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Vaccination against Shigellosis with Attenuated *Shigella flexneri* 2a Strain SC602

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The *Shigella flexneri* 2a SC602 vaccine candidate carries deletions of the plasmid-borne virulence gene *icsA* (mediating intra- and intercellular spread) and the chromosomal locus *iuc* (encoding aerobactin) (S. Barzu, A. Fontaine, P. J. Sansonetti, and A. Phalipon, Infect. Immun. 64:1190–1196, 1996). Dose selection studies showed that SC602 causes shigellosis in a majority of volunteers when 3×10^8 or 2×10^6 CFU are ingested. In contrast, a dose of 10^4 CFU was associated with transient fever or mild diarrhea in 2 of 15 volunteers. All volunteers receiving single doses of $\geq 10^4$ CFU excreted *S. flexneri* 2a, and this colonization induced significant antibody-secreting cell and enzyme-linked immunosorbent assay responses against *S. flexneri* 2a lipopolysaccharide in two-thirds of the vaccinees. Seven volunteers who had been vaccinated 8 weeks earlier with a single dose of 10^4 CFU and 7 control subjects were challenged with 2×10^3 CFU of virulent *S. flexneri* 2a organisms. Six of the control volunteers developed shigellosis with fever and severe diarrhea or dysentery, while none of the vaccinees had fever, dysentery, or severe symptoms ($P = 0.005$). Three vaccinees experienced mild diarrhea, and these subjects had lower antibody titers than did the fully protected volunteers. Although the apparent window of safety is narrow, SC602 is the first example of an attenuated *S. flexneri* 2a candidate vaccine that provides protection against shigellosis in a stringent, human challenge model.

Microbiological surveys in areas where diarrheal disease is endemic implicate *Shigella* species as etiologic agents in at least 20% of diarrheal cases. *Shigella flexneri* 2a is usually the most prevalent species and serotype in these areas (8, 14, 30). Shigellae are extraordinarily adept intestinal pathogens, as evidenced by their small infectious doses (7). *Shigella* infection is usually transmitted by the fecal-oral route and can be manifested as uncomplicated watery diarrhea. A more definitive manifestation of shigellosis is dysentery, i.e., frequent passage of small-volume stools with gross blood, mucus, and fecal leukocytes. Constitutional symptoms (e.g., fever, rectal tenesmus, and headache) also characterize severe disease. Colonoscopy of patients infected with either *Shigella dysenteriae* or *S. flexneri* reveals diffuse erythema, focal hemorrhages, and inflammatory changes resembling ulcerative colitis. Rectal biopsies taken during the early stages of infection reveal aphthoid lesions overlying small lymphoid follicles (23). These clinical findings are consistent with experimental observations made with the rabbit ileal loop model, suggesting that *Shigella* initiates intestinal infections by invading the follicle-associated membranous cells (28).

Experiments employing polarized epithelial cells as a model of the intestinal epithelium suggest that shigellae invade enterocytes through the basolateral membrane. Internalized bacteria subsequently spread within infected cells by organizing host cell actin into a cytoskeleton-based motor (10, 22). Genetic analysis has shown that this spreading phenotype is dependent upon a plasmid-borne virulence gene designated *icsA* (2) or *virG* (22). The 120-kDa protein expressed by this plasmid-carried gene acts as a recruiter for cytosolic nucleators of filamentous actin (10). This actin is concentrated at the distal poles of septating shigellae, and the resulting comet-like tails provide a motive force for the bacteria within the cytoplasm of infected epithelial cells (36). The mobilized bacteria impinge on the inner face of the host cell plasma membrane, and they spread into contiguous epithelial cells via membrane protrusions (10). Intragastric challenge of rhesus monkeys, a primate model of intestinal shigellosis, demonstrates that *icsA*-mediated intercellular spread of shigellae is a key step in pathogenesis. For example, endoscopy of asymptomatic animals challenged with an *icsA* mutant of *S. flexneri* serotype 5 reveals only scattered nodular abscesses rather than the hemorrhagic ulcerations and diffuse mucosal inflammation seen in animals challenged with the virulent parent strain (32). *icsA* mutants are also avirulent in the Sereny guinea pig keratoconjunctivitis model of suppurative *Shigella* infection (13, 26). Deletion of the *iuc* chromosomal locus (encoding aerobactin) partially attenuates *S. flexneri* in the Sereny guinea pig test and in the rabbit ileal loop model (24). Intragastric inoculation with either an *icsA* single mutant or an *icsA iuc* double mutant protects rhesus monkeys against subsequent challenge with the virulent *S. flexneri* 5 parent strain (32, 33).

Epidemiological and clinical studies indicate that an episode

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of shigellosis elicits substantial immunity against subsequent disease caused by the same *Shigella* serotype (6, 8, 15, 19). In a rational approach to *Shigella* vaccine development, we and others have constructed genetically attenuated vaccines designed to establish asymptomatic infections that induce protective immune responses. However, the ideal balance of safety and efficacy in attenuated *Shigella* vaccines has been elusive (11, 15–18, 20, 25). Current research suggests that *icsA iuc* mutants could serve as attenuated *Shigella* vaccines, and the SC602 Δ*icsA* Δ*iuc S. flexneri* 2a candidate was constructed to test this combination of attenuating mutations in volunteers. Here we describe a preliminary dose selection study, two expanded dose selection studies, and an efficacy (challenge) study of SC602 that were performed in the clinical inpatient ward of the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Ft. Detrick, Md.

