# Oral Immunization of Mice with Attenuated *Salmonella enteritidis* Containing a Recombinant Plasmid Which Codes for Production of the B Subunit of Heat-Labile *Escherichia coli* Enterotoxin

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We used *Salmonella enteritidis* serotype dublin strain SL1438, a nonreverting, aromatic-dependent, histidine-requiring mutant, as a recipient for a recombinant plasmid coding for production of the nontoxic B subunit of the heat-labile *Escherichia coli* enterotoxin. The *S. enteritidis* derivative EL23 produced heat-labile enterotoxin subunit B that was indistinguishable from heat-labile enterotoxin subunit B produced by strains of *E. coli* or *Salmonella typhi* harboring the same plasmid. Mice immunized orally with strain EL23 developed progressively increasing mucosal and serum antibody responses to both heat-labile enterotoxin subunit B and to the lipopolysaccharide of the vaccine strain. The mucosal antibody response was shown to be immunoglobulin A specific and to be capable of neutralizing the biological activities of both *E. coli* heat-labile enterotoxin and cholera enterotoxin in vitro.

Cholera occurs when a sufficient number of virulent *Vibrio cholerae* are ingested, survive passage through the gastric acidity, and interact with and colonize the epithelium of the proximal small bowel. The ensuing extensive diarrhea results from a net outpouring of fluid and electrolytes into the lumen of the bowel in response to a potent exo-enterotoxin produced by these organisms. This enterotoxin (choleragen) is an 84,000-dalton polymeric protein composed of two major, noncovalently associated, immunologically distinct regions or domains (cholera-A and cholera-B) (10). Of these, the 56,000-dalton B region, or choleragenoid (CT-B), is responsible for binding of the toxin to the host-cell membrane receptor and facilitating penetration of the toxic A region into the cell membrane (9, 11). The A subunit causes the activation of adenylate cyclase and a subsequent increase in intracellular levels of cyclic AMP. The B subunit is nontoxic and appears to be the immunodominant moiety of the holotoxin (16, 21, 24).

Recent efforts to develop an effective immunoprophylactic for cholera have employed mutagenesis (2, 12, 17) or recombinant DNA technology (18, 19, 23) to construct strains of *V. cholerae* genetically incapable of producing the toxic A fragment of the enterotoxin. These genetically altered organisms, which produce CT-B but lack CT-A, have potential for use as live oral vaccines—colonization of the intestinal epithelium with these organisms should elicit a mucosal immune response to the organisms and to CT-B. Some of these strains have provided protection against challenge with *V. cholerae*, with an overall reduction in the occurrence of diarrhea, but none is completely safe and effective.

We have recently constructed a potential multivalent live oral vaccine for both typhoid fever and the cholera-*Escherichia coli*-related diarrheas (3) utilizing the galactose epimeraseless (*gal E*) mutant of *Salmonella typhi* (14) which has been shown to be a safe, effective live oral vaccine for typhoid fever (15, 30). This strain, *S. typhi* Ty21a, has previously been used by Formal et al. (13) to construct a potential bivalent dysentery-typhoid vaccine, whose safety

and antigenicity have recently been demonstrated in volunteers (28). We used *S. typhi* Ty21a as a recipient for a recombinant plasmid containing the gene for production of the nontoxic B subunit of the heat-labile enterotoxin of *E. coli* (LT-B). This protein shares extensive sequence and immunologic homology with the B subunit of choleragen (4–6, 8). The *S. typhi* derivative strain SE12 was shown to contain a 3.5-kilobase recombinant LT-B plasmid and produced LT-B that possessed no demonstrable biological activity and was structurally and immunologically indistinguishable from the LT-B produced by *E. coli* strains harboring the same plasmid. The derivative strain was rapidly cleared after intraperitoneal (i.p.) injection into mice, caused no diarrhea or other manifestations when inoculated orally (p.o.) into guinea pigs, and retained the galactose sensitivity characteristic of the parent *S. typhi* Ty21a. More importantly, mice injected i.p. with SE12 developed a significant antitoxin antibody response which could be specifically boosted with a subsequent injection of either viable organisms or purified LT-B.

Although this recombinant strain has potential for use as a live oral multivalent vaccine for both typhoid fever and the cholera-*E. coli*-related diarrheas in humans, the limited host range of the parent *S. typhi* precluded a thorough investigation of the full potential of this vaccine candidate or of this approach to development of immunity to enteric pathogens. The ability of this strain when delivered p.o. to induce a specific mucosal antitoxin response cannot be tested except in human volunteers. Fortunately, several of the >1,700 serotypes of *Salmonella enteritidis*, including serotypes typhimurium and dublin, are able to cause a systemic infection after oral inoculation of mice analogous to that caused by *S. typhi* in man. Aromatic-dependent mutants of these strains have been shown to protect mice and cattle against subsequent challenge with virulent wild-type *Salmonella* species (26).

