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Development of Mucosal Protection against the Heat-Stable Enterotoxin (ST) of *Escherichia coli* by Oral Immunization with a Genetic Fusion Delivered by a Bacterial Vector

LUCIA CÁRDENAS AND JOHN D. CLEMENTS*

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112

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An LT-B–ST (LT-B/ST) fusion peptide was constructed by genetically joining the 5′ terminus of a synthetic gene coding for the heat-stable enterotoxin (ST) of *Escherichia coli* to the 3′ terminus of the gene coding for the binding subunit of the heat-labile enterotoxin (LT-B) of *E. coli*. An eight-amino-acid, proline-containing linker was included between the LT-B and ST moieties. An *aroA* mutant of *Salmonella dublin* transformed with a plasmid carrying this genetic construct was shown to express a fusion peptide with antigenic determinants of both LT-B and ST. Mice were immunized orally with this strain or with a control strain expressing just LT-B from the same plasmid. Sera and mucosal secretions were obtained and analyzed for the presence of serum immunoglobulin G and mucosal immunoglobulin A that were able to recognize LT-B and ST by enzyme-linked immunosorbent assay (ELISA) and, more importantly, were able to neutralize native ST in the suckling mouse assay. Sera and mucosal secretions from animals immunized with the strain expressing the LT-B/ST fusion exhibited detectable ELISA reactivity against LT-B but not against native ST. However, even in the absence of detectable ELISA reactivity, both sera and mucosal secretions from these animals were able to neutralize the biological activity of native ST in the suckling mouse assay. These findings are important because they demonstrate the development of mucosal protection against ST by oral immunization with a genetic fusion delivered by a bacterial vector.

Escherichia coli causes diarrheal disease by a variety of mechanisms, including production of one or more enterotoxins. In addition to the high-molecular-weight, heat-labile enterotoxin (LT), *E. coli* may also produce a low-molecular-weight (ca. 2,000), plasmid-mediated, heat-stable enterotoxin (ST) which functions by stimulating guanylate cyclase. The relative contribution of each enterotoxin to the global problem of *E. coli*-mediated diarrhea has never been clearly established. What is clear is that in some specific regions ST-producing *E. coli* organisms are the predominant enterotoxigenic *E. coli* organisms identified in indigenous populations as well as in foreign visitors to those areas. Furthermore, it is now clear that other genera of bacteria can produce enterotoxins immunologically related to both LT and ST, including *Salmonella* spp., noncholera vibrios, *Yersinia* spp., *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp. (22, 28, 35). It is therefore important to include protection against ST in an overall program of complete immunological protection against enteric disease.

Because of its small size, ST tends to be poorly immunogenic. It can, however, become immunogenic when coupled to an appropriate carrier in a standard hapten-carrier configuration. Furthermore, ST can be chemically coupled to either LT or its binding subunit, LT-B. Not only is the product of such a coupling immunogenic, but the toxicity of the ST molecule may be reduced as a function of the cross-linking (27). As an alternate approach to the development of a suitable ST toxoid, a number of research groups have constructed genetic fusions between ST and one or more subunits of cholera enterotoxin or LT (9, 23, 36–38). These fusion polypeptides have many potential advantages over chemically derived conjugates, including a precisely defined and homogeneous protein structure and the possibility of delivering the antigens via a live oral vaccine.

Because of the site of action of enterotoxigenic *E. coli*, an effective immunoprophylactic against ST must be capable of eliciting protective immunity at the level of the mucosal surfaces of the small bowel. One method for induction of antigen-specific mucosal antibodies is delivery of antigens to the gut-associated lymphoid tissues by use of avirulent derivatives of *Salmonella* spp. as carriers of plasmids that code for relevant determinants of foreign antigens. This has been shown to be an effective means of inducing significant serum and secretory antibody responses in a number of animal species, including humans (29, 30), cattle (41), sheep (31), rabbits (34), fowl (4, 5), and mice (2, 3, 6, 10, 13, 16, 18–20, 25, 26, 33, 40). The effectiveness of this system is attributed to the fact that these *Salmonella* spp. reach the Peyer's patches, where they present their array of antigens directly to the T and B lymphocytes of the gut-associated lymphoid tissues, thereby priming the mucosal immune system to produce antigen-specific immune responses. This has prompted many investigators to consider and test the use of live attenuated *Salmonella* spp. as carriers of genes coding for virulence determinants from other pathogenic microorganisms (recently reviewed by Cárdenas and Clements [6]).

