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IMMUNOLOGIC INTERRELATIONSHIPS OF COLIFORM HEAT-LABILE AND HEAT-STABLE ENTEROTOXINS

Annual Report

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The goal of these studies is the development of an immunization program to prevent acute diarrheal disease due to intestinal contamination of enterotoxigenic strains of *Escherichia coli*. We have demonstrated (a) that the holotoxin is the most immunogenic form of the *E. coli* heat-labile enterotoxin, (b) that immunization with this material protects rats against challenge with heterologous serotypes of *E. coli*, and (c) that the degree of protection is influenced by the route, schedule and dosage of both the primary and booster immunizations.

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FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

INTRODUCTION

This annual report describes research investigations conducted during a fourth year of support from the USAMRDC and the Office of Naval Research. Our research efforts during the past three years have been directed principally at developing an effective program of immunization against acute diarrheal disease caused by enterotoxigenic strains of *Escherichia coli* (ETEC). Three years ago (1978) we showed that immunization of rats with a purified preparation of the polymyxin-release form of the *E. coli* heat-labile enterotoxin (LT) provides protection against direct challenge in ligated ileal loops of the immunized rats with either the toxin itself or viable strains of bacteria which produce LT either along (LT^+/ST^-) or together with the heat-stable enterotoxin (LT^+/ST^+). We also established the fact that peroral immunization with this antigen is effective only if gastric secretions are ablated by pretreatment with cimetidine (ref 8). Two years ago (1979), we evaluated the effect on the degree of protection achieved of using different routes of administration with this LT immunogen and found that an immunization schedule of parenteral primary immunization followed by four peroral booster immunizations is the only approach that yields the combination of both maximum immediate and extended protection (refs 13,14). We also confirmed the similarity of these results (which employed ligated ileal loops) to those obtained under conditions which resemble more closely those of the actual disease state by showing that this immunization program yields protection against the secretory action of monocontamination with ETEC strains in gnotobiotic rats (refs 7,15).

During the past year (1980), we have investigated three additional aspects of the immunization program using LT toxin as immunogen. (1) The first was related to developing an optimal immunization schedule by means of identifying the relative contributions to protection of the primary and booster immunizations in terms of route of administration, number of immunizations and dosage required to give maximal protection (ref 17). (2) The second was to identify the optimal form of LT immunogen by determining the relative immunogenicity of the polymyxin-release form of LT, the LT holotoxin, and the holotoxin B subunit (ref 18). (3) The third study was concerned with establishing the fact that immunization with LT protects against direct challenge with viable organisms of all different somatic serotypes (ref 20); this study also evaluated the relative immunogenicity of LT produced by an LT^+/ST^- strain versus toxin derived from an LT/ST^+ strain. (4) Finally, we have commenced investigations concerned with developing an immunization program that affords protection against ETEC strains which produce just the heat-stable toxin (LT^-/ST^+ strains).

(1) RESPECTIVE CONTRIBUTIONS TO PROTECTION OF PRIMARY AND BOOSTER IMMUNIZATION WITH *Escherichia coli* POLYMYXIN-RELEASE LT IN RATS.

Although studies conducted during the previous year (1979) showed that an immunization schedule of parenteral primary immunization followed by four peroral booster immunizations yields the combination of maximum immediate and extended protection, they provided no information regarding the respective contributions of the primary or booster immunizations to the observed protection. Further, we felt that the number of immunizations (five) required by this schedule were excessive to be considered practical. In order to establish the

optimal approach for immunization, we next conducted a series of investigations in which the respective contributions to protection of the route and dosage of the primary and booster immunizations were evaluated. Immunization was with the polymyxin-release form of LT. The degree of protection in immunized animals was determined by challenge with toxin or viable bacteria in ligated ileal loops and the serum antitoxin (AT) response was assayed by an enzyme-linked immunosorbent assay (ELISA).

