INFECTION AND IMMUNITY, May 1995, p. 1617–1623
0019-9567/95/$04.00+0

Vol. 63, No. 5

Dissociation of *Escherichia coli* Heat-Labile Enterotoxin Adjuvanticity from ADP-Ribosyltransferase Activity

BONNY L. DICKINSON AND JOHN D. CLEMENTS*

Department of Microbiology and Immunology, Tulane University Medical Center, New Orleans, Louisiana 70112

Received 2 September 1994/Returned for modification 12 December 1994/Accepted 31 January 1995

The heat-labile enterotoxin (LT) of *Escherichia coli* is immunologically and physiochemically related to cholera enterotoxin. A number of studies have been performed to determine the relationship of the ADP-ribosylating enzymatic activity of these enterotoxins to toxicity and adjuvanticity. These studies have generally examined the effect of abolishing the ADP-ribosyltransferase activity of A_1 by a variety of chemical or genetic manipulations. In every case, loss of enzymatic activity was associated with loss of biological activity and also with the ability of the molecules to function as oral adjuvants. Consequently, we explored an alternate approach to detoxification of LT without altering its adjuvanticity. Specifically, we generated a novel mutant form of LT by genetic modification of the proteolytically sensitive residues that join the A_1 and A_2 components of the A subunit. This mutant contains a single amino acid substitution within the disulfide subtended region joining A_1 and A_2. This mutant toxin, designated $LT_{(R192G)}$, is not sensitive to proteolytic activation, has negligible activity on mouse Y-1 adrenal tumor cells, and is devoid of ADP-ribosyltransferase activity. Nonetheless, $LT_{(R192G)}$ retains the ability to function as a mucosal adjuvant, increasing the serum immunoglobulin G (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 coadministered 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.

Secretory immunoglobulin A (sIgA) antibodies directed against specific virulence determinants of infecting organisms play an important role in overall mucosal immunity (6). In many cases, it is possible to prevent the initial infection of mucosal surfaces by stimulating production of mucosal sIgA directed against relevant virulence determinants of an infecting organism. sIgA 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. While extensive research has been conducted to determine the role of cell-mediated immunity and serum antibody in protection against infectious agents, less is known about the regulation, induction, and secretion of sIgA. Parenterally administered inactivated whole-cell and whole-virus preparations are effective at eliciting protective serum IgG and delayed-type hypersensitivity reactions against organisms that have a significant serum phase in their pathogenesis. However, parenteral vaccines are not effective at eliciting mucosal sIgA responses and are ineffective against bacteria that interact with mucosal surfaces and do not invade.

Oral immunization 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. The Peyer's patches contain helper T (TH) cells that mediate B-cell isotype switching directly from IgM cells to IgA B cells. The patches also contain T cells that initiate terminal B-cell differentiation. The primed B cells then migrate to the mesenteric lymph nodes and undergo differentiation, enter the thoracic duct and then the general circulation, and subsequently seed all of the secretory tissues of the body, including the lamina propria of the gut and respiratory tract. IgA is then produced by the mature plasma cells, complexed with membrane-bound secretory component, and transported onto the mucosal surface, where it is available to interact with invading pathogens (31, 32). The existence of this common mucosal immune system explains in part the potential of live oral vaccines and oral immunization for protection against pathogenic organisms that initiate infection by first interacting with mucosal surfaces.

A number of strategies for oral immunization have been developed, including the use of attenuated mutants of bacteria (i.e., *Salmonella* spp.) as carriers of heterologous antigens (4, 5), encapsulation of antigens into microspheres composed of poly-DL-lactide-glycolide and proteinlike polymers (proteinoids, gelatin capsules, and different formulations of liposomes [1, 16, 17]), adsorption onto nanoparticles, use of lipophilic immune stimulating complexes (ISCOMS) (26), and addition of bacterial products with known adjuvant properties (12, 14, 22, 23, 34). The two bacterial products with the greatest potential to function as oral adjuvants are cholera toxin (CT), produced by various strains of *Vibrio cholerae*, and the heat-labile enterotoxin (LT) produced by some enterotoxigenic strains of *Escherichia coli*. Although LT and CT have many features in common, they are clearly distinct molecules with biochemical and immunologic differences which make them unique (11, 13, 34).

Both LT and CT are synthesized as multisubunit toxins with A and B components. After the initial interaction of the toxin with the host cell membrane receptor, the B region facilitates the penetration of the A subunit through the cell membrane. On thiol reduction, this A component dissociates into two smaller polypeptide chains. One of these, the A_1 piece, catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein ($G_{S\alpha}$) in the adenylate cyclase enzyme complex on the basolateral surface of the epithelial cell, and this results in increasing intracellular levels of cyclic AMP (cAMP). The re-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Tulane University Medical Center, 1430 Tulane Ave., New Orleans, LA 70112. Phone: (504) 588-5070. Fax: (504) 588-5144. Electronic mail address: jdclemen@tmcpop.tmc.tulane.edu.

sulting increase in cAMP causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms involving (i) NaCl cotransport across the brush border of villous epithelial cells and (ii) electrogenic Na-dependent Cl secretion by crypt cells (15). The A subunit is also the principal moiety associated with immune enhancement by these toxins. This subunit then becomes a likely target for manipulation in order to dissociate the toxic and immunologic functions of the molecules (3, 18, 19, 21, 23, 25, 27, 33). The report by Lycke et al. (23) indicated that alterations that affect the ADP-ribosylating enzymatic activity of the toxin and alter the ability to increase intracellular levels of cAMP also prevent the molecule from functioning as an adjuvant. Consequently, another approach to detoxification was explored.

