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Investigation of E. coli Enterotoxins

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The results of the present investigation indicate a new approach to the development of a single vaccine formula which may ultimately be used to confer protection against both cholera and E. coli diarrheal disease in man and domestic animals. Fundamentally, the design of the vaccine is based on the well-documented ability of cholera antitoxin to neutralize both cholera and heat-labile E. coli enterotoxins and on the ability of certain E. coli antigens to enhance the immune response to cholera toxoid and possibly whole-cell Cholera Vaccine, as well.

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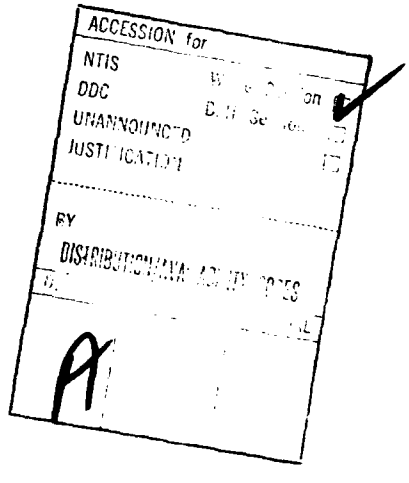

SUMMARY

A major objective of our program of investigation was the design of a vaccine which might be used to protect man (and domestic animals) against E. coli diarrheal disease. Because so little was known about the enterotoxin elaborated by disease-producing strains, the scope of our work included the development of methods for the measurement and isolation of the enterotoxin (ECT) as well as studies designed to improve our knowledge of its immunological properties and pathogenic mechanism. During the course of the program, reliable methods for assay, production and partial purification were developed and analyses of the problems associated with the ultimate purification of the polydisperse enterotoxin were delineated. With the use of analytical techniques, it was possible to characterize the various forms of the enterotoxin and to classify the distribution into two distinct categories: soluble (a single polypeptide chain of about 68,000 daltons) and insoluble (consisting of molecules ranging in size from about 200,000 daltons to about 20 x 10^6 daltons). The heterogeneity of the latter group of molecules was attributed, in part, to the fact that the enterotoxin was complexed with endotoxin. Later, studies on the time course of release of ECT by enterotoxigenic strains led us to consider that insoluble ECT might represent fragments of outer membrane dispersed into the medium during cultivation, and that the majority of ECT molecules were integral components of these fragments or complexes. Further, we discovered that most ECT molecules released by E. coli were not fully active, since treatment with trypsin resulted in a substantial increase in toxicity. This finding explained several outstanding questions and enabled us to establish a role for in vivo proteolytic activation in pathogenicity.

The hypothesis that insoluble ECT represented fragments of outer membrane led us to propose that killed enterotoxigenic E. coli might be capable of eliciting antitoxin. Accordingly, comparative antigenicity studies in the rabbit revealed that insoluble ECT and killed E. coli were each capable of eliciting antitoxin which was specific for ECT, but which showed little or no ability to neutralize cholera toxin (CT). In contrast, cholera toxoid was found to produce antitoxin equally capable of neutralizing both ECT and CT. By combining the E. coli antigen (insoluble ECT or the organisms) with cholera toxoid, it was possible to demonstrate dramatic protection when immunized rabbits were challenged with either enterotoxin (ECT or CT) or live, enterotoxigenic strains (E. coli or vibrios). In addition to providing specific ECT-antitoxin, the E. coli antigens acted as potent adjuvants for cholera toxoid, resulting in extraordinary levels of cholera antitoxin. The evidence also indicated that E. coli antigens may act as adjuvants for Cholera Vaccine (i.e., vibrios). As a result of these studies, we proposed that a combined antigen formula comprised of cholera toxoid and E. coli Vaccine may provide the basis for a vaccine with specificity for both cholera and E. coli diarrheal disease, and that this same formula, together with Cholera Vaccine, might be favored in cholera-endemic areas.

I. Introduction

This report summarizes the major findings of the research program entitled "Investigation of E. coli Enterotoxins" which was carried out under the auspices of the U.S. Army Medical Research & Development Command (contract no. DAMD 17-74-C-4007) during the period from August of 1973 through September of 1977. The principal areas of investigation included: assay, production and purification (of enterotoxin); pathogenicity; and immunogenicity. Since detailed accounts of our studies have been described previously in a series of quarterly and annual reports (1-4), it is the purpose of this report to present a general description of the salient findings in each area of investigation and thereby provide an overview of the achievements of the program.

