

Transcutaneous Immunization Using Colonization Factor and Heat-Labile Enterotoxin Induces Correlates of Protective Immunity for Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) diarrheal disease is a worldwide problem that may be addressed by transcutaneous delivery of a vaccine. In several human settings, protective immunity has been associated with immune responses to *E. coli* colonization factors and to the heat-labile toxin that induces the diarrhea. In this set of animal studies, transcutaneous immunization (TCI) using recombinant colonization factor CS6 and cholera toxin (CT) or heat-labile enterotoxin (LT) as the adjuvant induced immunoglobulin G (IgG) and IgA anti-CS6 responses in sera and stools and antibody responses that recognized CS6 antigen in its native configuration. The antitoxin immunity induced by TCI was also shown to protect against enteric toxin challenge. Although immunization with LT via the skin induced mucosal secretory IgA responses to LT, protection could also be achieved by intravenous injection of the immune sera. Finally, a malaria vaccine antigen, merzoite surface protein 1₄₂ administered with CT as the adjuvant, induced both merzoite surface protein antibodies and T-cell responses while conferring protective antitoxin immunity, suggesting that both antiparasitic activity and antidiarrheal activity can be obtained with a single vaccine formulation. Overall, our results demonstrate that relevant colonization factor and antitoxin immunity can be induced by TCI and suggest that an ETEC traveler's diarrhea vaccine could be delivered by using a patch.

Enterotoxigenic *Escherichia coli* (ETEC) diarrhea is a worldwide problem that is responsible for 400,000 to 800,000 deaths per year (20). It is a primary cause of morbidity and mortality in children less than 5 years old (3, 39) and is a significant cause of disease among travelers and military personnel deployed to areas of endemicity (51). The diarrheal disease caused by ETEC is a sequela of disruption of fluid homeostasis at the level of the epithelia of the small intestine due to the actions of toxins secreted by ETEC (35). It is generally thought that after ETEC is ingested, the bacteria adhere to the epithelia of the small intestine through colonization (31, 48). The enterotoxins, heat-labile enterotoxin (LT) and heat-stable toxin (ST), are then secreted into the gut lumen and attach to specific gut receptors, resulting in aberrations in the epithelial cells' fluid homeostasis mechanisms (35, 38). Children acquire natural immunity to ETEC as they age (10), but the factors contributing to this protection, as determined by immune responses and epidemiology, are complex and debated. In more controlled settings, human challenge studies with live organisms have resulted in complete resistance to disease upon rechallenge with organisms that have a homologous colonization factor (CF) (36). Data obtained in these and other studies suggest that immunity to CF and other cell wall antigens contributes to protection (15). The narrowest confirmation of the role of protective CF immunity has come from the successful use of orally ingested CF anti-

body to protect humans against challenge organisms expressing the same CF (17), although this strategy clearly has practical limitations for prophylaxis against ETEC (47). More traditional studies have also suggested that CF immunity is important for protection (15), as well as antitoxin immunity (6). In animal studies, antitoxin immunity to cholera toxin (CT), which has 85% amino acid homology to LT and a nearly identical three-dimensional structure and mechanism of action, has been shown to completely protect against both intestinal toxin and live organism challenges (19, 40, 42).

Identification of target immune responses useful for vaccine development has been aided by extensive characterization of the worldwide distribution of ETEC CFs and the toxins that ETEC produces (48). Vaccines comprising killed whole cells with a variety of CF-expressing strains and adjuvanted with the CT B subunit are in field trials (8). ETEC subunit vaccine trials using CFs are also under way. Although there are many CFs, effective immunity to CFs A/I, A/II, and A/IV could account for approximately 80% of worldwide isolates (48). Addition of anti-LT toxin immunity to a vaccine would further extend this coverage (48). CF A/IV is composed of CS6 with or without CS4 and CS5 and accounts for a significant portion of ST-related ETEC diarrhea (5). The recent cloning of CS6 and the extensive distribution of this antigen have made it an important candidate for a subunit ETEC vaccine (50).

Transcutaneous immunization (TCI) has been shown to induce both serum and mucosal immune responses (14, 22–25). The recent demonstration of the feasibility of using this approach in humans with a simple patch suggests that an ETEC vaccine delivered by a patch is a viable concept (24). Induction

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of robust responses to topical immunization depends on the use of adjuvants that activate resident Langerhans cells and greatly enhance immune responses to vaccine antigens coadministered with these compounds (45). LT and CT are widely used adjuvants (7, 18) and are very effective in the context of skin immunization (45). In the present animal studies, we explored the potential for inducing relevant immune responses to ETEC vaccine components. We found that topical application of CS6 and LT can induce robust and protective immune responses, which suggests that use of a multivalent vaccine with a simple patch may be feasible. Potential protective correlates for immunity were also observed in a guinea pig model used for toxicology studies; antibody-secreting cells and antigen-specific secretory immunoglobulin A (IgA) were detected in the stools of immunized mice. Our data also demonstrate that an adjuvant induces relevant antitoxin immunity in a malaria vaccine delivered by TCI, indicating that the response to an adjuvant in non-ETEC vaccines delivered by TCI may play a protective role against toxin-mediated disease.

MATERIALS AND METHODS

Animals. BALB/c and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice that were 6 to 10 weeks old were maintained in pathogen-free conditions and fed rodent chow and water ad libitum. Female Hartley guinea pigs that were 4 to 6 weeks old were procured from Charles River Laboratories, Inc. (Wilmington, Mass.) and were maintained in pathogen-free conditions; these animals received food and water ad libitum.

Antigens and adjuvants. CT was purchased from LIST Biologicals (Campbell, Calif.). LT was purchased from SSVI (Berne, Switzerland).

To prepare recombinant CS6 (rCS6), the complete four-gene CS6 operon (approximately 5 kb) was cloned into *E. coli* strain HB101 (49, 50) on a pUC19-derivatized plasmid containing the gene for kanamycin resistance. rCS6 was produced by using this clone and a BioFlo 3000 fermentor (New Brunswick Scientific, Edison, N.J.). The fermentation broth was harvested by centrifugation, and the rCS6 was purified by tangential flow filtration followed by precipitation in ammonium sulfate (49). The rCS6 was then buffer exchanged with phosphate-buffered saline (PBS). The resulting purified rCS6 was stored at -30°C until immunization. The purity of rCS6 was determined to be $>98\%$ by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), Coomassie blue staining, and densitometric scanning (4, 44).

Clinical ETEC strains E8775 and E9034A were utilized as the sources of native CS6 (nCS6) and native CS3, respectively. Heat and saline extracts of these two strains grown on CFA agar (16) were treated with ammonium sulfate sequentially at 10% saturation intervals (50). The material that precipitated at 60 and 30% ammonium sulfate saturation contained the greatest quantity and highest purity of nCS6 and native CS3, respectively, as determined by SDS-PAGE and an enzyme-linked immunosorbent assay (ELISA) (data not shown).

Plasmodium falciparum merozoite surface protein 1 (MSP-1) (3D7) was expressed from *E. coli* BL21(DE3) (Novagen) and was purified almost to homogeneity by using three chromatographic steps (nickel affinity chromatography, Q anion-exchange chromatography, and carboxymethyl cellulose cation-exchange chromatography).

Vaccination and challenge. (i) **Vaccination.** Mice were immunized transcutaneously as described previously (45). Briefly, each animal was shaved on the dorsum with a no. 40 clipper, which left no visible irritation or changes in the skin, and then rested for 48 h. Mice and guinea pigs were anesthetized in the hind thigh intramuscularly or intraperitoneally with a ketamine-xylazine mixture during the immunization procedure to prevent self-grooming. The exposed skin surface was hydrated with water-drenched gauze for 5 to 10 min and then lightly blotted with dry gauze prior to immunization. For each animal 25 to 100 μl of immunizing solution was placed on the shaved skin in a 2-cm² area for 1 h. The animals were then extensively washed, tails down, under running tap water for approximately 30 s, patted dry, and washed again.

Passive immunization was accomplished by tail vein injection of pooled hyperimmune serum from a matched strain of mice having an anti-LT IgG titer of more than 10,000 ELISA units. Naive BALB/c mice were injected with 0.5 ml of serum 1 h before oral challenge with LT or bicarbonate buffer. Naive C57BL/6

mice were also passively immunized by using the same procedure 12 h before challenge.

(ii) **Challenge.** An oral exotoxin challenge model that has been described previously (43) was used. BALB/c mice were fed LT (10 μg in 500 μl) suspended in a 10% sodium bicarbonate (NaHCO_3) solution. C57BL/6 mice were fed LT (100 μg per g in 500 μl of 10% NaHCO_3) based on body weight. Control animals received 500 μl of 10% NaHCO_3 alone. To prevent coprophagy, the mice were transferred to cages with wire mesh flooring. The mice were given 10% glucose water but no food for 12 h before challenge and during challenge. Six hours after the challenge, the animals were weighed and sacrificed. The small intestines were then dissected (pyloric valve to ileal-cecal junction), tied off to prevent fluid loss, and weighed. Fluid accumulation (FA) was calculated by using the following formula: $\text{FA} = [G/(B - G)] \times 1,000$, where G is the weight of the gut and fluid (in grams) and B is the body weight (in grams). As determined by this formula, the baseline fluid accumulation in untreated or bicarbonate-fed animals was 30 to 150, depending on the initial body weight of the animal.

