Oral Adjuvant Therapy in the Development of Immunological Protection Against Mucosal Pathogens

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We report the development of a non-toxic adjuvant for orally administered antigens that can elicit the production of both serum IgG and mucosal IgA against antigens with which it is delivered. This adjuvant is a mutant form of the heat-labile cholera-like toxin of E. coli. This adjuvant, designated LT(R192G), acts as a mucosal adjuvant, increasing the serum IgG and mucosal IgA responses to co-administered antigen beyond that achieved with administration of antigen alone. Further, LT(R192G) prevented the induction of tolerance to antigen and did not induce tolerance against itself as demonstrated by the presence of significant serum anti-LT IgG and mucosal anti-LT IgA antibodies in immunized mice. In addition to its potential use as an adjuvant for unrelated antigens, use of this non-toxic adjuvant as one component of a whole-cell/toxoid vaccine against cholera-related enteropathies should provide more epitopes for induction of neutralizing antibodies as well as adjuvant activity not associated with B subunit alone. In addition, this mutant LT provides for the first time a model system in which to examine the role of proteolytic processing with respect to the enterotoxic and immunologic properties of ADP-ribosylating toxins both in vitro and in vivo.

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FINAL REPORT


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OBJECTIVE: To develop a safe, effective oral adjuvant for use in human vaccines.

APPROACH: Secretory IgA (sIgA) antibodies directed against specific virulence determinants of infecting organisms play an important role in overall mucosal immunity. In many cases, it is possible to prevent the initial infection of mucosal surfaces by stimulating production of mucosal sIgA levels directed against relevant virulence determinants of an infecting organism. Secretory IgA may prevent the initial interaction of the pathogen with the mucosal surface by blocking attachment and/or colonization, neutralizing surface acting toxins, or preventing invasion of the host cells. Oral vaccines can be effective for induction of specific sIgA responses if the antigens are presented to the T and B lymphocytes and accessory cells contained within the Peyer's patches where preferential IgA B-cell development is initiated. A number of strategies have been developed for inducing active secretory immunity, including the use of attenuated mutants of bacteria and viruses as carriers of foreign antigens, incorporation of antigens into liposomes, use of lipophilic immune stimulating complexes, and addition of bacterial products with known adjuvant properties (see below).

We have isolated and extensively characterized a protein from E. coli with potential for use as an oral adjuvant. We discovered the adjuvant properties of this protein, designated LT, while studying the phenomenon of immune tolerance following oral administration of antigen under a previous ONR contract (ONR Contract No. N00014-83-K-0192, R&T 441e018). We examined a number of parameters, including the effect of LT on oral tolerance to OVA and the role of the two subunits of LT in the observed response, the effect of varying the timing and route of delivery of LT, the effect of prior exposure to OVA on the ability of LT to influence tolerance, the use of LT as an adjuvant with two unrelated antigens, and the effect of route of immunization on anti-OVA (Clements, et al., Vaccine 6:269, 1988).

Both LT and the closely related cholera toxin (CT) have significant immunoregulatory potential, not only as means of preventing the induction of tolerance but also as adjuvants for orally administered antigens. This raises the possibility of an effective immunization program against a variety of pathogens involving the oral administration of killed or attenuated agents or relevant virulence determinants of specific agents in conjunction with LT or CT. However, the fact that these "toxins" can stimulate a net lumenal secretory response may prevent their use. This problem could be resolved if the molecules could be detoxified without diminishing their adjuvant properties. A number of attempts have been made to alter the toxicity of LT or CT using site-
directed mutagenesis to change amino acids associated with the crevice where NAD binding and catalysis is thought to occur. Both Glu 112 and Ser 61, which share a side-chain hydrogen bond, are important for catalysis while the side chain for Arg 7 forms the binding cleft. Replacement of any of these amino acids by site-directed mutagenesis has been shown to alter ADP-ribosyltransferase activity with a corresponding loss of toxicity in a variety of biological assay systems. In addition, it has been shown that exchanging Lys for Glu 112 not only removes ADP-ribosylating enzymatic activity, but cAMP activation and adjuvant activity as well. A logical conclusion is that ADP-ribosylation and induction of cAMP are essential for the adjuvant activity of these molecules. Consequently, another approach to detoxification was explored.

Both CT and LT are synthesized with a trypsin sensitive peptide bond that joins the A1 and A2 pieces. This peptide bond must be nicked for the molecule to be "toxic". If the A1-A2 bond is not removed, either by bacterial proteases or intestinal proteases in the lumen of the bowel, the A1 piece cannot reach its target (adenylate cyclase) on the basolateral surface of the intestinal epithelial cell. In contrast to CT, LT is not biologically active when first isolated from the cell. LT also requires proteolysis to be active and the proteolytic activation does not occur inside of the bacterium. Therefore, one means of altering the toxicity of the molecule without directly affecting the NAD binding and catalytic site of the molecule would be to remove by genetic manipulation the trypsin sensitive amino acids that join the A1 and A2 components of the A subunit. If the molecule cannot be proteolytically cleaved, it should not induce net secretion in the intestine. It should, however, retain its immunologic function.