II. Assay, Production and Purification of E. coli Enterotoxin

At the beginning of our investigation of E. coli enterotoxin (ECT), the only reliable assay available was the adult rabbit ligated ileal loop test (5) which was developed in the study of cholera pathogenesis. Another assay pertaining to cholera, the rabbit skin test for increased vascular permeability (6), was also examined for its ability to measure ECT --- with negative results. Sack et al (7), for example, reported that ECT did not exhibit vascular permeability factor (PF) activity, and Donta (8) had not yet reported on the mouse Y-1 adrenal tissue culture assay for enterotoxin. Because of the striking biological similarity between ECT and cholera toxin (CT), and because of the recognized limitations of the ileal loop test, we proposed to examine the ability of ECT to alter vascular permeability despite Sack's previous report.

Using culture filtrates prepared from enterotoxigenic strains, it was determined that ECT did, in fact, increase vascular permeability as measured by the appearance of blue lesions at 18 hrs (1 hr after the i.v. administration of blue dye) after the intradermal inoculation of ECT into shaved rabbit backs. It was soon apparent, however, that the titers of ECT-containing filtrates were orders of magnitude less than those produced by filtrates prepared from cultures of Vibrio cholerae. And, perhaps as a consequence of the low titers, the quality of bluing was inferior to that produced by CT (possibly leading Sack et al to the conclusion that ECT did not alter vascular permeability). Subsequently, in November of 1973, Evans et al (9) published their report on the production of vascular permeability factor by enterotoxigenic E. coli isolated from man. Their findings, which established the validity of the rabbit skin test for assay of ECT, were in total agreement with our own unpublished results. On this basis, then, the rabbit PF test became the principal means for assay of ECT in our laboratory.

Using this assay, an investigation of problems associated both with the production and the purification of ECT was initiated. A variety of

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media (including minimal, defined and complex) and different growth conditions (aerobic and anaerobic) were examined. Although most of the conditions supported some level of ECT production, it was found that the yeast extract-supplemented casamino acids-salt medium advocated by Evans et al (9), together with vigorous aeration (rotary shaking) reproducibly supported maximal levels of PF production. Nevertheless, as mentioned above, the PF titers of ECT-culture filtrates were generally orders of magnitude (100 to 1000-fold) less than those of comparable filtrates prepared from cultures of Vibrio cholerae. This finding indicated that either ECT was 100 to 1000 fold less active, molecule for molecule, than CT or that E. coli strains produced 100 to 1000 times less toxin than the most commonly employed cholera strain, Inaba 569B. A major interest of the program, therefore, was either to find naturally-occurring E. coli strains which produced, like Inaba 569B, unusually high levels of toxin or to explore mutagenesis as a means of isolating such a strain. At about this time, Dr. Mark Levner of our laboratories became interested in the E. coli program and offered to collaborate on the problems of increasing enterotoxin production. Dr. Levner suggested exploring drug-resistant, enterotoxigenic strains for increased PF production, since it was known that colicinogenic factor E_1 (a bacteriocin) plasmid DNA increases substantially when certain strains of E. coli are grown in the presence of the antibiotic, chloramphenicol (10). Using the skin test to assay for ECT production, Dr. Levner discovered that lincomycin-resistant enterotoxigenic E. coli produced at least tenfold more extracellular ECT than did parent strains. This discovery was an important breakthrough not only because of the possible handle it offered for investigating the genetic control of toxin production, but because it improved the prospects for isolation and characterization of the toxin. Later, Dr. Levner extended his discovery to Vibrio cholerae, showing that lincomycin also induced increased production of CT. Dr. Levner's results have been published (11) and patented (U.S. Patent Nos. 3,984,285 & 4,076,590).

