**Evaluation of the Safety, Tolerability, and Immunogenicity of an Oral, Inactivated Whole-Cell Shigella flexneri 2a Vaccine in Healthy Adult Subjects**

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Shigella causes bacillary dysentery, a severe, intensely inflammatory gastrointestinal disease affecting the distal regions of the colon and the rectum. Shigellosis is a low-incubum (10 to 100 organisms) infection that is readily transmitted by direct fecal-oral contact. Most of the infections are associated with poor sanitation and contaminated water. Naturally acquired Shigella infection elicits antibody-mediated serotype-specific protective immunity (1, 2). According to the Global Enteric Multicenter Study (GEMS), Shigella was one of the most common pathogens responsible for the diarrheal disease burden and death in children ages 12 to 59 months in areas of Shigella endemicity (3, 4). Mortality from shigellosis has diminished significantly in the past 2 to 3 decades (3, 6), largely because of the virtual disappearance of the highly virulent Shiga toxin-producing Shigella dysenteriae 1 serotype worldwide. However, the pediatric morbidity due to other species of Shigella remains substantial (7, 8). In the United States, 10,382 and 10,898 cumulative cases of shigellosis were reported in 2014 and 2015 (just through August of 2015), respectively (9). World Health Organization guidelines recommend antibiotic treatment for clinical dysentery (diarrhea with gross blood). However, Shigella frequently acquires resistance to antibiotics that were previously effective in reducing disease severity and duration along with pathogen excretion (8, 10). This background of the enormous global burden of Shigella, augmented by the growing rate of antimicrobial resistance, makes a compelling case that development of a Shigella vaccine is essential to reduce pediatric shigellosis. The real challenge in making such a vaccine is that all four species of Shigella (Shigella flexneri, Shigella sonnei, Shigella boydii, and Shigella dysenteriae) can cause dysentery, and there are more than 50 serotypes and subserotypes of Shigellae. However, different epidemiological studies have shown that the distribution of species/serotypes that most affect children <5 years of impoverished populations in areas where Shigella is endemic are S. sonnei and S. flexneri 2a (4, 7). Vaccines against these two species/serotypes and against S. flexneri 3a and 6 should cover 88% of Shigella infections in developing countries through homologous protection and cross protection among the S. flexneri serotypes based on shared type and group O antigen determinants (7, 11, 12). In GEMS and in the study conducted by von Seidlein et al., Shigella flexneri was isolated most frequently among the species, and Shigella flexneri 2a was the most frequent serotype (4, 7). Since protective immunity is serotype specific, a vaccine against S. flexneri serotype 2a, or a multivalent vaccine which includes S. flexneri 2a, will be required to control morbidity and mortality related to shigellosis.

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A critical question in the evaluation of vaccine candidates is which antigen-specific responses are associated with protection. Data from previous studies indicate that O-specific antibodies play an important role in protection (13–15). Antibodies against invasion plasmid antigens (Ipa) are also produced after natural and experimental infection and may also contribute to protection (16, 17).

To be acceptable to countries where Shigella is endemic, a vaccine should be safe, inexpensive, stable, and easy to deliver. At present, there is no licensed vaccine against Shigella, although a variety of vaccines have been shown to confer protection in experimental models of shigellosis (18–21). Inactivated whole-cell vaccines (WCV) are a potentially important complement to the broad range of approaches that are currently under development (22–25). By this strategy, multiple, often unidentified, antigens are delivered safely to the gut mucosa to stimulate mucosal and systemic immunity. Preparation is relatively easy and inexpensive, and administration does not require needles. Further, this type of vaccine may not require a buffer for delivery and can be stored in liquid formulations. Although only the inactivated whole-cell oral cholera vaccine Shanchol has been licensed and is in use (26), other WCV have the potential for global use. For example, vaccination with inactivated whole cells has shown promise against enteric diseases caused by enterotoxigenic Escherichia coli (27).

Several early studies reported that an inactivated WCV was protective against shigellosis (28, 29), but little information is available about the composition of these vaccines, and large oral doses were required for efficacy. An inactivated WCV vaccine for Sh. sonnei formulated using a complicated fermentation process with $2 \times 10^{10}$ vaccine particles (vp)/ml in a three-dose schedule was found to be effective in a phase I trial (30).

Preclinical studies were undertaken to manufacture formalin-inactivated whole-cell vaccine against Shigella flexneri 2a (Sf2aWC). An immunogenicity and protection study using a murine pulmonary model showed that animals vaccinated with three doses of $10^{7}$ or $10^{8}$ vp/ml of Sf2aWC were significantly protected (>93%) from an intranasal challenge with a lethal dose (1.5–10^7 CFU) of S. flexneri 2a strain 2457T. Notably, Sf2aWC formulations containing double mutant heat-labile toxin (dmLT) showed minimal improvement or, in some cases, a decrease of the magnitude of the immune response but were still highly protective in both the mouse and guinea pig models (31).

The current study was undertaken to evaluate the feasibility of using an orally delivered formalin-inactivated whole-cell vaccine Sf2aWC against Shigella flexneri 2a. We determined the safety and characterized the mucosal and systemic immune responses in a dose-escalation study in adult volunteers from the United States.

