Protective Effect of Active Immunization with Purified
Escherichia coli Heat-Labile Enterotoxin in Rats

FREDERICK A. KLIPSTEIN* AND RICHARD F. ENGERT

Gastroenterology Unit, Department of Medicine, University of Rochester School of Medicine and Dentistry,
Rochester, New York 14642

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The protective effect of active immunization by different routes with a purified preparation of the polymyxin-release form of Escherichia coli heat-labile toxin was evaluated in rats. Immunized animals were challenged by placing toxin into ligated ileal loops at dosages which produced either 50% or the maximum secretary response in unimmunized rats. Immunization exclusively by the parenteral route yielded significant protection. Rats were also protected when parenteral priming was followed by boosting given either directly into the duodenum or perorally 2 h after intragastric cimetidine, but not when the peroral boosts were given with bicarbonate. Immunization administered entirely by the peroral route with cimetidine yielded protection but only when the immunizing dosage was fivefold greater than that found effective in the parenteral-peroral approach. Rats immunized exclusively by the parenteral route and those boosted perorally with cimetidine were also tested, and found to be protected, against challenge with viable organisms of strains that produce either heat-labile toxin alone or both heat-labile and heat-stable toxin, but they were not protected against a strain which produces just heat-stable toxin. Geometric mean serum antibody titers were increased by 16-fold or more over control values in those groups of rats in which protection was achieved, with the exception of those immunized exclusively by the peroral route. These observations demonstrate that (i) active immunization with purified E. coli heat-labile toxin results in significant protection against both this toxin as well as viable organisms which produce it, but not against viable strains which produce heat-stable toxin only, and (ii) concomitant ablation of gastric secretion by the use of cimetidine renders the peroral route of immunization effective. They suggest that prophylactic immunization against diarrheal disease caused by heat-labile toxin-producing strains of E. coli may be feasible in humans.

Both Vibrio cholerae and toxigenic strains of Escherichia coli cause acute diarrheal disease by means of elaborating enterotoxin(s) during intestinal colonization. Because immunization with somatic antigens of these bacteria has proven to be of only limited protective value (2, 13), recent investigations concerned with the development of prophylactic immunization against these disorders have focused on immunological aspects of the enterotoxins. Cholera toxin and its two subunits are antigenic (18), as is the large-molecular-weight, heat-labile toxin (LT) produced by E. coli which is antigenically similar to, although not identical with, cholera toxin (14, 17, 27, 39). The low-molecular-weight, heat-stable form of E. coli toxin (ST) is nonimmunogenic when impure forms from human strains are used for immunization (7, 22), although purified ST from a porcine strain has been found to be weakly antigenic (1).

Knowledge concerning the protective value of immunization with cholera toxin is well advanced. Active immunization yields complete protection in experimental animals against intestinal challenge with either the toxin or viable V. cholerae (19, 29). Parenteral immunization, with the presently available form of toxoid has yielded only transient, limited protection in field trials (4), however, and attention is now being directed toward identifying the optimal immunizing agent (18) and route of administration (31). Considerably less is known about the protective value of immunization with E. coli LT because this has been evaluated mainly in terms of passive protection. The addition of hyperimmune antiserum developed in rabbits to impure forms of this toxin has been found to protect against the action of the toxin in evoking fluid secretion in rabbit ligated ileal loops (15, 17), in inducing diarrhea in infant rabbits (38) or...
tobiotic pigs (23), and in causing fluid secretion during in vivo perfusion in the rat jejunum (21). Attempts at active immunization, which employed crude toxin preparations, have either failed to demonstrate any protective effect when ligated loops of immunized rabbits were challenged with toxin (6, 39) or achieved protection only when large doses were used for immunization (35). Pierce has recently shown that active immunization of rats with cholera toxoid is also protective when ligated loops are challenged with *E. coli* LT (27).

In the present study, we evaluated the protective value of active immunization in rats with the purified polymyxin-release form of *E. coli* LT. Groups of animals were immunized by five different routes of administration, including parenteral priming followed by boosts given parenterally, intraduodenally, or perorally, and bicarbonate or after cimetidine, as well as exclusively by the peroral route. The protection afforded by these approaches was evaluated by challenge in which graded doses of the toxin were added to ligated loops. Additional rats immunized by those two approaches involving parenteral priming followed by either parenteral or peroral boosting which had proved to be most effective were also challenged by exposing ligated loops to graded concentrations of separate strains of viable *E. coli* which produce just LT, both LT and ST, or only ST. The antibody response in all groups was assessed by determining antitoxin titers to *E. coli* LT in the serum and an extract of the surface epithelium of the intestinal mucosa.