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MATERIALS AND METHODS

Vaccine construction and manufacture. *S. flexneri* 2a strain 454, from the Centre National de Reference des Shigelles, Unité des Entérobactéries, Institut Pasteur, was the SC602 progenitor. The Δ*icsA* Δ*iuc* double mutant was constructed in the Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, as described previously (1). The *iuc* mutation was generated by recombination of *iuc*::Tn*10* into the chromosome by using phage P1 transduction. Spontaneous excision of the tetracycline resistance gene, and its flanking regions including the *iuc* locus, was selected by growth on fusaric acid medium. The *icsA* gene was inactivated by double recombination with a kanamycin resistance (Km^r)-sucrose sensitivity (*sacB*) cartridge carrying flanking regions of *icsA*. Deletion of the Km^r-*sacB* cartridge was selected by growth on sucrose, and the resistant clones were screened for retention of the invasive phenotype in HeLa cells. An isolate designated SC602 had suffered a deletion of the entire *icsA* gene along with substantial flanking sequences (total deletion is approximately 10 kb). This SC602 isolate was expanded into a master cell bank and was manufactured as a lyophilized product under current good manufacturing procedures at the Walter Reed Army Institute of Research pilot vaccine production facility in Forest Glen, Md. The product was dispensed as a 5-ml fill in 50-ml serum bottles and was stored at −80°C. The reconstituted product yielded 5×10^{10} to 1×10^{11} CFU per vial (with approximately 30% viability). This organism was invasive for tissue culture cells, and preclinical studies demonstrated its safety and efficacy in guinea pig and rhesus monkey models. Clinical trials of SC602 were conducted under a Food and Drug Administration investigational new drug application.

Subject selection. Volunteers were recruited from the local community, and written, informed consent was obtained under protocols approved by internal review boards within USAMRIID. Potential volunteers were excluded if they reported previous exposure to shigellae; were allergic to quinolones; had any significant gastrointestinal abnormality; were pregnant; were HLA B27, human immunodeficiency virus, or hepatitis B surface antigen positive; were currently being treated with antibiotics, theophylline, iron, zinc, histamine H_2-receptor antagonist blockers, or proton pump inhibitors; or had a febrile illness within 48 h of admission. Because of the theoretical possibility of vaccine excretion after release from the ward, food handlers, day care workers, and individuals who live with a child less than 2 years of age were also excluded.

Vaccination, challenge, and safety assessment. Volunteers fasted for 90 minutes before and after vaccination (and before and after challenge in the subsequent efficacy study). Lyophilized SC602 vaccine was reconstituted in sterile, deionized water and was diluted in phosphate-buffered saline to achieve the target number of CFU in 1-ml volumes. This inoculum was mixed with 30 ml of sodium bicarbonate buffer (2 g of $NaHCO_3$ per 150 ml of sterile, deionized water) and was ingested by each volunteer 2 min after ingestion of 120 ml of the sodium bicarbonate solution (19). Placebo controls received sodium bicarbonate buffer with no added bacteria. The challenge inoculum, containing approximately 10^3 CFU of virulent *S. flexneri* 2a strain 2457T, was prepared at the Center for Vaccine Development, University of Maryland School of Medicine, and was administered with sodium bicarbonate as described previously (19). All subjects who were vaccinated or challenged with *S. flexneri* were treated with ciprofloxacin (500 mg, twice daily for 5 days), and passage of two consecutive stools with no cultivable *S. flexneri* was a prerequisite for discharge. Statistical significance between study groups was determined by a two-tailed Fisher's exact test.

Reactions to vaccination (and challenge) were determined by daily clinical assessment and were graded as mild (i.e., no limitation of activity), moderate (i.e., mild to moderate limitation of activity), or severe (i.e., significant limitation of normal activities). Vital signs were recorded three times a day, and fever was defined as an oral temperature of >100.5°F. All stools were collected, weighed, assessed for presence of blood, and graded as firm (normal), soft (normal), thick liquid (abnormal), opaque watery (abnormal), or rice water (abnormal). Diarrhea was defined as two or more abnormal stools within 48 h totaling ≥200 ml, or a single abnormal stool of >300 ml within 24 h (19). Dysentery was defined as an abnormal stool with gross blood. Reportable constitutional symptoms included headache, myalgia, arthralgia, loss of appetite, and fatigue. Reportable intestinal symptoms included abdominal cramps, nausea, emesis, tenesmus, and gas. Shigellosis was defined as a temperature of >101°F, diarrhea and/or dysentery, more than one severe intestinal symptom, and more than one severe constitutional symptom. Severe shigellosis was defined as a temperature of >101°F, more than five abnormal stools, more than one severe constitutional symptom, and more than one severe intestinal symptom. Shigellosis was also considered severe, even if constitutional and intestinal symptoms were mild, when fever was >101°F and abnormal stools totaled >10. Ciprofloxacin treatment was initiated early if volunteers met the clinical definition of shigellosis. Oral rehydration was started as soon as a volunteer developed diarrhea or had signs suggestive of volume depletion. Any volunteer unable to maintain adequate hydration by the oral route would have been treated with intravenous D5 Ringer's lactate, although none of the patients in our studies required intravenous hydration.

