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Construction and Characterization of Bivalent *Shigella flexneri* 2a Vaccine Strains SC608(pCFAI) and SC608(pCFAI/LTB) That Express Antigens from Enterotoxigenic *Escherichia coli*

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An invasive strain of *Shigella flexneri* 2a (SC608) has been developed as a vector for the expression and delivery of heterologous antigens. SC608 is an aspartate semialdehyde dehydrogenase (*asd*) derivative of SC602 (*icsA iuc*), a well-characterized live attenuated vaccine strain which has undergone several clinical trials in human volunteers. When administered orally at a single 10^4 (CFU) dose, SC602 is both immunogenic and efficacious against shigellosis. Using *asd*-based plasmid vectors, we designed SC608 to express the enterotoxigenic *Escherichia coli* (ETEC) fimbrial subunit CfaB (CFA/I structural subunit) alone or in combination with the *E. coli* B subunit of heat-labile enterotoxin (LTB). The expression of each heterologous protein in SC608 was verified by immunoblot analysis. Each strain was comparable to the parent strain, SC602, in a HeLa cell invasion assay. After intranasal immunizations of guinea pigs, serum and mucosal immune responses were detected against both *Shigella* lipopolysaccharide and heterologous ETEC antigens by enzyme-linked immunosorbent assay and ELISPOT analysis. All immunized animals were subsequently protected against a challenge with wild-type *S. flexneri* 2a in a keratoconjunctivitis Sereny test. Serum antibodies generated against LTB and CfaB demonstrated antitoxin and agglutination activities, respectively. These results suggest that CfaB and LTB expressed in SC608 retain important conformational epitopes that are required for the generation of antibodies that have functional activities. These initial experiments demonstrate that a fully invasive *Shigella* vaccine strain can be engineered to deliver antigens from other diarrheal pathogens.

Diarrheal diseases are a significant global problem resulting in high levels of morbidity and mortality in the developing world, especially in young children. Two of the most prominent bacterial agents that cause diarrheal disease are *Shigella* spp. and enterotoxigenic *Escherichia coli* (ETEC). Current measures for preventing and treating these diseases are insufficient in that over 375 million cases occur each year, with an estimated 1.48 million deaths annually (17).

Shigella organisms are invasive pathogens that can cause disease after the ingestion of as few as 10 to 100 bacteria (14, 36). The pathogenic mechanisms of *Shigella* have been deduced from experiments using in vitro tissue cultures as surrogates of the colonic epithelium and from various animal models (reviewed in reference 9). Once ingested, invasive *Shigella* bacteria are taken up by specialized M cells lining the distal colon and the rectum. With the aid of specific virulence factors, the bacteria escape from the phagocytic vacuoles of the macrophages by inducing cell death (27). During the process of entry and dissemination within the epithelia, the pathogen initiates an intense mucosal inflammatory response. This inflammation is elicited by proinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1β, leading to the recruitment of neutrophils and macrophages at the site of infection and resolution of the disease (reviewed in reference 28). Thus, shigellosis or bacillary dysentery is characterized by an inflammatory condition of the colon with accompanying fever, vomiting, severe abdominal pain, diarrhea, and passage of characteristic blood- and mucus-containing stools (24).

ETEC is also transmitted through contaminated food or water, but the infectious dose for ETEC is much higher (16). Once ingested, ETEC cells attach to mucosal epithelial cells of the small intestine by using one or more proteinaceous fimbriae or colonization factor antigens (CFAs or CFs). The production of the plasmid-encoded fimbrial antigen CFA/I of ETEC requires two DNA regions, CFA/I region 1 and CFA/I region 2. These two regions are separated by about 40 kb on the wild-type plasmid. The CFA/I operon located in region 1 contains four genes (*cfaABCE*) which are important for proper fimbria biogenesis (reviewed in references 19 and 47). The CfaA gene appears to function as an atypical chaperone and is necessary for the periplasmic expression of CfaB, the major subunit of CFA/I fimbriae. Sequence homology to related colonization factors and preliminary data suggest that CfaC and CfaE are the usher and tip adhesion components of the CFA/I fimbriae and, together with CfaA, are involved in the secretion and assembly of the CFA/I fimbriae on the bacterial surface (45). Region 2 contains *cfaD*, which encodes a protein belonging to the AraC family of positive regulators and is necessary for the production of CFA/I synthesis (46). ETEC secretes up to two enterotoxins, designated heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) (reviewed in reference 30). Immune responses to ETEC infections indicate that secretory

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immunoglobulin A (sIgA) directed towards CFs can provide protective immunity against homologous fimbrial types (16), and antitoxin immunity has been shown to provide at least short-term protection (8, 43).

Strains with mutations in key biosynthetic pathways resulting in auxotrophic mutants of *Shigella* have been extensively used as live attenuated vaccine strains. These mutations tend to reduce or eliminate intracellular replication of the bacteria. These vaccines are immunogenic only when given at very high doses, and sometimes more than one dose is required (31, 34, 35). A greater understanding of *Shigella* pathogenesis has led more recently to the targeted deletion of specific virulence factors in live *Shigella* vaccines, such as strains SC602, WRSS1, WRSd1, and others (26, 52; also reviewed in reference 28). Attenuated strains of *Shigella* have also been used as vectors for the delivery of genes encoding protective antigens from other pathogens (1, 3–6, 33, 42). Several factors must be considered during the development of such multivalent vaccine strains. The heterologous antigen must be expressed at an optimum level so that an immune response to it is maximized without affecting the phenotype and subsequent immunogenicity of the carrier strain. The cellular localization of the antigen will also determine how effectively it is seen by the immune system. Previous studies have indicated that periplasmic and extracellular antigens are more immunogenic than antigens retained in the cytoplasm (29, 41).

There are several reports of *Shigella* expressing ETEC-related antigens. Such multivalent vaccines have the potential to protect against both *Shigella* and ETEC-induced diarrhea (1, 2, 4, 33, 37, 42, 57). Strain CVD1204 (Δ*guaBA*) was engineered to express several ETEC fimbrial antigens as well as mutant heat-labile toxin (mLT) (1, 2, 4, 33). The approach used in these studies was to clone the entire fimbrial operon, composed of several genes, under the control of an inducible promoter. The resulting *Shigella* strain expresses the ETEC fimbriae on its surface. To date, attenuated *Shigella* strains that express the CFA/I, CS2, CS3, and CS4 operons as well as detoxified mLT (LThK63 or LThR72) have been described. Guinea pigs immunized with mixed inocula containing five different *Shigella* strains, each expressing individual ETEC antigens, showed serum and mucosal antibody responses to both the *Shigella* vector and ETEC fimbriae (4).