### MATERIALS AND METHODS

**Formulation and vaccine preparation.** Sf2aWC vaccine was prepared at the pilot production facility at the Walter Reed Army Institute of Research, Washington, DC, from S. flexneri 2a strain 2457T, lot 1617S, following standard current good manufacturing practice guidelines. S. flexneri 2a strain 2457T is known to be virulent in North American volunteers and to cause diarrhea, fever, and dysentery. The vaccine was prepared as described by Kaminski et al. in 2014 (31). Viability, stability, and antigen expression of Ipa proteins (IpaB, IpaC, and IpaD) and lipopolysaccharide (LPS) were monitored at 3, 6, 9, 12, and 18 months, and they remained at acceptable levels throughout the course of the phase 1 trial. Randomly selected vials were tested for appearance, particle count, sterility, and pH by slide agglutination and Western blotting with specific antiserum for 2a LPS identity and by Western blotting with specific monoclonal antibody for detection of IpaB, IpaC, and IpaD. Vaccine immunogenicity, potency, and efficacy (after challenge with S. flexneri 2a) were assessed in guinea pigs. Sf2aWC vaccine lot 1656 was formulated as a sterile liquid suspension of formalin-killed bacterial cells in phosphate-buffered saline (PBS) (pH 6.5 to 7.8). After removal from refrigerated storage, the vaccine was kept on ice until diluted in sodium bicarbonate (1.34%) for administration to volunteers. A 30-ml aliquot of this buffer was added to a separate container, and 1 ml of the Sf2aWC vaccine was added to the container for ingestion by individual subjects.

**Regulatory approval.** The clinical protocol was performed under IND 14630 at the Center for Immunization Research, Johns Hopkins Bloomberg School of Public Health (JHSPH). The protocol was approved by the Western Institutional Review Board (Olympia, WA) for JHSPH and PATH and by the institutional biosafety committee of the Johns Hopkins institutions. The study was registered in clinicaltrials.gov under NCT01509846.

**Study design.** This single-site, phase 1, double-blind, randomized, placebo-controlled trial was designed to evaluate the safety and immunogenicity of increasing dosages of orally administered Sf2aWC. Healthy adults between ages 18 and 45 years with body mass indices (BMIs) between 19.0 and 34.0 kg/m2 were eligible. Subjects were screened for study eligibility via medical history review, physical examination, human leukocyte antigens (HLA-B27), routine metabolic and hematologic indices, urine toxicity screen, urinalysis by dipstick, and serologic tests for hepatitis B, hepatitis C, and human immunodeficiency virus. Additional exclusion criteria included a history of diarrhea in the 7 days prior to vaccination; regular use of laxatives or antacids; history of vaccination against or ingestion of Shigella within 3 years; symptoms consistent with travelers’ diarrhea concurrent with travel to countries where Shigella is endemic within 2 years; and use of proton pump inhibitors, H2 blockers, or antacids within 48 h prior to dosing. Each participant signed an informed consent form and was required to score ≥70% on a study comprehension assessment prior to undergoing study-specific procedures. Eighty-two subjects were enrolled into four separate cohorts and were randomized to receive oral Sf2aWC or placebo. The study was conducted between March 2011 and November 2012. The placebo preparation was bicarbonate buffer. The number and allocation of subjects in each cohort are shown in Table 1.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>No. of vaccine recipients</th>
<th>No. of placebo recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>5</td>
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</table>

Subjects in cohort 1 received a single oral Sf2aWC dose of $2.6 \pm 0.8 \times 10^{8}$ vaccine particles (vp)/ml or placebo. Subjects in cohorts 2, 3, and 4 received Sf2aWC in increasing respective dosages of $2.6 \pm 0.8 \times 10^{9}$, $2.6 \pm 0.8 \times 10^{10}$, and $2.6 \pm 0.8 \times 10^{11}$ vp/ml or placebo at 0, 1, and 2 months (Table 1). With each dose, each volunteer drank 120 ml of bicarbonate buffer solution (1.33%) to neutralize stomach acid; approximately 1 min later, they ingested the Sf2aWC vaccine (or placebo) diluted in 30 ml of the same buffer. Subjects were required to fast for 90 min before and after ingestion of the investigational product. The first dose, for each dose level, was given to volunteers when admitted to the inpatient unit of the Center for Immunization Research, where they could be carefully observed for 72 h following receipt of the vaccine.

Subsequent doses were administered on an outpatient basis. Before enrolling subjects in subsequent cohorts, safety data from the previous cohorts through study day 7 were evaluated and reviewed by the safety review committee.
Safety evaluation. The primary safety endpoints were all adverse events (AEs) following dosing with Sf2aWC (through day 28 for cohort 1 and through day 63 after the last vaccination for cohorts 2, 3, and 4), and they were evaluated via focused medical interviews, standardized vaccination report cards (VRCs), laboratory tests, vital signs, and physical examinations. For all cohorts, systemic reactogenicity data associated with vaccination 1 were collected by study staff during the inpatient phase on study days 0 to 3 and then by study subjects during the outpatient phase via diary card on study days 4 to 7. For subjects who received two additional vaccinations in cohorts 2 to 4, systemic reactogenicity data were recorded on diary cards for 7 days after each vaccination. Solicited AEs included oral body temperature, loose stools, diarrhea, abdominal pain or cramping, bloating, defecation urgency, nausea, and vomiting. Data on other systemic AEs, including headache, malaise, chills, myalgia, reactive arthritis, excess gas, constipation, and dysentery were also collected. Approximately 6 months following receipt of the last dose of vaccine (or placebo), subjects were contacted by study staff to inquire about any chronic health conditions or serious health events.