Histopathology. Histopathological studies were performed by Gary M. Zaucha of the Comparative Pathology Division of the Walter Reed Army Institute of Research. Two guinea pigs per treatment group and one control animal were used for the pathology study. The animals were euthanized on day 2 after exposure for each of the three vaccination protocols, and each animal was subjected to a complete gross necropsy. The histopathologic examination of the members of the high-dose group included examination of a full complement of tissues, and the skin (skin with hair and the dorsal lumbar exposure site) and liver were evaluated for the members of the remaining groups. The following tissues were collected and fixed with formalin for the high-dose group: brain, pituitary gland, tongue, lung, trachea, esophagus, thyroid, thymus, heart, pancreas, spleen, liver (with associated gallbladder), stomach, small intestine, cecum, colon, mesenteric lymph node, kidney, adrenal gland, urinary bladder, ovary, uterus, salivary glands, submandibular lymph node, bone marrow (sternum), skin with hair, dorsal lumbar exposure site, and gross lesions. The histopathologic findings for individual animals were graded on a scale of 1 to 5 (1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe).

Antibody assays. (i) **ELISA.** Levels of antibodies against CT, LT, native CS3, nCS6, rCS6, and MSP-1₄₂ were determined by ELISA. Immulon-2 polystyrene plates (Dyflex Laboratories, Chantilly, Va.) were coated with 0.1 μg of antigen per well, incubated at room temperature overnight, blocked with a 0.5% casein buffer in PBS, and washed; serial dilutions of specimens were applied; and the plates were incubated for 2 h at room temperature. IgG was detected by using horseradish peroxidase-linked goat anti-mouse IgG(H+L) (Bio-Rad, Richmond, Va.) for 1 h. Anti-LT-specific IgA levels were determined as described above by using horseradish peroxidase-linked goat anti-mouse IgA (Zymed, South San Francisco, Calif.) as the secondary antibody. Secretory IgA antibody levels were also measured by ELISA; in this assay LT-coated plates were sequentially incubated with stool, lung wash, or vaginal wash samples from naive and immunized animals, purified rabbit anti-secretory chain antibody (16 to 24 h at 4°C) (11), and peroxidase-labeled goat anti-rabbit IgG(H+L) (Kirkegaard and Perry, Gaithersburg, Md.) (2 h at room temperature). Bound antibody was revealed by using 2,2'-azino-di-(3-ethylbenzothiazoline sulfonic acid) (ABTS) substrate (Kirkegaard and Perry), and the reaction was stopped after 30 min by using a 1% SDS solution. Plates were read at 405 nm. Antibody titer data are reported below in either units of optical density at 405 nm or ELISA units, which were defined as the inverse dilution of the serum that yielded an optical density of 1.0. Guinea pig anti-rCS6 ELISAs were performed as described above with peroxidase-conjugated goat anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, Pa.) included in the detection step. Anti-secretory chain secondary antibody reacted with antigen-coated (rCS6) plates, resulting in high background values, which made this assay unsuitable for anti-rCS6 SC detection.

(ii) **Immunodot blotting.** One microliter of rCS6 (0.5 μg), nCS6 (1.6 μg), and native CS3 (0.5 μg) was spotted onto nitrocellulose strips (Schleicher and Schuell, Keene, N.H.) and dried overnight. The strips were blocked by incubation in PBS-0.05% Tween 20 (PBS/TW) (Sigma Chemical Co., St. Louis, Mo.) containing 1% bovine serum albumin for 2 h. Primary mouse antibody was diluted 1:1,000 and 1:4,000 and incubated with the strips for 1 h, and this was followed by three washes (1, 5, and 10 min) in PBS/TW. The strips were then incubated in goat anti-mouse IgG labeled with horseradish peroxidase (1:5,000 in PBS/TW, 30 min). After the strips were washed in PBS three times (10 min each), they were developed with 3,3'-diaminobenzidine (Sigma), hydrogen peroxide (Sigma), and cobalt chloride (Mallinckrodt, Paris, Ky.) as described by Harlow and Lane (28). All incubations and washes took place on an orbital shaker at room temperature.

(iii) **Antibody-secreting cell assays in guinea pigs.** Seven days after the last immunization, mononuclear cells were isolated from the spleens and superficial

ventral cervical nodes and washed in RPMI 1640 with 50 mg of gentamicin per ml prior to use in the ELISPOT assay as previously described (29, 30). Washed spleen and lymph node cells were counted and diluted in culture medium (RPMI 1640 with 2 mM glutamine, 50 mg of gentamicin per ml, and 10% fetal bovine serum) to a density of 2.5×10^6 cells/ml. One hundred milliliters of each cell suspension was inoculated into microwells previously coated with 0.1 μ g of CS6 antigen per ml in carbonate coating buffer (pH 9.6) or with coating buffer alone. Each sample was assayed in quadruplicate. After incubation at 37°C for 4 h, the plates were washed, and rabbit anti-guinea pig IgG (1:1,200), IgA (1:700), or IgM (1:800) (ICN Laboratories, Costa Mesa, Calif.) was added. After overnight incubation at 4°C, the plates were washed, and alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma) at a dilution of 1:1,200 was added. After incubation for 2 h at 37°C, the plates were washed, and spots were visualized by adding 100 ml of molten agarose containing 100 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml. Spot-forming cells were then counted with a stereomicroscope.

Preparation of samples. Blood contamination was not apparent upon visual inspection of freshly collected murine stool, lung wash, or vaginal wash specimens. Further testing with Hemastix (Bayer, Elkhart, Ind.) strips indicated that the levels of blood contamination were ≤ 5 to 20 intact red blood cells per μ l or ≤ 0.015 to 0.062 mg of free hemoglobin per dl.

(i) **Stool collection.** Stool pellets were collected the day before challenge after spontaneous defecation, weighed, homogenized in 1 ml of PBS per 100 mg of fecal material, and centrifuged, and the supernatant was collected and stored at -20°C .

(ii) **Lung wash.** Each mouse was exsanguinated, the trachea was transected, 22-gauge polypropylene tubing was inserted, and PBS was gently infused to inflate the lungs. The infused material was then withdrawn and reinfused; a total of three cycles was used, and the material was stored at -20°C .

(iii) **Vaginal wash.** The vaginal cavity was gently lavaged by repeated insertion and aspiration of PBS (80 μ l) into the vaginal cavity a total of three times. The vaginal material was centrifuged for 10 min at $10,000 \times g$, and the supernatant was transferred to a clean container and stored at -20°C .

Proliferation assays. BALB/c mice were immunized on the skin with MSP-1₄₂ alone or with CT and MSP-1₄₂ at zero time and after 4, 8, and 12 weeks. Spleen and draining lymph node (inguinal) tissues were removed 24 weeks after the primary immunization. Single-cell suspensions were prepared from spleen tissue from individual mice or from lymph nodes pooled for each group. Cells (4×10^5 cells per well) were cultured in 96-well plates for 5 days at 37°C in the presence of 5% CO₂ and in the presence or absence of 10 μ g of MSP-1₄₂ antigen per ml. Concanavalin A at a concentration of 5 μ g/ml was used as a positive control. The culture medium contained RPMI 1640 (BioWhittaker Inc., Walkersville, Md.), 10% fetal calf serum (Gibco BRL, Grand Island, N.Y.), penicillin (10 U/ml; BioWhittaker), streptomycin (100 μ g/ml; BioWhittaker), L-glutamine (2 mM; Sigma), and HEPES (10 μ M; Bio-Rad, Hercules, Calif.). [³H]thymidine (1 μ Ci/well) was added to the cultures during the last 20 h of the 5-day culture period. Thymidine incorporation was assessed by harvesting cellular DNA on glass fiber filters, followed by liquid scintillation counting.

CD4⁺ cell purification. CD4⁺ cells were isolated from pooled spleen cells obtained from the CT-MSP-1₄₂ immunization group by using a CD4⁺ T-cell selection column as recommended by the manufacturer (R & D Systems, Minneapolis, Minn.). Cells eluted from the column (CD4⁺) were cultured in 96-well plates (1×10^5 cells per well) in the presence or absence of 3×10^5 irradiated (3,000 rads) feeder cells from naïve mice. Proliferation assays were conducted in the presence or absence of antigen stimulation as described above.

Statistical analysis. Unless otherwise indicated, the ELISA data shown below are the geometric means of values obtained with individual animals, and the error bars represent 2 standard deviations of the mean. Comparisons between antibody titers and fluid accumulation values for groups were performed by using an unpaired, two-tailed Student's *t* test, and *P* values of <0.05 were considered significant.