The *Shigella flexneri* 2a vaccine strain SC602 has deletions in both *icsA* and *iucA* (6). SC602 is highly invasive in HeLa cells, but it cannot spread intercellularly due to the *icsA* mutation. Unlike wild-type strains, *icsA* mutants do not elicit a characteristic keratoconjunctivitis reaction (Sereny test) when applied to the eyes of guinea pigs. SC602 has been used for several inpatient and outpatient clinical trials with North American volunteers, and it has demonstrated safety, immunogenicity, and protection against shigellosis after a single oral dose of 10^4 CFU (10, 32). Higher doses proved more reactogenic, but with the identification of several new virulence factors such as chromosomal and plasmid-based enterotoxins, further attenuation of SC602 is feasible and is being pursued. The *Shigella sonnei* vaccine strain WRSS1, with a single deletion in *icsA*, behaves similarly to SC602 in human volunteers. The safety and immunogenicity profiles of SC602 and WRSS1 indicate that the strategy of targeting specific and appropriate virulence factors that maintain the invasiveness of the bacterial strain for attenuation can result in safe, effective, low-dose, oral, live *Shigella* vaccines. Furthermore, such vaccines can also be directed to deliver antigens from other diarrheal pathogens. This study discusses the development of the bivalent vaccine strains SC608(pCFAI) and SC608(pCFAI/LTB), which use an *asd* derivative of SC602 as a vector to express ETEC antigens. The expression plasmids for CfaB and the B subunit of LT (LTB) represent a novel method for fimbrial and toxin expression and provide a strategy for the inclusion of other CFs in a single plasmid while still maintaining the level of invasiveness of the parent strain. The *Shigella* vaccine strains SC608(pCFAI) and SC608(pCFAI/LTB) are both immunogenic, and both provide significant protection when administered intranasally to guinea pigs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. SC608 is an aspartate semialdehyde dehydrogenase (*asd*) knockout of SC602 (6) and was created by using pCVD442 and standard suicide vector mutagenesis techniques (11). SC608 lacks the *asd* gene and requires diaminopimelic acid at a concentration of 50 μg/ml for growth in Luria-Bertani (LB) medium. The endpoints of the *asd* deletion in SC608 have been mapped by sequencing the region. *Shigella* strains were propagated on LB medium supplemented with diaminopimelic acid (50 μg/ml) when required, unless otherwise noted.

Plasmid construction. The recombinant plasmid used for CfaB expression was constructed in a single step. PCR primers (CFA1-2, 5′-GATCAAGCTTCCATGAAAAAGGAGGGATGTA-3′; and CFA-9, 5′-GATCCCATGGGCATGCATAAATTATTCTATTTACTAAGT-3′) were used to amplify the CfaA and CfaB genes from genomic DNA of the CFA/I-producing strain of ETEC H10407. The PCR product was digested with NcoI and HindIII and then purified (QIAGEN). The pYA3098 plasmid was digested with the same enzymes and then purified (QIAGEN). The fragments were ligated by the use of T4 DNA ligase (NEB) and were transformed into strain SC608. Colonies were selected on LB agar plates, and those containing the proper insert were verified by restriction digestion.

The recombinant plasmid used for CfaB and LTB expression was constructed in two steps. A DNA fragment containing the CfaA and CfaB genes, including the signal sequence, was amplified from ETEC strain H10407 genomic DNA by use of the PCR primers 5′-TACATGCCATGGATAAATTATTCTATTTACT-3′ and 5′-ATTCTGTTATATATGTCAACCTGCAGGAGGGATGTATAAACATACC-′3. A separate fragment containing the LTB gene was amplified from the same template by use of the primers 5′-GGTATGTTTATACATCCCTCCTGCAGGTTGACATATATAACAGAAT-3′ and 5′-TTACCCAAGCTTAGACATGCTTTTAAAGCAAA-3′. Both PCR products were gel purified (QIAGEN) and used in a second round of PCR to generate a fusion or single PCR product that consisted of the CfaA and CfaB genes linked to the LTB gene. The resulting PCR product was cut with NcoI and HindIII and then purified (QIAGEN). This fragment was ligated into pYA3098 cut with the same enzymes. The ligation product was transformed into *E. coli* strain *x*6212 (20). Colonies containing the correct insert were identified by restriction digestion and then transformed into SC608 by electroporation.

Protein expression and colony blots. *Shigella* strains were grown to late log phase (optical density at 600 nm [OD_{600}] of 1.0 to 1.5) and then harvested by centrifugation (9,000 × *g* for 7 min at 4°C). The bacterial pellets were resuspended in 1× NuPage loading buffer (Invitrogen) and stored at −20°C. Lysates were prepared by sonicating the pellets (three times for 15 s each, output 3), followed by boiling for 5 min. Protein samples were loaded in a 4 to 12% NuPage Bis-Tris gel (Invitrogen) and electrophoresed for 1 h at 180 V. Control proteins, including purified *E. coli*-derived LT (Swiss Serum and Vaccine Institute, Berne, Switzerland) and purified CFA/I fimbriae, were diluted to 50 and 200 ng/μl, respectively, and 5 μl was loaded along with bacterial lysates in a 4 to 12% NuPage Bis-Tris gel. Gels were either stained with Coomassie blue R-250 to check protein levels or transferred to nitrocellulose membranes for Western blot analysis. Blots were blocked with 2% casein (3.5 g of NaCl/liter, 0.58 g of Tris-HCl/liter, 20 g of casein/liter, 1 g of sodium azide/liter) and then incubated with a primary antibody (either anti-LT or anti-CFA/I) for 1 to 14 h. The blots were washed three times with Tris-buffered saline (TBS; 1.58 g of Tris-HCl/liter, 0.9 g of NaCl/liter, pH 7.4). A protein A-alkaline phosphatase conjugate solution (Sigma) was diluted 1:500 in 2% casein and added to the blots for 1 h. The blots

were washed three times in TBS and developed with Naphthol/Fast red solution in 50 mM Tris-HCl, pH 8.0 (Sigma).

For colony blots, *Shigella* vaccine strains were spotted onto LB agar plates and grown for 18 h at 37°C. Nitrocellulose membranes were placed on the plates, and proteins were adsorbed for 5 min at room temperature. The colonies were washed off the membrane with TBS, blocked with 2% casein, and incubated with a primary antibody (either rabbit anti-LT or rabbit anti-CFA/I) for 1 to 14 h. The blots were washed three times with TBS. A protein A-alkaline phosphatase conjugate solution (Sigma) was diluted 1:500 in 2% casein and added to the blots for 1 h. The blots were washed again in TBS and developed with Naphthol/Fast red solution in 50 mM Tris-HCl, pH 8.0 (Sigma). This protocol was adapted from protocols described by Szakal et al. (48).

HeLa cell invasion assays. Gentamicin protection assays with HeLa cells were performed as previously described (15), with some minor modifications. HeLa cell (ATCC CCL-2) monolayers were grown to semiconfluence in 75-cm^2 flasks in complete minimum essential medium (cMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin, and streptomycin (180 μg/ml for both). One flask was trypsinized with 0.25% trypsin (Gibco), and the concentration of cells was adjusted to 2×10^5 cells/ml in cMEM. Twenty-four-well plates were seeded with 2 ml of HeLa cells, which were grown overnight at 37°C in 5% CO_2 to an approximate confluence of 90%. HeLa cells were washed, and the cMEM was changed 2 h before the addition of bacteria. Log-phase cultures of bacteria (grown in LB medium) were added at an estimated multiplicity of infection of 10. After the addition of bacteria, the 24-well plates were centrifuged in a Sorvall swinging bucket rotor at 3,000 rpm for 10 min at 25°C. The plates were incubated at 37°C in 5% CO_2 for 1.5 h. The plates were washed three times with Hanks' balanced salt solution (Gibco) and then incubated with cMEM containing gentamicin (50 μg/ml) for 2 h at 37°C in 5% CO_2. HeLa cells were lysed in a 0.1% Triton X-100 solution for 10 min. The bacteria were plated onto LB agar plates, and bacterial colonies were counted after growth at 37°C for 18 h.