As an alternative approach to dissociation of enterotoxicity from adjuvanticity, we constructed a mutant of LT by using site-directed mutagenesis to create a single amino acid substitution within the subtended disulfide region of the A subunit. This single amino acid change altered the proteolytically sensitive site within this region, rendering the mutant insensitive to trypsin activation. We have analyzed the physical characteristics of this mutant by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), examined its biological activity on mouse Y-1 adrenal tumor cells, determined its enzymatic properties in an in vitro NAD:agmatine ADP-ribosyltransferase assay, and analyzed its immunomodulating capabilities by testing for both the retention of adjuvanticity and the ability to influence the development of systemic tolerance.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM83(pJC217) *ara* Δ*lac-proAB rpsL* ϕ80δ*lacZ* ΔM15 is a K-12 derivative transformed with a 3.5-kb plasmid (pJC217) which contains the gene for production of LT-B (8). *E. coli* BMH 71-18 *mutS thi supE* Δ(*lac-proAB*) [*mutS*::Tn*10*] [F′ *proA*$^+$*B*$^+$ *lacI*^{q} ZΔM15] (Promega, Madison, Wis.) is a highly transformable mismatch repair-minus strain used in site-directed mutagenesis to obtain a high mutation efficiency. Plasmid pLTA210 is a pUC19 derivative containing the LT-A gene encoded on an *Nco*I-*Sst*I fragment cloned within the polylinker region (generously supplied by Hugh S. Mason, Institute of Biosciences and Technology, Texas A&M University, Houston). Plasmid pGEM-5Zf(+)LTA is a pGEM-5Zf(+) (Promega) derivative containing the LT-A gene from pLTA210 cloned across the *Nco*I-*Sst*I sites (this study). Plasmid pBD94 is a pUC18 derivative containing both the LT-A and the LT-B genes cloned in the same reading frame and under control of the *lac* promoter (this study). Plasmid pBD95 is isogenic to pBD94 except for a single point mutation within the A subunit.

Media. Transformants of *E. coli* JM83 were screened on Luria-Bertani agar plates containing 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma). Transformants of *E. coli* BMH 71-18 were screened on Luria-Bertani agar plates containing 0.5 mM IPTG and either 125 μg of ampicillin or 12 μg of tetracycline per ml. The native and mutant heat-labile enterotoxins were purified from cultures grown overnight in 10-liter fermentors containing Trypticase soy broth (BBL, Cockeysville, Md.) with 100 μg of ampicillin per ml.

Restriction enzymes. *Hin*dIII, *Sst*I, *Sph*I, *Xba*I, *Sma*I, *Kpn*I, *Bgl*II, and *Nco*I were obtained from GIBCO BRL (Gaithersburg, Md.). *Sac*I was purchased from New England BioLabs, Inc. (Beverly, Mass.).

Transformation. *E. coli* JM83 was made competent and transformed by the calcium chloride transformation procedure (2). *E. coli* BMH 71-18 was made competent by a modification of the rubidium chloride technique as prescribed by the manufacturer. Briefly, cultures were grown to an A_{600} of 0.13 to 0.15, the cells were recovered by centrifugation, and the pellet was resuspended in a solution containing 10 mM MOPS (morpholinepropanesulfonic acid) and 10 mM rubidium chloride (pH 7.0). Cells were then centrifuged as described above; resuspended in a solution containing 100 mM MOPS, 50 mM calcium chloride, and 10 mM rubidium chloride (pH 6.5); and incubated in an ice-water bath. The cells were recovered as described above and resuspended in 2 ml of the latter solution, and 200-μl aliquots were used in transformation reactions. Transformation was achieved by adding dimethyl sulfoxide and plasmid DNA to 200 μl of competent cells and then incubating the cells in an ice-water bath. After a brief heat shock at 42°C, the cells were incubated in Luria-Bertani broth at 37°C to allow for recovery prior to the addition of ampicillin and overnight growth at 37°C.

DNA sequencing. DNA sequencing was performed on a 6% acrylamide–bisacrylamide gel, using a Sequi-Gen Nucleic Acid Sequencing Cell (Bio-Rad, Richmond, Calif.) and a Sequenase Version 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio), which is a variation of the dideoxynucleotide chain termination method (30). The single nucleotide mutation was confirmed by sequencing with a specific 20-mer oligonucleotide (5′ GAGAGAAGAACCCT GGATTC 3′) designed to anneal 30 nucleotides upstream of the mutation site. Primers specific for the SP6 and T7 regions of plasmid pGEM-5Zf(+) were used to confirm the LT-A gene insert in pGEM-5Zf(+)LTA.

Electrophoresis. Agarose gel electrophoresis was performed in 0.04 M Tris–0.2 M sodium acetate–0.0002 M EDTA (pH 7.8).

Site-directed mutagenesis. A mutagenic oligonucleotide was designed to incorporate a single nucleotide substitution within the A subunit which would replace the wild-type arginine residue (AGA) with a glycine (GGA) at amino acid 192 from the N terminus. A second mutagenic oligonucleotide was designed to create a unique restriction site in the polylinker of pBD94. One such mutant, designated *E. coli* JM83(pBD95), was identified by this procedure, and the plasmid was found to contain the arginine-to-glycine mutation upon DNA sequencing.

Purification of LT and $LT_{(R192G)}$. *E. coli* JM83(pBD94) and *E. coli* JM83(pBD95) were cultured and LT and $LT_{(R192G)}$ were purified by agarose affinity chromatography as previously described (11). CT used in this study had been previously prepared in this laboratory.

