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Safety and immunogenicity of attenuated *Salmonella enterica* serovar *Typhimurium* delivering an HIV-1 Gag antigen via the *Salmonella* Type III secretion system ☆

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Abstract

Background: CKS257 (*Salmonella typhimurium* SL1344 *ΔphoP/phoQΔ aroA Δasd ΔstrA/strB* pSB2131) is a live oral vaccine vector expressing HIV Gag.
Methods: HIV Gag was expressed as a fusion protein of a *Salmonella* Type III secretion system protein SopE, from a balanced lethal *asd*-based plasmid. Eighteen healthy adults were given single escalating oral doses of 5×10^6 to 1×10^{10} CFU of CKS257 and were monitored for clinical events, shedding and immune responses.
Results: Adverse events were mild except at the highest dose. Volunteers shed the organism an average of 5.1 days (range 0–13 days). Eighty-three percent (15/18) of subjects had a mucosal immune response to *Salmonella* LPS and flagella by IgA ELISPOT assay. Seventy-two percent (13/18) of subjects seroconverted to *Salmonella* antigens. No volunteer had a response to recombinant Gag as measured by serology, IgA ELISPOT, or immediate *ex vivo* γ-interferon ELISPOT response to Gag peptide pools. Two volunteers responded to Gag peptides by IL-2 ELISPOT, and 4 of 10 volunteers receiving $\geq 5 \times 10^8$ CFU had a response to HIV peptides in a cultured γ-interferon ELISPOT assay.
Conclusions: Although immunogenicity of the HIV antigen needs augmentation, the attenuated *Salmonella* strain proved to be an excellent platform for vaccine development.
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Keywords: *Salmonella*; Vaccine; HIV; Mucosal; Immunity

1. Introduction

Salmonella vaccines bearing foreign antigens can induce strong protective immune responses against a wide variety of infectious diseases in animal models. Live bacterial vaccines influence the type of immunity derived from vaccination, augmenting mucosal and cellular immunity compared with parenteral administration [1–3]. Previous human work with bacterial vectors expressing foreign antigens demonstrated modest immune responses to the vectored heterologous antigens [4]. Even vaccine constructs with promising immunogenicity profiles in animals have not been highly successful when directly transferred to *S. enterica* serovar *Typhi* (*S. typhi*) and studied in humans. Clinically, *S. enterica* serovar *Typhimurium* (*S. typhimurium*) produces a more vigorous and

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durable intestinal colonization than *S. typhi* [5], potentially increasing mucosal immunologic stimulation. We previously studied a *S. typhimurium* strain deleted for *phoP*/*phoQ* in humans, which appeared promising but inadequately attenuated [6].

Mucosal, humoral, and cell-mediated immunity all help protect against HIV [7]; as *Salmonellae* are able to elicit all these responses [8], they may be excellent vaccine vectors for HIV antigens. The intestinal immune system appears to be the site of intense HIV replication, persistence, and $CD4^+$ T-cell loss [9,10]. HIV Gag is a clinical relevant, structurally important conserved protein that has been frequently used in other vaccine constructs designed to elicit cellular immune responses [11,12].

The Type III secretion system (TTSS) of *Salmonella* is an important virulence mechanism that can be exploited for vaccine development. The *Salmonella* TTSS creates a macromolecular needle-like apparatus that "injects" proteins into host cells [13], directly accessing MHC class I-restricted antigen processing pathways. Fusion proteins of the *Salmonella* TTSS effector proteins with H-2 restricted influenza epitopes effectively present influenza antigens to MHC class I-restricted T-cell hybridoma cells *in vitro* and *in vivo* [14,15]. Mice immunized with *Salmonella* delivering an LCMV epitope via its TTSS were fully protected against lethal challenge [15]. In macaques, *phoP*/*phoQ*-deleted *Salmonella* vectors delivering a SopE–SIV–Gag fusion protein via the TTSS were able to prime antigen-specific $CD4^+$ and $CD8^+$ responses against SIV antigens [14]; after boosting with modified vaccinia Ankara delivering SIV–Gag, these animals exhibited high levels of antigen-specific T-cells that expressed the $\alpha_4\beta_7$ integrin, indicating a significant mucosal immune response. To further investigate the utility of both *S. typhimurium* vectors and the TTSS system in humans, we undertook a Phase I dose escalation, safety, tolerability and immunogenicity trial of an attenuated live *S. typhimurium* (*ΔphoP*/*ΔphoQ ΔaroA ΔstrA*/*ΔstrB Δasd*) strain delivering a SopE–HIV-1 Gag fusion protein. The investigational strain was found to be safe and induced a strong immune response to *Salmonella* antigens, with modest immune responses to the HIV Gag antigen.

2. Methods

2.1. Bacteriology

S. typhimurium SL1344 is a wild-type strain with a 50% lethal dose of <20 CFU intraperitoneally in BALB/c mice [16]. The *ΔphoP*/*ΔphoQ* deletion was introduced into strain SL1344 [17–19]. Non-polar, in-frame deletions in *aroA*, *strA*/*strB*, and *asd* genes were constructed using the suicide vector pCVD442 [20], overlap extension PCR, and sequencing confirmation.