Primary immunization was effective only when given by the parenteral route; large dosages of toxin given for primary immunization by either the peroral route (after pretreatment with cimetidine) or directly into the duodenum failed to yield significant degrees of protection when followed by peroral boosters. Further, we found that the parenteral immunization must be given as the primary immunization since it is ineffective when given during or after peroral immunizations. For this reason, all additional studies employed parenteral primary immunization. Although rats in this study were primed parenterally by means of an intraperitoneal injection, subsequent studies have shown that primary parenteral immunization given by the subcutaneous route is equally effective.

The effect of varying the dosage of either the primary or booster immunizations over a 25-fold range was determined in rats who were primed parenterally and boosted by the parenteral (IP/IP) or peroral (IP/PO) routes. The degree of the protection was enhanced by a fivefold increase in dosage from 10 to 50 μg) in the primary parenteral immunization when followed subsequently by constant dosages of either parenteral (IP/IP) or peroral booster immunizations (IP/PO), but further increases in dosage of the primary immunization did not enhance the degree of protection. In contrast, the degree of protection rose progressively when dosages of the booster immunizations (either parenteral or peroral) were increased over a 25-fold range in rats given a constant dosage of primary immunization. Four weekly peroral booster immunizations (IP/PO), but only two biweekly parenteral (IP/IP) booster immunizations, were necessary to achieve strong protection. In view of the strong protection achieved by only two parenteral booster immunizations plus the previously demonstrated need for peroral booster immunizations to achieve extended protection, we evaluated a combination of two biweekly combined parenteral and peroral booster immunizations. This regimen yielded both strong immediate and extended protection over a two month period that was equivalent to that obtained using a parenteral primary immunization followed by four peroral booster immunizations.

The degree of protection against the toxin correlated with that against viable bacteria (LT^+/ST^- and LT^+/ST^+ strains) and with elevated serum AT titers. All seven groups with a protection index of > 5 against challenge with the toxin also had strong protection (> 50% reduced secretion) against LT-producing strains and a fourfold greater increase in the AT titer, whereas none of the nine groups with a PI < 3 had a similar degree of protection against challenge bacteria or an equivalent AT response. Although these observations showed a correlation between serum antitoxin titers and the degree of protection, it is noteworthy that we did not evaluate another key parameter of protection: mucosal antibody production. It is highly likely that mucosal production of IgA antitoxin played a significant role in the protection observed in rats immunized with peroral boosters, and direct observations of mucosal antitoxin production are included in our proposed studies for 1981.

These observations demonstrated (a) that primary immunization must be given by the parenteral (either intraperitoneally or subcutaneously) route; (b) that once an adequate primary parenteral immunization is given, then both the serum antitoxin response and the degree of protection are influenced principally by the dosage of the booster immunizations (either parenteral or peroral), the necessary number of which is dependent on the route of the administration; and (c) that a combination of parenteral and peroral immunization offers the maximum degree of immediate and extended protection with the minimum number of necessary immunizations. The results of this study were published in January, 1981 (ref. 17).

(2) COMPARATIVE IMMUNOGENICITY OF TOXIN FORMS OF THE *Escherichia coli* HEAT-LABILE (LT) ENTEROTOXIN.

In order to identify that form of LT which has maximum immunogenicity, the protective effect of immunization with the polymyxin-release form of LT (PM LT), the LT holotoxin (HT), and the holotoxin B subunit were compared by immunizing rats with graded dosages (based on protein concentration) over a 25-fold range, given exclusively by the parenteral (IP/IP) or peroral (PO/PO) routes or by a parenteral primary immunization followed by peroral booster immunizations (IP/PO). The holotoxin and its B subunit were obtained from *E. coli* strain 711 F1LT (a transformed K-12 derivative bearing LT gene(s) of the Ent plasmid) and prepared and assayed by published methods (Infec Immun 24:760, 1979; 29:91, 1980). The degree of protection in immunized rats was evaluated by challenge in ligated ileal loops with toxin and with viable LT^+/ST^- and LT^+/ST^+ strains, and the serum antitoxin (AT) response was determined by an enzyme-linked immunosorbent assay (ELISA) using the homologous toxin form as the antigen.

When given by the PO/PO route, each of the three LT antigens provided only weak protection against the toxin and virtually none against viable LT-producing strains. The maximum degree of protection (a protection index of 3.8) was achieved by immunization with the holotoxin. Serum AT titers were not significantly increased in any of these groups.