Laboratory methods. A measured sample from each collected stool (or a rectal swab if no stool was passed within 24 h) was suspended in buffered glycerol saline, diluted in phosphate-buffered saline, and plated for quantitative colony count on Hektoen enteric agar (Difco Laboratories, Detroit, Mich.). Non-lactose-fermenting colonies were identified as *S. flexneri* 2a by slide agglutination in homologous antiserum (Difco). Ten colonies had to test negative in 2a antiserum before a Hektoen enteric agar plate was recorded as negative for *S. flexneri*. Randomly selected *S. flexneri* 2a isolates were confirmed to be the SC602 *icsA* deletion mutant by Southern blotting of bacterial DNA extracted and digested with *Eco*RI and *Sal*I. The blotted DNA fragments were hybridized with a radiolabeled probe consisting of a nick-translated PCR product amplified from an internal portion of the *icsA* structural gene. The enzyme-linked immunospot assay (35) was used to enumerate immunoglobulin A (IgA), IgG, and IgM antibody-secreting cells (ASC) per 10^6 peripheral blood lymphocytes (PBL) in samples obtained on days 0, 5, 7, and 9. The means plus 3 standard deviations (SD) of numbers of ASC recognizing *S. flexneri* 2a lipopolysaccharide (LPS) on day 0 were 5.6 (IgA), 7.1 (IgG), and 7.6 (IgM). Antibody responses against *S. flexneri* 2a LPS and *Shigella* invasion plasmid antigen (Ipa) proteins (27) were assessed as IgM, IgA, and IgG enzyme-linked immunosorbent assay (ELISA) titers in serum collected on days 0, 7, 14, and 28. Titers of antibody against Ipa proteins were determined by endpoint dilution with seroconversion defined as a fourfold rise in titer (12). Titers of antibody against *S. flexneri* 2a LPS were determined in serum and urine samples by using endpoint titers derived from a linear regression analysis of eight doubling dilutions by using adjusted optical densities of 0.3 for serum and 0.1 for urine (3). Secretory IgA (sIgA) recognizing *S. flexneri* 2a LPS in urine was quantified by ELISA, and the titer was adjusted for urine concentration by using the creatinine concentration as a divisor (4). Antibody titers in urine collected on days 7, 14, and 28 were considered significant if there was a fourfold increase in titer and if the peak titer exceeded mean day 0 values by 3 SD.

RESULTS

SC602 dose selection studies. Thirty-three subjects (aged 19 to 46 years) were enrolled in the initial, placebo-controlled dose selection trial. Eighteen subjects received the SC602 vaccine (Table 1) and fifteen received sodium bicarbonate placebo. The objective of this trial was to determine the maximal tolerated vaccine dose. The first group of volunteers was inoculated with a single dose of 10^2 CFU and was treated with ciprofloxacin on day 3 postvaccination. Five additional cohorts (three vaccinees and three placebo controls per group) were inoculated according to a double-blinded, placebo-controlled protocol. Cohorts 2 and 3 received doses of vaccine on days 0 and 3. Cohorts 2 and 3 showed that the first inoculation of SC602 achieved adequate intestinal colonization; therefore, groups 4, 5, and 6 received only one dose of vaccine. On day 8 postvaccination, or earlier if clinically indicated, ciprofloxacin treatment was initiated. Doses of 10^2 to 10^7 CFU were well tolerated in that no vaccine recipients developed diarrhea. However, transient fever was observed in 20% of these vaccinees, including one subject in group 2 who had ingested 10^4 CFU. A control volunteer in the same group also had fever, and one control volunteer in group 3 had diarrhea. Severe headache (7% of vaccinees), moderate headache (20%), and

TABLE 1. Summary of phase 1 trial of *S. flexneri* 2a SC602 vaccination

Group[a]	*S. flexneri* 2a SC602 ingested (CFU)		No. of vaccinees displaying:	
	Day 0	Day 3	Diarrhea[b]	Fever[c]
1	1.6×10^2	NA[d]	0	0
2	1.6×10^4	3.2×10^4	0	1
3	2.4×10^5	1.2×10^5	0	1
4	2.0×10^6	NA	0	0
5	2.3×10^7	NA	0	1
6	2.9×10^8	NA	2	2

[a] Three volunteers per group.
[b] Two or more grade 3 stools totaling at least 200 ml or one grade 3 stool of 300 ml within 48 h.
[c] At least 100.5°F.
[d] NA, not applicable.

moderate abdominal cramping and loss of appetite (7%) were reported by vaccinees in groups 2 through 5. Moderate headache was reported by 16% of controls. Within the first day after vaccination with 2.9×10^8 CFU, two of three vaccinees in group 6 experienced diarrhea, fever, and severe intestinal and constitutional symptoms. These subjects were treated with ciprofloxacin on day 1. This initial study established 10^8 CFU as a reactogenic endpoint. The subsequent, expanded dose selection trials assessed the safety and immunogenicity of a maximal tolerated dose defined as being 100-fold below the experimentally determined reactogenic dose.

During the first expanded dose selection trial, 15 volunteers were vaccinated in an open-label study with a target dose of 10^6 CFU of SC602. Within 48 h of vaccination, diarrhea (47% of vaccinees), fever (33%), severe constitutional symptoms (40%), and severe intestinal symptoms (40%) were reported (Table 2). Volunteer G was treated with ciprofloxacin on day 3, and the remaining volunteers were treated as scheduled on day 12 to terminate vaccine excretion. The 10^6 CFU dose of SC602 was judged to be too reactogenic for use as a vaccine, and a 100-fold-lower dose was chosen for the next trial. Twelve volunteers were vaccinated with 10^4 CFU in a second, open-label trial. This dose of vaccine was well tolerated in that no volunteer experienced fever, severe constitutional symptoms, or severe intestinal symptoms. One subject (volunteer S/N) (Table 3) met the definition of displaying diarrhea with loose stools on days 3, 4, 8, and 9 (total of 1,341 g). This vaccinee had no additional symptoms, and he passed formed stools on the intervening days. Moderate nausea (8%), loss of appetite (8%), abdominal cramping (8%), gas (33%), headache (8%), and fatigue (17%) were also reported. Other vaccinees noted milder symptoms including headache, gas, myalgia, fatigue (33%), mild nausea (25%), loss of appetite (25%), and abdominal cramping (17%). All the volunteers were treated with ciprofloxacin on day 8.

