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Vaccine 24 (2006) 3727–3734
Vaccine
www.elsevier.com/locate/vaccine

Immunogenicity of multivalent *Shigella*-ETEC candidate vaccine strains in a guinea pig model

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Available online 22 July 2005

Abstract

Shigella and enterotoxigenic *Escherichia coli* continue to be significant causes of diarrheal disease in infants and young children in developing countries as well as prevalent agents of traveler's diarrhea. A vaccine which provides protection against disease caused by both pathogens would serve common at-risk populations. Such a vaccine would require inclusion of multiple *Shigella* strains as well as multiple ETEC antigens. The use of attenuated strains of *Shigella* as live vectors for the expression of ETEC antigens is one strategy for the development of such a multivalent vaccine. Live attenuated strains of *S. flexneri* 2a, *S. sonnei* and *S. dysenteriae* 1 containing deletions in *guaBA* biosynthetic pathway genes as well as in genes encoding enterotoxins, were constructed. Each strain was subsequently used as a live vector for the expression of one or two critical ETEC antigens. The resulting three *Shigella* derivative strains were tested for immunogenicty and protective capacity alone or as mixtures in the guinea pig model. *S. flexneri* strain CVD 1208(pCFA/I-CS3), *S. sonnei* strain CVD 1233(pCS4-LThK63) and *S. dysenteriae* 1 strain CVD 1252(pCS2) were able to elicit serum and mucosal antibody responses against the live vector as well as the guest ETEC antigens. Vaccination with combinations of two or three of these strains was able to elicit specific immune responses against each live vector as well as each ETEC antigen represented in the mixture. These studies demonstrate the potential of the use of mixtures of live *Shigella* derivatives expressing ETEC antigens to serve as an immunogenic multivalent vaccine.

Keywords: *Shigella* vaccine; ETEC vaccine; Live vector

1. Introduction

Shigella and enterotoxigenic *E. coli* (ETEC) continue to be two of the most prevalent causes of diarrhea and death in young children in developing countries [1,2]. These pathogens are also two of the most important agents of travelers' diarrhea in individuals from industrialized countries traveling to less developed regioins. Additionally, disease caused by ETEC and *Shigella* is of special concern for military personnel deployed to endemic regions [3–5]. While several different strategies have been employed towards the development of vaccines against these pathogens [6], no licensed vaccine currently exists.

These pathogens are transmitted by ingestion of contaminated water or food (ETEC and *Shigella*) or by direct fecal oral contact (*Shigella*). *Shigella* invade the intestinal epithelium via M cells and subsequently spread intracellularly, resulting in an inflammatory response, cell death and dysentery. ETEC do not invade intestinal cells but instead, attach to the small bowel mucosa via fimbriae (appendages on their surfaces). Once attached, ETEC elaborate heat-stable (ST) and/or heat-labile (LT) toxins, which disrupt the normal absorptive and secretory functions of intestinal cells and results in watery (sometimes profuse) diarrhea characteristic of ETEC infection.

Multiple species and serotypes of *Shigella* are important causes of disease including *S. flexneri* with at least 15 different serotypes and subserotypes, *S. sonnei* with a single serotype and *S. dysenteriae* 1 (which is unique in its ability to cause explosive outbreaks and pandemics characterized by severe disease and high case fatality rates [1]. *S. flexneri* is the most prevalent cause of endemic disease in less industrialized countries where a broad distribution of serotypes is found among clinical isolates [7]. *S. sonnei* is the most frequent agent of shigellosis in industrialized countries, particularly

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doi:10.1016/j.vaccine.2005.07.013

in day care centers and institutional settings with suboptimal hygiene; it is also common among travelers. *S. dysenteriae* 1, the cause of devastating pandemics [8] is currently of concern as a potential agent of bioterror. Epidemiologic and volunteer studies have revealed that protective immunity against *Shigella* is directed against the LPS or O-specific antigen and is therefore related to serotype. The use of attenuated strains of *Shigella* as live oral vaccines has been demonstrated to induce protective efficacy [9–11]. A vaccine that aims to confer broad-spectrum coverage would require inclusion of all of the important *Shigella* serotypes. Noting the presence of shared group- and type-specific antigens within *S. flexneri* serotypes, we hypothesized that cross protection could be achieved with a vaccine that includes three *S. flexneri* serotypes; *S. flexneri* 2a, 3a and 6 [12]. Addition of *S. sonnei* and *S. dysenteriae* 1 would result in a vaccine consisting of five strains that would allow broad coverage against *Shigella*.

Epidemiologic and volunteer studies have indicated that protective immunity against ETEC is targeted to the fimbrial antigens [13–15]. Different strains of ETEC express distinct fimbriae on their surface. Seven antigenic types are most prevalently identified on clinical isolates including CFA/I and CS2 to CS6. Recent reports have identified other fimbriae on clinical isolates with increasing frequency including CS17 [16]. A vaccine allowing broad coverage against ETEC would require inclusion of these seven fimbrial types. In addition, responses against LT have been shown to afford at least short-term protection against disease caused by LT-producing ETEC strains [17]; thus, an antigen to elicit anti-LT responses would be beneficial.