SDS-PAGE. SDS-PAGE was performed by the technique of Laemmli (20). Each well contained 100 μg of protein, and gels were stained with 0.05% Coomassie brilliant blue. When indicated, LT and $LT_{(R192G)}$ were activated by incubation for 45 min at 37°C in the presence of 0.1 μg of trypsin in a final reaction volume of 100 μl. Sample buffer containing dithiothreitol was added, and samples were heated to 100°C for 5 min prior to SDS-PAGE analysis.

Y-1 adrenal cell assay. Mouse Y-1 adrenal tumor cells (ATCC CCL-79) were seeded in 96-well round-bottom plates at a concentration of 10^4 cells per well (29). LT and $LT_{(R192G)}$ were treated with trypsin and serially diluted prior to incubation with Y-1 adrenal tumor cells at 37°C in 5% CO_2 overnight. The following day, the cells were examined by light microscopy for typical cell rounding. Titer is defined as the reciprocal of the minimum concentration of toxin required to give greater than 50% cell rounding.

NAD:agmatine ADP-ribosyltransferase assay. The NAD:agmatine ADP-ribosyltransferase assay was performed as described before (25, 28). Briefly, CT, LT, and $LT_{(R192G)}$ were either trypsin activated or left untreated and incubated for 30 min at 30°C with 50 mM glycine–20 mM dithiothreitol in TEAN buffer (11), after which the following were added and the reaction was continued for 90 min at 30°C: 0.1 μg of soybean trypsin inhibitor, 50 mM potassium phosphate, 10 mM agmatine, 20 mM dithiothreitol, 100 mM magnesium chloride, 100 mM GTP, 3 mM dimyristoylphosphatidylcholine, 0.2% cholate, 0.03 mg of ovalbumin, 100 μM (ca. 80,000 cpm) NAD (U-^{14}C-adenosine; Du Pont NEN, Boston, Mass.), and water to a final volume of 300 μl. Results are expressed as femtomoles of ADP-ribosylagmatine produced per minute.

Determination of adjuvanticity and oral tolerance. The procedures for determination of adjuvanticity and oral tolerance were essentially the same as those described by Clements et al. (12). Ovalbumin for inoculation was Calbiochem 5× crystalline egg albumin (OVA; Behring Diagnostics, La Jolla, Calif.). Groups of BALB/c mice were inoculated intragastrically with a blunt-tip feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.). Oral inoculations consisted of 0.5 ml of phosphate-buffered saline (PBS; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl [pH 7.2]), 0.5 ml of PBS containing 5 mg of OVA, or 0.5 ml of PBS containing 5 mg of OVA and 25 μg of LT or $LT_{(R192G)}$. Following the oral inoculations, animals were boosted intraperitoneally with 1 μg of OVA in 20% Maalox (William H. Rorer, Inc., Washington, Pa.). One week after the intraperitoneal inoculation, animals were sacrificed, and assays for serum IgG and mucosal IgA antibodies directed against OVA and LT were performed by enzyme-linked immunosorbent assay (ELISA). The number of animals in each immunization group is included in the legend to each figure.

Antibody assay. Animals were bled prior to euthanasia, and sera were stored at −20°C until assayed. The small intestine from duodenum to ileal-cecal junction was excised and homogenized in a solution containing 50 mM EDTA and 0.1 mg of soybean trypsin inhibitor (Sigma) per ml. Samples were homogenized with a Tekmar Tissuemizer, clarified by centrifugation, lyophilized, resuspended in 1 ml of TEAN buffer, dialyzed against TEAN buffer, adjusted to a constant volume, and stored at −20°C until assayed.

ELISA. Reagents and antisera for the ELISA were obtained from Sigma. Samples for ELISA were serially diluted in PBS–0.05% Tween 20. For anti-LT determinations, microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III) per well and then with 1 μg of purified LT per well. Anti-OVA was determined on microtiter plates precoated with 10 μg of OVA per well. Anti-LT and anti-OVA IgG levels in serum were determined with rabbit antiserum against mouse IgG conjugated to alkaline phosphatase. Mucosal anti-LT and anti-OVA IgA were assayed with goat antiserum against mouse IgA (alpha chain specific) followed by rabbit antiserum against goat IgG conjugated to alkaline phosphatase. Reactions were stopped with 3 N NaOH. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins [MOPC 315, γA(IgAλ2); MOPC 21, γG1]).

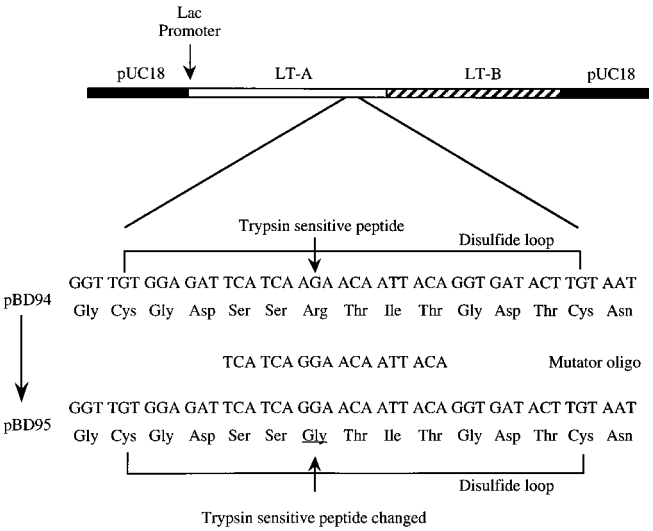

FIG. 1. Schematic diagram of plasmid pBD94, which encodes both subunits A and B of LT under control of the *lac* promoter. Plasmid pBD95 contains the single base substitution in amino acid residue 192 of subunit A, coding for Gly rather than Arg, which preserves the reading frame but eliminates the proteolytic site. The amino acid sequence corresponding to the region of trypsin sensitivity and the site of amino acid change Arg-192 to Gly-192 is shown.

