LIVE ATTENUATED SHIGELLA AS A DIARRHEAL VACCINE AND/OR MUCOSAL DELIVERY VECTOR FOR DNA VACCINES

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ABSTRACT

Several live attenuated *Shigella* vaccines of different serotypes have been shown to be safe, immunogenic, and in one case effective against challenge with virulent strains. The ability to invade epithelial cells remains critical for the success of these vaccine candidates. Live, orally administered *Shigella* vaccine derivatives are also being evaluated as multivalent mucosal vaccines able to deliver both bacterial antigens and eukaryotic genes to the gut associated lymphoid tissues of the common mucosal immune system. Fimbrial and enterotoxin antigen genes from enterotoxigenic *E. coli* (ETEC) were cloned into a plasmid encoding the gene for aspartate semialdehyde dehydrogenase (*asd*) and expressed within SC608, an *asd* mutant of *Shigella flexneri* 2a vaccine strain SC602. Guinea pigs immunized intranasally with SC608 expressing the ETEC antigens demonstrated serum and mucosal immune responses to antigens from both diarrheal pathogens. SC608, in the absence of an *asd*-based plasmid, lyses within epithelial cells after invasion. This phenotype has also been used to deliver plasmid DNA vaccines containing eukaryotic genes into the host cell cytoplasm for expression. Splenocytes from mice immunized intranasally with *asd* mutants of *Shigella* containing plasmid HIV DNA vaccines demonstrated HIV antigen-specific IFN-γ ELISPOTS after *in vitro* expansion. These preliminary results are encouraging and suggest that live, attenuated *Shigella* vaccines can be engineered to deliver both prokaryotic and eukaryotic antigens to the mucosal system.

INTRODUCTION

Infectious diseases such as diarrhea and dysentery pose a significant threat to military operational effectiveness. During Operation Desert Shield (ODS)/Storm, diarrheal disease became a major threat to U.S. forces—57 percent of troops had at least one episode and 20 percent reported they were temporarily incapacitated. The leading cause of lost duty time during Operation Rescue Hope was acute diarrhea (1998 Army Science and Technology Master Plan, www.fas.org/man/dod-101/army/docs/astmp98/eb16.htm). *Shigella* and enterotoxigenic *E. coli* (ETEC) were the two principal causes of diarrhea (60% of the isolates) in ODS. Both pathogens are transmitted orally by contaminated food and water. Together they are responsible for 375 million annual episodes of diarrhea worldwide with 1.5 million deaths, predominantly in children <5 years in the developing world. While antibiotics have been the drugs of choice there is serious concern that most isolates are becoming increasingly and rapidly resistant to multiple antibiotics, which includes recent reports of ciprofloxacin-resistant *Shigella*. Therefore, vaccines against these pathogens are a top priority for USAMRMC.

Live, attenuated vaccine strain of *S. flexneri* 2a, SC602, which has a deletion of the plasmid *virG (icsA)* gene, required for bacterial spreading to contiguous host cells, and the chromosomal aerobactin gene *iuc*, required for binding iron, has recently undergone several Phase I trials for safety and immunogenicity in N. American volunteers (1, 2). SC602 was shown to be both safe and immunogenic at a single, oral dose of 10,000 CFU, and elicited substantial IgA and serum ELISA response against *Shigella* 2a LPS. A subset of these vaccinees were challenged with a virulent *S. flexneri* 2a strain, 2457T and found to be significantly protected against the more severe symptoms of shigellosis such as fever and dysentery (1). The invasive phenotype retained by SC602 is thought to be an important feature of the vaccine, responsible for generating the protective immune response. *Shigella* vaccines of other serotypes such as *S. sonnei* vaccine strain WRSS1 as well as *S. dysenteriae* 1 vaccine strain WRSd1, have also undergone successfully and are going through Phase I studies respectively, in human volunteers (3-5).

The development of successful live attenuated *Shigella* vaccines allows one the opportunity to expand their use as carriers of other mucosal antigens from heterologous bacterial or viral pathogens. Mucosal immunogens can induce cellular responses in both the systemic and mucosal immune compartments whereas even strong systemic immunogens tend to induce poor mucosal immune responses, if they induce mucosal responses at all (6). Attenuated *Shigella* vaccines offer several key advantages as mucosal vaccine delivery vectors (Fig. 1). *Shigella* vaccines are administered orally and are selectively passaged through the gut-associated lymphoid tissue (GALT) in the colonic mucosal surface where local inflammatory responses provide an opportunity to induce not only *Shigella*-

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 DEC 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Live Attenuated Shigella As A Diarrheal Vaccine And/Or Mucosal Delivery Vector For Dna Vaccines	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Walter Reed Army Institute of Research, Silver Spring, MD 20910; US Military HIV Research Program, HMJF, Rockville, MD. 20850;	

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**UU**	**8**	

specific secretory IgA but also long-lasting humoral and cell-mediated mucosal immunity against antigens from other pathogens expressed or carried by the *Shigella* vector (7-14).

