Epitope Maps of the *Escherichia coli* Heat-Labile Toxin B Subunit for Development of a Synthetic Oral Vaccine

ICHIRO TAKAHASHI,^{1,2*} HIROSHI KIYONO,¹ RAYMOND J. JACKSON,¹ KOHTARO FUJIHASHI,¹
HERMAN F. STAATS,³ SHIGEYUKI HAMADA,² JOHN D. CLEMENTS,⁴
KENNETH L. BOST,⁴ AND JERRY R. MCGHEE¹

Mucosal Immunization Research Group, Immunobiology Vaccine Center, Departments of Microbiology and Oral Biology, University of Alabama at Birmingham Medical Center, Birmingham, Alabama 35294¹; Department of Oral Microbiology, Osaka University, Faculty of Dentistry, Osaka 565, Japan²; Duke University Medical Center, Durham, North Carolina 27710³; and Department of Microbiology and Immunology, Tulane University, School of Medicine, New Orleans, Louisiana 70112⁴

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Linear B- and T-cell epitopes spanning all 103 amino acids of the *Escherichia coli* heat-labile toxin B subunit (LT-B) were assessed in mice orally immunized with native LT or with recombinant *Salmonella enteritidis* expressing LT-B. Oral administration of native LT induced mucosal immunoglobulin A (IgA) antibodies reactive with an epitope at residues 85 to 91, while IgA induced by recombinant *Salmonella* LT-B reacted with an epitope at residues 36 to 44. Serum IgG anti-LT-B antibodies from mice orally immunized with either LT or with recombinant *Salmonella* LT-B were directed to both epitopes. A single T-cell epitope spanning residues 34 to 42 was identified by T-cell proliferative and cytokine responses. When a 20-mer peptide (residues 26 to 45) with B- and T-cell epitopes was given orally to BALB/c (H-2^d) and B10 congenic (I-A^d, I-A^b, and I-A^k) mice, significant fecal IgA and serum IgG anti-LT-B antibodies were induced. The peptide also induced LT-B-specific T-cell proliferative responses in these mice. Orally administered LT-B peptide (residues 26 to 45) induced a cytokine profile indicative of both T helper 1- and 2-type cells. The remarkable immunogenicity of this 20-mer peptide makes it a candidate for a vaccine to protect against enterotoxigenic *E. coli*.

The majority of pathogenic viruses and bacteria initiate disease at mucosal surfaces. This finding has led to a greater awareness of the importance of mucosal immunity, especially in vaccine development. In this regard, induction of antigen-specific T- and B-cell responses in gut-associated lymphoreticular tissues may provide immunity to microorganisms which invade through these tissues (18, 19). Enterotoxigenic *Escherichia coli* (ETEC) is a major causative agent of diarrhea among children in developing countries as well as travellers to these areas (3). ETEC produces a heat-labile enterotoxin termed heat-labile toxin (LT) which consists of an A subunit (LT-A) and a pentameric B subunit (LT-B) (28). The A and B subunits have different molecular and biological characteristics which are important in host pathogenesis and immunity. For example, the B subunit binds to gangliosides which are localized in the membrane of eukaryotic cells (28). The A1 polypeptide portion of the A subunit catalyzes the ADP ribosylation of G proteins and leads to activation of adenylate cyclase and increased cyclic AMP in epithelial cells with the subsequent secretion of water and chloride, which is clinically manifested as a watery diarrhea (28).

The high-affinity binding of LT-B to gangliosides on intestinal epithelial cells is thought to be important in immunogenicity (26, 28). Epitope-mapping studies with monoclonal anti-LT antibodies have revealed that pentameric LT-B contains most of the immunodominant epitopes which result in LT-specific antibody responses (2). In addition, patients suffering from infections caused by ETEC develop brisk intestinal secretory immunoglobulin A (S-IgA) antibody responses to LT-B (30).

The induction of LT- and LT-B-specific antibody responses provides partial protection against ETEC infection in humans (33), and the disease itself results in significant mucosal S-IgA and serum IgG anti-LT antibody responses (28). Furthermore, oral immunization of mice with only microgram quantities of LT induces intestinal IgA and serum IgG anti-LT-B antibody responses in mice (8, 24, 36). Another unique feature of LT is the ability to serve as an oral adjuvant for the enhancement of mucosal S-IgA and serum IgG responses to coadministered proteins which normally provide low levels of immunogenicity when given alone (8, 18, 19). However, little information concerning the molecular basis for the adjuvant effect of LT is currently available.

It has been shown that oral immunization is an effective way to stimulate CD4⁺ T helper (Th) and B cells for IgA responses in mucosal inductive sites such as the Peyer's patches (PP). This initial response is followed by migration of B and T cells to mucosal effector sites for antigen-specific S-IgA responses (i.e., lamina propria regions of the intestine, upper respiratory, and genitourinary tracts as well as exocrine glands [18, 19]). Recent studies have provided evidence that induction of CD4⁺ Th2 cells and derived cytokines is an essential component for the generation of antigen-specific IgA responses in orally immunized mice (37, 38). In this regard, Th cells can be functionally divided into at least two subsets according to the profile of cytokines produced (21, 31). The Th1-type cells selectively produce interleukin-2 (IL-2), gamma interferon (IFN- γ), and tumor necrosis factor beta, while Th2-type cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (21, 31). The Th1 subset promotes cell-mediated immunity, while Th2 cells are important for the regulation of IgG1, IgE, and IgA B-cell responses.

Clones of Th1- and Th2-type have also been obtained from humans suffering from infectious diseases, immunodeficient

* Corresponding author. Mailing address: Department of Oral Microbiology, Osaka University Faculty of Dentistry, 1-8 Yamadaoka, Suita-Osaka, Japan 565. Phone +81-6-878-4755. Fax: +81-6-878-4755. Electronic mail address: snatum@dent.osaka-u.ac.jp.

cies, or allergic disorders (25). Our studies of mice have shown that oral immunization with the protein tetanus toxoid and the mucosal adjuvant cholera toxin (CT) resulted in the preferential induction of IL-4 and IL-5 secreting CD4⁺ Th2-type T cells in PP and elevated numbers of antigen-specific IgA antibody-producing cells in the intestinal lamina propria (14, 38). These results have shown that appropriate induction of antigen-specific Th2-type T cells regulates the resultant antigen-specific IgA B-cell responses. However, the mechanisms whereby orally administered adjuvants such as CT or LT induce antigen-specific Th2-type cells for support of IgA B-cell responses remain to be elucidated.