A "balanced lethal" plasmid stabilization system using the *asd* locus encoding aspartate semialdehyde dehydrogenase [21] was constructed to express the fusion protein from pSB2131 [14]. pSB2131 is a pWSK29 derivative, a low copy number plasmid [22]. The ampicillin resistance cassette (*bla*) in the isogenic precursor plasmid pSB2065 was replaced with the *asd* cassette from plasmid pYA280 [21].

2.2. Preclinical evaluation of bacterial strains

Bacterial cultures were grown overnight in 0.3 M NaCl Luria–Bertani (LB) broth (for increased fusion protein expression [23,24]) with aromatic amino acid supplementation. Western blotting detected the expected fusion protein [6]. Culture supernatants were concentrated by precipitation with 20% trichloroacetic acid, separated by SDS-PAGE, and blotted to nitrocellulose. Murine monoclonal antibodies directed against either the M45 protein tag [25] or HIV-1 Gag p24 (NIH AIDS Reference Reagent Program) were used, followed by a secondary sheep anti-mouse antibody conjugated to horseradish peroxidase (Amersham), then chemiluminescent substrate (LumiGlo, KPL).

Intracellular survival of CKS257 was evaluated in J774A.1 murine macrophage-like cells (ATCC TIB-67) and human U937 macrophage-like cells (ATCC CRL-1593.2) using gentamicin exclusion assays [26,27]. Eukaryotic cells were infected at multiplicities of infection of 20–30:1 for 30 min at 37 °C. The intraperitoneal 50% lethal dose (LD_{50}) of the investigational strain CKS257 was measured in 10-week-old BALB/c mice [6,28,29].

The ability of CKS257 to deliver the fusion protein with an influenza nucleoprotein (NP) epitope via the class I-restricted antigen presenting pathway was evaluated *in vitro* [15]. Murine RMA thymoma cells ($H\text{-}2^b$) were infected with *S. typhimurium* and then mixed with the cognate T-cell hybridoma 12.164. Influenza NP epitope presentation by class I-restricted mechanisms was quantified by interleukin-2 (IL-2) secretion by the T-cell hybridoma (IL-2 ELISA, Pharmingen) [15].

2.3. Clinical study

The study was an open-label, dose-escalation design. The study was approved by the IRB at the Massachusetts General Hospital, overseen by an independent Data Safety Monitoring Board, and conducted under FDA IND #11239. All subjects provided written informed consent. Healthy volunteers 18–45 years old and at low risk for acquiring HIV were screened [6]. Subjects were HLA B-27 and HIV negative. Fasting volunteers were given single, oral, escalating doses of the live attenuated bacterial strain (5.0×10^6 to 1×10^{10} CFU), administered in 30 ml of 0.9% saline after an antacid solution (2 g of $NaHCO_3$). Subjects were monitored for 14 days as inpatients. Blood cultures were obtained daily on days 1–10. Stools were counted and graded for consistency [6]. Diarrhea was defined as four or more bowel movements (based upon our experience over the past decade in querying healthy individuals). Up to three stools were cultured each day. On

days when a volunteer did not have a bowel movement, a rectal swab was obtained. Volunteers returned for six weekly outpatient visits.

2.4. Immunologic assays

2.4.1. Antigens

Vaccine-specific antigens included purified *S. typhimurium* lipopolysaccharide (LPS) (Sigma), *S. typhimurium* flagella, p24 protein (the core antigen capsid of HIV Gag) (Protein Sciences), and synthetic peptides. Flagella were purified from wild-type *S. typhimurium* SL1344 [6]. A complete set of HIV consensus subtype B Gag 15-mer peptides, overlapping by 11 amino acids and >80% pure, was obtained (NIH AIDS Research and Reference Reagent Program). An additional 12 peptides were synthesized when the sequence of the fusion protein varied from the clade B sequence, as were 19 aminoterminal SopE 16–19-mer peptides, overlapping by 10 amino acids and >80% pure (Partners AIDS Research Center). EBV-derived HLA class I-restricted CTL peptides were kindly provided by Dr. Christian Brander [30]. Pools of 9–11 peptides were used, at a final concentration of 5 μg/ml per peptide.

2.4.2. ELISAs

End-point dilution ELISAs were performed to evaluate serum IgG against vaccine antigens [18]. End-point dilutions were defined as the serum dilution at which the $OD_{405\,nm}$ was ≥0.15. Seroconversion for *S. typhimurium* antigens was defined ≥4-fold increase in the end-point titer. ELISA results were compared to paired serum samples drawn 14 days apart from nine healthy adults who received parenteral influenza vaccine in 2004. Similar research laboratory ELISA testing examined IgG against recombinant HIV p24 antigen in the three highest dose cohorts. All subjects had standard hospital blood bank HIV ELISAs utilizing the HIV-1/HIV-2 (rDNA) EIA (Abbott Laboratories).