We have been pursuing the strategy of developing a multivalent *Shigella*-ETEC vaccine by utilizing attenuated strains of *Shigella* as live vectors for the expression of antigens from ETEC. Combinations of attenuated *Shigella* strains each expressing multiple ETEC antigens allows a mixed formulation containing relevant immunogens from both pathogens. Recent clinical trials have demonstrated the safety and immunogencity of *S. flexneri* 2a strain CVD 1208, which harbors attenuating mutations in *guaBA*, *set* and *sen*. Application of this attenuating strategy to other *Shigella* strains, in conjunction with construction of expression plasmids for the ETEC antigens, allows a rational approach to construction of a multivalent vaccine. Towards this end, we have constructed prototype attenuated strains, each containing expression plasmids encoding one or two ETEC antigens. Preclinical immunization experiments in the guinea pig have demonstrated the immunogenicity of single strains as well as strain mixtures in engendering responses against both *Shigella* and ETEC vaccine components.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Shigella strains were grown on tryptic soy agar (TSA) with Congo Red and guanine (0.001% final concentration). *E. coli* strains were grown on LB agar. Kanamycin was used, when necessary, at a concentration of 40 μg/mL. *S. flexneri* 2a strain 2457T [18] was the wild type parent of the derivative strain CVD 1208. *S. sonnei* strain 53G [19] was the wild type parent of strain CVD 1233. *S. dysenteriae* 1 strain 1617 [20] was the wild type parent of strain CVD 1252.

2.2. Molecular genetic techniques

Restriction enzymes, ligase and polymerase were purchased from New England Biolabs (Beverly, MA) and used according to the manufacturers' instructions. All transformations were performed in competent *E. coli* DH5α (BRL, Gaithersburg, MD). *Shigella* strains were made electrocompetent by growing cells to a mid log OD_{600} value of 0.5–0.8 at 37 °C. Cells were then washed twice with sterile, cold 10% glycerol in distilled H_2O and resuspended in 1/75 the original culture volume in sterile, cold 10% glycerol in distilled H_2O. Electroporation conditions were 1.75 kV, 600 Ω and 25 μF.

2.3. Strain construction

Deletions in the *guaBA*, and *sen* genes in *S. sonnei* 53G and *S. dysenteriae* 1 1617 strains were introduced using the suicide plasmids pFM726A and pFM307 containing the deleted alleles with flanking DNA as previously described [12,21]. *S. dysenteriae* 1 strain CVD 1252 also contains deletions in *virG* and *stxA*. The *virG* deletion was introduced using the suicide plasmid pSHΔ*virG* as previously described [22]. The deletion in the shiga toxin A subunit was introduced using the suicide plasmid pSHΔ*stxA* which contains the *stxA* allele deleted of 495 bp flanked by approximately 1 kb of 5′ and 3′ DNA sequences (a plasmid containing the deleted *stxA* allele plus flanking DNA was kindly provided by Alison O'Brien, Uniformed Services University of the Health Sciences). Allelic exchange with the wild type *stxA* gene was performed as described for *virG* deletion.

Deleted alleles were confirmed by PCR analysis using primers outside of the sequences used in allelic exchange procedures. Deletion of the *guaBA* genes was confirmed by auxotrophy in strains CVD 1233 and CVD 1252 which were unable to grow on minimal medium containing (per liter) 0.4 g of NaCl, 8.4 g of K_2HPO_4, 3.6 g of KH_2PO_4, 0.8 g of $(NH_4)_2SO_4$, 2.5 g of glucose, 0.05 g of nicotinic acid, 0.05 g of aspartic acid and 0.05 g of serine which was progressively supplemented with aromatic amino acids (50 mg each of L-tryptophan, L-tyrosine, and L-phenylalanine).

2.4. Construction of expression plasmids

The vector used for expression of the ETEC genes in *Shigella*, pGA2 [23], was derived from plasmid pEXO1, an expression plasmid derived from pGEN222 that carries a two-component plasmid maintenance system comprised of the *hok-sok* post-segregational killing system plus the *parA* plasmid partitioning system; the *hok-sok* and *parA* components

have been shown to work in concert to minimize plasmid loss from a population of actively growing bacteria, and to lyse any bacteria from which plasmids have segregated [24]. pEXO1 is expected to be present at approximately 15 copies per chromosomal equivalent, and to drive expression of ETEC antigen-encoding genes from the osmotically responsive *ompC* promoter.

