Immunization of Rats with Heat-Labile Enterotoxin Provides Uniform Protection Against Heterologous Serotypes of Enterotoxigenic Escherichia coli

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Rats immunized with Escherichia coli heat-labile (LT) enterotoxin, either in the form of the holotoxin derived from a transformed K-12 strain or the polymyxin-release form obtained from human strains which produce LT toxin alone (LT+/ST− [ST is heat-stable toxin]) or together with ST toxin (LT+/ST+) were challenged with viable organisms of 10 different serotypes, 5 LT+/ST+ and 5 LT+/ST−. The serum antitoxin response was monitored by enzyme-linked immunosorbent assay, and the degree of protection was determined by challenge in ligated ileal loops. Immunization with the holotoxin provided a strong antitoxin response and protection against all 10 challenge strains. Immunization with toxin from the LT+/ST+ strain provided equally strong protection against all 10 strains, but immunization with toxin from the LT+/ST− strain yielded only a weak antitoxin response, moderate protection against challenge with LT+/ST− strains, and no protection against LT+/ST+ strains, increasing by fivefold the immunization dosage of the LT+/ST− toxin failed to enhance protection. These observations (i) establish the fact that immunization with the LT holotoxin provides uniformly strong protection against heterologous serotypes and (ii) indicate that, for reasons which remain to be determined, the immunogenicity of the polymyxin-release LT from an LT+/ST+ strain differs from that of an LT+/ST− strain.

Acute diarrheal disease caused by transient contamination of the small bowel by noninvasive, enterotoxigenic strains of Escherichia coli (ETEC) is widespread, particularly among children in underdeveloped countries and visitors to such places (21). The only practical measure for control of this disorder is the development of an immunization program capable of preventing either intestinal colonization by these strains or the secretory abnormality that their enterotoxins produce. Such a program would need to provide protection against all ETEC strains, including heterologous somatic serotypes which produce the heat-labile (LT) and heat-stable (ST) toxins, either singly or together.

Only one of the three E. coli antigens that have been shown to provide immunological protection against contamination by ETEC strains in experimental animals appears to afford consistent protection against heterologous somatic serotypes. (i) Immunization with the somatic antigen evokes protection by inhibiting bacterial growth, but only against the homologous serotype (13). (ii) Immunization with specific fimbrial antigens (colonization factors) provides protection against heterologous somatic serotypes by inhibiting adherence and colonization of the bacteria on the surface of the intestinal mucosa, but it extends only to strains having homologous fimbrial antigens (1, 17), and human ETEC strains are recognized to have at least two, and probably more, antigenically dissimilar fimbrial antigens (4, 15). (iii) Immunization with the LT toxin arouses an antitoxin response which protects against ETEC strains which produce LT, either alone (LT+/ST−) or in combination with ST (LT+/ST+) (10−12). The LT toxin produced by different somatic serotypes is thought to be antigenically homogeneous (21), but the evidence establishing that immunization with LT provides protection against all heterologous serotypes is confined to the demonstration of variable degrees of passive neutralization of crude LT toxin by antisera raised against either intact organisms or crude preparations of LT toxin (7, 9, 22).

This study had two purposes. The first was to determine whether immunization with a pure preparation of the LT holotoxin (HT) provides a uniform degree of protection against direct challenge with viable ETEC strains of different somatic serotypes. To accomplish this, rats immunized with HT derived from a transformed strain were directly challenged with viable
strains of 10 different serotypes, five LT+/ST− and five LT−/ST+. The second was to compare the immunogenicity of LT toxin from strains which produce only this toxin versus those which elaborate both LT and ST toxins. To evaluate this, rats immunized with the polymyxin-release form of LT toxin obtained from either an LT+/ST− or an LT−/ST+ strain were challenged with toxin and with viable strains of 10 different serotypes. The serum antitoxin response in all of the immunized groups was monitored by an enzyme-linked immunosorbent assay (ELISA) and the degree of protection was ascertained by challenge using the ligated ileal loop technique.