**MATERIALS AND METHODS**

**Bacterial strains used.** LT was prepared from *E. coli* strain H-10407 (serotype O78:H11) which has been used by Evans et al. for this purpose (9, 10); this strain, which was isolated from an individual with acute diarrhea in Bangladesh, produces LT and ST (7). Immunized rats were challenged with this and two other strains of *E. coli*: PB-258 (serotype O115:H2) which produces LT only (kindly supplied by D. J. Evans, Jr.), and 214-4, a nontypable strain which elaborates only ST; it was isolated from a person with acute diarrhea in Mexico (26) and produces acute diarrhea when given perorally to volunteers (24) (kindly provided by M. M. Levine).

**Preparation of enterotoxin.** LT was obtained by the polymyxin-release technique (9) and subsequently purified by ammonium sulfate precipitation and back extraction followed by adsorption to, and elution from, Affi-Gel 202 (Bio-Rad, Richmond, Calif.) as recently described in detail by Evans and his co-workers (10). All preparations were assayed for potency by means of the spectrophotometric method of passive immune hemolysis (8) with a specially processed cholora antitoxin preparation (Swiss Serum and Vaccine Institute, Bern, Switzerland), which was kindly provided by D. G. Evans, Jr., as the antiserum.

**Immunization procedure.** Groups of from 12 to 46 Sprague-Dawley weanling rats (Charles River Breeding Laboratories, Wilmington, Mass.) were immunized by one of five different combinations of parenteral and/or intestinal routes. The priming dose was 100 µg, and the boosting dose was 50 µg in all instances except where specified. For parenteral immunization, the priming dose was dissolved in 0.25 ml of saline, emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and administered intraperitoneally; for boosting doses, the toxin was given either in saline alone or mixed with Freund incomplete adjuvant (FIA). For intestinal administration, the toxin was dissolved in 0.5 ml of saturated sodium bicarbonate and delivered by one of three methods: (i) directly into the duodenum by means of a laparotomy, (ii) perorally at 10 min after the peroral administration of 0.5 ml of the same bicarbonate solution, or (iii) perorally at 2 h after the peroral administration of cimetidine (Tagamet; Smith, Kline and French Laboratories, Carolina, Puerto Rico) at a dosage of 50 mg/kg of body weight, an amount which has been shown to ablate gastric secretions in the rat (3). All administrations referred to as peroral were given directly into the stomach via an orogastric tube. Those animals which received either parenteral or intraduodenal boosts were boosted twice, at 2 and 4 weeks after priming; rats which were boosted perorally received doses at weekly intervals for 1 month after priming.

**Challenge procedures.** Rats were challenged at 1 week after the final boost by the instillation of either the LT or viable organisms, each at those concentrations which yielded either 50% maximum secretory response (ED50 dosage) or the maximum secretory response in unimmunized animals, into a single 10-cm ligated loop of distal ileum for 18 h by previously described techniques (27, 35). The toxin was administered in a final volume of 0.5 ml of sterile saline. For challenge with viable cultures, the bacteria were cultured for 5 h at 37°C in Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.), the growth was centrifuged at 10,000 × g for 20 min, and the bacterial pellet was resuspended in sterile saline to the desired concentration, which is expressed as a multiple of the original concentration. A 0.1 ml amount of the various concentrations was instilled into ligated loops, and 20 mg of streptomycin sulfate was administered into the peritoneal cavity to prevent peritonitis in case of bacterial spillage.