Statistical analysis. The standard error of the mean was calculated for all data, and means of variously immunized groups were compared by the Student *t* test. Statistical significance was considered to be $P \leq 0.05$.

RESULTS

Construction of $LT_{(R192G)}$. The first step in construction of the mutant LT was to isolate the LT-A and LT-B genes separately from plasmids pLTA210 and pJC217, respectively, and to recombine them in cassette form into pUC18, thus creating pBD94 (Fig. 1). Subcloning the LT-A and LT-B genes under control of the *lac* promoter of pUC18 permitted the purification of reasonable quantities of LT and derived mutants. By using site-directed mutagenesis, a glycine residue was substituted for the arginine at amino acid 192 from the amino terminus of the A_1 subunit (Fig. 1). Removal of this trypsin-sensitive arginine residue should eliminate the proteolytic site, rendering the molecule trypsin insensitive. This was accomplished by constructing two mutagenic oligonucleotides: one to incorporate a single nucleotide substitution within the A subunit, thereby converting a wild-type arginine residue (AGA) to a glycine (GGA), and a second to convert the *Kpn*I site in the pUC18 polylinker to a *Bgl*II site to facilitate screening. The mutagenic oligonucleotides were first annealed to single-stranded pBD94, and an in vitro synthesis reaction was performed. Recombinant plasmids were then transformed into *E. coli* BMH 71-18 to allow for strand segregation and amplification. Upon recovery, the plasmids were transformed into *E. coli* JM83 and individual colonies were screened for the desired mutation by restriction analysis with *Kpn*I and *Bgl*II. *Bgl*II-sensitive clones were screened for production of LT by ELISA and for the desired nucleotide substitution by DNA sequencing. One such recombinant plasmid containing the single nucleotide substitution was identified and designated pBD95.

Mutant LT containing the glycine 192 substitution was designated $LT_{(R192G)}$ and was purified from JM83(pBD95) by agarose affinity chromatography (11) and then analyzed by SDS-PAGE for modification of the trypsin-sensitive bond (Fig. 2). LT and $LT_{(R192G)}$ were examined following thiol reduction with and without trypsin treatment. As shown in lane 1 of Fig. 2, thiol reagents completely cleaved the 28,000-molecular-weight A subunit of trypsin-treated native LT into its major A_1 peptide (21,000 molecular weight) and its A_2 peptide (7,000 molecular weight [not visible on SDS–10% PAGE]). In the absence of trypsin treatment, the A subunit of native LT remained intact (lane 2). In contrast, the A subunit of trypsin-treated $LT_{(R192G)}$ did not dissociate into its A_1 and A_2 components upon thiol reduction and heating in the presence of SDS (lane 3). Lane 4 contains $LT_{(R192G)}$ in the absence of trypsin treatment. This demonstrated that sensitivity to this protease had been removed in $LT_{(R192G)}$.

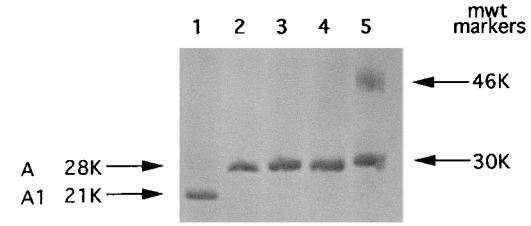

FIG. 2. Analytical discontinuous SDS-PAGE of thiol-reduced LT and $LT_{(R192G)}$ with and without trypsin treatment. Thiol reagents completely cleaved the 28,000-molecular-weight A subunit of trypsin-treated native LT into its major A_1 peptide (21,000 molecular weight) and its A_2 peptide (7,000 molecular weight) (lane 1). In the absence of trypsin treatment, the A subunit of native LT remained intact (lane 2). In contrast, the A subunit of trypsin-treated $LT_{(R192G)}$ did not dissociate into its A_1 and A_2 components upon thiol reduction and heating in the presence of SDS (lane 3). Lane 4 contains $LT_{(R192G)}$ in the absence of trypsin treatment. Molecular weight (mwt) markers are shown in lane 5.

Effect of $LT_{(R192G)}$ on Y-1 adrenal cells. Next, the effect of $LT_{(R192G)}$ on mouse Y-1 adrenal tumor cells was examined. We have previously shown that unnicked LT is more than 1,000-fold less active on Y-1 adrenal cells than is CT and that trypsin-activated LT has the same biological activity as CT (11, 13). It was presumed that the residual activity of LT observed in this assay in the absence of in vitro trypsin activation was a function of some residual protease activity which could not be accounted for. For instance, trypsin is used in the process of subculturing Y-1 adrenal cells. It was therefore reasonable to assume that LT that could not be nicked would be completely inactive in the Y-1 adrenal cell assay, as is isolated recombinant LT-B (8). However, as shown in Table 1, $LT_{(R192G)}$ retained a basal level of activity in the Y-1 adrenal cell assay even though it could not be proteolytically processed. CT and native LT treated with trypsin have the same level of activity (15 pg) on Y-1 adrenal cells. By contrast, $LT_{(R192G)}$ (48,000 pg) was >1,000-fold less active than CT or native LT and could not be activated by trypsin, as shown by SDS-PAGE (Fig. 2). The residual basal activity undoubtedly reflects a pathway of adre-

TABLE 1. Mouse Y-1 adrenal cell activity of CT, native LT, and $LT_{(R192G)}$

Toxin	Trypsin activated[a]	Sp act (pg per well)[b]
CT	−	15
LT	+	15
$LT_{(R192G)}$	−	48,800
$LT_{(R192G)}$	+	48,800

[a] LT and $LT_{(R192G)}$ were activated by incubation for 45 min at 37°C in the presence of 0.1 μg of trypsin in a final reaction volume of 100 μl.
[b] Minimum dose required to produce significant (>50%) cell rounding.