Immunogenicity evaluation: collection of samples. Blood and fecal samples were collected from volunteers for immunological evaluations of total IgA (fetal IgA only) and antigen-specific IgA or IgG to LPS and Ipa proteins.

Blood specimens. Venous blood was collected from volunteers from cohorts 2, 3, and 4 on study days –1 (before immunization), 7, 28, 35, 56, and 63, and 112, and serum samples were separated to measure systemic immune responses. Serum IgG and IgA responses were measured using enzyme-linked immunosorbent assay (ELISA) and were analyzed by the level of response (geometric mean titers) as well as the frequency with which subjects seroconverted, as measured by the maximum dilution of sera which showed reactivity to LPS or Ipa protein antigens by more than a 2-fold increase over the preimmunization levels.

Antibody responses from the mucosal immune system were determined by ELISA using antibody from lymphocyte supernatant (ALS) specimens. This assay measures antibodies secreted by peripheral blood mononuclear cells (PBMCs) circulating in the peripheral blood following immunization. Venous blood was collected in a BD vacutainer CPT with heparin (cell preparation tubes) (Becton Dickinson, Franklin Lakes, NJ, USA) on day 0 (before vaccination) and on days 7, 28, 35, 56, and 63 after vaccination. The PBMCs, isolated by gradient centrifugation, were resuspended at 1 × 10^9 viable lymphocytes per milliliter and then incubated at 37°C, 5% CO₂ for 72 h with no antigenic stimulation. The supernatant fluid was cryopreserved and used later in an IgA ELISA. An ALS response was defined as a >4-fold increase over preimmunization levels.

Fecal samples. Stool specimens were collected on the day before vaccination and on days 7, 35, and 63 days after vaccination. Specimens were stored at −70°C until they were received. Since some specimens were brought to the clinic during outpatient follow-up visits, the duration between collection and freezing varied up to 18 h. Antibodies were extracted by the following procedure. Four grams of thawed stool was mixed with 16 ml of a solution containing soybean trypsin inhibitor (STI) (100 μg/ml) (Sigma, St. Louis, MO), EDTA (0.05 M) (Merck, NJ), and Pefablock (final concentration, 0.35 mg/ml) (Roche, NJ) dissolved in PBS (pH 7.2) supplemented with 0.05% Tween 20 (PBS-Tween). The mixture was left to stand on the bench at room temperature for 15 min with intermittent shaking. Next, the mixture was centrifuged at 12,000 × g for 30 min. Bovine serum albumin (BSA) (final concentration, 0.1% [wt/vol]) was added to the supernatant after the pellet was discarded. Aliquots of the supernatant were stored at −70°C until they were assayed by ELISA for total and specific IgA antibody contents. A fecal response was defined as a >4-fold increase over baseline.

ELISAs with serum, ALS, or fecal samples were performed according to standard protocols. Ninety-six-well microtiter plates were first coated with S. flexneri 2a LPS or Ipa (Ipa B and Ipa D) in PBS. The plates were then washed twice with PBS, blocked with 0.1% BSA-PBS for 30 min and washed three times with PBS-0.05% Tween 20. Serum, ALS, and fecal samples were diluted 3-fold in the plates using 0.1% BSA-PBS-Tween as a diluent. After overnight incubation at 4°C, the plates were washed three times with PBS-Tween. Then 100 μl of anti-human IgG or anti-human IgA conjugated with horseradish peroxidase (KPL, Baltimore, MD) diluted in 0.1% BSA-PBS-Tween was added to each well, and the plates were incubated for 90 min. Finally, after washing three times with PBS-Tween, o-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO) was added at a concentration of 100 μl per well. After 20 min, the plates were read at 450 nm in an automated ELISA reader. Antibody titers for fecal specimens were expressed as units per milligram of IgA, and these titers were calculated by using the specific titer (in units per milliliter) divided by the total IgA contents (in micrograms per milliliter) and then multiplying by 1,000. The total IgA contents for given samples were determined by ELISA using a standard IgA preparation (Sigma, St. Louis, MO) with a known IgA concentration (1 mg/ml).

Titeres were calculated to interpolate the dilution of serum, which yielded an optical density (OD) above baseline of 0.2 for serum samples and of 0.4 for ALS and fecal samples. Prevaccine and postvaccine sera were tested simultaneously in the same plate. LPS and Ipa were supplied by E. Oaks of the Walter Reed Army Institute for Research.