In the present study, we have used native LT from *E. coli* and recombinant LT-B expressed in attenuated *Salmonella enteritidis* to orally immunize mice in order to elucidate the nature of the antibody responses to LT-B which occurs in external secretions or in serum. The resultant T-cell-dependent mucosal IgA and serum IgG anti-LT-B antibody responses were used for mapping of both T- and B-cell epitopes of LT-B and for construction of a synthetic peptide which elicited both mucosal and serum anti-LT-B antibody responses.

MATERIALS AND METHODS

Mice. BALB/c (H-2^d) and B10 congenic (including B10.D2 [I-A^d], B10 [I-A^b], and B10.BR [I-A^k]) mice which had been barrier maintained and were antibody negative for a panel of mouse viral and bacterial pathogens were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, Md.), maintained in horizontal laminar flow cabinets, and provided sterile food and water ad libitum. All mice used in this study were between 8 and 12 weeks of age.

LT, LT-B, and synthetic peptides. LT of ETEC was purified from the ETEC H10407 serotype O78:H11, a human isolate, by Sepharose 4B chromatography (Sigma Chemical Co., St. Louis, Mo.) as described previously (6). LT-B from ETEC was isolated from an *E. coli* K-12 strain JM83 transformant containing the plasmid (pJC217) for the LT-B gene (7) and purified by Sepharose 4B chromatography. Purified LT-B was found to be structurally identical to the native B subunit since sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of both preparations revealed an 11.8-kDa protein band. No demonstrable A subunit was seen in the purified recombinant LT-B preparation as determined by silver staining following SDS-PAGE.

We also used an avirulent *S. enteritidis* serotype Dublin strain EL23 as a vehicle for the delivery of LT-B to mucosa-associated tissues (9). *Salmonella dublin* EL23 is a nonreverting aromatic-dependent and histidine-requiring (*aro his*) mutant which harbors the plasmid (pJC 217) incorporating the LT-B gene. Recombinant LT-B expressed in *S. dublin* EL23 was found to be principally cell associated (94%) (9). On the basis of the predictive amino acid sequence of LT-B derived from ETEC H10407 (39), a series of overlapping decapeptides (94 species) covering the LT-B molecule (103 amino acids in length for the mature form) were synthesized by a solid-phase procedure (20, 35) in order to elucidate T-cell- and linear B-cell-specific epitopes in the LT-B molecule. In this study, the distance in the number of residues between amino-terminal ends of two overlapping peptides was 1. Purity was assessed by high-performance liquid chromatography (HPLC) and amino acid analysis. We also synthesized a 20-mer peptide which included residues 26 to 45 of the LT-B molecule (referred to as peptide 26-45) by the solid-phase procedure. A cysteine was placed at the carboxy terminus of the synthetic peptide. Purity was also assessed by HPLC and amino acid analysis.

Coupling of synthetic peptide to OVA. A selected synthetic peptide (i.e., peptide 26-45) was coupled to ovalbumin (OVA; Sigma) by a previously described method (22). Briefly, 5.0 mg of OVA in 50 mM phosphate buffer (pH 8.0) was reacted with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce Chemical Co., Rockford, Ill.) dissolved in dimethyl sulfoxide at an OVA-MBS molar ratio of 10:40, and the solution was stirred for 30 min at room temperature. Unreacted MBS was removed by passage through a Sephadex G50 column. Five mg of peptide 26-45 was added to MBS-activated OVA in 50 mM phosphate buffer (pH 7.2). The solution was stirred at room temperature for 3 h. The final product (peptide 26-45 coupled to OVA) was isolated by Sephadex G50 column chromatography. Conjugation ratios were determined by Ellman's method (10). The number of moles of peptide per mole of OVA was 7 to 11.

Oral immunization of mice. Groups of five mice of either sex were immunized orally by a standard protocol (14). Briefly, mice were gavaged with 0.5 ml containing 8 parts isotonic Hanks balanced salt solution and 2 parts 7.5% sodium bicarbonate 2 h before immunization. For oral immunization, 25 µg of LT was administered in 0.25 ml of phosphate-buffered saline (PBS) on day 0. For oral

immunization with *S. dublin* EL23, 10¹⁰ CFU of strain EL23 was administered in 0.25 ml of saline on days 0, 1, and 2. Inocula for the oral immunization were prepared from log-phase cultures of strain EL23 grown at 37°C on a rotary shaker in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing ampicillin (100 µg/ml) and 2,3-dihydroxybenzoic acid (Sigma) (1 µg/ml) (9). Bacteria were washed and suspended in sterile saline to a final volume of 4 × 10¹⁰ CFU/ml. Oral immunization with the synthetic peptide coupled to OVA was performed with 50 µg of the peptide conjugate administered in 0.25 ml of PBS at weekly intervals (days 0, 7, and 14). Fecal extracts were prepared as described previously (14), and serum samples were obtained from individual mice, monitored for anti-LT-B antibodies, and subsequently used for epitope mapping.

ELISA. Antibody titers in serum and fecal extracts were determined by enzyme-linked immunosorbent assay (ELISA) (14). Briefly, ELISA plates (Becton Dickinson, Oxnard, Calif.) were coated with an optimal concentration of LT-B (100 µl of 5 µg/ml) or synthetic peptides (100 µl of 100 µg/ml) in PBS. Blocking was accomplished with 1% bovine serum albumin (BSA; Sigma) in PBS. Serial twofold dilutions of sera or fecal extracts (100 µl per well) were added. After overnight incubation at 4°C, unbound antibodies were removed with PBS containing 0.05% Tween 20 (PBS-T), and a 1:1,000 dilution of biotinylated goat anti-mouse IgM, IgG, or IgA heavy-chain-specific antibodies (Southern Biotechnology Associates [SBA], Birmingham, Ala.) in a 1% BSA solution in PBS-T was added. The plates were incubated and washed with PBS-T, and the detection enzyme horseradish peroxidase-conjugated streptavidin (Zymed Labs, South San Francisco, Calif.) was added in 1% BSA-PBS-T. The plates were incubated, washed with PBS-T, and developed with 1.1 mM ABTS [(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid))] in citrate-phosphate buffer (pH 4.2) containing 0.01% H₂O₂. Endpoint titers were expressed as the reciprocal log₂ of the last dilution which gave an optical density at 414 nm (OD₄₁₄) of ≥0.1. For epitope scanning, a 1:10 dilution of fecal extract or a 1:1,000 dilution of serum in 1% BSA-PBS was prepared, 100 µl was added to the wells in duplicate, and the plates were incubated for 2 h at 37°C. The procedure to detect antibody epitopes was described previously (12, 35). Data were expressed as A₄₁₄ after 15 min of incubation with the substrate.