2.4.3. ELISPOTs

ELISPOT studies were performed using freshly isolated peripheral blood mononuclear cells (PBMCs) [6,18] on days 0, 7, 10, 14, and 28. A positive vaccine-specific IgA-bearing cell result was considered to be ≥6 spots/10^6 PBMC [18]. For γ-IFN ELISPOT studies, MAIPS4510 plates (Millipore) were coated overnight with primary monoclonal mouse anti-human recombinant IFN-γ antibody (Endogen). 2×10^5 PBMCs were applied with peptide pools (HIV Gag, SopE, or EBV) at 5 μg/ml, or phytohemagglutinin (PHA) 5 μg/ml. A biotin-conjugated secondary mouse anti-human IFN-γ antibody (Endogen) and streptavidin-alkaline phosphatase solution with substrate were used to develop plates. Spots were counted using an AID ELISPOT reader system (Cell Technology, Inc). Responses were considered positive if both ≥50 spot-forming cells (SFCs) were detected per 10^6 cells after subtraction of the negative control and SFCs were ≥2-fold the negative control [31]. IL-2 ELISPOT studies were performed similarly at the IAVI Core Immunology laboratory using an IL-2 antibody ELISPOT pair (BD Biosciences).

2.4.4. Cultured ELISPOT

An *in vitro* expansion assay (cultured ELISPOT) was developed to enhance the sensitivity of the γ-IFN ELISPOT [32–34]. Frozen PBMCs were resuspended in R10-AB, composed of RPMI 1640 (Sigma) with heat-inactivated human AB serum (Sigma), L-glutamine (Sigma) recombinant human IL-7 (Biosource International), penicillin and streptomycin (Sigma). The peptide pools (above) were condensed into four "super-pools" of 44–48 peptides. Cells were cultured for 10 days with peptides. On days 3 and 7, recombinant human IL-2 (Biosource International) was added to a final concentration of 20 Units/ml. On day 10, cells were washed and rested. On day 11, 40,000 cells/well were analyzed in the standard ELISPOT procedure. Responses were considered positive if both ≥250 SFCs were detected per 10^6 cells (after subtraction of the negative control) and SFCs were ≥4-fold above the negative control, similar to criteria used with another HIV-1 Gag vaccine [34].

3. Results

3.1. Preclinical bacteriology

A balanced lethal plasmid, pSB2131, encoding a fusion protein between the first 104 amino acids of SopE and a mutant HIV Gag protein that has been optimized for Type III secretion (Chen and Galán, submitted for publication) as shown in Fig. 1 was constructed. The chimeric protein also contains an M45 monoclonal antibody tag (MDRSRDRLPPFETETRIL) [35] and an influenza A nucleoprotein peptide (QIASNENMETMESSTLELRSR), to monitor pro-

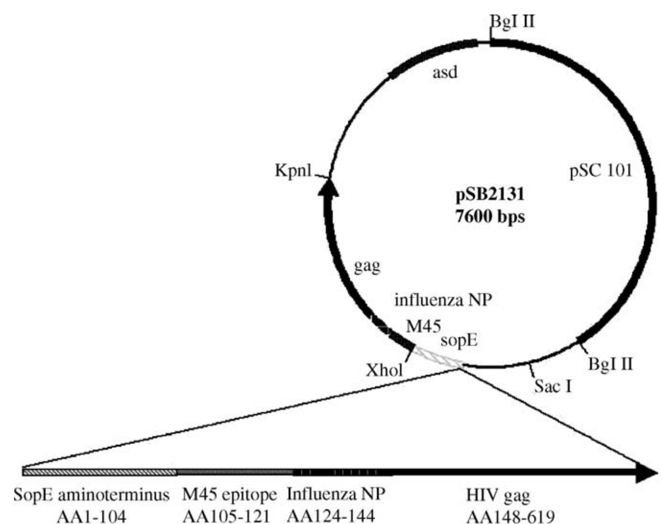

Fig. 1. Plasmid map of pSB2131, expressing fusion protein antigen: SopE–M45–FluNP–HIV Gag (619 amino acids). The plasmid contains an *asd* cassette at the former site of a *bla* cassette.

tein expression and MHC Class-I-restricted antigen presentation, respectively.

All anticipated chromosomal deletions in the bacterial vector organism were confirmed by sequencing and were as expected with one exception. The *ΔphoP*/*ΔphoQ* mutation was expected to encompass a 954 bp deletion, based upon Southern blotting [6], but the actual deletion present in this and other *ΔphoP*/*ΔphoQ* mutants [6,18] is 1209 bp. SL1344 is streptomycin resistant, a phenotype conferred by the *strA*/*strB* gene [36], which was deleted and the deletion mutant was streptomycin susceptible.

Western blotting was used to demonstrate that CKS257 secreted the expected 78 kDa SopE–HIV Gag fusion antigen in culture supernatant (Fig. 2); no protein was detected in *Salmonella* with the "empty plasmid". For unclear reasons, a prototype strain lacking the *asd* mutation and containing a plasmid encoding ampicillin resistance (lane 3, Fig. 2) consistently produced significantly more antigen than the subsequent *asd*-deleted, balanced lethal plasmid strain (lane 5).