Serum cytokine measures. We determined serum cytokines as an extrapolatory assay in this phase 1 study. We aimed to understand if there were any significant levels of cytokine changes after vaccination with an inactivated vaccine Sf2aWC. We used the ultrasensitive human proinflammatory 9-plex and the ultrasensitive human IL-17A single-plex assays from Meso-Scale Discovery ( MSD), Gaithersburg, MD. Serum samples were collected from vaccinees and from those who received placebo in cohorts 3 and 4 on the day before vaccination and at 8, 24, 48, and 72 h on days 56, 58, and 63 postvaccinations. The MSD multiplex array was run according to the manufacturer’s protocol, with minor modifications. All samples were run in duplicate. Briefly, plates were preincubated with 25 μl of supplied human serum diluents for 30 min, with shaking, at room temperature. Calibration curves were prepared in the diluents and ranged from 2,500 pg/ml to 0.15 pg/ml. Following the 30-min incubation period, 25 μl of serum sample or calibrator was added to the wells. Plates were then incubated at room temperature for 2 h with shaking. Plates were then washed with PBS and 0.05% Tween and then incubated with 25 μl of detection antibody for 2 h at room temperature with shaking. After washing plates with PBS and 0.05% Tween, 150 μl of detection antibody was added. Plates were read using the MS2400 imager ( MSD). The lowest limit of quantification (LLOQ) was defined as the lowest calibrator value at which the coefficient of variance of concentration was less than 25% and recovery of calibrator was within 25% of the expected value. All cytokine values that were below the LLOQ were considered undetectable and assigned a value equal to the plate-specific LLOQ for statistical analyses.

Statistical analyses. Clinical and safety data were captured using electronic case report forms (CRFs). For analysis of immune responses, all of the placebos were combined and compared with different doses of Sf2aWC. The threshold for definition of a positive serological or ALS response was derived by investigating the apparent level of responses in the placebo recipients. For between-group comparisons of titer magnitude at each visit, the Kruskal-Wallis test was used; if that test was statistically significant, the Wilcoxon rank sum test (2-sided t test approximation) was used to make pairwise comparisons. Within-group change from baseline at postvaccination follow-up visits was assessed using the signed-rank test. In cytokine analyses, comparisons of dichotomous outcomes, such as fold increase or fold decrease were performed with the chi-square test or Fisher’s exact test if cell counts were below 5. For comparisons of cytokines at baseline versus cytokines at postvaccination follow-up, Student’s t test was performed using the pooled method or the Welch-Satterthwaite method, depending on whether the variance was equal or not. GraphPad Prism (GraphPad, CA) was used to analyze the data. Results of statistical analyses were considered significant only if the P value was less than 0.05.
RESULTS

Study enrollment, retention, and demographics. A total of 132 subjects were screened for this vaccine study, of which 82 subjects were enrolled. No significant differences between participants (n = 82) and nonparticipants (n = 50) were observed for age, gender, or race/ethnicity (data not shown). Seven subjects were vaccinated in the first cohort. In cohorts 2 to 4, all 75 (100%) subjects completed the first dose, and 72 (96.0%) and 70 (93.3%) subjects completed the second and third doses, respectively (Fig. 1). Vaccine administration was discontinued after two doses for one subject in the 10^11vp/ml dose group due to pregnancy. The reasons for the few dropouts were assessed to be unrelated to the study product administration. Of the 82 subjects enrolled, 23 were female and 59 were male. The majority (n = 72) race was black or African American. The mean age was 33 years (range, 18 to 45 years). The disposition of study subjects is shown in Table 2.

Safety assessment. Overall, the vaccine was well tolerated at all four dose levels, and no safety signals were identified. At the lowest dose, in the five subjects who received only one vaccination, no reactogenicity events were reported. At the highest dose, mild/moderate nausea and mild/moderate flatulence were the most common vaccine-related events (Table 3). All laboratory abnormalities were mild in severity and transient in duration. There were no vaccine-related severe adverse events and no other serious adverse events reported. Of the 77 subjects randomized to receive three vaccinations or placebo in cohorts 2 to 4, two (3.3%) subjects had moderate nausea in the vaccine group compared to one (5.9%) in the placebo group. Three (5.0%) vaccinated and two (11.8%) placebo subjects had moderate abdominal pain. Moderate excessive flatulence was found in five (8.3%) of the vaccinated subjects and in two (11.8%) of the placebo subjects. One subject in each group—vaccinated (1.7%) or placebo (5.9%)—had moderate anorexia; one (1.7%) vaccinated subject had moderate urgency of defecation (Table 3). There were no discernible trends in safety laboratory values, which included basic hematologic and metabolic parameters.

TABLE 2 Subject demographics

<table>
<thead>
<tr>
<th>Demographic variable</th>
<th>Value by cohort and Sf2aWC dose (vp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cohort 1, 2.6 ± 0.8 × 10^8 (n = 5)</td>
</tr>
<tr>
<td>No. (%) by sex</td>
<td>Male 3 (60.0) 14 (70.0) 18 (90.0) 12 (60.0) 12 (70.6) 59 (72.0)</td>
</tr>
<tr>
<td></td>
<td>Black or African American 4 (80.0) 19 (95.0) 14 (70.0) 19 (95.0) 16 (94.1) 72 (87.8)</td>
</tr>
<tr>
<td></td>
<td>0 (0.0) 1 (5.0) 0 (0.0) 0 (0.0) 1 (5.9) 2 (2.4) 0 (0.0) 1 (5.0)</td>
</tr>
<tr>
<td>No. (%) by race</td>
<td>Mean (range) age, yrs 29.0 (25–34) 31.7 (18–44) 36.5 (20–45) 32.3 (18–44) 31.3 (20–45) 32.7 (18–45)</td>
</tr>
</tbody>
</table>
TABLE 3 Proportional maximum gastrointestinal reactogenicity after vaccination with different doses of Sf2aWC or placebo

