central nervous system (31). It has also been suggested that the potentiating effect may be caused by inactivation of endotoxin clearance mechanisms by SPE (6, 17, 30). To provide further understanding of the mechanism underlying the potentiating effect, the present study examines the physiologic basis for the toxic synergism of the two toxins.

MATERIALS AND METHODS

Preparation of SPE. Strain SF42-100,081 of Streptococcus pyogenes (T-type 12) provided by the Osaka Prefectural Institute of Public Health, Osaka, Japan, was used for toxin production. SPE was prepared by the method of Kim and Watson (24). Briefly, the bacteria were cultivated in a Todd-Hewitt dialysate medium (20), and SPE was extracted from the culture supernatant by repeated precipitations with ethanol and resolubilizations in acetate-buffered saline. The method has been shown to be effective for eliminating streptolysins O and S, streptococcal enzymatic activities, and endotoxin (24). The extracted SPE induced a monophasic fever response in rabbits, and its minimal pyrogenic intravenous (i.v.) dose was approximately 1 μg/kg of body weight.

Endotoxin. The endotoxin preparation used was lipopolysaccharide derived from E. coli (strain UKT-B; provided by the Research Institute for Microbial Disease, Osaka University, Osaka, Japan). The lipopolysaccharide was prepared by the method of Westphal and Lüderitz (36) with our modifications (23).

Animals. Male Japanese White rabbits weighing about 3 kg were used throughout this study. They were fed and watered ad libitum unless otherwise indicated.

Treatment of animals. A series of studies was conducted to assess SPE-induced potentiation of endotoxin lethality. Rabbits were given an i.v. dose (5 μg/kg) of endotoxin at 3 h after they received various i.v. doses of SPE. Deaths were recorded over 48 h. Another series of studies was conducted to elucidate several pathophysiologic changes induced by the two toxins in concert. The experimental protocol was similar to that described above except that the doses of SPE and endotoxin were 10 and 1 μg/kg, respectively. Control animals were administered endotoxin alone (1 or 100 μg/kg) or an equivalent volume of saline. At various times after SPE and endotoxin injections, blood samples were obtained via a marginal ear vein. Plasma was obtained from the chilled, heparinized blood samples by centrifugation and stored at −20°C for subsequent assays.

Assays. Blood glucose level was measured by the o-toluidine method (21) with Glucose-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Blood lipoperoxide level was measured by the thiobarbituric acid method of Yagi (38), which estimates the production of malondialdehyde as the measure of lipid β-oxidation. Plasma β-glucuronidase activity was determined by the method of Fishman (14) with 3-nitrophenyl-β-D-glucuronide as a substrate, and the activity is expressed as nanomoles of phenolphthalein liberated per hour at 37°C.
Endotoxin concentration in blood was measured by the method of Obayashi (27) with a chromogenic Limulus test, the Toxicolor Test (Seikagaku Kogyo Co., Ltd., Tokyo, Japan), which was recently devised as a novel method for an accurate and objective endotoxin assay. Briefly, platelet-rich plasma was prepared by centrifuging heparinized blood at 110 × g for 10 min at 4°C. Limulus test-interfering factors were removed by exposing 100 μl of the platelet-rich plasma to 200 μl of 0.26 M perchloric acid for 5 min at 60°C. After centrifugation, the supernatant was neutralized with 0.14 M NaOH. The test sample was added to an equal volume of the Toxicolor Test reagent (a mixture of factor G-free Limulus amoebocyte lysate and a chromogenic substrate, 1-butyloxy carbonyl-Leu-Gly-Arg-p-nitroanilide) and incubated for 30 min at 37°C. A₅₄₅ was measured after diazotization.

Statistics. Statistical significance was assessed in all cases by the Student’s t test.