Fig. 1 Advantages of using *Shigella* as a mucosal delivery vector.

Here we describe our efforts at using live attenuated *Shigella* vaccine strains to deliver both bacterial antigens on *asd*-based plasmids as well as eukaryotic genes on plasmid DNA vaccines.

I. LIVE ATTENUATED SHIGELLA VACCINES EXPRESSING ANTIGENS FROM ETEC

ETEC is an important cause of travelers' diarrhea and diarrheal deaths in children living in developing countries. ETEC is transmitted through contaminated food or water. However unlike *Shigella* infections which have a very low multiplicity of infection (10-100 colony forming units or cfu of bacteria can cause disease) the infectious dose for ETEC is in the range of 10^8-10^9 cfu (15). Once ingested, ETEC attach to mucosal epithelial cells of the small intestine using one or more plasmid-encoded fimbrial antigens or colonization factor antigens (CFAs or CFs). Several families of CFAs have been defined such as CFA/I, CFAII and CFAIV. Each family contains several related colonization factor sequences. ETEC secrete up to two enterotoxins designated heat-stable toxin (ST) and heat-labile toxin (LT). During the past 25 years, vaccine development efforts have been focused on induction of protective immunity against the CFs and the LT. Immune responses to ETEC infections indicate that secretory IgA (sIgA) directed towards CFs can provide protective immunity against homologous fimbrial type (15) and antitoxin immunity has been shown to provide at least short-term protection (16). At WRAIR we have utilized live *Shigella* vaccines to express ETEC antigens and tested them in animal models.

The production of CFA/I antigen requires two DNA regions: region 1 and region 2. These two regions are separated by about 40 kb on the wild-type plasmid of CFA/I-expressing ETEC. Region 1 contains the CFA/I operon composed of four genes (cfaABCE), which are important for proper fimbriae biogenesis (Fig. 2). The CfaA gene appears to function as an atypical chaperone and is necessary for periplasmic expression of CfaB, the major subunit of CFA/I fimbriae. CfaC and CfaE are considered 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 (18). Region 2 contains a regulatory protein CfaR belonging to the AraC family of positive regulators and is necessary for CFA/I synthesis (18).
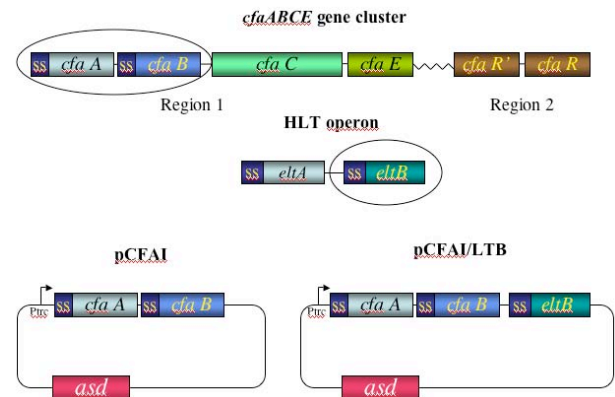

Fig. 2 Schematic representation of the CFA/I and heat-labile toxin operons. Below are the *asd*-based plasmids (pCFAI and pCFAI/LTB) derived from both operons.

A previously characterized *asd*-based 'balanced-lethal' system allows for the plasmid-based expression of genes in *asd* mutants without using an antibiotic selection for the maintenance of the plasmid (19). *asd* mutations result in lack of growth of the bacteria in LB media unless diaminopimelic acid (DAP) is added or the mutant is complemented with a plasmid bearing the *asd* gene. SC608, an *asd* mutant of SC602, was complemented with two *asd*-containing plasmid clones pCFAI and pCFAI/LTB, generating strains SC608(CFAI) and SC608(CFAI/LTB) (Fig. 2). The CfaA and CfaB genes were amplified with their signal sequences from ETEC strain H10407 and cloned downstream from the Ptrc promoter in pYA3098 generating pCFAI. The LT-B gene was separately amplified and a second round of PCR was used to create a single DNA fragment containing the CfaA, CfaB and the LTB ORFs. This DNA fragment was then cloned into pYA3098 generating plasmid pCFAI/LTB. pCFAI, pCFAI/LTB and the empty vector pYA3098 were electroporated into SC608 and the recombinants were tested for antigen expression and immune response in animal models.

