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Safety, Immunogenicity, and Transmissibility in Humans of CVD 1203, a Live Oral *Shigella flexneri* 2a Vaccine Candidate Attenuated by Deletions in *aroA* and *virG*

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We evaluated the safety and immunogenicity of attenuated *Shigella flexneri* 2a vaccine candidate CVD 1203, which harbors precise deletions in the plasmid gene *virG* and in the chromosomal gene *aroA*. CVD 1203 invades epithelial cells but undergoes minimal intracellular proliferation and cell-to-cell spread. Fasting healthy volunteers, aged 18 to 40 years, were randomly allocated (double-blind design) to receive either CVD 1203 vaccine or placebo, along with sodium bicarbonate buffer, on days 0 and 14, as follows. At the time of the first inoculation, 10 subjects received placebo (group 1) and 22 subjects received either 1.5×10^8 (group 2; 11 subjects) or 1.5×10^9 (group 3; 11 subjects) CFU of CVD 1203. Fourteen days later, subjects from group 1 received 1.2×10^6 CFU of CVD 1203 and subjects from groups 2 and 3 received 1.2×10^8 vaccine organisms. Clinical tolerance was dose dependent. After a single dose of CVD 1203 at 10^6, 10^8, or 10^9 CFU, self-limited (<48-h duration) objective reactogenicity (fever, diarrhea, or dysentery) developed in 0, 18, and 72% of subjects, respectively, and in no placebo recipients. CVD 1203 induced immunoglobulin G seroconversion to *S. flexneri* 2a lipopolysaccharide (LPS) in 30, 45, and 36% of subjects from groups 1, 2, and 3, respectively, and stimulated immunoglobulin A-producing anti-LPS antibody-secreting cells in 60, 91, and 100% of subjects, respectively. After vaccination, significant rises in tumor necrosis factor alpha concentration in serum (groups 1, 2, and 3) and stool (group 2) samples were observed. We conclude that engineered deletions in *virG* and *aroA* markedly attenuate wild-type *S. flexneri* but preserve immunogenicity; however, less reactogenic vaccines are needed.

The potential public health benefits of controlling shigellosis with immunization have long been appreciated. Prevention of infection requires improvements in sanitation and hygiene that are not feasible in many less developed areas of the world. Widespread occurrence of multiresistant strains, especially of *Shigella dysenteriae* type 1 in Africa and the Indian subcontinent, threatens the availability of a simple, safe, affordable treatment (34, 38) and renders the need for a *Shigella* vaccine even more urgent. However, despite intensive efforts, development of a well-tolerated, genetically stable, effective vaccine capable of inducing durable immunity in humans has not yet been achieved (3, 7, 10, 15, 16, 20, 24, 26).

By applying knowledge of *Shigella* pathogenesis, recombinant genetic techniques allow the construction of *Shigella* vaccine strains which express critical antigens in their native form but harbor specific mutations that cripple undesirable pathologic processes. CVD 1203 is a new *Shigella flexneri* 2a vaccine candidate attenuated with precise deletions in the chromosomal gene *aroA* and in the plasmid gene *virG*, also referred to as *icsA* (30). *aroA* is a gene of the aromatic amino acid biosynthesis pathway that is necessary for the synthesis of *para*-aminobenzoic acid (PABA), a metabolite that is not available within human cells in sufficient amounts to sustain bacterial intracellular proliferation (21, 36). Reports of Lindberg and colleagues indicate that inactivation of the *aro* pathway partially attenuates *S. flexneri* (13, 21). The 120-kDa protein encoded by *virG* mediates the process by which *Shigella* spreads to adjacent cells by using directed intracellular movements via reorganization of the host cell cytoskeleton (2). The resultant live, Δ*aroA* Δ*virG* double-mutant *Shigella* vaccine candidate, CVD 1203, invades epithelial cells but undergoes minimal intracellular proliferation and no cell-to-cell spread (30). Here we report the results of a clinical trial to evaluate the safety, immunogenicity, and transmissibility of CVD 1203 in adult volunteers.

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MATERIALS AND METHODS

Subject selection. Healthy young adults 18 to 40 years of age were recruited from the Baltimore-Washington community to participate in this study. Subjects were excluded from participation if any of the following criteria were met: (i) acute or chronic medical or psychiatric illness detected by medical history, physical examination, psychological interview, and a panel of screening clinical laboratory tests; (ii) known allergy to ciprofloxacin or sulfa drugs; and (iii) HLA-B27 haplotype (which is associated with development of reactive arthritis after *Shigella* infection) (14). Informed, written consent was obtained according to the guidelines of the Institutional Review Board of the University of Maryland at Baltimore.

Study design. Thirty-two volunteers were admitted to the Center for Vaccine Development's Research Isolation Ward and, in a double-blind fashion, were randomly allocated to one of three groups to receive a two-dose regimen of either vaccine or placebo on days 0 and 14 (Table 1). On day 0, 10 subjects received placebo (group 1) and 22 subjects received CVD 1203 vaccine at a dose of either 1.5×10^8 (group 2; 11 subjects) or 1.5×10^9 (group 3; 11 subjects) CFU. The starting doses were selected on the basis of the reported experience with the vaccine candidate SFL1070, a single (*aroD*)-deletion mutant of *S. flexneri* 2457T

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TABLE 1. Clinical response after inoculation with CVD 1203 or placebo[a]

Group	No. of subjects	Response to dose 1 (day 0)					Response to dose 2 (day 14)				
		Inoculum (CFU)	No. of subjects (%)				Inoculum (CFU)	No. of subjects (%)			
			Any illness	Fever	Diarrhea	Dysentery		Any illness	Fever	Diarrhea	Dysentery
1	10	Placebo	0[b,d]	0	0	0	1.2×10^6	0	0	0	0
2	11	1.5×10^8	2 (18)[c,d]	1 (9)	2 (18)	1 (9)	1.2×10^8	4 (36)	0	4 (36)	1 (9)
3	11	1.5×10^9	8 (72)[b,c]	7 (64)	3 (27)	3 (27)	1.2×10^8	1 (9)	1 (9)	1 (9)	0

[a] A three-way comparison (Bonferroni corrected; significance, $P < 0.017$) was performed to determine whether there was a dose response in the occurrence of any illness after the first dose of vaccine.
[b] Group 1 versus group 3, $P < 0.001$ (Fisher's exact test, one tailed).
[c] Group 2 versus group 3, $P = 0.015$ (Fisher's exact test, one tailed).
[d] Group 1 versus group 2, $P = 0.26$ (Fisher's exact test, one tailed).