RESULTS

Effect of SPE pretreatment on endotoxin lethality. Treatment of rabbits with SPE before challenge with endotoxin rendered them more sensitive to the lethal effect of endotoxin (Table 1). A pretreatment SPE dose of 10 μg/kg in combination with 5 μg of endotoxin per kg caused death in 60% of the animals, although 50% lethality was obtained in the absence of SPE with about 500 μg of endotoxin per kg. The pretreatment thus enhanced lethality by a factor of more than 100. Deaths occurred in just under 24 h with pretreatment, and when high doses of SPE and endotoxin were given, the animals succumbed 0.5 to 2 h after receiving endotoxin.

In the following experiment, we examined pathophysiologic changes induced by the two toxins in concert. The studies were conducted with a sublethal combination of the toxins (10 μg of SPE and 1 μg of endotoxin per kg) to examine physiologic changes over 24 h and to avoid potential secondary effects of lethal shock. The combination doses caused death in 10% of the rabbits (Table 1).

Effects of SPE and endotoxin on blood glucose level. Among a number of pathophysiologic disturbances induced by endotoxin, glucose dysshomeostasis is one of the most prominent and serves as a consistent index for metabolic disorders (10). In an initial experiment, therefore, changes in blood glucose level were examined in rabbits treated with SPE and endotoxin. In this experiment, animals were fasted overnight to obtain stable data on blood glucose levels. A fast of this duration did not in itself alter the susceptibility of animals to endotoxin.

Administration of SPE (10 μg/kg) alone induced no significant alteration in blood glucose level (Fig. 1). Rabbits given only a small dose of endotoxin (1 μg/kg) showed a transient and slight hyperglycemia. A large dose of endotoxin (100 μg/kg) alone caused a pronounced hyperglycemia followed by a significant hypoglycemia. When 1 μg of endotoxin per kg was injected into SPE-pretreated rabbits, the induced change in blood glucose level was essentially analogous to that for animals treated with 100 μg of endotoxin per kg alone. The early hyperglycemic phase was not as pronounced as in the group which received a large dose of endotoxin, although the response was more evident than that in the group which received a small dose. The subsequent hypoglycemic phase, however, was more rapidly progressive and profound than that in the group which received the large dose of endotoxin. These results indicate that pretreatment with SPE potentiates the glucose dysshomeostasis induced by endotoxin.

Effect of SPE on endotoxin-induced elevation of blood lipoperoxide level. It has recently been suggested that lipid peroxidation is an important mechanism involved in endotoxin toxicity (28). Therefore, to examine whether SPE could potentiate other pathophysiologic effects of endotoxin, the blood lipoperoxide level was measured as another index. In rabbits pretreated with SPE, the blood lipoperoxide level was elevated in response to 1 μg of endotoxin per kg, notably at 24 h after endotoxin was administered (Table 2). SPE injection itself, even a large dose (1 mg/kg), did not produce a significant increase in the lipoperoxide level.

Effect of SPE on endotoxin-induced increase of plasma β-glucuronidase activity. Plasma β-glucuronidase activity was determined to assess the effect of SPE on a more acute response to endotoxin, since the plasma lysosomal enzyme level is known to increase within 1 h after endotoxin administration (15, 22). In rabbits pretreated with SPE, there were marked elevations of plasma β-glucuronidase activity in response to 1 μg of endotoxin per kg (Fig. 2). These elevations were significant as early as 1.5 h after endotoxin administration, and they were comparable with those in rabbits given a large dose of endotoxin (100 μg/kg) alone. SPE injection itself showed some tendency to increase the enzyme levels, but even with a large dose (1 mg/kg), the increase that was induced was not significant (data not shown).

Vascular clearance of the injected endotoxin. It has been suggested that SPE enhancement of endotoxin lethality may involve suppression of endotoxin clearance from the circulation (6, 17, 30). Therefore, we examined the effect of SPE pretreatment on the vascular clearance of injected endotoxin. The vascular clearance of a large dose of endotoxin (100 μg/kg) was significantly depressed in rabbits pretreated with SPE (10 μg/kg), as compared with that in control animals which were given endotoxin only (Fig. 3). All of the SPE-pretreated animals died less than 3 h after receiving the large dose of endotoxin. However, the SPE pretreatment failed to affect the clearance of a small dose of endotoxin (1 μg/kg), even though the pathophysiologic effect of the dose of endotoxin was potentiated by SPE, as described above.