We have previously reported the construction of plasmids for the stabilized expression of operons encoding individual ETEC antigens including CFA/I [25], CS3 [26], CS4 [23], CS2 [26], and LThK63 [25]. LThK63 is a humanized version of LT holotoxin that is devoid of enzymatic activity by virtue of the amino acid substitution serine to lysine at position 63 [25]. These plasmids served as the source of operons that were cloned in bicistronic fashion to result in pCFA/I-CS3 and pCS4-LThK63.

2.5. Western immunoblot analysis

Bacterial broth cultures were diluted to an OD_{600} value of 1.0, and then concentrated 10-fold. Bacterial samples were mixed 1:1 (v/v) with Laemmli sample buffer and 5% β-mercaptoethanol and then boiled for 7 min. Aliquots of 5 μl of each sample were electrophoresed on SDS-15% polyacrylamide gels. Gels were stained with Fast-Page Blue Stain Reagent (Gibco-BRL) for visualization of protein bands or transferred to polyvinyl difluoride membranes (Millipore Corp., Bedford, MA) for Western immunoblot analysis. Membranes were probed with absorbed polyclonal rabbit anti-sera specific for each antigen. Western immunoblots were developed using the Immun-Star™ substrate system (BioRad Laboratories).

2.6. Immunizations and sample collection

Overnight cultures of the immunizing strains were harvested from TSA guanine plates (with kanamycin as required) with PBS to a concentration of 10^{10} CFU/ml. After sedation with a 1:1 solution of ketamine/xylazine, randomized, female, Hartley guinea pigs (8 weeks old) were immunized intranasally with 50 μl of the bacterial suspension. An identical booster dose was administered 14 days later. Tears, elicited from guinea pigs as previously described [27], were collected in 50 μl capillary tubes. Blood was obtained from anaesthetized guinea pigs by cardiac puncture on days 0, 14, and 28 post-immunization. All animal procedures were approved by the University of Maryland School of Medicine IACUC.

2.7. ELISA

Antigens used in enzyme-linked immunosorbent assay (ELISA) included hot water phenol-extracted lipopolysaccharide (LPS) from *S. flexneri* 2a 2457T, *S. sonnei* 53G or *S. dysenteriae* 1 CVD 1252. ETEC antigens included purified CFA/I from strain H10407, purified CS3 from strain E9034A, purified CS4 from strain E11881E, purified CS2 from strain C91F and LTh (Berna Biotech, Berne, Switzerland). IgA antibodies in tears were measured by ELISA using rabbit anti-guinea pig IgA α-chain-specific antibody (Bethyl Laboratories Inc., Montgomery, TX). Specific IgG antibodies in sera of guinea pigs were determined by ELISA using goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories, Gaitherburg, MD) conjugate.

To measure serum IgG antibodies, 96-well immulon 2 plates were coated with 100 μl of the indicated antigen at the indicated concentration for 3 h at 37 °C and then blocked overnight with 10% milk (Nestle USA Inc., Glendale, CA) in PBS at 4 °C. After each incubation, plates were washed five times with PBS containing 0.05% Tween 20 (PBST). To determine the endpoint titer eight 2-fold dilutions of sera in 10% milk PBST were added to the plates and incubated for 1 h at 37 °C. After a washing step, plates were incubated with 100 μl of peroxidase-labeled rabbit anti-guinea pig IgG (Kirkegard & Perry Laboratories, Gaithersburg, MD) for 1 h at 37 °C.

For IgA measurement, goat anti-guinea pig IgA was added to plates and incubated for 1 h at 37 °C followed by incubation with a second antibody, peroxidase-labeled rabbit anti-goat IgG, for 1 h at 37 °C. The substrate solution was TMB Microwell Peroxidase (KPL Kirkegard & Perry Laboratories). After a 15-min incubation, the reaction was stopped by the addition of 100 μl H_2PO_4, and the $OD_{450\,nm}$ was determined in an ELISA microplate reader (Multiscan Ascent; Thermo Labsystems, Helsinki, Finland). Sera and tears were run in duplicate. Linear regression curves were plotted for each serum or tear sample, and titers were calculated (through equation parameters) as the inverse of the specimen dilution that produces an OD of 0.2 above the blank.

2.8. Protection assay

Wild type *Shigella* strains were harvested from TSA plates into PBS to a concentration of 5.5×10^9 CFU/ml. Groups of vaccinated and sham immunized guinea pigs were challenged by the Sereny keratoconjunctivitis test by administering 10 μl of the bacterial suspension into the conjunctival sac of one eye [19]. Guinea pigs were examined for 5 days by an observer who was unaware of the immunization status of the animals, and the degree of inflammatory response, if any, was graded according to the following scale: 0 = normal eye indistinguishable from contralateral uninoculated eye, 1 = lacrimation or eyelid edema, 2 = 1 plus mild conjunctival hyperemia, 3 = 2 plus slight exudates, and 4 = full purulent keritoconjunctivitis.