We have reported previously the construction of a fusion peptide in which the 5′ terminus of the gene coding for ST was genetically fused to the 3′ terminus of the gene coding for the binding (B) subunit of LT (9). The ST gene used for those studies was constructed synthetically with appropriate restriction sites to permit in-frame, downstream insertion of the oligonucleotide. Maximum expression of ST antigenicity was obtained when a seven-amino-acid, proline-containing

* Corresponding author.

linker was included between the LT-B and ST moieties. The LT-B:linker:ST gene fusion coded for production of a fusion polypeptide with antigenic determinants of both LT and ST but without toxicity. Animals immunized parenterally with either crude or purified preparations containing the hybrid molecule produced antibodies that were able to recognize native ST in vitro and neutralize the biological activity of ST in the suckling mouse assay. Here we report the development of neutralizing mucosal antibodies against ST by oral immunization with a second LT-B:linker:ST fusion peptide in which an eight-amino-acid, proline-containing linker was included between the LT-B and ST moieties. This fusion peptide was delivered to the mucosal immune system by an attenuated bacterial carrier.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM83(pJC217) *ara* Δ*lac-pro strA thi* 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 (11, 13). The vehicle used for oral delivery of the fusion peptide was *Salmonella dublin* SL1438, a nonreverting, aromatic-dependent, histidine-requiring mutant (41) (kindly provided by B. A. D. Stocker, Stanford University School of Medicine). We have used this and other equivalently attenuated *Salmonella* spp. extensively as carriers of foreign antigens in orally administered vaccines (3, 6, 10, 11, 13, 40). *S. dublin*(pBL1) is a derivative of SL1438 containing a plasmid that codes for production of an LT-B:linker:ST fusion peptide (this study).

Isolation of plasmid DNA and restriction endonuclease digestion. Plasmid DNA was isolated by standard techniques (1). Restriction enzymes, ligase, and reagents were obtained from GIBCO-Bethesda Research Laboratories and used according to the manufacturer's directions.

DNA synthesis and DNA sequencing. Oligonucleotides were synthesized on a Biosearch Cyclone DNA Synthesizer by the phosphoramidite method. DNA sequence determinations were made by the dideoxy-chain-terminating method (39).

Electrophoresis. Agarose gel electrophoresis was performed on 0.9% slab gels in 0.04 M Tris–0.2 M sodium acetate–0.002 M EDTA (pH 7.8). Phage λ DNA fragments generated by *Hin*dIII digestion and phage φX174 replicative-form DNA fragments generated by *Hae*III digestion were used as molecular weight standards.

Transformation. Transformation was carried out by electroporation, using a Bethesda Research Laboratories Cell-Porator and Voltage Booster as prescribed by the manufacturer.

ELISA. Reagents for the enzyme-linked immunosorbent assay (ELISA) were obtained from Sigma Chemical Co. Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS-Tween). For determination of LT-B or ST antigen in the fusion peptides, microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III) per well in carbonate-bicarbonate coating buffer (pH 9.6) and then incubated with samples serially diluted in PBS-Tween. Determinations were made on cell lysates from overnight cultures grown in ML medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) and lysed with lysozyme-EDTA and by freeze-thaw. Precoating the plates with mixed gangliosides permits the LT-B:linker:ST fusion peptide to bind to the microtiter plates via the LT-B moiety for which G_{M1} is the natural receptor. This procedure eliminates background and increases the sensitivity of the assay. For determination of LT-B antigen, reactions were further developed with affinity-purified monospecific goat hyperimmune antiserum to LT-B together with rabbit anti-goat immunoglobulin G (IgG) conjugated to alkaline phosphatase. For determination of ST antigen in the fusion peptide, reactions were further developed with hyperimmune anti-ST antiserum raised in rabbits in conjunction with goat anti-rabbit IgG conjugated to alkaline phosphatase. Goat anti-LT-B was produced in the investigators' laboratory, as was the hyperimmune anti-ST antiserum.

