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Arousal of Mucosal Secretory Immunoglobulin A Antitoxin in Rats Immunized with *Escherichia coli* Heat-Labile Enterotoxin

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Specific serum and mucosal antitoxin levels were determined by enzyme-linked immunosorbent assays in rats immunized with *Escherichia coli* heat-labile enterotoxin (LT). Immunization by means of a parenteral prime followed by peroral boosts was the only approach that aroused titers of both serum immunoglobulin G (IgG) antitoxin and mucosal secretory IgA antitoxin that were increased fourfold or more over control values. Primary parenteral immunization was effective when given either intraperitoneally or subcutaneously with either Freund complete adjuvant or alum as the adjuvant. The magnitude of the mucosal secretory IgA antitoxin response and the degree of protection against challenge with either LT or viable LT-producing organisms were related to the number and dosage of peroral boosts. LT antigenicity, as determined by enzyme-linked immunosorbent assay, was progressively reduced by toxoiding it with increasing amounts of glutaraldehyde or a carbodiimide; when LT antigenicity was reduced by >50%, the effectiveness of the toxoid in stimulating mucosal antitoxin and providing protection was compromised. Strong protection extended for more than 6 weeks only in rats immunized with a sufficient peroral dosage of LT to arouse mucosal secretory IgA antitoxin titers at least fourfold greater than those of controls. These observations indicate that the ability of LT to stimulate a mucosal secretory IgA antitoxin response is dependent on the antigenicity, route, and dosage of this immunogen; they suggest that the duration of protection in animals immunized by the peroral route is related to the extent of arousal of mucosal secretory IgA antitoxin.

Protection against the secretory effect of intestinal contamination by some species of enterotoxigenic enteric pathogens has been achieved in experimental animals by immunization with the appropriate toxin or toxoid (10, 14, 20). The nature of the protective antitoxin (AT) response depends on the route of delivery: in the case of cholera toxin (CT), immunization given by the parenteral route arouses primarily a serum immunoglobulin G (IgG) AT response, whereas immunization by the intestinal route evokes principally an IgA AT response within the intestinal mucosa. The respective AT titers correlate with the degree of protection in both instances (11, 14, 16, 23). Cholera toxoid differs from CT in that primary parenteral immunization is necessary for effective subsequent intestinal immunization (15, 17, 19, 20).

Immunization of rats with the *Escherichia coli* heat-labile enterotoxin (LT) provides protection against challenge with homologous or heterologous serotypes of *E. coli* which elaborate LT, either alone (LT^+/ST^-) or together with the heat-stable toxin (ST) (LT^+/ST^+) (4, 8, 10). This immunogen resembles cholera toxoid in that, in this animal model at least, it also requires primary parenteral immunization for peroral (p.o.) immunization to be effective (4–7). Immunization with LT given just by the parenteral route, or by a parenteral prime followed by p.o. boosts, has been found to yield dose-dependent increases in serum IgG AT levels which correlate with the degree of protection (6, 7), but the mucosal AT response to immunization with this toxin has not been ascertained so far.

In the present study, we used the enzyme-linked immunosorbent assay (ELISA) to quantitate both the serum and the mucosal AT responses to immunization with LT. Factors affecting the mucosal secretory IgA (sIgA) AT response, including the antigenicity, route of administration, and dosage of the LT immunogen, were identified, and the relationship between levels of this AT and the degree of imme-

diate and extended protection was evaluated.

MATERIALS AND METHODS

Immunogens. The LT holotoxin was prepared in purified form by the methods of Clements and Finkelstein (2) from *E. coli* 711 (F1LT), a transformed K-12 derivative bearing the LT gene(s) of the Ent plasmid from porcine strain P307. Its homogeneity was confirmed by polyacrylamide gel electrophoresis. Concentrations of LT and its toxoids are expressed on the basis of protein concentration as determined by the method of Lowry et al. (12).