3. Results

3.1. Shigella strain construction

Attenuated *S. flexneri* 2a strain CVD 1208 containing deletion mutations in the *guaBA* operon as well as the *set* and *sen*

genes has been demonstrated to be safe and immunogenic in volunteers [28]. The *guaBA* deletion renders the strain auxotrophic for guanine while the deletions in *sen* and *set* prevent production of *Shigella* enterotoxins 1 (ShET1) and ShET2. Attenuated strains of *S. sonnei* and *S. dysenteriae* 1 were constructed containing the fundamental *guaBA* deletion as well as deletion of *sen*. The *set* gene is only present in *S. flexneri* 2a strains. Additional deletions in the *stxA* gene encoding the shiga toxin A subunit as well as in the *virG* gene were introduced into *S. dysenteriae* 1 for additional safety. The resulting attenuated vaccine candidate strains *S. sonnei* CVD 1233 and *S. dysenteriae* 1 CVD 1252, which were demonstrated to be nonreactogenic in the Sereny test (data not shown), were used as live vectors for the expression of antigens from ETEC.

Expression plasmid pCFA/I-CS3 was constructed to encode both CFA/I and CS3 fimbriae. The operons encoding each fimbria were cloned into the stabilized plasmid vector pGA2. The operons are oriented in bicistronic fashion with CFA/I preceding CS3 (Fig. 1). This plasmid was electroporated into *S. flexneri* 2a vaccine strain CVD 1208. Expression of both fimbriae in CVD 1208 was first assessed by agglutination. Antiserum specific for CFA/I or CS3 was able to agglutinate CVD 1208(pCFA/I-CS3) whereas the CVD 1208 control containing the empty plasmid was not agglutinated. Positive control strains included wild type ETEC strains H10407, a CFA/I expressing strain, and E9034A expressing CS3. Western blot analysis confirmed the expression of CFA/I and CS3 by the CVD 1208 live vector. The 17-kDa structural subunit of CFA/I was visualized in whole cell lysates of CVD 1208(pCFA/I-CS3) following incubation with anti-CFA/I antibody (Fig. 2, panel A).

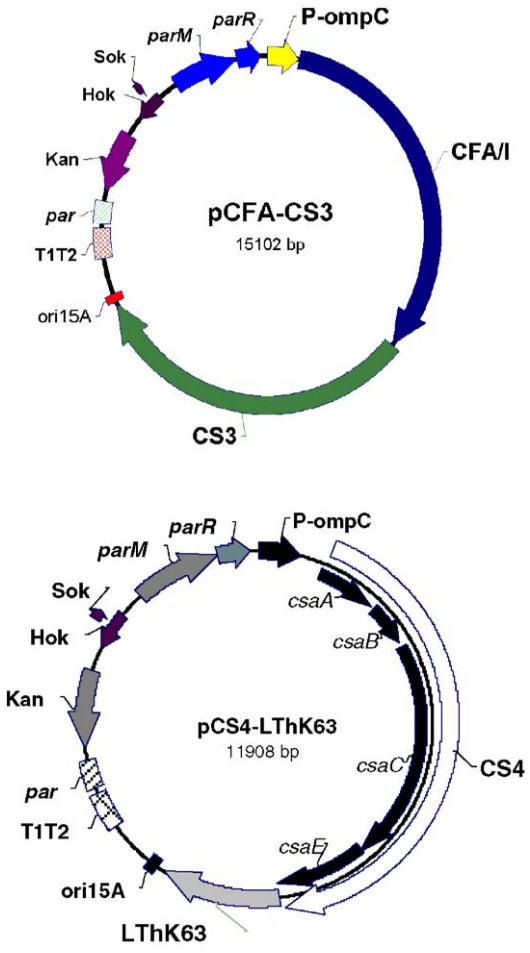

Fig. 1. Plasmid maps of the bicistronic constructs pCFA/I-CS3 driving expression of two ETEC fimbriae and pCS4-LThK63 driving expression of ETEC fimbria CS4 as well as detoxified LTh.

Expression plasmid pCS4-LThK63 was constructed to encode ETEC fimbria CS4 as well as the detoxified LTh variant K63 [25,29]. The operons are oriented in bicistronic fashion with CS4 preceding LThK63 (Fig. 1). This plasmid was electroporated into *S. sonnei* strain CVD 1233 where expression of CS4 was assessed by agglutination with rabbit anti-CS4 antibody. Western immunoblot analysis confirmed the expression of CS4 as well as LThK63 in CVD 1233. Whole cell lysates of CVD 1233(pCS4-LThK63) probed with anti CS4 antibody allowed visualization of the 17-kDa structural subunit band as seen in wild type ETEC control strain E11881E (Fig. 2, panel C, lanes 3 and 7). The approximately 11-kDa B subunit of LT is also visible in CVD 1233(pCS4-LThK63) as well as in the purified LT control lane following probing with anti-LTB specific antibody (Fig. 2, panel D, lanes 3 and 7).