Working with late stationary phase culture filtrates prepared from lincomycin-induced and standard cultures, methods for concentration and purification of ECT were explored. It was determined that reproducible and quantitative concentration of the enterotoxin could be achieved either by ammonium sulfate precipitation (90% saturation) or by ultrafiltration (using a 50,000 dalton molecular weight cutoff membrane) (1,2). Irrespective of the concentration method, however, subsequent purification by molecular sieve chromatography revealed that enterotoxin activity was associated with a distribution of molecules ranging in size from about 68,000 daltons to over 20 X 10^6 daltons (2,3). Molecular weight heterogeneity of ECT had been reported by numerous investigators (12-18) who employed a variety of conditions and strains, and in our laboratory, it was observed to occur in both drug-resistant and standard cultures.

Because of the relative ease with which the higher molecular weight

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forms of ECT could be separated from the bulk of soluble factors found in culture filtrate (by high speed sedimentation or by gel chromatography), our efforts were initially directed at purification and characterization of these particular forms. The high molecular weight forms were designated "insoluble" in order to distinguish them from a less complex, soluble enterotoxin and because they were opalescent in appearance. It was determined that insoluble enterotoxin consisted of molecules ranging in size from about 200,000 daltons to about 20 X 10^6 daltons and that they were complexed with endotoxin (2-4). In contrast, soluble ECT was later found to be a homogeneous protein and in this respect, it more closely resembled CT than did insoluble ECT. The relative distribution of soluble and insoluble ECT varied somewhat from culture to culture making it difficult to decide which of the forms was the predominant species. Even under optimal conditions, the concentration of soluble ECT was sufficiently low that its quantitative recovery from complex media proved difficult on a preparative scale.

However, with the aid of analytical techniques such as acrylamide gel electrophoresis (with and without SDS) and immunodiffusion, it was possible to examine the properties and relationships of the two forms of the enterotoxin. Soluble enterotoxin was found to be a single polypeptide chain, about 68,000 daltons, since it migrated on SDS-acrylamide gels just ahead of human serum albumin (68,450 daltons) (3). In this respect, it differs from CT which consists of two non-identical polypeptide chains (19-22). In contrast, insoluble ECT consists of numerous electrophoretic components (when electrophoresed in SDS gels), one of which migrates with a mobility equivalent to that of soluble ECT (3,23). Immunologically, both forms of ECT were neutralized by cholera antitoxin and by E. coli antitoxin. Because of its physical properties, soluble ECT was more readily studied than insoluble ECT by immunodiffusion techniques and it was found to be immunologically related to CT. When the two proteins (soluble ECT and CT) were diffused against cholera antitoxin, a precipitin line of partial identity was formed; whereas when they were diffused against E. coli antitoxin, a precipitin line of identity was formed (3,23). These observations indicate that soluble ECT shares at least one common antigenic determinant with CT. In addition, numerous rabbit antisera raised against insoluble ECT (see section IV) were capable of neutralizing soluble ECT and this was regarded as evidence that the two forms of the enterotoxin (soluble and insoluble) are immunologically related. Other studies, concerned with the time course of release of ECT by enterotoxigenic strains, and with the antigenic properties of killed enterotoxigenic E. coli, led us to propose that insoluble ECT might represent fragments of outer membrane dispersed into the medium during growth (23).

III. Pathogenicity

During the course of attempts to purify ECT, it was discovered that treatment of ECT with trypsin resulted in a substantial increase in PF

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activity and in the ability to stimulate growth hormone secretion by monolayer cultures of rat pituitary cells (24). However, no difference in the activity of control and trypsin-treated ECT samples could be demonstrated in the rabbit ileal loop test (2,24). It was determined that the reason for the discrepancy between the assays was that control ECT was activated to the same extent as trypsin-treated ECT by proteolytic enzymes in vivo. Short term incubation (and withdrawal) of enterotoxigenic culture filtrates in ligated intestinal segments of the rabbit, for example, resulted in elevated levels of PF activity (24). Further, rabbit intestinal fluid could be substituted for trypsin in vitro with the same effect (i.e., activation of PF and tissue culture activity) (24).