In vitro stimulation of LT-B-specific T cells. In order to assess proliferation and cytokine profiles of LT-B-specific T cells, groups of 7 to 10 BALB/c mice were orally immunized with 25 µg of LT. One week later, the PP were carefully removed from the small intestines of the mice and dissociated into single-cell suspensions by use of the neutral protease enzyme Dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in Joklik's modified medium (Gibco, Grand Island, N.Y.) as described previously (11). Single-cell suspensions were washed extensively to remove the enzyme and resuspended in RPMI 1640 (Whittaker Bioproducts Inc., Walkersville, Md.) supplemented with HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 15 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (40 µg/ml), and 10% fetal calf serum (complete medium). The cells were incubated for 6 h at 37°C in 5% CO₂ in air to allow full reexpression of surface molecules and to remove adherent cells prior to further T-cell purification. The CD3⁺ T cells were enriched by negative panning to avoid T-cell stimulation by use of goat anti-mouse IgG F(ab')₂ (20 µg/ml) as previously described (1). Cell suspensions were subjected to three consecutive rounds of panning for complete removal of macrophages and B cells. The PP CD4⁺ T cells were further purified by positive panning on petri plates coated with monoclonal anti-L3T4 (GK1.5) antibody (20 µg/ml). This procedure resulted in a cell fraction containing >99% CD4⁺ CD8⁻ T cells.

The CD4⁺ T cells (10⁵ per well) were cultured in flat-bottom 96-well micro-culture plates (Corning Glass Works, Corning, N.Y.) in the presence of T-cell-depleted, irradiated (3,000 rads) splenic feeder cells (10⁴ cells per well) and 20 µg of synthetic peptides per well for 4 days. Peptide-specific CD4⁺ T-cell proliferative responses were analyzed after the addition of 0.5 µCi of [³H]thymidine (Amersham Corp., Arlington Heights, Ill.) to all wells 6 h prior to harvest. The uptake of [³H]thymidine was determined by standard liquid scintillation counting. Controls consisted of PP CD4⁺ T cells and feeder cells without added peptides. The results are expressed as the stimulation index (ratio of the experimental counts per minute to control counts per minute). For analysis of antigen-specific Th1- and Th2-cell responses, culture supernatants were harvested after 4 to 6 days and stored at -20°C until use in cytokine-specific ELISAs.

Cytokine-specific ELISA. For the determination of IFN-γ and IL-5 produced by LT-specific CD4⁺ T cells, the culture supernatants from individual wells were assayed by cytokine-specific ELISA. Briefly, flat-bottomed 96-well microtiter plates (Nunc Inc., Naperville, Ill.) were coated with anti-IL-5 monoclonal antibody TRFK-5 (Pharmingen, San Diego, Calif.) at 1 µg/ml in PBS and incubated overnight at 4°C. Following three washes in PBS, the plates were blocked with 1% BSA in PBS for 1 h at 37°C. Different dilutions of culture supernatants in 1% BSA-PBS-T were added. A standard curve was generated by using recombinant murine IL-5 (Pharmingen) at 100 U/ml in serial twofold dilutions in PBS-T containing 1% BSA. Samples were incubated at 4°C overnight, and following thorough washing with PBS-T, biotinylated anti-IL-5 monoclonal antibody TRFK-4 (Pharmingen) was added to the wells at 1 µg/ml in PBS-T with 1% BSA and left for 2 h at 37°C. After subsequent washing with PBS-T, a 1:1,000 dilution of horseradish peroxidase-conjugated streptavidin in 1% BSA-PBS-T was added. After incubation for 2 h at room temperature, the reaction was developed with a substrate solution consisting of 1.1 mM ABTS in citrate-phosphate buffer (pH

4.2) containing 0.01% H_2O_2 . The color was quantitatively assessed in a plate reader at A_{414} (model EL312; Bio-Tek Instruments, Winooski, Vt.). The concentration of IFN- γ was also determined by an identical protocol, except that 2.5 μ g of an anti-IFN- γ monoclonal antibody (R4-6A2; Lee Biomolecular, San Diego, Calif.) per ml and 1 μ g of biotin-conjugated monoclonal antibody anti-IFN- γ (XMG 1.2) per ml were used as coating and detection antibodies, respectively. A standard curve was generated with appropriate dilutions of recombinant murine IFN- γ (PanData, Rockville, Md.) at 400 U/ml in 1% BSA-PBS-T.

SDS-PAGE and Western immunoblotting. A standard SDS-PAGE was performed by the method of Laemmli (16). One microgram of purified LT-B was subjected to SDS-PAGE and transferred electrophoretically to a nitrocellulose sheet. The sheet was then treated either with a 1:10 dilution of fecal extracts from mice orally immunized with LT, peptide 26-45, or peptide 26-45 coupled to OVA or OVA for 16 h at 4°C, respectively. A 1:10 dilution of fecal extracts from sham-immunized mice was used as a control. The antibody bound to the immobilized replica proteins was incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgA antibody for 2 h at 25°C. The sheet was then washed with PBS-T and developed with 2.8 mM 4-chloro-1-naphthol (Sigma) in 10 mM Tris-HCl buffer (pH 7.5) containing 0.015% H_2O_2 .

LT-B and peptide binding assays to GM₁ ganglioside. To assess the ability of peptide 26-45 to bind to GM₁ ganglioside, flat-bottomed 96-well ELISA plates (Nunc) were coated with 10 μ g of GM₁ ganglioside (Sigma) per ml and incubated overnight at 4°C. Following three washes with PBS, the plates were blocked with 1% BSA in PBS for 1 h at 37°C. Three dilutions (1 mg/ml, 100 μ g/ml, and 10 μ g/ml) of the peptide or LT-B were added and incubated for 2 h at 37°C. Following four washes with PBS-T, biotinylated LT-B at 10 μ g/ml in 1% BSA-PBS-T was added to the wells and incubated for 2 h at 37°C. After subsequent washings with PBS-T, a 1:1,000 dilution of horseradish peroxidase-conjugated streptavidin in 1% BSA-PBS-T was added. The plates were incubated for 1 h at room temperature and washed with PBS-T, and the substrate ABTS in 0.1 M citrate-phosphate buffer (pH 4.2) was added. The percentage inhibition of binding of LT-B to GM₁ ganglioside was calculated by the following formula: % inhibition = $\{[OD_{414}$ of biotin-conjugated LT-B - (OD_{414} of biotin-conjugated LT-B + peptide 26-45 or LT-B)]/ OD_{414} of biotin-conjugated LT-B $\} \times 100$.