TABLE 2. ADP-ribosyltransferase activity of CT, native LT, and $LT_{(R192G)}$

Toxin	Activity (fmol/min)			
	Expt 1	Expt 2	Expt 3	Mean ± SEM
None	9.12	5.63	14.17	9.64 ± 2.48
CT				
1 μg	17.81	17.60	25.75	20.39 ± 2.68
10 μg	107.32	111.28	104.04	107.55 ± 2.09
100 μg	351.55	361.73	308.09	340.46 ± 16.45
LT, 100 μg	17.32	14.48	13.86	15.22 ± 1.07
LT, 100 μg, + trypsin	164.10	189.89	152.96	168.98 ± 10.94
$LT_{(R192G)}$, 100 μg	14.58	12.34	9.30	12.07 ± 1.53
$LT_{(R192G)}$, 100 μg, + trypsin	14.73	8.90	10.47	11.37 ± 1.74

nal cell activation heretofore unknown and different from that requiring separation of the A_1-A_2 linkage.

ADP-ribosylating enzymatic activity of $LT_{(R192G)}$. The NAD:agmatine ADP-ribosyltransferase assay was employed to determine if the mutant retained its ADP-ribosylating enzymatic activity. This assay measures ADP-ribosyltransferase activity and uses agmatine as an ADP-ribose receptor. As shown in Table 2, CT induced a dose-dependent increase in ADP-ribosylagmatine in this assay. At the highest level tested, 100 μg, CT produced an average of 340.46 ± 16.45 fmol of ADP-ribosylagmatine per min. In contrast, $LT_{(R192G)}$ lacked any detectable ADP-ribosylating enzymatic activity either with (11.37 ± 1.74 fmol of ADP-ribosylagmatine per min) or without (12.07 ± 1.53 fmol of ADP-ribosylagmatine per min) trypsin activation, even though the enzymatic site had not been altered and $LT_{(R192G)}$ demonstrated a basal level of activity in the Y-1 adrenal cell assay. By comparison, native LT required trypsin activation to exhibit ADP-ribosylating enzymatic activity, whereas the unnicked form, like $LT_{(R192G)}$, did not exhibit any enzymatic activity. The ADP-ribosylagmatine activities of nicked and unnicked $LT_{(R192G)}$ as well as that of unnicked native LT were not significantly different from those of negative (no toxin) controls (Student's *t* test).

Adjuvant activity of $LT_{(R192G)}$. Since $LT_{(R192G)}$ possessed no demonstrable ADP-ribosyltransferase activity, it should lack adjuvant activity. As mentioned previously, the report by Lycke et al. (23) makes it clear that alterations that affect the ADP-ribosylating enzymatic activity of the toxin and alter the ability to increase intracellular levels of cAMP also prevent the molecule from functioning as an adjuvant. $LT_{(R192G)}$ has no ADP-ribosylating enzymatic activity and only some undefined basal activity in Y-1 adrenal cells.

As shown in Fig. 3, animals primed orally with OVA and LT developed a significantly higher serum IgG anti-OVA response following subsequent parenteral immunization with OVA (484 ± 171 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (no detectable anti-OVA response). Significantly, animals primed orally with OVA and $LT_{(R192G)}$ also developed a significantly higher serum IgG anti-OVA response following subsequent parenteral immunization with OVA (203 ± 72 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA.

Similar results were obtained when anti-OVA IgA responses were compared within the same groups of animals. Also shown in Fig. 3, animals primed orally with OVA and LT developed a significantly higher mucosal IgA anti-OVA response following subsequent parenteral immunization with OVA (4,620 ± 1,633 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (9 ± 3 ng/ml). As above, animals primed orally with OVA and $LT_{(R192G)}$ also developed a significantly higher mucosal IgA anti-OVA response following subsequent parenteral immunization with OVA (6,866 ± 2,428 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA.

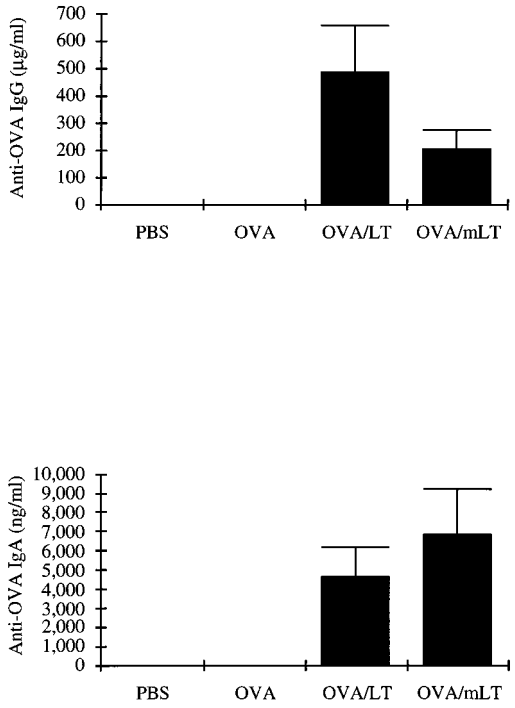

FIG. 3. Ability of $LT_{(R192G)}$ to act as an immunological adjuvant. Mice were primed orally with PBS, OVA, or OVA in combination with LT or $LT_{(R192G)}$ (mLT) at weekly intervals as indicated. Animals were boosted intraperitoneally with OVA, and levels of serum anti-OVA IgG and mucosal anti-OVA IgA were determined by ELISA. Bars represent mean antibody response ± standard error in each group 1 week after boost. Each group contained eight mice.

