Safety, Immunogenicity, and Efficacy in Monkeys and Humans of Invasive Escherichia coli K-12 Hybrid Vaccine Candidates Expressing Shigella flexneri 2a Somatic Antigen

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A live, oral Shigella vaccine, constructed by transfer of the 140-MDa invasiveness plasmid from Shigella flexneri 5 and the chromosomal genes encoding the group- and type-specific O antigen of S. flexneri 2a to Escherichia coli K-12, was tested in humans. Designated EcSf2a-1, this vaccine produced adverse reactions (fever, diarrhea, or dysentery) in 4 (31%) of 13 subjects who ingested a single dose of 1.0 × 10^6 CFU, while at better-tolerated doses (5.0 × 10^6 to 5.0 × 10^7 CFU), it provided no significant protection against challenge with S. flexneri 2a. A further-attenuated arov mutant derivative, EcSf2a-2, was then tested. Rhesus monkeys that received EcSf2a-2 in three oral doses of ca. 1.5 × 10^7 CFU experienced no increase in gastrointestinal symptoms compared with a control group that received an E. coli K-12 placebo. Compared with controls, the vaccinated monkeys were protected against shigellosis after challenge with S. flexneri 2a (60% efficacy; P = 0.001). In humans, EcSf2a-2 was well tolerated at inocula ranging from 5.0 × 10^6 to 2.1 × 10^7 CFU. However, after a single dose of 2.5 × 10^8 CFU, 4 (17%) of 23 subjects experienced adverse reactions, including fever (3 subjects) and diarrhea (209 ml) (1 subject), and after a single dose of 1.8 × 10^10 CFU, 2 of 4 subjects developed dysentery. Recipients of three doses of 1.2 to 2.5 × 10^8 CFU had significant rises in serum antibody to lipopolysaccharide (61%) and invasiveness plasmid antigens (44%) and in gut-derived immunoglobulin A antibody-secreting cells specific for lipopolysaccharide (100%) and invasiveness plasmid antigens (60%). Despite its immunogenicity, the vaccine conferred only 36% protection against illness (fever, diarrhea, or dysentery) induced by experimental challenge (P = 0.17). These findings illustrate the use of an epithelial cell-invasive E. coli strain as a carrier for Shigella antigens. Future studies must explore dosing regimens that might optimize the protective effects of the vaccine while eliminating adverse clinical reactions.

Each year Shigella species cause an estimated 250 million cases of diarrheal illness and more than 600,000 deaths worldwide (15). Because of the limited efficacy of current control efforts, a public health priority for nearly three decades has been the development of safe, genetically stable, and effective oral vaccines that can be adapted to large-scale production without critical alterations in immunogenicity. A variety of strategies have been attempted, including producing attenuated Shigella mutants (23, 27) and mutant hybrids (Shigella species attenuated by the insertion of Escherichia coli genes) (4, 5). Another approach to Shigella vaccine development has been the use of orally administered carrier bacteria to deliver immunizing Shigella antigens to intestinal lymphoid tissue. In the 1970s, a noninvasive E. coli carrier vaccine expressing Shigella flexneri 2a somatic antigen on its surface was constructed by conjugal transfer of the chromosomal genes for the expression of S. flexneri 2a group 3,4 and type II lipopolysaccharide (LPS) into a commensal E. coli 08 strain (21). Although this vaccine was safe and was able to colonize the intestines of volunteers for several days, the antibody response to 2a LPS, which has been associated with protection (1, 3), was relatively meager. In addition, the vaccine did not confer immunity in adults experimentally challenged with the homologous Shigella serotype (21).

