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Effects of Exogenous Agents on the Action of *Bordetella parapertussis* Heat-Labile Toxin on Guinea Pig Skin

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Injection of sonic extracts of *Bordetella parapertussis* into the shaved backs of guinea pigs produced hemorrhagic necrosis, which previously has been attributed to the action of heat-labile toxin. As heat-labile toxin was purified from this crude mixture, its ability to induce hemorrhagic lesions decreased significantly. However, ischemic lesions were apparent after injection of the purified toxin. These lesions, while not hemorrhagic in nature, were marked by erythema surrounded by a region in which the ischemia was apparent. Exogenous agents were found to alter the nature of the skin lesion induced by heat-labile toxin. The lipid A portion of endotoxin in combination with heat-labile toxin caused hemorrhagic lesions surrounded by a ring of ischemia, whereas bovine serum albumin increased the area of erythema. While the nature of lesions induced by heat-labile toxin was affected by exogenous agents, the diameter of ischemia produced by the toxin was found to be independent of the presence of these agents and was linear with toxin dose. These results indicate that induction of hemorrhagic necrosis may not be a reliable indicator of heat-labile toxin activity. Instead, measurement of the ischemic lesion produced by heat-labile toxin may be a useful assay for the toxin.

Virulent *Bordetella* spp. produce diseases of the upper respiratory tract in both animals and humans. Whooping cough (pertussis) and a mild whooping cough-like syndrome are produced in humans by *Bordetella pertussis* and *Bordetella parapertussis*, respectively. *Bordetella bronchiseptica* has been identified as the etiologic agent of kennel cough in dogs and atrophic rhinitis in pigs (13), whereas *Bordetella avium* causes rhinotracheitis in birds (17, 26). While the host ranges of these organisms differ, the bacteria produce strikingly similar clinical symptoms and pathological changes in the respiratory tract (3, 14, 15, 19, 21). The diseases produced by these organisms are often followed by serious secondary infections (3, 11, 24).

Heat-labile toxin (HLT) is a virulence factor which may play an important role in bordetellosis, since this toxin is one of only two toxins known to be produced by all virulent *Bordetella* spp. (11, 12). Bordet and Gengou, who were the first to describe the action of this toxin, noted that *B. pertussis* cells were dermonecrotizing and lethal when injected into animals (2). After the cells were heated for 30 min at 56°C, all dermonecrotizing activity and a large part of the lethal toxicity were destroyed. Cell homogenates of *B. parapertussis*, *B. bronchiseptica*, and *B. avium* produce similar dermonecrotic lesions (5, 9, 11). The HLTs of *B. parapertussis* and *B. bronchiseptica* appear to have similar sizes, as estimated by gel filtration chromatography (5, 9).

Many of the studies of HLT have utilized crude toxin preparations consisting of either whole cells or lysates of cells. Intracutaneous injection of guinea pigs or suckling mice with such preparations yields a hemorrhagic dermonecrosis which has been considered one of the hallmarks of HLT action (4, 18, 20). In fact, HLT has also been called dermonecrotic toxin and hemorrhagic toxin (1, 4, 22). This toxin, however, does not always produce hemorrhagic skin lesions in animals. Kurokawa et al. observed that the lesions produced in rabbits were ischemiclike, with or without hemorrhage (18).

Since this virulence factor may play a critical role in the pathogenesis of *Bordetella* spp., it is important to understand the biological effects of purified HLT. Therefore, we examined the ability of purified preparations of HLT to induce hemorrhagic necrosis. In this report, we describe findings which indicate that purification of this toxin results in a loss of the ability of toxin preparations to induce hemorrhagic necrosis in guinea pigs. Instead, these preparations produced ischemic lesions. We further investigated the ability of HLT to induce ischemia and examined the ability of exogenous agents to alter the biological activity of HLT.

MATERIALS AND METHODS

Materials. A standard *Escherichia coli* endotoxin available in the Pregel kit supplied by Seikagaku-Kogyo Co., Tokyo, Japan, was used. Synthetic lipid A (compound 506) was obtained from Daiichi Pure Chemicals, Tokyo, Japan. Bovine serum albumin, fraction V (BSA), was from Sigma Chemical Co., St. Louis, Mo.