For determination of murine antibodies directed against LT-B resulting from the immunizations, microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III) per well and then with 1 μg of purified LT-B per well (11). For determination of antibodies directed against ST, microtiter plates were precoated with 1 μg of purified ST (a gift from Donald C. Robertson, University of Kansas, Lawrence) per well. Serum and mucosal secretions were obtained as described previously (13). Briefly, the small intestine from the duodenum to the ileal-cecal junction was excised and homogenized in a solution containing 50 mM EDTA and 0.1 mg of soybean trypsin inhibitor per ml. Samples were homogenized with a Tekmar Tissuemizer, clarified by centrifugation, lyophilized, resuspended in 2 ml of TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3, 0.2 M NaCl, pH 7.5) (12), dialyzed against TEAN buffer, and stored at −20°C until assayed. Serum was collected by cardiac puncture, prepared by centrifugation through serum separators (Becton Dickinson), and stored at −20°C until assayed.

Samples from immunized mice or control samples were serially diluted in PBS-Tween and added to the microtiter wells. The presence of serum anti-LT-B or anti-ST IgG was determined by using rabbit antiserum against mouse IgG conjugated to alkaline phosphatase. Mucosal anti-LT-B or anti-ST IgA was assayed with goat antiserum against mouse IgA (alpha chain specific) and then with rabbit antiserum against goat IgG conjugated to alkaline phosphatase. The assays for ST were developed with hyperimmune rabbit anti-ST as a control and then by substituting serum or mucosal samples from immunized mice and then the appropriate anti-mouse specific conjugate. Values for IgG and IgA were determined from a standard curve with purified mouse myeloma proteins [MOPC 315, γA(IgAλ2); MOPC 21, γG1].

Animals. Female BALB/c mice 6 to 10 weeks of age were purchased from Charles River. Mice received water and food ad lib and did not receive treatment with antibiotics.

Immunization. For primary immunization, groups of mice were immunized orally with two doses containing 10^{10} CFU each of the *S. dublin* derivative or control strains on days 0 and 4. Inocula for immunizations were prepared from log-phase cultures grown at 37°C on a gyratory shaker in Trypticase soy broth (BBL Microbiology Systems) containing 100 μg of ampicillin and 1 μg of 2,3-dihydroxybenzoic acid (Sigma) per ml. Cells were washed and resuspended in sterile normal saline to a final volume containing 2×10^{10} CFU/ml and administered intragastrically in 0.5-ml doses with a feeding tube. This regimen was repeated for each animal at 21 and 25 days after primary immunization. Animals were sacrificed 2 weeks after the final boost. The time points for the boosters and animal sacrifices were chosen because extensive studies in this and other laboratories have indicated that with this regimen of immunization initial levels of both serum and mucosal antibody peak between 3 and 4 weeks after primary immunization and approximately 2

weeks following the booster immunization (6, 8, 10, 11, 13, 16, 17, 24, 32).

Bioassays. The suckling mouse assay for ST was performed as described by Giannella (21). Newborn CD-1 suckling mice (1 to 3 days old) were separated from their mothers immediately before use and randomly divided into groups. To develop a standard curve, each mouse was inoculated intragastrically with 0.1 ml of sterile normal saline containing amounts of purified ST ranging from 1 to 400 ng. There were three animals for each group. At 3 h after inoculation, the mice were killed, the abdomen was opened, and the entire intestine was removed with forceps. The intestine from each mouse was weighed, and the ratio of the gut weight to remaining carcass weight was calculated. The mean gut weight/carcass weight ratio was then determined for each group. In our study, gut weight/carcass weight ratios of ≥ 0.09 are considered positive.

Toxin neutralization in the suckling mouse assay was determined as follows. A selected dose of toxin (100 ng) in sterile normal saline was mixed with an equal volume of sera or mucosal secretions pooled from groups of five to seven immunized or control mice. The final dilution of the sample was 1/10. Following a preincubation of 1 h at room temperature, samples were administered to groups of suckling mice as described above. The minimum amount of ST necessary to produce a positive response in the suckling mouse assay is 1 ng.

Guidelines used for recombinant DNA experiments. The experiments reported here were performed under conditions specified in the *Guidelines for Recombinant DNA Technology* published by the National Institutes of Health, Bethesda, Md.