Because of the relative avirulence of such aromatic-dependent mutants, they have been proposed as immunologic vehicles for delivery of antigens from a variety of organisms. Antigens expressed by these strains would pre-

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sumably be delivered directly to the antibody-forming cells in the gut-associated lymphoid tissues. This should be an effective means of stimulating significant levels of specific mucosal immunoglobulin A (IgA) directed against the carrier strain and the antigen and would stimulate production of serum antibodies as well. The purpose of the current investigation was to determine the efficacy of this mechanism of antigen delivery for production of specific mucosal antitoxin IgA, with delivery of the toxoid antigen to be accomplished by utilizing an avirulent mouse strain of *Salmonella* sp. This study should provide information about the protective potential of the *S. typhi*–LT-B hybrid and should also serve as a model system for evaluation of this approach to development of immunity and to the production of mucosal and serum antibodies to a variety of antigens.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The vehicle used for the delivery of antigen to the target cells was an *S. enteritidis* serotype dublin mutant, strain SL1438, a nonreverting aromatic-dependent, histidine-requiring (*aro his*) mutant (kindly provided by B. A. D. Stocker, Stanford University School of Medicine). Strain SL1438 was derived from a virulent *S. enteritidis* serotype dublin parent, S4454, by transducing in the *aroA554*::Tn*10* transposon and backselecting tetracycline-sensitive variants. One such nonreverting variant, SL1438, was avirulent for mice and effective as a parenterally administered vaccine for mice and calves (26). *E. coli* JM83(pJC217) *ara* Δ(*lac-pro*) *rpsL thi* φ80d*lacZ*ΔM15 is a K-12 derivative transformed with a 3.5-kilobase plasmid (pJC217) which contains the gene for production of LT-B (3). *S. typhi* SE12 is an *S. typhi* Ty21a derivative also transformed with plasmid pJC217.

**Isolation of plasmid DNA.** Plasmid DNA was isolated by the procedure of Birnboim and Doly (1).

**Restriction endonuclease digestion.** Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Reactions were carried out as prescribed by the manufacturer.

**Electrophoresis.** Agarose gel electrophoresis was performed on 1% horizontal slab gels in 0.04 M Tris–0.2 M sodium acetate–0.002 M EDTA, pH 7.8. Bacteriophage λ DNA fragments generated by *Hin*dIII digestion were used as molecular weight standards. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the technique of Laemmli (20).

**Transformation.** Transformation was carried out as previously described (3). The plasmid coding for production of LT-B was purified from *S. typhi* SE12 by cesium chloride gradient centrifugation and used to transform strain SL1438. Transformants were screened for resistance to ampicillin and assayed by enzyme-linked immunosorbent assay (ELISA) (see below) for LT-B production. All ampicillin-resistant transformants tested produced LT-B. One such isolate, designated EL23, was selected for further study.

**Immunization.** For primary immunization with EL23, female BALB/c mice (Harlan/Timco, Houston, Tex.) were immunized p.o. with two doses containing $10^{10}$ CFU each of strain EL23 on days 0 and 4. Inocula for immunizations were prepared from log-phase cultures of strain EL23 grown at 37°C on a New Brunswick Scientific gryatory shaker in Trypticase soy broth (BBL Microbiology Systems, Cockeysville. Md.) containing 100 μg of ampicillin per ml and 1 μg of 2,3-dihydroxybenzoic acid (Sigma Chemical Co., St. Louis. Mo.) per ml. Cells were washed and suspended in sterile

normal saline to a final volume containing $10^{11}$ CFU/ml and administered intragastrically in 0.1-ml doses with a feeding tube. At 21 days post-primary immunization, animals to be boosted were given $10^{10}$ CFU of EL23 prepared as indicated above, 100 μg of LT-B i. p. in sterile normal saline, or 100 μg of LT-B p.o. in sterile normal saline. Animals receiving primary immunization with LT-B only received two 0.1-ml doses containing 64 μg each of LT-B in sterile normal saline on days 0 and 4. This amount of LT-B was chosen because it represents the amount of LT-B present in $10^{10}$ CFU of strain EL23.

**Antibody assay.** Groups of animals were sacrificed at weekly intervals and analyzed by ELISA for production of antibodies to LT-B and, in some cases, to the lipopolysaccharide (LPS) of the vaccine strain. Animals were bled before euthanasia, and sera were stored at −20°C until assayed. The small intestine from the doudenum to the ileal-cecal junction was excised and homogenized in a solution containing 50 mM EDTA and 0.1 mg of soybean trypsin inhibtor (Sigma Chemical Co.) per ml. Samples were homogenized with a Tekmar Tissuemizer, clarified by centrifugation, lyophilized, suspended in 1 ml of TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN₃, 0.2 M NaCl, pH 7.5) (4), dialyzed against TEAN buffer, and stored at −20°C until assayed.