MATERIALS AND METHODS

Antigens used for immunization. HT was obtained from E. coli strain 711 (F1LT), a transformed K-12 derivative bearing the LT gene(s) of the Ent plasmid from porcine strain P307, and prepared in purified form by the methods described by Clements and Finkelstein (3). Suitable quantities of pure HT could not be obtained from human strains H 10407 (LT+/ST−) and PB 258 (LT+/ST−), necessitating the use of the polymyxin-release form of LT toxin from these strains; this material was purified by the methods described by Evans and his co-workers (5). The polymyxin-release LT toxin derived from strain H 10407 is referred to as LT/ST toxin; that from strain PB 258 is referred to as LT/− toxin. Toxin dosages are expressed in micrograms of protein as determined by the method of Lowry et al. (16).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (3) confirmed the homogeneity of the HT but showed the polymyxin-release toxins to consist of multiple protein bands. LT/ST toxin had (per microgram of protein) 1.6 μg, and LT/− toxin had 0.11 μg, of material that gave a positive reaction for 2-keto-3-deoxyoctonate (18), indicating the presence of lipopolysaccharide (LPS); the HT had none.

The HT was considerably more active in biological assay systems than the polymyxin-release LT toxins. (i) A positive response in the Y1 adrenal cell assay (19) was elicited, after trypsin activation, by 0.04 ng of HT, 9.8 ng of LT/ST toxin, and 156 ng of LT/− toxin. (ii) The 50% effective dose for stimulation of fluid secretion in ligated ileal loops of immunized rats was 180 μg for HT, 105 μg for LT/ST toxin, and 220 μg for LT/− toxin. (iii) The reciprocal of the maximum titer of goat hyperimmune serum to HT, as detected by using 1 μg of each antigen in ELISA, was 102,000 for HT, 1,600 for LT/ST toxin, and 400 for LT/− toxin.

LPS was prepared from strains H 10407 and PB 258 by the method of Westphal and Jahn (25), using multiple ultracentrifugations for purification.

Immunization procedures. Sprague-Dawley weaning rats were immunized by parenteral primary immunization followed by four weekly peroral booster immunizations. The parenteral immunization was given intraperitoneally, using Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The peroral immunizations were given via an intragastric tube 2 h after the peroral administration of cimetidine (Tagamet; Smith Kline & French Laboratories, Carolina, P.R.) as a dosage of 50 mg per kg of body weight, an amount shown to ablate gastric secretions in rats (2). The primary immunization dosage was 100 μg in all instances; unless otherwise specified, peroral booster dosages were 250 μg of LT/− and LT/ST toxins, 50 μg of HT in rats challenged with viable strains, and 250 μg of HT in those challenged with toxin.

Challenge procedures. Rats were challenged 1 week after the final booster immunization by instilling into a single 10-cm ligated loop of distal ileum, for 18 h, either toxin or viable organisms, using previously described techniques (10). Each datum point was determined by tests in two or more rats. Protection against toxin was determined by challenge with graded dosages of LT/ST toxin; this approach has been shown to yield equivalent results for protection after immunization with different LT toxin forms (12). The protection index was determined by dividing that dosage in immunized rats which yielded the same secretion as the 50% effective dose in unimmunized rats by the 50% effective dose for unimmunized rats. For challenge with viable cultures, 0.1 ml of a culture broth containing 10⁶ organisms per ml was used; this concentration has been found to be the minimum amount necessary to yield maximum secretion in ligated loops of unimmunized rats (10). The values reported are the mean ± standard error of the mean for the degree of reduced secretion in immunized rats as compared with unimmunized rats challenged with viable organisms of the same strain.

Challenge strains. Strains of all 10 serotypes were positive in the Y1 adrenal cell assay for LT, and all five of the LT+/ST− strains were positive in the sucking mouse assay (6) for ST. (Eight strains were kindly provided by Bernard Rowe.)

Antitoxin assays. Serum antibody titers were determined by ELISA (12), using HT as the antigen for sera from rats immunized with the LT toxins and the specific LPS preparations for sera from rats immunized with this material. The optimum concentration of each antigen was determined by checkerboard titration, HT against goat hyperimmune serum to HT and the LPS preparations against rabbit antiserum to E. coli serotype O78 or O15 (Difco). Values reported are the increase in the reciprocal of the geometric mean titer in immunized rats compared with the value for control sera, derived from 20 unimmunized rats, assayed with that specific antigen.