DISCUSSION

In the present study, we first confirmed that pretreatment of rabbits with SPE renders them more sensitive to the lethal effect of endotoxin. We further investigated the physiologic basis for the toxic synergism of the two toxins. It was demonstrated that SPE potentiates endotoxin-induced glucose dysshomeostasis characterized by transient hyperglycemia and subsequent hypoglycemia. These disturbances in

| TABLE 1. Potentiation of endotoxin lethality induced by SPE pretreatment in rabbits |
|---------------------------------|------------------|------------------|
| SPE/endotoxin treatment* (μg/kg) | No. dead**/total | Lethality (%)    |
| 0/500                           | 5/10             | 50               |
| 0/50                            | 0/5              | 0                |
| 1/5                             | 1/5              | 20               |
| 10/5                            | 3/5              | 60               |
| 100/5                           | 5/5              | 100              |
| 10/1                            | 0/5              | 0                |
| 0/5                            | 1/10             | 10               |

*Rabbits were pretreated i.v. with SPE at 3 h before endotoxin injection.
**Deaths were recorded over 48 h.
carbohydrate metabolism are distinctive features in endotoxemia and gram-negative sepsis, and the hypoglycemia has been implicated as the cause of death (12, 16, 37). It seems probable, therefore, that SPE-induced deterioration of the glucose dyshomeostasis is involved in the potentiating effect on endotoxin lethality. The exaggerated hypoglycemic response has been demonstrated in most experimental models characterized by a hypersensitivity to endotoxin. For example, animals pretreated with carbon tetrachloride (CCl₄) develop a rapidly progressive and profound hypoglycemia with an absence of the early hyperglycemia following i.v. injection of relatively small doses of endotoxin (9). In this model, liver damage due to CCl₄ was suggested as a cause of the hypoglycemia since the liver is the chief organ for gluconeogenesis. In our study, SPE pretreatment enhanced not only the hypoglycemia but also the early hyperglycemia induced by endotoxin. In addition, it seems less likely that SPE causes severe liver damage since the administration of SPE alone resulted in no significant alterations in blood lipoperoxide level and plasma β-glucuronidase activity. Schlierver et al. (30) have reported that SPE does not produce progressive necrosis of the liver in rabbits. It is probable, therefore, that SPE does not directly cause the hepatocellular dysfunction which is responsible for the glucose dyshomeostasis but rather that it merely potentiates the toxic effects of endotoxin on the metabolic regulation of blood glucose.

In the present study, we demonstrated that SPE potentiates the endotoxin-induced elevation of blood lipoperoxide level and a more-acute response, such as the increase of plasma β-glucuronidase activity. The lipid peroxidation is known to arise in the lipid moiety of cell membranes in response to the local release of an excess number of free radicals and to be extremely damaging to biological systems (29). Endotoxin-induced increases in the levels of lysosomal enzymes in the circulation are presumed to be a reflection of cellular injury, and the major sources are believed to be the viscera (especially the liver and pancreas) and leukocytes sequestered in capillaries (8, 25). Although the extent to which these responses are responsible for the lethal shock is unclear, the fact that SPE potentiates these endotoxin-induced pathophysiologic changes, as well as glucose dyshomeostasis, suggests that SPE renders rabbits more sensitive to extensive pathophysiologic effects of endotoxin. Thus the potentiating effect of SPE on endotoxin lethality may involve a general potentiation of physiologic failures.