Statistical method. Significant differences in $\log_2$ ELISA antibody titers were determined by Student's *t* test.

RESULTS

Induction of LT-B-specific mucosal IgA responses. In the initial studies, we determined the kinetics, levels, and isotypes of anti-LT-B antibody responses in BALB/c (H-2^d) mice orally immunized with LT or with *S. dublin* (strain EL23) producing LT-B. A single dose of 25 μ g of LT given orally was sufficient to induce IgA anti-LT-B antibody responses in the gastrointestinal tract (Fig. 1). Fecal IgA antibodies appeared within 1 week following oral immunization, and peak titers occurred after 2 weeks (Fig. 1A); however, this LT-B-specific IgA response declined by week 3. Transient fecal IgG anti-LT-B antibody responses were also observed at week 2, but the level of IgG anti-LT-B responses was lower than that of IgA. No fecal IgM anti-LT-B antibody responses were observed throughout this 3-week period. In contrast to oral immunization with soluble LT, three consecutive daily oral doses of *S. dublin* EL23 expressing the LT-B gene were required for the induction of anti-LT-B specific IgA responses (Fig. 1B). Low but significant levels of fecal IgA anti-LT-B antibody responses were observed at 3 and 4 weeks following oral immunization (Fig. 1B); however, no LT-B-specific fecal IgG or IgM responses were detected during these intervals. Thus, a single oral dose of LT was sufficient to elicit significant fecal IgA and transient IgG anti-LT-B responses, while three oral doses of *S. dublin* EL23 gave lower IgA titers, which were maximal by week 3 following oral administration (Fig. 1).

Isotype of serum anti-LT-B antibody responses. Oral administration of one dose of LT induced detectable serum anti-LT-B responses by week 1 and maximum responses by week 2 (Fig. 2A). The major isotype was IgG, followed by IgM and IgA (Fig. 2A). Interestingly, oral recombinant strain EL23 elicited detectable responses by week 1; however, IgG anti-LT-B antibodies continued to increase and reached maximum titers by week 4 (Fig. 2B). Thus, maximum IgG responses to oral administration of LT and recombinant strain EL23 oc-

FIG. 1. Time course of fecal antibody responses in BALB/c mice orally immunized once with 25 μ g of LT (A) or with 10^{10} recombinant *S. dublin* expressing LT-B on days 0, 1, and 2 (B). The isotype and endpoint titers of antibody responses in fecal extracts were determined by ELISA. Samples were taken at the indicated times before and after oral immunization. Data are the means $\pm$ standard deviations of $\log_2$ ELISA antibody titers obtained from five mice per group. Significant differences in $\log_2$ ELISA antibody titers between before (day 0) and after the immunization were determined by Student's *t* test (*, $P < 0.05$). The results presented are representative of four separate experiments.

curred after 2 and 4 weeks, respectively (Fig. 2). Taken together, these results indicate that oral immunization with soluble LT and recombinant LT-B expressed in recombinant *S. dublin* are effective for induction of mucosal and serum antibody responses to LT-B; however, the former oral regimen was more efficient for induction of antigen-specific mucosal IgA responses, since a single dose of LT induced high titers of LT-B-specific IgA antibodies in fecal samples.

B-cell epitopes based on mucosal IgA antibodies. Since oral administration of LT induces relatively high levels of antigen-specific IgA antibodies in fecal extracts, we used this antibody to map linear epitopes of LT-B, which are responsible for induction of the observed IgA response. For this purpose, we synthesized 94 species of overlapping decapeptides by a solid-phase procedure. In this study, fecal IgA anti-LT-B was reacted with this series of decapeptides covering the entire LT-B molecule. LT-B-specific fecal IgA antibodies reacted with four peptides: amino acid sequences for residues 82 to 91, 83 to 92, 84 to 93, and 85 to 94 (Fig. 3A). Thus, the minimum linear amino acid motif which reacted with LT-B-specific fecal IgA antibodies was a 7-mer of Leu-Cys-Val-Trp-Asn-Asn-Lys,

FIG. 2. Time course of isotype and titer of serum antibody responses to LT-B from BALB/c mice orally immunized with LT (A) or with 10^{10} *S. enteritidis* EL23 on days 0, 1, and 2 (B). Samples were taken at the times indicated following the first oral dose with recombinant *S. dublin* EL23 or the single dose of LT. The isotypes and titers of LT-B-specific serum antibody responses were determined by ELISA and are expressed as indicated in the legend to Fig. 1. The results are representative of four separate experiments.

since overlapping decapeptides containing residues 85 to 91 always gave maximum reactivity by ELISA. On the other hand, a different reaction pattern of peptide-specific IgA antibodies was noted in the fecal extracts from mice orally immunized with recombinant *Salmonella* LT-B. In this case, fecal IgA anti-LT-B antibodies were reactive with decapeptides spanning residues 35 to 45 (epitope 36-44; see below). The linear amino acid motif for this LT-B epitope was a 9-mer of Glu-Met-Val-Ile-Ile-Thr-Phe-Lys-Ser (Fig. 3B). These results indicate that peptides located in the central region (e.g., residues 36 to 44) and the carboxy-terminal region (e.g., residues 85 to 91) of the LT-B molecule serve as major epitopes for induction of IgA responses after oral immunization with native LT and recombinant *S. dublin* expressing LT-B, respectively.