Results are expressed as microliters of fluid secretion per centimeter of gut length. Each datum point was derived from values in from five to eight rats. There was no secretion after the instillation of (i) 0.5 ml of saline alone in 20 rats, (ii) 150 µg of LT after exposure to 100°C for 30 min in 5 rats, or (iii) a 15-fold concentration of the three strains of viable bacteria which had been exposed to 100°C for 90 min, each tested in 3 rats. Previous investigations from other laboratories, which have used ligated loops to determine immunological protection against LT, have reported their results in terms of an index of protection which is calculated by dividing that dosage of toxin in...
immunized rats which yields the same secretion as the ED₅₀ in unimmunized rats by the ED₅₀ dosage of toxin in unimmunized rats (27, 35). Such did not prove to be possible in the present study because fluid secretion in several groups of immunized animals was less, even at the maximum challenge dosage used, than that at the ED₅₀ for unimmunized rats. For this reason, the results of challenge in immunized rats was expressed by comparing the amount of fluid secretion in immunized rats to that in unimmunized rats at those challenge concentrations which yielded either 50% or the maximum secretory response in unimmunized rats. These results are expressed as either (i) the difference in means between the two groups, in which case the significance of this difference was determined by Student's 't' test for two independent means, or (ii) as the percentage reduction of secretion values in immunized animals as compared to values at the same concentration of challenge material in unimmunized, control rats.

Reference antisera. Rabbit hyperimmune serum to LT was prepared in the identical manner and with the dosages as described by Evans et al. (11). The standard used to determine antitoxin units was purified cholera antitoxin lot number EC 3(A-2/67)-B (Swiss Serum and Vaccine Institute, Bern, Switzerland) (kindly supplied by Carl Miller, National Institute of Allergy and Infectious Diseases). This antitoxin has been used as a standard reference in other laboratories where it has been arbitrarily assigned a value of 1,000 antitoxin units per ml to E. coli LT (27, 35, 41). It yielded a titer of 4,096 when assayed by passive immune hemolysis with 250 μg of E. coli LT as the antigen.

Antitoxin assay. Sera were inactivated by heat at 56°C for 30 min after separation. Tissue for antitoxin determinations was obtained from the 30 cm of ileum proximal to the challenge site and processed by the method of Kaur et al. (20) of the method described by Stern and Jensen (40). The material yielded by this procedure which was assayed was the cell extract of the surface epithelial cells. The antibody activity of the sera and surface cell extracts was assayed by passive immune hemolysis as described by Evans et al. (8, 11) with the modification that hemolysis was quantitated spectrophotometrically from serial dilutions in tubes. Serial twofold dilutions were assayed in duplicate. Optimal concentrations for assay of the cellular extracts were established by testing serial concentrations; this indicated that 200 μg of Lowry protein per ml (25) yielded values which were within the linear range of analysis. The optical density of the supernatant was read at 420 nm in a Hitachi Perkin-Elmer model 139 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) versus a blank containing phosphate-buffered saline in place of the test material. Hemoglobin release was quantitated against freshly prepared serial dilutions of ovine hemoglobin (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline, pH 7.2. Hemoglobin release of greater than 50 μg was considered a positive response. A positive control was included in each assay consisting of rabbit hyperimmune antiserum which consistently had a titer of 128.

The values reported are derived from assays in 20 unimmunized rats and from 10 to 18 rats in each of the immunization groups. Results are expressed as the geometric mean antibody titer (GMT) for the sera and as units of antitoxin per milligram for the surface epithelial cell tissue. Antitoxin units in the cellular extracts were determined by converting from micrograms of hemoglobin released per microgram of protein based on the fact that 1 unit of standard purified cholera antitoxin released 4.167 μg of hemoglobin.

RESULTS

Protection against challenge with LT. Rats immunized by parenteral priming followed by parenteral boosts given in saline were significantly protected when challenged at either that toxin concentration which yielded 50% or the maximum secretory response in unimmunized rats (Fig. 1). The administration of the same dosage of parenteral boosts which were mixed with FIA instead of saline resulted in significantly lower (P < 0.001) water secretion at the maximum challenge concentration of toxin. Immunization by a toxin preparation which had been inactivated by exposure to 100°C for 30 min yielded no protection.