**ELISA.** Reagents and antisera for the ELISA were obtained from Sigma Chemical Co. Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.2)–0.05% Tween 20. For anti-LT-B determinations, 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. Anti-LPS was determined on microtiter plates precoated with 10 μg of LPS per well purified from *S. enteritidis* serotype dublin strain SL1438 by the technique of Sutherland and Wilkinson (27). Serum anti-LT-B or anti-LPS IgG was determined by using rabbit antiserum against mouse IgG conjugated to alkaline phosphatase. Mucosal anti-LT-B or anti-LPS IgA was assayed by using 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; Litton Bionetics, Inc., Charleston, S.C.]. Cross-reactivity was determined by crossing reagents (i.e., IgG was bound to plates to check antiserum against IgA). Cross-reactivity was 2.4% for IgG and 6.7% for IgA. Values reported are corrected for cross-reactivity. For studies on the effect of boosting, mucosal IgA values are further corrected for contamination of mucosa with serum {corrected mucosal IgA = mucosal IgA − [serum IgA × (mucosal IgG/serum IgG)]}. Although the values obtained by this procedure were higher than anticipated, they were reproducible and accurately reflect changing responses within this system.

**Bioassays.** Toxin neutralization assays in mouse Y1 adrenal cells (25) were performed as previously described (5). Briefly, a selected dose of toxin was mixed with an equal volume of serial dilutions of pooled mucosal samples. After preincubation for 1 h at 37°C, samples were applied to a monolayer of mouse Y1 adrenal cells (ATCC CCL79), and the incubation continued for 18 h. The titer was defined as the reciprocal of the highest dilution showing complete neutralization of the toxin (approximately 10 rounding doses) in the microtiter well. Specificity of the neutralization reaction was determined by preincubating the mucosal pool

with either goat antiserum against mouse IgA (Sigma Chemical Co.) or rabbit antiserum against mouse IgG (Sigma Chemical Co.).

**Purification of cholera toxin, LT, and LT-B.** Cholera toxin was prepared as described by Mekalanos et al. (22). The culture conditions and purification of LT and LT-B were as previously described (6). Organisms were cultured overnight at 37°C with vigorous aeration and agitation after inoculation with $10^6$ viable bacteria per ml. The bacteria were harvested by centrifugation at 4°C, and the cells were suspended in TEAN buffer and lysed with a French pressure cell. The crude lysate was then dialyzed against TEAN buffer and, after centrifugation, applied directly to columns of Sepharose 4B (Sigma Chemical Co.) equilibrated with TEAN buffer.

**Guidelines used for recombiant DNA experiments.** The experiments reported here were performed under conditions as 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 S. enteritidis serotype dublin strain EL23.** We have previously described the construction of a plasmid (pJC217) containing the gene for production of LT-B from a human isolate of *E. coli* H10407. This gene is under control of the *lac* promoter of plasmid pUC8 (29) and codes for production of LT-B which is structurally identical to the native B subunit when examined by SDS-PAGE, immunologically identical in ELISA and double immunodiffusion, and nontoxic. This is the plasmid that was used to transform *S. typhi* Ty21a in our previous study (3). Plasmid DNA from *S. typhi* SE12 was purified by cesium chloride gradient centrifugation and used to transform *S. enteritidis* serotype dublin strain SL1438. Transformants were selected for re-
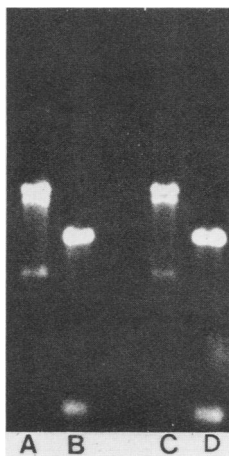
FIG. 1. Agarose gel electrophoresis of plasmid DNA purified by cesium chloride gradient centrifugation. Lanes: A, *S. typhi* SE12; B, *S. typhi* SE12 cleaved with *Hind*III; C, *S. enteritidis* serotype dublin derivative EL23; and D, *S. enteritidis* serotype dublin derivative EL23 cleaved with *Hind*III. Agarose gel electrophoresis was performed in 0.04 M Tris–0.2 M sodium acetate–0.002 M EDTA (pH 7.8). Phage λ DNA fragments generated by *Hind*III digestion were used as molecular weight standards.
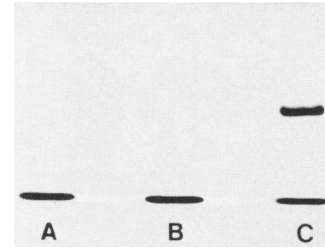
FIG. 2. Analytical discontinuous electrophoresis in SDS-PAGE of agarose affinity-purified preparations of LT-B from *S. enteritidis* serotype dublin strain EL23 (lane A), *E. coli* JM83(pJC217) (lane B), and LT (lane C). Preparations derived from *S. enteritidis* serotype dublin strain EL23 and *E. coli* JM83(pJC217) contained a band corresponding to monomeric LT-B. Neither contained a band corresponding to LT-A (lane C, upper band). SDS-PAGE was performed in 10% slab gels by the technique of Laemmli (20). Each well contained 100 μg of protein and was heated to 100°C for 3 min in 0.1% SDS before loading.

sistance to ampicillin and assayed by ELISA for production of LT-B. All ampicillin-resistant transformants tested produced LT-B. One such isolate, designated EL23, was selected for further study.

**Characterization of S. enteritidis serotype dublin derivative EL23.** Strain EL23 was first examined for the presence of plasmid pJC217. Plasmid DNA was prepared from the transformant and examined by agarose gel electrophoresis. Strain EL23 contained a 3.5-kilobase plasmid which, when cut with the restriction endonuclease *Hind*III yielded the same 2.7- and 0.8-kilobase fragments, representing pUC8 and the LT-B gene, respectively, previously seen with plasmid pJC217 (3) (Fig.1).