Further development of the Shigella vaccine carrier concept has focused on strains that invade and proliferate within the intestine to more effectively deliver critical Shigella antigens to the local immune system, thereby simulating the protective immune response that follows natural infection (5, 9, 14). Second-generation E. coli carrier vaccine strains containing the invasiveness plasmid of S. flexneri as well as the chromosomal genes encoding the S. flexneri 2a LPS were thus constructed (10, 29). In rabbit ileal loops, these hybrid vaccines penetrate the intestinal epithelium, where they stimulate a mild inflammatory response in the lamina propria. However, these strains do not express the critical S. flexneri chromosomal genes necessary for full Shigella virulence (e.g., lacA and kcpA) and do not produce keratoconjunctivitis when inoculated into the guinea pig conjunctival sac (Sereny test). Furthermore, when fed to rhesus monkeys in doses of 10^10 to 10^11 CFU, an invasive hybrid vaccine...
strain designated EC104 conferred significant protection against diarrhea and dysentery when the monkeys were challenged with virulent S. flexneri 2a (10). The present report describes studies of monkeys and humans to assess the safety, immunogenicity, and efficacy of a kanamycin-sensitive analog of EC104, designated EcSF2a-1, and an aroD mutant derivative, designated EcSF2a-2.

**MATERIALS AND METHODS**

Preparation of vaccine and placebo strains. The vaccines were constructed at the Walter Reed Army Institute of Research (WRAIR) by conjugal transfer of the 140-MDa invasiveness plasmid of S. flexneri 5 (strain M907) into an E. coli K-12 recipient (strain 395-1), using the temperature-sensitive mobilizing plasmid PMT999 carrying the Hg2+-resistant transposon Tn501 (29). Subsequent conjugal transfer from the S. flexneri 2a Hfr donor (strain 256) of the his, pro, and arg chromosomal markers in separate matings allowed for expression of the S. flexneri 2a LPS surface antigen and stabilized expression of the plasmid-encoded invasiveness phenotype. The resultant hybrid strain, designated EcSF2a-1, was formulated at WRAIR as lyophilized organisms in sealed glass vials containing 8 x 10^15 viable organisms (lot 2) and stored at -70°C until use.

A further-attenuated derivative, EcSF2a-2, was constructed by P1 transduction of a tetracycline resistance transposon (Tn10)-inactivated aroD gene followed by selection of a tetracycline-sensitive strain with a growth requirement for para-aminobenzoic acid, a precursor of folic acid not available in mammalian cells. The EcSF2a-2 vaccine and an E. coli K-12 placebo (the parent strain for the vaccine) were maintained at WRAIR as master seeds in 12% glycerol at -70°C.

A placebo-controlled safety-efficacy trial of EcSF2a-2 was performed with monkeys. For this study, the master seeds of the vaccine and the placebo were inoculated onto tryptic soy agar (Difco Laboratories, Detroit, Mich.), grown overnight at 37°C, and subcultured onto brain heart infusion agar (BHI; Difco Laboratories) with overnight incubation at 37°C. The strains were harvested from the BHI plates into BHI broth immediately before the inoculation of the monkeys.

For human studies, a lyophilized production seed of EcSF2a-2 (lot 5) was prepared at WRAIR and transferred to the Salk Institute in Swiftwater, Pa., where a single lot, 5-1-90, of fermentor-grown lyophilized vaccine mixed with a dextran-sucrose-glycerol stabilizer was packaged in sealed vials containing 4.0 x 10^10 viable organisms and stored at -70°C. One safety-efficacy study of humans using EcSF2a-1 (lot 2) and two studies using EcSF2a-2 (lot 5-1-90) were performed. The placebo strain was not used in human studies.

Prior to each vaccination study, vaccine stability was ascertained by determining that colony counts, Congo red binding, and agglutination with specific antisera were unchanged. Stable phenotype expression in HeLa cell invasion (11) assays was confirmed for vaccine strains isolated from several randomly selected subjects in each human study.

**Design of monkey studies.** Forty rhesus monkeys weighing 3 to 5 kg were used in a study that was carried out in the animal facilities of the Division of Product Quality Control, Office of Biologics, Food and Drug Administration. The animals were individually caged. When handled for procedures, they were sedated with 50 mg of ketamine hydrochloride. To enable the exclusion of Shigella carriers, three stool specimens from each animal were cultured for Shigella strains during the week before vaccination.