Parenteral priming followed by intraduodenal boosting evoked significant protection, but parenteral priming followed by peroral boosting given 10 min after intragastric bicarbonate yielded no protection whatsoever (Fig. 2). In

![Fig. 1. Results of challenge with toxin in rats immunized by parenteral priming and boosting. IP signifies intraperitoneal administration. Values are the mean ± standard error of the mean. P values compare the results in immunized rats versus unimmunized animals at each challenge concentration. PNS signifies not statistically significant from values in unimmunized mice.](https://journals.asm.org/journal/iai)
In contrast, peroral boosting at the same toxin dosage of 50 μg/week, but given 2 h after intragastric cimetidine, resulted in reduced values in water secretion which were comparable to those obtained by means of intraduodenal boosting. In an additional group of rats immunized by the parenteral-peroral route with cimetidine the dosage of the toxin used for peroral boosting was increased fivefold from 50 to 250 μg weekly. Secretion was further reduced in this group; values were 3 ± 3 μl/cm at the ED50 challenge concentration and 10 ± 14 μl/cm at the maximum concentration, a value which was significantly lower (P < 0.001) than in rats immunized with a dosage of 50 μg/week.

Immunization given exclusively by the peroral route, in which both the priming and weekly boosts were given with cimetidine, afforded no protection when the dosage of toxin used was 50 μg/week (Fig. 3). There was significant protection, however, when the immunizing dosage was increased to 250 μg/week.

**Protection against challenge with viable organisms.** Groups of rats immunized with either (i) parenteral priming followed by parenteral boosting using FIA or (ii) parenteral priming followed by peroral boosting (at a weekly dosage of 50 μg) with cimetidine were challenged with viable organisms of three different strains of *E. coli* at concentrations of the broth cultures which evoked either 50% or the maximum fluid response in unimmunized rats. Both of these immunization regimens yielded significant protection against challenge with strain PB-258 which elaborates just LT (Fig. 4), as well as against challenge with a strain H-10407, which produced both LT and ST (Fig. 5). The reduction in water secretion at the maximum challenge concentration in rats immunized exclusively by the parenteral route was significantly less (P < 0.001) in rats challenged by the LT- and ST-producing strain than in those challenged by either the toxin itself or the LT-only-producing strain. Neither group of immunized rats showed any evidence of protection when challenged with strain 214-4 which elaborates ST only (Fig. 6).

There was no significant difference between the degree of bacterial growth in fluid recovered from the intestinal loops at the termination of challenge in control, unimmunized rats versus immunized animals challenged by any of the three strains tested.

**Antibody response.** Among the two groups in which no protection was achieved by immunization, the value for the serum GMT to LT was the same in rats immunized parenterally with heat-inactivated toxin as that in control, unimmunized animals, and it was twofold higher in rats primed parenterally and boosted perorally with bicarbonate (Table 1). The serum GMT was increased by 16-fold or more over

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**Fig. 2.** Results of challenge with toxin in rats immunized by parenteral priming and intestinal boosting. IP signifies intraperitoneal administration, and PO indicates peroral administration. NS, Not statistically significant.

**Fig. 3.** Results of challenge with toxin in rats immunized by peroral priming and boosting with cimetidine. The two groups of immunized rats were immunized by the same route, but different dosages of toxin were used. Abbreviations are the same as those in Fig. 2.
Antitoxin concentrations of the surface epithelium of the intestinal mucosa were significantly greater ($P < 0.01$) than the value in unimmunized rats in those four groups of immunized rats in which the serum GMT was increased by 16-fold or more.

**DISCUSSION**

The results of this study establish the fact that active immunization with a purified preparation of the polymyxin-release form of *E. coli* LT, when administered by several different routes, produces significant protection against challenge by this toxin. Exclusively parenteral immunization yielded protection which appeared to be greater when the boosting doses were given with FIA rather than saline. We were led to evaluate intestinal routes of administration by the facts that (i) this approach has been found to yield protection in the case of cholera toxin (12, 16, 29), and (ii) Pierce and his colleagues have shown that parenteral priming followed by intraintestinal boosting yields the most prolonged immunological protection in the case of cholera toxin (31, 32). Our initial observation that parenteral priming followed by giving the boosting doses directly into the duodenum yielded protection, whereas intragastric boosting was ineffective despite the concomitant administration of bicarbonate, led us to the conclusion that the antigenicity of the LT was being destroyed by gastric secretions. For this reason, we evaluated the effect of ablating gastric secretions...
by pretreatment with peroral cimetidine; this approach yielded protection. Because pretreatment with cimetidine is a relatively simple approach, we believe this observation may have application to other programs of immunization against enteric pathogens in which an oral approach is regarded as optimal. Immunization with cholera toxin given exclusively by the oral route has also been found to yield protection (12, 16), and we found that a similar approach using *E. coli* LT given with cimetidine also achieved protection but only when the dosage of toxin used for immunization was fivefold greater than that found to be effective in peroral boosting after parenteral priming.