Since the effectiveness of this vaccine is dependent in part upon the structural and immunologic relatedness of LT and choleragen, it was essential that LT-B produced by EL23 be structurally and immunologically identical to LT-B produced by *E. coli*. LT-B was purified from EL23 by agarose affinity chromatography (6) and examined by SDS-PAGE and ELISA. In SDS-PAGE (Fig. 2), LT contained two bands, one at ca. 28,000 daltons representing the toxic A subunit (LT-A), and one at ca. 11,500 daltons representing the monomeric from of LT-B (Fig. 2C). Preparations from both EL23 (Fig. 2A) and JM83(pJC217) (Fig. 2B) contained a band corresponding to monomeric LT-B. Neither preparation contained a band corresponding to LT-A. Both preparations were then assayed by ELISA (Fig. 3), and LT-B produced by EL23 was indistinguishable from LT-B produced by JM83(pJC217). Relative distribution was determined, and LT-B was found to be principally cell associated (94.4%) in strain EL23, a value similar to that observed with *E. coli* (>96.7%) and with *S. typhi* derivative SE12 carrying the same plasmid (Table 1).

**Effect of primary immunization with LT-B.** Mice immunized p.o. with LT-B developed progressively increasing levels of serum and mucosal antitoxin antibodies throughout the course of the experiment, 5 weeks post-primary immunization (Fig. 4). Serum and mucosal antitoxin levels for unimmunized controls were zero (Fig. 4, legend). Antitoxin serum IgG (Fig. 4A) increased to a maximum of 1,819 μg/ml by week 3, leveling off at 1,655 μg/ml by the end of week 5. Similarly, mucosal antitoxin IgA (Fig. 4B) increased to a maximum of 3,439 ng/ml by week 1 and decreased slightly thereafter. The difference between the value observed for mucosal antitoxin IgA at week 1 (3,439 ng/ml) and that at week 5 (2,646 ng/ml) is not statistically significant.
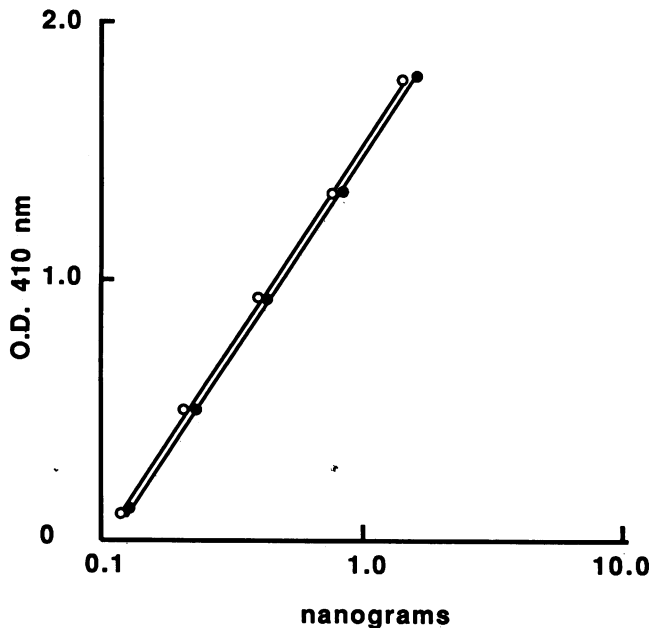
FIG. 3. ELISA comparison of agarose affinity-purified preparations from *S. enteritidis* serotype dublin strain EL23 (●) and *E. coli* JM83(pJC217) (○) with monospecific antiserum to LT-B. Microtiter plates were precoated with 1.5 μg of mixed gangliosides (type III; Sigma Chemical Co.) per well. Samples to be assayed were serially diluted in phosphate-buffered saline (pH 7.2)–0.05% Tween 20. O.D., optical density.

**Effect of primary immunization with EL23.** Mice immunized p.o. with strain EL23 also developed progressively increasing levels of antitoxin serum and mucosal antibodies throughout the 5 weeks of the experiment. Antitoxin serum IgG (Fig. 4A) increased to 370 μg/ml by week 3, reaching a maximum 671 μg/ml by the end of week 5. The differences in antitoxin serum IgG levels between values observed for immunization with LT-B and those seen for immunization with EL23 are significantly different at each time point. Mucosal antitoxin IgA (Fig. 4B) increased throughout the experiment to a maximum of 836 ng/ml by week 5. The values observed after immunization with EL23 for the first 3 weeks are significantly different from those for immunization with LT-B. However, by week 5, the differences are no longer significant. Thus, immunization with LT-B produces significantly higher levels of antitoxin serum IgG and initially higher levels of mucosal secretory IgA. The differences in IgA levels are no longer significant by week 5.

Mucosal and serum anti-LPS titers after immunization with EL23 also increased over the same time course. By week 5, anti-LPS values increased to 8 μg/ml for serum anti-LPS IgG and 112 ng/ml for mucosal anti-LPS IgA (data not shown).