Western blots of whole cell extracts using antiserum generated against intact CFA/I fimbriae showed that both SC608(CFAI) and SC608(CFAI/LTB) were expressing nearly equivalent amount of the 15-kDa CfaB protein (Fig. 3). When the same extracts were probed with antiserum to *E. coli* LT a ~12-kDa band corresponding to LTB was seen only in the extracts of SC608(CFAI/LTB).

Colony immunoblot assays in the absence and presence of lysozyme indicated that the ETEC antigens were most likely localized in the periplasm although more stringent methods may be needed to prove this definitively (Fig. 3). Expression of the heterologous antigens did not appear to affect the invasiveness of the *Shigella* vaccine since SC608(pYA3098), SC608(CFAI) and SC608(CFAI/LTB) were comparable to wild type *S. flexneri* 2a strain 2457T in the HeLa cell invasion assay.
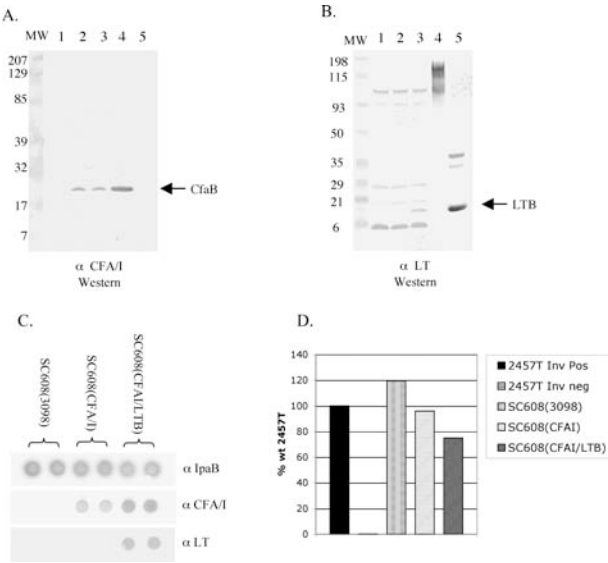

Fig. 3 Characterization of *Shigella*-ETEC hybrid strains. (A and B) Protein expression. Whole-cell extracts were prepared using log phase cultures from strains lane 1, SC608(3098), lane 2, SC608(CFAI), lane 3, SC608(CFAI/LTB). Lane 4 had 50 ng purified CFA/I fimbriae, lane 5, 50ng purified LT. Western blots were probed using the indicated rabbit polyclonal antibody. (C) Colony immunoblots. Colonies were grown on LB agar plates and lifted onto nitrocellulose, blocked with casein buffer 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 using the gentamicin protection asssay. Two assays were done on different days and the results were averaged. The invasiveness is plotted as a percentage of a wild-type 2457T invasion positive isolate. 2457T Inv Pos indicates a congo red positive colony, while Inv negative indicates a congo red negative colony.

Guinea pigs ocularly infected with 2457T develop an intense inflammatory reaction with purulent exudate resembling keratoconjunctivitis within 24-48. This assay, referred to as the Sereny test, evaluates intercellular dissemination of the bacteria after the initial invasion of corneal epithelial cells and is routinely used to assay both the virulence as well as the immunogenicity of *Shigella* strains. SC602, SC608(pYA3098), SC608(CFAI) and SC608(CFAI/LTB) are fully invasive in cultured epithelial cells but are negative in the Sereny test because they lack the VirG(IcsA) protein required for intercellular dissemination. However, SC602 mounts a strong immune response after either intraocular or intranasal immunization in guinea pigs. This immune response protects the eyes from developing keratoconjunctivitis upon a subsequent ocular challenge by 2457T.

Groups of guinea pigs were immunized twice intranasally with approximately 1 x 10^7 cfu per nare of freshly harvested SC608(pYA3098), SC608(CFAI) or SC608(CFAI/LTB) to test for immunogenicity and efficacy against challenge. The immunizations were spaced two weeks apart (Fig. 4).

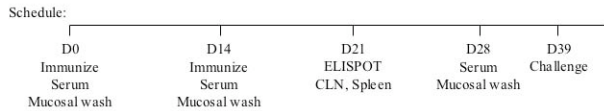| Group (# Guinea Pigs)	Vaccine Strain	Dose	Route
1 (14)	Naïve	2-5 x 10^7	IN
2 (14)	SC608/vector	2-5 x 10^7	IN
3 (14)	SC608/CFAI	2-5 x 10^7	IN
4 (14)	SC608/CFAI/LTB	2-5 x 10^7	IN

Fig.4 Schematic of guinea pig immunization protocol for *Shigella*-ETEC hybrid strains.