The plasmid expressing ETEC fimbria CS2, pCS2, was electroporated into *S. dysenteriae* 1 strain CVD 1252. Agglutination with anti-CS2 antiserum verified expression of CS2 on the surface of CVD 1252(pCS2). Western immunoblot analysis confirmed the expression of the 17-kDa structural subunit of CS2 in CVD 1252(pCS2) as well as in the ETEC control strain E9034A (Fig. 2, panel B, lanes 4 and 7).

3.2. Immunization studies

The attenuated *Shigella* live vector strains were used to immunize guinea pigs individually or as mixtures. In a first experiment, groups of guinea pigs were immunized intranasally with either CVD 1208(pCFA/I-CS3), CVD 1233(pCS4-LThK63), or a mixture of the two strains. Animals were immunized intranasally with two doses and tears and serum were collected prior to each dose and 14 days following the second dose to assess mucosal and serum antibody responses. Strong serum and mucosal immunoglobulin responses were elicited against *S. flexneri* 2a in Group A, or *S. sonnei* in Group B, live vectors following immunization with a single strain (Fig. 3). The group of guinea pigs immunized with the combination of the two strains, Group C, responded with equally strong responses to both the *S. flexneri* 2a and *S. sonnei* components of the formulation. Strong serum and mucosal responses were also elicited against each of the ETEC antigens expressed by the live vectors. Animals in Group C responded to all four antigens contained within the mixed inoculum (data not shown).

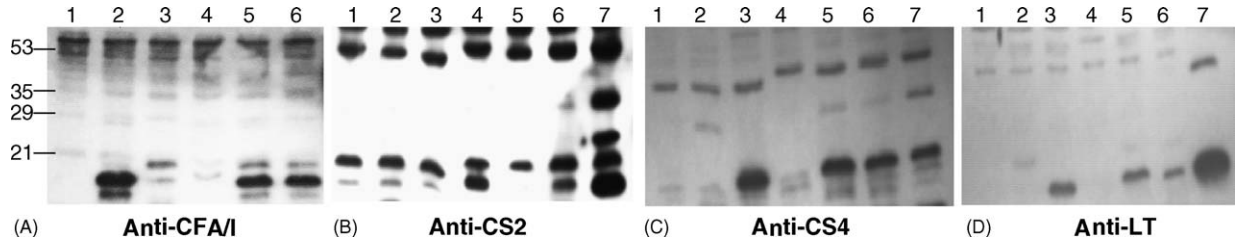

Fig. 2. Western immunoblot analysis of *Shigella* strains expressing ETEC antigens. Whole cell lysates of *Shigella* strains expressing ETEC antigens were subject to SDS-PAGE, transferred to PVDF membrane and probed with the indicated antibodies. Lane 1, CVD 1208; lane 2, CVD 1208(pCFA/I-CS3); lane 3, CVD 1233(pCS4-LT); lane 4, CVD 1252(pCS2); lane 5, mix of strains CVD 1208(pCFA/I-CS3) plus CVD 1233(pCS4-LT); lane 6, mix of three strains CVD 1208(pCFA/I-CS3) plus CVD 1233(pCS4-LT) plus CVD 1252(pCS2); and lane 7, positive controls. Lane B7 contains CS2 expressing ETEC strain C91F, lane C7 contains CS4 expressing ETEC strain E11881A, and lane D7 contains purified LT. Antibodies used for antigen detection were: panel A, anti-CFA/I antibody; panel B, anti-CS2 antibody; panel C, anti-CS4 antibody; and panel D, anti LT antibody.