Immunizations and challenge assay. *Shigella* strains used for immunization were streaked onto LB agar plates and grown to confluence for 24 h at 37°C. Bacteria were scraped off of the plates and resuspended in normal saline (NS) solution (0.9% [wt/vol] NaCl). The concentration was adjusted to 2×10^8 to 5×10^8 CFU/ml with NS. Male Hartley guinea pigs (150 to 200 g of body weight) were sedated with a 2:1 mixture of ketamine and xylazine and then immunized intranasally with 50 μl of bacterial suspension per naris. Guinea pig immunizations were spaced 2 weeks apart. Three weeks after the last immunization, the animals were sedated as described above, and 5×10^8 CFU of wild-type *S. flexneri* 2a 2457T in 25 μl of NS was inoculated into each eye of immunized animals and only the left eye of unimmunized animals. Sereny reactions were monitored for 4 days and were scored on day 3. The reactions were scored as follows: 0, no inflammation or mild disease; 1, mild keratoconjunctivitis; 2, keratoconjunctivitis without purulence; and 3, severe keratoconjunctivitis with purulence. With respect to protection, a score of 0 was considered protection and 1 was considered partial protection. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals*.

ELISAs. Antigens used for enzyme-linked immunosorbent assays (ELISAs) included purified *S. flexneri* 2a lipopolysaccharide (LPS), purified CFA/I fimbriae from ETEC strain H10407, and purified *E. coli*-derived LT (Swiss Serum and Vaccine Institute). Antigens were diluted in coating buffer (50 mM sodium carbonate, 30 mM sodium bicarbonate, pH 9.6) and allowed to adsorb to 96-well plates (1 μg/well) overnight at 4°C (Coster). The plates were blocked with 2% casein for 1 h at room temperature. Primary antibodies derived from sera were obtained via ear pricks of immunized guinea pigs. Primary antibodies in mucosal secretions were obtained by washing the conjunctival sacs of guinea pig eyes with 100 μl of phosphate-buffered saline (PBS; 10.75 mM sodium phosphate, 145 mM NaCl). Primary antibodies were diluted in 2% casein and incubated with the antigen-coated plates for 1 h. After three washes in PBS with 0.05% Tween 20 (PBS-Tween), the plates were probed with a rabbit anti-guinea pig IgG–alkaline phosphatase conjugate (diluted 1:1,000 in 2% casein) (Sigma) or rabbit anti-guinea pig IgA (diluted 1:800 in 2% casein) (ICN Laboratories). Plates that were probed with IgA were washed with PBS-Tween and incubated with the anti-rabbit IgG–alkaline phosphatase conjugate (diluted 1:1,000 in 2% casein) (Sigma). After a 1-h incubation with the conjugated antibody, the plates were washed three times with PBS-Tween and 100 μl of the BluePhos Microwell phosphatase substrate system (Kirkegaard & Perry). The OD_{595} was measured on a Molecular Devices ELISA plate reader. The plates were read at 15, 30, and 60 min. Time points and dilutions at which OD_{595} values were approximately 1.0 for the highest average response are presented. Average OD_{595} values for eight animals were calculated (with 95% confidence intervals).

Antibody-secreting cell (ASC) analysis. Animals were sacrificed by injection with sodium pentobarbital, and spleens and cervical lymph nodes were harvested and placed immediately in RPMI 1640 medium (Gibco) containing 50 μg of gentamicin/ml. The tissues were homogenized, and the cell suspensions were filtered through a sterile 70-μm-pore-size screen to remove debris. The cells were washed once in RPMI 1640 medium containing 50 μg of gentamicin/ml, and the erythrocytes were lysed with erythrocyte lysis buffer (Sigma). The cells were washed an additional time with RPMI 1640 medium and finally resuspended in complete RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 50 μg of gentamicin/ml at a concentration of 2.5×10^6 cells/ml.

The antigen-specific ASC cell responses from splenocytes and cervical lymphocytes were determined as described previously (25). Briefly, 96-well microtiter plates (Immuno Maxisorb plates; Nunc) were coated as described above and then blocked with RPMI 1640 containing 5% FBS for 1 h prior to use. Immediately before use, the plates were washed three times with PBS. The cell suspension was dispensed, and the plates were incubated overnight at 37°C in 5% CO_2. After incubation, the plates were washed four times with PBS-Tween, and rabbit anti-guinea pig IgG, IgA, or IgM (diluted in 2% casein to 1:1,200, 1:800, and 1:800, respectively) (ICN Laboratories) was added and incubated for 2 h. After incubation, the plates were washed three times with PBS-Tween, and an anti-rabbit IgG–alkaline phosphatase conjugate (Sigma) was added at a dilution of 1:1,200 in 2% casein. After a 2-h incubation, the plates were washed three times with PBS-Tween, and a melted agarose substrate overlay (0.7% type I, low-EEO agarose; Sigma) containing BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium was added. Antigen-specific ASCs were visualized and counted under a stereomicroscope. Counts for six guinea pigs were averaged and plotted (with 95% confidence intervals).

LT inhibition assay. LT inhibition was assessed by the extent of Chinese hamster ovary (CHO-K1) cell elongation in the presence of LT, essentially as described previously (51). CHO cells (ATCC CCR-61) were maintained in complete Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% FBS, penicillin, and streptomycin (180 μg/ml for both) at 37°C in 5% CO_2. Semiconfluent monolayers grown in 75-cm^2 flasks were trypsinized with 0.25% trypsin (Gibco), and the concentration was adjusted to 8.75×10^4. For determination of the lowest concentration needed for maximum CHO cell elongation, LT purified from *E. coli* was serially diluted in PBS, pH 7.2 (Gibco), and added directly to trypsinized CHO cells in four-well chamber slides (Lab-Tek). The slides were grown for 20 h at 37°C in 5% CO_2. The cells were fixed with methanol and stained with Giemsa stain, and three fields per concentration were photographed at a magnification of ×10. A concentration of 25 ng/ml was chosen for neutralization experiments. The serum used for inhibition assays was prepared by pooling equal volumes of sera collected on day 28 from each vaccination group (eight guinea pigs per group). The inhibition of LT-mediated elongation was determined by incubating serially diluted serum (10 μl) in the presence of LT (25-ng/ml final concentration) at 37°C for 30 min in a volume of 100 μl. Toxin-antiserum mixtures were added to trypsinized CHO cells and assayed for elongation as described above. The minimum dilution used for inhibition was 1:100.