B-cell epitopes based on serum IgG antibodies. It was important to also compare the distribution of linear epitopes in the LT-B molecule responsible for induction of serum IgG antibodies. To this end, we tested the reactivity of overlapping peptides with immune sera of mice orally immunized with either LT or recombinant *Salmonella* LT-B (Fig. 4). Oral administration of LT induced serum IgG antibodies which reacted strongly with four overlapping peptides (residues 85 to

94, 86 to 95, 87 to 96, and 88 to 97), which is the same epitope recognized by mucosal IgA antibodies from this mouse group (Fig. 2A). Furthermore, these antibodies showed less reactivity with peptides starting at residues 35 and 36 (epitope 36-44) as well as peptides beginning at residues 67, 68, and 69 (epitope 69-76). The minimum linear amino acid motif for the major epitope was Trp-Asn-Asn-Lys-Thr-Pro-Asn, since decapeptides containing residues 88 to 94 always gave elevated IgG antibody reactivity. On the other hand, recombinant LT-B induced serum IgG recognizing decapeptides of Thr-Phe-Lys-Ser-Gly-Ala-Thr-Phe-Gln, which corresponds to residues starting at 40 and 41 and the decapeptide starting at residue 64 as well as decapeptides starting at residues 87 and 88. These findings suggested that LT-B-specific IgG antibodies induced by the oral immunization with soluble LT and recombinant LT-B expressed in *S. dublin* both recognized one major epitope (residues 88 to 94) and a second epitope containing residues 41 to 44; however, recombinant *Salmonella* LT-B also induced reactivity to a third epitope spanning residues 64 to 73 (Fig. 4).

Analysis of antigenic epitopes for induction of LT-specific T-cell and cytokine responses. To gain insight into the molecular mechanisms for LT-induced mucosal IgA and serum IgG antibody responses, it was important to determine the T-cell epitopes which result in CD4⁺ T-cell responses in IgA-inductive tissues (e.g., PP) after oral immunization. For this purpose, purified CD4⁺ T cells from the PP of BALB/c (H-2^d) mice orally immunized with 25 μ g of LT were stimulated with the overlapping synthetic peptides, and the proliferative responses to individual peptides were assessed (Fig. 5). Maximum re-

FIG. 3. Specificity of fecal S-IgA antibodies from BALB/c mice orally immunized with LT (A) or with recombinant (r) *S. dublin* expressing LT-B (B) for overlapping peptides of LT-B. The assay for reactivity with decapeptides was performed with a 1:10 dilution of fecal IgA from groups of five mice. The results are expressed as the OD₄₁₄ after 15 min of incubation with the substrate. The residue number shown at the bottom corresponds to the position of the amino-terminal residue of the indicated peptide in the LT-B molecule.

FIG. 4. Reactivities of anti-LT (A) or anti-recombinant LT-B from *S. dublin* EL23 (B) serum IgG antibodies from BALB/c mice with 94 species of overlapping decapeptides spanning the entire LT-B molecule. The binding of synthetic peptides to a 1:1,000 dilution of anti-LT-B antibodies from mice orally immunized with LT (A) or with recombinant *S. dublin* EL23 (B) were determined by ELISA. The data are expressed as the OD₄₁₄ after 15 min of incubation with the substrate. The residue number shown corresponds to the position of the amino-terminal residue of the indicated peptide in the LT-B molecule.

sponses were induced by peptides spanning residues 33 to 42 and 34 to 43, suggesting the presence of only one T-cell epitope. In other studies, CD4⁺ T-cell cultures were stimulated with overlapping peptides, and after 4 days of incubation, culture supernatants were assessed for levels of IFN- γ and IL-5 as an indication of Th1- and Th2-type cytokines, respectively. CD4⁺ T-cells cultured with peptides spanning residues 34 to

FIG. 5. Antigen-specific CD4⁺ T-cell proliferative responses in PP of mice orally immunized with LT. The PP CD4⁺ T cells were isolated from BALB/c mice 1 week after oral immunization with 25 μ g of LT and stimulated in vitro for 4 days with each of the 94 species of overlapping decapeptides. Six hours before cell harvest, 0.5 μ Ci of [³H]thymidine was added to each well. The proliferative responses of CD4⁺ T cells in culture were determined by [³H]thymidine incorporation. The results are expressed as a stimulation index (ratio of experimental counts per minute/control counts per minute). The stimulation index induced by incubation with 10 μ g of LT-B per test was 6.2. Control cultures ranged from 2,300 to 2,430 cpm.

FIG. 6. Level of IFN- γ (A) or IL-5 (B) production by PP CD4⁺ T cells reacted with synthetic peptides. One week after oral immunization with 25 μ g of LT, mice were sacrificed, and the PP CD4⁺ T cells were isolated and stimulated in vitro for 4 days with each of the 94 species of overlapping decapeptides. The levels of IFN- γ and IL-5 in the respective culture supernatants were determined by IFN- γ - and IL-5-specific ELISA, respectively. Data are expressed as the mean units of activity per milliliter from three separate experiments. Cytokine levels induced by culture with LT-B (10 μ g per test) were 151 U/ml (IFN- γ) and 8.2 U/ml (IL-5). The residue numbers shown at the bottom corresponds to the position of the amino-terminal residues of the indicated peptide in the LT-B molecule.

43 produced large amounts of IFN- γ (Fig. 6A). Furthermore, this same peptide also induced the production of IL-5 (Fig. 6B). These findings indicated that the peptides spanning residues 33 to 42 and 34 to 43 of the LT-B molecule are T-cell epitopes.

Immune responses to 20-mer peptide containing T- and B-cell epitopes. In order to insure that residues 34 to 43 and 36 to 44 contain both T- and B-cell epitopes, we synthesized a 20-mer peptide (peptide 26-45) which encompassed linear epitopes for both B and T cells. This 20-mer was conjugated to OVA and was orally administered to BALB/c mice. Oral immunization of mice with the OVA-peptide conjugate at weekly intervals resulted in peptide-specific serum IgG and fecal IgA responses (Table 1). It should be noted that the peptide alone induced levels of peptide-specific mucosal IgA and serum IgG responses comparable to those in mice orally immunized with the OVA-peptide conjugate.

In the next series of experiments, groups of B10 (I-A^d), B10.D2 (I-A^b), and B10.BR (I-A^k) mice were orally immunized with the peptide. Elevated LT-B-specific fecal IgA and serum IgG responses were also noted in these B10 congenic mice (Table 1). This finding clearly shows that oral immunization with the 20-mer peptide alone or the peptide coupled to OVA induced serum IgG and mucosal IgA antibodies not only to the peptide in mice of different haplotypes but also to the LT-B molecule itself (Table 1). This finding was confirmed by using immunoblots in which peptide-specific IgA antibodies were found to bind to native LT-B (Fig. 7). The peptide-