RESULTS

Immunization on the skin with bacterial adjuvant and CF CS6 results in a protective antibody response. To determine if topical administration is an effective method for inducing relevant ETEC immune responses, mice were immunized four times by TCI with CT and rCS6, assayed for anti-CS6 responses, and subsequently challenged orally with CT holotoxin, and the degree of acute intestinal swelling (fluid accumulation)

was determined 6 h later. A positive control group was immunized by the intramuscular route with 5 μ g of rCS6 in alum, and a negative control group received rCS6 alone on the skin. Antibodies that reacted with rCS6 antigen were apparent after the first immunization in animals that received either a low dose (10 μ g) or a high dose (100 μ g) of adjuvant, and the titers continued to rise after the first and second booster immunizations (Fig. 1A). The immune response to CS6 in the presence of adjuvant (10 or 100 μ g) was greater ($P < 0.05$) than the response to antigen alone delivered by TCI at 12 weeks. The geometric mean anti-CS6 titers were greatest in the high-dose group (100 μ g of CT) following the third immunization, and a higher geometric mean anti-CS6 titer was obtained when TCI was used than when intramuscular immunization was used, but neither difference was statistically significant. Anti-CT titers were elevated in both of the CT-adjuvanted groups at all time points (Fig. 1B). Animals immunized with CS6 alone on the skin failed to develop a consistent antibody response to the antigen or the adjuvant.

Groups of naïve mice and mice that received antigen alone or CT (100 μ g) plus CS6 (100 μ g) on the skin were selected for oral challenge with CT after TCI. The group that received CS6 alone and the group that received CT plus CS6 were boosted 11 weeks after the third immunization (i.e., after 19 weeks). Two weeks later, the animals were fed either bicarbonate buffer alone (10% NaHCO₃) or bicarbonate buffer containing 10 μ g of CT, and the resulting intestinal swelling was measured as described in Materials and Methods. The results of the toxin challenge are shown in Fig. 1C. The intestines from naïve mice fed bicarbonate alone had a baseline fluid accumulation value of 103 (range, 78 to 146). Oral administration of CT to naïve mice resulted in a twofold increase in the fluid accumulation value (mean, 209; range, 164 to 359). Similarly, mice vaccinated with rCS6 alone and subsequently fed CT also exhibited an approximately twofold increase in the fluid accumulation value (mean, 192; range, 119 to 294). In contrast, mice vaccinated with CT by TCI developed negligible intestinal swelling following challenge (mean fluid accumulation value, 105; range, 84 to 120), and the fluid response was indistinguishable from that observed for the naïve group fed bicarbonate buffer alone ($P < 0.5$).

Comparable adjuvant effects of CT and LT for topically administered CS6 antigen. Use of LT as an adjuvant for an ETEC vaccine may be desirable, as LT is the causative agent in a significant number of cases of ETEC diarrhea (51) and thus can function both as an antigen and an adjuvant. To test the relative potencies of CT and LT as adjuvants for rCS6, mice were immunized on the skin three times at 4-week intervals with 100 μ g of rCS6 and different doses of CT or LT (10, 20, and 100 μ g). The resulting serum anti-rCS6 and adjuvant titers were assessed 2 weeks after the final immunization. As expected, anti-adjuvant (CT or LT) IgG titers were apparent with all adjuvant doses, and the titers were highest and most consistent for the high-dose animals (the animals that received 100 μ g) (Fig. 2). In contrast, while the members of all of the CS6 and LT-CT groups developed elevated anti-CS6 titers, the responses were greatest at the lowest LT doses (10 versus 100 μ g) and generally comparable to the responses observed previously when animals were immunized intramuscularly.

Serum antibodies from mice immunized with rCS6 and LT

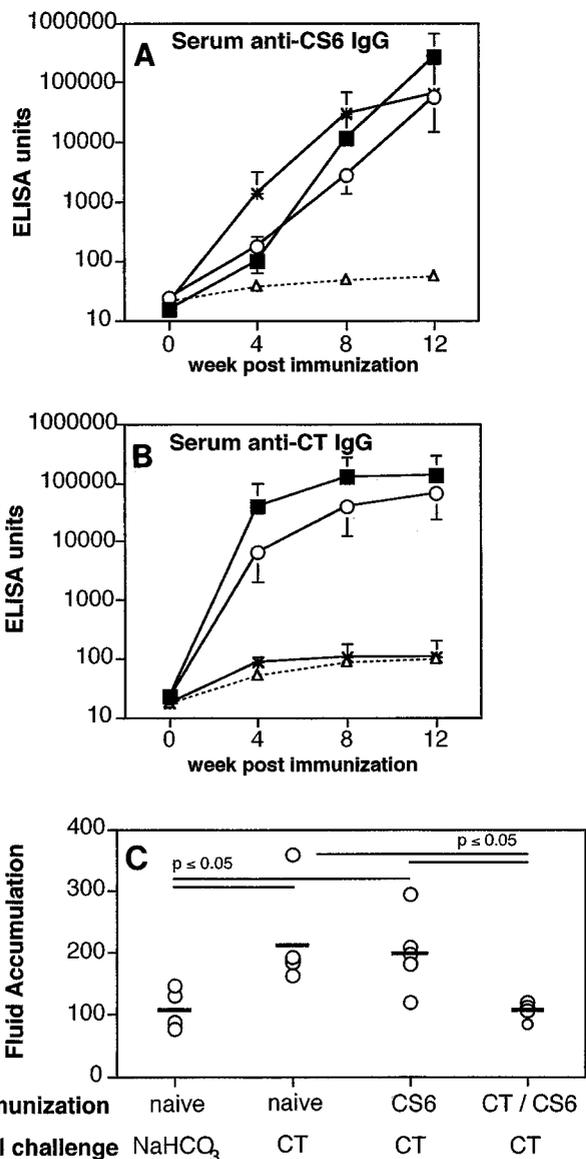


FIG. 1. Systemic antibody responses (A and B) and protection from oral challenge (C) following skin immunization with rCS6 and CT. (A and B) Five C67BL/6 mice were immunized on the skin at zero time and after 4 and 8 weeks with 100 µg of rCS6 (△) or with 100 µg of rCS6 and 10 µg of CT (○) or 100 µg of CT (■). A control group received 5 µg of rCS6 in alum intramuscularly (*). Serum collected prior to immunization and 4, 8, and 12 weeks later was analyzed for rCS6-specific IgG (A) and CT-specific IgG (B) by ELISA. The data points are the geometric mean titers for each group at zero time and after 4, 8, and 12 weeks. (C) Groups of animals selected for challenge were boosted with antigen and adjuvant 11 weeks following the third immunization. Two weeks after the last boost (study week 21), mice were fed 10% bicarbonate buffer with or without 10 µg of CT. Six hours later, fluid accumulation in the small intestine was assessed as described in Materials and Methods. Each circle represents the fluid accumulation in an individual animal. Statistically significant differences ($P < 0.05$) in fluid accumulation are indicated by lines.

recognize native CS6. As shown in Fig. 1 and 2, mice immunized with rCS6 produced a high titer of serum IgG that reacted with the recombinant protein used in the ELISA. While these results suggested that topical immunization might effec-

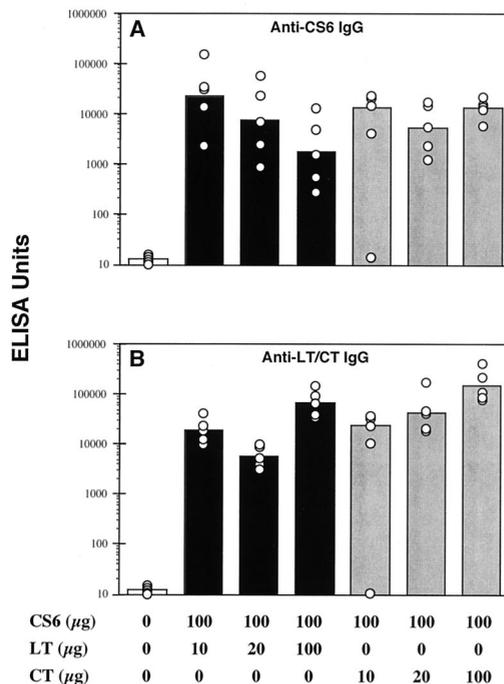


FIG. 2. Adjuvant effects of LT and CT for topically administered rCS6 antigen. Five C57BL/6 mice were immunized on the skin with 100 µg of rCS6 and LT (10, 20, or 100 µg) or CT (10, 20, or 100 µg) at zero time and after 4 and 8 weeks. Serum was collected 2 weeks after the last immunization. CS6-specific IgG titers were determined by ELISA. LT- or CT-specific titers were determined depending on the adjuvant used for immunization. Each circle represents the titer of an individual animal. Open bars, naïve mice; solid bars, LT-immunized mice; gray bars, CT-immunized mice.

tively attenuate ETEC infection and disease, it was important to determine if the induced antibodies reacted with the nCS6 present on *E. coli* isolates. To test the specificity of the anti-CS6 response, sera from rCS6-immunized mice were analyzed for reactivity to nCS6 protein by ELISA (Fig. 3) and immunodot blot assays (Fig. 4). In the ELISA, each of the three samples with reactivity to rCS6 and LT (Fig. 3A, C, and E) exhibited specificity for nCS6 protein but not for native CS3 protein (Fig. 3B, D, and F). Similarly, as determined by the immunodot blot assays, mice immunized with rCS6 and LT reacted with both the immunizing rCS6 antigen and partially purified nCS6 (Fig. 4B to D, strips 1 and 2), and little or no reaction was observed with preimmune sera (strips 3 and 4). None of the mouse sera reacted with the native CS3 antigen control. Serum from a mouse that was immunized intramuscularly with rCS6 (Fig. 4A) responded in similar ways to both nCS6 and rCS6, as did sera from topically immunized mice; in addition, a BALB/c mouse (Fig. 4D) responded like the C57BL/6 mice (Fig. 4A to C). Thus, skin immunization with rCS6 induced a serum antibody capable of recognizing native antigen.