Purification of HLT. *B. parapertussis* was grown as previously described (10). HLT was purified from a sonic extract of the bacteria by using a modification of the scheme devised for purification of HLT from *B. bronchiseptica* (5). Briefly, the bacteria were collected by centrifugation for 60 min at 4,500 × *g*. The bacterial pellet was washed with distilled H_2O, centrifuged, and resuspended in 100 ml of distilled H_2O (10^{12} cells per ml). The bacterial suspension at 0°C was sonicated for 6 min at 10 kHz in an Insonator (Model 200 M; Kubota Instruments, Inc., Tokyo, Japan). After centrifugation at 4°C for 50 min at 27,000 × *g*, the supernatant (180 ml) was collected. Portions of the supernatant (30 ml) were each layered on top of 5 ml of sucrose (1.15 g/cm^3) and were centrifuged for 5 h at 50,000 × *g*. HLT was found in the low-density layer (1.01 g/cm^3) above the sucrose solution and was carefully separated from the turbid layer found at the interface between the high- and low-density layers. The

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following steps were conducted at 4°C. The fraction (117 ml) containing HLT was applied to a column (60 by 1,230 mm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M Tris hydrochloride (pH 8.0) containing 0.5 M NaCl and was eluted at a flow rate of 80 ml/h. The fractions (20 ml) were assayed for A_{280} and for ischemia-inducing activity. The HLT-rich fractions at a position between two major protein peaks were pooled, concentrated by using YM-10 Diaflo ultrafiltration membranes (Amicon Corp., Lexington, Mass.), and applied to a column containing Sephadex G-150 (60 by 1,200 mm; Pharmacia) which had been equilibrated with 0.1 M Tris hydrochloride (pH 8.0) containing 0.5 M NaCl. The column was eluted with the same buffer. Fractions were collected and assayed for HLT activity. Those fractions containing HLT were pooled, the concentration of NaCl was adjusted to 0.1 M, and the pooled fractions were applied to a QAE-Sephadex A-50 column (25 by 200 mm) which had been equilibrated with 0.1 M Tris hydrochloride buffer (pH 8.0) containing 0.1 M NaCl. Protein which did not bind to the column (which included HLT) was collected, concentrated by ultrafiltration, and dialyzed against 0.01 M Tris hydrochloride (pH 8.0) containing 30% sucrose. Bio-Lyte 5/7 carrier ampholytes (Bio-Rad Laboratories, Richmond, Calif.) were added to this fraction to give a 1% (wt/vol) solution. The fraction was layered on an LKB type 8100-2 isoelectric focusing column (440 ml; LKB Instruments, Inc., Bromma, Sweden) under the condition of a continuous gradient of sucrose from 60 to 10% and was fractionated in a pH 2 to 12 density gradient formed at 800 V for 48 h at 0°C. Each fraction (10 ml) was tested for pH and assayed for ischemia-inducing activity. The HLT-containing fractions at pH 6.9 were pooled and concentrated as a final product. In this report, "partially purified HLT" refers to a preparation of HLT which was obtained after sucrose gradient centrifugation, Sepharose 4B chromatography, and Sephadex G-150 chromatography but which was not processed through the final purification steps.

Ischemia-inducing and dermonecrotizing activities. Ischemia-inducing and dermonecrotizing activities of HLT were quantified by using female Hartley guinea pigs weighing 300 to 350 g. Samples (100 μl) of an HLT preparation which had been diluted logarithmically in phosphate-buffered saline were injected intracutaneously in the shaved back of the animal. After 8 h, the diameter of the ischemia was measured. After 24 h, the colored lesions, ranging from light reddish (erythema) to purple (hemorrhagic necrosis), were measured. The minimum quantity of protein producing an ischemic lesion 10 mm in diameter is defined as the minimum ischemia-inducing dose (MID). Values reported for MID are means calculated from the dose-response curves for three test animals.

Assay of endotoxin. Endotoxin was measured by a *Limulus* amebocyte lysate assay, using the Pregel kit from Seikagaku-Kogyo Co. The assay was conducted according to the instructions of the manufacturer.

Statistical methods. Data were tested for statistical significance by Student's *t* test.

RESULTS

HLT was purified to remove traces of endotoxin contamination. The specific activities and endotoxin content of HLT preparations at various stages in the purification process are given in Table 1. The purified preparation of HLT used in this study contained less than 0.033 μg of endotoxin per mg of protein.

TABLE 1. Purification of HLT

Purification stage	Sp act (MIDs[a]/μg of protein)	Endotoxin content (μg/mg of protein)
Sonic extract	10	100
Partially purified HLT[b]	300	0.33
Purified HLT[b]	3,000	<0.033

[a] MID is defined as the minimum quantity of protein producing an ischemic lesion 10 mm in diameter. Thus 1 MID of the purified HLT preparation is equal to 0.33 ng of protein.

[b] Partially purified and purified HLT preparations were obtained as described in Materials and Methods.