When the toxins were given after a parenteral primary immunization with booster immunizations given by either the parenteral or peroral routes, each LT antigen provided a dose-related increase in serum AT titers and in the degree of protection against the toxin as well as against viable strains which produce LT alone (LT^+/ST^-) or in combination with the heat-stable toxin (LT^+/ST^+) (with the exception that immunization with the B subunit by the IP/IP route failed to give protection against the LT^+/ST^+ strain). When expressed on the basis of molar equivalents, HT provided significant protection (a protection index of > 5 against toxin challenge and > 50% reduced secretion with bacterial challenge) with four to 15 times fewer mols than PM LT and up to 50 times fewer mols than the B subunit (Fig. 1).

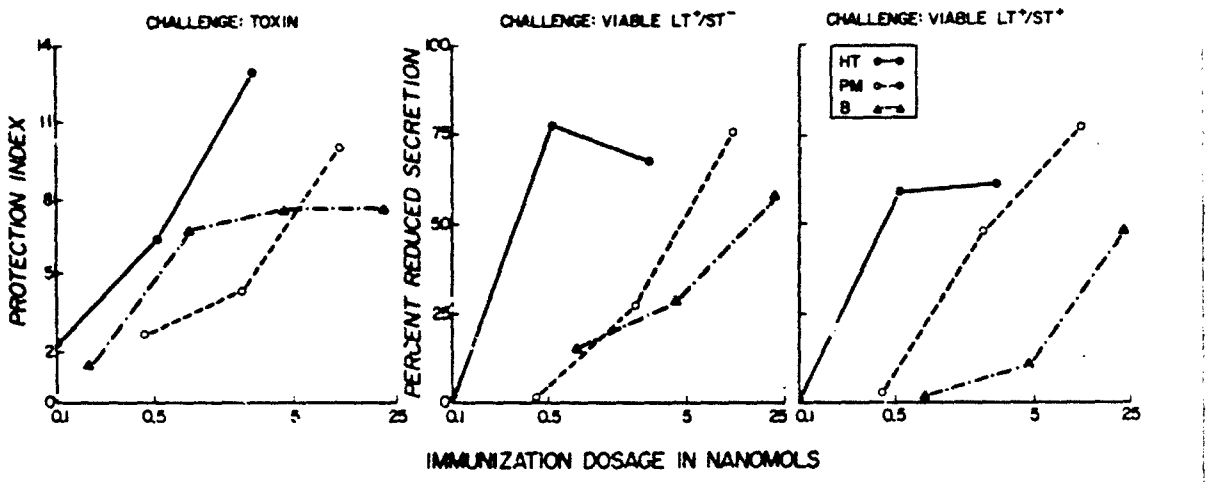

FIG. 1. Protection against challenge afforded by immunization with the LT toxin forms given by parenteral primary immunization followed by peroral booster immunizations (IP/PO). Booster dosages are expressed in nanomols.

These observations indicate that, on the basis of molar equivalents, the holotoxin (which contains one A plus five or six B subunits) is a more potent immunogen than either PM LT (which contains probably one B subunit) or the B subunit. This finding has led us to use purified holotoxin as the immunogen in subsequent studies. Its incorporation into a final immunization program for possible use in humans, however, is based on the premise that it can be modified so that it will lose most of its toxic properties without losing a significant degree of its immunogenicity. In the event that such should not prove to be possible, then the B subunit could be used since, on the basis of protein concentration, it proved to be an equally potent immunogen.

The results of this study were published in January, 1981 (ref 18).

(3) PROTECTIVE EFFECT OF IMMUNIZATION OF RATS WITH *Escherichia coli* HEAT-LABILE ENTEROTOXIN AGAINST HETEROLOGOUS SEROTYPES.