Half of the monkeys were assigned to receive the vaccine and half were assigned to receive the placebo suspended in 20 ml of BHI broth. The monkeys were fed three doses of ca. 1.5 x 10^11 CFU of the vaccine or the placebo by pediatric gastric tube during a 7-day period. Eighteen days after the last dose, each monkey was challenged via gastric tube with 3.2 x 10^10 CFU of S. flexneri 2a (strain 2457T) suspended in 20 ml of BHI broth. After vaccination and challenge, the monkeys were observed twice daily for signs of diarrhea, defined as one or more loose stools during 2 consecutive days, or dysentery, defined as one or more bloody stools. Stool samples were obtained daily for culture. Animals that became ill were treated with chloramphenicol when necessary. Animals that died were subjected to complete necropsy.

**Design of human studies.** Healthy adult volunteers aged 18 to 40 years recruited from the Baltimore, Md., community were admitted to the Isolation Ward of the Center for Vaccine Development, University of Maryland Hospital. Informed consent was obtained from the volunteers, and guidelines for human experimentation of the U.S. Department of Health and Human Services, the Human Volunteers Research Committee of the University of Maryland, and the Surgeon General’s Human Subjects Research Review Board of the Department of the Army were followed in the conduct of the clinical research. The volunteers fasted for 90 min before and after administration of the vaccine or the challenge strains. They were evaluated daily by a physician for evidence of illness, and all stools were examined for blood, consistency, and weight and then sent for culture. Diarrhea was defined as the passage of two or more loose (grade 3 or higher) stools totaling at least 200 ml within 48 h or a single 300-ml loose stool (18). Dysentery was defined as one or more loose stools containing gross blood. Illness was defined as diarrhea, dysentery, or fever (oral temperature of 100.0°F [37.8°C] or greater).

Prior to administration, the vaccine was reconstituted in sterile water and diluted in phosphate-buffered saline to achieve the desired inoculum. Two grams of NaHCO3 was suspended in 150 ml of distilled H2O. Subjects drank 120 ml of the NaHCO3 solution and then 1 min later ingested the reconstituted vaccine suspended in the remaining 30 ml. Colony counts of the reconstituted vaccine were performed before and after vaccination, and the results were averaged to estimate the inoculum.

One month after receiving the first dose of vaccine, a group of vaccinees and a group of unimmunized control subjects were challenged with a single oral dose of ca. 10^9 virulent S. flexneri 2a (strain 2457T) organisms suspended in 45 ml of skim milk (6). All subjects received a 5-day course of ciprofloxacin (500 mg every 12 h) beginning 5 days after challenge. Treatment was initiated earlier in the event of illness at the discretion of the investigator.

**Bacteriology.** Stool samples were inoculated into buffered glycerol saline for transport to the laboratory (28). If stools were not available from a human subject in a 24-h period, a rectal swab was obtained and inoculated into gram-negative broth for transport. Swabs and stools were plated directly onto MacConkey’s agar containing 500 μg of streptomycin per ml for recovery of the streptomycin-resistant vaccine strain or onto Salmonella-Shigella species and MacConkey’s agar without antibiotics for recovery of the Shigella challenge strain. Each specimen was also inoculated into gram-negative enrichment broth and incubated overnight at

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**E. COLI-S. FLEXNERI 2a HYBRID VACCINE**

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35°C before being plated onto the above enteric media. Lactose-negative colonies identified after 24 or 48 h of incubation at 35°C on solid media were inoculated onto triple sugar iron agar slants. Those producing an alkaline slant and an acid butt with little gas production were verified by using agglutination by Shigella group B antisera (Difco Laboratories). The level of excretion of the vaccine strain was measured quantitatively by using serial dilutions of a suspension of 1 g of stool in 9 ml of sterile saline plated onto MacConkey’s agar with streptomycin.