Within the disulfide subtended region that separates the A1 and A2 pieces is a single Arginine residue which is believed to be the site of cleavage necessary to activate the enterotoxic properties of the molecule. We proposed to change this region by site-directed mutagenesis is such a way as to render the molecule insensitive to proteolytic digestion and, consequently, nontoxic. We then examined the biologic and immunologic properties of the resulting construct.

ACCOMPLISHMENTS: Our first step was to substitute another amino acid for Arg (GGA = Gly replaces AGA = Arg), thus preserving the reading frame while eliminating the proteolytic site. LT was then purified by agarose affinity chromatography from one mutant (pBD95) which had been confirmed by sequencing. This mutant LT, designated LT(R192G) was then examined by SDS-polyacrylamide gel electrophoresis for modification of the trypsin sensitive bond. Samples were examined with and without exposure to trypsin and compared with native (unmodified) LT. As we predicted, LT(R192G) did not dissociate into A1 and A2 when incubated with trypsin, thereby indicating that sensitivity to protease had been removed.

Further characterization revealed that LT(R192G) is not sensitive to proteolytic activation by trypsin, chymotrypsin or pepsin, is devoid of in vitro ADP-ribosyltransferase activity, retains a basal level of activity on mouse Y-1 adrenal tumor cells and is able to induce production of cyclic adenosine monophosphate by those cells. Importantly, this mutant retains the ability to function as a mucosal adjuvant, increasing the serum IgG and mucosal IgA responses to
coadministered antigen above that achieved by administration of antigen alone. Moreover, LT(R192G) prevented the induction of oral tolerance both to itself and to coadministered antigen as evidenced by the presence of significant serum and mucosal anti-LT antibodies in inoculated mice. LT(R192G) is non-toxic in the patent mouse model and is capable of inducing anti-toxin immunity which protects mice against challenge with native toxin.

CONCLUSIONS: The mutant LT(R192G) differed from those previously reported in that the mutation in LT(R192G) affects A1-A2 cleavage and not the putative NAD-catalytic site. In a model in which the cellular target is located on the basolateral surface of polarized intestinal epithelial cells, the inability to dissociate A1 from A2 could prevent access to the substrate adenylate cyclase thereby reducing or eliminating cAMP accumulation and the ensuing events associated with secretion. In non-polarized lymphoid tissues this would presumably not be the case and adjuvant activity would not be affected. On the other hand, single amino acid changes in the catalytic site may have undetermined conformational effects which influence adjuvanticity in addition to ADP-riboseyltransferase activity, but the two may not, in fact, be the same. LT(R192G) retained the ability to act as a mucosal adjuvant, increasing the serum IgG and mucosal IgA responses to coadministered antigen (OVA) beyond that achieved with administration of that antigen alone. Further, LT(R192G) prevented the induction of tolerance to that antigen and did not induce tolerance against itself as demonstrated by the presence of significant serum anti-LT IgG and mucosal anti-LT IgA antibodies in immunized mice. This is an important finding because it provides a means of inducing the production of antibodies directed against both A and B subunits of LT and CT without the associated toxicity of the holotoxin. In addition to its potential use as an adjuvant for unrelated antigens, use of this non-toxic adjuvant as one component of a whole-cell/toxoid vaccine against cholera-related enteropathies should provide more epitopes for induction of neutralizing antibodies as well as adjuvant activity not associated with B subunit alone. In addition, this mutant LT provides for the first time a model system in which to examine the role of proteolytic processing with respect to the enterotoxic and immunologic properties of ADP-ribosylating toxins both in vitro and in vivo.

SIGNIFICANCE:

Human pathogens that initiate disease following infection of mucosal surfaces represent the single largest cause of morbidity and mortality among the world's populations. Diarrheal and respiratory illness are among the most common debilitating infectious diseases afflicting people of all ages around the globe. One major limitation to the development of effective immunoprophylaxis against mucosal pathogens is the inability to stimulate significant levels of mucosal antibody directed against specific virulence determinants of those pathogens. The development of a non-toxic adjuvant for orally administered antigens that can elicit the production of both serum IgG and mucosal IgA against antigens with which it is delivered raises the possibility of an effective immunization program against a variety of pathogens involving the oral administration of killed or attenuated agents or relevant virulence determinants of specific agents.
PATENT INFORMATION:

Two patents are pending -

U.S. Application No. 08/000,906
"Pharmaceutical composition of Escherichia coli heat-labile enterotoxin adjuvant and methods of use"
J.D. Clements

U.S. Application No. 08/296,648
"Mutant enterotoxin effective as a non-toxic oral adjuvant"
J.D. Clements, B.L. Dickinson

PUBLICATIONS AND ABSTRACTS:


Clements, J.D. 1993. Background of LT oral adjuvant research; data on the peptide antigen ovalbumin; genetic manipulation of LT. LT oral adjuvant workshop. Navy Medical Research Institute.


Dickinson, B.L., and J.D. Clements. 1995. Dissociation of Escherichia coli heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. 95th Annual Meeting of the American Society for Microbiology.