Injection of sonic extracts of *B. parapertussis* into the shaved backs of guinea pigs produced the typical hemorrhagic necrosis that has been associated with HLT action (Fig. 1). As HLT was purified from this crude mixture, its ability to induce hemorrhagic lesions decreased significantly. Ischemic lesions were produced by the purified toxin. These lesions, while not hemorrhagic in nature, were marked by erythema surrounded by a region in which ischemia was apparent. In contrast, the purified preparations of HLT did induce hemorrhagic necrosis when injected into suckling mice (data not shown).

The ability of the purified HLT preparation to induce ischemia in guinea pig skin was sensitive to heat. HLT (100 MIDs) which had been heated to 56°C for 10 min was unable to induce any lesion when injected into the animal (data not shown).

The time course of HLT action is shown in Fig. 2. Small quantities (0.1 MID) of purified HLT induced a transient ischemic response which lasted for several hours. Larger doses of HLT resulted in ischemia of larger diameter and of longer duration. The larger doses of toxin produced a region of erythema surrounded by a ring of ischemia. The diameter of the lesion was dependent not only upon the dose of HLT but upon the injection volume. An increase in injection volume from 0.025 ml to 0.2 ml resulted in an increase in diameter of both erythema and ischemia (data not shown). A linear dose-response curve for ischemia was obtained when 1 to 30 MIDs of HLT were injected in a total volume of 0.1 ml. In all subsequent experiments, an injection volume of 0.1 ml was used.

Since other workers have previously described an effect of endotoxin on the size of hemorrhagic lesions produced by HLT preparations in guinea pigs (18), we examined the ability of purified lipopolysaccharide from *E. coli* to induce hemorrhagic necrosis. Endotoxin alone did not produce any

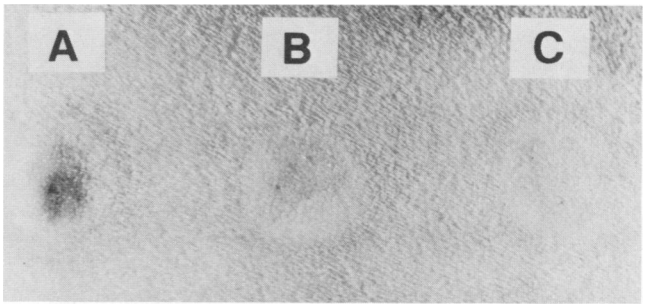

FIG. 1. Guinea pig skin reaction 24 h after injection with HLT preparations. Sonic extract (A), partially purified HLT (B), or purified HLT (C) was injected (100 MIDs of HLT activity per site in a volume of 0.1 ml).

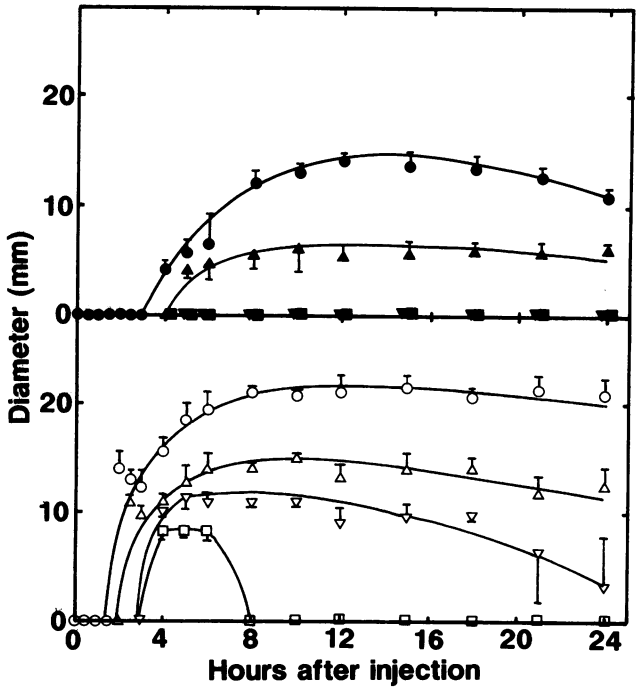

FIG. 2. Time course of HLT action. The diameters of erythemic (closed symbols) and ischemic (open symbols) lesions were measured at the indicated times after intracutaneous injection of 3 (● and ○), 1 (▲ and △), 0.3 (▼ and ▽), or 0.1 (■ and □) MID of purified HLT in a total volume of 0.1 ml. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations.

type of lesion when injected into the shaved backs of guinea pigs (data not shown). However, endotoxin in combination with purified HLT produced hemorrhagic lesions (Fig. 3). This action of endotoxin could be mimicked by the lipid A portion of endotoxin (Fig. 4). While induction of hemorrhagic necrosis depended on the presence of endotoxin in the preparation, the size of the ischemic lesion produced by HLT was not affected by this molecule (Fig. 5).