For calculations of the extent of inhibition, CHO cell elongation resulting from 25 ng of LT/ml (which usually caused 88 to 90% elongation) was set at zero and the percent elongation in the absence of toxin (approximately 15 to 20%) was set as 100% inhibition. The results were averaged (with 95% confidence intervals) for three separate fields each, with 50 to 150 cells per field.

Bacterial agglutination. For slide agglutination tests, loopfuls of bacteria from LB plates grown overnight at 37°C or for 3 days at 16°C were resuspended in 1× PBS on glass slides. Samples of pooled antiserum (described above) were mixed with the suspensions, and agglutination of the bacteria after 1 min at room temperature was considered a positive result.

Statistical analysis. Statistical computations were performed with InStat software (version 3.0b; GraphPad Software, San Diego, Calif.). One-way analysis of variance and an unpaired *t* test were used for analyses of both ASC and serological data.

RESULTS

Construction of pCFAI and pCFAI/LTB expression plasmids. ETEC strain H10407 (serotype O78:H11) is considered a prototypic human ETEC strain and was therefore used as a template for PCR amplification of the *cfaA* and *cfaB* open reading frames (ORFs). H10407 was administered at 10^8 to 10^9 CFU to human volunteers in a challenge model and was shown to cause diarrhea in 80% of volunteers (M. Wolf, personal communication).

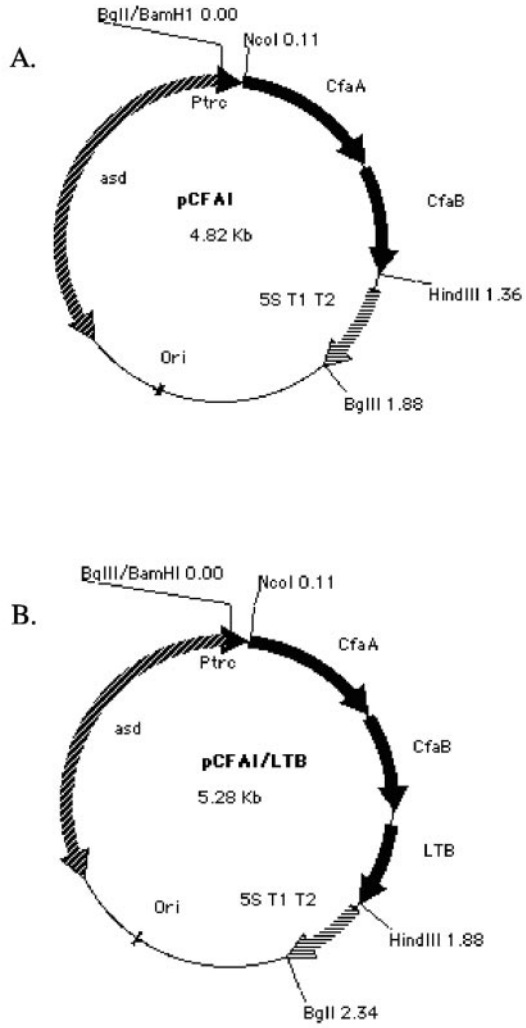

FIG. 1. Diagram of *asd*-based expression plasmids pCFAI and pCFAI/LTB. (A) Map of pCFAI. The DNA fragment encoding the CfaA and CfaB genes was inserted at the NcoI and HindIII restriction sites of pYA3098. (B) Map of pCFAI/LTB. The DNA fragment encoding the CfaA, CfaB, and LTB genes was inserted at the NcoI and HindIII restriction sites of pYA3098. 5ST1T2 is a transcriptional terminator. Maps include approximate locations of restriction sites, genes, and the origin of replication.

The PCR primers used for the amplification of *cfaA* and *cfaB* were designed to include the signal sequence for each protein as well as appropriate restriction sites for cloning into the *asd*-based vector pYA3098 downstream of the P*trc* promoter. The resulting plasmid (pCFAI) is a unique construct for the expression of CfaB (Fig. 1A). Previous constructs employed for heterologous CFA/I expression in *Shigella* and *Salmonella* have included the entire CFA/I operon, allowing for not only expression but also the export and assembly of CFA/I fimbriae on the bacterial surface (21, 33, 55). By removing the CfaC and CfaE open reading frames, we prevented the production of entire fimbriae on the surfaces of *Shigella* organisms, as this tends to reduce *Shigella* invasiveness (1). In order to include a component of the heat-labile toxin in the same construct, LTB was also amplified by the use of appropriate primers and the H10407 template as described in Materials and Methods. A second round of PCR was used to create a single DNA fragment containing the CfaA, CfaB, and LTB ORFs. This DNA fragment was inserted into pYA3098 downstream of the P*trc* promoter, generating the plasmid pCFAI/LTB (Fig. 1B). pCFAI and pCFAI/LTB, along with an empty vector control (pYA3098), were electroporated into SC608 to obtain SC608(p3098), SC608(pCFAI), and SC608(pCFAI/LTB).

Protein expression of ETEC antigens in *Shigella* vaccine strain SC608. CfaB expression in these new strains was initially evaluated in whole-cell extracts prepared from log-phase cultures. Immunoblotting with a CFA/I-specific antiserum generated against intact fimbriae demonstrated that both SC608 (pCFAI) and SC608(pCFAI/LTB) expressed nearly equivalent amounts of the 15-kDa CfaB protein (Fig. 2A, lanes 2 and 3). The same extracts were probed with an antiserum to *E. coli* LT which detected an ~12-kDa band corresponding to LTB in the extracts of SC608(pCFAI/LTB) only (Fig. 2B, lane 3). As expected, no band of this size was detected in the lanes of the control strains that lacked the LTB ORF (Fig. 2B, lanes 1 and 2).

Previous studies with *Salmonella* as a mucosal delivery vector have indicated that antigens located in the periplasm and the extracellular fluid are much more immunogenic than antigens retained in the cytoplasm (29). In this study, a simple colony blot technique, adapted from previous studies with *Shigella* (48), was used to demonstrate the surface expression of the CfaB and LTB proteins in SC608(pCFAI) and SC608(pCFAI/LTB). This technique allows for the adsorption of extracellular proteins directly onto nitrocellulose for detection by immunoblotting. In previous studies, we noticed a correlation between positive colony blots and immunogenicity in guinea pigs. As a control, the colonies on nitrocellulose filters were also evaluated for the expression of IpaB, a *Shigella* protein that is critical for the invasion phenotype (Fig. 2C). All strains had similar levels of IpaB, while only SC608(pCFAI) and SC608(pCFAI/LTB) were positive for CfaB (Fig. 2C). Interestingly, SC608(pCFAI) had slightly reduced levels of CfaB compared to SC608(pCFAI/LTB) in colony blots (Fig. 2C) but very similar levels in whole-cell extracts (Fig. 2A). As expected, only SC608(pCFAI/LTB) was positive for LTB expression (Fig. 2C). Interestingly, the detection of CfaB and LTB in the colony blot assay was somewhat unexpected since the expression of both CfaB and LTB is expected to remain periplasmic. Further investigations which used a brief pretreatment of the colonies with lysozyme to expose the periplasmic proteins more efficiently showed that the signals corresponding to CfaB and LTB were simply intensified along with nonspecific cross-reacting proteins (data not shown). This indicates that perhaps a percentage of the two ETEC proteins in the SC608 background is also localized in the periplasm. Culture supernatants from log-phase cultures failed to reproducibly demonstrate the presence of the two ETEC antigens, indicating that perhaps CfaB and LTB are distributed between the periplasm and bacterial surface without being actively secreted into the culture medium (data not shown). More precise subcellular distribution studies with these strains are the subject of ongoing experiments.