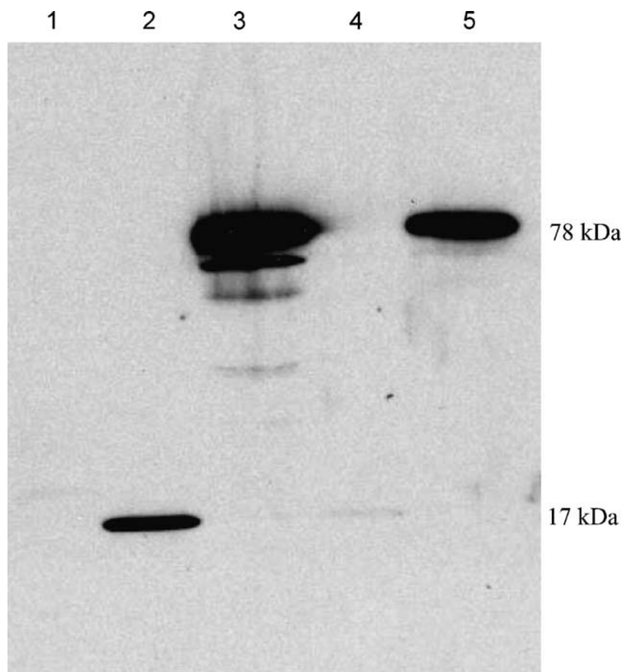

Fig. 2. Western blot of SopE–HIV Gag fusion protein. Concentrated bacterial culture supernatants from the indicated strains were separated on a 10% polyacrylamide gel and blotted to nitrocellulose. A monoclonal antibody directed against the M45 protein tag was used to probe the blot. Each lane contains approximately 10 μg of protein. Lane 1: SL1344 wild type (negative control); lane 2: SL1344 *ΔphoP*/*ΔphoQ* *ΔaroA* *ΔstrA*/*ΔstrB* pSB1784 (*bla* plasmid without HIV fusion protein); lane 3: SL1344 *ΔphoP*/*ΔphoQ* *ΔaroA* *ΔstrA*/*ΔstrB* pSB2065 (similar to pSB2131 but with *bla* not *asd*); lane 4: SL1344 *ΔphoP*/*ΔphoQ* *ΔaroA* *ΔstrA*/*ΔstrB* *Δasd* pSB2128 (*asd* plasmid without HIV fusion protein); lane 5: SL1344 *ΔphoP*/*ΔphoQ* *ΔaroA* *ΔstrA*/*ΔstrB* *Δasd* pSB2131 (investigational strain). The lighter bands below the very large band in lane 3 likely represent degraded protein. The fusion antigen was detected in cultures with the SopE–HIV Gag plasmid at about 78 kDa. A much smaller SopE–M45-influenza NP protein was appropriately detected in strains with the "empty plasmid" (lane 2, pSB1784, lacking the HIV *gag* sequence) and no protein was detected in *Salmonella* without the plasmid (lane 1). Strains in lanes 2 and 3 contain plasmid stabilized by the *bla* cassette; strains in lanes 4 and 5 contain a plasmid stabilized by the *asd* balanced lethal system. Plasmids stabilized by the *bla* gene appeared to reproducibly secrete more fusion antigen.

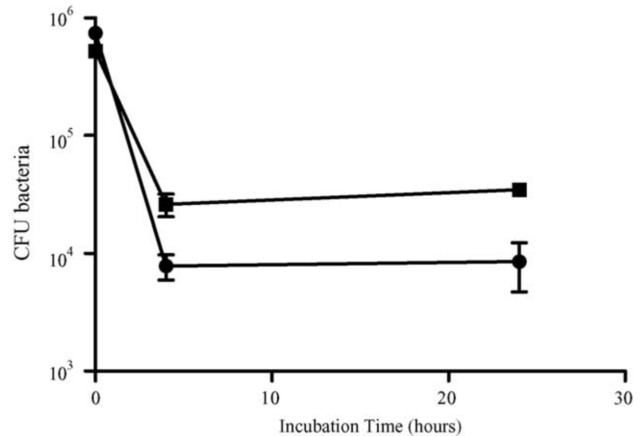

Fig. 3. Invasion and persistence of *S. typhimurium* within U937 human macrophage-like cells. Wild-type (SL1344, ■) and investigational (CKS257, ●) strains were grown overnight in 0.3 M NaCl Luria–Bertani broth with aromatic amino acid supplementation. Suspension cultures of 5×10^5 U937 cells were infected at a multiplicity of infection of 25:1 and incubated for 30 min at 37 °C in the presence of 5% CO_2. The medium was removed and the cells were washed twice with phosphate buffered saline. Fresh medium containing gentamicin 15 μg/ml was added and incubated for a further 30 min. At this point (0 h) and at 4 and 24 h, triplicate cultures were washed to remove excess gentamicin, and eukaryotic cells were lysed with 1% deoxycholate to release intracellular bacteria. Lysates were serially diluted and plated in triplicate on LB agar plates containing aromatic amino acids and CFU counted. Data shown are mean values ± S.E.M. of CFU/ml. The investigational strain survived less well in human macrophage-like cells than wild-type organisms.