It seemed essential to determine whether the immunized rats were also protected against challenge with viable strains of toxigenic *E. coli* because the polymyxin-release form of toxin used for immunization has a considerably smaller molecular weight of 20,000 (10) than the value of 102,000 reported for LT purified by other methods (5) and it may well not represent the LT holotoxin. Rats immunized either exclusively by the parenteral route as well as those boosted perorally with cimetidine were significantly protected against challenge by strains which produced either just LT or both LT and ST. The data in the parenterally immunized groups suggest that protection was less effective against the LT/ST-producing strain than against the LT toxin itself or the strain which produces just LT. This may have been attributable to the presence of ST, because neither group of immunized animals was protected against an ST-only-producing strain; however, this observation awaits confirmation by additional studies in which a more quantitative assessment of protection is obtained by determining the protection index when immunized rats are challenged at even higher concentrations of bacteria.

The antibody response of the serum and intestinal mucosa to immunization with cholera toxin or toxoid has been the subject of extensive investigations (19, 28-31). Protection after repeated parenteral immunization is related principally to serum-derived antitoxin of the immunoglobulin G immunoglobulin class, and a correlation exists between the titer of this antitoxin and the degree of protection (4, 29). Antitoxin in the small bowel is derived both from the serum antibody as well as from antibody of the immunoglobulin A immunoglobulin class that is locally produced by the intestinal immune system in response to direct intraintestinal, or peroral, immunization (19, 30, 31). Each of these antitoxins plays a protective role against challenge with cholera toxin. Although information concerning *E. coli* LT is limited, the available evidence suggests that immunological protection is also mediated by systemic and local antibodies. Passive protection against the secretory effect of LT in various animal models is afforded by the addition of hyperimmune serum to the toxin (21, 23, 38); serum antibody levels were increased in rabbits protected by parenteral immunization with a crude toxin preparation (35); and exclusively intestinal exposure to toxin was found to produce a decrease in the secretory response of canine Thiry-Vella loops to LT in the absence of a rise in serum antitoxin levels, suggesting the presence of an exclusively local intestinal immune response (37). Our experience was similar. Parenteral priming followed by parenteral or effective intestinal boosting yielded increased serum titers which were associated with the presence of protection. Antitoxin levels in the...
surface epithelium of the intestinal mucosa mirrored serum titers; based on studies of the response of mucosal antibodies to cholera toxoid (30), it would seem likely that the antitoxin that we assayed in this material was derived from the serum. We also observed what appeared to be an exclusively local intestinal immune response in the absence of a serum response among rats immunized entirely by the peroral route. Immunological protection appeared to be related to the arousal of antibody specific to the purified LT because immunization with a similarly prepared but heat-inactivated toxin preparation failed to arouse either an antibody response or protection. It would seem unlikely that the protection observed was related to antibodies to cross-reacting antigens common to E. coli because immunization with the purified LT yielded protection against viable strains of LT-producing organisms but not against one that produces just ST.

Because LT produced by diverse serotypes appears to be antigenically homogeneous (36), our results indicate that active immunization with either LT itself, or a toxoid derivative, may represent a feasible approach to immunological control of diarrheal disease caused by toxigenic E. coli. A number of considerations remain to be clarified, however. First, the present studies were conducted shortly after completion of immunization, and it remains to be determined as to which immunization route will provide the most prolonged protection. Second, this immunization program did not protect against strains that produce just ST. We have previously suggested that an immunological relationship may exist between E. coli LT and ST based on our observation that the addition of hyperimmune rabbit antiserum to impure LT partially inhibits the secretory effect of ST in the rat jejunum (21, 22). Our present results indicate that such is not relevant when rats immunized against purified LT are challenged with a viable ST-only-producing organism. Although uncommon, diarrheal disease due to strains which produce just ST has been described in humans (33, 34). This means that a totally protective immunization program against toxigenic E. coli would require the addition of an ST antigen to the LT preparation that was used. Although impure preparations of ST derived from human strains have shown no evidence of antigenicity (7, 22), the recent demonstration that purified ST from a porcine strain is weakly immunogenic (1) raises the possibility of developing an actively antigenic preparation of this toxin form.

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LITERATURE CITED