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 the LT-B:linker:ST gene fusion. The plasmid encoding LT-B was isolated from *E. coli* JM83(pJC217) (11) and modified to permit the downstream, in-frame insertion of DNA sequences for the expression of fusion peptides at the carboxy terminus of LT-B as described previously (9). A polylinker containing restriction sites for the enzymes *Nco*I, *Stu*I, and *Eco*RV and termination codons in all three reading frames was added. Additional restriction sites downstream from the original polylinker are present in the modified LT-B plasmid, including a unique *Hin*dIII site. Plasmid pJC700 is a pUC18 derivative containing a synthetic structural gene for ST cloned across the *Eco*RI and *Sal*I sites of pUC18 and was used as the source of the ST gene (9). A synthetic linker containing 24 nucleotides with *Nco*I- and *Eco*RI-compatible ends was constructed with nucleotides coding for two proline residues to ensure maximum expression of ST antigenicity. We have shown previously that, in the absence of such a linker, no ST antigen is detected in these LT-B:linker:ST fusions (9).

The modified LT-B plasmid was digested with *Nco*I and *Hin*dIII to permit a forced cloning insertion of the ST gene and synthetic linker. Next, plasmid pJC700 was digested with *Eco*RI and *Hin*dIII and the ST gene was purified by electroelution. This ST gene fragment, together with the *Nco*I-*Eco*RI compatible linker, was inserted across the *Nco*I-*Hin*dIII region of the modified LT-B plasmid (Fig. 1). This plasmid construct was designated pBL1, and the sequence was confirmed by dideoxy sequencing. The complete nucleotide sequence and inferred amino acid sequence of the fusion peptide with the eight-amino-acid linker are shown in Fig. 2. This construct differs from our previous LT-B:linker:ST construct in that the current linker contains three additional nucleotides (chloramphenicol acetyltransferase= histidine) at the beginning of the linker. This change was made to facilitate ongoing studies in our laboratory investigating potential means of increasing the antigenicity and immunogenicity of the ST moiety. *E. coli* JM83 was transformed with plasmid pBL1, and transformants were shown by ELISA to synthesize LT-B- and ST-related antigen (Fig. 3).

Immunogenicity of the LT-B/ST fusion peptide. We have shown previously that mice immunized parenterally with an LT-B:linker:ST fusion peptide developed serum antibodies capable of recognizing native ST and neutralizing the biological activity of ST in the suckling mouse assay. However, to be an effective immunogen, the fusion peptide would need to be administered in a manner capable of inducing protective antibodies on relevant mucosal surfaces. In order to accomplish this, an attenuated *aro* mutant of *S. dublin* was transformed with plasmid pBL1. Transformants were screened for expression of LT-B- and ST-related antigen by ELISA. One LT-B–ST (LT-B/ST) antigen-positive transformant, designated *S. dublin*(pBL1), was selected and used to immunize groups of BALB/c mice orally.

Mice were immunized orally with this strain or with a control strain expressing just LT-B from the same plasmid. Sera and mucosal secretions were obtained and analyzed for the presence of serum IgG and mucosal IgA that were able to recognize LT-B and ST by ELISA and, more importantly, to neutralize native ST in the suckling mouse assay. Sera and mucosal secretions from animals immunized with *S. dublin*(pBL1) expressing the LT-B:linker:ST fusion exhibited detectable ELISA reactivity against LT-B but not native ST. The serum IgG anti-LT-B response in this group of animals was 150 ± 56 μg/ml, and the mucosal IgA anti-LT-B response was 513 ± 119 ng/ml. There was no ELISA-detectable serum or mucosal anti-ST in these animals. However, even in the absence of detectable ELISA reactivity against ST, both sera and mucosal secretions from these animals were able to neutralize the biological activity of native ST in the suckling mouse assay (Fig. 4). The biological activity of ST was partially neutralized by sera and completely neutralized by mucosal secretions from animals immunized orally with *S. dublin*(pBL1). ST was not neutralized by control sera or mucosal secretions or samples from normal unimmunized mice. These findings are important because they demonstrate the development of mucosal protection against ST by oral immunization with a genetic fusion delivered by a bacterial vector. They also demonstrate that this system can be used to elicit a protective immune response against antigens that are not normally immunogenic but which can be made immunogenic when genetically fused to an appropriate carrier.