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>10^5 vp/ml (n = 20)</th>
<th>10^6 vp/ml (n = 20)</th>
<th>10^7 vp/ml (n = 20)</th>
<th>No. of reactogenicity events with placebo (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Excess flatulence</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Urgency</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Loose stool, mild</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4 IgA and IgG response* rates to S. flexneri 2a LPS or Ipa elicited by vaccine doses

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sample type</th>
<th>10^5 (n = 20)</th>
<th>10^6 (n = 20)</th>
<th>No. with response/total no. tested (%) with placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>IgA Serum</td>
<td>3 (15)</td>
<td>14 (70)</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td></td>
<td>IgG Serum</td>
<td>3 (15)</td>
<td>14 (70)</td>
<td>3/17 (18)</td>
</tr>
<tr>
<td></td>
<td>IgA ALS</td>
<td>9 (45)</td>
<td>19 (95)</td>
<td>1/17 (6)</td>
</tr>
<tr>
<td></td>
<td>IgG ALS</td>
<td>NDb</td>
<td>15 (75)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td></td>
<td>IgA Fecal</td>
<td>8 (40)</td>
<td>10 (50)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>Ipa protein</td>
<td>IpaB IgA ALS</td>
<td>ND</td>
<td>4 (20)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td></td>
<td>IpaB IgG ALS</td>
<td>ND</td>
<td>2 (10)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>IpaD IgA ALS</td>
<td>ND</td>
<td>2 (10)</td>
<td>0/12 (0)</td>
</tr>
</tbody>
</table>

*Greater than fold (in serum) or greater than 4-fold (in ALS and fecal) increase in titers from baseline on any day was considered a response.

**ND, not done.

Immunology: dose escalation and immune responses. We investigated the magnitude and kinetics of the immune responses to the Sf2aWC vaccine in this dose-escalation study. Analyzing the immune responses, we found that the IgA and IgG responses to LPS in serum and ALS samples were minimal in cohort 3. Therefore, we decided not to perform any immunology analysis on the samples from the vaccinees who received lower doses, i.e., cohorts 1 (immunization with 10^5 Sf2aWC) and 2 (immunization with 10^6 Sf2aWC). Cohorts 3 (immunization with 10^6 Sf2aWC) and 4 (immunization with 10^7 Sf2aWC) showed dose-dependent immune responses in serum, ALS, and fecal samples (Table 4, Fig. 2, 3, and 4).

Serum. Blood samples collected and processed for serum were assayed by ELISA to determine IgA and IgG endpoint titers. In cohort 4, most vaccinees seroconverted with anti-LPS IgG and IgA (Table 4). Seventy percent (14 of 20) of the vaccinees showed a >2-fold increase for both IgA and IgG to LPS compared to none for IgA and three subjects for IgG in the placebo group. The fold increase of the IgG responses in the three placebo subjects were below 2.3-fold and occurred on day 35 (7 days after the second dose). All three of these placebo subjects were in cohort 3. For LPS IgA, 93% (13 of 14) of the vaccinees who responded had a >2-fold increase on day 7 after the first dose. In contrast, for LPS IgG, only 50% (7 of 14) of the vaccinees who responded had >2-fold increase after the first dose. We also analyzed the data by considering >2.5-fold and ≥4-fold increases as the cut points for response. For LPS IgA in serum samples, 12 of 20 (60%) and 9 of 20 (45%) of the vaccinees responded when using >2.5-fold and ≥4-fold increases, respectively, as the cut points. For LPS IgG, 8 of 20 (40%) and 2 of 20 (20%) of the vaccinees responded when using >2.5-fold and ≥4-fold increases, respectively, as the cut points. The geometric mean titer (GMT) for LPS IgA was highest at 7 days after the first dose (range, 176 to 3,242) and was 3.2-fold (P < 0.0001) higher than the baseline (highest fold increase of 30) (Fig. 2A). Subsequent titers for LPS IgA also significantly increased from prevaccination to 7 days after the second and third doses, although the increase was lower after each one: the fold increase was 2.3 (P = 0.0006) (highest fold increase of 10) and was 2.0 (P = 0.0072) (highest fold increase of 7) at 7 days after vaccinations 2 and 3, respectively (Fig. 2B). The GMTs of vaccinees at all the time points postvaccination were significantly higher than those for placebos. For LPS IgG, the GMT was highest 7 days after the second dose compared to baseline and increased by 1.96-fold (P < 0.0005) (Fig. 2B). However, the GMT of LPS IgG was not significantly different from placebo. The highest fold increase was 17 compared to the baseline. The titer range on day 35 was 845 to 11,361 (Fig. 2A). In placebos, the GMTs for IgA and for IgG did not change much throughout the study period.