**Effect of boosting.** Since both serum antitoxin IgG and mucosal antitoxin IgA levels plateaued at approximately 3 weeks post-primary immunization, a second group of animals was similarly immunized with EL23 and boosted at that time period. At 21 days post-primary immunization, animals to be boosted were given $10^{10}$ CFU of EL23, 100 μg of LT-B i.p., or 100 μg of LT-B p.o.

**Serum antitoxin IgG.** The serum antitoxin IgG levels in all treatment groups increased to a maximum at 5 weeks and gradually declined thereafter (Fig. 5A). Serum antitoxin IgG levels determined at 11 weeks post-primary immunization

remained significantly elevated after i.p. boost with LT-B compared with those after p.o. boost with LT-B or EL23. Differences between p.o. boost with LT-B and EL23 were also significant.

**Mucosal antitoxin IgA.** All treatment groups reached a maximum level of mucosal antitoxin IgA by 4 weeks and gradually declined thereafter (Fig. 5B). By 11 weeks post-primary immunization there was no significant difference between animals boosted i.p. with LT-B and those receiving a p.o. boost with EL23. Mucosal antitoxin IgA levels after a p.o. boost with LT-B were significantly higher than levels after a p.o. boost with EL23. Although IgA levels after a p.o. boost with LT-B were slightly higher, the differences between i.p. and p.o. boost with LT-B were not significant.

**Serum antitoxin IgA.** All treatment groups reached a maximum level of serum antitoxin IgA at 5 weeks and gradually declined thereafter (Fig. 5C). By 11 weeks post-primary immunization, serum antitoxin IgA levels were significantly higher in animals boosted i.p. with LT-B over those in animals boosted p.o. with LT-B or those in animals boosted p.o. with EL23. Differences observed between animals boosted p.o. with LT-B and those boosted p.o. with EL23 were not significant.

**Mucosal antitoxin IgG.** The mucosal antitoxin IgG levels in all treatment groups remained consistently low relative to specific serum antitoxin IgG, probably representing contamination of mucosal preparations during sample processing (Fig. 5D).

**Serum anti-LPS IgG.** Animals receiving a p.o. boost with EL23 reached maximum serum anti-LPS IgG levels at 8 weeks post-primary immunization, while those receiving p.o. LT-B reached a maximum at 11 weeks (Fig. 6A). Differences between treatment groups at 11 weeks post-primary immunization were not statistically significant.

**Mucosal anti-LPS IgA.** Animals receiving a p.o. boost with EL23 reached maximum mucosal anti-LPS IgA levels at 4 weeks post-primary immunization, while animals boosted either i.p or p.o. with LT-B reached a maximum at 8 weeks, (Fig. 6B). Differences between treatment groups at 11 weeks post-primary immunization were not statistically significant.

Therefore, mice receiving strain EL23 p.o. developed progressively increasing mucosal and serum antibody responses to both LT-B and the LPS of the vaccine strain. Boosting i.p. with LT-B gave the highest sustained levels of antitoxin serum IgG and serum IgA, while boosting i.p. or p.o. with LT-B gave equivalent mucosal antitoxin IgA responses.

**In vitro toxin neutralization.** Samples of mucosa from nonboosted animals from the week 5 sacrifice were pooled and tested for the ability to neutralize the biological activities of cholera toxin and LT in the mouse Y1 adrenal cell assay.

TABLE 1. Comparison of distribution of LT-B produced by *E. coli*, *S. typhi* derivative SE12, and *S. enteritidis* serotype dublin derivative EL23

| Strain | Amt (μg/ml) of LT-B produced (%)[a] | |
| --- | --- | --- |
| | Cell associated | Extracellular |
| JM83(pJC217) | 13.5 (96.7) | 0.460 (3.30) |
| SE12 | 6.60 (99.9) | 0.001 (0.02) |
| EL23 | 4.73 (94.4) | 0.281 (5.60) |