Thus, both Western blot analysis and colony immunoblotting indicated that CfaB was expressed on the surfaces of SC608(pCFAI) and SC608(pCFAI/LTB) cells even though

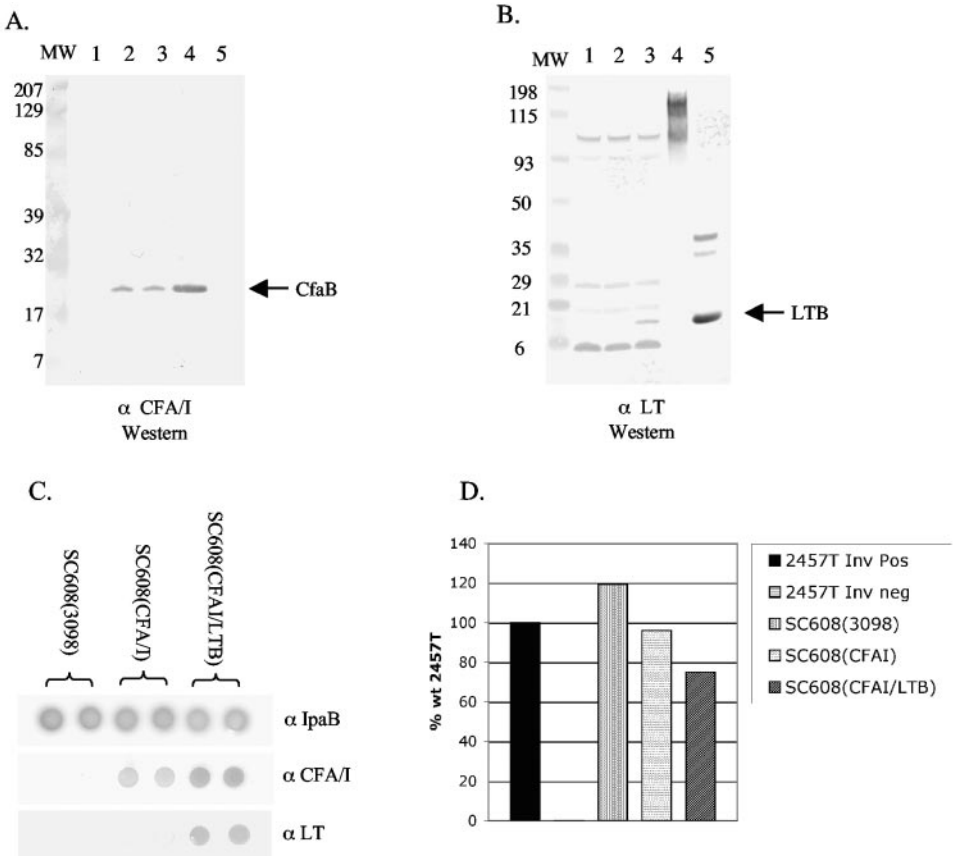

FIG. 2. Protein expression, as measured by Western blots of whole-cell extracts from *Shigella* vaccine strains expressing ETEC antigens. (A) Western blot probed with rabbit anti-CFA/I; (B) Western blot probed with rabbit anti-LT. Lanes: MW, molecular size markers in kilodaltons (indicated on the left); 1, SC608(p3098); lanes 2, SC608(pCFAI); lanes 3, SC608(pCFAI/LTB); lanes 4, 50 ng of purified CFA/I fimbriae; lanes 5, 50 ng of purified LT. (C) Colony immunoblots. The indicated strains were spotted onto LB agar plates in duplicate and grown at 37°C, lifted onto nitrocellulose, washed extensively, and probed with the indicated rabbit polyclonal antibody. (D) HeLa cell invasion assay. *Shigella* vaccine strains were tested for the ability to invade HeLa epithelial cells in a gentamicin protection assay. Invasiveness is plotted as percentages of the invasiveness of a wild-type 2457T invasion-positive isolate. 2457T Inv Pos indicates a Congo red-positive colony, while Inv Neg indicates a Congo red-negative colony.

pCFAI lacked the *cfaC* and *cfaE* genes. Furthermore, the replacement of *cfaC* and *cfaE* with LTB allowed for the expression of both CfaB and LTB from a single P*trc* promoter.

Evaluation of HeLa cell invasion by *Shigella* hybrid vaccine strains. It is generally accepted that live attenuated strains of *Shigella* must retain the ability to invade nonphagocytic cells in order for them to generate protective immune responses in the host. SC608(p3098), SC608(pCFAI), and SC608(pCFAI/LTB) were evaluated for their invasiveness by use of a gentamicin protection assay with HeLa cells (15). *Shigella* cells that invade and become intracellular are protected from the bactericidal effects of gentamicin, whereas extracellular organisms are killed. The control strains for this assay were Congo red-positive (positive control) and Congo red-negative (negative control) isolates of the wild-type *S. flexneri* 2a strain 2457T. Results for the test strains were calculated as percentages of the result for the 2457T Congo red-positive isolate. The results of this assay indicated that SC608(p3098), SC608(pCFAI), and SC608(pCFAI/LTB) are comparable to wild-type 2457T for HeLa cell invasion (Fig. 2D). The invasion assay results demonstrated that expression of the ETEC antigens CfaB and LTB in SC608(pCFAI) and SC608(pCFAI/LTB) does not appear to significantly impair the invasiveness of *Shigella* for HeLa cells ($P = 0.65$ and 0.35, respectively).

Evaluation of immune responses in guinea pigs immunized with hybrid vaccine strains. The immunogenicity and protective efficacy of each vaccine strain were tested in guinea pigs. Fifty-six guinea pigs were separated into groups (14 animals per group) and intranasally immunized with two doses of either SC608(p3098), SC608(pCFAI), SC608(pCFAI/LTB), or normal saline. One week after the last immunization, six guinea pigs per group were euthanized, and an ELISPOT assay was used to detect local immune responses to *Shigella* and ETEC antigens in the spleens and cervical lymph nodes. *S. flexneri* LPS-specific IgG, IgA, and IgM ASCs were consistently detected and did not vary significantly ($P = 0.3$) between vaccine strains (Fig. 3, top panel). ASCs were also measured by use of the ETEC antigens CFA/I and LT. When intact CFA/I fimbriae were used as an antigen, ASCs were detected in both SC608(pCFAI)- and SC608(pCFAI/LTB)-vaccinated animals, with IgG being the dominant isotype (Fig. 3, middle panel). Interestingly, the number of combined CFA/I-specific ASCs for SC608(pCFAI/LTB)-immunized animals was significantly ($P < 0.0001$) lower than that for SC608(pCFAI)-immunized