In the cohort 3 immunization with 10^6 vp/ml of Sf2aWC, 15% (3 of 20) of the vaccinees seroconverted for both LPS IgA and IgG (Table 4). All of the responders seroconverted on day 7 after the first dose for IgA and at 28 days after the first dose for IgG (Fig. 2A and B).

Although Sf2aWC induced strong serum antibody responses to LPS, antibody responses directed against IpaB and IpaD were low. A subset of samples randomly selected from cohort 4 was tested for IgA and IgG of IpaB and IpaD in serum. Twenty-nine percent of the vaccinees mounted responses, with both IgA and IgG to IpaB. For IpaD, 14% and 29% of the vaccinees responded for IgA and IgG, respectively (data not shown).

ALS. With respect to IgA responses directed to LPS in ALS samples, immunization with 10^11 vp/ml of Sf2aWC (cohort 4) resulted in significantly higher endpoint titers than immunization with 10^10 vp/ml of Sf2aWC (cohort 3). In both cohorts 3 and 4, the titers for LPS IgA and IgG significantly increased on day 7 after the first dose but declined to the baseline level by day 28. The titers again increased 7 days after the second and third doses, but the magnitudes were much lower than the first peak (Fig. 3A and B). For the IgA to LPS in cohort 4, the GMT increased 24-fold on day...
7 (7 days after the first dose), with the highest fold increases of 290, 6-fold on day 35 (7 days after the second dose) and 2.9-fold on day 63 (7 days after the third dose); for LPS IgG, the GMT increased 7.8-fold on day 7 (with the highest fold increase of 76), 2-fold on day 35, and 1.5-fold on day 63 compared to the preimmunization level. In cohort 4, for LPS IgA and IgG, 95% (19 of 20) and 75% (15 of 20), respectively, of the vaccinees showed 4-fold or higher increases in their ALS titers. In the placebo group, one subject responded to LPS IgA, and none responded to IgG (Table 4).

In cohort 3, only 45% (9 of 20) of the vaccinees responded for IgA to LPS in ALS, with the highest peak on day 7 of a GMT increase of 2.5-fold over the baseline (Table 4, Fig. 3). Due to lower responses for IgA to LPS in ALS samples, immune responses to IgG in ALS were not done for cohort 3 samples.

The antigen-specific responses for IgA and IgG to IpaB and IpaD in ALS were low. Twenty percent (4 of 20) of the vaccinees in cohort 4 had a >4-fold increase and 30% (6 of 20) had a >2-fold increase in titers from baseline for IgA to IpaB. For IgG to IpaB, 10% (2 of 20) of the vaccinees had a >4-fold increase and 55% (11 of 20) had a >2-fold increase in titers compared to baseline (Table 4). For IgA to IpaD, 10% of vaccinees (2 of 20) had a >4-fold increase and 20% (4 of 20) had a >2-fold increase in cohort 4. None in the placebo group responded for IgA and IgG to IpaB or for IgA to IpaD.

Fecal IgA. The fecal extracts were evaluated using ELISA for...
FIG 4 IgA-to-LPS geometric mean (95% confidence interval) fold increase of titers compared to baseline in fecal samples after immunization with either 2.6 × 10^10 or 2.6 × 10^11 vp/ml of Sf2aWC or placebo. The x axis indicates days of immune responses measured: day 7 (7 days after first dose), day 35 (7 days after second dose), or day 63 (7 days after third dose). The dotted line shows 4-fold increase. Data are presented in logarithmic scale (log10). *, P < 0.05 (responses compared to baseline).

IgA to LPS in cohorts 3 and 4. In cohorts 3 and 4, 40% (8 of 20) and 50% (10 of 20), respectively, of the vaccinees showed 4-fold or higher responses for IgA to LPS; two subjects responded in the placebo group (Table 4). In cohorts 3 and 4, the GMTs at 7 days after the first and second doses were similar (Fig. 4).

Cytokine responses in serum. We investigated the inflammatory response to vaccination by measuring the levels of proinflammatory cytokines and chemokines in serum. The 9-plex proinflammatory cytokines included gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-2, IL-6, IL-8, IL-10, IL-12p70, and the chemokine granulocyte macrophage colony-stimulating factor (GM-CSF). We also tested the serum for IL-17A in a singleplex assay. For analysis, we compared vaccinees in cohorts 3 (n = 20) and 4 (n = 20) with all of the placebo from both cohorts combined (n = 10).

Among subjects in cohort 4, 16 of 20 (80%) had a ≥2-fold increase in levels of IL-17 at 8 h after the first dose, compared to only one subject in the placebo group (P < 0.0004). Forty percent of the subjects in the vaccine group had a ≥4-fold increase compared to none in the placebo group (P < 0.0288) (Fig. 5A). The magnitude of the response was also much higher in the vaccine group than in the placebo group (P < 0.0001). The increased levels of IL-17A were transient and decreased at 24 h after the first vaccination; IL-17A levels were not substantially increased again following subsequent vaccine dosing.

Similarly, the levels of Th1-type cytokine IL-2 were significantly increased (P < 0.003) compared to the baseline (Fig. 5B) at 8 h after vaccination of the first dose. Forty percent (8 of 20) vaccinees had a ≥2-fold increase compared to one subject in the placebo group. IFN-γ secretion was also significantly higher at 8 h (P < 0.0337) and at 72 h (P < 0.014) after the first vaccination compared to baseline (Fig. 5C). Compared to baseline, mean levels of TNF-α in serum samples were higher at 8, 24, and 72 h after first dose (P < 0.0043, < 0.0482, and < 0.0479, respectively) and were higher than the levels among placebo recipients (data not shown).