TABLE 1. Immunogenicity of synthetic peptide 26-45 coupled to OVA^a

Mouse strain	Immunogen	Log ₂ ELISA antibody titer			
		Fecal IgA		Serum IgG	
		Peptide 26-45	LT-B	Peptide 26-45	LT-B
BALB/c (H-2 ^d)	Peptide 26-45-OVA	7.1 ± 0.4	3.7 ± 0.2	8.4 ± 0.4	7.2 ± 0.2
	Peptide 26-45	7.4 ± 0.1	4.7 ± 0.3	8.6 ± 0.4	8.8 ± 0.4
	OVA	<3	<3	<3	<3
	PBS	<3	<3	<3	<3
B10.D2 (I-A ^d)	Peptides 26-45	7.8 ± 0.4	5.8 ± 0.2	8.6 ± 0.3	9.1 ± 0.4
	PBS	<2	<2	<3	<3
B10 (I-A ^b)	Peptide 26-45	8.5 ± 0.4	4.7 ± 0.5	8.0 ± 0.4	8.3 ± 0.4
	PBS	<2	<2	<3	<3
B10.BR (I-A ^k)	Peptide 26-45	7.0 ± 0.5	5.0 ± 0.5	7.1 ± 0.4	8.1 ± 0.4
	PBS	<2	<2	<3	<3

^a BALB/c mice were orally immunized with 50 µg of peptide 26-45 alone, peptide 26-45 coupled to OVA, or OVA only at day zero and boosted orally with the same regimen 1 week and 2 weeks later. In addition, B10 congenic mice were also orally immunized with 50 µg of peptide 26-45. As controls, other groups of mice received PBS. Serum and fecal samples were collected 1 week following the last oral boost, and titers were determined by ELISA with wells coated with peptide 26-45 or with LT-B. Neither fecal IgA nor serum IgG anti-LT-B(26-45) antibodies recognized a random peptide (EPTPTTRTPDQAEPNKPPTC). Data are the means ± standard deviations of log₂ ELISA antibody titers for five mice per group.

specific IgA antibodies recognized an 11.8-kDa peptide corresponding to LT-B (Fig. 7). These findings show that the LT-B peptide spanning residues 26 to 45 can be used as an oral immunogen for induction of both LT-B-specific mucosal IgA and serum IgG responses.

To directly prove that the 20-mer peptide contained the original B-cell epitope spanning residues 34 to 43, 35 to 44, and 36 to 45, these original peptides were tested with immune sera from B10 mice which had been orally immunized with the 20-mer peptide. Significant reactivity occurred with peptide 36-45, and less with peptide 34-43 suggesting that the major B-cell epitope spans residues 36 to 45 (Table 2). Interestingly, fecal IgA antibodies induced to peptide 26-45 did not react with the 10-mer peptide spanning residues 33 to 45 (Table 2).

Inasmuch as oral administration of the 20-mer LT-B peptide induced mucosal IgA and serum IgG responses, it was impor-

tant to assess antigen-specific T-cell responses. CD4⁺ T cells isolated from PP of BALB/c and B10 congenic mice orally immunized with the peptide 26-45 were restimulated in vitro with the 20-mer (Fig. 8). Peptide-specific T-cell proliferative responses were noted regardless of the expression of the major histocompatibility complex class II haplotype. On the other hand, PP CD4⁺ T cells from unimmunized mice did not respond to the peptide. Furthermore, T-cell proliferative responses to the original peptides spanning residues 33 to 42 and 34 to 43 were also induced, confirming that this region is indeed a major T-cell epitope (data not shown). These findings suggested that the LT-B 20-mer peptide 26-45 contains a T-cell epitope which exhibits a broad range of major histocompatibility complex class II-mediated T-cell responses.

Binding of synthetic peptide 26-45 to GM1. The precise locale for binding of LT-B to gangliosides is not yet known; however, multiple binding sites have been postulated (28). We have shown that peptide 26-45 inhibited binding of the LT-B molecule to solid-phase GM₁ in a dose-dependent manner (Table 3). On the other hand, an irrelevant LT-B peptide (residues 5 to 24) did not inhibit binding of the native molecule to GM₁. Although the 20-mer LT-B peptide 26-45 inhibited the binding ability of LT-B to GM₁, the level of inhibition by the 20-mer peptide 26-45 was less than that with LT-B alone (Table 3), again suggesting that multiple sites exist for LT-B

FIG. 7. Western blot analyses of LT-B reacted with fecal IgA anti-LT (lane 1), anti-peptide 26-45 (lane 2), anti-peptide-OVA (lane 3), anti-OVA (lane 4), or fecal extracts from sham-immunized mice (control) (lane 5). One microgram of purified LT-B was analyzed by 15% SDS-PAGE and transferred onto nitrocellulose sheets by electroblotting. The protein on the sheets was allowed to react with the respective fecal IgA antibodies and detected by horseradish peroxidase-conjugated goat anti-mouse IgA. Molecular sizes are indicated on the left.

TABLE 2. Epitope mapping with antisera to peptide 26-45 with decamer peptides of LT-B to determine the fine specificity of the epitope^a

Sample tested	<i>A</i> ₄₁₄ (10 ³)			
	Peptide 33-42	Peptide 34-43	Peptide 36-45	Peptide 26-45
Serum IgG	246 ± 14	220 ± 14	463 ± 33	2,265 ± 18
Serum IgG1	14 ± 6	92 ± 1	357 ± 6	3,179 ± 199
Fecal IgA	142 ± 2	162 ± 3	148 ± 1	644 ± 21
Control IgG in PBS	<2	<2	<2	<2

^a Groups of B10.D2 mice were orally immunized with peptide 26-45 as described in Table 1, footnote *a*. Data are the means ± standard deviations.

FIG. 8. LT-B peptide specific CD4⁺ T-cell proliferative responses in PP of mice orally immunized with the 20-mer peptide 26-45. The PP CD4⁺ T cells were isolated from BALB/c and B10 congenic mice 1 week following the final oral immunization with the peptide 26-45. The detailed immunization protocol is described in Table 1, footnote a. Purified CD4⁺ T cells were then cultured either in the presence or absence of different concentrations of the peptide (20 µg [■], 10 µg [▨], 5 µg [▩], and 2.5 µg [□]). The detailed culture conditions are described in the legend to Fig. 5. Control cultures ranged from 1,900 to 2,250 cpm.

binding to the GM₁ ganglioside (28). We conclude that the remarkable immunogenicity of peptide 26-45 when given orally to mice may be due in part to its affinity for GM₁.