Because activation of ECT did not take place when trypsin or rabbit intestinal fluid were treated with Lima Bean Trypsin Inhibitor (LBTI), we then examined the effect of trypsin inhibitor on the secretory response to cell-free enterotoxin and to enterotoxigenic strains. The results showed that the ability of enterotoxigenic culture filtrates to elicit a secretory response in the rabbit was significantly reduced in the presence of LBTI (3). The presence in most culture filtrates of preexisting toxicity appeared to account for the residual secretory responses which were observed in the presence of some doses of LBTI (3). A consideration of the possible utility of trypsin inhibitors in the treatment of E. coli diarrheal disease led us to investigate the effect of LBTI on the rabbit secretory response to live enterotoxigenic strains. Although a certain percentage of the animals studied (about 30%) did not respond, the administration of LBTI together with live, enterotoxigenic strains, resulted in the diminution or the abolition of the secretory response in the majority of animals (3). In fact, the effect was dose dependent since a linear relationship between the relative reduction in response and the concentration of inhibitor was observed. Further, the result could not be attributed to an inhibitory effect of LBTI on either enterotoxin production or on viability (3). Several explanations were considered to account for the group of non-responding animals. These included: 1) inaccessability of proteolytic enzymes (to LBTI) in the niches and folds of the intestinal epithelium; 2) activation of the enterotoxin by an enzyme not inhibited by LBTI; and 3) degradation of inhibitor by an intestinal factor.

The role of proteolytic activation in pathogenicity was also examined in a number of in vitro studies. By following the time course of release of ECT by enterotoxigenic strains, it was determined that the majority of enterotoxin molecules are released only after the organisms enter the period of decline (23). Further, it was found that the bulk of the enterotoxin so released was largely inactive since subsequent trypsin treatment of culture filtrates (collected at various times during cultivation) resulted in at least a tenfold increase in activity (23). Similarly, cultures grown in the presence of trypsin contained at least tenfold more activity than did standard cultures (23).

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Other studies concerning the biochemical mechanism of activation have shown that both forms of the enterotoxin, soluble and insoluble, are each activated by trypsin (23). In the case of soluble enterotoxin, activation by trypsin does not appear to be accompanied by a change in molecular weight, nor is there any evidence that a peptide fragment is liberated (23). In the case of insoluble enterotoxin, however, activation is accompanied by the release of an inactive molecule, which is thought to be a protein of about 80,000 to 100,000 daltons and which, as far as is known, does not bear any resemblance to soluble enterotoxin (2,3,23). Although other evidence, from experiments concerned with the time course of release of ECT, indicated that trypsin promoted the early release (from the organisms) of a small percentage (about 10% of the final yield) of ECT molecules which resembled soluble ECT, it is possible that the majority of ECT molecules are sufficiently complexed with components of outer membrane to preclude release by trypsin alone.

Taken together, the results of in vivo and in vitro studies are consistent with the idea that activation of ECT by proteolytic enzymes is likely to play an important role in the pathogenicity of E. coli strains not only by promoting increased toxicity of ECT, but perhaps also by facilitating its release.

IV. Immunogenicity

Another facet of our investigation of E. coli enterotoxins included an evaluation of certain E. coli antigens for their ability to produce antitoxin and also for their ability to elicit protection against enterotoxin or live organism challenge in animal models. For these experiments, the insoluble form of the enterotoxin was employed because it was more readily isolated from culture filtrate than soluble enterotoxin and because it was relatively nontoxic (in its unactivated form, this antigen exhibited a level of toxicity equivalent to that exhibited by cholera (glutaraldehyde) toxoid (25) which is known to be safe for human use). Using rabbits and a parenteral immunization schedule previously employed in the evaluation of cholera toxoid (26), it was determined that the E. coli antigen was an effective and reproducible antigen: 50-60 mcg doses elicited levels of E. coli antitoxin comparable to cholera antitoxin levels produced by similar doses of cholera toxoid. In addition, it was established that trypsin-activated ECT was at least six times more antigenic than untreated antigen, possibly due to the intrinsic immunostimulatory properties of enterotoxin (27). Further, it was determined that the antitoxin produced was specific for E. coli (soluble) enterotoxin since it showed little or no ability to neutralize CT. In contrast, cholera toxoid produced antitoxin which was just as capable if not more so, in neutralizing ECT as it was in neutralizing CT (3,4).