Laboratory evaluation of dose selection studies. Robust and prolonged intestinal colonization by *S. flexneri* 2a was observed in all volunteers who had ingested the SC602 vaccine. For example, 100% of volunteers who had ingested 2×10^6 CFU excreted shigellae for 7 days, and 57% excreted the organisms until treatment began 12 days later. Likewise, 92% of the volunteers who had ingested 10^4 CFU shed shigellae until treated on day 8. The peak excretion of vaccine was 10^4 to 10^6 CFU/g of stool regardless of the dose ingested. The first stools yielding *S. flexneri* 2a were passed by 93% of volunteers within 24 h of ingesting 10^6 CFU of SC602, and 100% of volunteers excreted these organisms within 12 h of ingesting 10^8 CFU. In both cases, symptoms of shigellosis coincided with vaccine excretion. In contrast, only 42% of volunteers who ingested 10^4 CFU excreted *S. flexneri* 2a within 24 h. The proportion of excretors gradually increased to 58% on day 2, 83% on day 3, and 91% on day 4.

Stability of the *icsA* deletion in SC602 was confirmed by Southern blot analyses showing no *icsA* sequences in colonies grown from the cGMP vaccine ampoules used for inoculation of groups 4, 5, and 6 of the first phase 1 dose selection trial nor in 16 stool isolates shed by vaccinees in these groups (data not shown). None of the placebo control volunteers were colonized with *S. flexneri* 2a, even though they shared living space and toilet facilities with vaccinees who were excreting SC602.

Clinical and immunological data from the 15 volunteers who participated in the 10^6 CFU phase 1 trial are summarized in Table 2. IgA ASC that recognized *S. flexneri* 2a LPS were present in 13 of these subjects and 11 also had positive IgG

TABLE 2. Volunteer symptoms and immune responses against *S. flexneri* 2a LPS or Ipa elicited by a 2×10^6-CFU dose of SC602 vaccine

Volunteer	Symptoms		Peak[a] no. of ASC/10^6 PBL anti-LPS		Fold increase[a] in serum anti-LPS concn			Urine anti-LPS[b] (IgA)	Serum anti-Ipa[b] (IgA or IgG)
	Fever temp (°F)	Diarrhea vol (ml)	IgA	IgG	IgA	IgG	IgM		
C			0	1	1	<1	<1	−	−
P			8	1	<1	<1	<1	−	−
D		617	11	5	1	<1	<1	−	−
B	101.9	571	61	0	4	<1	<1	+	+
M			105	71	2	2	<1	+	+
A			132	80	6	2	<1	−	+
J		592	249	60	3	2	<1	−	+
K	101.3		305	92	3	4	<1	+	−
E	103.7	253	406	146	10	4	3	+	+
N		506	479	37	4	<1	<1	−	+
L	101.3		621	416	4	3	<1	+	−
G	102.6	1,354	706	158	4	2	3	−	+
W		1,185	916	287	9	3	4	−	+
H			1,109	108	5	<1	<1	−	+
R			1,208	762	20	7	2	+	−

[a] ASC levels peaked on day 7 and ELISA titers peaked on day 14.
[b] +, positive response of ≥4-fold increase over baseline; −, negative response.

TABLE 3. Summary of immune response against *S. flexneri* 2a LPS elicited by vaccination with SC602 or by challenge with *S. flexneri* 2a strain 2457T

Volunteer	Reaction to SC602 (10^4 CFU)	Peak[a] ASC/10^6 PBL			Fold increase[a] in serum ELISA titer			Urine[b] IgA	Reaction to 2457T (2×10^3 CFU)	Peak[a] ASC/10^6 PBL		Fold increase[a] in serum ELISA titer		Urine[b] IgA
		IgA	IgG	IgM	IgA	IgG	IgM			IgA	IgG	IgA	IgG	
Vaccinees														
B/D[c]	None	0	0	6	<1	<1	<1	−	Diarrhea	10	14	<1	<1	−
J/G	None	0	0	24	2	<1	<1	−	Diarrhea	114	0	28	6	+
S/N	Diarrhea	40	1	49	<1	1	<1	−	Diarrhea	2	1	1	<1	−
C/K	None	75	85	109	3	<1	<1	−	None	41	22	3	<1	+
K/S[d]	None	370	149	146	35	4	4	+	None	4	0	1	<1	−
E/A	None	524	82	796	34	9	2	+	None	240	45	6	1	+
G/C[d]	None	556	21	611	13	4	4	+	None	360	22	4	<1	+
H	None	1	3	4	<1	<1	<1	−						
A	None	2	6	0	<1	<1	<1	−						
D	None	2	3	6	<1	<1	<1	−						
T	None	35	1	75	3	<1	<1	−						
N[d]	None	45	47	56	15	4	4	+						
Controls														
H									None	0	0	<1	<1	−
P									Shigellosis	67	226	7	6	+
B									Shigellosis	242	0	16	<1	+
J									Shigellosis	484	0	13	2	+
M									Shigellosis	632	42	ND[e]	ND	+
E									Shigellosis	>800	254	23	5	+
R									Shigellosis	>1,200	169	200	27	+

[a] ASC levels peaked on day 9 and ELISA titers peaked on day 14.
[b] +, positive response of ≥4-fold increase over baseline ELISA optical density; −, negative response.
[c] The cohort that subsequently volunteered for an efficacy trial was assigned a second code letter in the challenge study.
[d] Vaccinees G and K had ASC responses against *Shigella* Ipa proteins, and these two subjects, along with vaccinee N, had IgA ELISA responses against Ipa proteins.
[e] ND, not determined.