Intracellular survival within macrophages is important to the pathogenesis and persistence of *Salmonellae* [37–40]. CKS257 had defective survival within the human macrophage-like monocytic cell line U937 as compared to wild-type organisms (Fig. 3) and in murine J774.1 macrophages (data not shown). A comprehensive analysis of intracellular survival of Salmonellae within U937, J774, and primary murine and human macrophages showed that although elutriated macrophages are more metabolically active, nontyphoidal Salmonella survival trends are similar in U937 and elutriated macrophages [41].

To demonstrate that *S. typhimurium* is capable of delivering the SopE–HIV Gag fusion protein to the HLA-restricted, class I antigen presenting pathway via the TTSS, the RMA assay was used [15,42] (Table 1). *ΔphoP*/*phoQ* mutants induced greater IL-2 secretion; TTSS proteins are *phoP*-repressed genes. Deletion of *aroA*, *asd*, and *strA*/*strB* loci did not significantly alter this phenotype. *S. typhimurium* strains derived from SL1344 consistently resulted in greater IL-2 responses than ATCC14028 strains (data not shown).

The murine LD_{50} for CKS257 delivered i.p. was calculated to be 2×10^8. An *S. typhimurium phoP*/*phoQ aroA* mutant ($LD_{50} = 1.25 \times 10^7$), is more attenuated than either single mutant ($LD_{50} = (1–3) \times 10^6$), and the addition of the

Table 1
Quantification of class I Ag presentation of an influenza epitope contained within the SopE–M45–NP–HIV fusion antigen

S. typhimurium SL1344		Plasmid/peptide	IL-2 in pg/ml ± S.E.M.
CKS171	*ΔstrA*/*ΔstrB*	pSB2065 Amp^R	252 ± 16
CKS91	*ΔphoP*/*ΔphoQ*	pSB2065 Amp^R	390 ± 20
CKS118	*ΔphoP*/*ΔphoQ ΔaroA*	pSB2065 Amp^R	375 ± 14
CKS173	*ΔphoP*/*ΔphoQ ΔaroA ΔstrA*/*ΔstrB*	pSB2065 Amp^R	326 ± 19
CKS257	*ΔphoP*/*ΔphoQ ΔaroA ΔstrA*/*ΔstrB Δasd*	pSB2131 *asd*	352 ± 15
	Negative control	RPQASGVYM	<10 pg/ml
	Positive control	ASNENMETM	1846 + 78 pg/ml

Fusion antigens were secreted from deletion mutants of SL1344 as indicated. All fusion antigens contain the influenza NP epitope, as an immunological tag. Mouse (H-2^b) thymoma cells were infected with mutant *Salmonella* expressing the secreted fusion antigens. The secreted proteins are processed and peptides are exported to the cell surface in conjunction with MHC class I molecules. Thymoma cells are then mixed with a cognate T-cell hybridoma, which recognizes the influenza NP epitope tag and becomes activated, resulting in IL-2 secretion. ELISA quantifies IL-2 secretion from cell culture supernatants. When antigens are not "injected" via the Type III system, they are not processed, and the hybridoma does not produce IL-2. IL-2 secretion was reproducibly greater in mutants deleted for the *phoP*/*phoQ* locus as compared with wild type. Sequential deletion of the *aroA*, *strA*/*strB*, or *asd* loci did not substantially alter IL-2 production, nor did switching from the plasmid with an ampicillin resistance gene to an isogeneic plasmid when the *bla* was replaced by *asd*. Each condition was tested in triplicate culture wells. Negative controls used RMA cells loaded with an irrelevant lymphocytic choriomeningitis virus peptide, RPQASGVYM. As previously described [15], positive controls used RMA cells loaded with the cognate peptide ASNENMETM from the influenza virus nucleoprotein.

SopE–HIV Gag plasmid and corresponding chromosomal *asd* deletion results in further attenuation by approximately 1 log CFU.

3.2. Clinical responses

Seven volunteers felt completely well; eight had mild constitutional symptoms. Three had gastroenteritis. Volunteer 14 (5×10^9 CFU) had gastroenteritis (5–10 grade 4 bowel movements/day for 3 days) with a temperature peak of 100.4 °F (38.0 °C). Volunteers 17 and 18 (highest dose, 1×10^{10} CFU) developed diarrhea with fevers of 103.0 °F (39.4 °C) and 101.0 °F (38.3 °C) acutely at 17 and 22 h after vaccination. Symptoms resolved within 5 and 2 days after dosing. Volunteer 17 received ciprofloxacin and intravenous fluids. Volunteer 18 recovered with oral fluids. No volunteer had blood cultures that were positive for the investigational organism, including additional cultures obtained at the time of fever.