DISCUSSION

E. coli ST is an important virulence determinant in enterotoxin-mediated diseases caused by *E. coli*. However, the small size and poor immunogenicity of the molecule have hampered any serious study to determine whether immunization against ST can significantly influence the occurrence of diarrheal disease due to organisms that produce this toxin. There have been a number of efforts directed at developing

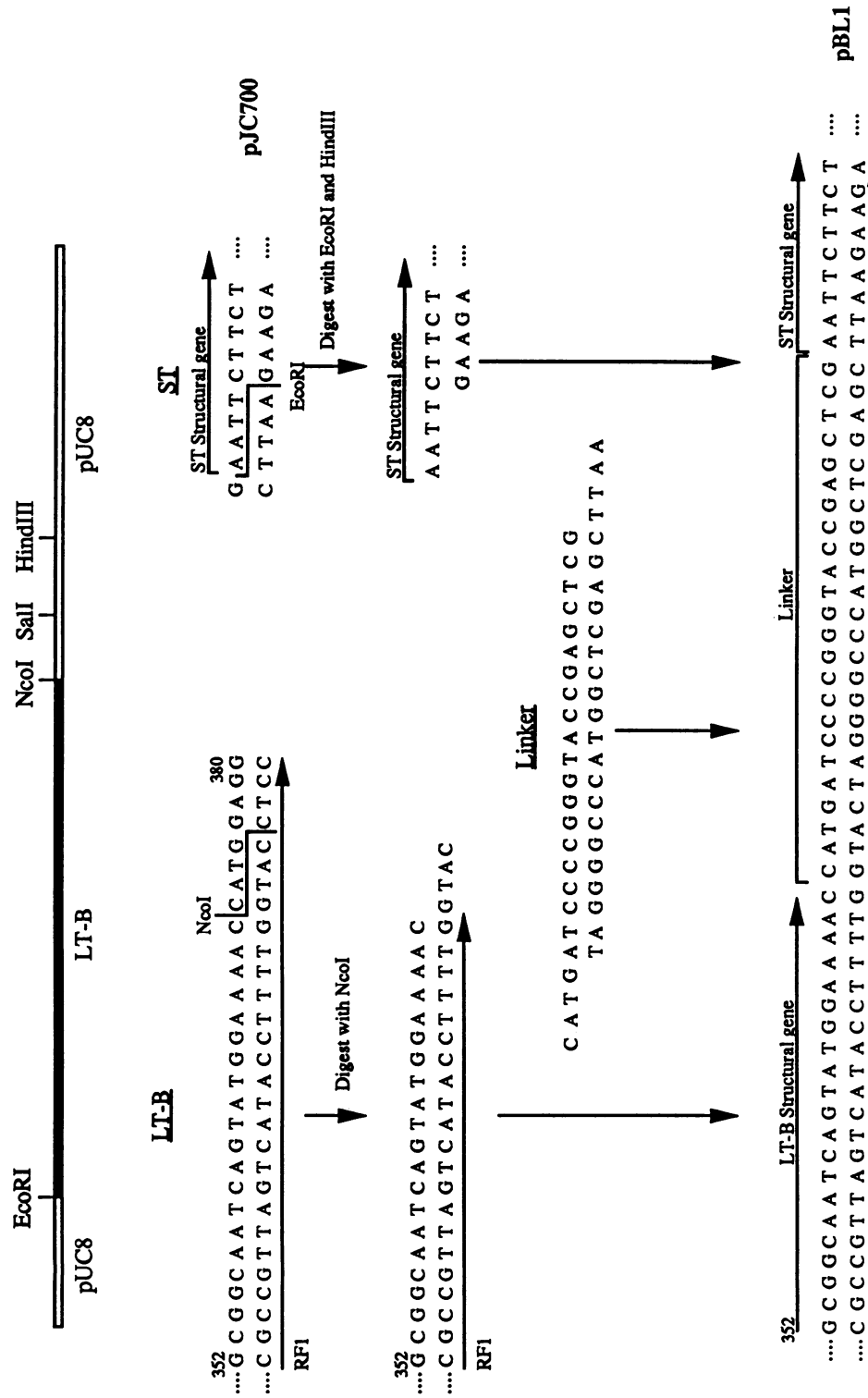

FIG. 1. Construction of the LT-B:linker:ST gene fusion. A synthetic oligonucleotide constituting the structural gene for ST was cloned into plasmid pUC18 across the *Eco*RI and *Sal*I sites. This plasmid was designated pJC700 and was used as the source of the ST gene. A synthetic linker containing 24 nucleotides with *Nco*I- and *Eco*RI-compatible ends was constructed with nucleotides coding for two proline residues to ensure maximum expression of ST antigenicity. For the initial insertion, the modified LT-B plasmid was digested with *Nco*I and *Hin*dIII to permit a forced cloning insertion of the ST gene and synthetic linker. Next, plasmid pJC700 was digested with *Eco*RI and *Hin*dIII, and the ST gene was purified by electroelution. This ST gene fragment, together with the *Nco*I-*Eco*RI-compatible linker, was inserted across the *Nco*I-*Hin*dIII region of the modified LT-B plasmid. This construct was designated pBL1.

ATG	AAT	AAA	GTA	AAA	TGT	TAT	GTT	TTA	TTT	ACG	GCG
met	asn	lys	val	lys	cys	tyr	val	leu	phe	thr	ala
TTA	CTA	TCC	TCT	CTA	TGT	GCA	TAC	GGA	GCT	CCC	CAG
leu	leu	ser	ser	leu	cys	ala	tyr	gly	ala	pro	gln
TCT	ATT	ACA	GAA	CTA	TGT	TCG	GAA	TAT	CGC	AAC	ACA
ser	ile	thr	glu	leu	cys	ser	glu	tyr	arg	asn	thr
CAA	ATA	TAT	ACG	ATA	AAT	GAC	AAG	ATA	CTA	TCA	TAT
gln	ile	tyr	thr	ile	asn	asp	lys	ile	leu	ser	tyr
ACG	GAA	TCG	ATG	GCA	GGC	AAA	AGA	GAA	ATG	GTT	ATC