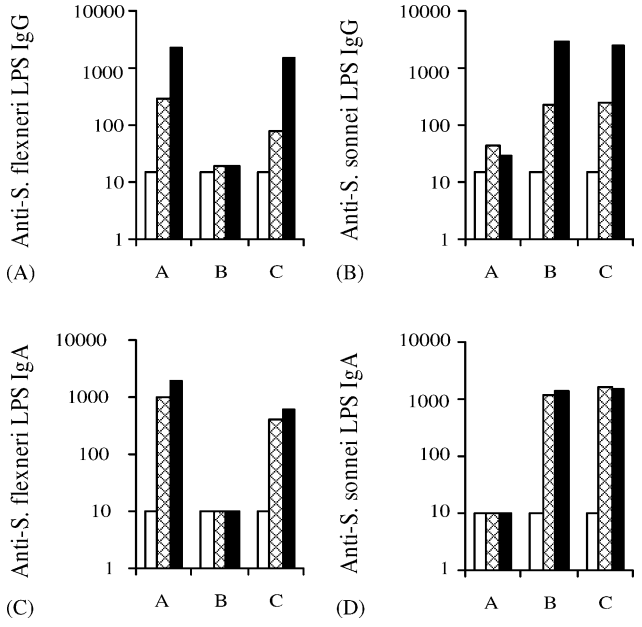

Fig. 3. Serum and mucosal antibody responses from experiment 1. Antibody titers are shown as geometric mean titers for each group prior to immunization (open bars) and following dose 1 (hatched bars) and dose 2 (black bars) each of two doses. Panel A, serum anti-*S. flexneri* 2a LPS IgG response; panel B, serum anti-*S. sonnei* LPS IgG responses; panel C, mucosal IgA anti-*S. flexneri* 2a LPS responses; and panel D, mucosal IgA, anti-*S. sonnei* LPS responses. Immunization groups were Group A, CVD 1208(pCFA.I-CS3); Group B, CVD 1233(pCS4-LThK63); and Group C, CVD 1208(pCFA/I-CS3) + CVD 1233(pCS4-LThK63).

In a second experiment, guinea pigs were immunized with CVD 1208(pCFA/I-CS3), CVD 1233(pCS4-LThK63), CVD 1252(pCS2), or a combination of the three strains. Animals in each group demonstrated strong mucosal and serum antibody responses to the live vector(s) contained in the inoculum (Fig. 4). Animals in Group D, who received the mixture of three strains, responded to all three *Shigella* strains represented within the multivalent formulation. Strong responses were also elicited against each of the ETEC antigens expressed by the live vectors. Animals in Group A achieved high titers against both CFA/I and CS3, while animals in Group B responded with anti-CS4 and anti-LT antibodies and animals in Group C demonstrated strong anti-CS2 responses (Fig. 5). Most importantly, animals in Group D, who received a mixture of three strains, responded with strong serum and mucosal antibody titers against all five ETEC antigens represented in the mixed inoculum.

3.3. Challenge studies

Challenge experiments were performed with all groups of immunized guinea pigs as well as control, sham-immunized animals. All challenges were performed with wild type *Shigella* at 10^7 CFU in 10 μl delivered to the conjunctival sac of one eye. In experiment 1, two animals from each group were challenged with wild type *S. flexneri* 2a and 2 animals from each group were challenged with wild type *S. sonnei*. In experiment 2, two animals from each group were challenged with *S. flexneri* 2a, two were challenged with *S. sonnei*, and two were challenged with *S. dysenteriae* 1. The results are combined in Table 1 to demonstrate the serotype specificity of protection afforded by homologous vaccination/challenge. Animals were protected against challenge with the homologous *Shigella* strain used for vaccination but were not protected against heterologous challenge (Table 1). Animals vaccinated with mixtures of *Shigella* strains were generally protected against all strains represented in the mixed inoculum. Eight out of ten animals immunized with a mixed inoculum were protected against challenge with all representative strains contained in the inoculum. One out of the two animals immunized with the double mix and challenged with *S. sonnei* was not protected and one out of two animals immunized with the triple mix and challenged with *S. flexneri* was not protected.

4. Discussion

A vaccine that can provide broad protection against disease caused by *Shigella* and ETEC requires the inclusion of multiple antigens representative of each pathogen. One strategy for the production of such a formulation is the use of live attenuated strains of *Shigella* each expressing one or two critical ETEC antigens. We have hypothesized that inclusion