The chief advantage of using the *E. coli* LT toxin as the immunogen in a program of immunologic protection against ETEC strains is the fact that the LT produced by different somatic serotypes is thought to be uniform, so that immunization with this material should provide protection against all of the *E. coli* heterologous serotypes. This concept is based, however, exclusively on previous studies which examined only passive protection and employed impure antigens for immunization and challenge. Sack and Froelich, for example, showed that the in vitro addition of hyperimmune serum derived from rabbits immunized with a crude LT preparation yielded highly variable degrees of neutralization of the secretory effect (in rabbit ligated ileal loops) of crude toxin preparations derived from heterologous serotypes of *E. coli* (Infec Immun 5:750, 1977). To date, there have been no studies regarding the protective effect of active immunization with a purified preparation of LT against direct challenge with viable organisms or multiple heterologous serotypes.

We examined this by immunizing rats with purified preparations of either the LT holotoxin (HT) obtained from strain 711 F1LT (a transformed K-12 derivative bearing LT gene(s) of the Ent plasmid). The protective effect of immunization with toxin from strains which elaborate either LT alone or in combination with ST was also evaluated because previous studies conducted elsewhere in volunteers given various ETEC strains of *E. coli* have raised a question concerning whether the LT produced by these two different types of ETEC strain is antigenically homologous in terms of cross reactive protection (Infec Immun 23:729, 1979). For this purpose, the polymyxin-release form of LT (PM LT) produced by strains H-10407 (LT^+/ST^+) and PB 258 (LT^+/ST^-) was used since it proved technically difficult to purify the holotoxin produced by these strains. Rats immunized by these three toxins (given by parenteral primary immunization followed by four weekly peroral booster immunizations) were challenged with toxin and with viable organisms of ten different serotypes, five of which produce just LT (LT^+/ST^-), and five, both LT and ST (LT^+/ST^+). Serum antitoxin titers were assayed against the homologous antigen by means of an enzyme-linked immunosorbent assay (ELISA).

Immunizations with either 50 µg (protein concentration) of HT or 250 µg of PM LT derived from the LT^+/ST^+ strain resulted in a significant increase in serum AT titers (> 4-fold increase over control values) and significant protection (> 50% reduced secretion in immunized rats) against challenge with viable organisms of all ten somatic serotypes irrespective of whether they were LT^+/ST^- or LT^+/ST^+ (Fig. 2). In contrast, PM LT derived from the LT^+/ST^- strain proved to be only weakly immunogenic. In rats immunized with 250 µg of this material, the serum AT titer rose only twofold over control values, the protection index against challenge with toxin was 2.6 versus values of 10.0 in rats immunized with PM LT from the LT^+/ST^+ strains and 13.0 in those immunized with HT (Fig. 3), and there was only weak protection against viable LT^+/ST^- organisms and virtually none at all against the LT^+/ST^+ strains. Increasing the immunization dosage of this toxin 5-fold to 1,250 µg failed to increase the degree of protection against challenge with either toxin or viable organisms.

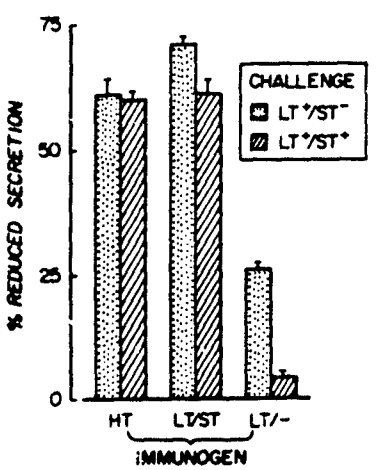

FIG. 2. Protection afforded by immunization with LT preparations from different sources against challenge with viable organisms. HT signifies the holotoxin from strain 711, LT/ST the polymyxin-release toxin from a LT/ST^+ strain and LT/- the toxin from a LT^+/ST^- strain. Values are the mean ± SEM for challenge with five LT^+/ST^- and five LT^+/ST^+ strains.

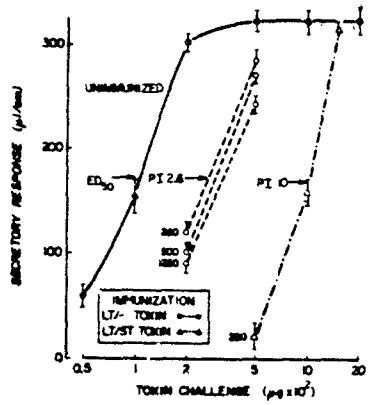

FIG. 3. Results of challenge with LT/ST toxin in rats immunized with either LT/- or LT/ST toxin. ED_{50}, 50% effective dose. PI, the protection index.