Antibodies to LT actively and passively protect mice against oral challenge with LT. Although LT, the causative agent of LT-mediated ETEC disease, is highly homologous to CT and exhibits cross-protection with CT B-subunit antibodies (6), direct protection against LT oral challenge using LT antibodies

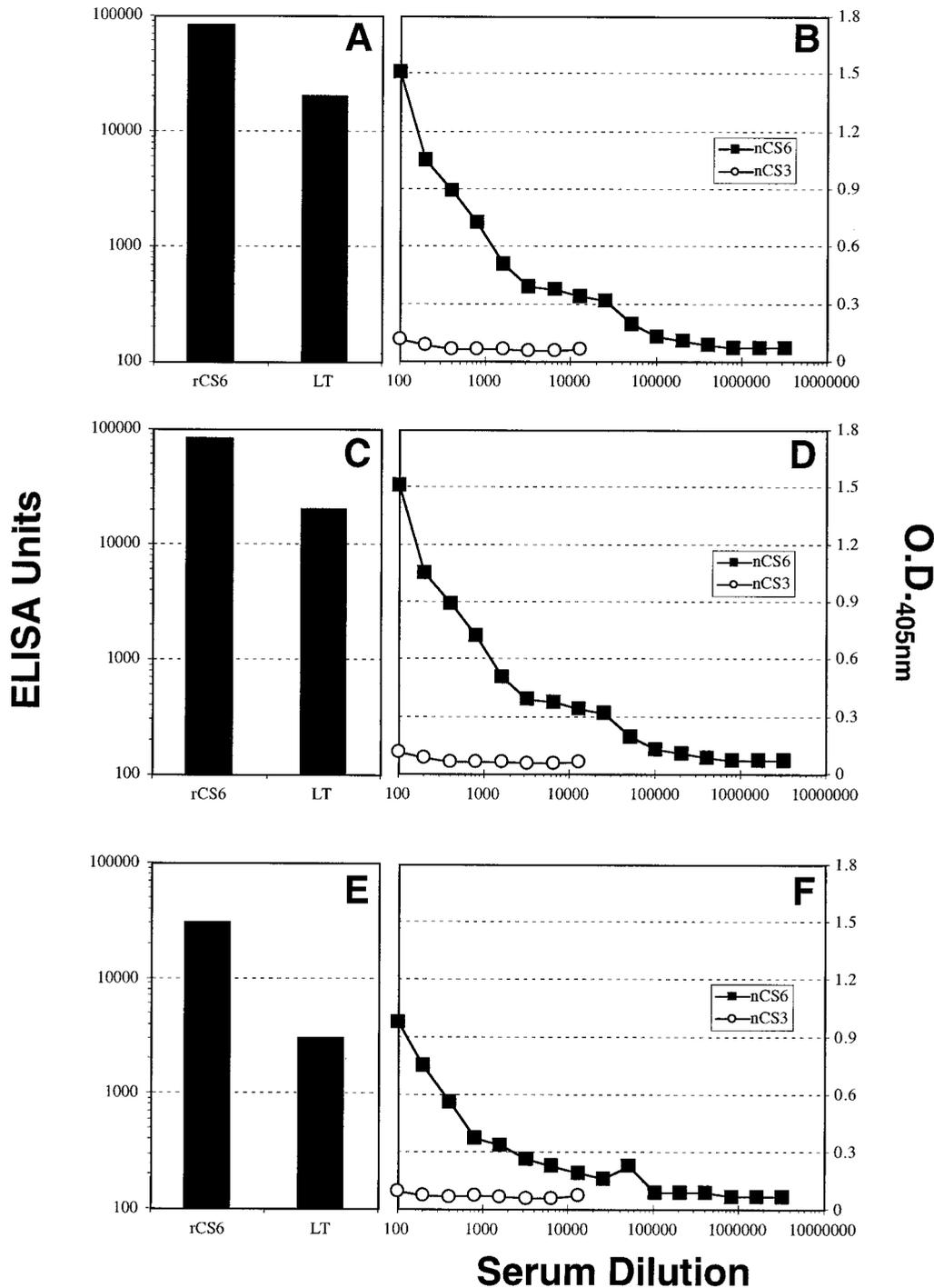


FIG. 3. Antibodies from rCS6-vaccinated animals recognize nCS6 antigen in a liquid phase ELISA. C57BL/6 (A to D) and BALB/c (E and F) mice were topically immunized with rCS6 and LT three times at 3-week intervals. Sera were collected 4 weeks after the final immunization and assayed by ELISA for IgG specific for rCS6, LT, nCS6, and native CS3 (nCS3). (A, C, and E) LT and rCS6 serum IgG responses of individual animals, expressed in ELISA units. (B, D, and F) nCS6 and native CS3 responses of individual animals, expressed as titration curves. The data are representative of the data obtained for immunized mice with serum reactivities specific for rCS6, LT, nCS6, and native CS3. O.D._{405nm}, optical density at 405 nm.

has not been shown. Mice immunized with LT and rCS6 by TCI were orally challenged with LT as described above. High levels of anti-LT IgG were detected in the sera of immunized mice (geometric mean for BALB/c mice, 36,249 ELISA units;

geometric mean for C57BL/6 mice, 54,792 ELISA units). For the oral toxin challenge experiments, two strains of mice with different sensitivities to challenge were used. C57BL/6 mice are much more sensitive to the effects of LT toxin challenge than

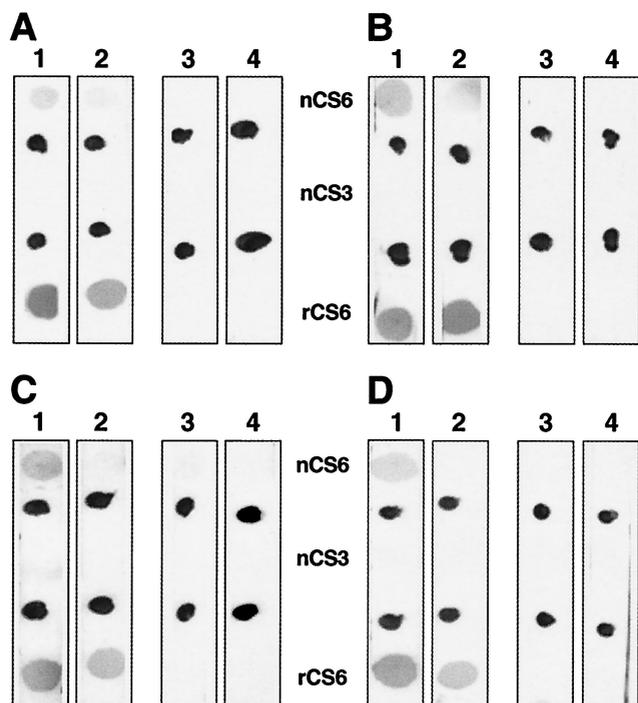


FIG. 4. Mouse serum response to nCS6 and rCS6. Each strip was spotted with 1 μ l of each of three antigens; the top protein (above the higher black marker spot) was nCS6 (1.6 μ g), the middle protein (between the two black marker spots) was native CS3 (nCS3) (0.5 μ g), and the bottom spotted protein (below the lower black marker spot) was rCS6 (0.5 μ g). Strips 1 and 2 were treated with the postimmune mouse serum at dilutions of 1:1,000 (strip 1) and 1:4,000 (strips 2), respectively, while strips 3 and 4 were treated with preimmune serum from the same individual at dilutions of 1:1,000 and 1:4,000, respectively. (A) Serum from mouse 417; (B) serum from mouse 354; (C) serum from mouse 357; (D) serum from mouse 432. Mouse 417 was immunized intramuscularly with rCS6 and alum; all other mice were immunized transcutaneously with rCS6 and LT. Mouse 432 was a strain BALB/c mouse. All other mice were strain C57BL/6 mice.