Four volunteers developed abnormal liver function tests. Volunteer 4 had the most significant increase, with a rise in aminotransaminases starting on day 4. The transaminases peaked on day 12, with a SGPT at 2.25 times the upper limit of normal (ULN); this test returned to normal by day 18. Total and direct bilirubin, alkaline phosphatase, and GGT remained normal. Volunteer 7 had a small isolated increase in aminotransaminases starting on day 10, with a SGPT 14 points above the ULN, and a return to normal on day 12. Volunteers 10 and 14 (women), had mild, transient transaminase increases that would have been within normal range for men; ours is one of the few institutions that has sex-based ranges.

3.3. Shedding

All but one volunteer (lowest dose) had investigational bacteria in stool cultures. Average shed time was 5.4 days, range 0–13 days (Fig. 4). Volunteer 9 was given ciprofloxacin per protocol on day 14 for a positive stool culture on day 13 to hasten clearance. All volunteers had negative stool cultures by day 14 and at six weekly visits thereafter.

3.4. Immune responses to Salmonella typhimurium

Serum titers of IgG against LPS and flagella are the peak values after vaccination, which occurred at either day 10 or 14 (Table 2). 13/18 subjects seroconverted to *S. typhimurium* LPS (Table 2). 7/18 responded to *S. typhimurium* flagella (data not shown). None of nine parenteral influenza vaccines paired sera had a 4-fold increase in either flagella or LPS titer (13 of 18 versus 0 of 9, $P = 0.001$ by the Mann–Whitney U-test).

IgA ELISPOT responses to *Salmonella* antigens were vigorous (Table 2). Fifteen of 18 volunteers had evidence of mucosal immune responses to *Salmonella* LPS by IgA ELISPOT (range 12–2500 antigen secreting cells (ASC)/10^6 PBMCs), and 12/18 volunteers had positive IgA ELISPOT

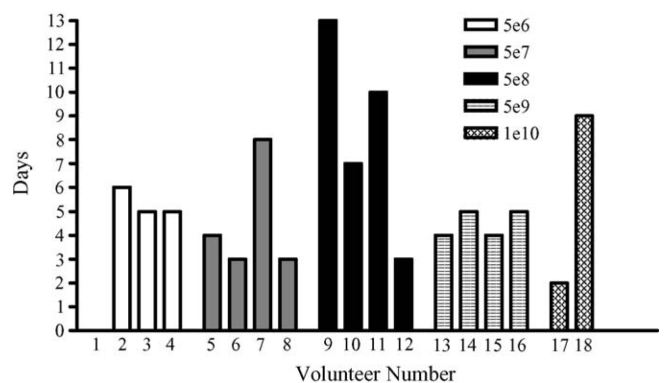

Fig. 4. Fecal shedding of individual volunteers. Stool cultures were performed by both direct plating and plating after incubation in selenite enrichment broth. On average, over a broad dose range, volunteers shed the investigational organism for 5.4 ± 3.1 days, range 0–13 days. Volunteers 14, 17, and 18 had gastroenteritis. Volunteers 9 and 17 received oral antibiotics for prolonged secretion and symptomatic illness, respectively (see text for details).

Table 2
IgA ELISPOT and IgG ELISA response to *Salmonella* vector antigens

Volunteers	Actual dose	IgA ASC/10^6 PBMC[a]		Serum IgG titer vs. LPS		
		vs. LPS	vs. Flagella	Preimmune	Postvaccine	Fold increase
1	$(3–5) \times 10^6$	11	3	1:10	1:40[b]	4×
2		3	1	<1:10	<1:10[c]	–
3		1	0	<1:10	1:<10[c]	–
4		68	4	1:20	1:80[b]	4×
5	5×10^7	963	438	<1:10	1:1280[b]	>128×
6		2500	850	1:10	1:40[b]	4×
7		44	17	<1:10	1:40[b]	>4×
8		2	1	1:20	1:40[c]	2×
9	$(4–6) \times 10^8$	1025	140	1:20	1:80[b]	4×
10		263	160	1:20	1:160[b]	8×
11		505	413	<1:10	1:160[b]	>16×
12		12	3	<1:10	1:<10[c]	–
13	$(4–5) \times 10^9$	883	863	<1:10	1:80[b]	>8×
14		675	600	1:10	1:320[b]	32×
15		290	163	1:10	1:20[c]	2×
16		655	375	1:10	1:40[b]	4×
17	1.0×10^{10}	2100	1100	1:40	1:1280[b]	32×
18		488	160	1:10	1:640[b]	64×

Serum, salivary, and fecal IgA were not evaluated.
[a] More than six antibody-secreting cells/10^6 PBMC cells is a positive result.
[b] Significant increase (four-fold or greater increase in titer).
[c] NS, not significant.

results to *S. typhimurium* flagella (range 17–1100 ASC/10^6 PBMCs). The magnitude of the responses did not appear to be consistently dose-related.