Spleens, cervical lymph nodes (CLNs), blood and eyewashes were collected for evaluating immune responses. IgG and IgA-specific immune responses in serum and mucosal secretions were measured using enzyme-linked immunosorbent assay (ELISA) on days 0, 14, and 28 after immunizations. These responses were measured against *S. flexneri* 2a LPS, intact CFA/I fimbriae and LT antigens. All groups immunized with an SC608 derivative induced significant LPS-specific serum IgG and IgA responses. Significant serum IgG responses to CFA/I were detected for SC608(CFAI) and SC608(CFAI/LTB) immunized animals and serum IgG response to LT in SC608(CFAI/LTB) was also significant (data not shown, 8). Serum IgA-specific immune responses to CFA/I and LT, although reduced in magnitude, were still considered significant in SC608(CFAI) and SC608(CFAI/LTB) when compared to SC608(3098) immunized animals. An ELISPOT assay for measuring antibody-secreting cells (ASCs) was used to detect local immune response to *Shigella* and ETEC antigens in spleen and CLNs (Fig. 5).
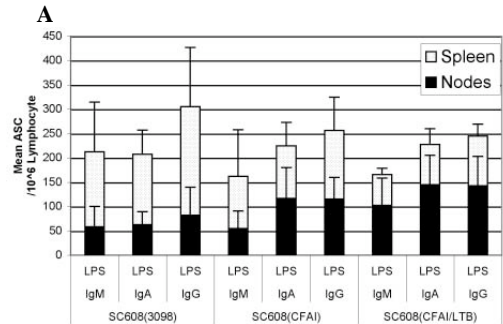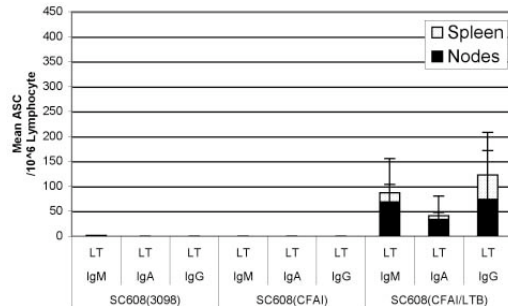
B
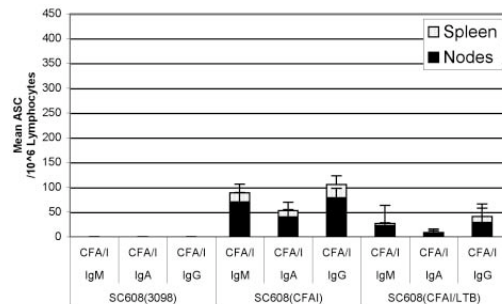
C

Fig. 5 Animal study using *Shigella* hybrid vaccine strains. Fifty-six male Hartley guinea pigs were intranasally immunized with 2-5x10^7 CFU per dose of Shigella strains spaced two weeks apart. One week after the second immunization spleens and cervical lymph nodes (CLNs) were harvested from 6 animals per vaccine group. Antigen-specific antibody secreting cells (ASCs) from CLNs (Nodes) and splenocytes (Spleen) were measured by ELISPOT analysis. Lymphocytes from individual animals were tested for response to A) *S. flexneri* 2a LPS B) purified CFA/I fimbriae and C) *E. coli* LT. The results are given as a mean ASC per 10^6 lymphocytes. Error bars indicate the standard deviation for each group.

S. flexneri LPS-specific IgG, IgA, and IgM antibody secreting cells (ASCs) were consistently detected and did not vary significantly ($P = 0.3$) between vaccine strains (Fig. 5, A). Using intact CFA/I fimbriae as an antigen, ASC's were detected in both SC608(CFAI) and SC608(CFAI/LTB) vaccinated animals with IgG being the dominant isotype (Fig 5, B). As expected LT-specific ASCs were detected for SC608(CFAI/LTB) only, and IgG dominated the isotype distribution (Fig. 5, C). A subset of vaccinated guinea pigs in all groups, including those that received normal saline as controls, were challenged three weeks after the final immunization with $5x10^8$ CFU of 2457T using the Sereny test. All groups immunized with a SC608 derivative were protected against severe keratoconjunctivitis (Table 1). SC608(CFAI)-immunized animals demonstrated the best protection.