BALB/c mice, and protection in both strains suggests that the protective effect might be observed in more genetically diverse settings. As shown in Fig. 5A and B, significant protection against LT challenge was observed in both strains ($P < 0.05$).

Studies of dog and human ETEC disease suggest that serum antibody contributes to protection against diarrhea due to intact bacteria as well as isolated toxin (40–42). Consistent with this suggestion, we and other workers have previously reported that a transcutaneously elicited serum factor protects animals against a lethal intranasal challenge with CT (2, 22). Thus, we postulated that a serum factor, presumed to be antitoxin antibody, might also contribute to the prevention of toxin-induced intestinal swelling in topically immunized mice. The host-protective role of antitoxin antibody serum was evaluated by quantifying the intestinal swelling elicited by oral LT challenge of naïve and passively immunized mice that received serum from transcutaneously immunized animals. The effect of passive immunization was evaluated in both BALB/c and C57BL/6 mice. Oral administration of LT to naïve mice consistently induced fluid accumulation that was apparent upon visual inspection (Fig. 5C and D). In contrast, passively immunized mice devel-

oped negligible fluid accumulation, and the magnitude of this fluid accumulation was comparable to that observed in the groups fed buffer alone (Fig. 5C and D). Thus, the passively immunized mice given antibody from topically immunized mice were protected from the sequelae of oral toxin challenge. Together, these results indicate that transcutaneously immunized mice produce serum antibodies capable of protecting animals from toxin exposure.

Mucosal IgG, IgA, and secretory IgA responses to ETEC antigen following topical immunization. While serum IgG responses are associated with host protection against many infectious agents, mucosal immune responses are considered important for attenuation and prevention of mucosally acquired pathogens, particularly intestinal pathogens, such as ETEC. To determine if topical immunization with rCS6 induces mucosally detectable antibody responses, IgG and secretory IgA responses were analyzed in fecal, lung, and vaginal specimens harvested from mice immunized on the skin with rCS6 and adjuvant (Fig. 6 and 7). In the first experiment, C57BL/6 mice were immunized three times with CS6 alone, LT plus CS6, or CT plus CS6. CS6-specific IgG levels were evaluated in fecal, lung, and vaginal wash specimens collected 9 weeks after the third immunization. Immunization with rCS6 alone did not induce elevated CS6-specific IgG levels in fecal, lung, or vaginal wash specimens. In contrast, both lung and vaginal wash specimens of all three animals in the CT-CS6 group contained detectable anti-CS6 IgG (Fig. 6). Similarly, CS6-specific IgG antibody was observed in lung and vaginal specimens from the LT-adjuvanted group and in fecal specimens from the CT- and LT-adjuvanted groups, although the responses were less consistent. The method used to collect fecal samples may have affected the consistency of the fecal antibody results, especially if the responses were modest, and other collection methods are being investigated.

Locally produced IgA is typically a dimeric protein associated with a secretory chain that allows transport across the epithelial membranes. To determine if topical immunization could induce secretory IgA production, animals were immunized twice on the skin with LT, and the antigen-specific IgG, IgA, and secretory IgA titers in mucosal specimens were evaluated by ELISA (Fig. 7). Compared to specimens from naïve animals, immunization with LT induced antigen-specific IgG and IgA in the fecal and vaginal specimens of all 10 immunized mice. More importantly, secretory IgA was readily detected in all 10 fecal and vaginal specimens tested (Fig. 7).

Induction of protective antitoxin immunity following coadministration of CT and a malarial vaccine antigen. Vaccination targeted against multiple infectious agents is desirable in developing countries where relatively low life expectancies and high morbidity and mortality rates are associated with infection of individuals with more than one pathogen. To determine whether TCI might be used to induce protection against multiple unrelated infectious agents, mice were simultaneously vaccinated with CT and a C-terminal 42-kDa fragment of *P. falciparum* MSP-1 (MSP-1₄₂). In these experiments, CT (0, 10, or 100 μ g) and MSP-1₄₂ (100 μ g) were applied to the skin at zero time and after 4, 8, and 13 weeks. Mice were considered responsive to MSP-1₄₂ if the postimmunization titer was threefold or more greater than the optical density measured in the preimmunization serum at a dilution of 1:100. Based on this

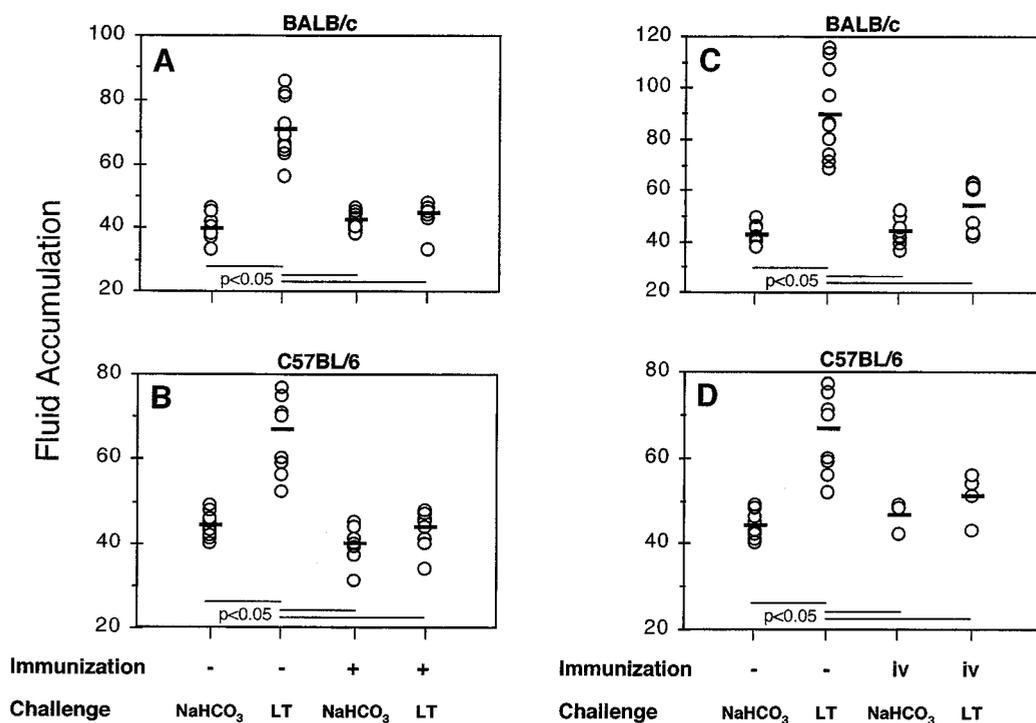


FIG. 5. Serum from topically immunized mice passively protects naïve mice from oral toxin challenge. BALB/c and C57BL/6 mice were vaccinated on the skin with LT at zero time and after 2 weeks (A and B) or were passively immunized by transfer of serum from topically immunized mice (C and D). Naïve and immunized (actively and passively) animals were subdivided into two groups and fed either 10% NaHCO₃ alone or LT in 10% NaHCO₃. Actively immunized mice (A and B) ($n = 10$) were challenged 2 weeks after the second immunization. Passively immunized animals were challenged either 12 h after intravenous injection of the immune serum (C57BL/6) ($n = 4$) or 1 h after intravenous (iv) injection (BALB/c) ($n = 10$). Fluid accumulation was assessed 6 h after the challenge. Statistically significant differences ($P < 0.05$) in fluid accumulation are indicated by lines.

criterion, MSP-1₄₂ antibodies (Fig. 8A) were detected in serum from mice immunized with CT and MSP-1₄₂ together but not in serum collected from the control group (MSP-1₄₂ alone) or serum harvested prior to immunization (prebleed serum). To evaluate the effectiveness of the anti-CT antibody response in the mice immunized with both proteins, animals that received CT (100 μ g) plus MSP-1₄₂ (100 μ g) and developed high levels of anti-CT antibodies were orally challenged with CT, and the degree of intestinal swelling (fluid accumulation) was compared with the degree of intestinal swelling induced in mice vaccinated with MSP-1₄₂ alone. All of the animals immunized on the skin with CT and MSP-1₄₂ together exhibited lower fluid accumulation levels ($P < 0.01$) than comparably challenged mice in the group exposed to MSP-1₄₂ alone (Fig. 8B). Moreover, spleen and draining lymph node cells from the immunized mice exhibited strong antigen-specific proliferative responses in vitro (Fig. 8C and D), to which CD4⁺ T cells contributed (Fig. 8E and F). These results suggest that the antibodies to the adjuvant may provide protection against LT-mediated disease while they function as an adjuvant for other antigens, such as candidate malaria vaccine antigens.