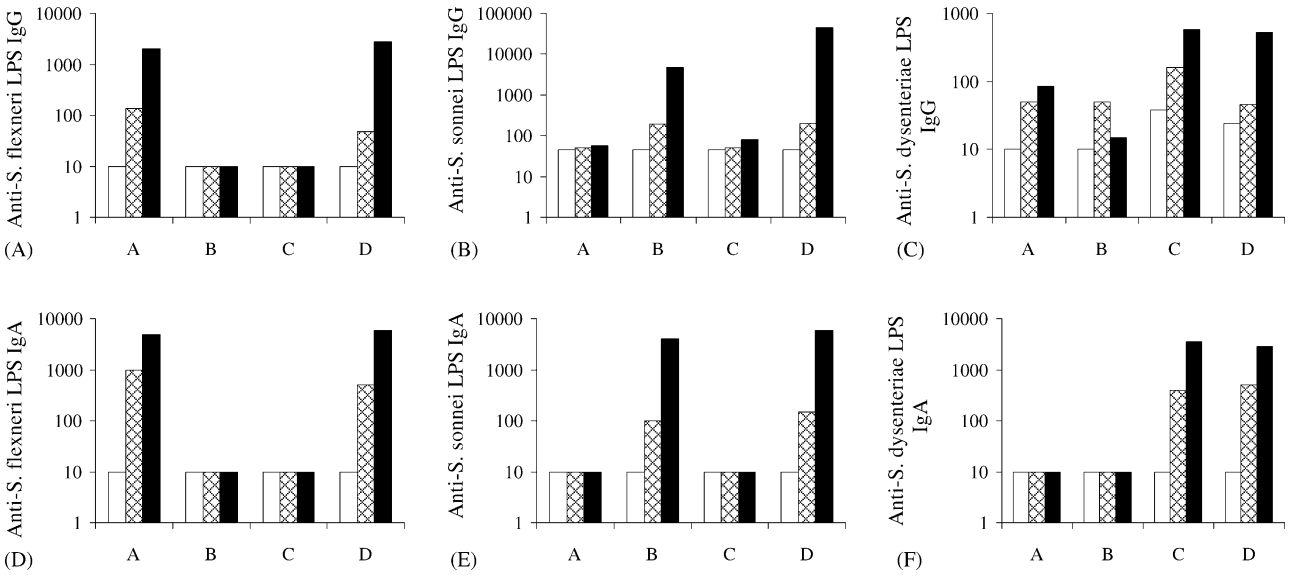

Fig. 4. Serum and mucosal antibody responses to live vectors from experiment 2. Antibody titers are shown as geometric mean titers for each group prior to immunization (open bars) or following dose 1 (hatched bars) or dose 2 (black bars). Panel A, serum anti-*S. flexneri* 2a LPS IgG response; panel B, serum anti-*S. sonnei* LPS IgG responses; panel C, serum anti-*S. dysenteriae* LPS responses; panel D, mucosal IgA anti-*S. flexneri* 2a LPS responses; panel E, mucosal IgA, anti-*S. sonnei* LPS responses; and panel F, mucosal IgA anti-*S. dysenteriae* LPS responses. Immunization groups were Group A, CVD 1208(pCFA.I-CS3); Group B, CVD 1233(pCS4-LThK63); Group C, CVD 1252(pCS2); and Group D, CVD 1208(pCFA/I-CS3) + CVD 1233(pCS4-LThK63) + CVD 1252(pCS2).

of just five *Shigella* strains, *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6, *S. sonnei*, and *S. dysenteriae* 1 would elicit protective immunity against approximately 80% of shigellosis strains. Critical ETEC antigens include fimbrial antigens CFA/I and CS1 through CS6 plus an antigen to elicit LT neutralizing responses. Previously we have demonstrated that individual ETEC fimbriae can be expressed with the correct morphology on the surface of attenuated *Shigella* strains and induce relevant serum and mucosal responses following immunization in a guinea pig model [23,25,26,30]. This strategy was successful due to the use of a specialized, stabilized plasmid expression system which ensures inheritance and maintenance of expression plasmids within the live vector [24]. Herein, we have extended these studies to investigate the ability of single expression plasmids to direct concomitant synthesis of two distinct ETEC antigens in the *Shigella* live vector. Two bicistronic plasmids were constructed. Expression plasmid pCFA/I-CS3 directs the synthesis of two fimbriae while pCS4-LThK63 directs synthesis of one fimbrial antigen plus an LT antigen. Both plasmids were stably maintained within *Shigella* live vector strains and able to elicit serum and mucosal antibody responses against both antigens when delivered via the live vector in the guinea pig model.

In order to investigate the use of a mixed strain inoculum, two immunization experiments were performed. Guinea pigs were immunized with a single *Shigella* live vector expressing one or two ETEC antigens or a mixture of two or three *Shigella* strains each expressing ETEC antigens. All immunized animals responded with strong antibody titers against the live vector strain used for inoculation. This is important in confirming that the co-expression of one or two guest antigens does not hinder the ability of the live vector to elicit relevant LPS responses. More importantly, however, was the ability of a mixed strain inoculum composed of *S. flexneri* plus *S. sonnei* plus *S. dysenteriae* attenuated derivatives to elicit strong serum and mucosal responses against each live vector component. There was no indication of immune dominance of any one strain and responses were equivalent to those elicited by each individual strain. Furthermore, the quality of these responses was confirmed by the ability of the immunized

Table 1
Protection of immunized animals following challenge with wild type *Shigella* in the Sereny test

Immunizing strain	Number of animals protected against keratoconjunctivitis/number of animals challenged with the indicated *Shigella* strain		
	S. flexneri 2a	*S. sonnei*	*S. dysenteriae*
CVD 1208(pCFA/I-CS3)	4/4	0/2	0/2
CVD 1233(pCS4-LThK63)	0/2	4/4	1/2
CVD 1252(pCS2)	0/2	0/2	2/2
CVD 1208(pCFA/I-CS3) + CVD 1233(pCS4-LThK63)	2/2	1/2	ND
CVD 1208(pCFA/I-CS3) + CVD 1233(pCS4-LThK63) + CVD 1252(pCS2)	1/2	2/2	2/2
Controls	0/6	0/6	0/2