(21). At a dose of 10^8 CFU, SFL1070 was considered to be well tolerated, while at 10^9 CFU, four of nine subjects developed symptoms, including cramps, diarrhea, and fever. We anticipated that the double mutation in CVD 1203 would eliminate the symptoms observed with SFL1070 at these dose levels.

On day 14, all subjects were scheduled to receive an inoculation of 10^9 CFU of CVD 1203. However, because of the reactogenicity observed after the first inoculation, the following changes were made to the protocol by investigators who remained blinded to the volunteers' group assignments. Subjects from group 1 received CVD 1203 at a dose of 1.2×10^6 CFU, and vaccine recipients from groups 2 and 3 were inoculated with CVD 1203 at a dose of 1.2×10^8 CFU. Observations with the streptomycin-dependent *S. flexneri* 2a vaccines that dose-dependent reactions occurred most frequently after the first dose of vaccine even when increasing inocula were given in subsequent doses led us to believe that a dose of 10^8 CFU would be better tolerated in the second inoculum than in the first (19, 25).

The inoculum was administered with 2 g of sodium bicarbonate buffer to volunteers who fasted for 90 min before and after inoculation, as previously described (15). Replicate colony counts were performed before and after vaccination to determine the actual inoculum of test strain ingested.

During their 21-day stay in the isolation ward, the volunteers underwent careful clinical assessment by observers who were blinded to their group assignments. This included the measurement of oral temperature every 6 h and daily evaluation by a physician. Every stool passed was cultured, examined for blood, and graded for consistency by using a standardized scale (18); diarrheal stools were weighed to estimate volume. Each subject was interviewed daily to elicit the occurrence of constitutional symptoms. Headache and cramps were graded for severity on a four point scale as follows: 0, absent; 1, scarcely noticed or easily noticed but could continue activity; 2, cannot take mind off symptom but continued same activity; 3, gone to bed but able to read, watch television, or other activity; 4, gone to bed, with no activity. To eradicate excretion before discharge from the ward, those who were shedding vaccine on day 20 received ciprofloxacin, 500 mg by mouth every 12 h, for 5 days.

Illness after vaccination was defined as the presence of either diarrhea (two or more loose [grade 3 to 5] stools totaling 200 ml in 48 h or one loose stool totaling 300 ml), dysentery (gross blood in a loose stool), or fever (defined as an oral temperature of 100°F or higher measured at two readings 5 min apart). This analysis includes events which occurred during the 5 days after each inoculation.

Laboratory evaluation. (i) ASC. To assess the vaccine's ability to prime the mucosal immune system, we performed ELISPOT before vaccination and on days 7, 10, 14, and 21 after vaccination to enumerate immunoglobulin A (IgA) antibody-secreting cells (ASC) circulating in the peripheral blood that recognize *S. flexneri* 2a lipopolysaccharide (LPS) and invasion plasmid antigens (IPA) (39). A count that was ≥3 standard deviations (SDs) above the mean prevaccination ASC count for all subjects was considered to be a positive response.

(ii) Serum antibody. The IgG, IgA, and IgM responses to LPS and IPA were measured in serum samples before vaccination and on days 14, 21, 28, and 42 after vaccination by enzyme-linked immunosorbent assay (ELISA) using previously described methods (3, 31). Seroconversion was defined as a fourfold rise in titer after vaccination.

(iii) Fecal IgA. A stool specimen was collected before vaccination and on days 7, 14, 21, 28, and 42 after vaccination for measurement of total and antigen-specific (LPS and IPA) IgA by previously described methods (22). Similarly prepared stool specimens from a negative control population (North American infants, aged 6 months to 2 years, who participated in rotavirus vaccine trials) were tested to define the minimum value, in optical density (OD) units, indicating the presence of specific antibody. The mean plus 2 SDs above the mean for 20 stool samples run at a total IgA concentration of 20 mg/100 ml against the *Shigella* antigens resulted in a cutoff OD unit of 0.2 for both antigens. For each volunteer specimen, the antigen-specific titer was the highest dilution that demonstrated a net OD of ≥0.2.

All specimens from volunteers in this study were assayed by using an initial total IgA concentration of at least 2 mg/100 ml. Any specimen containing IgA below this concentration without a positive absorbance was considered to be an insufficient sample. All results were corrected back to a stool concentration of 20 mg of total IgA per 100 ml. A response was defined as a fourfold rise in antibody after vaccination.

(iv) Bacteriology. The duration of vaccine excretion was measured by culturing all stools from the time of admission until discharge and weekly thereafter for 3 weeks. A swab of the stool was inoculated into buffered glycerol saline and maintained at 4°C until transport to the laboratory. A rectal swab (inoculated into gram-negative broth [Becton Dickinson, Cockeysville, Md.]) was collected from any volunteer who failed to produce a stool in a 24-h period. Swabs and stools were cultivated on *Salmonella-Shigella*, MacConkey's, and XLD enteric media (Becton Dickinson) (plated on solid media either directly or after incubation in gram-negative enrichment broth) to identify lactose-negative colonies that were verified for agglutination by *Shigella* group B antiserum (Difco Laboratories, Detroit, Mich.), as previously described (17).