LT toxoids were obtained by exposing LT either to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) for 18 h at 4°C at EDAC-toxin ratios of from 10 to 75 by weight (4.8×10^3 to 35.8×10^3 M) or to glutaraldehyde for 96 h at 37°C at molar ratios of from 100 to 600, which are the same as those that have been used to toxoid CT (21). The toxoids were subsequently exhaustively dialyzed against water to remove the toxoiding agents. The toxicity of the toxoids was compared with that of unattenuated LT by testing twofold serial dilutions of trypsinized LT and toxoids in the Y1 adrenal cell assay (22). Values for the toxoids are expressed as percentages of the minimum dosage of that material compared with that of LT which yielded a positive response. The antigenicity of the toxoids was assessed in an ELISA by comparing the response of twofold serial dilutions of LT or the toxoids laid down as the solid phase against goat monospecific hyperimmune antiserum to LT. Concentrations yielding an optical density of 0.600 at 410 nm were compared, and the results for the toxoids were expressed as percentages of the values for unattenuated LT (Fig. 1).

Immunization procedures. Eight-week-old Sprague-Dawley rats were used. Unless otherwise specified, all parenteral immunizations were administered by the intraperitoneal (i.p.) route with Freund complete adjuvant for primary immunization and Freund incomplete adjuvant for boosting. LT alum adjuvant was prepared by mixing 1 volume of LT with 1 volume of 10% lactose and 18 volumes of 1% aluminum potassium sulfate. The pH was then adjusted until maximum turbidity was achieved; this occurred at a pH of 5.8. For p.o. immunization, the toxin was given via an intragastric tube 2 h after the p.o. administration of cimetidine (Tagamet; Smith Kline & French Laboratories, Philadelphia, Pa.) at a dosage of 50 mg/kg of body weight, an amount that has been shown to be sufficient to ablate gastric secretion in rats (1). Unless otherwise specified, immunizations were given at 4-day intervals.

Challenge procedures. Rats were challenged 1 week after the final boost by the instillation of test material into a single 10-cm ligated loop of distal ileum for 18 h. Challenge dosages were 500 pg of LT or 0.1 ml of a culture containing 10^9 organisms per ml of *E. coli* LT^+/ST^- strain PB258 ($O15:H^-$); these dosages have been shown to arouse maximum secretion in unimmunized rats (4, 9). Each datum point was determined for three to five rats, and the values reported are for the mean ± standard error of the mean of the degree of reduced secretion in immunized rats as compared with unimmunized rats similarly challenged. Under these challenge conditions, a 50% reduction in secretion is referred to as significant protection since it represented a statistically significant difference ($P < 0.001$ as determined by Student's t test for two independent means) between the values in immunized and control animals.

AT titers. Serum and mucosal AT titers to the LT holotoxin were determined by ELISA (24) with immobilized LT. In these experiments, rabbit anti-rat IgG together with goat anti-rabbit antiserum conjugated to alkaline phosphatase and goat anti-rat sIgA together with rabbit anti-goat antiserum conjugated to alkaline phosphatase (Miles Laboratories, Inc., Elkhart, Ind.) were used. The AT titer was the maximum dilution which gave an optical density of 0.100 or more measured spectrophotometrically at 410 nm. Values reported are for the increase in the reciprocal of the geometric mean titer for samples from five or more rats in each test group over the value of 1:2 obtained in a control group of 10 unimmunized rats; thus, a fivefold increase in immunized animals represented a titer of 1:64.

Mucosal AT titers were measured in intestinal washings. Ligated loops were emptied, rinsed with 5 ml of normal saline, and centrifuged; the supernatant was sterile filtered and then lyophilized and stored at −60°C until it was reconstituted at a constant volume for assay. To exclude the possibility that AT levels might be spuriously reduced by binding of the AT to the antigen that was placed in the challenge loops, AT titers were compared in washings from both challenge and adjacent loops in each rat; the values were the same.

None of the immunized rats had increased titers of serum sIgA or mucosal IgG AT; therefore, these determinations are not reported. AT values referred to as serum AT indicate IgG AT, and those referred to as mucosal AT indicate sIgA AT.