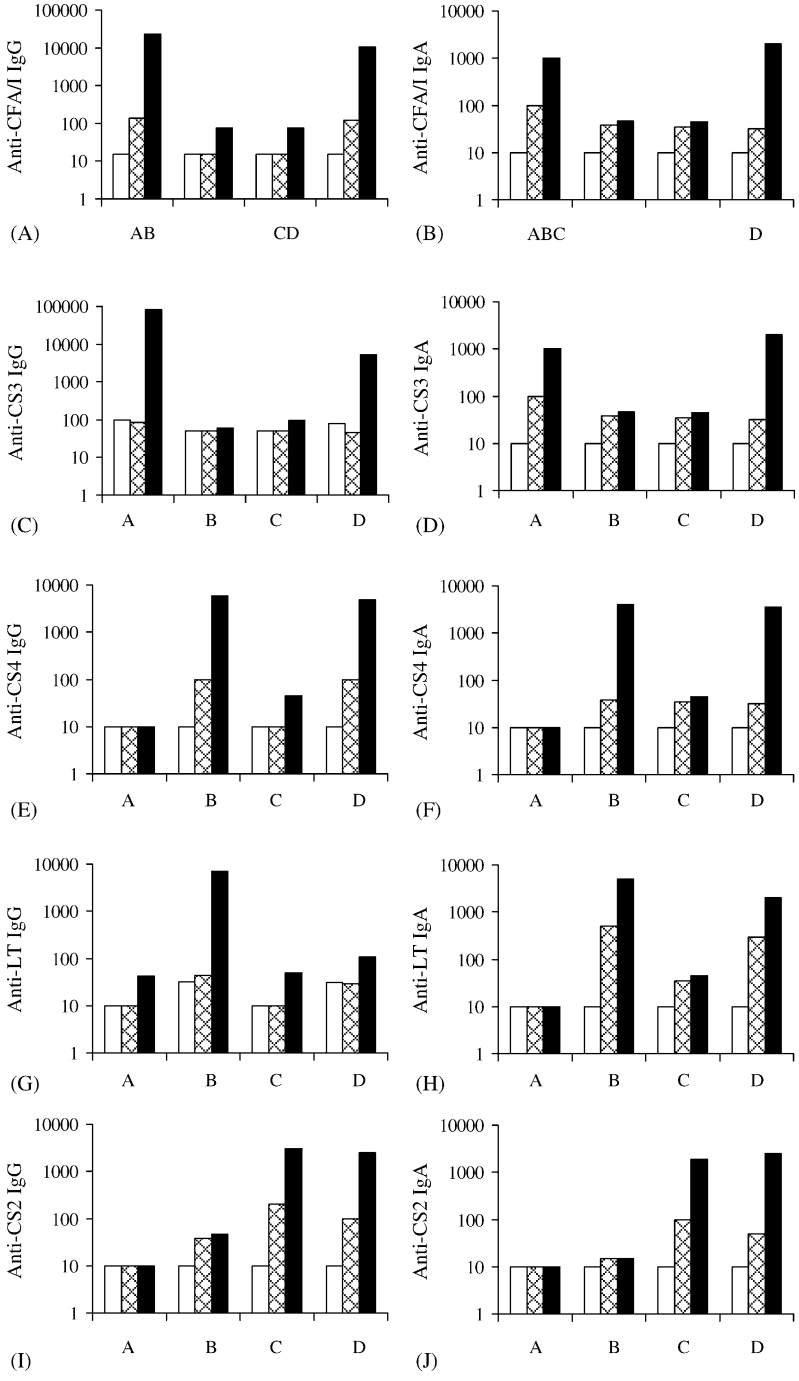

Fig. 5. Serum and mucosal antibody responses to ETEC antigens from experiment 2. Antibody titers are shown as geometric mean titers for each group prior to immunization (open bars) or following dose 1 (hatched bars) or dose 2 (black bars). Panels A, C, E, G, and I are serum IgG responses to the indicated antigens. Panels B, D, F, H, and J are mucosal IgA responses to the indicated antigens. Immunization groups were Group A, CVD 1208(pCFA.I-CS3); Group B, CVD 1233(pCS4-LThK63); Group C, CVD 1252(pCS2); and Group D, CVD 1208(pCFA/I-CS3) + CVD 1233(pCS4-LThK63) + CVD 1252(pCS2).

animals to be protected against wild type challenge. Animals were protected against homologous wild type challenge but not against heterologous challenge. Animals vaccinated with the mixed inoculum were generally protected against challenge with each component wild type parent. Two out of ten animals were not protected in these studies. This may be an indication of a suboptimal response in an individual animal and requires further investigation.

Finally, the mixed strain inoculum was able to engender both serum and mucosal responses against each of the five guest ETEC antigens expressed by the live vector strains. The immunogencity of this prototype multivalent formulation is

encouraging for further investigation of this strategy for the development and refinement of a combined *Shigella*-ETEC vaccine.

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