TNF-α determinations for serum and stool samples. Fresh stool specimens collected in sterile containers before vaccination and on days 2, 4, 7, 14, 21, and 28 after vaccination were immediately diluted in phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (0.5 g of stool graded 1 or 2 or 0.5 ml of stool graded 3 to 5 in 5 ml of PBS) and centrifuged at 20,000 × *g* for 20 min. Supernatant fluids were collected and kept frozen at −70°C until processed. Serum specimens were collected before vaccination and on days 1, 2, 3, 14, 15, 16, and 17 after vaccination and kept frozen at −70°C until processed. Serum and stool tumor necrosis factor alpha (TNF-α) levels were measured in duplicate wells with a commercial sandwich enzyme immunoassay (Predicta TNF-α kit; Genzyme Diagnostics, Cambridge, Mass.) (level of sensitivity, 10 pg/ml). TNF responses were defined as follows. We calculated the mean and SD of the difference between the two prevaccination replicate runs for all subjects. For each subject, we derived a net TNF OD for each of the postvaccination days selected for sampling by subtracting the prevaccination mean of replicates from the mean OD measured on each postvaccination day. Net ODs that were >3 SDs above the mean of differences between prevaccination replicates were considered to be responses.

Preparation of vaccine and placebo. CVD 1203 was constructed by Noriega and coworkers by double homologous recombination using suicide plasmid deletion cassettes containing Δ*aroA* and Δ*virG* alleles and exchanged for the wild-type genes in *S. flexneri* 2a strain 2457T (30). Inocula were derived from a frozen master seed stock and plated onto Trypticase soy agar (Becton Dickinson) containing Congo red dye (0.01%; Sigma Chemical Co., St. Louis, Mo.) and 0.2 g of PABA per liter. After incubation at 37°C for 18 to 24 h, single, isolated Congo red colonies that exhibited characteristic *Shigella* morphology were confirmed as *S. flexneri* with specific antisera. Then additional well-isolated Congo red colonies were picked and suspended in sterile saline. For heavy growth, the saline suspension was used to inoculate PABA-supplemented Trypticase soy agar plates which were incubated overnight at 37°C. Overnight growth from the Trypticase soy agar plates was harvested into sterile PBS (pH 7.4). The heavy bacterial suspension was diluted with additional sterile PBS to produce a suspension with an OD at 660 nm corresponding to the desired bacterial count per milliliter. The placebo consisted of sterile broth to which powdered milk was added to match the turbidity of the vaccine inoculum.

Statistical methods. Associations between dichotomous variables were analyzed by Fisher's exact test. Geometric means of data that included zeroes were calculated by adding 1 before log transformation (35). Correlations between immune responses and selected variables were measured by using Spearman's test. Comparisons which involved paired responses were analyzed by using Wilcoxon's signed-ranks test or Student's *t* test, as appropriate, and unpaired data were compared by using the Kruskal-Wallis test. Two-tailed hypotheses were evaluated unless otherwise stated. Resulting *P* values of <0.05 were considered statistically significant. Bonferroni adjustments were made for two-way comparisons in the analysis of the serum TNF-α response after vaccination and for three-way comparisons in the analysis of the differences in attack rate of clinical

symptoms by dose. *P* values of <0.025 and <0.017 were considered statistically significant for two- and three-way comparisons, respectively; since the question of interest in these analyses was whether vaccination elicited an increase in the frequency or magnitude of the outcome event, one-tailed hypotheses were evaluated.

RESULTS

Clinical response to vaccination. After receiving a single oral dose of vaccine, 10 of 32 subjects experienced objective adverse reactions consisting of either diarrhea, dysentery, or fever (Table 1). The mean incubation period until the first symptom was 20.4 h, with 7 of 10 reactions occurring within 24 h of inoculation. Fever was the predominant symptom (eight subjects). Diarrhea occurred in five subjects, with stool volumes ranging from 205 to 579 ml, and dysentery occurred in four subjects. No illnesses were observed among recipients of either 1.2×10^6 CFU or placebo. The clinical symptoms were self-limited (<48-h duration) and dose dependent, with objective reactogenicity developing in 0, 18, and 72% of subjects after one dose of CVD 1203 at 10^6, 10^8, and 10^9 CFU, respectively. The frequency of objective reactions experienced by groups 1 and 2 was significantly lower than the frequency of reactions experienced by group 3 (Table 1).

After the second (1.2×10^8 CFU) dose of vaccine, four subjects from group 2 and one subject from group 3 manifested adverse events (Table 1). Two of these five volunteers had also experienced reactions after the first dose of vaccine, but those after the second dose were milder. Again the symptoms had a sudden onset and brief duration; however, this time watery diarrhea was the predominant finding (all five subjects). One of the five subjects also fulfilled the criteria for dysentery by having only a single 5-ml loose stool with gross blood. Another subject had diarrhea and fever, but the temperature elevation consisted of a single episode of 100.0°F.

In the analysis of subjective complaints, it was found that headache or cramps sufficiently severe to interfere with daily activities in the isolation ward (grade 3 or 4) were reported by four (40%), three (27%), and seven (64%) subjects from groups 1, 2, and 3, respectively, after a single dose of vaccine and by one (10%) placebo recipient. After the second dose of vaccine, three (27%) group 2 subjects and no subjects from group 3 experienced these symptoms.

Shedding of vaccine strain. Vaccine excretion was detected after a single dose of vaccine in stool specimens from 8 of 10 subjects from group 1 (80%), all 11 members of group 2 (100%), and 10 of 11 volunteers belonging to group 3 (91%). No excretion was detected after the ingestion of placebo. The median duration of shedding after a single dose was 5 days, but excretion persisted for as long as 13 days in one volunteer. There was no correlation between the duration of vaccine excretion after the first inoculation and either the vaccine inoculum, the geometric mean day 7 IgA ASC response, or the peak geometric mean IgG titer after the first dose of vaccine among subjects in groups 2 and 3. Twenty-one of 22 subjects (96%) who received the booster dose of 1.2×10^8 CFU of CVD 1203 excreted vaccine.