We also compared the abilities of LT and $LT_{(R192G)}$ to elicit an anti-LT antibody response in the same animals. This was important in that it would provide an indication of whether the mutant LT was able to prevent induction of tolerance to itself in addition to functioning as an adjuvant for other proteins. As shown in Fig. 4, animals primed orally with OVA and LT developed a significantly higher serum IgG anti-LT response following subsequent parenteral immunization with OVA (724 ± 256 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (no detectable anti-LT response). Animals primed orally with OVA and $LT_{(R192G)}$ also developed a significantly higher serum IgG anti-LT response following subsequent parenteral immunization with OVA (597 ± 211 μg/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA.

Similar results were obtained when anti-LT IgA responses were compared within the same groups of animals. Also shown in Fig. 4, animals primed orally with OVA and LT developed a significantly higher mucosal IgA anti-LT response following subsequent parenteral immunization with OVA (18,250 ± 6,452 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA (no detectable anti-LT response). As above, animals primed orally with OVA and $LT_{(R192G)}$ also developed a significantly higher mucosal IgA anti-LT response following subsequent parenteral immu-

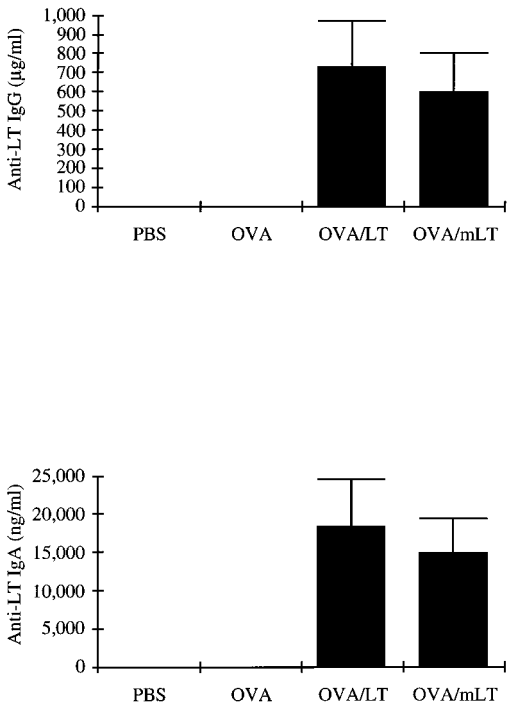

FIG. 4. Ability of $LT_{(R192G)}$ to prevent induction of oral tolerance to LT. Mice were primed orally with PBS, OVA, or OVA in combination with LT or $LT_{(R192G)}$ (mLT) at weekly intervals as indicated. Animals were boosted intraperitoneally with OVA, and levels of serum anti-LT IgG and mucosal anti-LT IgA were determined by ELISA. Bars represent mean antibody response ± standard error in each group 1 week after boost. Each group contained eight mice.

nization with OVA (14,326 ± 5,065 ng/ml) than those primed with OVA alone and subsequently immunized parenterally with OVA.

DISCUSSION

Both LT and CT have significant immunoregulatory potential, not only as a 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.

These two bacterial enterotoxins have many features in common. LT and CT have the same subunit number and arrangement, the same biological mechanism of action, and the same specific activity in many in vitro assays (11, 13). However, there are significant differences between these molecules that influence not only their enterotoxic properties but also their ability to function as adjuvants. To begin with, unlike CT produced by *V. cholerae*, LT is not fully biologically active when first isolated from the bacterial cell. Consistent with the A-B model for bacterial toxins, LT requires proteolysis and disulfide reduction to be fully active. In the absence of proteolytic processing, the enzymatically active A_1 moiety is unable to dissociate from the A_2 component and cannot reach its target substrate (adenylate cyclase) on the basolateral surface of the intestinal epithelial cell. This difference in activation of the isolated material results in differences in response thresholds for LT and CT in biologic systems. For instance, CT induces detectable net fluid secretion in the mouse intestine at a dose of 5 to 10 μg. LT induces detectable net secretion in this assay at 50 to 100 μg. In the rabbit ligated ileal loop, the difference is more dramatic and clear-cut. Significantly, when LT is exposed to proteolytic enzymes with trypsinlike specificity, the molecule becomes indistinguishable from CT in any biologic assay system. This was most clearly demonstrated by Clements and Finkelstein (11). In addition, LT has an unusual affinity for carbohydrate-containing matrices. Specifically, LT binds to an array of biological molecules containing galactose, including glycoproteins and lipopolysaccharides. This lectinlike binding property of LT results in a broader receptor distribution on mammalian cells for LT than for CT, which binds only to GM1 (1a, 13). The two molecules also have many immunologic differences, as demonstrated by immunodiffusion studies (9, 10), in vitro neutralization studies, and the partial protection against LT-associated *E. coli* diarrhea in volunteers receiving B-subunit whole-cell cholera vaccine (7). LT and CT have also been shown to induce different helper T-lymphocyte responses (24). When used as a mucosal adjuvant, CT selectively induces TH2-type cells in Peyer's patches and spleens as manifested by TH cells which produce interleukins 4 and 5 but not interleukin 2 or gamma interferon, while LT induces both TH1 and TH2 cells and predominantly antigen-specific IgA responses. Taken together, these findings demonstrate that LT and CT are unique molecules, despite their apparent similarities.

