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

Eileen M. Barry*, Jin Wang, Tao Wu, Tamar Davis, Myron M. Levine

Center for Vaccine Development, University of Maryland, Baltimore, 685 West Baltimore Street, MD 21201, USA

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.

© 2005 Elsevier Ltd. All rights reserved.

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 regions. 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
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 CS7 [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 immunogenicity of S. flexneri 2a strain CVD 1208, which harbors attenuating mutations in gutR, 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 OD600 value of 0.5–0.8 at 37 °C. Cells were then washed twice with sterile, cold 10% glycerol in distilled H2O and resuspended in 1/75 the original culture volume in sterile, cold 10% glycerol in distilled H2O. Electroporation conditions were 1.75 kV, 600 μF and 25 μF.

2.3. Strain construction

Deletions in the gutR, set 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. flexneri 2a strain CVD 1252 also contains deletions in virG and stxA. The virG deletion was introduced using the suicide plasmid pSHΔvirG as previously described [22]. The deletion in the shiga toxin A subunit was introduced using the suicide plasmid pSHΔstxA 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 gutR4 gene 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 K2HPO4, 3.6 g of KH2PO4, 0.8 g of (NH4)2SO4, 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]. pEXO3 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 pCFAI-CS3 and pCS4-LThK63.

2.5. Western immunoblot analysis

Bacterial broth cultures were diluted to an OD600 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 Microwell Peroxidase (KPL Kirkegard & Perry Laboratories, Gaithersburg, MD) for visualization of protein bands. Membranes were probed with absorbed polyclonal rabbit anti-sera specific for each antigen. Western immunoblots were developed using the Immun-StarTM substrate system (BioRad Laboratories).

2.6. Immunizations and sample collection

Overnight cultures of the immunizing strains were harvested from TSA plates and incubated 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 (Kirkegaard & 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 Kirkegaard & Perry Laboratories). After a 15-min incubation, the reaction was stopped by the addition of 100 μl H2PO4, and the OD450 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 109 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 keratoconjunctivitis.

3. Results

3.1. Shigella strain construction

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

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); 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* LPS IgG response; panel B, serum anti-*S. sonnei* LPS IgG response; 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-LTbK63); and Group C, CVD 1208(pCFA/I-CS3)+CVD 1233(pCS4-LTbK63).

In a second experiment, guinea pigs were immunized with CVD 1208(pCFA/I-CS3), CVD 1233(pCS4-LTbK63), 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 10⁷ CFU in 10µl 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
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 strain [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>
<thead>
<tr>
<th>Immunizing strain</th>
<th>Number of animals protected against keratoconjunctivitis/number of animals challenged with the indicated Shigella strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri 2a</td>
<td>S. sonnei</td>
</tr>
<tr>
<td>CVD 1208pCFA/I-CS3</td>
<td>4/4</td>
</tr>
<tr>
<td>CVD 1233pCS4-LThK63</td>
<td>0/2</td>
</tr>
<tr>
<td>CVD 1252pCS2</td>
<td>0/2</td>
</tr>
<tr>
<td>CVD 1208pCFA/I-CS3 + CVD 1233pCS4-LThK63</td>
<td>2/2</td>
</tr>
<tr>
<td>CVD 1208pCFA/I-CS3 + CVD 1233pCS4-LThK63 + CVD 1252pCS2</td>
<td>1/2</td>
</tr>
<tr>
<td>Controls</td>
<td>0/6</td>
</tr>
</tbody>
</table>
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 immunogenicity of this prototype multivalent formulation is...
encouraging for further investigation of this strategy for the development and refinement of a combined Shigella-ETEC vaccine.

References