Immediate *ex vivo* γ-IFN ELISPOT was performed on days 0, 7, 10, 14, and 28 using SopE peptides. Native SopE is present within the investigational strain, in addition to the aminoterminal SopE present in the SopE–HIV Gag fusion antigen. Three volunteers (numbers 6, 14, and 17) had borderline results (range 27–48 SFCs/10^6 PBMCs; all more than twice background); no volunteers had positive responses by study criteria (data not shown). Cultured γ-IFN ELISPOT assays on day 14 showed an unequivocal response to SopE peptides in 1/9 volunteers tested (number 11; 500 SFCs/10^6 PBMCs and 10 times background) and borderline response in volunteers 9 (1925 SFCs/10^6 PBMCs) and 17 (2075 SFCs/10^6 PBMCs), both 2.6 times background.

3.5. Immune responses to HIV p24 antigen

Standard HIV ELISAs performed by the hospital blood bank remained negative in all volunteers through day 56. Serum IgG ELISAs with HIV p24 antigen performed in the research lab were negative in the 10 volunteers tested. IgA-ELISPOT responses against recombinant HIV p24 antigen were not detected.

Immediate *ex vivo* γ-IFN ELISPOT responses to HIV Gag peptides were borderline positive in two volunteers (volunteer 3 at 5×10^6 and volunteer 9 at 5×10^8). Immediate *ex vivo* IL-2 ELISPOT responses were positive in volunteers 14 and 18 (65 SFC and 56 SFC/10^6 PBMCs, respectively) and were borderline in volunteer 17 (71 SFCs but only 1.8 times background); these three volunteers had gastroenteritis.

Cultured γ-IFN ELISPOT assays were positive in response to at least one of the pools of HIV Gag peptides in 4 of 10 volunteers tested using stringent criteria (bars with asterisks, Fig. 5). A high response to the H3 pool in the day 0 samples was occasionally seen (volunteers 11 and 18, Fig. 5), presumably due to peptides that cross-react with commonly recognized epitopes. Others have noted the same phenomenon (personal communication, Dr. Mark Boaz, IAVI and [34]). None of the four volunteers with positive results were positive by the H3 pool.

4. Discussion

This study represents the second report of *S. typhimurium* used as a vaccine vector in humans and is the first human trial of a live oral *Salmonella* vaccine delivering a foreign antigen via the TTSS of *Salmonella*. The vector is markedly attenuated by deletion of the *phoP*/*phoQ* virulence regulatory locus and the *aroA* locus. Both of these attenuating mutations have been extensively studied in animal and human trials [6,18,19,43]. The strain chosen for study was phenotypically stable, free of antibiotic resistance genes, secreted immunoreactive heterologous antigen, had defective survival in macrophage-like cell lines, and was markedly attenuated in mice.

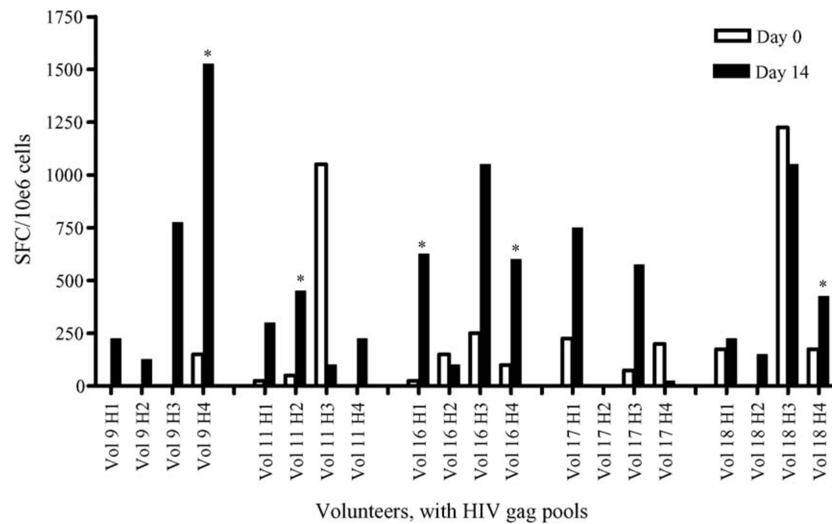

Fig. 5. Cultured ELISPOT data. An *in vitro* expansion assay (cultured ELISPOT) was developed to enhance the sensitivity of the γ-IFN ELISPOT. Frozen PBMCs harvested before and 14 days after inoculation (open and filled bars, respectively) were thawed, resuspended in media, and cultured for 10 days in the presence of HIV Gag peptides. The original 12 HIV Gag peptide pools (above) were condensed into four "super-pools" (H1–H4), each containing three of the original pools. On day 11, the standard ELISPOT procedure was followed. Responses for cultured ELISPOTs were considered to be positive if both ≥250 SFCs were detected per 10^6 cells after subtraction of the negative control (background well) and SFCs were ≥4-fold than those in the negative control. Positive results are designated by an asterisk.