It is well known that pretreatment of animals with several colloidal agents which block the phagocytic function of the reticuloendothelial system (RES) renders them more sensitive to endotoxin (2, 3). Hanna and Watson (17) found that SPE depresses RES phagocytic function, as defined by carbon clearance, and speculated that SPE allows greater time for endotoxin to exert its deleterious effect before it is phagocytized by the RES. The speculation is consistent with our findings on pathophysiologic changes suggesting that SPE potentiates endotoxin toxicity extensively. Schlierver et al. (30) recently reported that SPE practically inhibits the clearance of endotoxin from the circulation. Also, in the present study, we confirmed that SPE pretreatment depresses the vascular clearance of a large and lethal dose of endotoxin. However, SPE pretreatment failed to affect the clearance of a small dose of endotoxin, although pathophys-

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**TABLE 2. Effect of SPE pretreatment on endotoxin-induced elevation of blood lipoperoxide level in rabbits**

<table>
<thead>
<tr>
<th>SPE/endotoxin treatment* (µg/kg)</th>
<th>Blood lipoperoxide (nmol of MDA/ml) at time h:</th>
<th>No. dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3</td>
<td>4.5</td>
</tr>
<tr>
<td>0/Saline</td>
<td>1.07 ± 0.17</td>
<td>1.05 ± 0.11</td>
</tr>
<tr>
<td>10/Saline</td>
<td>1.02 ± 0.11</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>1,000/Saline</td>
<td>1.16 ± 0.05</td>
<td>1.11 ± 0.06</td>
</tr>
<tr>
<td>0/1</td>
<td>1.03 ± 0.06</td>
<td>1.61 ± 0.30</td>
</tr>
<tr>
<td>0/100</td>
<td>1.17 ± 0.08</td>
<td>2.10 ± 0.28*</td>
</tr>
<tr>
<td>10/1</td>
<td>1.12 ± 0.11</td>
<td>3.12 ± 0.61*</td>
</tr>
</tbody>
</table>

* Rabbits were pretreated with SPE at 3 h before endotoxin injection.
* MDA, Malondialdehyde. All values are expressed as the mean ± the standard error of the mean. Sample collection time before or after the injection of endotoxin or saline.
* P < 0.05, as compared with control treated with saline alone.
The pathophysiologic effects of the endotoxin dose were potentiated. Several possible interpretations of these findings are discussed below. First, the major sites of endotoxin deposition and detoxification may differ with the size of endotoxin dose, e.g., RES tissues versus plasma components (33), so that SPE may not affect the clearance mechanism which contributes to the sequestration of a small quantity of endotoxin. Second, with a lethal dose, hemodynamic changes incident to fatal shock may depress the endotoxin clearance since the clearance rate of phagocytizable particles is determined not only by the phagocytic capacity of the RES but also by such factors as blood flow through the liver (4). Third, endotoxin by itself may depress the clearance function since endotoxin has been reported to cause an initial depression of RES phagocytic function (17). In any event, our findings indicate that the potentiating effect of SPE on pathophysiologic changes induced by a small dose of endotoxin may not be due to a decrease in the vascular clearance of endotoxin. It was concluded, therefore, that the potentiation of lethal endotoxin shock by SPE could not be readily explained solely on the basis of the decreased clearance of endotoxin.

It has been suggested that the functional status of the RES exerts a determinant effect on host susceptibility to endotoxin, whereas the mechanism and locus of this effect are not phagocytic function per se (5). Fisher (13) has also reported that the increased sensitivity to endotoxin in RES-blocked animals is a function of delayed disposal, not phagocytosis, of endotoxin. As for SPE, Schlievert et al. (30) have found that the toxin inhibits RNA synthesis in Kupffer cells. SPE could therefore reduce the ability of RES cells (e.g., Kupffer cells) to detoxify endotoxin by impairing the synthesis of inducible detoxifying enzyme. On the other hand, RES macrophages have been shown to release such injurious substances as a monokine with insulinlike activity (11), hydrogen peroxide and oxygen radicals (26), and lysosomal enzymes (34) in response to endotoxin, which are probably...
involved in the pathophysiologic changes assessed in the present study. Therefore, the possibility that SPE enhances endotoxin sensitivity by altering these macrophage functions is an interesting area for future studies. In any event, RES involvement still seems to be important in the potentiation of lethal endotoxin shock by SPE.

ACKNOWLEDGMENT

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LITERATURE CITED