Fasting volunteers swallowed gelatin string capsules (Enterotox; HEDECO, Mountain View, Calif.) 24 and 48 h after each dose of vaccine to detect duodenal colonization with the vaccine. The bile-stained distal portion of the duodenal string was removed after 4 h, inoculated into gram-negative enrichment broth, and incubated overnight at 35°C before being cultured on enteric media as described above.

Measurement of serum and jejunal antibodies in humans. Sera were collected before and after the first dose of vaccine or the challenge strain was ingested for the measurement of immunoglobulin A (IgA), IgM, and IgG antibodies. For the measurement of intestinal secretory IgA antibodies, intestinal incubation was performed to collect jejunal fluid before and 14 days after ingestion of the first dose of the vaccine and before and 14 days after ingestion of the challenge strain (1). Serum and jejunal antibodies to the invasiveness plasmid antigens (IPA) and LPS O antigens of S. flexneri 2a were measured by an enzyme-linked immunosorbent assay (ELISA), as previously described (1, 30).

Detection of antibody-secreting cells in humans. Heparinized blood was collected before and 7 and 10 days after the first dose of the EcSf2a-2 vaccine or the challenge strain for the measurement of circulating IgA antibody-secreting cells (ASC) trafficking from the gut-associated lymphoid tissue to other lymphoid sites as previously described (34).

Statistical analysis. Percent vaccine efficacy (VE) was calculated as follows: VE = [(attack rate of illness in controls − attack rate of illness in vaccinees)/attack rate of illness in controls] × 100. Because the question of interest was whether the vaccine reduced the rate of illness, the one-tailed Fisher exact test was used to compare vaccinees and controls. Two-tailed Fisher’s exact and chi-square tests were used to compare proportions among groups of vaccinees; for comparison of ASC responses, $t$ tests were performed on log-transformed data.

RESULTS

Clinical evaluation of EcSf2a-1 in humans. (i) Dose-response safety studies. Only 1 of the 17 volunteers who ingested three doses of ca. 5.0 × 10⁹ or 5.0 × 10¹⁰ vaccine organisms developed diarrhea (225 ml); this occurred following the third dose of the vaccine. In contrast, 4 (31%) of 13 recipients of a single inoculum of 1.0 × 10⁹ CFU had adverse reactions: one subject developed fever (101.4°F [ca. 38.4°C]), one had mild diarrhea (435 ml), one passed a dysenteric stool, and one experienced both fever (100.0°F; [37.8°C]) and dysentery. Because of these adverse reactions, further doses of 1.0 × 10⁹ CFU were not given.

(ii) Bacteriology. Recipients of the two lower doses shed vaccine organisms after each of the three administrations of the vaccine, so vaccine organisms were recovered by co-culture for a total of 7 to 12 days. The pattern of shedding after a single inoculation could be estimated by observing the 13 recipients of a single dose of 1.0 × 10⁹ CFU; all but 1 vaccinee shed vaccine organisms for 3 to 5 days. Duodenal cultures were all negative.

(iii) Immunology. One recipient (6%) of the lower doses of the vaccine exhibited a fourfold rise in antibody to LPS, and two (11%) seroconverted to IPA. In comparison, significant responses to LPS and IPA were detected with four (30%) and five (38%), respectively, of the recipients of 1.0 × 10⁹ CFU.

(iv) Efficacy. One month after vaccination, 10 recipients of EcSf2a-1, including 8 who received three doses of ca. 5.0 × 10⁹ CFU, one who received three doses of ca. 5.0 × 10⁹ CFU, and one who received a single dose of 1.0 × 10⁹ CFU of vaccine, were challenged with virulent S. flexneri 2a along with 8 unimmunized controls to assess the protective efficacy of the vaccine. Illness occurred in 4 of 10 vaccinees and 4 of 8 unimmunized controls (VE, 20%; $P = 0.5$).