Immune response to vaccination. (i) ASC response. Since the ASC responses invariably returned to baseline by 14 days postvaccination, it was possible to evaluate the response to the first and second doses of vaccine separately. After only a single dose, CVD 1203 elicited an IgA ASC response to *S. flexneri* 2a LPS antigen (defined as greater than three ASC per 10^6 peripheral blood mononuclear cells [PBMC]) in 6 (60%), 10 (91%), and 11 (100%) subjects from groups 1, 2, and 3, respectively (Table 2). An IgA response to IPA (defined as greater than or equal to six ASC per 10^6 PBMC) was detected in 4 volunteers from group 1 (40%), 7 volunteers from group 2 (64%), and 10 volunteers from group 3 (91%). Responses were seen in both IgA1 and IgA2 subclasses (Table 3).

TABLE 2. IgA ASC response to *S. flexneri* 2a LPS and IPA after vaccination with CVD 1203 on days 0 and 14[a]

IgA response	Day	Group[b]	No. of subjects with response (%)	Geometric mean ASC[c]
Anti-LPS	7	1	0	0.3
		2	10 (91)	43 (63)
		3	11 (100)	175 (175)
	21	1	6 (60)	13 (70)
		2	8 (73)	8 (17)[d]
		3	7 (64)	5 (12)[d]
Anti-IPA	7	1	0	0
		2	7 (64)	12 (39)
		3	10 (91)	77 (108)
	21	1	4 (40)	7 (93)
		2	4 (36)	2 (29)
		3	3 (30)	3 (8)

[a] Response was defined as ≥3 SDs above the geometric mean prevaccination level as follows: for LPS, ≥3 ASC; for IPA, ≥6 ASC.
[b] On day 0, group 1 received placebo, group 2 received 1.5×10^8 CFU of CVD 1203, and group 3 received 1.5×10^9 CFU of CVD 1203. On day 14, group 1 received 1.2×10^6 CFU of CVD 1203 and groups 2 and 3 received 1.2×10^8 CFU of CVD 1203.
[c] Per 10^6 PBMC, as measured by ELISPOT. Parenthetical data are for responders only.
[d] $P < 0.05$, Wilcoxon's signed-ranks test, for day 21 versus day 7 geometric mean ASC for the same group and antibody response.

The relationship between vaccine dose and ASC response 7 days after inoculation was examined for total IgA, IgA1, and IgA2 against LPS and IPA. A statistically significant positive correlation was observed by Spearman's test in each instance ($P < 0.05$). There was a significant positive relationship between these ASC responses and the occurrence of adverse clinical reactions after vaccination ($P \leq 0.01$).

The magnitude and frequency of the ASC response after the first inoculation were greater than those after the second (Table 2). However, three subjects with low (<10 ASC per 10^6 PBMC) anti-LPS responses after primary vaccination im-

TABLE 3. IgA subclass ASC response to *S. flexneri* 2a LPS and IPA 7 days after a single dose of CVD 1203 vaccine[a]

IgA subclass	Response to:	Group[b]	No. of subjects with response/total no. of subjects (%)	Geometric mean ASC[c]
IgA1	LPS	1	6/10 (60)	7 (30)
		2	9/10 (90)	20 (28)[d]
		3	10/11 (91)	60 (90)
	IPA	1	6/10 (60)	5 (17)
		2	8/10 (80)	6 (11)
		3	9/10 (90)	29 (43)
IgA2	LPS	1	6/10 (60)	9 (42)
		2	9/10 (90)	30 (45)[d]
		3	10/11 (91)	69 (106)
	IPA	1	5/10 (50)	3 (18)
		2	6/10 (60)	8 (35)
		3	9/10 (90)	39 (60)

[a] Response (defined as ≥3 SDs above the geometric mean prevaccination level) to IgA1 was ≥2 and ≥1 ASC for LPS and IPA, respectively, and response to IgA2 was ≥1 ASC for both LPS and IPA.
[b] See Table 2, footnote *b*.
[c] See Table 2, footnote *c*.
[d] $P = 0.04$, Wilcoxon's signed-ranks test.

TABLE 4. Local and systemic antibody responses to *S. flexneri* 2a LPS and IPA after vaccination with CVD 1203 on days 0 and 14[a]

Response to:	Type of sample and Ig	Group[b]	No. of subjects with response/total no. of subjects (%)	Geometric mean peak titer[c]
LPS	Serum IgA	1	1/10 (10)	29
		2	4/10 (40)	50
		3	4/11 (36)	42
	Serum IgM	1	0/10 (0)	76
		2	0/10 (0)	83
		3	1/11 (9)	100
	Serum IgG	1	3/10 (30)	62
		2	5/11 (45)	107
		3	4/11 (36)	129
	Fecal IgA	1	2/9 (22)	ND[d]
		2	7/10 (70)	ND
		3	5/11 (45)	ND
IPA	Serum IgA	1	1/10 (10)	41
		2	7/10 (70)	113
		3	4/11 (36)	74
	Serum IgM	1	1/10 (10)	18
		2	2/10 (20)	24
		3	1/11 (9)	18
	Serum IgG	1	1/10 (10)	429
		2	7/10 (70)	584
		3	6/11 (54)	483
	Fecal IgA	1	1/9 (11)	ND
		2	4/10 (40)	ND
		3	0/11 (0)	ND

[a] Response was defined as at least a fourfold rise in antibody titer after vaccination.
[b] See Table 2, footnote *b*.
[c] For calculations of geometric means of serum antibody, a value equal to half the starting dilution was used for undetectable titers. The starting dilution was 25 for anti-LPS IgA, anti-LPS IgG, anti-IPA IgA, and anti-IPA IgM; the starting dilution was 100 for anti-LPS IgM; the starting dilution was 200 for anti-IPA IgG.
[d] ND, not done.

proved their responses after the booster dose and three of six subjects who did not respond to IPA after primary vaccination did so after the booster dose. It is of interest that a response to LPS was not seen after either dose of 10^8 CFU of vaccine for one of the two volunteers who experienced reactions after both inoculations. There was no association between the ASC response 7 days after the first vaccine dose and the occurrence of adverse clinical reactions after the booster dose.