thr	glu	ser	met	ala	gly	lys	arg	glu	met	val	ile
ATT	ACA	TTT	AAG	AGC	GGC	GCA	ACA	TTT	CAG	GTC	GAA
ile	thr	phe	lys	ser	gly	ala	thr	phe	gln	val	glu
GTC	CCG	GGC	AGT	CAA	CAT	ATA	GAC	TCC	CAA	AAA	AAA
val	pro	gly	ser	gln	his	ile	asp	ser	gln	lys	lys
GCC	ATT	GAA	AGG	ATG	AAG	GAC	ACA	TTA	AGA	ATC	ACA
ala	ile	glu	arg	met	lys	asp	thr	leu	arg	ile	thr
TAT	CTG	ACC	GAG	ACC	AAA	ATT	GAT	AAA	TTA	TGT	GTA
tyr	leu	thr	glu	thr	lys	ile	asp	lys	leu	cys	val
TGG	AAT	AAT	AAA	ACC	CCC	AAT	TCA	ATT	GCG	GCA	ATC
trp	asn	asn	lys	thr	pro	asn	ser	ile	ala	ala	ile
AGT	ATG	GAA	AAC	CAT	GAT	CCC	CGG	GTA	CCG	AGC	TCG
ser	met	glu	asn	his	asp	pro	arg	val	pro	ser	ser
AAT	TCT	TCT	AAC	TAC	TGC	TGT	GAA	CTT	TGT	TGT	AAT
asn	ser	ser	asn	tyr	cys	cys	glu	leu	cys	cys	asn
CCT	GCC	TGT	ACA	GGA	TGT	TAC	GTA	TAG			
pro	ala	cys	thr	gly	cys	tyr	val	STOP			

FIG. 2. Nucleotide sequence of the LT-B:linker:ST fusion gene with inferred amino acid sequence. The sequence shown is for the construction with an eight-amino-acid (His-Asp-Pro-Arg-Val-Pro-Ser-Ser) linker inserted between the LT-B gene and the synthetic ST gene. DNA sequence determinations were made by the dideoxy-chain-terminating method described by Sanger et al. (39).

a toxoid suitable for immunization against ST, including chemical coupling to appropriate carrier proteins (27) and the construction of genetic fusions between ST and one or more subunits of cholera enterotoxin and LT (9, 23, 36–38). In general, these genetic fusions have either retained some degree of ST-associated toxicity or been incapable of eliciting neutralizing antibodies. Moreover, none of these fusion polypeptides has been shown to be effective at eliciting mucosal antibodies that could either recognize ST antigenically or neutralize the biological activity of ST in a relevant animal model.