These observations establish the fact that immunization with the LT holotoxin obtained from a transformed strain provides uniform protection against direct challenge with heterologous serotypes of *E. coli*. They have also identified a quantitative or qualitative difference in the immunogenicity of LT produced by strains which elaborate this toxin alone versus those which produce both LT and ST. The results of this study have been published (ref. 20).

(4) DEVELOPMENT OF A PROGRAM OF IMMUNOLOGICAL PROTECTION AGAINST THE HEAT-STABLE ENTEROTOXIN (ST) OF *E. coli*.

Although immunization with LT provides protection against strains which produce LT alone (LT^+/ST^-) or in combination with ST (LT^+/ST^+) (in which circumstance we presume the protection is directed against the LT produced by these strains), it fails to provide protection against strains which produce just ST (LT^-/ST^+). Since LT^-/ST^+ strains are recognized to be a cause of acute diarrheal disease in humans (Lancet 2:239, 1975; New Eng J Med 295:849, 1976), any effective program of immunological protection against all enterotoxigenic strains of *E. coli* will have to include protection against such strains.

The difficulty encountered in the past regarding incorporating ST as an antigen along with LT in a program of immunological protection has been related to the facts that techniques for purifying ST had not been developed and that the low molecular weight ST toxin was not thought to be immunogenic. Recently, however, several laboratories have described purification procedures for bovine, porcine or human strains of ST (Infec Immun 19:1021, 1978; 25:978, 1979; 26:173, 1979; 28:469, 1980; J Biol Chem 255:4716, 1980) and several studies have shown that immunization with ST in rabbits or goats yields weak antitoxin titers as determined by neutralization studies using the suckling mouse assay (Infec Immun 19:1021, 1978; 28:469, 1980; Nature 284, 473, 1980). Further, immunization of a goat with ST conjugated to bovine IgG by the carbodiimide method has been reported to yield high antitoxin titers as detected by radioimmunoassay (Gastroenterology 78:1172, 1980).

Starting in September (1980), we commenced to make ST from strain Texas 452, an LT^-/ST^+ strain which has been well characterized (New Eng J Med 295:849, 1976). using the methodology described by Staples and Giannella (J Biol Chem 255:4716, 1980) which employs growth in minimal medium, Amberlite XAD-C chromatography, acetone fractionation, Sephadex G-25, DEAD-Sephacel ion exchange chromatography, and Sephadex G-25 gel filtration. The potency of the material produced at each purification step, including the eluate fractions obtained from column chromatography, was monitored by the suckling mouse assay (Infec Immun 14:95, 1976). By late November, we had achieved the production of a highly potent toxin material (minimal effective dose in the suckling mouse assay of 2 ng) that appears to be homogeneous in the elution from the final G-25 Sephadex column chromatography and resembles the purified ST toxin obtained by Staples and Giannella when subjected to amino acid analysis (kindly performed by Dr. George Abrahams of the Department of Medicine).

The purified ST toxin coupled with porcine immunoglobulin G was used to immunize a goat. After two biweekly intramuscular immunizations, the goat developed an antibody titer of 1:8192 to ST, as determined by ELISA using ST-BSA (Bovine Serum Albumin) as the antigen. We have now immunized a rabbit with this material in order to obtain a second source of antibody to ST and, as of this writing (after a single immunization), an antibody titer of 1:1000 has been achieved. This second source of antibody should permit us to assay ST by means of the double sandwich ELISA technique, an approach which should provide a simple in vitro test for assaying ST, either during purification procedures or from clinical isolates, thus replacing the time-consuming and cumbersome suckling mouse assay.

At the present time, ST coupled with porcine IgG is being used to immunize rats by approaches which we have already developed for LT. The degree of protection achieved will be ascertained by direct challenge with ST and with viable LT^-/ST^+ and LT^+/ST^- organisms.

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