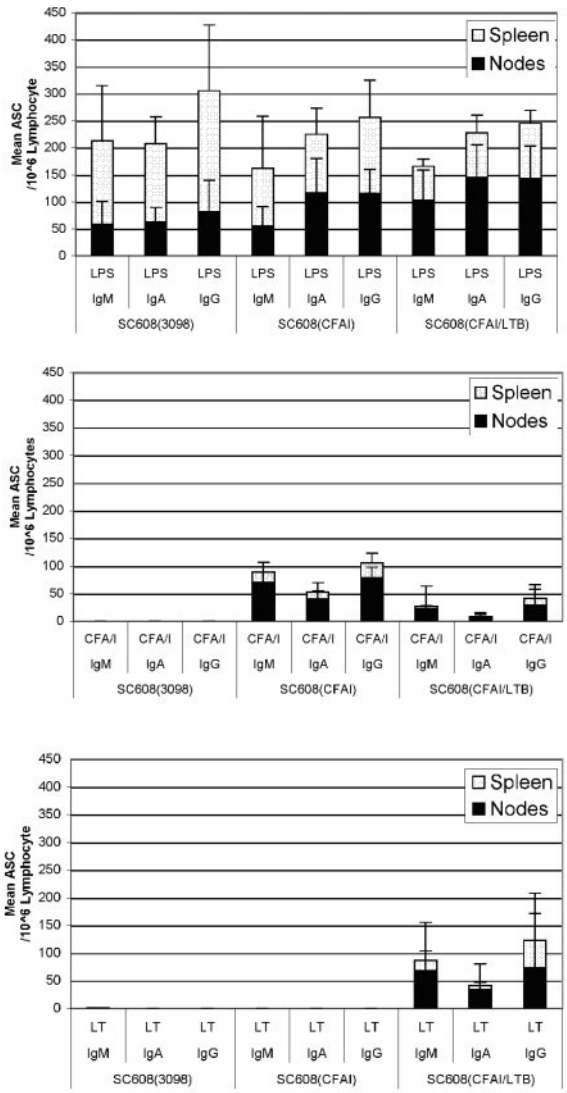

FIG. 3. Antigen-specific ASCs from cervical lymph nodes (nodes) and splenocytes (spleen), as measured by ELISPOT analysis. One week after the second immunization, spleens and cervical lymph nodes were harvested from six animals per vaccine group. Lymphocytes from individual animals were tested for their response to the indicated antigen according to a protocol described by Hartman et al. (25). The results are given as mean numbers of ASC per 10^6 lymphocytes. Error bars indicate standard deviations.

animals. As expected, LT-specific ASCs were detected for SC608(pCFAI/LTB)-immunized animals only, and IgG dominated the isotype distribution (Fig. 3, bottom panel).

Immune responses in serum and mucosal secretions were measured by ELISAs on days 0, 14, and 28. Serum IgG- and IgA-specific immune responses against *S. flexneri* LPS, intact CFA/I fimbriae, and LT were measured. Consistent with the ELISPOT assay results, all groups that were immunized with an SC608 derivative induced significant LPS-specific serum IgG and IgA responses (Fig. 4A and B). Interestingly, only serum IgA-specific LPS responses were detected on day 14. Significant serum IgG responses to CFA/I were detected for SC608(pCFAI) ($P = 0.006$)- and SC608(pCFAI/LTB) ($P = 0.02$)-immunized animals, and the serum IgG response to LT for SC608(pCFAI/LTB)-immunized animals was also significant ($P = 0.0008$) (Fig. 4A). Serum IgA-specific immune responses to CFA/I and LT, although reduced in magnitude, were still considered significant for SC608(pCFAI)- and SC608(pCFAI/LTB)-immunized animals compared to SC608 (p3098)-immunized animals (Fig. 4B).

Mucosal immune responses were measured for *S. flexneri* LPS-, CFA/I-, and LT-specific sIgA in eye washes of guinea pigs (Fig. 4C). ELISAs were used to quantitate sIgA antibodies, with an initial starting dilution of 1:30. All animals that were immunized with an SC608 vector had high levels of sIgA antibodies against *S. flexneri* LPS, even on day 14 (Fig. 4C). While significant levels of LT-specific sIgA antibodies were detected in animals that were vaccinated with SC608(pCFAI/LTB) ($P = 0.0031$), the levels of sIgA antibodies specific for CFA/I were marginal for SC608(pCFAI)- and SC608(pCFAI/LTB)-vaccinated animals.

Challenge assay. All vaccinated guinea pigs as well as normal saline controls were challenged 3 weeks after the final immunization with 5×10^8 CFU of homologous wild-type *Shigella* 2457T and were scored by the Sereny test. Guinea pigs (eight per group) were inoculated in the conjunctival sac and assayed for keratoconjunctivitis for 5 days. All groups that were immunized with an SC608 derivative were protected against severe keratoconjunctivitis (Table 1). SC608(pCFAI)-immunized animals demonstrated the highest level of protection (Table 1).

Functional evaluation of antibodies produced against ETEC after *Shigella* vaccination. *E. coli*-derived LT is the prototypical $\alpha_1\beta_5$ enterotoxin that causes elevation of the intracellular levels of cyclic AMP, resulting in the watery diarrhea which is associated with ETEC infections (reviewed in reference 30). In vitro, LT induces elongation of cultured Chinese hamster ovary (CHO-K1) cells (22), most likely due to changes in the cytoskeleton as a result of high cyclic AMP levels. This LT-induced change in cellular morphology was used to measure the neutralizing properties of the antibodies induced by SC608 derivatives. The minimal concentration of LT needed for maximal CHO cell elongation in this assay was determined to be 25 ng/ml. Sera collected from guinea pigs (eight per group) on day 28 were pooled and tested for the ability to inhibit LT elongation in CHO cells. Only sera from animals immunized with SC608(pCFAI/LTB) were able to inhibit LT-mediated elongation (Fig. 5F). This inhibitory effect could be titrated upon dilution of the pooled sera. These results demonstrate that antibodies raised against the B subunit of LT in SC608(pCFAI/LTB)-immunized animals are functional and can neutralize the toxicity of LT in vitro (Fig. 5G).