RESULTS

Immunization with LT toxins. (i) Challenge with toxin. The protection index against challenge with toxin was 13.0 in rats immunized with HT, 10.0 in those immunized with LT/ST toxin, and 2.6 in those immunized with LT/− toxin. Increasing the booster dosage of LT/− toxin, by either twofold to 500 μg or fivefold to 1,250 μg, failed to produce a significant increase in the protection index (Fig. 1).

(ii) Challenge with viable organisms. Rats
immunized with either HT or LT/ST toxin were strongly protected against challenge with all 10 serotypes (Table 1). The degree of protection was the same against LT"/ST" and LT"/ST+ strains (Fig. 2). In contrast, rats immunized with LT− toxin had only weak protection against the five LT"/ST" serotypes and virtually none against the five LT"/ST+ strains. Raising the booster dosage of LT− toxin fivefold to 1,250 μg failed to enhance the degree of protection against an LT"/ST" strain or to provide any protection whatsoever against an LT"/ST+ strain.

(iii) Serum antibody response. Serum antitoxin titers rose to a titer of 64 (a fivefold increase over the value in controls) in rats immunized with LT or LT/ST toxin, but only to 8 in rats immunized with LT− toxin, using either 250- or 1,250-μg booster dosages. Antibody titers to the LPS present in the polymyxin-release LT preparations were not increased over control values.

**Immunization with LPS.** To exclude the possibility that the LPS contaminant of the polymyxin-release LT preparations was responsible for the protection they afforded, groups of rats were immunized by the parenteral/peroral route with (i) the LT/ST and LT− toxins, (ii) the LT/ST toxin after exposure to 100°C for 30 min before use for immunization, and (iii) LPS from the two strains from which toxins were obtained. LPS dosages (in dry weight) were 100 μg for primary immunization and 150 μg for the booster immunization.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Results of challenge with LT/ST toxin in rats immunized with either LT− or LT/ST toxin. ED50, 50% effective dose; PI, protection index.

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Protection afforded by immunization with LT preparations from different sources against challenge with viable organisms. Values are the mean ± standard error for challenge with five LT"/ST" and five LT"/ST+ strains.

<table>
<thead>
<tr>
<th>Challenge strain</th>
<th>Serotype</th>
<th>Toxin</th>
<th>Protection after immunization with*:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
</tr>
<tr>
<td>PB 258</td>
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<td>LT&quot;/ST−</td>
<td>67 ± 4</td>
</tr>
<tr>
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<td>O114:H49</td>
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</tr>
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<td>58 ± 4</td>
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</tbody>
</table>

* Values are the mean ± standard error percent reduced secretion in immunized rats as compared with the value in unimmunized rats for each strain.

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**Table 1.** Protective effect of immunization with toxins against challenge with viable heterologous serotypes.
peroral boosters. The immunized rats were challenged with viable organisms of three strains, each of which has different enterotoxigenic properties.

Immunization with LT/ST toxin provided strong protection against the LT+/ST+ and LT+/ST- strains but none against the LT-/ST- strain, whereas immunization with LT- toxin provided weak protection against only the LT+/ST+ strain (Table 2). The protective effect of both toxins was abolished by heat inactivation. Rats immunized with LPS had weak to moderate protection against challenge with the homologous strains but none at all against heterologous strains. Immunization of an additional group of rats with LPS from the LT+/ST- strain, given in larger dosages of 500 μg by the parenteral route on two occasions, evoked stronger protection against challenge with the homologous strain (secretion was reduced by 48 ± 3%) but none against the heterologous strains. Serum antibody titers to the homologous LPS preparation were increased to 16 (twofold over control values) in rats immunized by either the peroral or parenteral route.