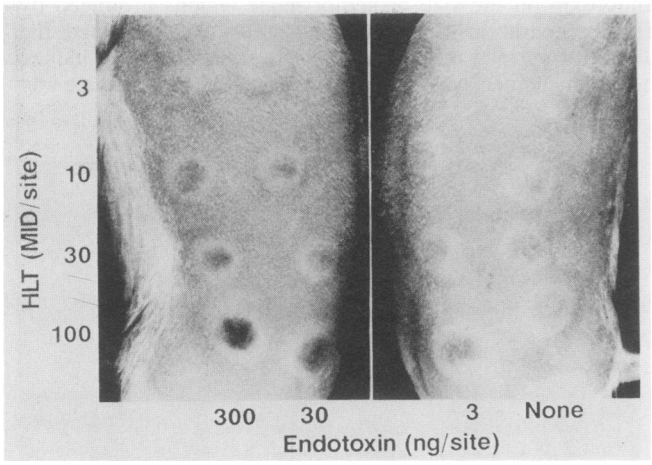

FIG. 3. Effect of endotoxin on HLT action. The indicated amounts of purified HLT and endotoxin were mixed in a total volume of 0.1 ml and were injected into the shaved backs of guinea pigs. The lesions resulting after 24 h are shown.

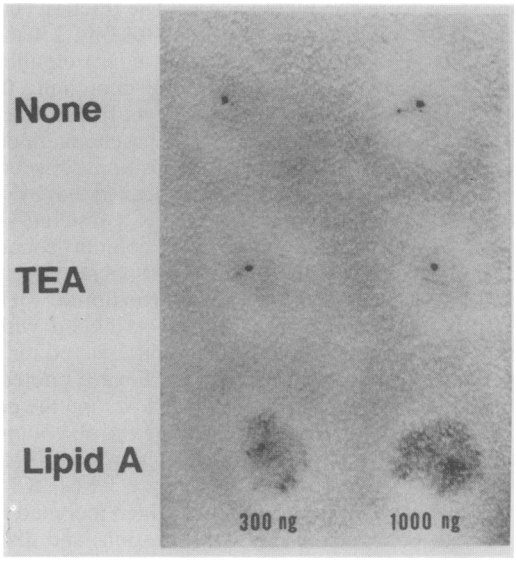

FIG. 4. Effect of synthetic lipid A (compound 506) on HLT action. Purified HLT (100 MIDs) in a total volume of 0.1 ml of either PBS (None) or PBS containing 2% triethylamine (TEA), each shown in duplicate, or TEA containing either 300 or 1,000 ng of lipid A as indicated was injected into sites on the shaved back of a guinea pig. The lesions resulting after 24 h are shown.

Since protein contaminants are present in crude preparations of HLT, we examined the ability of an exogenously added protein, BSA, to alter the ability of HLT to produce skin lesions. BSA had no effects on the diameter of ischemic lesions produced by HLT (Fig. 6). However, the region of the erythema inside the ring of ischemia was significantly larger when BSA was present.

DISCUSSION

The data reported in this study suggest that purified preparations of HLT produce ischemic skin lesions in guinea pigs. The vasoconstrictive activity of HLT may be its

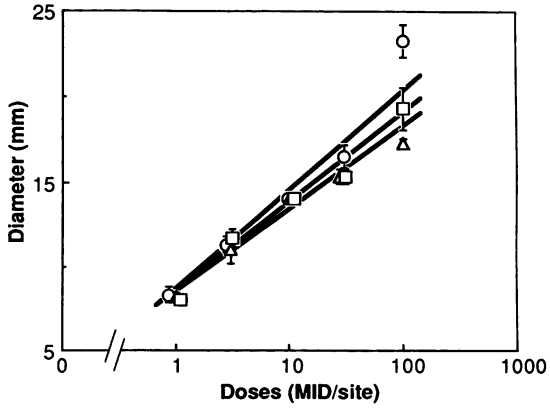

FIG. 5. Effect of endotoxin on ischemia-inducing activity of purified HLT. The indicated quantities of HLT in the absence (□) or presence of 30 (△) or 300 (○) ng of endotoxin in a total volume of 0.1 ml were injected into the shaved backs of guinea pigs. After 8 h, the diameter of the ischemia produced was measured. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations. The lines determined by each set of points were not significantly different ($P > 0.05$).