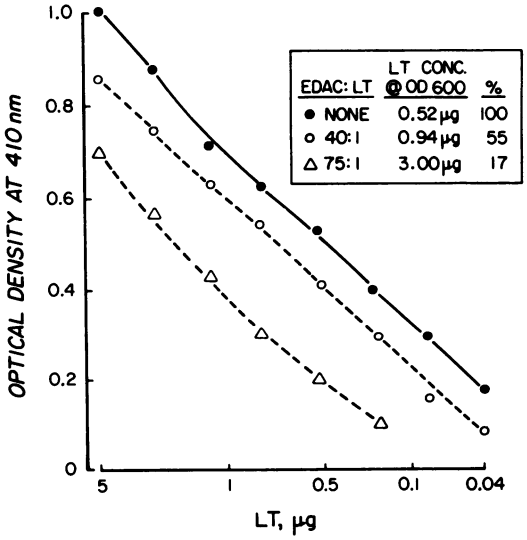

FIG. 1. Antigenicity of LT exposed to various amounts of the carbodiimide EDAC, as determined by ELISA. OD 600, Optical density at 600 nm.

TABLE 1. Influence of route of immunization on antitoxin response and degree of protection

Route[a]	Dosage (μg)[b]	% Reduced secretion[c]		AT titer[d]	
		Toxin	LT^+/ST^-	Serum	Mucosal
i.d./i.d.	250/500	3 ± 3	6 ± 2	0	0
p.o./p.o.	350/350	2 ± 2	4 ± 3	0	0
i.p./i.p.	100/500	92 ± 3	69 ± 2	5	6
i.p./i.p.	350/350	52 ± 2	39 ± 4	5	0

[a] Prime/boosts. i.d., Direct intraduodenal administration.
[b] Prime/each of two boosts.
[c] Mean ± standard error of the mean percent reduced secretion in immunized rats compared with similarly challenged unimmunized animals. Reduced secretion of >50% represents a significant difference ($P < 0.001$) between the two groups.
[d] Fold increase over the value in unimmunized rats.

RESULTS

Factors influencing immediate protection. (i) Route of immunization. Rats were immunized by four different approaches with a total dosage of between 1,050 and 1,250 μg given in divided dosages on three occasions, at 10-day intervals by direct instillation or at 4-day intervals by the other approaches (Table 1). Rats immunized either exclusively by the p.o. route or by the direct instillation of the toxin into the duodenum by means of a laparotomy had no AT response and were not protected against challenge. In contrast, those immunized with two p.o. boosts after primary i.p. immunization had at least fivefold elevations of both serum and mucosal AT titers and were significantly protected against challenge with both the toxin and viable bacteria. Immunization exclusively by the parenteral route aroused only a serum AT response; animals so immunized had significant protection against challenge with the toxin.

(ii) Site of parenteral prime and type of adjuvant used. Rats were immunized with a 100-μg parenteral prime given at different sites followed by two p.o. boosts of 500 μg each (Table 2). When Freund complete adjuvant was used, rats primed by either the i.p. or subcutaneous (s.c.) route had at least fivefold increases in serum and mucosal AT titers and were significantly protected. Rats primed by the s.c. route with alum as the adjuvant had the same degree of AT response and protection when the priming dose was doubled to 200 μg. Without any adjuvant, s.c. priming failed to evoke a significant increase in AT titers and provided only a slight degree of protection.

(iii) Number and dosage of p.o. boosts. Rats were given a primary immunization of 100 μg by the i.p. route with Freund complete adjuvant followed by either two or four p.o. boosts of various dosages (Fig. 2). A total p.o. dosage of 200 μg failed to raise AT titers or to provide protection when given in two boosts, but this dosage did yield a fourfold increase in serum AT titers and a significant degree of protection when it was divided into four boosts. Immunizations with both two and four boosts aroused at least fourfold increases in serum and mucosal AT titers associated with significant protection when given at a total dosage of 500 μg. Doubling the total p.o. dosage to 1,000 μg increased AT titers in both groups further, to at least five or six times their original levels, and produced an increased degree of protection in those immunized with two boosts.