In contrast, the level of IL-8 decreased significantly 8 h after the first dose and remained low at 72 h. On day 56 (28 days after the second dose), the level was significantly increased and returned to baseline. The level was again decreased 2 days after the third dose (Fig. 5D). In the placebo group, the changes in the level of IL-8 were random.

IL-10 was significantly increased at 8 h after the first dose, remained elevated at 72 h, and then decreased to the baseline level at day 56 (Fig. 5D). Another cytokine that was upregulated (≥2-fold in vaccinees) after the first vaccination was IL-6 (25%). The trend of responses to all of the cytokines measured was similar but of lower magnitude in cohort 3. Cytokine responses in serum for GM-CSF, IL-12p70, and IL-1β in cohort 4 and in addition for IFN-γ in cohort 3, were below detectable limit and were excluded from analysis.

We also examined associations between cytokine responses in serum samples and LPS-specific immune response in serum, ALS, and fecal samples after vaccination. Among subjects in cohort 4, subjects with elevated IFN-γ levels also had ≥2-fold increases in titers against IgA and IgG to LPS in serum samples and ≥4-fold increases in titers in fecal extracts for IgA.

**DISCUSSION**

The present study demonstrated that an orally delivered, formalin-inactivated, whole-cell *Shigella flexneri* 2a vaccine (Sf2aWC) with a dose of 2.6 ± 0.8 × 10^11 vaccine particles/ml was safe and was able to induce both systemic and mucosal immune responses. Sf2aWC was well tolerated at all four dose levels, ranging from 10^8 to 10^11 inactivated cells. There were no significant differences in the frequency or severity of symptoms in vaccinees compared to placebo recipients. No fever or serious gastrointestinal symptoms were noted by any of the vaccinated subjects. Thus, our data suggest that Sf2aWC, which represents a major *Shigella* species of clinical importance, can be used safely at a high dose in humans.

An obvious concern regarding live attenuated candidates is identifying the appropriate balance between immunogenicity and adverse effects, while the primary concern for an inactivated WCV is immunogenicity. *Shigella* are invasive enteric bacteria, and cellular invasion is said to be the key to efficient antigen delivery. However, in our study, three doses of Sf2aWC were able to induce considerable mucosal and systemic immunity. All vaccinated subjects had serum or mucosal IgA or IgG responses to LPS. In serum, most of the vaccinated subjects had IgA responses to LPS, and the highest GMT was after the first dose. Interestingly, IgG responses to LPS were delayed, and the highest GMT was after the second dose. The increase observed in GMTs of IgG to LPS was lower than that detected in the IgA isotype. Although IgA titers to LPS increased after every dose, the largest increase over baseline occurred following the first dose. Since there was little or no boosting of immune responses after the third dose, two doses of 10^11 vp/ml of Sf2aWC might be sufficient. The oral inactivated WCV for cholera with a 10^13 dose in a two-dose schedule was found to be effective (26).

Notably, the serum LPS antibody responses elicited by Sf2aWC were stronger than the response elicited by the live attenuated vaccine SC602, an *S. flexneri* strain attenuated by deletions in icgA and *icsA*, which encode aerobactin. Administration of 10^8 CFU of SC602 led to a 3-fold increase in the serum IgA GMT to LPS titers.
in half of the subjects and to a <2-fold increase in anti-LPS-specific IgG (21, 32). Despite these modest immune responses, SC602 was protective in a challenge study in the United States. The immune response to LPS with Sf2aWC in our study was also comparable to the oral inactivated whole-cell vaccine for Shigella sonnei (SsWC), where four of seven vaccinees (57%) had IgG and IgA responses to LPS (30).

Based on the human challenge-rechallenge studies (19, 33, 34), primate studies, epidemiological field studies (13), and seroepidemiological surveys (14, 15), LPS is believed to be of critical importance in protection against Shigella infections. In field settings, preexisting IgA and IgG anti-LPS serum antibodies have been associated with protection against shigellosis following natural exposure (14). Using active surveillance at the community levels, Ferrecio et al. followed a cohort of children less than 4 years of age in Santiago, Chile, for 30 months and observed that an initial bout of shigellosis afforded 72% protection against subsequent clinical illness caused by the homologous serotype ($P = 0.05$). However, protection against a second clinical illness due to a heterologous serotype was low ($<30%; P$ value not significant) (13). The importance of humoral immunity to LPS is also supported by the protection provided in the SC602 trials (21, 32). It has been postulated that local secretory IgA against LPS may help mediate this protection by blocking the interactions that occur between Shigella and mucosal epithelial cells which ultimately lead to invasion. Another mechanism might be the antibody-dependent cellular cytotoxicity against Shigella observed in vitro (35).