(ii) Serologic response. Vaccination elicited a fourfold rise in either IgA, IgM, or IgG antibody to LPS in serum samples from four volunteers from group 1 (40%), seven volunteers from group 2 (64%), and six volunteers from group 3 (55%). A fourfold rise in serum anti-IPA antibody was detected in a total of two subjects from group 1 (20%), nine subjects from group 2 (82%), and six subjects from group 3 (55%). The class-specific responses are shown in Table 4. There was no association between the occurrence of a fourfold rise in serum antibody to LPS or IPA and a positive ASC IgA response to the corresponding antigen.

The anti-LPS response occurred by day 14 after a single dose of vaccine in 14 of 17 seroconverters (82%). The anti-IPA response occurred by day 14 in 10 of 17 seroconverters (59%).

(iii) Fecal IgA response. A fecal anti-LPS IgA response was detected in 2 of 9 group 1 subjects (22%), 7 of 10 group 2 subjects (70%), and 5 of 11 group 3 subjects (45%) (Table 4). An anti-IPA response was detected in 1 of 9 subjects (11%) from group 1, 4 of 10 subjects (40%) from group 2, and no subjects from group 3. There was no significant association between a fecal IgA response and a positive IgA ASC response to either LPS or IPA.

Serum and fecal TNF-α responses to vaccination. A rise in mean serum TNF-α concentration occurred after each inoculation with vaccine but not with placebo (Fig. 1). The peak response was observed 3 days after inoculation and was most pronounced after the second dose. The difference between the day 0 and mean peak serum TNF-α concentrations reached statistical significance for all groups after a single dose of vaccine but not after placebo administration. The difference between the day 0 and mean peak serum TNF-α concentrations after the second vaccination was highly significant ($P < 0.001$) for groups 2 and 3. Overall, a serum TNF-α response was observed in 70, 91, and 100% of subjects from groups 1, 2, and 3, respectively; a response was detected after only a single dose of vaccine in 70, 46, and 64% of subjects from groups 1, 2, and 3, respectively.

A rise in mean stool TNF-α concentration was detected 2 to 4 days after a single dose of vaccine (Fig. 2). No response was detected after the second dose; however, the earliest measurement was not obtained until 7 days after the second dose. The difference between the day 0 and mean peak stool TNF-α concentrations after a single dose of vaccine reached statistical significance for group 2 ($P = 0.03$) and approached significance for group 3 ($P = 0.08$). Overall, a single dose of vaccine elicited a fecal TNF-α response in 0, 27, and 18% of subjects from groups 1, 2, and 3, respectively.

There was no correlation detected between the peak serum or stool TNF-α OD and either the occurrence of objective reactogenicity, the peak IgA ASC response to LPS or IPA, or the peak serum titer of anti-LPS IgA or IgG.

DISCUSSION

These data provide an important basis for further efforts to develop a promising live oral attenuated *Shigella* vaccine. We have previously shown that an oral dose of 10^3 CFU of wild-type *S. flexneri* 2a administered with buffer induces full-blown clinical illness (fever, diarrhea, and/or dysentery) in 85 to 90% of volunteers after oral challenge (17). In contrast, in this study we observed that a 1,000-fold-higher (10^6 CFU) oral dose of CVD 1203 caused no objective adverse clinical responses; the adverse reactions seen when much higher doses of vaccine were administered (10^8 or 10^9 CFU) were clearly milder and of shorter duration than the illnesses observed with wild-type *S. flexneri* 2a. Furthermore, a clear-cut dose response was detected, with objective reactions developing in 0, 18, and 72% of subjects after primary inoculation with 1.2×10^6 CFU, 1.5×10^8 CFU, and 1.5×10^9 CFU of CVD 1203, respectively. At the lowest dose, reactogenicity was limited to nonspecific constitutional symptoms (cramps and headache). The importance of these symptoms is difficult to interpret in the setting of an inpatient isolation ward that lacks many of the stimulating and distracting events which occur in a natural environment. Nonetheless, these findings suggest that research must continue to construct vaccine strains that are inherently further attenuated.

The onset of illness after challenge with high doses of vaccine was abrupt (mean, 20.4 h) compared with that (44.6 h) after challenge with 1,000- to 10,000-fold-lower doses of virulent *S. flexneri* 2a (unpublished observations), suggesting that the initial process of intestinal invasion by large numbers of vaccine organisms may have been responsible for the symptoms. This temporal pattern of illness observed in volunteers is reminiscent of the response of CVD 1203 in the Sereny test, in which 4 of 11 guinea pigs developed short-lived mild conjunctival inflammation at 24 h that resolved spontaneously by the