ASC responses against LPS. Nine of the ASC responders had a ≥4-fold rise in serum IgA titer, and three volunteers had a positive serum IgG response against 2a LPS. Nine vaccinees had IgA or IgG serum ELISA responses against Ipa proteins. The latter responses were predominately of the IgA serotype, but a majority of volunteers also had IgG anti-Ipa responses. The presence of IgA ASC that recognize *Shigella* antigens in the peripheral circulation indicates that intestinal colonization by SC602 stimulates an IgA response in the gut-associated lymphoid tissue. However, sIgA in urine was also evaluated as a direct measurement of mucosal immune responses against LPS. Six volunteers had fourfold increases in urinary anti-LPS sIgA titers that exceeded 3 SD of the mean baseline titer. Only two subjects (C and P) failed to mount a measurable antibody response against *Shigella* Ipa and/or LPS antigens.

Symptoms and immune response data from the 12 volunteers who ingested 10^4 CFU of SC602 are summarized in Table 3. Eight vaccinees had IgM ASC responses against 2a LPS and seven had IgA ASC responses, with five of these vaccinees also having IgG responses. Compared to the response seen after vaccination with 10^6 CFU, these responses were delayed by 48 h, i.e., ASC first appeared in the peripheral circulation around day 7 and the numbers peaked on day 9, while ASC appeared by day 5 and peaked around day 7 in volunteers who had ingested the larger dose of vaccine. Four vaccinees receiving 10^4 CFU had ≥4-fold increase in IgA and IgG anti-LPS, and two additional subjects had threefold increases. Only vaccinees G/C and K/S had ASC responses against Ipa proteins, and these two subjects, along with vaccinee N, also had serum IgA responses against Ipa proteins (data not shown). The relatively modest responses against Ipa, compared to vigorous responses seen after a 10^6 CFU dose, probably reflect the reduced severity of infection that followed ingestion of the lower dose of vaccine. Four vaccinees receiving the 10^4 CFU dose (K/S, E/A, G/C, and N) had ≥4-fold increases in urinary IgA, and this subset of vaccinees also had positive serum ELISA responses in all antibody isotypes.

Clinical evaluation of phase 2b efficacy trial. The dose selection trials established 10^4 CFU of SC602 as a relatively safe vaccine dose that induces measurable immune responses in a majority of volunteers. Subjects who received this dose were eligible to volunteer for an efficacy trial that was scheduled 8 weeks after inoculation. The seven subjects who volunteered were readmitted to the inpatient ward along with seven unvaccinated controls. All volunteers were treated with ciprofloxacin on day 5, or sooner if they met the clinical criteria for shigellosis. Following experimental challenge, the symptoms of dysentery, fever, and severe shigellosis were confined to the unvaccinated control group (Table 4). The absence of these symptoms in vaccinees allowed statistical differentiation of the two groups on the basis of fever and severe shigellosis ($P = 0.005$). Volunteer H in the control group (Table 3) failed to excrete the challenge organism, and this individual had no symptoms of shigellosis. Interestingly, this volunteer was the only subject who had a significant number of circulating ASC against *S. flexneri* 2a LPS at the time of challenge (9 IgA and 14 IgM/10^6 PBL). With the exception of volunteer H, all controls had severe shigellosis, as illustrated by a mean of eight diarrheal stools per day during the acute phase of disease and a mean total of 11 diarrheal stools (Table 4).

Three SC602 vaccinees met the definition of displaying clinical diarrhea (Tables 3 and 4). Vaccinee B/D passed two diar-

TABLE 4. Symptoms and colonization of SC602 vaccinees and unvaccinated control subjects after challenge with *S. flexneri* 2a strain 2457T

Characteristic	Controls	Vaccinees
No. of volunteers	7	7
Volunteers excreting *S. flexneri* 2a	6	6
Volunteers with diarrhea	6	3
Mean no. of diarrheal stools (range)	11 (6–20)	4 (2–6)
Volunteers with dysentery	4	0
Volunteers with fever (mean temp)	6 (102.7°F)	0[a]
Volunteers with severe shigellosis	6	0[a]

[a] $P = 0.005$, vaccinees versus controls.

rheal stools on day 2 that totaled 258 g, barely qualifying as clinical diarrhea. Vaccinee J/G passed a total of five diarrheal stools occurring on days 4 (130 g), 5 (343 g), 6 (246 g), and 7 (19 g). Vaccinee S/N passed a total of five diarrheal stools on days 0 (210 g), 1 (248 g), 5 (522 g), and 6 (168 g). The latter volunteer had also passed diarrheal stools after vaccination. We conclude that vaccination with SC602 either completely protects volunteers from shigellosis or ameliorates the symptoms of disease so that the vaccinee requires no medical intervention.