A compelling rationale for the oral delivery of live attenuated vaccines is the induction of mucosal immunity, which has been shown to correlate well with the transient appearance of LPS-specific IgA antibody-secreting cells (ASC) in the peripheral blood (36). In the SC602 Shigella vaccine trial, a rise in IgA ASCs to LPS was correlated with protection (21). In our phase I trial, oral administration of Sf2aWC resulted in high levels of IgA and IgG to LPS in ALS. All but one vaccinee responded to IgA, and 75% responded to IgG in the highest dose. The GMT was increased after each dose, but the highest pick was after the first dose.

The immune responses to LPS in fecal samples were modest,

![Figure 5](cvi.asm.org)
with 50% of the vaccinees exhibiting a >4-fold increase after the first dose. The other WCV vaccine, SsWC, yielded a relatively similar 60% (3 of 5 volunteers) IgA antibody response to LPS in fecal samples after immunization. IgA responses measured using fecal samples might have a role in assessing protection; however, an association has not yet been established (8, 34).

Despite many studies, the potential for other antigens, such as the Ipa proteins, to elicit protective immunity remains unclear. In our study, Sf2aWC vaccine elicited low IgA and IgG immune responses to Ipa in serum and ALS samples. The preclinical studies with Sf2aWC also observed relatively meager humoral responses for IpaB, IpaC, or IpaD after immunization with inactivated whole Shigella cells in mice regardless of the different conditions used to inactivate the vaccine (31).

Oral immunization with inactivated Sf2aWC resulted in the rapid production of several cytokines, including the signature Th1 and Th17 cytokines IFN-γ and IL-17A, respectively. The serum cytokine responses occurred very quickly (within 8 h) after the first vaccine dose and would likely have been missed without this 8-h specimen. To our knowledge, this is the first study to measure the cytokine responses after vaccination with Shigella in humans where a time point before 24 h was analyzed. Interestingly, several cytokines exhibited peak expression at 8 h after the first vaccination and then declined by 24 h postvaccination. Thus, the studies where cytokines were measured starting at 24 h or later might have missed the earlier responses.

Our vaccine induced IL-17A after the first dose. In a pulmonary mouse model, Selge et al. in 2010 showed that IL-17A might play an important modulatory role in anti-Shigella protective immunity to Shigella infection (37). TNF-α contributes to epithelial destruction in experimental shigellosis. Interestingly, in our study, the Sf2aWC vaccine induced only a transient significant increase in TNF-α levels after the first dose. IFN-γ as an indicator of Th1 immunity was significantly induced by Sf2aWC. Similar results were also observed after experimental challenge of naive U.S. volunteers with Shigella sonnei, where IFN-γ, IL-2, and TNF-α were significantly increased after infection (38).

We also observed a relationship between IFN-γ levels and serum and fecal antibody responses. Earlier studies showed that Ifngr1-deficient mice have impaired mucosal immune responses (39).

Several studies have revealed the central role played by IL-8 in neutrophil recruitment and inflammation in experimental shigellosis. In a previous rabbit study using ligated ileal loops, IL-8 was shown to be expressed in infected intestinal cells exposed to a noninvasive Shigella mutant. This implies that a significant proportion of IL-8 was produced in response to extracellular activation either by LPS or, indeed, by inflammatory cytokines (40). Interestingly, in our study, IL-8 expression level was never increased over baseline but rather decreased at 8 h after the first dose and came back to baseline at day 56 (28 days after the second dose). The decrease of IL-8 levels in our study might be due to recruitment of the IL-8--expressing and/or -responding cells from the periphery to the tissues, taking free IL-8 with them. A decrease of IL-8 was also shown to be associated with the trivalent inactivated influenza vaccine (41). Notably, the anti-inflammatory cytokine IL-10 showed a similar pattern but in the opposite direction. IL-10 levels were significantly increased at 8 h after the first dose and were decreased at day 56 when the proinflammatory cytokine IL-8 was increased. IL-10 is known to inhibit production of a wide range of cytokines, including IL-8 (42). Cytokine responses for GM-CSF, IL-12p70, and IL-1β could not be detected in this study. This might be because we missed related time points.

Overall, these data suggest that this vaccine rapidly induced a profile of serum cytokines which are known to play an important role in generating protective immunity to infection. For most of these cytokines, responses were transient. These short-lived responses were consistent with the excellent safety profile observed for the vaccine, since persistent increases would have likely been associated with a higher degree of systemic and/or local gastrointestinal side effects. A very similar pattern was observed in volunteers immunized with injectable influenza vaccine, where frequent blood samples postimmunization demonstrated an early cytokine increase within 3 h of dosing that were back to baseline within 24 h (41). In a follow-up to these initial observations, further studies of this inactivated vaccine will be needed to better appreciate the significance of these cytokine changes and the impact on safety and immunogenicity.

In conclusion, this phase I trial demonstrated that Sf2aWC vaccine, a relatively simple economical and safe approach, effectively presented LPS and other accessible antigens of Shigella to the majority of immunized subjects. When given orally, it was extremely well tolerated and induced both systemic and mucosal immune responses to LPS. These immune responses were comparable to those induced by a live attenuated Shigella vaccine which was protective in prior human challenge studies performed by other investigators. Thus, the Sf2aWC vaccine deserves further evaluation as a Shigella vaccine candidate alone or in combination with other clinically important species and serotypes to prevent S. flexneri epidemics.

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