[a] Distribution of LT-B was determined by ELISA as previously described (7) after overnight growth at 37°C on a gyratory shaker. Cells were separated by centrifugation, and supernatants were filter sterilized before testing. Amounts of LT-B in each sample were determined by extrapolation from a standard curve of a known quantity of purified *E. coli* LT-B.
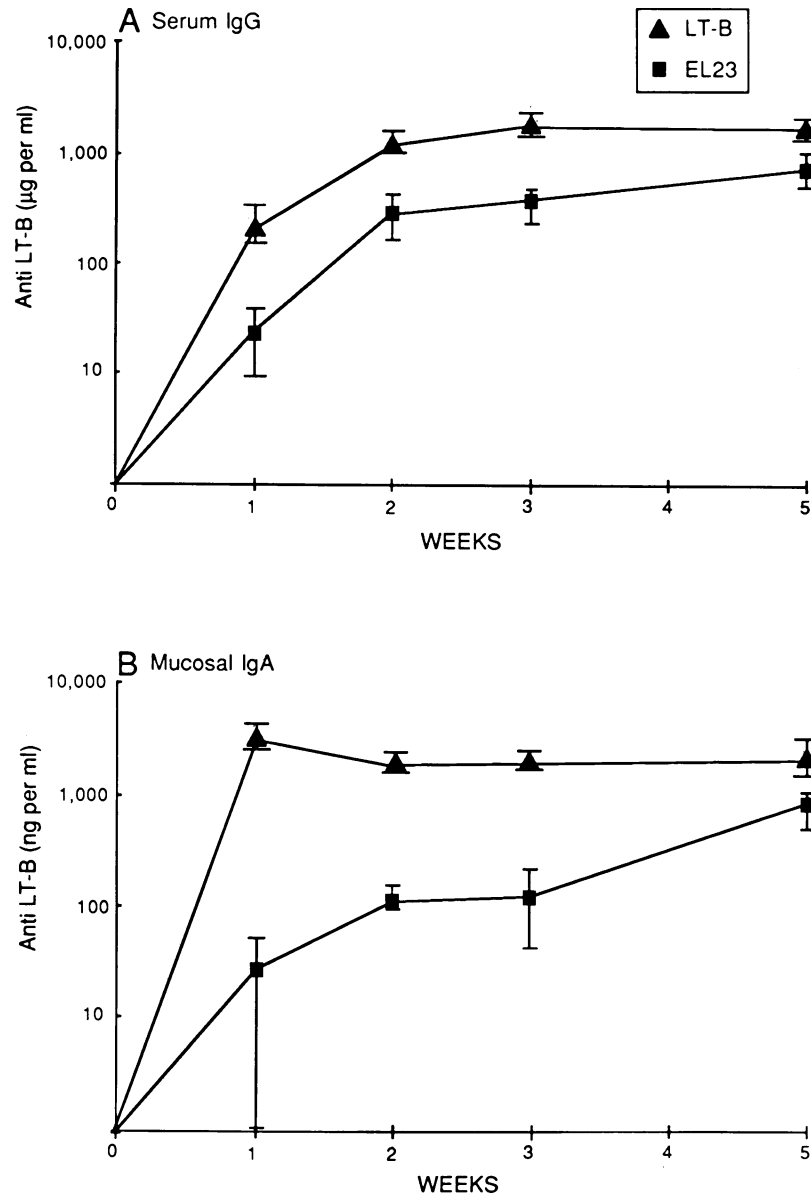
FIG. 4. Serum IgG and mucosal IgA antitoxin responses after p.o. immunization with either LT-B or *S. enteritidis* serotype dublin strain EL23. Female BALB/c mice were immunized p.o. with two doses containing either 64 $\mu$g of LT-B or $10^{10}$ CFU each of strain EL23 on days 0 and 4. Groups of animals were sacrificed at weekly intervals thereafter and analyzed for production of antibodies to LT-B. For details see the text. Serum and mucosal antitoxin levels were zero for control animals throughout the study. Each datum point represents the mean of determinations in four to six animals. Standard error bars are shown.

Mucosal IgA was capable of completely neutralizing both of the toxins in this system. Mucosal antitoxic IgA titers against LT and CT were 256 and 128, respectively (Table 2). Determination that neutralization was indeed due to mucosal antitoxic IgA and not to contaminating IgG was accomplished by preincubating the mucosal pool with goat antiserum against mouse IgA, which reduced the neutralizing capacity of the pool, or with rabbit antiserum against mouse IgG, which did not reduce the neutralizing capacity of the pool.