(iv) Antigenicity of the LT immunogen. Exposure of LT to increasingly larger amounts of either glutaraldehyde or EDAC resulted in a progressive decline in antigenicity (Fig. 3) as

TABLE 2. Influence of injection site and adjuvants used for primary parenteral immunization

Prime[a]			% Reduced secretion[b]		AT titer[b]	
Site	Dose (μg)	Adjuvant	Toxin	LT^+/ST^-	Serum	Mucosal
i.p.	100	FCA	92 ± 3	69 ± 3	5	6
s.c.	100	FCA	72 ± 1	59 ± 2	5	5
s.c.	100	None	23 ± 3	3 ± 3	1	1
s.c.	100	Alum	49 ± 1	37 ± 2	2	2
s.c.	200	Alum	68 ± 1	57 ± 1	4	5
s.c.	300	Alum	80 ± 1	60 ± 1	4	5

[a] Primary immunizations were followed by two 500-μg p.o. boosts. FCA, Freund complete adjuvant.
[b] Values are expressed as in Table 1.

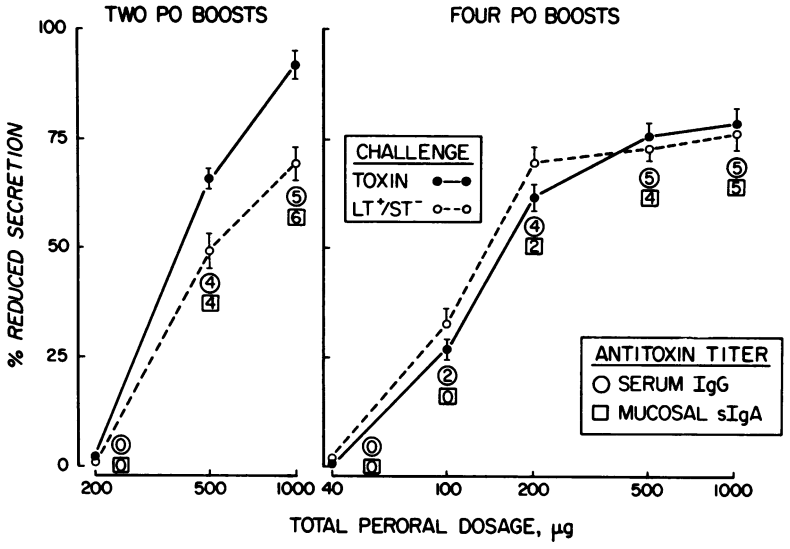

FIG. 2. Relationship between the number and total dosage of p.o. boosts and the AT response and degree of protection. All groups received an i.p. prime of 100 μg.

well as in toxicity; at the maximum amount of toxoiding agent used, the glutaraldehyde toxoid had 32% residual LT toxicity, and the EDAC toxoid had <1% residual toxicity. To determine whether reduced antigenicity, as determined by ELISA, correlated with compromised effectiveness as an immunogen, an LT toxoid which had 43% retained antigenicity after exposure to EDAC at a ratio of 45:1 was used to immunize rats. The effectiveness of this toxoid was compared with that of LT when both were given as a 100-μg i.p. prime followed by two p.o. boosts of various dosages (Fig. 4). Four times more toxoid than unattenuated LT was needed to achieve fourfold elevations of serum and mucosal AT titers and to afford a significant degree of protection.