The generation of a protective immune response against ETEC strains likely requires the induction of antibodies that are able to block the CFA/I-mediated binding of ETEC to receptors located on the small intestinal epithelium. Agglutination assays using ETEC strain H10407 demonstrated that only sera from the SC608(pCFAI)- and SC608(pCFAI/LTB)-immunized guinea pigs were able to agglutinate bacteria grown on LB medium at 37°C. As a control, these sera were tested and found to be negative for agglutination with H10407 grown at 16°C, a temperature that inhibits fimbrial protein synthesis (data not shown). These results, in combination with the results of the toxin neutralization experiments, demonstrate that an-

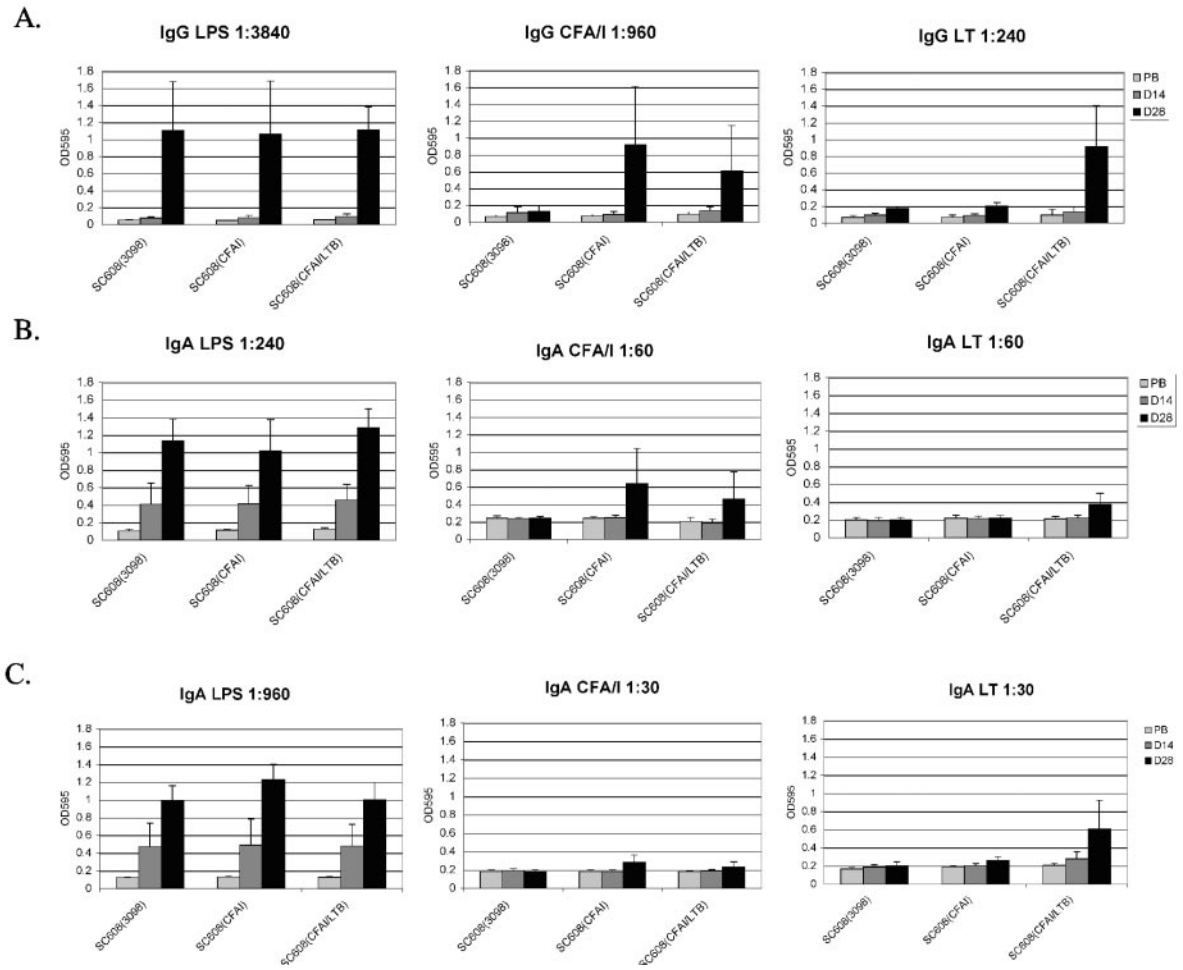

FIG. 4. Serum and mucosal antibody responses from guinea pigs immunized on days 0 and 14 with SC608 or its derivatives. Reactions to *S. flexneri* 2a LPS, CFA/I, or LT were measured in sera (A and B) and mucosal secretions (C) by ELISA. The bars represent the mean OD_{595} values (*y* axis) for each vaccination group (*x* axis) consisting of eight individual guinea pigs. Error bars indicate standard deviations. The isotype, antigen, and dilution factor are indicated at the top of each graph, and the immunizing strain is indicated at the bottom of each graph.

tibodies generated against LTB and CfaB have both antitoxin and agglutination activities and therefore have significant potential to provide protection against natural ETEC infections.

DISCUSSION

The development of an effective combination vaccine targeting *Shigella* and ETEC would have a significant global impact on reducing morbidity due to diarrheal episodes in both developed and developing countries. Such a vaccine must be inexpensive and easy to manufacture, be able to generate long-lasting protection against both *Shigella* and ETEC (preferably in a single dose), be easy to administer, have minimal side effects, and be effective in children. A live attenuated *Shigella* vaccine vector that can simultaneously deliver ETEC antigens to the mucosal immune system has the potential to fulfill these criteria. Several laboratory groups are pursuing the development of a *Shigella*-ETEC hybrid vaccine strain.

Previous constructs employed for heterologous CFA/I expression in *Shigella* and *Salmonella* have included the entire CFA/I operon, allowing for not only expression but also the

TABLE 1. Protection after challenge with wild-type *S. flexneri* 2a 2457T

Immunizing strain	No. of eyes inoculated	No. of eyes with the indicated rating[a]				% Protection[b]		
		0	1	2	3	F	P	C
Normal saline	8	0	1	1	6	0	0	0
SC608(p3098)	16	9	5	2	0	56.3	31.2	87.5
SC608(pCFAI)	16	13	3	0	0	81.3	18.7	100
SC608(pCFAI/LTB)	16	12	2	2	0	75.0	12.5	87.5

[a] 0, no inflammation or mild disease; 1, mild keratoconjunctivitis; 2, keratoconjunctivitis without purulence; 3, severe keratoconjunctivitis with purulence.
[b] F, full; P, partial; and C, combined.