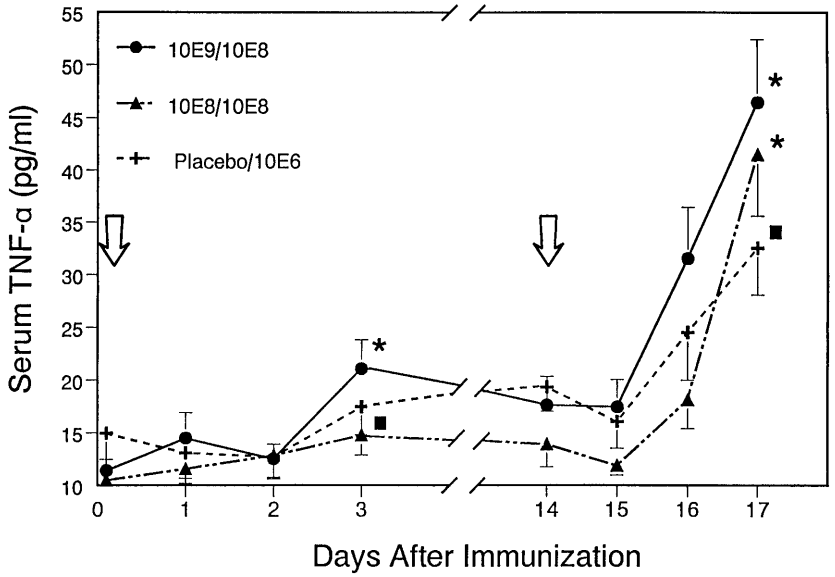

FIG. 1. Serum TNF-α levels after immunization with *S. flexneri* 2a strain CVD 1203. Volunteers were immunized orally with CVD 1203 at various doses (10^9 organisms on day 0 followed by 10^8 organisms on day 14 [10E9/10E8; group 3], 10^8 organisms on day 0 followed by 10^8 organisms on day 14 [10E8/10E8; group 2], and placebo on day 0 followed by 10^6 organisms on day 14 [Placebo/10E6; group 1]). Arrows denote the days of vaccination. The levels of TNF-α in serum samples were measured at the indicated times after vaccination by ELISA. Data are means ± standard errors. *, $P < 0.001$; ■, $P < 0.02$.

next day, in contrast to the progressive inflammation that followed wild-type inoculation (30). The self-limited natures of these illnesses suggest that the duration and extent of intracellular injury were limited by the *aroA* and *virG* deletions.

The ability of CVD 1203 to colonize the intestine for a prolonged period, up to 13 days in one subject, is a surprising occurrence for an auxotrophic mutant. Spontaneous repair of the large chromosomal *aroA* deletion would require bacterial conjugation and would be an unlikely event. It is possible that vaccine organisms were able to scavenge sufficient PABA from the intraluminal contents to sustain growth at the mucosal surface but were prevented from inducing further cellular injury or clinical illness. The addition of minute quantities of PABA restored the normal growth rate of another vaccine candidate with aromatic auxotrophy, strain CVD 906, an Δ*aroC* Δ*aroD* derivative of *Salmonella typhi* (11). Despite active colonization, transmission of CVD 1203 to placebo recipients did not occur in the closed setting provided by the ward.

It is notable that 80% of the adverse clinical reactions that followed the second inoculation occurred in the group that had received two doses of 10^8 CFU, suggesting that the recipients of 10^9 CFU may have benefitted from greater protective im-

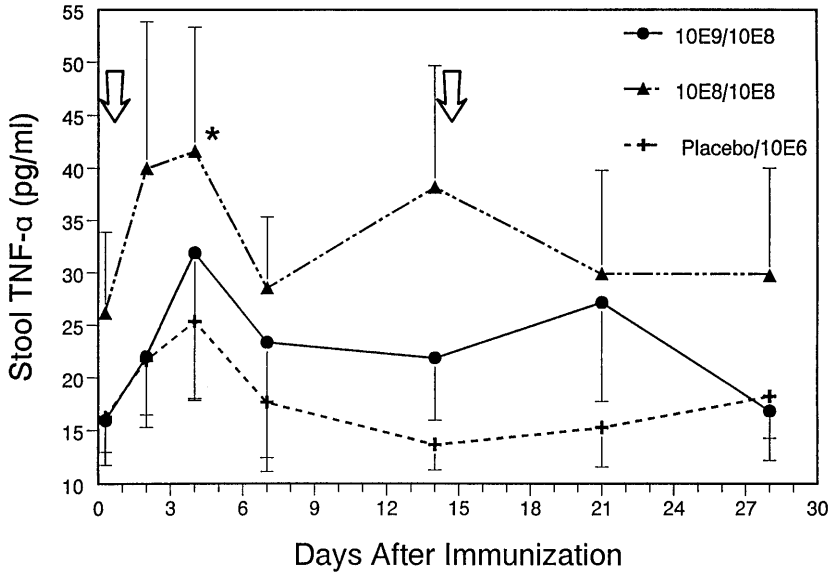

FIG. 2. Stool TNF-α levels after immunization with *S. flexneri* 2a strain CVD 1203. Volunteers were immunized orally with CVD 1203 at various doses (10^9 organisms on day 0 followed by 10^8 organisms on day 14 [10E9/10E8; group 3], 10^8 organisms on day 0 followed by 10^8 organisms on day 14 [10E8/10E8; group 2], and placebo on day 0 followed by 10^6 organisms on day 14 [Placebo/10E6; group 1]). Arrows denote the days of vaccination. The levels of TNF-α in stool samples were measured at the indicated times after vaccination by ELISA. Data are means ± standard errors. *, $P < 0.05$.

munity against illness resulting from the booster dose. One component of the vaccine's reactogenicity, which was especially apparent during the more modified clinical responses that followed the booster dose, was its ability to produce watery diarrhea. A recently discovered chromosomal operon and a plasmid gene, encoding *Shigella* enterotoxins ShET1 (8) and ShET2 (27), respectively, are thought to be involved in the pathogenesis of the watery diarrhea that often precedes *Shigella* dysentery and is sometimes the only clinical manifestation of shigellosis. The deletion of genes involved in the production of these enterotoxins may therefore provide additional attenuation.

Overall, CVD 1203 elicited vigorous local (circulating IgA ASC and fecal IgA) and systemic (serum antibody) immune responses that were dose dependent. The immunologic determinants of protection against shigellosis remain contentious; the same responses that have been associated with protection are also correlated with development of clinical illness after vaccination or challenge (6, 16). Nonetheless, the strength of the anti-LPS IgA ASC response 7 days after vaccination has been correlated with protective efficacy against experimental challenge with virulent *Shigella* organisms (16) and preexposure serum anti-LPS levels have been significantly associated with immunity to shigellosis after both natural (4) and experimental (3) challenge. In these measures, the immunogenicity of CVD 1203 at 10^9 CFU was comparable to that seen after wild-type challenge with 10^3 CFU of *S. flexneri* 2a, which induces 70% serotype-specific protective immunity (17). Even in the lowest-dose group (10^6 CFU), an IgA anti-LPS ASC response occurred in the majority of subjects (60%), although the day 7 geometric mean ASC count was significantly lower than those for the higher-dose groups. Our finding that additional responders could be recruited among subjects who received a second dose of CVD 1203 suggests that it is possible to enhance the immune response to lower, better-tolerated dosage levels of this vaccine by administering two or three oral inoculations. Furthermore, the presence of a vigorous ASC response to both IgA1 and IgA2 antibody subclasses suggests that CVD 1203 is capable of inducing responses in various compartments of the mucosal immune system, a desirable characteristic for a vaccine construct that can be used as a carrier for expressing foreign antigens (23, 29).