## DISCUSSION

A variety of cholera vaccines have been developed since the identification of the causative organism by Koch, the most widely used being parenterally administered killed whole-cell vaccines (21). Unfortunately, parenterally administered vaccines are not effective at stimulating mucosal antibody production. Since cholera toxin and its B subunit are good mucosal antigens for development of IgA responses, live oral cholera vaccines should be effective at stimulating mucosal antibody production. This has proven to be the case for some of the mutant strains tested, although immunity is primarily antibacterial and none is completely safe and effective. An alternative in the development of live oral vaccines was demonstrated by Formal et al. (13). In that study, the plasmid responsible for form I antigen synthesis in *Shigella sonnei* was transferred to *galE S. typhi* Ty21a, which invades normally but does not survive owing to lack of Vi antigen and to lysis resulting from toxicity of accumu-
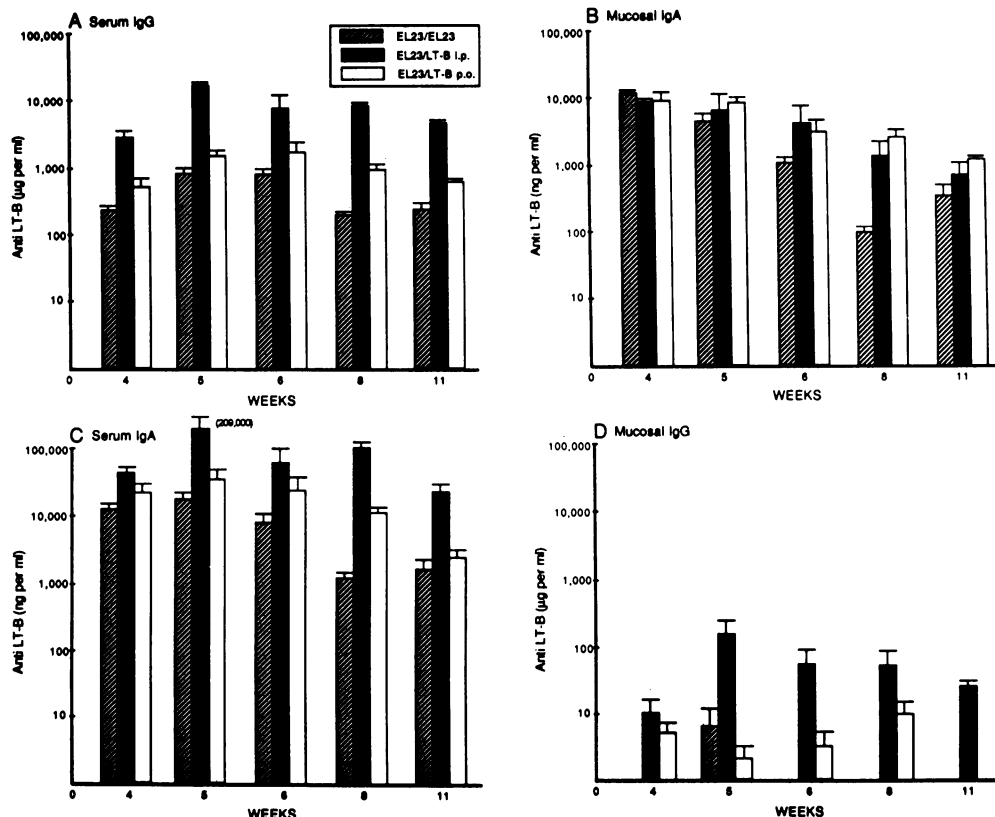
FIG. 5. Serum and mucosal antitoxin responses after oral immunization with *S. enteritidis* serotype dublin strain EL23. Female BALB/c mice were immunized p.o. with two doses containing $10^{10}$ CFU each of strain EL23 on days 0 and 4. At 21 days post-primary immunization, groups of animals were boosted with $10^{10}$ CFU of EL23, 100 μg of LT-B given i.p., or 100 μg LT-B given p.o. Groups of animals were sacrificed at weekly intervals thereafter and analyzed for production of antibodies to LT-B by ELISA. Values shown begin at week 4 post-primary immunization (i.e., 1 week after booster immunizations). For details, see the text. Serum and mucosal antitoxin levels were zero for control animals throughout the study. Each datum point represents the mean of determinations in four to six animals. Standard error bars are shown.

lated galactose-1-phosphate and UDP-galactose (15). The transconjugant was shown to express the form I antigen and to provide protection against both *Shigella sonnei* and *S. typhi* challenge. In that study, the authors suggested that the *galE S. typhi* strain or other such attenuated mutants might be effective carriers for other potentially protective antigenic determinants.

We subsequently cloned the gene coding for production of LT-B into Ty21a. The extensive immunologic and sequence homology between LT and cholera toxin (4–6, 8) made the transformant a potential multivalent live oral vaccine for both typhoid fever and the cholera-*E. coli*-related diarrheas. The *S. typhi* derivative SE12 produced LT-B and induced a significant serum antitoxin antibody response when injected parenterally into mice and guinea pigs. We were not, however, able to test the full potential of this strain as a live oral vaccine or of this method of antigen delivery as a means of stimulating mucosal antitoxin antibodies, owing to the limited host range of *S. typhi*.

We recently obtained an avirulent mouse *Salmonella* strain capable of producing an infection after oral inoculation of mice analogous to the events associated with oral inoculation of humans with Ty21a. We reasoned that this might provide a suitable model for testing the theory of direct stimulation of the antibody-forming cells in the gut-associated lymphoid tissues with antigen as a means of stimulating significant levels of mucosal antibodies. In this

study, *S. enteritidis* serotype dublin strain SL1438 was transformed with plasmid pJC217, characterized for production of LT-B, and examined for efficacy as an oral vaccine in mice. The derivative strain, designated EL23, produced LT-B that was, by all criteria, identical to the LT-B produced by *E. coli* or by *S. typhi* SE12.

Initial priming studies comparing oral immunization with LT-B with oral immunization with strain EL23 showed that immunization with LT-B produced significantly higher levels of antitoxin serum IgG and initally higher levels of antitoxin mucosal secretory IgA. The differences in IgA levels were no longer significant by week 5. Mice receiving strain EL23 p.o. also developed progressively increasing mucosal and serum antibody responses to the LPS of the vaccine strain. The effect of boosting mice primed p.o. with strain EL23 was also investigated, and boosting i.p. with LT-B gave the highest sustained levels of antitoxin serum IgG and serum IgA, while boosting i.p. or p.o. with LT-B gave equivalent mucosal antitoxin IgA responses. The mucosal antibody response was also shown to be IgA specific and to be capable of neutralizing the biological activities of both LT and cholera toxin in vitro.