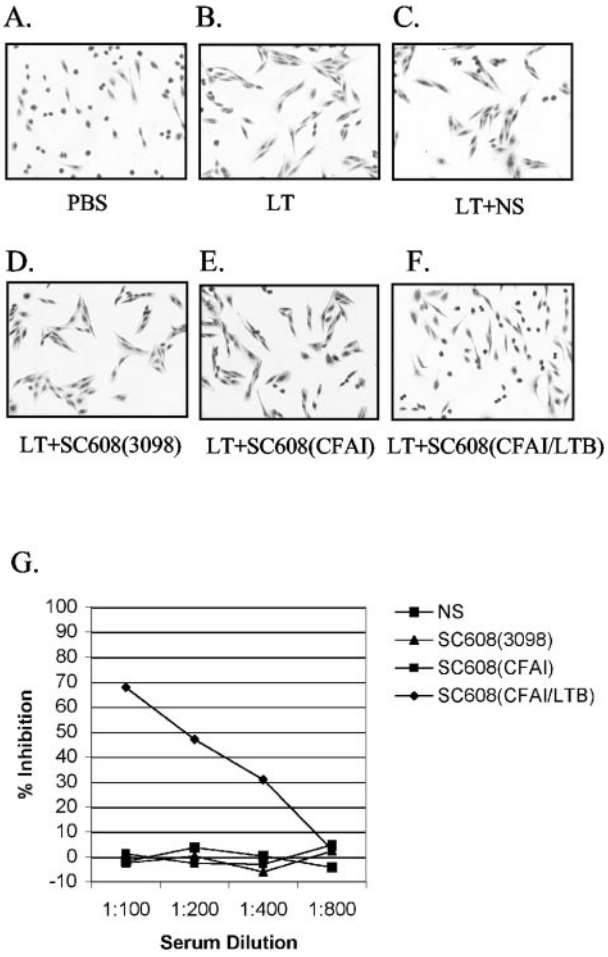

FIG. 5. Inhibition of LT-mediated elongation by antisera generated in animals immunized with SC608 or its derivatives. The images show CHO cells treated with PBS (A), LT (25 ng/ml) (B), LT mixed with pooled sera from guinea pigs immunized with normal saline (NS) (C), LT plus SC608(p3098) (D), LT plus SC608(pCFAI) (E), and LT plus SC608(pCFAI/LTB) (F). (G) Antisera were serially diluted in PBS and mixed with a constant amount (25 ng/ml) of LT. The percent inhibition was calculated by taking elongation with 25 ng of LT/ml alone as 0% inhibition and the extent of elongation without LT as 100% inhibition.

export and assembly of CFA/I fimbriae on the surfaces of *Shigella* and *Salmonella* cells (21, 33, 55). In fact, strain *S. flexneri* CVD1204 (*guaBA*) harboring a CFA/I expression construct has been shown to produce fimbriae with a typical morphology similar to those produced by ETEC strains (33). However, *guaBA* mutants are significantly impaired in HeLa cell invasion assays (7), and the expression of ETEC fimbriae in *guaBA* mutants further reduces their invasiveness by several orders of magnitude compared to wild-type strains (1, 42). However, in a model using guinea pig eyes, these strains protected against challenge.

Field trials with vaccines composed of avirulent, noninvasive strains such as T-32 Istrati and streptomycin-dependent strains have indicated that while these noninvasive vaccines may be safe and effective, multiple doses of large numbers of organisms are required to induce resistance to subsequent challenges (18, 39, 40). In contrast, a single, low dose of SC602 with mutations in defined virulence factors was sufficient to provide protection against shigellosis (10). The ability of an invasive *Shigella* strain to deliver antigen in a predictable fashion to the mucosal cells of the lamina propria should contribute to its effectiveness as a vaccine. Thus, for the present study a different approach was used to make bivalent *Shigella* vaccines in which a fully invasive attenuated strain of *Shigella* (SC608) was used to express ETEC antigens. By including only the chaperone and the major subunit of the CFA/I operon in SC608, we bypassed the surface expression of ETEC fimbrial structures, maintaining the invasiveness of the *Shigella* strain without affecting the serum immune response to ETEC antigens. This was clearly demonstrated in guinea pigs, in which intranasal immunizations with SC608(pCFAI) and SC608(pCFAI/LTB) elicited strong serum immune responses to both *Shigella* LPS and ETEC antigens. Mucosal immune responses, however, were less consistently detected. Efforts are currently ongoing to increase the expression of the ETEC antigens by using higher-copy-number vectors and stronger promoters. Secretion vectors such as the hemolysin system and autotransporters are also being studied to ensure that these antigens are presented on the bacterial surface. In contrast to the SC608-based vaccines described in this work, strain CVD1204 expressing ETEC fimbriae demonstrated strong mucosal antibody responses to ETEC antigens in guinea pigs but had significantly lower antibody and mucosal responses to *Shigella* LPS (33).

ETEC causes disease by the secretion of LT, ST, or both (30). Thus, colonization factors and both enterotoxins are obvious choices for incorporation into a *Shigella*-ETEC vaccine. ST is a nonimmunogenic peptide of 18 or 19 amino acids and for this reason has not been considered a high priority for inclusion into vaccine preparations. LT, however, is a well-characterized mucosal adjuvant that enhances immunity to antigens when it is either expressed in live attenuated bacteria or coadministered with purified vaccine antigens (reviewed in reference 56). LT's obvious toxicity in humans has spurred research into nontoxic forms of the protein, leading to the use of various detoxified mutants (mLT) and recombinant LTB. Numerous mLT derivatives have been constructed and shown to be devoid of toxic activity while retaining their adjuvanticity (12, 13, 44). Studies looking at the adjuvant effect of LTB have been less conclusive than those for mLT (23, 49, 50, 53, 54). In this study, immune responses to *Shigella* and ETEC antigens in guinea pigs administered SC608(pCFAI) or SC608(pCFAI/LTB) did not indicate an increased immune response to *Shigella* LPS or CFA/I when the antigen was coexpressed with LTB.

Sera from guinea pigs immunized with SC608(pCFAI/LTB) showed both mucosal IgA and serum IgG responses to LT. A CHO cell elongation assay was used to characterize the antitoxin activities of serum antibodies (22). Pooled sera from immunized guinea pigs nearly eliminated the elongating properties of LT on CHO cells. This provides a proof of principle that LTB expressed in SC608 can generate an immune response that inhibits LT activity. This is consistent with results for the use of the Stx2 B subunit from Shiga toxin (38).

The healthy human gastrointestinal tract (GIT) is remarkably diverse in its content of aerobic and anaerobic bacteria. Pathogens that colonize the GIT, such as ETEC, or that invade the enterocytes, such as *Shigella*, have to compete with the

beneficial effect of the normal microfloral content, which normally excludes potential pathogens. In order to compete in the highly competitive microenvironment of the GIT, individual pathogens have evolved to possess a distinct set of virulence factors and surface characteristics that determine the anatomic location of the pathogen within the GIT as well as the clinical and pathological outcome of the diseases they cause. ETEC at infecting doses of 10^8 to 10^9 CFU adheres with the help of its fimbrial antigens to the upper small intestine, an area with no normal resident flora. As few as 10 to 100 *Shigella* organisms containing a large virulence plasmid penetrate the mucosal epithelial cells, primarily in the rectum and the distal regions of the colon, where they invade the mucosa and cause characteristic dysenteric symptoms. After oral administration at low doses in humans, SC608(pCFAI) and SC608(pCFAI/LTB), like SC602, are expected to show natural host tissue tropism, effectively delivering antigens directly to the gut-associated lymphoid tissue and inducing strong immune responses to the *Shigella* antigens as well as the ETEC antigens. The colonization fate of *Shigella* expressing ETEC fimbriae on its surface is unclear at this time. Human testing will show whether either strategy will be effective at eliciting a sufficient immune response against both sets of antigens.

Our approach for expressing ETEC antigens in *Shigella* can be extended to the expression of other antigenically distinct ETEC colonization factors, the most common of which include CFA/I, CS17, and CS6. Moreover, these expression constructs can be easily incorporated into *Shigella* vaccine strains of different serotypes to obtain a *Shigella*-ETEC combination vaccine that is able to protect against the most prevalent serotypes and antigenic forms of *Shigella* and ETEC, respectively. A single *Shigella* vaccine strain that expresses LTB in addition to multiple antigenically distinct colonization factors would also be significant progress towards a *Shigella*-ETEC combination vaccine. Future studies will address the immunogenicities of these strains in nonhuman primates as well as in humans.

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