DISCUSSION

The reemergence of infectious diseases and the emergence of antibiotic-resistant microorganisms clearly point to the need for either new or improved vaccines. Furthermore, since most viral and bacterial pathogens affect the gastrointestinal, upper respiratory, or genitourinary tract, e.g., mucosal effector tissues, more emphasis has been placed on vaccines which induce mucosal immunity (29). In this regard, there is a clear need for effective mucosal delivery systems as well as adjuvants which enhance both mucosal and serum antibody responses. The two most widely used adjuvants to date for enhancing mucosal immune responses are CT and the related molecule LT. Although both CT and LT induce mucosal and serum antibody responses to CT-B or LT-B as well as to coadministered proteins, the majority of these studies have been done with CT. Thus, it was important to place more emphasis on LT and in particular to better define the linear B- and T-cell epitopes which occur in the 103-amino-acid structure of the B subunit.

We have used 94 overlapping 10-mer peptides of the entire LT-B molecule to determine linear epitopes. Our rationale for this approach was that both immunogens used, e.g., native LT or recombinant LT-B produced by *S. dublin*, were given orally. Thus, we would expect more degradation of the LT-B molecule in the milieu of the gastrointestinal tract and that linear epitopes have more relevance in this system. This was indeed the case, since our studies with either fecal IgA or serum IgG anti-LT-B antibodies clearly mapped two major and one minor B-cell epitope in this molecule.

Of interest was the finding that oral immunization with native LT induced IgA antibodies mainly to the peptide 85-91 sequence, while recombinant *S. dublin* EL23 expressing LT-B induced IgA antibodies mainly to epitope 36-44. The reasons for this response pattern are not yet known; however, we speculate that this pattern results from differences in the antigen presentation pathways. Native LT would be expected to bind to epithelial cells, including M cells which cover the epithelium of

the PP, prior to interactions with B and T cells. The native LT molecule would also be more protected (less degradation) in the gastrointestinal tract, and residues 85 to 91 may represent a major epitope. On the other hand, recombinant *Salmonella* LT-B would be taken up by macrophages, and epitope 36-44 likely results from presentation by macrophages. A less likely explanation is that native LT acts as an adjuvant for enhancement of the B-cell response to epitope 85-91. In this case, it would seem to be equally likely that LT as an adjuvant would also boost responses to epitope 36-44 as well.

Analysis of serum IgG anti-LT-B antibodies with overlapping peptides again supported the results with mucosal IgA antibodies showing that epitopes at residues 36 to 44 and 85 to 91 are present. Serum IgG from mice orally immunized with either LT or recombinant *Salmonella* LT-B showed significant reactivity with epitope 88-94 and somewhat less to the epitope 36-44 determinant. Of interest was the finding of a third epitope at residues 64 to 73, which could be detected with serum IgG of mice orally immunized with recombinant *S. dublin*. It is not clear why serum IgG anti-LT-B responses to two or even three epitopes occur, while IgA antibodies are induced to only one B-cell epitope. The questions raised by this study, when carefully addressed, should provide new information concerning the mechanisms for antigen presentation in mucosal inductive sites.

Our study is also the first to show that specific LT-B peptides covering residues 33 to 42 and 34 to 43 are essential for the induction of antigen-specific CD4⁺ T cells. Specifically, the PP of mice orally immunized with LT contained specific CD4⁺ T cells which underwent proliferative responses when stimulated with either peptide 33-42 or 34-43. The proliferating CD4⁺ PP T cells produced both IFN-γ and IL-5, suggesting a mixed Th1 and Th2 phenotype. As discussed above, LT-B peptides which contain residues 36 to 44 are also major linear epitopes for LT-B-specific B-cell responses.

Our past work has shown that Th2-type cells are important for the induction of antigen-specific mucosal S-IgA responses (38). In the present study, we showed that a mixed Th1- and Th2-type response may also be involved in the induction of antibodies to LT-B. We cannot eliminate the possibility that Th0-type cells are also induced by oral immunization with LT since our cytokine analysis was performed by the assessment of IFN-γ and IL-5 levels in culture supernatants harvested from different peptide-stimulated CD4⁺ T-cell cultures. In order to directly address the issue, it will be important to derive peptide-specific CD4⁺ T-cell clones from orally immunized mice. In separate studies, we have generated CD4⁺ Th-cell clones from mice orally immunized with LT-B peptide 26-45. Our preliminary results suggest that an approximately equal fre-

TABLE 3. Peptide 26-45 inhibits LT-B binding to GM₁ ganglioside^a

Inhibitor	% Inhibition with dose (µg/test) of:		
	1,000	100	10
Peptide 26-45	64 ± 2	39 ± 4	9 ± 0.1
Peptide 26-45-OVA	17 ± 1	15 ± 2	8 ± 1
Peptide 5-24	3 ± 2	1 ± 1	1 ± 1
LT-B	100	100	27 ± 3

^a Comparisons of the ability of the T- and B-cell epitope peptide 26-45 and nonrelevant peptide 5-24 to bind to GM₁ ganglioside as assessed by a competitive ELISA assay. The percentage inhibition of binding of biotinylated LT-B to GM₁ ganglioside was calculated by the following formula: % inhibition = [(OD₄₁₄ of biotin-conjugated LT-B - (OD₄₁₄ of biotin-conjugated LT-B + peptide 26-45, LT-B peptide 26-45-OVA, or LT-B))/OD₄₁₄ of biotin-conjugated LT-B] × 100. Results are the means ± standard deviations.

quency of peptide-specific Th1- and Th2-cell clones were generated from PP of mice orally immunized with the peptide (33a).

Since LT-B peptide 26-45 induces both Th1- and Th2-type responses, we speculate that the Th1-type response may be important in antigen presentation of the peptide by antigen-presenting cells in the PP as well as in mucosal effector sites such as the intestinal lamina propria. In this regard, IFN- γ increases major histocompatibility complex class II expression and may enhance the antigen-presenting capacity of antigen-presenting cells, including S-IgA⁺ B cells, macrophages, dendritic cells, or enterocytes for induction of Th2-type cells which provide cytokines (e.g., IL-5 and IL-6) necessary for IgA responses. Thus, IFN- γ stimulated S-IgA⁺ B cells may effectively interact with IL-5- and IL-6-producing Th2-type cells. Studies to support this suggestion are currently underway in our laboratory.