However, the fact that LT can stimulate a net lumenal secretory response may hinder investigation into its potential or prevent its use under appropriate conditions. This problem could be resolved if the molecule could be detoxified without diminishing its adjuvant properties. A number of attempts to alter the toxicity of LT or CT have been made, using site-directed mutagenesis to change amino acids associated with the crevice where NAD binding and catalysis are thought to occur. The arginine residue at position 7 is proposed to interact with the nicotinamide ring and stabilize the position of NAD in the binding cleft, while glutamate 112 is important for catalysis (13a). Replacement of either 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 (3, 18, 19, 21, 23, 25, 27, 27a, 33). In addition, Lycke et al. (23) have shown that exchanging lysine for glutamate 112 removes not only ADP-ribosylating enzymatic activity but cAMP activation and oral adjuvant activity as well. They concluded that ADP-ribosylation and induction of cAMP were essential for the adjuvant activity of these molecules.

Our findings are in direct contrast to those of Lycke et al. (23) in that $LT_{(R192G)}$, while devoid of ADP-ribosyltransferase activity and enterotoxicity, retains the ability to function as an oral adjuvant. The assay system used in the current study to determine ADP-ribosyltransferase activity was the same as that employed by Lycke et al. (23). There are a number of possible explanations for this difference in findings. The mutant created in this study differed from those previously reported in that the mutation in $LT_{(R192G)}$ affects A_1-A_2 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 A_1 from A_2 could prevent access to the substrate adenylate cyclase, thereby reducing or eliminating cAMP accumulation and the ensuing events associated with secretion. In nonpolarized lymphoid tissues, this would presumably not be the case and adjuvant activity would not be affected. This ability to interact with nonpolarized cells would account for the residual Y-1 adrenal cell observed in the present study. Such activity could also result from in vivo ADP-ribosyltransferase activity not detect-

able in the ADP-ribosylagmatine assay or an alternate intracellular proteolytic processing event. If that is the case, then ADP-ribosylation and induction of cAMP may indeed be essential for the adjuvant activity, as suggested by Lycke et al. (23). Stabilization of the A_1-A_2 linkage by site-directed mutagenesis may alter the enterotoxic properties of the molecule by preventing dissociation of A_1 from A_2 and thereby preventing separation of A_1 from the A_2-B_5 complex or by preventing A_1 from assuming the conformation necessary to bind and cleave NAD.

It should also be noted that Grant et al. (17a) recently reported construction of an identical LT mutant in which Arg-192 was replaced with Gly. In their hands, $LT_{(R192G)}$ exhibited undiminished levels of cytotonic activity for CHO cells compared with native LT and a marked delay in the ability to increase intracellular levels of cAMP in Caco-2 cells. They concluded that the R192G mutation reduces the enzymatic and toxic activity by an amount insufficient to be useful in the development of nontoxic analogs for vaccine purposes. They did not, however, report enterotoxicity or adjuvanticity for their construct.

In the study reported here, we demonstrated that $LT_{(R192G)}$ retains 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 nontoxic 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 the B subunit alone. In addition, this mutant LT provides 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.

ACKNOWLEDGMENTS

This work was supported by Office of Naval Research grant N00014-92-J-1980. B.L.D. is a recipient of a Department of Defense Augmentation Award for Science & Engineering Research Training (ASSERT), grant N00014-93-1-1071.