Immune correlates of protection in the phase 2b efficacy trial. Immune correlates of vaccine efficacy against diarrhea and severe shigellosis included a significant IgA ASC response and a threefold or greater rise in serum IgA antibody against *S. flexneri* 2a LPS (Table 3). Other correlates of protection against all symptoms included urinary sIgA responses against 2a LPS in addition to IgG ASC and IgG serum responses. Subjects B/D and J/G evidenced only IgM ASC responses against the *S. flexneri* 2a LPS after vaccination, and these volunteers experienced mild diarrhea after challenge, even though both were protected from severe shigellosis. Secretory IgA is locally produced and is actively transported into the colon rather than into the peripheral circulation (29); therefore, it is possible that protective levels of secretory IgA could be present on the colonic epithelium of vaccinees who did not demonstrate measurable IgA responses in the peripheral circulation. For example, subject S/N had substantial IgM and IgA ASC responses after vaccination, but no serum antibody response was detected. Although S/N passed some diarrheal stools, *S. flexneri* 2a was not excreted by this vaccinee after challenge, suggesting a substantial degree of immunity.

DISCUSSION

Site-directed genetic mutation of *Shigella* has allowed construction of stable, attenuated, candidate vaccines that retain the invasive phenotype. These vaccines are designed to initiate abortive intestinal infections, efficiently delivering protective *Shigella* antigens through follicle-associated membranous cells into the underlying gut-associated lymphoid tissue without eliciting clinical shigellosis. For the present study, the SC602 *S. flexneri* 2a candidate vaccine was attenuated by deletion of the *icsA* gene, resulting in the loss of intracellular motility and the intercellular spreading phenotype. The secondary aerobactin mutation (*iuc*) is not sufficient to attenuate shigellae for vaccine use, but it may moderate the reactogenicity of SC602.

Others have attenuated candidate vaccines with an *aro* mutation (16) or with combinations of *aro* and *icsA* mutations (*virG*) (20). We have evaluated an *aro* hybrid of *Escherichia coli* K-12 and *S. flexneri* 2a (18) that also has some characteristics of an *icsA* (*virG*) mutant (25). Auxotrophic *aro* mutants require PABA (*para*-aminobenzoic acid) for intracellular growth (16), and they do not survive intracellularly since PABA is not a constituent of mammalian cytosol. The *aro* mutants are clearly attenuated and demonstrably safe at doses of 10^6 or 10^7 CFU (16). When the dose of *aro* vaccines was increased to 10^8 or 10^9 CFU, however, a significant proportion of volunteers suffered intestinal or systemic reactions within the first 24 h. These reactions, which included diarrhea and fever, were similar to those observed after volunteers ingested 10^6 CFU of SC602.

In contrast to the 10^6-CFU dose of SC602, a 10^4-CFU dose was not associated with serious adverse events, although one subject developed transient fever and one volunteer passed occasional diarrheal stools (13% reactogenicity). Mild to moderate constitutional and intestinal symptoms were also experienced by some subjects, but these subclinical symptoms did not affect normal activities. In the context of previous studies with *aro* mutants, the reactogenicity of a 10^4-CFU dose of SC602 is roughly comparable to a 7×10^8-CFU dose of the *aroD* hybrid EcSf2a-2 and to a 10^7-CFU dose of the SFL1070 *aroD* mutant (16). The reactogenicity of this dose also falls within the spectrum of reactions reported after ingestion of 10^8 and 10^6 CFU of CVD1203 Δ*aroA* Δ*virG* double mutant (20). A significant immune response against *S. flexneri* 2a LPS was detected in approximately two-thirds of volunteers ingesting any of the above vaccines. Only the EcSf2a-2 and the SC602 vaccines have been evaluated for efficacy by challenge with virulent *S. flexneri* 2a 2457T. The former vaccine elicited no significant protection against fever, dysentery, or diarrhea (17, 18). In contrast, SC602 gave significant protection against fever and severe shigellosis. The mild diarrhea that was experienced by some of the challenged vaccinees did not inhibit normal activities and did not indicate antibiotic treatment. Solid protection against severe shigellosis, with occasional mild diarrhea or fever, was also reported in experimental rechallenge studies of volunteers who had experienced previous clinical shigellosis (15, 19, 29).

The mean time to excretion of *S. flexneri* 2a by vaccinees who ingested 10^4 CFU of SC602 was 72 h, while the mean time to excretion by volunteers who ingested 10^6 CFU was only 22 h. We speculate that the lower vaccine inoculum allows gradual invasion of the colonic epithelium by SC602 while limiting the cumulative inflammatory response to a subclinical threshold in most subjects. Of the five volunteers who excreted substantial numbers of shigellae within 24 h of ingesting a 10^4-CFU dose, two experienced presumptive vaccine reactions (fever of 100.9°F or mild diarrhea). In contrast, none of the vaccinees with delayed colonization had clinical symptoms. An expanded outpatient safety trial of SC602 has recently been performed to further assess the safety and immunogenicity of the 10^4 CFU dosage (5). In this trial, six volunteers (19%) experienced mild diarrhea or fever lasting less than 24 h. These data suggest a narrow window of safety for *icsA* vaccines, and further attenuation of SC602 would be desirable if the efficacy of this strain can be maintained.