Analysis of antigenic epitopes of the LT-B molecule revealed that different oral vaccine delivery systems, e.g., soluble LT versus recombinant LT-B expressed by attenuated *S. dublin*, may account for the induction of antigen-specific responses which recognize different epitopes of LT. On the basis of the above discussion, several possible explanations for this different response pattern can be suggested. First, differences in the ligand densities generated by the various vaccine delivery systems could result in the recognition of certain antigenic epitopes (23). Second, the form of the immunogen could exert different effects on the antigen-presenting cell itself; for example, live salmonellae may be taken up by macrophages and could alter fusion of lysosomes (5) or, alternatively, could induce stress protein responses in this cell type (4) which could affect the outcome of the antibody response regulated by T cells. Various types of antigen-presenting cells present antigens in different ways to Th cells; for example, it is possible that antigen presentation by S-IgA⁺ B cells could lead to the induction of Th2 responses, while macrophages may favor Th1-cell induction. Therefore, the peptide repertoire preferentially presented by live recombinant salmonellae may be different from that of the antigen epitopes presented by soluble protein antigens.

Another major finding of this study was that oral immunization with a partial peptide only (or coupled to OVA) spanning residues 26 to 45 (containing both T- and B-cell epitopes) induced intestinal peptide-specific S-IgA antibody responses. This 20-mer peptide induced mucosal S-IgA antibodies which cross-reacted with the native LT-B molecule. It is important that the peptide also induced serum IgG responses not only to the peptide itself but also to the native LT-B. LT-B peptide 26-45 partially inhibited the binding of the LT-B molecule to the GM₁ ganglioside, one of the receptor molecules for LT-B which is expressed in the membranes of eukaryotic cells (Table 3). Thus, from the results obtained in this study, one can propose that LT-B peptide 26-45 may be an appropriate vaccine candidate for oral immunization to induce mucosal and systemic immunity to ETEC.

The 20-mer peptide 26-45 was selected for the following reasons. Epitope mapping of the LT-B molecule revealed that a peptide spanning residues 36 to 44 of the LT-B molecule strongly reacted with serum IgG and fecal S-IgA antibodies induced following oral immunization with recombinant *Salmonella* LT-B (Fig. 3B). A partial peptide of residues 34 to 43 also stimulated PP CD4⁺ T cells to proliferate and to produce both IFN- γ and IL-5. To our knowledge, this is the first study which shows that oral immunization with a synthetic peptide of only 20 amino acids resulted in the induction of LT-B-specific mucosal S-IgA and serum IgG responses.

It should be emphasized that oral immunization with LT-B peptide induced antigen-specific antibody responses in the absence of mucosal adjuvant. It is well established that most soluble proteins or peptide antigens require mucosal adjuvants in order to induce antigen-specific immune responses (13). Although we do not yet have direct proof, possible reasons may be given to explain why this peptide was immunogenic when given orally. First, this peptide contains both a T- and B-cell epitope (Fig. 4, 5, and 8), and second, the T-cell epitope consisting of residues 33 to 42 or 34 to 43 stimulated both Th1- and Th2-type cytokine synthesis (Fig. 6). It is also important to emphasize that LT-B 20-mer peptide 26-45 universally stimulated CD4⁺ T cells from mice of different haplotypes (Fig. 8). Thus, antigen-specific CD4⁺ T cells were induced by oral immunization of BALB/c (H-2^d) or B10 congenic mice including B10.D2 (I-A^d), B10 (I-A^b), and B10.BR (I-A^k). Levels of antigen-specific mucosal IgA and serum IgG antibodies were also comparable among these different mouse strains (Table 1).

From our results, it appears that the B- and T-cell epitopes both span residues 33 to 45 of the LT-B molecule. In this regard, others (15) have reported that the major T- and B-cell epitopes for the P1 protein of *Mycoplasma pneumoniae* adhesin closely associated in the molecule; i.e., the T cell-stimulating amino acid sequence (starting at amino acid residue 845) was located at the carboxy-terminal end of a B-cell epitope (starting at amino acid residue 842). More recently, Lehner et al. (17) reported that oro-rectal immunization of rhesus monkeys with the recombinant p27 Gag protein of simian immunodeficiency virus induced rectal S-IgA antibodies and Gag p27-specific T-cell responses in CD4⁺ T cells from the regional lymph nodes as well as from the peripheral blood. Epitope mapping analysis of Gag p27 revealed that two immunodominant T- and B-cell epitopes spanned the polypeptide residues 51 to 80 and 121 to 150, respectively. Adjacent linkage of a specific T-cell epitope with a B-cell epitope may be sufficient to induce CD4 Th cells for B-cell responses.

Despite numerous studies with LT and CT for the induction of mucosal immune responses, the precise cellular and molecular mechanisms for LT and CT adjuvant responses remain unknown. In this study, we have provided evidence that partial peptides located at the central and carboxy-terminal regions of the LT-B molecule were involved in the potent immunogenicity of LT-B for mucosal and serum antibody responses. From the X-ray crystallography studies of the LT-B molecule, the immunogenic peptides shown in our study are located on the exposed surface and beta-turn structure of the LT-B molecule and constitute a small cavity of the β -pentamer, which is presumably involved in ganglioside binding (26, 27). On the other hand, we could not detect antibody responses (including serum IgG or fecal IgA antibodies) to the carrier protein OVA in mice orally immunized with the 20-mer coupled to OVA (data not shown). These findings suggest the possibility that different molecular domains of the LT-B molecule may possess immunogenic or adjuvant components. In this regard, Sun et al. (32) have reported that CT-B, a LT-B-related molecule, induces oral tolerance (systemic unresponsiveness) to an unrelated protein antigen (human gamma globulin) when co-administered orally. We also found that low-dose, multiple feedings of LT-B peptide 26-45 in mice induced systemic unresponsiveness to the relevant antigen (34).

In summary, our results show that oral immunization with LT-B and/or a partial peptide 26-45 of the LT-B molecule induces both Th1- and Th2-type cytokine synthesis as well as brisk mucosal IgA and serum IgG antibody responses. The induction of antigen-specific mucosal IgA and systemic IgG by an orally administered peptide would provide two lines of

defense against ETEC infection. Our findings suggest that induction of mixed Th1- and Th2-cell development may be a key element for vaccine efficacy without toxic side effects. Since CT has both immunomodulating properties in mucosal and systemic sites as well as toxic effects such as induction of diarrhea, IgE-type hypersensitivity, and eosinophilia, use of LT peptide derivatives may be more beneficial. In this regard, our studies of oral immunization with partial peptide 26-45 of LT-B were not associated with any adverse effects, such as the induction of an IgE-type response (data not shown). The LT-B 20-mer peptide 26-45 is also capable of universally inducing antigen-specific mucosal IgA and serum IgG responses in mice of different haplotypes. Thus, the selection of peptides from the LT molecule such as the 20-mer LT-B peptide 26-45 may provide a promising oral vaccine for ETEC infections.

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