In our study of the comparative antigenicity of E. coli antigen and cholera toxoid, we also immunized animals with a combination of both

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antigens. Somewhat unexpectedly, it was observed that the combined antigens exhibited synergism: when administered together, they elicited levels of antitoxin far in excess of the additive titers produced by each antigen alone (3), and also in excess of titers produced by toxoid plus aluminum salt adjuvants (26). Further, the antitoxin was characteristic of cholera antitoxin since it was equally capable of neutralizing both CT and (soluble) ECT. Since it was known that the E. coli antigen was complexed with endotoxin, a likely explanation of the effect was that the endotoxin moiety exerted an adjuvant effect on the toxoid. Subsequently, it was shown that "purified" endotoxin, obtained commercially, did, in fact, exert an adjuvant effect on toxoid resulting in about a fivefold increase in antitoxin compared with toxoid alone (4). However, the even greater increases in antitoxin produced by the combination of E. coli antigen and cholera toxoid (at least tenfold and in some cases, as much as 20-30 fold) led us to consider that the E. coli antigen was acting as a "super" adjuvant by virtue of containing two separable components: the endotoxin moiety and the enterotoxin moiety. Further, the added advantage that the antigen also was capable of producing E. coli-specific antitoxin suggested that it might be an ideal adjuvant for cholera toxoid.

Subsequently, a comparative study of the ability of the E. coli antigen, cholera toxoid, and the combined antigens to elicit protection against enterotoxin challenge in the rabbit ileal loop model was conducted. The results showed that the combined antigen formula provided superior protection against both CT and ECT challenge than did either antigen alone (4). In the case of CT challenge, the protection was so great that the highest challenge dose (of a series) did not elicit a measurable response. This level of protection (greater than 10 mcg) was of an order of magnitude not previously observed by ourselves or by other investigators (28).

As mentioned earlier, we had proposed that insoluble enterotoxin might represent fragments of outer membrane dispersed during cultivation of the organisms (23). If this were the case, then it was reasoned that thoroughly washed and killed enterotoxigenic E. coli might be capable of producing not only bacterial antibodies, but antitoxin as well. Using two different enterotoxigenic strains (inactivated under mild conditions with glutaraldehyde), rabbits were immunized as before. The results showed that killed-enterotoxigenic organisms were indeed capable of eliciting antitoxin (4). The antitoxin levels produced by nine or ten logarithms of the organisms were equivalent to those levels produced by 50 to 60 mcg of insoluble ECT. This result led us to consider that killed organisms might substitute for insoluble ECT as an adjuvant for cholera toxoid both with regard to antibody levels and protection. If such a substitution could be made, then it would provide the basis for the design of a simple and economically feasible vaccine which might be useful against both cholera and

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E. coli diarrheal disease. The toxoid, for which reliable production methods are available (25), would be expected to provide antitoxic immunity against CT and ECT and the organisms, in addition to their adjuvant properties for the toxoid, might be expected to provide antibacterial immunity against certain enterotoxigenic strains. Further, the incorporation of cholera vibrios in the formula could provide a vaccine of broad specificity.

In order to test the adjuvant properties of killed E. coli, groups of rabbits were immunized with cholera toxoid (100 mcg) alone and together with the organisms (at doses of 5×10^9 or 1×10^{10}). In addition, some rabbits received equivalent doses of toxoid and non-toxigenic (killed) organisms or non-toxigenic organisms alone. The results of the study showed that the enterotoxigenic organisms elicited antitoxin, which like the antitoxin produced by insoluble ECT, was specific for (soluble) ECT; whereas the non-toxigenic organisms, as expected, did not produce antitoxin (4). Further, both types of organisms (toxigenic and non-toxigenic) acted as adjuvants for the toxoid (4). In both cases, the levels of antitoxin produced were similar to those previously generated by the combination of toxoid plus insoluble ECT.