We have reported previously the construction of a fusion peptide in which the 5′ terminus of the gene coding for ST was genetically fused to the 3′ terminus of the gene coding for LT-B (9). The LT-B and ST moieties were joined with a seven-amino-acid, proline-containing linker. This LT-B:linker:ST gene fusion coded for production of a fusion polypeptide with antigenic determinants of both LT and ST but without toxicity. Immunogenicity of the fusion peptide was demonstrated in animals immunized parenterally with either crude or purified preparations containing the hybrid molecule. Sera from these animals contained antibodies that were able to recognize native ST in vitro and neutralize the biological activity of ST in the suckling mouse assay.

An important aspect of these earlier genetic fusion studies was the finding that maximum antigenicity of the fused peptide was obtained when an appropriate linker was included between the LT-B and ST moieties. Presumably, the included proline residues interrupt the α-helix and create a rigid kink or bend which affects the flexibility and antigenicity of the fused antigen. In the present study, we developed a second LT-B:linker:ST construct in which an eight-amino-acid, proline-containing linker was included between LT-B and ST. The primary aim of the study reported here was to

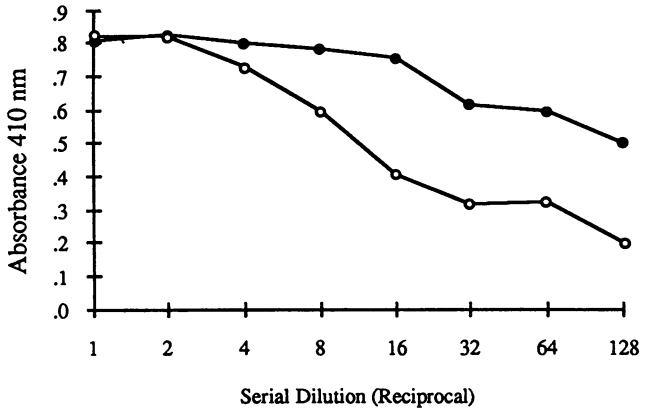

FIG. 3. ELISA determination of LT-B- and ST-related antigen. *E. coli* JM83 was transformed with plasmid pBL1, and transformants were shown to synthesize LT-B (●)- and ST (○)-related antigen by ELISA. Determinations were made on cell lysates from overnight cultures grown in ML medium and lysed with lysozyme-EDTA and by freeze-thaw. For additional details, see Materials and Methods.

examine delivery of this fusion peptide to the gut-associated lymphoid tissues in such a manner as to develop significant mucosal protection against ST.

Parenterally administered vaccines are not effective for eliciting mucosal secretory IgA responses and are generally ineffective against organisms that colonize mucosal surfaces and do not invade. Orally administered vaccines, especially live attenuated vaccines, have been shown to be effective in inducing specific secretory IgA responses (7, 14, 15). One application of this approach has been to employ avirulent derivatives of *Salmonella* spp. as carriers for plasmids that code for antigenic determinants of heterologous mucosal pathogens. This technique has been examined with a number of antigens and has been shown to be an effective means of stimulating significant levels of specific mucosal secretory IgA directed against the carrier strain and the foreign antigen. In the study reported here, an *aroA* mutant of *S. dublin* was transformed with the LT-B:linker:ST fusion plasmid and used to immunize mice orally. Sera and mucosal secretions from animals immunized with this strain exhibited detectable ELISA reactivity against LT-B but not native ST. However, even in the absence of detectable ELISA reactivity, both sera and mucosal secretions from these animals were able to neutralize the biological activity of native ST in the suckling mouse assay. These findings constitute the first demonstration of the development of mucosal antibody capable of neutralizing ST by oral immunization with a genetic fusion delivered by a bacterial vector.