Relationship of mucosal AT to extended protection. Serum and mucosal AT titers and the

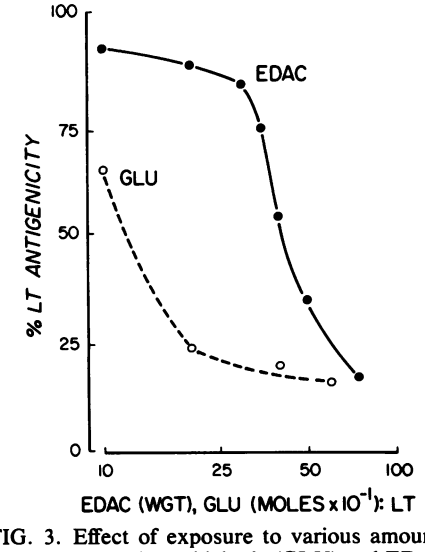

FIG. 3. Effect of exposure to various amounts of toxoiding agents glutaraldehyde (GLU) and EDAC on the antigenicity of LT as determined by ELISA. Values are expressed as percentages of antigenicity of unattenuated LT.

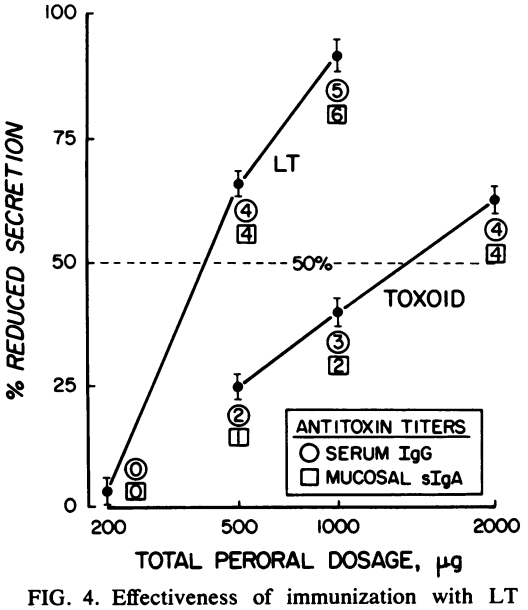

FIG. 4. Effectiveness of immunization with LT toxoided with an EDAC ratio of 45:1 by weight. This toxoid had a retained antigenicity of 43% as determined by ELISA. Challenge was with 500 pg of LT.

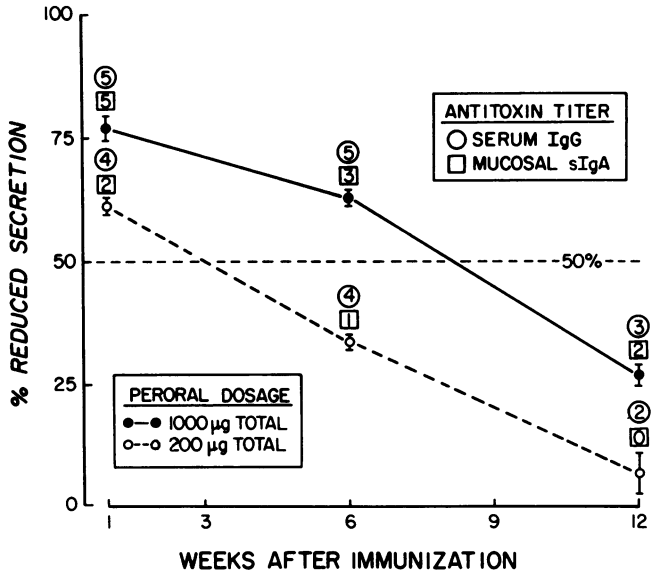

FIG. 5. Influence of mucosal sIgA levels on extended protection. Both groups were immunized with a 100-μg i.p. prime followed by four p.o. boosts of either 50 or 250 μg. Challenge was with 500 pg of LT.

degree of protection were monitored over a 3-month period in two groups of rats (Fig. 5). Both groups were immunized by an i.p. prime followed by four p.o. boosts of two different dosages. Both groups had at least fourfold elevations of serum AT titers and were significantly protected immediately after immunization, but only those which had received a total p.o. dosage of 1,000 μg had a fourfold increase or more in mucosal AT titers.

Serum AT titers remained unchanged at 6 weeks after immunization in both groups, but significant protection persisted only in those with elevated mucosal AT titers. By the end of 12 weeks, serum and mucosal AT titers had fallen to less than fourfold increases, and the degree of protection was reduced in both groups. Based on the rate of fall in the degree of protection, a significant degree of protection persisted for approximately twice as long in those rats which had elevated mucosal AT titers.