Until this point in our investigation, the efficacy of different antigens or antigen combinations was based on their ability to protect immunized rabbits against cholera or E. coli enterotoxin challenge. In order to fully evaluate protection, however, it was necessary to test the antigens for their ability to protect against the appropriate challenge organisms, themselves,--- a situation more comparable to the disease. Since cholera antitoxin was capable of neutralizing both CT and ECT, it was of interest to test the single (toxoid) and combined (toxoid plus E. coli) antigens for their ability to protect against both cholera and E. coli diarrheal disease. And, as indicated above, it was also of interest to compare the efficacy of standard Cholera Vaccine (a mixture of Inaba and Ogawa serotypes) alone and together with the other antigens. Groups of rabbits were therefore immunized with cholera toxoid (100 mcg), E. coli Vaccine (5×10^9 organisms), and Cholera Vaccine (4×10^9 organisms), each administered alone and in all possible combinations (i.e., toxoid plus Cholera Vaccine; toxoid plus E. coli Vaccine; Cholera Vaccine plus E. coli Vaccine; and toxoid plus Cholera Vaccine plus E. coli Vaccine). In each case, antisera were obtained at the time of challenge (conducted during a 2 week period starting on the 12th day after the second of two inoculations spaced six weeks apart) and they were each tested for their ability to neutralize both ECT and CT.

The results confirmed all previous studies which showed that killed E. coli acted to elevate antitoxin titers produced by cholera toxoid, although the evidence also suggested that vibrios acted to depress the effect. Further, antitoxin levels correlated well with protection: those antigens or antigen combinations which exhibited the highest antitoxin

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levels also provided the greatest degree of protection, Of all the antigens tested, the combination of toxoid plus E. coli Vaccine provided the greatest degree of protection against both cholera (Ogawa 395) and E. coli (strain H74-114) challenge, whereas the same combination together with Cholera Vaccine provided the second best level of protection against cholera challenge (4). Although the combination of toxoid plus Cholera Vaccine provided better protection than did either antigen alone, the level of protection was not as great as that provided by toxoid plus E. coli Vaccine or toxoid plus E. coli Vaccine plus Cholera Vaccine (4). In fact, the superior protection provided by toxoid plus E. coli Vaccine as compared with toxoid plus Cholera Vaccine in this particular study suggested that antitoxic immunity might be the predominant factor in cholera immunity after all --- especially since Cholera Vaccine, in the same study, did not act as an adjuvant for the toxoid (4). Interestingly, the evidence also indicated that E. coli Vaccine acted as an adjuvant not only for cholera toxoid but for Cholera Vaccine as well, since better protection was provided against cholera challenge by the combination of E. coli and Cholera Vaccines than by Cholera Vaccine alone (4).

Although further studies would be required to establish the superiority of any particular combination of antigens, the results of the above investigation do indicate that the inclusion of an appropriate E. coli antigen (in this case, killed E. coli Vaccine) in a toxoid or toxoid/vibrio vaccine formula may make the difference between marginal and truly effective protection against cholera and, the simple combination of cholera toxoid and E. coli Vaccine may provide excellent protection against E. coli diarrheal disease. The use of the killed organisms obviates the need to produce and purify ECT, which it has been noted, is produced in exceedingly small amounts. Further, the organisms themselves are likely to contribute an antibacterial component to the immunity which is developed. Unfortunately, termination of contract support has precluded an examination of the specificity of the bacterial antibodies generated by the combined antigen formula.

Finally, it is noteworthy that both cholera toxoid (as prepared by these laboratories or by Burroughs-Wellcome) and Cholera Vaccine are safe for human use, and that combinations of the two are to be tested in field studies in Bangladesh (N. Pierce, personal communication). The present studies raise the question of the safety of E. coli Vaccine for man. As far as is known, the method selected for inactivation of the organisms (i.e., glutaraldehyde) appears to have produced an antigen with little or no demonstrable toxicity since 2×10^{10} organisms, when administered i.p. to mice, failed to prevent weight gain in accordance with the specifications of mouse toxicity tests for Bordetella pertussis. Since the diseases in question are confined to the gastrointestinal tract, it would clearly be of interest

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to determine the efficacy of the present preparations when they are administered by the oral route, or possibly, by a combination of parenteral (toxoid) and oral (toxoid plus E. coli Vaccine) routes. If significant protection could be demonstrated, then it would obviate many of the problems associated with the development of parenteral vaccine formulations. However, the present usage of Gram-negative bacteria in several parenteral vaccines (Vibrio cholerae, Bordetella pertussis, and Salmonella typhosa) provides a precedent for considering the potential incorporation of an appropriately detoxified E. coli antigen in a parenteral toxoid vaccine.

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