Immunizing strain	No. of eyes inoculated	No. of eyes with the indicated rating[a]				Percent Protection[b]		
		0	1	2	3	F	P	C
Normal Saline	8	0	1	1	6	0	0	0
SC608(3098)	16	9	5	2	0	56.3	31.2	87.5
SC608(CFAI)	16	13	3	0	0	81.3	18.7	100
SC608(CFAI/LTB)	16	12	2	2	0	75.0	12.5	87.5

[a] Infammation: 0 indicates no inflammation or mild disease, 1 indicates mild keratoconjunctivitis, 2 indicates keratoconjunctivitis without purulence, 3 indicates severe keratoconjunctivitis with purulence.

[b] F=Full, P=Partial, and C=Combined

Table 1. Protection study of *Shigella*-ETEC hybrid strains in guinea pigs.

The ability of an invasive *Shigella* strain to deliver antigen in a predictable fashion to the mucosal cells of the lamina propria contributes to their effectiveness as vaccines. In previous studies, highly attenuated strains of *Shigella* have been used to express CFA/I fimbriae on the bacterial surface (7). These strains have shown efficacy in animal models. In the current study a different approach has been used to make bivalent *Shigella* vaccines where a fully invasive attenuated strain of *Shigella* (SC608) has been used to express ETEC antigens. By including only the chaperone and the major subunit from the CFA/I operon in SC608, the surface expression of ETEC fimbrial structures has been bypassed, maintaining invasiveness of the *Shigella* strain without affecting the immune response to ETEC antigens. Our approach for expressing ETEC CFA/I antigens in SC608 can be extended to include other antigenically distinct ETEC colonization factors. 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.

II. LIVE ATTENUATED SHIGELLA VACCINES AS CARRIERS FOR DNA VACCINES

There are several encouraging reports demonstrating *Shigella*-mediated delivery of DNA vaccines in animal models. Using *Shigella* 2a strains with an assortment of attenuating lesions, humoral, proliferative, or CTL responses have been demonstrated against β-galactosidase (9), tetanus toxoid (10), measles virus haemmaglutinin, F antigen, nucleoprotein (11), influenza virus haemmaglutinin (12) SIV *nef* and HIV gp120 (13) and HIV *gag* (14). In each case the antigens were expressed via a eukaryotic expression cassette contained on a plasmid DNA vaccine. Since *asd* mutants like SC608 and 15D (*asd* mutant of 2457T), in the absence of an *asd*-based complementing plasmid (pasd in Fig. 6), lyses within mammalian cells which lack DAP, such mutants can directly deliver the DNA vaccine to the host cell cytoplasm (Fig. 6).
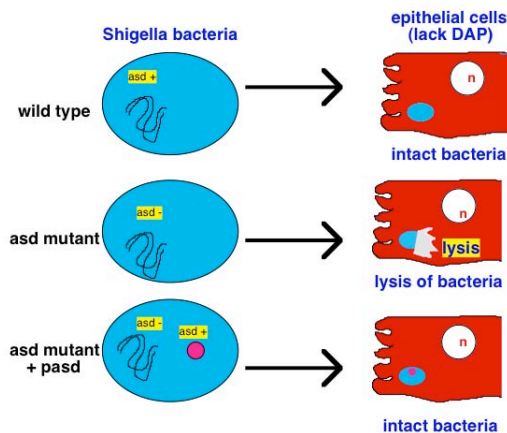
Growth Of Bacteria Within Mammalian Cells

Fig. 6. Schematic representation of the phenotype of wild type *Shigella* and *asd* mutants inside epithelial cells.