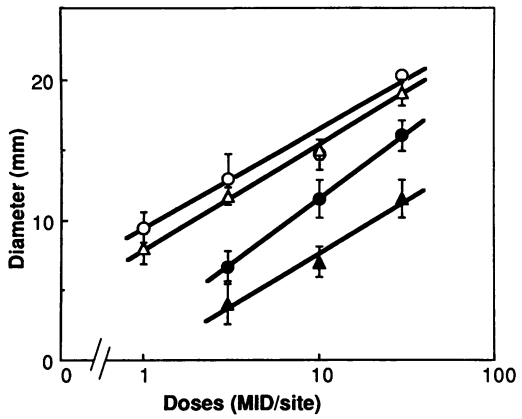

FIG. 6. Effect of BSA on ischemia- and erythema-inducing activities of purified HLT. HLT (1 to 30 MIDs per site) either with (○ and ●) or without (△ and ▲) BSA (1 mg) was injected in a total volume of 0.1 ml. The diameters of the ischemic (open symbols) and erythemic (closed symbols) lesions were measured at 8 and 24 h after the injection, respectively. Each symbol with a vertical line represents the mean ± standard deviation of triplicate determinations. The lines determined by the open symbols were not significantly different ($P > 0.05$). The lines determined by the closed symbols were significantly different ($P < 0.05$).

primary mode of action. Recently, HLT has been shown to constrict peripheral arterioles and venous capillaries in vivo (6) and was demonstrated to have a vasoconstrictive effect in perfused lungs isolated from guinea pigs (7). Moreover, this toxin induces contraction of vascular smooth muscle cells in culture (8, 9). The data presented here suggest that the hemorrhagic lesions in guinea pig skin which have previously been thought to be the typical result of HLT action are caused by HLT only in the presence of exogenous agents. We found that the addition of endotoxin, specifically the lipid A portion of the molecule, to preparations of HLT rendered these preparations capable of inducing hemorrhagic necrosis. The synergistic effect of HLT and endotoxin which resulted in hemorrhagic lesions may have been due in part to the ability of lipopolysaccharide to alter vascular permeability (27).

Previously, the skin lesions produced by HLT in guinea pigs have been described as hemorrhagic or necrotic (18, 23). These studies utilized crude preparations of HLT such as whole *Bordetella* cells or lysates of cells which would have contained significant quantities of endotoxin. Kurokawa et al. demonstrated that supernatants of sonicated *B. pertussis* cells as well as "further purified" preparations of HLT induced hemorrhagic lesions in guinea pigs. While the quantities of endotoxin present in the preparations utilized in that study were not determined, further addition of endotoxin to these preparations increased the sizes of the hemorrhagic lesions produced (18). These workers also showed that the HLT preparations which induced hemorrhagic lesions in guinea pigs produced ischemic lesions in rabbits either with or without hemorrhage. Thus, rabbits appeared to be less susceptible than guinea pigs to the hemorrhagic action of crude HLT preparations. In contrast to the findings of Kurokawa et al. (18), we did not find that our purified preparations of HLT produced hemorrhagic lesions in guinea pigs. Our purified preparations may have had a lower endotoxin content than those used in the earlier study. At the present time, the molecular mechanism by which purified HLT produces hemorrhagic lesions in suckling mice is not understood.

In addition to endotoxin, exogenously added protein may affect the lesion produced by purified HLT. In the presence of BSA, HLT produced a larger region of erythema, although the ischemia produced by HLT was unaffected by the added protein. BSA may intensify the inflammatory response at the site of injection, thus enlarging the region of erythema.

While exogenous agents alter the nature of the lesion produced by HLT in guinea pigs, the diameter of the ischemic lesion produced by either crude or purified preparations of HLT can be readily measured. Even the hemorrhagic lesions produced by crude preparations of HLT exhibit a clear ring of ischemia surrounding the necrotic lesion. Previously, the activity of HLT has been assayed by measuring the necrotic or hemorrhagic lesions induced by impure preparations of HLT (4, 11, 16, 25). The results reported here suggest that pure preparations of HLT should not be assayed in guinea pigs by a method in which induction of hemorrhagic necrosis is measured. Instead, an appropriate assay method for this toxin would involve determination of the ischemia-inducing activity of HLT preparations. The diameter of the ischemic region is independent of the presence of other agents and is linear with HLT dose. If the injection volumes of HLT preparations are standardized, the diameters of the resulting ischemic lesions can be used as a measure of active HLT. Thus, measurement of the ischemic activity of HLT in guinea pigs may be a useful assay for biological activity of this toxin.

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