In an attempt to identify laboratory correlates of reactogenicity and protective immunity that could be measured in animal models as predictors of the human response, we examined TNF-α in serum and stool samples after vaccination. TNF-α is a potent mediator of inflammatory and immune responses produced upon stimulation by monocytes/macrophages and other cell types, including T- and B-lymphocytes and NK cells (9, 37). LPS and other microbial products are believed to be the main stimuli for TNF-α production in vivo, but other cytokines, as well as antibodies and complement products, may also play an important role (9, 37). TNF-α acts by modulating the production of many cytokines, including those responsible for the clinical manifestations associated with bacterial invasion and septic shock syndrome, and by regulating the growth, differentiation, and function of numerous cell types, including endothelial cells, colonic epithelial cells, and cells in the hypothalamic centers that regulate body temperature (9, 37).

The exposure of human epithelial cells in vivo to invasive bacteria such as *S. dysenteriae* markedly increases the secretion of several proinflammatory cytokines, including TNF-α (12). The levels of TNF-α in stool samples and detection of TNF-α-producing cells in rectal biopsy have been correlated with clinical severity of acute shigellosis, degree of inflammation on histopathology, and local increases in other proinflammatory cytokines (28, 32, 33), but not with the occurrence of extraintestinal complications such as hemolytic uremic syndrome and leukemoid reaction (1, 5). In contrast, serum TNF-α levels have been inconsistently elevated during shigellosis (5, 33). Heretofore, no studies have explored TNF-α production at the local and systemic levels after oral vaccination of volunteers with attenuated or wild-type *Shigella* strains, a model amenable to plotting the precise kinetics of TNF-α production in relation to the inciting exposure. We detected significant rises in serum and stool TNF-α concentrations after vaccination, with the suggestion of a booster response in serum samples after the second dose. The TNF-α response in stool samples appeared to precede the serum response by approximately 1 day, suggesting that the TNF-α elevation in serum samples may have been derived from local intestinal production. Although these responses did not correlate with either the clinical or antibody response to vaccination, in this setting TNF-α may contribute to subclinical intestinal inflammation and may act alone or through modulation of the production of other molecules in the cytokine cascade to induce macrophage activation or other immunological responses that favor elimination of the infecting strain.

The clinical response to CVD 1203 can be broadly compared with the response of Swedish volunteers to SFL1070, an Δ*aroD* *S. flexneri* 2a vaccine candidate derived from the same 2457T parent strain as CVD 1203 (21). Both vaccines caused dose-dependent responses in clinical tolerance, with self-limited objective gastrointestinal manifestations occurring at high doses (10^9 CFU). Both elicited specific immune responses to *Shigella* antigens in most subjects. It is of interest that in guinea pigs, Δ*virG* added to the attenuation of Δ*aroA* (30).

We conclude that *aroA virG* deletions markedly attenuate wild-type *S. flexneri* 2a but preserve immunogenicity. These observations constitute progress toward developing a safe and effective *Shigella* vaccine. Future investigations will explore further attenuated recombinant mutant strains.

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REFERENCES

1. **Azim, T., R. C. Halder, M. S. Sarker, S. Ahmed, J. Hamadani, A. Chowdhury, F. Quadri, M. A. Salam, R. B. Sack, and M. J. Albert.** 1995. Cytokines in the stools of children with complicated shigellosis. Clin. Diagn. Lab. Immunol. **2:**492–495.
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. **Black, R. E., M. M. Levine, M. L. Clements, G. Losonsky, D. Herrington, S. Berman, and S. Formal.** 1987. Prevention of shigellosis by a *Salmonella typhi-Shigella sonnei* bivalent vaccine. J. Infect. Dis. **155:**1260–1265.
4. **Cohen, D., M. S. Green, C. Block, R. Slepon, and I. Ofek.** 1991. Prospective study of the association between serum antibodies to lipopolysaccharide O antigen and the attack rate of shigellosis. J. Clin. Microbiol. **29:**386–389.
5. **de Silva, D. G. H., L. N. Mendis, N. Sheron, G. J. M. Alexander, D. C. A. Candy, H. Chart, and B. Rowe.** 1993. Concentrations of interleukin 6 and tumour necrosis factor in serum and stool of children with *Shigella dysenteriae* 1 infection. Gut **34:**194–198.
6. **DuPont, H. L., R. B. Hornick, A. T. Dawkins, M. J. Snyder, and S. B. Formal.** 1969. The response of man to virulent *Shigella flexneri* 2a. J. Infect. Dis. **119:**296–299.