Immune responses to ETEC antigens in guinea pigs. To assess the capability of TCI to induce antibody-secreting cells, an established guinea pig antibody-secreting cell animal model was used. The guinea pig test system also provided a conventional model for assessing toxicological responses to topical

administration of adjuvant and antigen. In these studies, guinea pigs were exposed to different doses of LT (12 to 100 μ g) and rCS6 (25 to 200 μ g) on the skin at zero time and on days 21 and 42. Serum was collected for serological analysis on days 1, 20, 41, and 56, and antibody titers to the antigen and adjuvant were determined by ELISA. As in the mouse studies, TCI administration of the rCS6-LT vaccine resulted in induction of CS6 and LT antibody responses that appeared to be related to the CS6 and LT concentrations (Table 1). The presence of serum antibody to CS6 was confirmed by the presence of antibody-secreting cells specific for CS6 in spleen and draining lymph node tissues. An ELISPOT assay conducted with freshly isolated cells from sham PBS-immunized animals and animals immunized with CS6 and LT revealed that there was a significant increase ($P < 0.05$) in the number of rCS6-specific IgG-producing cells in all 4 of the animals exposed to antigen. The number of antigen-specific IgA- and IgM-producing cells also appeared to increase, although the actual number of cells detected was smaller and less consistent (Table 2).

Toxicology studies. For each of the three levels of exposure two guinea pigs from each treatment group and one guinea pig from the control group were used for a pathological analysis (Table 1). A full complement of tissues was subjected to histopathologic evaluation for the high-dose group. Only skin and liver tissues were collected from members of the remaining groups (control, low dose, and intermediate dose). A gross

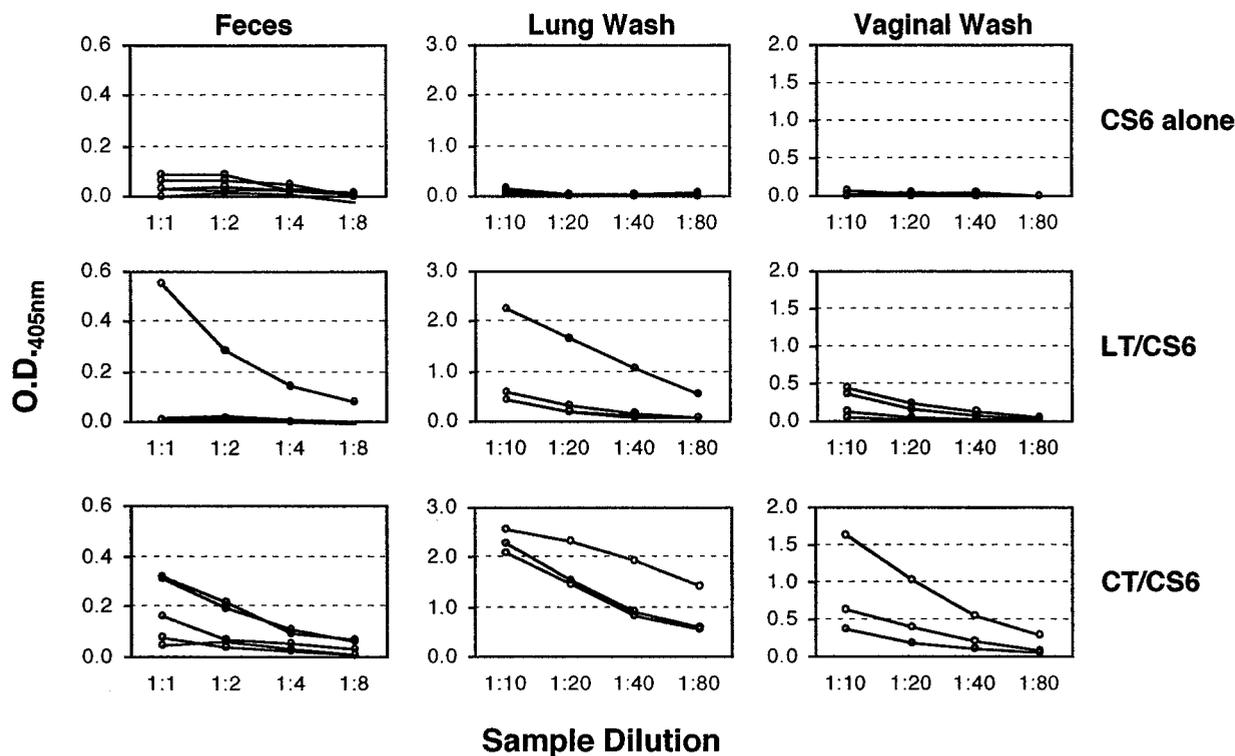


FIG. 6. Mucosal antibody responses in mice immunized on the skin with LT, CT, and/or CS6. Fecal (left panels), lung (middle panels), and vaginal (right panels) IgG responses to CS6. C57BL/6 mice ($n = 3$ to 5) were immunized on the skin with rCS6 alone (100 μg) (upper panels), 100 μg of LT plus 100 μg of rCS6 (middle panels), or 100 μg of CT plus 100 μg of rCS6 (lower panels) at zero time and after 3 and 8 weeks by TCI. Nine weeks after the last immunization, fecal, lung, and vaginal specimens were collected and analyzed for CS6-specific IgG by ELISA. The dilution curves for individual animals are shown. The group that received only rCS6 was the control group. O.D._{405nm}, optical density at 405 nm.

necropsy and histopathology analysis of the high-dose group revealed no systemic lesions that could be attributed to administration of the test preparations for any of the levels of exposure. Hepatic necrosis was observed grossly in all animals, including the PBS controls, but there was no correlation of the findings with the treatment groups. The serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sodium, potassium, and blood urea nitrogen contents were evaluated and were determined to be normal for all treatment groups (data not shown).

Topical application of LT plus CS6 resulted in minimal to mild inflammatory changes limited to the local sites of exposure, and increasing the dose to more than 25 μg of LT and 50 μg of CS6 had no appreciable effect on the severity of the local response. The typical results included infiltration of the superficial dermis by low numbers of granulocytes and lymphocytes (inflammation), mild thickening of the epidermis by hyperplasia of keratinocytes (acanthosis), and occasional small foci where epidermal cells had lost cohesion, resulting in the formation of intraepidermal vesicles containing free keratinocytes (acantholysis). Minimal changes were seen in the group that received the lowest dose (12 μg of LT and 125 μg of CS6) at the three time points, and no skin changes were observed in the controls exposed to PBS. In both the mice and guinea pigs, there was no clinical progression in the severity of the skin responses after repeated immunization, and when vesicles

were observed, they either resolved or crusted and resolved spontaneously over several days.

DISCUSSION

Needle-free delivery of vaccines has become a priority for the World Health Organization and other public health agencies. The potential advantages of needle-free immunization using a patch have been described elsewhere (24), but use of a vaccine patch for travelers requiring protection against infectious diarrhea may be a specific application of TCI. Furthermore, development of a stabilized, cold-chain-free patch may have great relevance for TCI against childhood and infant diarrheas in developing countries. The objective of the studies described here was to explore the feasibility of an ETEC vaccine delivered by TCI.

Although there are several potential approaches to formulation of an ETEC vaccine, such as whole-cell vaccines, we have focused on a subunit vaccine approach that incorporates elements of immunity against the toxin that causes the diarrhea and CFs that allow the ETEC organisms to become established in the intestine. Previous studies have shown that CT delivered by TCI can elicit strong and protective antitoxin immunity (22). We advanced this concept by investigating the role of anti-LT immunity elicited by TCI in an animal challenge model relevant to ETEC. We also examined induction of mucosal and