One of the characteristics of SC602 that sets it apart from the *aro* vaccines is the persistent colonization that is achieved even after ingestion of a 10^4-CFU dose of vaccine. For example, all volunteers were excreting shigellae 8 days after vaccination in the clinic-based studies described above, and the mean time of colonization in a subsequent community-based phase 1 trial was 12 days (34). In contrast, the auxotrophic *aro* vaccines are usually excreted for 5 days or less (16, 18, 20). We judge the hazard of secondary fecal-oral transmission of SC602 to be minimal. Secondary spread of *S. flexneri* 2a from an adult

index case of shigellosis occurs in only 5% of households (37), and no transmission to placebo controls was detected in the clinical trials of SC602 presented here. In addition, excretion of SC602 by Bangladeshi adults in ongoing phase 1 trials has been scarcely detectable, suggesting that secondary spread of the vaccine in areas of endemicity will also be unlikely. It should also be noted that the reactogenicity profile of live *Shigella* vaccines is ameliorated in partially immune adolescents and adults living in developing countries (21), and this is also the case with SC602.

Naturally acquired immunity against shigellosis is species specific (8), and it is anticipated that a multivalent product, or a series of vaccinations with different *icsA* mutant vaccines, would be required to make a significant impact on levels of shigellosis in developing countries where the disorder is endemic. Candidate *icsA* vaccines have been described for *S. dysenteriae* 1 (9) and *Shigella sonnei* (13), and phase 1 trials of these serotypes are planned. Although the initial consumers of commercially produced vaccines against shigellosis would be the 20 million civilian and military travelers who visit developing countries annually, the simple and economical manufacturing process required for these live vaccines would make local production in developing countries feasible. The single-dose regimen suggested by the present clinical trials is a distinct advantage for vaccine delivery to children at risk of shigellosis in areas of endemicity. By eliminating the need for medical intervention and antibiotic usage (31) in vaccinated populations, *icsA* vaccines could prove to be practical public health tools for the prophylactic control of shigellosis in areas where shigellosis is endemic.

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REFERENCES

1. **Barzu, S., A. Fontaine, P. J. Sansonetti, and A. Phalipon.** 1996. Induction of local anti-IpaC antibody response in mice by use of a *Shigella flexneri* 2a vaccine candidate: implications for use of IpaC as a protein carrier. Infect. Immun. **64:**1190–1196.
2. **Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86:**3867–3871.
3. **Cohen, D., S. Ashkenazi, M. S. Green, M. Gdalevich, G. Robin, R. Slepon, M. Yavzori, N. Orr, C. Block, I. Ashkenazi, J. Shemer, D. N. Taylor, T. L. Hale, J. C. Sadoff, D. Pavliakova, R. Schneerson, and J. B. Robbins.** 1997. Double-blind vaccine-controlled randomised efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet **349:**155–159.
4. **Cohen, D., N. Orr, G. Robin, R. Slepon, S. Ashkenazi, I. Ashkenazi, and J. Shemer.** 1996. Detection of antibodies to *Shigella* lipopolysaccharide in urine after natural *Shigella* infection or vaccination. Clin. Diagn. Lab. Immunol. **3:**451–455.
5. **Coster, T. S., et al.** Unpublished data.
6. **DuPont, H. L., R. B. Hornick, M. J. Snyder, J. P. Libonati, S. B. Formal, and E. J. Gangarosa.** 1972. Immunity in shigellosis. II. Protection induced by oral live vaccine or primary infection. J. Infect. Dis. **125:**12–16.
7. **DuPont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal.** 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159:**1126–1128.
8. **Ferreccio, C., V. Prado, and A. Ojeda.** 1991. Epidemiologic patterns of acute diarrhoea and endemic *Shigella* infections in children in a poor periurban setting in Santiago, Chile. Am. J. Epidemiol. **134:**614–627.
9. **Fontaine, A., J. Arondel, and P. J. Sansonetti.** 1990. Construction and evaluation of live attenuated vaccine strains of *Shigella flexneri* and *Shigella dysenteriae* 1. Res. Microbiol. **141:**907–912.
10. **Goldberg, M., and P. J. Sansonetti.** 1994. *Shigella* subversion of the cytoskeleton: a strategy for epithelial colonization. Infect. Immun. **61:**4941–4946.
11. **Hale, T. L.** 1995. *Shigella* vaccines, p. 179–203. *In* D. A. A. Ala'Aldeen and C. E. Hormaeche (ed.), Molecular and clinical aspects of bacterial vaccine development. John Wiley, Ltd., London, England.
12. **Hartman, A. B., C. J. Powell, C. L. Schultz, E. V. Oaks, and K. H. Eckels.** 1991. Small-animal model to measure efficacy and immunogenicity of *Shigella* vaccine strains. Infect. Immun. **59:**4075–4083.
13. **Hartman, A. B., and M. M. Venkatesan.** 1998. Construction of a stable attenuated *Shigella sonnei* Δ*virG* vaccine strain, WRSS1, and protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model. Infect. Immun. **66:**4572–4576.
14. **Henry, F. J.** 1991. The epidemiologic importance of dysentery in communities. Rev. Infect. Dis. **13**(Suppl. 4)**:**S238–S244.
15. **Herrington, D. A., L. Van De Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cruz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate *Shigella* vaccines: further experience with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.
16. **Karnell, A., A. Li, C. R. Xhao, K. Karlsson, N. B. Minh, and A. A. Lindberg.** 1995. Safety and immunogenicity study of the auxotrophic *Shigella flexneri* 2a vaccine SFL1070 with a deleted *aroD* gene in adult Swedish volunteers. Vaccine **13:**88–99.
17. **Kotloff, K. L., D. A. Herrington, T. L. Hale, J. W. Newland, L. Van De Verg, J. P. Cogan, P. J. Snoy, J. C. Sadoff, S. B. Formal, and M. M. Levine.** 1992. Safety, immunogenicity, and efficacy in monkeys and humans of invasive *Escherichia coli* K-12 hybrid vaccine candidates expressing *Shigella flexneri* 2a somatic antigen. Infect. Immun. **60:**2218–2224.
18. **Kotloff, K. L., G. L. Losonsky, J. P. Nataro, S. S. Wasserman, T. L. Hale, D. N. Taylor, J. W. Newland, J. C. Sadoff, S. B. Formal, and M. M. Levine.** 1995. Evaluation of the safety, immunogenicity, and efficacy in healthy adults of four doses of live oral hybrid *Escherichia coli-Shigella flexneri* 2a vaccine strain EcSf2a-2. Vaccine **13:**495–502.
19. **Kotloff, K. L., J. P. Nataro, G. A. Losonsky, S. S. Wasserman, T. L. Hale, D. N. Taylor, J. C. Sadoff, and L. L. Levine.** 1995. Protection against shigellosis following homologous reinfection using a modified volunteer challenge model in which the inoculum is administered with bicarbonate buffer: clinical experience and implications for *Shigella* infectivity. Vaccine **13:**1488–1494.
20. **Kotloff, K. L., F. Noriega, G. A. Losonsky, M. B. Sztein, S. S. Wassserman, J. P. Nataro, and M. M. Levine.** 1996. Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live, oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in *aroA* and *virG*. Infect. Immun. **64:**4542–4548.
21. **Li, A., P. D. Cam, D. Islam, N. B. Minh, P. T. Huan, Z. C. Rong, K. Karisson, G. Lindberg, and A. A. Lindberg.** 1994. Immune responses in Vietnamese children after a single dose of the auxotrophic, live *Shigella flexneri* Y vaccine strain SFL124. J. Infect. **28:**11–23.
22. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *Shigella flexneri* 2a. Cell **46:**551–555.
23. **Manthan, M. M., and V. I. Manthan.** 1991. Morphology of rectal mucosa of patients with shigellosis. Rev. Infect. Dis. **13**(Suppl. 4)**:**S314–S318.
24. **Nassif, X., M.-C. Mazert, J. Mounier, and P. J. Sansonetti.** 1987. Evaluation with an *iuc*::Tn*10* mutant of the role of aerobactin production in the virulence of *Shigella flexneri*. Infect. Immun. **55:**1963–1969.
25. **Newland, J. W., T. L. Hale, and S. B. Formal.** 1992. Genotypic and phenotypic characterization of an *aroD* deletion-attenuated *Escherichia coli-Shigella flexneri* hybrid vaccine expressing *S. flexneri* 2a somatic antigen. Vaccine **10:**766–776.
26. **Noriega, F. R., J. Y. Wang, G. Losonsky, D. R. Maneval, D. M. Hone, and M. M. Levine.** 1994. Construction and characterization of attenuated Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. Infect. Immun. **62:**5168–5172.
27. **Oaks, E. V., T. L. Hale, and S. B. Formal.** 1986. Serum immune response to *Shigella* protein antigens in rhesus monkeys and humans infected with *Shigella* spp. Infect. Immun. **53:**57–63.
28. **Perdomo, J. J., J. M. Cavaillon, M. Huerre, H. Ohayon, P. Gounon, and P. J. Sansonetti.** 1994. Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. J. Exp. Med. **180:**1307–1319.
29. **Prigent-Delecourt, L., B. Coffin, J. F. Colombel, J. P. Dehennin, J. P. Vaerman, and J. C. Rambaud.** 1995. Secretion of immunoglobulins and plasma proteins from the colonic mucosa: an *in vitro* study in man. Clin. Exp. Immunol. **99:**221–225.
30. **Rosemans, C., M. L. Bennish, and T. Wierzba.** 1988. Diagnosis and man-