Safety and efficacy of EcSf2a-2 in monkeys. After vaccination with three doses of EcSf2a-2, two monkeys in the vaccine group and two monkeys in the E. coli K-12 placebo group experienced diarrhea. All monkeys shed the vaccine strain for 3 or fewer days following the last dose of vaccine. After challenge, 13 (86%) of 15 monkeys in the placebo group became ill, with three deaths, and 5 (28%) of 18 monkeys in the vaccine group became ill, with two deaths (VE, 60%; $P < 0.001$). All monkeys shed the challenge strain. Seven monkeys were excluded from the analysis of efficacy, including one monkey who developed diarrhea shortly before challenge and six monkeys (four placebo recipients and two vaccinees) who exhibited no diarrhea or dysentery but who were treated with antibiotics after challenge because of lethargy and anorexia. Necropsies indicated that all the dead monkeys suffered from necrotizing enteritis characteristic of acute shigellosis.

Clinical evaluation of EcSf2a-2 in humans. (i) Dose-response safety studies. In the first safety study, no objective adverse reactions occurred among the 19 volunteers who ingested three doses of either ca. 5.0 × 10⁶ (n = 2), 5.0 × 10⁷ (n = 2), or 2.0 × 10⁸ (n = 15) CFU. However, approximately 72 h after the third dose of ca. 2.0 × 10⁹ CFU, two subjects complained of abdominal cramping, prompting a decrease in activity; one of these subjects also passed a single 37-ml loose stool. In contrast, the vaccine was overall reactogenic after a single dose of 1.8 × 10¹⁰ CFU, with two of four subjects developing dysentery. Because of this reactogenicity, the subjects were treated with ciprofloxacin, and subsequent doses at this level were not given. In preparation for a second vaccine efficacy study, a group of 23 volunteers was inoculated with three doses of ca. 2.0 × 10⁹ CFU. Reactions were seen with four individuals following the first dose of 2.5 × 10⁹ CFU, including fever in three individuals (101.2 to 102.4°F [ca. 38.4 to 39.1°C]) and diarrhea (209 ml) in one. No antibiotic therapy was initiated, and subsequent doses of 1.6 × 10⁹ and 1.8 × 10⁹ CFU given to these same volunteers were well tolerated.

(ii) Bacteriology. All 46 vaccine recipients excreted the vaccine strain on at least one occasion. Recipients of three spaced doses of ca. 2.0 × 10⁹ CFU shed ca. 10⁸ organisms per gram of stool (Table 1) for an average of 7 days (range, 1 to 12 days) (Fig. 1). Duodenal colonization was detected in five subjects, all recipients of ca. 2.0 × 10⁹ CFU.

(iii) Immunology. Overall, a fourfold increase in IgA or IgG antibody titers recognizing purified LPS was detected in 23 (61%), and a seroresponse to IPA was detected in 17 (44%), of the 38 recipients of ca. 2.0 × 10⁹ CFU. The IgM response was meager (data not shown). Circulating IgA ASCs were detected with four (30%) and five (38%), respectively, of the recipients of 1.0 × 10⁹ CFU.
Table 1. Immunologic and bacteriologic responses to oral vaccination with EcSf2a-2a

<table>
<thead>
<tr>
<th>Type of response</th>
<th>Study 1</th>
<th>Study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All vaccinees</td>
<td>Vaccines later challenged with S. flexneri 2a</td>
</tr>
<tr>
<td>Serum antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-LPS IgA</td>
<td>4/15 (27)</td>
<td>3/10 (30)</td>
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<tr>
<td></td>
<td>8/15 (53)</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>Anti-IPA IgA</td>
<td>4/15 (27)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td></td>
<td>6/15 (40)</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>Anti-LPS IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geometric mean peak ASC/10⁶</td>
<td>15/15 (100)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>PBMC (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IPA IgG</td>
<td>9/15 (60)</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>Geometric mean peak ASC/10⁶</td>
<td>5.0 (6.1)</td>
<td>2.1 (3.3)</td>
</tr>
<tr>
<td>PBMC (SD)</td>
<td></td>
<td></td>
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<tr>
<td>Jejunal IgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-LPS</td>
<td>1/10 (10)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Anti-IPA</td>
<td>0/10 (0)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Geometric mean peak vaccine excretion (CFU/g of stool)</td>
<td>5.5 × 10⁴</td>
<td>6.7 × 10⁵</td>
</tr>
</tbody>
</table>