While antitoxic immunity is an important component of the overall protective immunologic response to *V. cholerae*, there is ample evidence in the literature indicating the importance of antibacterial immunity as well. The study presented here is not intended to suggest that a multivalent
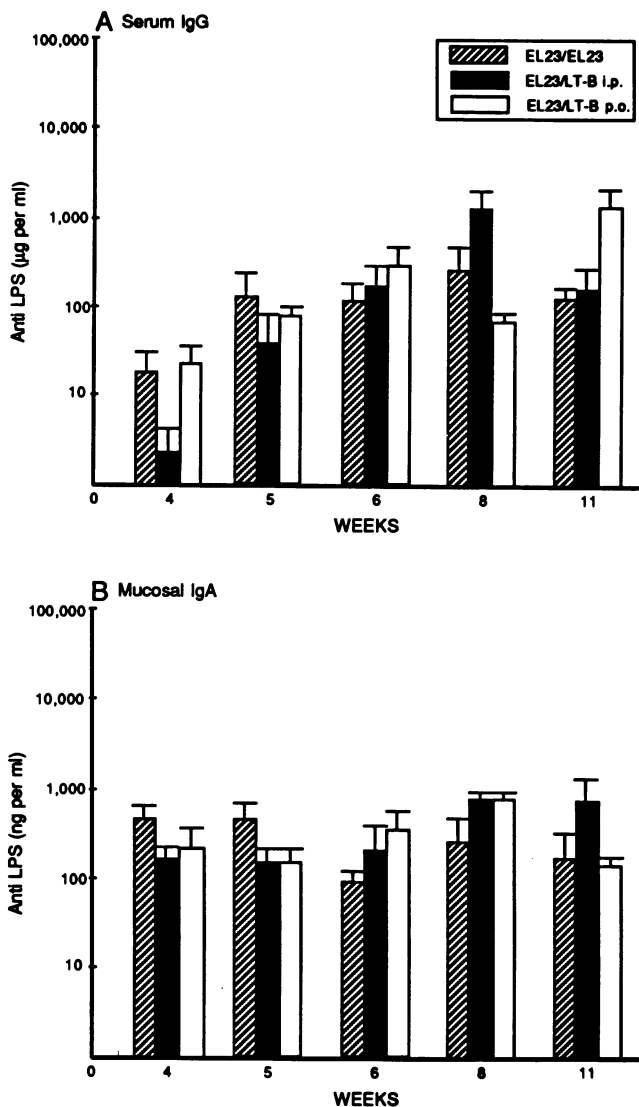
FIG. 6. Serum and mucosal anti-LPS responses after oral immunization with *S. enteritidis* serotype dublin strain EL23. Female BALB/c mice were immunized p.o. with two doses containing $10^{10}$ CFU each of strain EL23 on days 0 and 4. At 21 days post-primary immunization, groups of animals were boosted with $10^{10}$ CFU of EL23, 100 μg of LT-B given i.p., or 100 μg of LT-B given p.o. Groups of animals were sacrificed at weekly intervals thereafter and analyzed for production of antibodies to LPS of the vaccine strain by ELISA. Values shown begin at week 4 post-primary immunization (i.e., 1 week after booster immunizations). For details see the text. Serum and mucosal anti-LPS levels were zero for control animals throughout the study. Each datum point represents the mean of determinations in four to six animals. Standard error bars are shown.

vaccine consisting of *Salmonella* sp. as a carrier and LT-B as a sole cholera-related antigen represents a complete vaccine. Rather, this study was designed to serve as a model system for evaluation of this approach to development of mucosal immunity. It remains to be determined whether antitoxin antibodies developed by this vaccine approach will be more or less protective than those obtained by immunization with previously studied mutant strains of *V. cholerae* or those acquired by natural infection. Studies are currently under way in our laboratory to evaluate the effect of dosage

TABLE 2. Neutralization of adrenal cell activity[a] of cholera and
*E. coli* enterotoxins[b]

| Antiserum | Cholera toxin[c] | LT[c] |
|---|---|---|
| Pooled mucosa | 128 | 256 |
| Pooled mucosa + goat antiserum against mouse IgA | 16 | 32 |
| Pooled mucosa + rabbit antiserum against mouse IgG | 128 | 256 |

[a] The adrenal cell assay was conducted with mouse Y1 adrenal cells in miniculture (25).

[b] Approximately 10 minimal rounding doses were used.

[c] Reciprocal of highest serum dilution showing complete neutralization of biological activity. Pooled mucosa from control animals had no effect.

and timing on vaccine efficacy, to explore alternatives to Ty21a as a vehicle suitable for administration to humans, and to study the potential role of additional determinants (i.e., cholera somatic antigens) in formulation of an optimum vaccine. This study also provides information relative to development of serum antibodies after immunization by this technique. This may have significant implications for others proposing similar approaches with antigens in which a serum antibody response may have a protective role.

The impact of enteric disease on the world's populations is well recognized. A successful immunization program against these organisms would include the following: (i) the most appropriate antigen(s) delivered by the most effective route in a manner designed to stimulate a protective immune response, (ii) development of a minimal number of untoward side effects, and (iii) cost effectiveness. Use of a multivalent live oral vaccine for typhoid fever and cholera-*E. coli*-related diarrheas should meet all these criteria.

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