agement of dysentery by community health workers. Lancet **2:**552–555.
31. **Sack, R. B., M. Rahman, M. Yunus, and E. H. Khan.** 1997. Antimicrobial resistance in organisms causing diarrheal disease. Clin. Infect. Dis. **24**(Suppl. 1)**:**S102–S105.
32. **Sansonetti, P. J., J. Arondel, A. Fontaine, H. d'Hauteville, and M. L. Bernardini.** 1991. *ompB* (osmo-regulation) and *icsA* (cell-to-cell spread) mutants of *S. flexneri*: vaccine candidates and probes to study the pathogenesis of shigellosis. Vaccine **9:**416–421.
33. **Sansonetti, P. J., and J. Arondel.** 1989. Construction and evaluation of a double mutant of *Shigella flexneri* as a candidate for oral vaccination against shigellosis. Vaccine **7:**443–450.
34. **Teska, J. D., T. Coster, W. R. Byrne, J. R. Colbert, D. Taylor, M. Venkatesan, and T. L. Hale.** J. Lab. Clin. Med., in press.
35. **Van De Verg, L., D. A. Herrington, J. R. Murphy, S. S. Wasserman, S. B. Formal, and M. M. Levine.** 1990. Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with a bivalent *Salmonella typhi-Shigella sonnei* vaccine or infection by pathogenic *S. sonnei*. Infect. Immun. **58:**2002–2004.
36. **Vasselon, T., J. Mounier, R. Hellio, and P. J. Sansonetti.** 1992. Movement along actin filaments of the perijunctional area and de novo polymerization of cellular actin are required for *Shigella flexneri* colonization of epithelial Caco-2 cell monolayers. Infect. Immun. **60:**1031–1040.
37. **Wilson, R., R. A. Feldman, and J. Davis.** 1981. Family illness associated with Shigella infection: the interrelationship of age of the index patient and the age of household members in acquisition of illness. J. Infect. Dis. **143:**130–132.

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