Given as a single-oral dose, CKS257 was tolerated up to 5×10^9 CFU, with dose-limiting toxicity in 2/2 subjects who received 1×10^{10} CFU. Subjects tolerated a dose which was 2 log CFU higher than in our previous study with *S. typhimurium*, where two of six volunteers given (5–8) × 10^7 developed fever [6].

The duration of shedding of CKS275 (mean 5.4 days, range 0–13) was comparable to that seen in our previous study (mean 5.7 days, range 2–10) [6]. Shedding of *phoP*/*phoQ* mutants seems shorter than that seen with *S. typhimurium* TML *ΔaroC ΔssaV* (WT05), where shedding occurred for a mean of 12.2 days, range 3–23 (our calculation, based upon data presented) over a comparable dose range of 10^7 to 10^9 [5]. The duration of colonization did not appear to clearly correlate with symptoms or dose given (Fig. 4). The short shedding period in likely a result of deletion of *phoP*/*Q* (important for bacterial adaptation and persistence) and *aroA* (limits replication within mammals to 7–10 generations) [44]. The most vigorous immune response to the vectored HIV antigen was seen in volunteer 9, who shed the strain for the longest period. Administration of antibiotics did not cause relapse or prolonged shedding, nor abrogation of immune responses. A substudy performed during the course of this investigation compared fecal culture with rectal swabs and found rectal swabs to be 64% sensitive and 90% specific in detecting Salmonella [55].

We are unsure as to the etiology of asymptomatic abnormalities in serum transaminases, which were temporally related to inoculation and resolved spontaneously. In a retrospective review of 93 placebo subjects hospitalized for 14 days in Phase I trials, 20.4% of subjects showed at least one SGPT value above the upper limit of normal, and 7.5% had at least one value twice the upper limit of normal [45]. LFT abnormalities have also been linked to dietary changes, especially high-carbohydrate, high-calorie diets [46]; volunteer 4, who had a calculated basal caloric need of 1750 kcal/day, ate 3400+ kcal/day (55% carbohydrate). Alternatively, transaminase increases may reflect clearance of investigational organisms via the portal circulation.

CKS257 induced immune responses to the *Salmonella* vector over a wide dose range (10^6 to 10^{10}). Fifteen of 18 subjects had IgA ELISPOT mucosal immune responses to either *Salmonella* antigens and these responses were large, with most subjects having hundreds of cells recognizing LPS (Table 2). Increases in the number of vaccine-specific IgA-bearing cells by ELISPOT are believed to be a sensitive surrogate marker of mucosal immune responses to live oral bacterial vaccines [47]. These results are in contrast to spot numbers induced by the *ΔaroCΔssvA* mutant *S. typhimurium* [5], where the largest number of LPS-specific IgA-bearing cells in any volunteer was 131. Similar to other studies of live attenuated *S. typhi* vaccines [18,43,48], a peak IgA ELISPOT response was seen on day 7.

CKS257 was poorly immunogenic with respect to the foreign antigen. No subjects had detectable circulating mucosal immune responses or systemic humoral responses to HIV Gag. On immediate *ex vivo* γ-IFN ELISPOT analysis, no volunteer had a positive response to the HIV Gag peptides. Two volunteers had a positive response on the *ex vivo* IL-2 ELISPOT to HIV Gag peptides, and a third was borderline. The cultured γ-IFN ELISPOT assay demonstrated positive results to HIV Gag peptides in 4/10 volunteers. Recent studies suggest that *ex vivo* and cultured ELISPOT measure two different T-cell subsets, effector and central memory, respec-

tively [49,50]. Central memory T-cells likely have an important role in vaccination and protection.

The magnitude of the immune responses to *Salmonella* antigens did not appear to trend with dose or reactogenicity. Pre-existing immunity may play a role, as *S. typhimurium* is a common food-borne illness [51]. The importance of "vector-priming" (previous exposure to the vector) in humans is unclear. Some studies in mice have raised concerns that repeated use of *Salmonella* vectors would not be possible due to immunity to the vector itself, while others report that prior exposure leads to enhancement of immune responses [4].

The low level of cellular immune responses to the HIV antigen could be explained by the systemic rather than mucosal sampling of specific responses. In HIV exposed but uninfected subjects, higher levels of HIV specific $CD8^+$ T-cells were found in cervical samples than in peripheral blood samples [52]. That responses were only reliably detected in this study following *in vitro* expansion suggests that true responses at the mucosa may have been detectable *ex vivo* without expansion. Recent studies show that in addition to serving as a portal for entry, the gut may be a predominant site for initial HIV replication [53].

The single vaccination modality of this trial may also explain the low immunogenicity to the foreign antigen. When tested alone in human trials, other single-dose HIV vaccines have yielded low levels of *ex vivo* immunogenicity [34,54] which is augmented by booster vaccinations. Enhanced and accelerated responses to parenteral vaccination may be seen after mucosal priming, or *vice versa*. Several groups have proposed that live bacterial vectors may be most effective as mucosal priming vaccines, and we are pursuing this approach.

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