To confirm earlier observations, *Shigella* strains 15D, 15D(pCMVβgal), SC602(pCMVβgal), and SC608(pCMVβgal) were tested for the ability to invade HeLa cells and to deliver plasmid DNA (Fig. 7). pCMVβgal is a mammalian DNA expression plasmid, which contains the β-galactosidase gene under the control of the immediate early promoter and enhancer from the human cytomegalovirus (CMV). Invasion of HeLa cells was carried out by a standard invasion assay in the presence of DAP and gentamicin (Fig. 7).
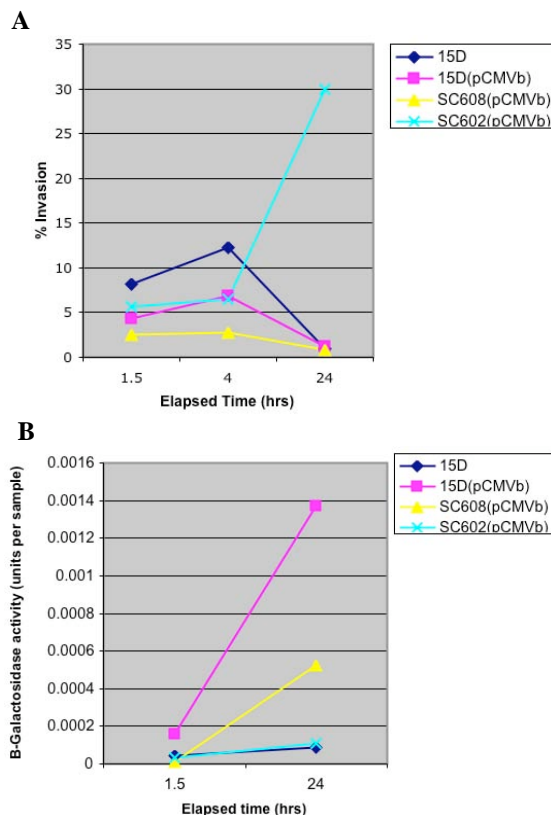

Fig. 7 HeLa cell invasion assay and b-galactosidase activity of

Shigella asd mutants carrying pCMVb (pCMVb in figure). HeLa cells were grown on cover slips in 24-well cell culture dishes to semiconfluence in MEM containing 10% calf serum and glutamine (MEM complete). Log phase cultures of bacteria grown in LB medium were resuspended in complete MEM with DAP (*asd* mutants) or without DAP (SC602) and added to individual wells. The medium in the wells also contained DAP where appropriate. The plates were incubated in a 37^0 CO2 incubator for 90 minutes. The HeLa cells were washed and fresh medium containing 50 ug/ml gentamicin was added and incubation continued for an additional 90 minutes. The cells were processed at this time which constituted the 1.5 hours time point. For the 4 and 24 hour time points, fresh MEM was added to the wells and incubation continued. A) For invasion assay the cover slips were stained with Giemsa stain and observed under the microscope. B) For b-galactosidase assay the cells were briefly trypsinized, the trypsin removed, and the cells collected in PBS. The cell pellet was treated with lysis buffer and enzyme activity was measured in cell extracts per manufacturer's instructions using a b-galactosidase reporter gene activity detection kit from Sigma.

Light microscopy of fixed and stained cells revealed that all three *asd* mutants could be recovered from 5-10% of the cells at 4 hours after invasion, but very few could be recovered after 24 hours. In contrast, upto 30% of the cells contained SC602(pCMVβgal) after 24 hours of invasion. Unlike the *asd* mutants, SC602 is expected to remain intact, invade and replicate within epithelial cells. However, when β-galactosidase activity was measured in HeLa cell extracts, increasing units of activity were detected in 15D(pCMVβgal) and to a lesser extent in SC608(pCMVβgal) indicating that the *asd* mutation results in release of the mammalian expression plasmid within the host cell cytoplasm. As expected, no β-galactosidase activity was detected with 15D alone or SC602(pCMVβgal) since SC602 is not expected to lyse following invasion of epithelial cells.

The ability of *Shigella* to deliver DNA vaccine constructs was assayed *in vivo* using an intranasal mouse model. *Shigella* strains SC608, 15D and 15G (15D without a kanamycin resistance cassette) were transformed with plasmid DNA pHIVA containing a gene encoding an immunogen designated HIVA (20, 21). The ampicillin determinant in pHIVA was replaced with a kanamycin resistance cassette generating pIHV003. The HIVA immunogen is a fusion protein derived from HIV-1, which consists of the consensus clade A sequences of *gag* p24[gag] and p17[gag] and a string of 25 partially overlapping CTL epitopes. The polyepitope also includes epitopes recognized by macaque and murine CTL (20, 21). 15Dsyngp120 was included in these studies as a positive DNA delivery control since previous observations have indicated that intranasal immunizations of Balb/c mice with 15Dsyngp120 (15D carrying a plasmid containing a synthetic gp120 sequence), elicited significant HIV gp120-specific IFN-γ responses (22). These responses could be attributed to T cells indicating DNA delivery and expression of syngp120 in mice. The frequencies of IFN-γ producing

T cells were comparable to those detected in mice immunized i.m. with purified syngp120 DNA vaccine (22).

Groups of Balb/c mice were immunized three times intranasally at 4 week intervals with $5x\ 10^6$ cfu of either SC608(pIHV003), 15D(HIVA), 15G(pIHV003) or 15Dsyngp120 as shown (Fig. 8).