DISCUSSION

Several techniques have been developed to document a specific mucosal IgA AT response to intestinal immunization with CT: the enumeration of specific antitoxin-containing cells within the lamina propria by immunofluorescence (11, 13–15), quantitation by ELISA of AT present in intestinal washings (15, 25), and determination by ELISA of the amount of AT produced during in vitro tissue culture of intestinal tissue (23). These approaches have shown that the magnitude of the mucosal IgA AT response to immunization with CT or toxoid is related to the number and dosage of intestinal immunizations and that mucosal AT levels correlate with the degree of protection against challenge with either toxin or viable *Vibrio cholerae*.

The results of the present study established the fact that intestinal immunization with LT also arouses a dose-dependent response of mucosal sIgA AT. It is well recognized that parenteral immunization can serve as an effective prime for a mucosal IgA response to a locally applied antigen (3, 15), and certain immunogens appear to require this when given by the intestinal route to achieve an immunological response. Cholera toxoid is effective as an intestinal immunogen only when given after parenteral primary immunization; this can be achieved by s.c. priming in dogs, but only priming by the i.p. route is effective in rats (15, 17, 20). Our past experience has been that LT also requires primary immunization by the parenteral route for p.o. immunization to be effective and that the p.o. route does not provide an effective prime for subsequent boosting by either the p.o. or the parenteral route (4–7). The results of the present study show that s.c. and i.p. sites are equally effective for primary immunization and that alum can serve as a satisfactory adjuvant for LT. Whether the requirement for parenteral priming is unique to the rat animal model used in the present and previous studies (4–7) or is a general attribute of LT as an immunogen is under investigation.

The effectiveness of LT as an intestinal immunogen was also found to be related to the antigenicity of the toxin preparation used. We

have previously shown that, on a molar basis, the LT holotoxin is a more effective immunogen than other forms of LT, such as that obtained by polymyxin release (6). Past experience with other toxins, such as CT (13, 21), has shown that toxoiding usually reduces the antigenicity of the toxin, the degree of which depends on the specific toxoiding agent and the amount and conditions used for toxoiding. We found that exposure of LT to increasing amounts of either of two toxoiding agents, glutaraldehyde and the carbodiimide EDAC, resulted in a progressive reduction in LT antigenicity as determined by an ELISA and that reduced antigenicity of the toxoid, as demonstrated by this assay, correlated with compromised effectiveness of the toxoided LT to evoke an AT response and provide protection in immunized animals. Our findings indicate that preliminary screening of toxoids for residual antigenicity by means of an ELISA can be used to expedite the evaluation of toxoiding agents and conditions by obviating the need to examine all toxoid preparations with the more cumbersome and time-consuming animal studies.

Although the respective contributions of serum and mucosal antibodies to protection against enterotoxigenic enteric pathogens remain to be clarified, the limited evidence available suggests that mucosal sIgA AT is primarily responsible for extended protection. Parenteral immunization yields only transient protection, whereas parenteral-p.o. immunization provides extended protection both in dogs immunized with cholera toxoid (15, 19, 20) and in rats immunized with LT (5, 7). In the present study, we found that strong protection extended for a longer period of time in rats which had received a sufficient p.o. dosage to achieve elevated mucosal sIgA AT levels. Previous studies have shown that the mucosal immunological system has immunological memory which is capable of rapid amnestic response to intestinal reexposure to the antigen: a single p.o. boost of LT given several months after immunization restores strong protection in rats (5), and subsequent intestinal boosts with cholera toxoid increase intestinal AT levels in dogs (18). Mucosal immunological memory appears to persist even after mucosal AT has fallen to undetectable levels since Pierce et al. found that dogs immunized with cholera toxoid by the s.c.-p.o. approach are protected against challenge with *V. cholerae* long after detectable serum and mucosal AT levels have disappeared (15).

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