REFERENCES

1. **Alving, C. R., R. L. Richards, J. Moss, L. I. Alving, J. D. Clements, T. Shiba, S. Kotani, R. A. Wirtz, and W. T. Hockmeyer.** 1986. Effectiveness of liposomes as potential carriers for vaccines. Applications to cholera toxin and human malaria sporozoite antigen. Vaccine **4:**166–172.
1a. **Ångström, J., S. Teneberg, and K.-A. Karlsson.** 1994. Delineation and comparison of ganglioside-binding epitopes for the toxins of *Vibrio cholerae*, *Escherichia coli*, and *Clostridium tetani*: evidence for overlapping epitopes. Proc. Natl. Acad. Sci. USA **91:**11859–11863.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York.
3. **Burnette, W. N., V. L. Mar, B. W. Platler, J. D. Schlotterbeck, M. D. McGinley, K. S. Stoney, M. F. Rhode, and H. R. Kaslow.** 1991. Site-specific mutagenesis of the catalytic subunit of cholera toxin: substituting lysine for arginine 7 causes loss of activity. Infect. Immun. **59:**4266–4270.
4. **Cárdenas, L., and J. D. Clements.** 1992. Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. Clin. Microbiol. Rev. **5:**328–342.
5. **Cárdenas, L., U. Dasgupta, and J. D. Clements.** 1994. Influence of strain viability and antigen dose on the use of attenuated mutants of *Salmonella* as vaccine carriers. Vaccine **12:**833–840.
6. **Cebra, J. J., J. A. Fuhrman, D. A. Lebman, and S. D. London.** 1986. Effective gut mucosal stimulation of IgA-committed B cells by antigen, p. 129–133. *In* F. Brown, R. M. Channok, and R. A. Lerner (ed.), Vaccines 86: new approaches to immunization. Developing vaccines against parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, P. K. Neogy, B. F. Stanton, N. Huda, M. U. Khan, B. A. Kay, M. R. Khan, M. Ansaruzzanan, M. Yunus, R. Rao, A. Svennerholm, and J. Holmgren.** 1988. Cross protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J. Infect. Dis. **158:**372–377.
8. **Clements, J. D., and S. El-Morshidy.** 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. Infect. Immun. **46:**564–569.
9. **Clements, J. D., and R. A. Finkelstein.** 1978. Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **22:**709–713.
10. **Clements, J. D., and R. A. Finkelstein.** 1978. Immunological cross-reactivity between a heat-labile enterotoxin(s) of *Escherichia coli* and subunits of *Vibrio cholerae* enterotoxin. Infect. Immun. **21:**1036–1039.
11. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24:**760–769.
12. **Clements, J. D., N. M. Hartzog, and F. L. Lyon.** 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine **6:**269–277.
13. **Clements, J. D., R. J. Yancey, and R. A. Finkelstein.** 1980. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli* cultures. Infect. Immun. **29:**91–97.
13a. **Domenighini, M., C. Magagnoli, M. Pizza, and R. Rappuoli.** 1994. Common features of the NAD-binding and catalytic site of ADP-ribosylating toxins. Mol. Microbiol. **14:**41–50.
14. **Elson, C. O.** 1989. Cholera toxin and its subunits as potential oral adjuvants. Immunol. Today **146:**29–33.
15. **Field, M.** 1980. Regulation of small intestinal ion transport by cyclic nucleotides and calcium, p. 21–30. *In* M. Field, J. S. Fordtran, and S. G. Schultz (ed.), Secretory diarrhea. Waverly Press, Baltimore.
16. **Garcon, N. M. J., and H. R. Six.** 1993. Universal vaccine carrier. Liposomes that provide T-dependent help to weak antigens. J. Immunol. **146:**3697–3702.
17. **Gould-Fogerite, S., and R. J. Mannino.** 1993. Targeted fusogenic proteoliposomes: functional reconstitution of membrane proteins through protein-cochleate intermediates, p. 261–276. *In* G. Gregoriadis (ed.), Liposome technology, 2nd ed., vol. III. Interaction of liposomes with the biological milieu. CRC Press, Inc., Boca Raton, Fla.
17a. **Grant, C. G., R. J. Messer, and W. Cieplak, Jr.** 1994. Role of trypsin-like cleavage at arginine 192 in the enzymatic and cytotonic activities of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **62:**4270–4278.
18. **Harford, S., C. W. Dykes, A. N. Hobden, M. J. Read, and I. J. Halliday.** 1989. Inactivation of the *Escherichia coli* heat-labile enterotoxin by *in vitro* mutagenesis of the A-subunit gene. Eur. J. Biochem. **183:**311–316.
19. **Häse, C. C., L. S. Thai, M. Boesman-Finkelstein, V. L. Mar, W. N. Burnette, H. R. Kaslow, L. A. Stevens, J. Moss, and R. A. Finkelstein.** 1994. Construction and characterization of recombinant *Vibrio cholerae* strains producing inactive cholera toxin analogs. Infect. Immun. **62:**3051–3057.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Lobet, Y., C. W. Cluff, and J. W. Cieplak.** 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **59:**2870–2879.
22. **Lycke, N., and J. Holmgren.** 1986. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology **59:**301–308.
23. **Lycke, N., T. Tsuji, and J. Holmgren.** 1992. The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur. J. Immunol. **22:**2277–2281.
24. **McGhee, J. R., M. Marinaro, I. Takahashi, R. J. Jackson, J. Clements, H. F. Staats, K. L. Bost, and H. Kiyono.** 1994. Vaccines for mucosal immunity: unique delivery systems and immune response analyses for TH1/TH2 and IgE/IgA B cells. Mucosal Immunol. Update **2:**21.
25. **Moss, J., S. J. Stanley, M. Vaughan, and T. Tsuji.** 1993. Interaction of ADP-ribosylation factor with *Escherichia coli* enterotoxin that contains an inactivating lysine 112 substitution. J. Biol. Chem. **268:**6383–6387.
26. **Mowat, A. M., and A. M. Donachie.** 1991. ISCOMS—a novel strategy for mucosal immunization. Immunol. Today **12:**383–385.
27. **Okamaoto, K., K. Okamoto, A. Miyama, T. Tsuji, T. Honda, and T. Miwatani.** 1988. Effect of substitution of glycine for arginine at position 146 of the A1 subunit on biological activity of *Escherichia coli* heat-labile enterotoxin. J. Bacteriol. **170:**2208–2211.

27a. **Pizza, M., M. Domeninghini, W. Hol, V. Giannelli, M. R. Fontana, M. M. Giuliani, C. Magagnoli, S. Peppoloni, R. Manetti, and R. Rappuoli.** 1994. Probing the structure-activity relationship of *Escherichia coli* LT-A by site-directed mutagenesis. Mol. Microbiol. **14:**51–60.
28. **Price, R. S., C. F. Welsh, R. S. Haun, S. J. Stanley, J. Moss, and M. Vaughan.** 1992. Effects of phospholipid and GTP on recombinant ADP-ribosylation factors (ARFs). J. Biol. Chem. **267:**17766–17772.
29. **Sack, D. A., and R. B. Sack.** 1975. Test for enteropathogenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. **11:**334–336.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Strober, W., and D. Jacobs.** 1985. Cellular differentiation, migration, and function in the mucosal immune system, p. 1–30. *In* J. I. Gallin and A. S. Fauci (ed.), Advances in host defense mechanisms, vol. 4. Mucosal immunity. Raven Press, New York.
32. **Tomasi, T. B., and A. G. Plaut.** 1985. Cellular differentiation, migration, and function in the mucosal immune system, p. 31–61. *In* J. I. Gallin and A. S. Fauci (ed.), Advances in host defense mechanisms, vol. 4. Mucosal immunity. Raven Press, New York.
33. **Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani.** 1990. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in loss of its toxic activity. J. Biol. Chem. **265:**22520–22525.
34. **Walker, R., and J. D. Clements.** 1993. Use of heat-labile toxin of enterotoxigenic *Escherichia coli* to facilitate mucosal immunization. Vaccine Res. **2:**1–10.