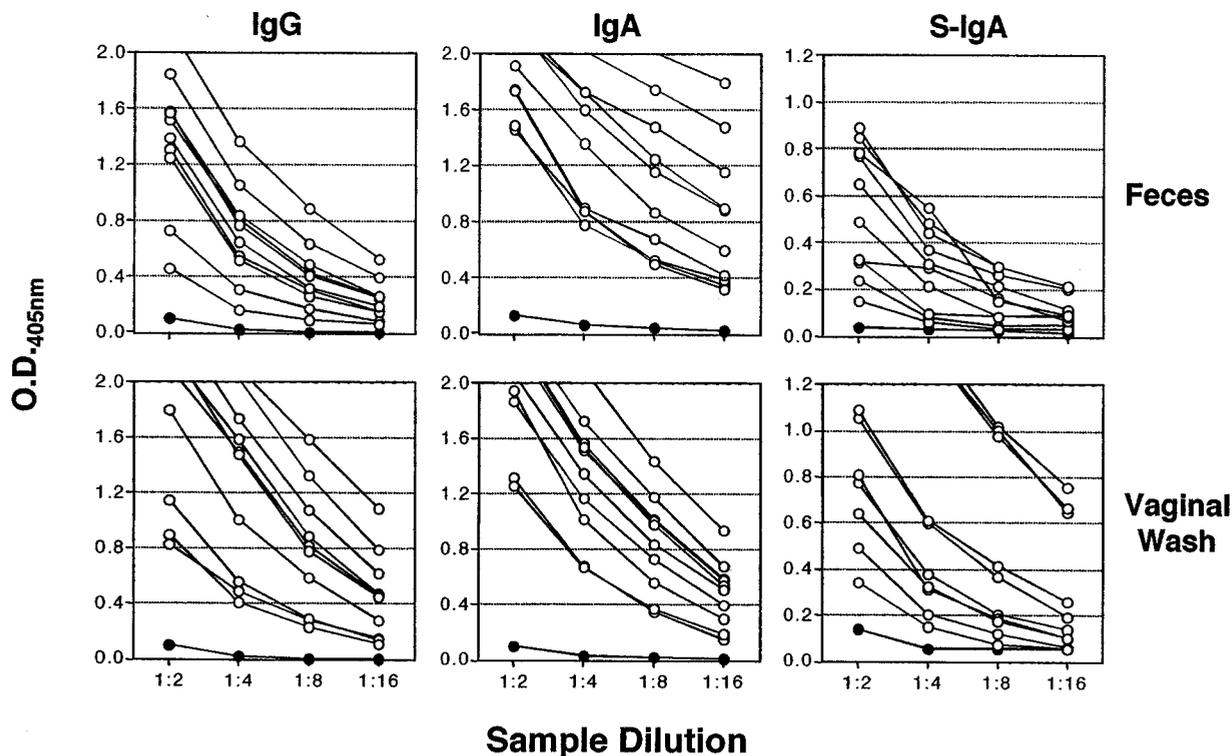


FIG. 7. Induction of secretory IgA (S-IgA) following TCI: fecal and vaginal anti-LT IgG (left panels), IgA (middle panels), and secretory IgA (right panels) responses. BALB/c mice ($n = 10$) were immunized on the skin with LT ($100 \mu\text{g}$) at zero time and after 2 weeks. Fecal and vaginal wash extracts were collected prior to immunization (naïve mice) and 3 weeks following the second immunization for analysis of LT-specific IgG, IgA, and secretory IgA. Symbols: ●, dilution curves for naïve mice ($n = 10$); ○, dilution curves for individual immunized animals ($n = 10$). O.D._{405nm}, optical density at 405 nm.

systemic antibodies to CS6, a widely distributed CF found in ST-secreting organisms (48).

Initial studies bridged previous experience with CT and showed that CT could act as an adjuvant and that a large antigen, such as rCS6, could be successfully delivered into the skin to elicit anti-CS6 immunity. The CS6-specific immunity was robust and comparable to the immunity obtained with antigen delivered intramuscularly. The results obtained were not fully expected, as the CS6 polymeric antigen ($>1 \text{ mDa}$) was larger than CT or LT and was the largest protein antigen used in studies to date. Because of this, relatively large doses of CS6 were used. More recent studies have shown that much smaller doses of antigen may be used (45; unpublished observations), and studies with whole viruses have extended the upper size limit of antigens delivered by TCI (14, 26). As LT is often the causative agent of ETEC diarrhea, we demonstrated that LT could also act as an adjuvant for CS6 delivered by TCI. The combination of LT immunity and CF immunity suggested that a traveler's vaccine delivered by TCI is feasible.

Animal models for testing the efficacy for ETEC using live organisms are not well established; however, the antitoxin immunity induced by TCI was found to protect against intestinal toxin challenge when LT was used as the challenge agent. This finding is consistent with previous animal data showing that anti-CT immunity is protective in several settings, including exposure to both toxin and live *Vibrio cholerae* (40). Interestingly, local intestinal immunity was not shown to be a feature

required for protection in the previous studies. Although TCI induced both serum and mucosal IgG and IgA responses, passive protection could be conferred by infusion of hyperimmune serum into mice prior to challenge, suggesting that the serum antitoxin immunity was sufficient to confer protection against challenge.

While it is clear that protective immunity against ETEC can be established (36), the role of local intestinal immunity in protection against ETEC disease has not been clearly demonstrated, and there is debate concerning the requirements for protective immunity against ETEC. However, it is likely that secretory IgA and antibody-secreting cells detected in peripheral blood play a role in protective immunity against ETEC disease. We and other workers have observed mucosal antibodies in animals immunized by TCI (14, 22, 23, 25, 45). We demonstrate here for the first time that the LT-specific IgA antibodies detected in stools and vaginal secretions contained secretory chain, suggesting that the anti-LT IgA antibodies are produced by local secretion. Additionally, antibody-secreting cells to CS6, thought to be a correlate for protection against ETEC, were detected in the guinea pig model. Using both immunoblot analysis and ELISA, we also showed that the antibodies to a recombinant antigen recognize the native antigen derived from a clinical ETEC strain. The data further support the concept that a recombinant ETEC vaccine delivered by TCI could induce protective immunity.