There are a number of possible explanations for the observed neutralization in the absence of a detectable ELISA response. In the first place, the ST moiety in the fusion peptide will undoubtedly assume a conformation different from that of native ST, so antibodies developed against the fusion peptide may not recognize native ST in a strictly antigen-driven assay such as the ELISA. It should be noted that antibodies to native ST do recognize ST antigen in the fusion peptide, implying some level of cross-reactivity. In addition, the critical factor for neutralization is development of antibodies directed against epitopes associated with toxicity (i.e., neutralizing antibodies). In these genetic fusions, ST is detoxified as a consequence of the covalent linkage of the amino terminus of ST to the carboxy terminus of LT-B. If ST is covalently linked to the amino terminus of LT-B, the toxicity of ST is not abated (23). It is also not clear how these antigens are processed by the antigen-presenting cells of the gut-associated lymphoid tissues following delivery to the Peyer's patches by attenuated *Salmonella* spp. or whether the ganglioside-binding ability of native LT-B plays a role in the subsequent immune response. Furthermore, the demonstration of protection against a bacterial pathogen in the absence of a detectable antibody response against a cloned antigen delivered by attenuated *Salmonella* spp. is not unique to this study. In a study reported by Strugnell et al. (42), the P.69 protective antigen of *Bordetella pertussis* was expressed in an *aroA aroD S. typhimurium* vaccine strain and used to immunize BALB/c mice orally and intravenously. These animals were protected against aerosol challenge with virulent *B. pertussis* even in the absence of demonstrable P.69 antibodies. There is also the possibility that the ELISA was unable to detect the presence of mucosal anti-ST for reasons unrelated to the biology of the immune response. These assays were developed by using hyperimmune rabbit anti-ST as a control and then substituting sera or mucosal samples from immunized mice and then the appropriate anti-mouse specific conjugate. Since there has been no other report of mucosal anti-ST, it is impossible

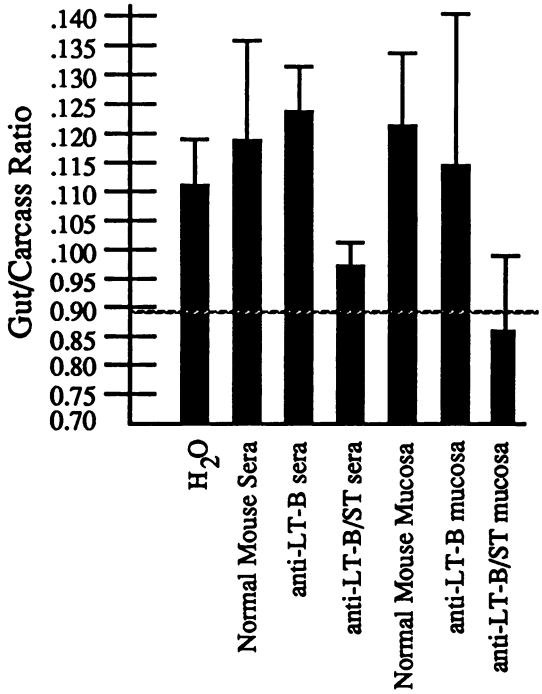

FIG. 4. Neutralization of ST by sera and mucosal secretions from animals immunized orally with *aroA S. dublin* transformed with plasmid pBL1. The biological activity of ST was partially neutralized by sera and completely neutralized by mucosal secretions from animals immunized orally with *aroA S. dublin* transformed with plasmid pBL1. ST was not neutralized by control sera or mucosal secretions or samples from normal unimmunized mice. A selected dose of toxin (100 ng) was mixed with an equal volume of sera or mucosal secretions pooled from groups of five to seven immunized or control mice. The final dilution of the sample was 1/10. The suckling mouse assay for ST was performed as described by Giannella (21). Gut/carcass weight ratios of ≥0.09 are considered positive.

to control that aspect of the reaction beyond the fact that samples were positive for anti-LT-B when appropriate.

The purpose of the study reported here was to test the ability of an LT-B:linker:ST fusion peptide to elicit mucosal protection against ST. Such an immunogen may be useful as one component of a live, oral, multivalent vaccine for typhoid fever, cholera, and the cholera-related enteropathies, such as enterotoxigenic *E. coli*.

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