*The subjects in study 1 were given 1.2 × 10⁶, 2.1 × 10⁶, and 1.8 × 10⁶ CFU on days 0, 3, and 6, respectively. The subjects in study 2 were given 2.5 × 10⁶, 1.6 × 10⁶, and 1.8 × 10⁶ CFU on days 0, 3, and 6, respectively.

Response defined as a fourfold rise in antibody by ELISA.

No response defined as 0 to 1 specific ASC per 10⁶ cells.

SD, standard deviation, which was back-transformed from logs; PBMC, peripheral blood mononuclear cells.

P = 0.05 (t test), comparing all vaccinees in study 1 with all vaccinees in study 2.

Specific for S. flexneri 2a LPS and for IPA were each detected in 29 (97%) and 18 (60%), respectively, of the 30 subjects tested. Jejunal fluid IgA (anti-LPS) was detected in 4 (17%) of the 28 subjects tested. The specific immune responses to vaccination with this regimen are depicted in Table 1. Every subject reacted in at least one immunologic assay.

(iv) Efficacy. After the S. flexneri 2a challenge strain was ingested in the first efficacy study, illness occurred in 3 (30%) of 10 recipients of three doses of ca. 2 × 10⁶ CFU, compared

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![FIG. 1. Daily geometric mean titer of fecal excretion and number of subjects excreting vaccine strain after inoculation with three oral doses of ca. 2.0 × 10⁶ CFU of EcSf2a-2. Arrows denote days of vaccination. To determine the number of subjects excreting vaccine (total n = 38), quantitative cultures were performed with stool specimens; in addition, specimens from subjects who did not pass a stool on a given day were cultured by rectal swab to get a qualitative estimate of vaccine excretion. Daily geometric mean titers were calculated only for subjects excreting vaccine in stool specimens.](http://iai.asm.org/)
TABLE 2. Efficacy of oral vaccination with EcSf2a-2 against homologous challenge with S. flexneri 2a*

<table>
<thead>
<tr>
<th>Parameter of study group(s)</th>
<th>Value for subjects with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Any illness</td>
</tr>
<tr>
<td>Study 1</td>
<td></td>
</tr>
<tr>
<td>No. (%) of vaccines (n = 10)</td>
<td>3 (30)</td>
</tr>
<tr>
<td>No. (%) of controls (n = 12)</td>
<td>7 (58)</td>
</tr>
<tr>
<td>VE (%)</td>
<td>48</td>
</tr>
<tr>
<td>P value*</td>
<td>0.18</td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
</tr>
<tr>
<td>No. (%) of vaccines (n = 20)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>No. (%) of controls (n = 9)</td>
<td>3 (33)</td>
</tr>
<tr>
<td>VE (%)</td>
<td>9</td>
</tr>
<tr>
<td>P value</td>
<td>0.59</td>
</tr>
<tr>
<td>Combined studies</td>
<td></td>
</tr>
<tr>
<td>VE (%)</td>
<td>36</td>
</tr>
<tr>
<td>P value</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Three doses of ca. 2 $\times 10^{10}$ CFU of EcSf2a-2 were administered on days 0, 3, and 6.

with 7 (58%) of 12 unvaccinated control subjects (VE, 48%; $P = 0.18$) (Table 2). Greater protection against individual symptoms, including diarrhea (66%), dysentery (80%), and fever (66%), was seen, although these trends were not statistically significant. However, VE was only 9% ($P = 0.59$) in the second challenge study, in which 6 (30%) of 20 recipients of three doses of ca. 2.0 $\times 10^{10}$ CFU developed illness, compared with 3 (33%) of 9 unvaccinated control subjects. Overall, the VE was 36%. Neither class-specific titers in prechallenge sera nor fourfold rises in antibody titer after vaccination could be correlated with protection against challenge (data not shown).