**DISCUSSION**

These results establish the fact that immunization with LT toxin, either in the form of HT produced by a transformed strain or in the polymyxin-release form derived from an LT+/ST+ strain, provides strong protection against direct challenge with viable heterologous serotypes of *E. coli* which produce LT toxin alone or in combination with ST toxin (in which case we presume that the protection is directed against the LT toxin). The protection provided by immunization with these toxins was clearly due to an antitoxin response and was unrelated to other immunogens present in some of the toxin preparations used for immunization. The HT was a homogeneous preparation. Although the LPS contained in the polymyxin-release toxin preparations may have exerted an adjuvant effect, any other role of this material can be excluded by the facts that (i) the immunogenicity of the toxins was heat labile, (ii) immunization with LPS derived from the strains used to make the toxins failed to provide protection against heterologous strains, and (iii) protected rats developed elevated serum antibody titers to the toxin used for immunization but not to LPS.

All previous immunization studies have used LT toxin preparations derived from an LT+/ST+ strain for the immunogen (10-12, 20, 22), and the effectiveness of immunization with LT toxin prepared from an LT+/ST+ strain has not been evaluated. The possibility that LT toxin produced by an LT+/ST- strain may differ antigenically from that produced by an LT+/ST+ strain was raised by studies in human volunteers who experienced different attack rates of diarrhea when reexposed to ETEC strains with differing enterotoxigenic properties (14). We found that, based on their protein concentrations, the polymyxin-release toxin from the LT+/ST- strain was less active than the toxin from the LT+/ST+ strain in the Y1 adrenal cell and ligated ileal loop assays and less potent as an immunogen in terms of its reactivity to goat antiserum to HT and its ability to provide a strongly protective antitoxin response in immunized rats. These observations suggest that there are quantitative or qualitative differences in the potency and immunogenicity of the LT toxin produced by these two strains; however, it is impossible to exclude the possibility that variable degrees of contamination with protein may have contributed to these differences and that an adjuvant effect of the variable amount of LPS contained in the toxin preparations could have contributed to their different degree of immunogenicity in immunized rats. This question will be settled only when procedures to produce pure, homogeneous LT holotoxin from the two strains are identified.

Immunization with purified *Vibrio cholerae* LPS yields cross-protection against growth of both major *V. cholerae* serotypes (24), and the addition of this material to cholera toxin or

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**Table 2. Protective effect of immunization with toxin or LPS derived from different strains**

<table>
<thead>
<tr>
<th>Challenge strain</th>
<th>Serotype</th>
<th>Toxin</th>
<th>Protection after immunization:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LT+/ST+ strain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toxin</td>
</tr>
<tr>
<td>PB 258</td>
<td>O15:H-</td>
<td>LT+/ST-</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>H 10407</td>
<td>O78:H11</td>
<td>LT+/ST-</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>TX 452</td>
<td>O78:H12</td>
<td>LT+/ST-</td>
<td>2 ± 2</td>
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</table>

* See Table 1.
* (H) indicates that the toxin material was exposed to 100°C for 30 min before use for immunization. Heat-treated toxin from the LT+/ST- strain yielded 2 ± 3% reduced secretion against challenge with strain PB 258.
toxoid for immunization yields synergistic antibacterial and antitoxin protective effect (23). This has led to the suggestion that a combination of V. cholerae LPS and the cholera toxin B subunit may represent the optimal antigen for immunological protection against cholera (8). Such does not appear to be the case for immunization against ETEC strains, however, since the E. coli LT toxin B subunit is considerably less antigenic on a molar basis than HT (12), and we found that immunization with purified preparations of E. coli LPS does not protect against challenge with heterologous serotypes. Although the LPS contaminant may have provided an adjuvant effect for the polymyxin-release LT toxins, the homogeneous HT, which contains no LPS, is the most immunogenic form of LT (12), and the use of adjuvants for this material that are less toxic than LPS would seem preferable.

ACKNOWLEDGMENTS

This study was supported by a grant from Johnson and Johnson Baby Products Co., Raritan, N.J., and by contracts DAMD 17-77-C-7032 from the U.S. Army Medical Research and Development Command and NR 204-060 from the Office of Naval Research.

LITERATURE CITED