SC608.003
15G.003
15D.HIVA
15Dsyngp120

Balb/c mice

Schedule:

D0	D28	D56		D133	D140
Immunize	Immunize	Immunize		Harvest	ELISPOT
				Spleen (IVS) & Serum	

Antigens for ELISPOT
IIIBV3 env (RGPGRAFVTI)
89.6env (IGPGRAFYAR)
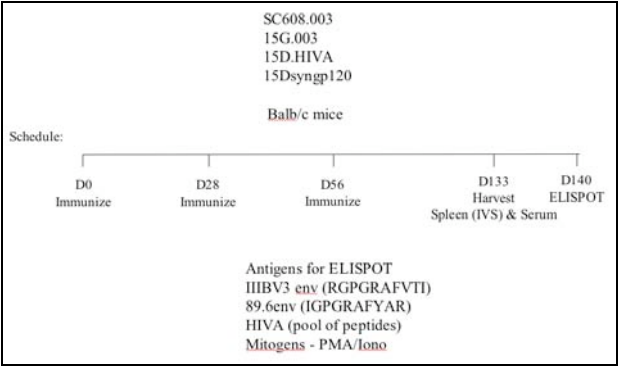HIVA (pool of peptides)
Mitogens - PMA/Iono

Figure 8. Schematic representation of schedule of mice immunization, harvesting of spleens and ELISPOT assay. *Shigella asd* mutants containing plasmid HIV DNA vaccines HIVA (15D.HIVA), pIHV003 (SC608.003 and 15G.003) and syngp120 (15Dsyngp120) were administered intranasally to Balb/c mice at days 0, 28 and 56. Peptides used for *in vitro* expansion of splenocytes are shown below the immunization schedule.

Mice were sacrificed 11 weeks after the last immunization. The control groups received either SC608 alone or only normal saline (Fig 8). HIV-specific T cell response was evaluated by enumerating IFN- γ producing cells (ELISPOT assay) (23). Freshly harvested splenocytes from immunized mice were stimulated with peptides which included IIIBV3 env (murine CTL epitope), HIVA p90 (pool of overlapping 15-mer gag peptides, does not contain V3) and the 89.6 peptide (one amino acid mismatch) specific for syngp120 protein (Figure 8). Splenocytes were pulsed with the peptides followed by addition of IL-2 for 7 days in culture. For each ELISPOT assay, 96-well nitrocellulose plates were coated with rat-anti-mouse-IFN-γ mAb AN18 and cells harvested from immunized mice were added to the wells. The assay was done in the presence of irradiated P815 target cells and respective peptide. As seen in Fig. 9, splenocytes from mice immunized with all four *asd* mutants carrying HIV DNA vaccine plasmids show HIV antigen-specific IFN- γ ELISPOTS indicative of T cell-mediated immune response to immunogens encoded on the DNA vaccine delivered by *Shigella*. Of interest, even though no significant ELISPOTs were observed in *ex vivo* splenocyte cultures, significant numbers of IFN- γ secreting cells were detected on stimulation with IL-2 for 7 days. This indicates that there are antigen-specific IFN- γ-producing cells at lower frequencies that could be expanded in culture to detect measurable response.

Serum antibody response to *Shigella* LPS was significant and comparable in all groups of mice (data not shown).