DISCUSSION

Previous efforts to develop a safe and protective Shigella vaccine have achieved some success. In the 1960s, protection against endemic shigellosis following oral immunization was accomplished with noninvasive attenuated Shigella mutant vaccines (23, 24, 27). Streptomycin-dependent mutant strains provided high-level safety and serotype-specific protective efficacy, as demonstrated in large-scale human trials conducted in Yugoslavia (24, 27) and corroborated in the volunteer model (5). Nonetheless, these noninvasive vaccines had drawbacks that may limit their usefulness in developing countries, including the apparent need for four large doses to induce primary immunity and the need to administer a booster after 12 months to maintain protection (23, 26). Another concern is the occasional occurrence of streptomycin-independent revertants, although in vivo revertants have remained avirulent (4, 20).

The bivalent Salmonella typhi-Shigella sonnei hybrid 5076-1C, a carrier vaccine containing heterologous Shigella antigens, also showed some success in volunteer studies (1). This attenuated S. typhi Ty21a vaccine strain, which carries the form I S. sonnei plasmid, was safe, and initial trials demonstrated considerable protective efficacy (1), but the results could not be replicated with subsequent lots of vaccine (14). In contrast to the E. coli hybrid vaccines we describe herein (29), the O-polysaccharide form I antigen of 5076-1C is loosely associated with the cell surface without covalent linkage to core lipid A (12, 33). In theory, this may have reduced the antigenicity of the O-polysaccharide.

At doses of $1 \times 10^{10}$ to $2 \times 10^{10}$ CFU, the intergeneric E. coli carrier vaccines described herein were highly immunogenic in humans, evoking a systemic immune response in nearly all recipients. After three doses, EcSf2a-2 induced a serum antibody response to LPS in 61% of its recipients, which is comparable to the 31% response seen after the challenge of unvaccinated controls with virulent S. flexneri (data not shown). This response was predominantly of immunoglobulin classes IgA and IgG, which have been associated with protection against illness in prospective seroepidemiologic studies of Shigella outbreaks among Israeli soldiers (3) and in Shigella challenge studies (1). Furthermore, all vaccinees exhibited an ASC IgA response, suggesting that priming of the intestinal immune system had occurred, although this response did not correlate with a strong secretory IgA response. It is disappointing, therefore, that despite its immunogenicity, the vaccine afforded little protection against challenge.

Furthermore, with the large inocula necessary to evoke a vigorous immune response, EcSf2a-2 was not sufficiently attenuated in humans. Nevertheless, it appeared less reactivogenic than its parent, EcSf2a-1, causing illness in only 4 (11%) of 38 recipients of ca. 2.0 $\times 10^{10}$ CFU compared with 4 (31%) of 13 recipients of 1.0 $\times 10^{10}$ CFU of the parent strain ($P = 0.07$). Karnell et al. also reported the occurrence of mild diarrhea in 4 (16%) of 25 subjects who ingested a single 109 CFU dose of an aroD mutant S. flexneri serotype Y vaccine (17). Together, these observations suggest that aromatic auxotrophy alone may not be sufficient to attenuate invasive Shigella vaccines.