In studies of TCI performed previously, adjuvants were a

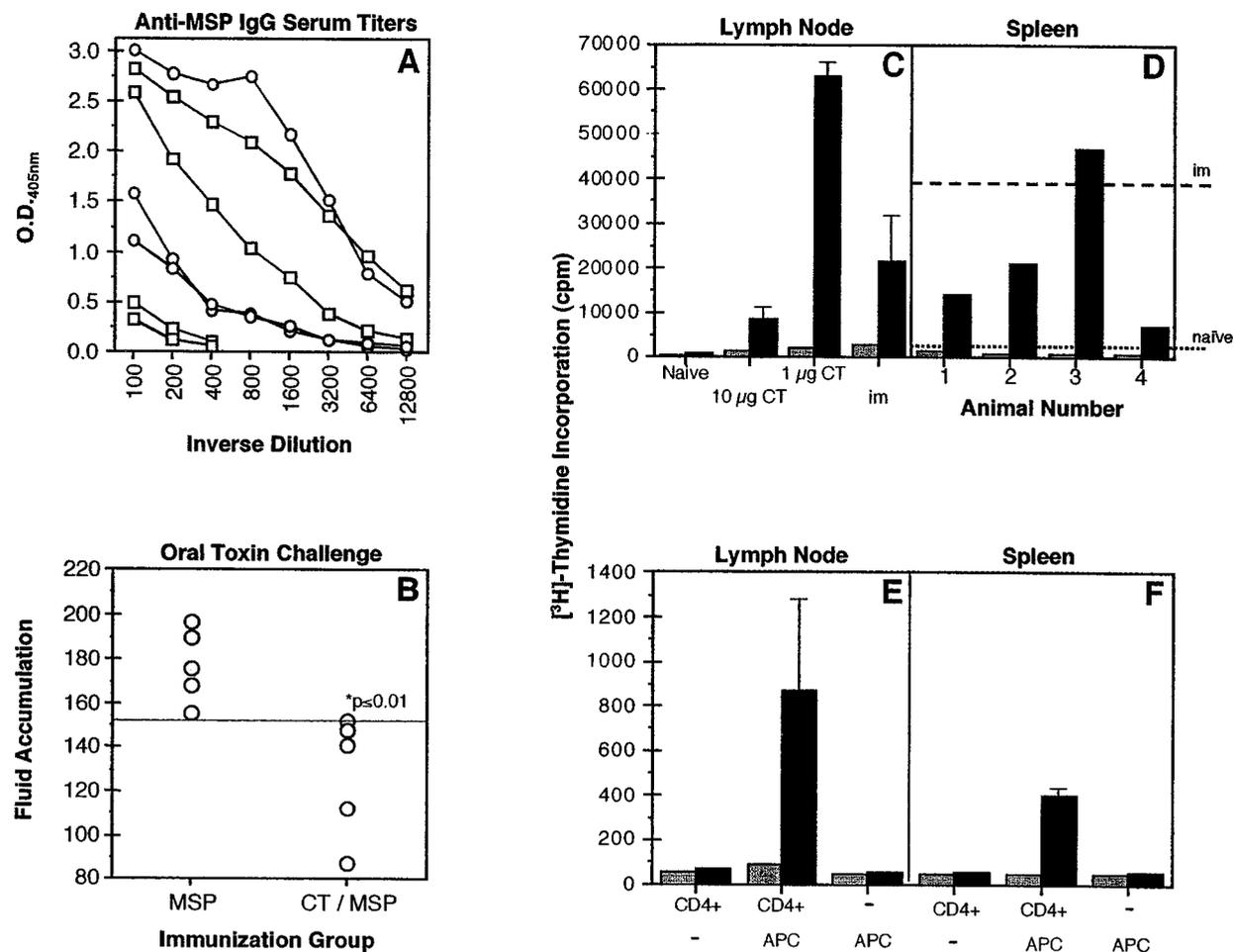


FIG. 8. Induction of malaria-specific responses and protective immunity against toxin challenge following coadministration of CT and a malarial vaccine antigen. (A and B) Systemic antibody responses (A) and protection against oral challenge (B) following skin immunization with MSP-1₄₂ and CT. C67BL/6 mice (*n* = 5) were immunized on the skin at zero time and after 4, 8, and 13 weeks with 100 µg of MSP-1₄₂ in the presence or absence of CT (10 or 100 µg). Serum collected prior to immunization and 15 weeks later was analyzed for MSP-1₄₂-specific IgG (A) and CT-specific IgG (data not shown) by ELISA. Animals that exhibited at least a threefold increase in optical density at 405 nm (O.D._{405nm}) at a 1:100 dilution following immunization were considered positive MSP-1₄₂ responders. (A) Serum antibody dilution curves for individual animals responding to MSP-1₄₂. Symbols: ○, 100 µg of CT; □, 10 µg of CT. (B) Results of oral challenge. Briefly, 4 weeks after the last boost (study week 17), mice were fed 10 µg of CT in 10% bicarbonate buffer. Five hours later, fluid accumulation in the small intestine was assessed as described in Materials and Methods. Each circle represents the fluid accumulation in an individual animal. (C to F) MSP-1₄₂-specific proliferation of draining (inguinal) lymph node (C and E) and spleen (D and F) cells collected from mice immunized on the skin with CT and MSP-1₄₂. BALB/c mice were immunized at zero time and after 4, 8, and 12 weeks. Proliferative responses were determined in cells harvested 13 weeks after the final immunization. Gray bars, *in vitro* response to media; solid bars, *in vitro* response to MSP-1₄₂ restimulation. Proliferation was measured in pooled inguinal lymph node cells from naïve mice, from mice immunized on the skin (1 or 10 µg of CT plus 100 µg of MSP-1₄₂), or from mice immunized intramuscularly (50 µg of MSP-1₄₂ adsorbed to alum) or in pooled spleen cells from individual naïve mice or mice immunized on the skin (1 µg of CT plus 100 µg of MSP-1₄₂). In panel D, the proliferation responses of antigen-stimulated spleen cells from naïve mice and mice that were immunized intramuscularly (im) are indicated by dashed lines. In panels E and F, the proliferation responses of CD4⁺ T cells purified from lymph node and spleen tissues of animals immunized by TCI (10 µg of CT plus 100 µg of MSP-1₄₂) are shown. CD4⁺ T-cell proliferation was examined as described in Materials and Methods. The error bars in panels C, D, E, and F indicate the standard deviations of the means. APC, antigen-presenting cells.

universal requirement for effective immunization via topical application (14, 21, 23, 27, 45). Antigens such as tetanus toxoid applied without an adjuvant can induce detectable antigen-specific antibodies. However, addition of even a small amount of adjuvant results in a several-log increase in antibody titers, and the data obtained with DS6 support this finding (45). Furthermore, although most data for TCI have focused on the bacterial ADP-ribosylating exotoxins, such as LT and CT, we

have found that a wide variety of adjuvants have the same effect topically; i.e., they enhance the immune response to a coadministered antigen, although few appear to have the potency of the bacterial ADP-ribosylating exotoxins (45). These data support our working hypothesis that immunization via the skin requires an adjuvant in order to induce a robust immune response, as confirmed by the data presented here.

We also evaluated whether CT could induce a protective

TABLE 1. Serum CS6 and LT IgG levels in guinea pigs immunized on the skin with CS6 and LT IgG

Adjuvant	Antigen	Antibody	Mean serum IgG level (ELISA units)			
			Prebleed	3 wk	6 wk	8 wk
LT (100 µg)	CS6 (200 µg)	Anti-CS6 IgG	30 (7) ^b	55 (7)	1,588 (5)	4,383 (3)
		Anti-LT IgG	57 (7)	104 (7)	1,258 (5)	3,764 (3)
LT (50 µg)	CS6 (100 µg)	Anti-CS6 IgG	23 (10)	55 (10)	1,155 (8)	12,471 (1)
		Anti-LT IgG	52 (10)	87 (10)	297 (8)	4,933 (1)
LT (25 µg)	CS6 (50 µg)	Anti-CS6 IgG	24 (6)	26 (6)	103 (4)	1,084 (2)
		Anti-LT IgG	43 (6)	77 (6)	106 (4)	381 (2)
LT (12 µg)	CS6 (25 µg)	Anti-CS6 IgG	13 (6)	26 (6)	68 (4)	243 (2)
		Anti-LT IgG	35 (6)	65 (6)	112 (4)	153 (2)
PBS		Anti-CS6 IgG	18 (6)	27 (6)	54 (5)	36 (2)
		Anti-LT IgG	42 (6)	73 (6)	113 (5)	95 (2)

^a Guinea pigs were immunized on the skin with LT and CS6 at zero time and after 3 and 6 weeks. A control group was given the same volume of PBS on the skin. Serum was collected the day before immunization and 2 weeks after the last immunization. CS6- and LT-specific IgG titers were determined by ELISA. Levels in serum are expressed in ELISA units (the inverse of the titer at which the optical density at 405 nm was equal to 1.0).

^b The numbers in parentheses are numbers of animals.

immune response against diarrheal disease in addition to acting as an adjuvant for a candidate malaria vaccine antigen. MSP-1, a blood stage malaria antigen, was adjuvanted with CT (6). Modest anti-MSP-1 antibody responses and potent T-cell responses were observed in conjunction with protective immunity against oral CT challenge. These findings suggest that the use of toxin-based adjuvants may confer simultaneous immunity against parasitic and diarrheal diseases, and further optimization of the anti-MSP-1 responses with adjuvants appears to be warranted.

The immunostimulation that results from parenteral, intranasal, or oral adjuvant use may be accompanied by adverse side effects; the most frequent side effects are local inflammation at the site of injection (33), rhinorrhea (12), and diarrhea in the case of oral administration of LT and its mutants (37). Interestingly, intranasal administration of LT in humans seems to be remarkably free of significant side effects (12). A few emulsion-based adjuvants have induced severe local reactions after injection; these adjuvants include incomplete Freund's adjuvant and DETOX (13, 32, 34, 46). The potent immunostimulation observed with adjuvants has raised concerns about long-term safety, including the possibility of inducing cancer and autoimmunity. However, a 35-year follow-up study of emulsion-based (i.e., adjuvanted) influenza vaccines failed to

reveal such effects (1). These findings are reassuring as emulsion-based adjuvants are extremely potent.

Because of the concerns regarding adjuvant safety, we evaluated the toxicity of the LT-CS6 preparation in guinea pigs. When relatively large doses of LT were used, no significant systemic pathological effects were observed. There were minimal to mild acute inflammatory responses on the skin that were restricted to the site of immunization. The etiology of these responses is not clear but may be related to the antigen, which is thought to bind to epithelial receptors in the gut (5), to skin manipulation and shaving, or to penetration of adjuvant into the dermis (9). Induction of skin reactions, including irritant and delayed-type hypersensitivity reactions, is common with transdermal drug delivery, and this is a potential effect of TCI in the clinical setting. Overall, our results support the observation that potent adjuvants, which have significant side effects when they are delivered by other routes, can be safely used for TCI (23).

Safety concerns associated with parenteral, oral, and nasal use of adjuvants involve systemic reactions that may not be relevant to topical administration. For example, concerns regarding the parenteral use of lipopolysaccharides (LPS) or LPS derivatives are not likely to be relevant for topical preparations because the adjuvants are apparently not absorbed into the circulation system. The requirement for low LPS concentrations in antigen preparations may not be a safety criterion for vaccines delivered by TCI. In fact, the presence of LPS in antigen preparations may enhance the adjuvant effect on the antigen. Similarly, while oral use of native LT is known to be diarrheagenic in humans (37), experience with TCI has been good with respect to vaccine-associated diarrhea, as might be expected (24). Although there are a variety of mutant toxins designed to alleviate this side effect of oral use, it appears that native toxins, unmatched for their adjuvanticity, can be used safely topically. The safe use of highly potent and inexpensive adjuvants opens new and previously restricted possibilities for vaccine development.

The promising combination of needle-free delivery and robust immunity to subunit antigens suggests that clinical studies to evaluate an ETEC vaccine delivered by a patch are warranted (48). The unique capacity for safe induction of robust

TABLE 2. Individual guinea pig anti-CS6 antibody-secreting cell and IgG antibodies induced by topical immunization^a

Immunization group	Animal	No. of antibody-secreting cells per 10 ⁶ cells		
		IgG	IgA	IgM
PBS	1	1	1	2
	2	2	0	4
CS6-LT	1	8	1	12
	2	42	3	1
	3	31	1	4
	4	63	5	7

^a Guinea pigs were immunized on the skin with LT (50 µg) and rCS6 (100 µg) at zero time and after 4 and 8 weeks. Seven days after the last immunization, spleen and lymph node cell suspensions were subjected to an ELISPOT assay to detect the number of anti-CS6 antibody-secreting cells induced by the topical immunization. The levels of IgG antibodies to CS6 were measured 7 days after the last immunization.

antitoxin immunity when the skin is used (23) may significantly enhance protection against this toxin-mediated disease. It remains to be shown in the human setting whether robust anti-LT immunity can confer protection against LT-secreting ETEC organisms. However, if LT immunity and CF immunity directed towards ST-secreting organisms can confer protection, then it is conceivable that a vaccine with broad coverage can be formulated by using this strategy (47).

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