A

	SC608	SC608.003	15G.003
p815-	0	1653	360
p815+	0	3647	3160

Stimulated with HIVA gag peptide pool

B

	SC608	SC608.003	15D.HIVA	15G.003
p815-	0	2140	2356	1186
p815+	0	3027	2000	3090

Stimulated with V3 peptide

C

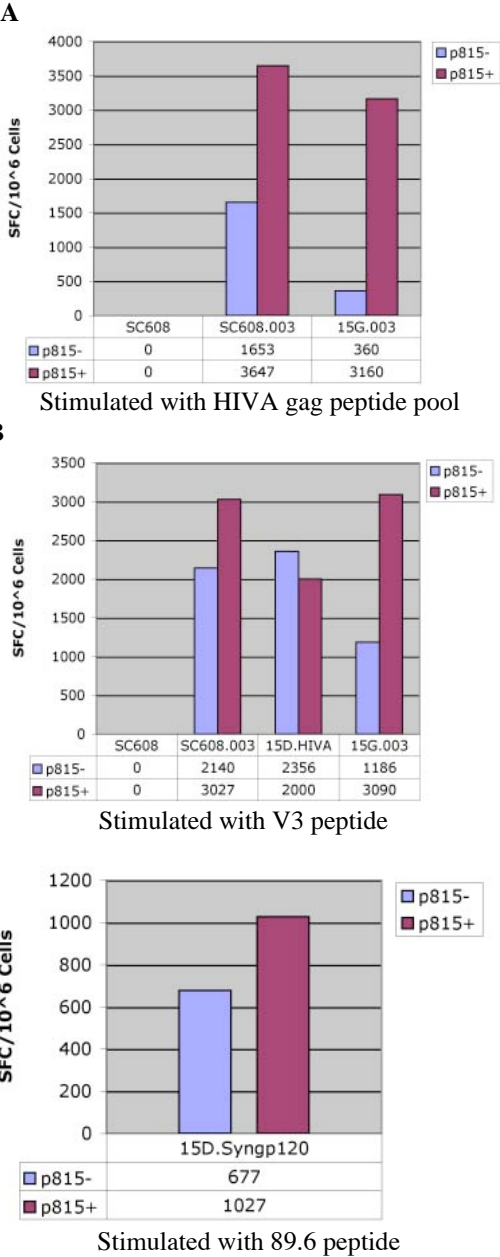|	15D.Syngp120
p815-	677
p815+	1027

Stimulated with 89.6 peptide

Figure 9. IFN-g-secreting cells from mice immunized with *Shigella asd* mutants carrying plasmid HIVA/pIHV003 DNA vaccine. Mice were immunized intranasally with 5×10^6 cfu of Shigella *asd* strains at weeks 0, 4, and 8 and spleens harvested 11 weeks after the last immunization. Spleen cells were pulsed with respective peptides followed by stimulation with IL-2 for 7 days *in vitro*. The peptides used were A) HIVA p90 pool of overlapping 15-mer gag peptides B) IIIBV3 env and C) 89.6. Splenocytes were added to 96-well plates coated with rat-anti-mouse-IFN-γ mAb AN18 and incubated with or without P815 cells. The production of IFN-γ by T cells was detected by addition of biotinylated rat anti-IFN-γ mAb. ELISPOTS were counted and expressed as SFC/10^6 spleen cells after subtraction of background number of SFC obtained in control saline mice splenocytes pulsed with the respective peptide during the assay. The actual SFC values after background subtraction, in the

presence and absence of P815 cells, are shown at the bottom of each graph.

This demonstrates that *Shigella* strains, with limited ability to replicate intracellularly, can induce good antibody response to itself as well as deliver DNA as measured by ELISPOT assay. These preliminary results are encouraging and demonstrate that live attenuated *Shigella* vaccine strains can be engineered to safely deliver HIV DNA vaccines in vivo.

A recent review on the design of an ideal HIV vaccine suggest that it may be necessary to build several vaccine components which collectively stimulate production of neutralizing antibodies, CTLs, mucosal immunity and the innate immune system (24). Live attenuated viral vaccines may be the most effective way to immunize against viral diseases but with HIV this is not possible. It has been a challenging task to develop a vaccine for HIV because of viral factors such as the hypervariability of sequences and masking of immunodominant epitopes. Selecting delivery systems has been an equally daunting task. Viral vectors are favored but they need to be delivered to mucosal sites; adjuvantation may be needed and have to be used in prime-boost regimens requiring multiple immunizations (25, 26, 27). Bacterial vectors (*Shigella*, *Salmonella*, *Listeria*, *BCG*) are attractive and advantageous as delivery vehicles for HIV antigens, particularly in developing countries, as they can be delivered orally, do not require additional adjuvants, and target antigens to mucosal sites, which may be important for HIV. Recently, attempts have been made to express HIV antigens in bacterial vectors and use them as delivery vehicles for DNA vaccines. Naked DNA vaccines by themselves as priming agents have shown limited success. The consensus opinion is that they can be improved with novel adjuvants. The results obtained with *Shigella asd* vectors containing pIHV003 or HIVA indicate that DNA vaccines can be efficiently delivered by attenuated bacteria after the system is optimized. Priming response to bacterially delivered DNA vaccines can be boosted with a variety of viral vectors expressing the same genes in a heterologous prime-boost system. It is proposed that optimization of the *Shigella* delivery systems should include testing a variety of attenuated bacterial strains in combination with different HIV plasmid DNA vaccine constructs. We believe that the preliminary results are encouraging and *Shigella* may prove to be a good self-adjuvant for enhancing humoral as well as adaptive and innate cellular immune responses to foreign antigens delivered by it.

ACKNOWLEDGEMENTS

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