The residual reactivogenicity of EcSf2a-2 may be related to the administration of a large inoculum of organisms that are able to invade the intestinal mucosa. Parenteral administration of LPS to mice causes a dose-related endothelial lesion in the lamina propria, with hemorrhage and fluid exudation into the intestinal lumen leading to diarrhea (22). Similar lesions in the rectal mucosa of adults with acute diarrhea have been observed (2). These findings suggest that in addition to the well-recognized systemic febrile response, LPS may play a role in the pathogenesis of diarrhea and dysentery. Alternatively, laboratory evaluation has disclosed the spontaneous appearance of kep* variants in the E. coli K-12 recipient that was used to construct EcSf2a-1. These variants were able to multiply within and spread to contiguous HeLa cells, thereby producing plaques in a confluent monolayer (31). It is also possible that the Arg locus or other uncharacterized genes encoding enterotoxin or cytotoxin activity contributed to the diarrheagenicity of the vaccine (7, 32). Although the aroD mutation renders the EcSf2a-2 strain incapable of forming plaques and of causing fluid secretion in the rabbit ileal loop (29), this attenuating effect may have been overcome in humans by the large inocula used or by the ability of this strain to scavenge para-aminobenzoic acid from extracellular sources, thereby regaining some of its virulence.

Ultimately, the safety and protective efficacy of a Shigella vaccine candidate must be determined by studies of humans. To select promising candidates for human trials, the rhesus monkey model has been extensively used, but the results of
safety studies are sometimes difficult to interpret because of high rates of Shigella infection among monkeys in captivity. Indeed, no adverse reactions attributable to vaccination with either hybrid vaccine were observed among monkeys receiving 100 times the dose that produced adverse reactions in humans. On the other hand, a challenge with comparable inocula of S. flexneri 2a produced shigellosis in most unvaccinated monkeys, suggesting that the hybrid vaccines were attenuated compared with virulent shigellae. As a model of vaccine efficacy, the monkey studies correctly predicted that partial protection against shigellosis could be achieved.

In the first of the two human trials with EcSF2a-2, the vaccine conferred modest protection against challenge with S. flexneri 2a. The 66% protection rate against watery diarrhea and 80% protection rate against dysentery are similar to the 64% level of protection provided by shigellosis itself in volunteer challenge studies with S. flexneri and the 50 to 60% level of protection in volunteers conferred by oral vaccines (5), which provided more than 80% protection in the field (25, 27). However, when the study was repeated with a second cohort of volunteers, the vaccine lacked efficacy. The differences in study outcomes could not be explained by changes in the relevant properties of the vaccine as measured by in vitro assays (colony counts, Congo red binding, and HeLa cell invasion) or by diminished immunogenicity in humans later challenged with virulent Shigella organisms (Table 1). It is notable, however, that the anti-LPS ASC response was significantly greater in the first study than in the second study. When this comparison was limited to vaccinees who were later challenged, the trend persisted, although the differences were no longer statistically significant. The ASC response to vaccination has been shown to correlate with protective efficacy in other oral vaccine trials (16).

Because of the limited protective efficacy of this construct in humans, it would be undesirable to reduce the dose to diminish reactogenicity. Thus, a potentially important observation is that the vaccine was well tolerated when given in inocula that did not exceed 2.1 \times 10^7 CFU. Dose-dependent reactions also occurred with the streptomycin-dependent S. flexneri 2a vaccines (19, 24). The reactions occurred most frequently after the first dose of vaccine and were minimized by decreasing the inoculum in the first dose and giving increasing inocula in subsequent doses. A similar strategy may be used in future trials involving EcSF2a-2. Furthermore, the closely spaced dosing regimens used in our studies may not be optimal. The schedule for immunization on days 0, 3, and 6 was selected because a similar schedule was effective with early lots of 5076-1C (1) and Ty21a live typhoid vaccine (8) and because an accelerated schedule has advantages for rapid immunization, such as that of travelers. However, for a Shigella vaccine, increasing the interval between doses may enhance the protective efficacy. In the guinea pig keratoconjunctivitis model, a threefold increase in the protective efficacy of the vaccine occurred by using a four-dose regimen with vaccination on days 0, 2, 14, and 15 rather than a three-dose regimen with vaccination on days 0, 2, and 4 (13). These observations provide the rationale for conducting further studies of EcSF2a-2 with humans with dosing regimens spaced to provide a secondary immune response and designed to diminish vaccine reactogenicity.

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REFERENCES

15. Institute of Medicine. 1986. The prospects for immunizing...