7. **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.
8. **Fasano, A., F. R. Noriega, D. R. Maneval, S. Chanasongcram, R. Russell, S. Guandalini, and M. M. Levine.** 1995. *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro. J. Clin. Invest. **95:**2853–2861.
9. **Fong, Y., and S. F. Lowry.** 1990. Tumor necrosis factor in the pathophysiology of infection and sepsis. Clin. Immunol. Immunopathol. **55:**157–170. (Review.)
10. **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.
11. **Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine.** 1992. Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. J. Clin. Invest. **90:**412–420.
12. **Jung, H. C., L. Eckmann, S. K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M. F. Kagnoff.** 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J. Clin. Invest. **95:**55–65.
13. **Kärnell, A., A. Li, C. R. Zhao, 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.
14. **Keat, A.** 1983. Reiter's syndrome and reactive arthritis in perspective. N. Engl. J. Med. **26:**1606–1614.
15. **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.
16. **Kotloff, K. L., G. A. 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 adults of four doses of live oral hybrid *Escherichia coli-Shigella flexneri* 2a vaccine strain EcSf2a-2. Vaccine **13:**495–502.
17. **Kotloff, K. L., J. P. Nataro, G. A. Losonsky, S. S. Wasserman, T. L. Hale, D. N. Taylor, J. C. Sadoff, and M. M. 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.
18. **Levine, M. M., R. E. Black, M. L. Clements, C. Lanata, S. Sears, T. Honda, C. R. Young, and R. A. Finkelstein.** 1984. Evaluation in humans of attenuated *Vibrio cholerae* El Tor Ogawa strain Texas Star-SR as a live oral vaccine. Infect. Immun. **43:**515–522.
19. **Levine, M. M., H. I. DuPont, E. J. Gangarosa, R. B. Hornick, M. J. Snyder, J. P. Libonati, K. Glaser, and S. B. Formal.** 1972. Shigellosis in custodial institutions. II. Clinical, immunologic and bacteriologic response of institutionalized children to oral attenuated *Shigella* vaccines. Am. J. Epidemiol. **96:**40–49.
20. **Levine, M. M., W. E. Woodward, S. B. Formal, P. Gemski, H. L. DuPont, R. B. Hornick, and M. J. Snyder.** 1977. Studies with a new generation of oral attenuated *Shigella* vaccine: *Escherichia coli* bearing surface antigens of *Shigella flexneri*. J. Infect. Dis. **136:**577–582.
21. **Lindberg, A. A., A. Kärnell, T. Pál, H. Sweiha, K. Hultenby, and B. A. D. Stocker.** 1990. Construction of an auxotrophic *Shigella flexneri* strain for use as a live vaccine. Microb. Pathog. **8:**433–440.
22. **Losonsky, G. A., M. B. Rennels, Y. Lim, G. Krall, A. Z. Kapikian, and M. M. Levine.** 1988. Systemic and mucosal immune responses to rhesus rotavirus vaccine MMU 18006. Pediatr. Infect. Dis. J. **7:**388–393.
23. **McGhee, J. R., and H. Kiyona.** 1993. New perspectives in vaccine development: mucosal immunity to infections. Infect. Agents Dis. **2:**55–73.
24. **Meitert, T., E. Pencu, L. Ciudin, and M. Tonciu.** 1984. Vaccine strain *Shigella flexneri* T_{32}-ISTRATI. Studies in animals and in volunteers. Antidysentery immunoprophylaxis and immunotherapy by live vaccine Vadizen (*Sh. flexneri* T_{32}-ISTRATI). Arch. Roum. Pathol. Exp. Microbiol. **43:**251–278.
25. **Mel, D., E. J. Gangarosa, M. L. Radovanovic, B. L. Arsic, and S. Litvinjenko.** 1971. Studies on vaccination against bacillary dysentery. 6. Protection of children by oral immunization with streptomycin-dependent *Shigella* strains. Bull. W.H.O. **45:**457–464.
26. **Mel, D. M., A. L. Terzin, and L. Vuksic.** 1965. Studies on vaccination against bacillary dysentery. 3. Effective oral immunization against *Shigella flexneri* 2a in a field trial. Bull. W.H.O. **32:**647–655.
27. **Nataro, J. P., J. Seriwatana, A. Fasano, D. R. Maneval, L. D. Guers, F. Noriega, F. Dubovsky, M. M. Levine, and J. G. Morris, Jr.** 1995. Identification and cloning of a novel plasmid-encoded enterotoxin in enteroinvasive *Escherichia coli* and *Shigella* strains. Infect. Immun. **63:**4721–4728.
28. **Nicholls, S., S. Stephens, C. P. Braegger, J. A. Walker-Smith, and T. T. MacDonald.** 1993. Cytokines in stools of children with inflammatory bowel disease or infective diarrhoea. J. Clin. Pathol. **46:**757–760.
29. **Noriega, F. R., G. Losonsky, J. Y. Wang, S. B. Formal, and M. M. Levine.** 1996. Further characterization of Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203 as a mucosal *Shigella* vaccine and as a live-vector vaccine for delivering antigens of enterotoxigenic *Escherichia coli*. Infect. Immun. **64:** 23–27.
30. **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.
31. **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.
32. **Raqib, R., A. A. Lindberg, B. Wretlind, P. K. Bardhan, U. Andersson, and J. Andersson.** 1995. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect. Immun. **63:**289–296.
33. **Raqib, R., B. Wretlind, J. Anderson, and A. A. Lindberg.** 1995. Cytokine secretion in acute shigellosis is correlated with disease activity and directed more to stool than to plasma. J. Infect. Dis. **171:**376–384.
34. **Shahid, N. S., M. M. Rahaman, K. Haider, H. Banu, and N. Rahman.** 1985. Changing pattern of resistant Shiga bacillus (*Shigella dysenteriae* type 1) and *Shigella flexneri* in Bangladesh. J. Infect. Dis. **152:**1114–1119.
35. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry. The principles and practice of statistics in biological research. W. H. Freeman and Co., San Francisco.
36. **Stocker, B. A. D.** 1988. Auxotrophic *Salmonella typhi* as a live vaccine. Vaccine **6:**141–145.
37. **Tracey, K. J.** 1994. Tumor necrosis factor-alpha, p. 289–304. *In* A. W. Thomson (ed.), The cytokine handbook, 2nd ed. Academic Press, New York.
38. **Tuttle, J., A. A. Ries, R. M. Chimba, C. U. Perera, N. H. Bean, and P. M. Griffin.** 1995. Antimicrobial-resistant epidemic *Shigella dysenteriae* type 1 in Zambia: modes of transmission. J. Infect. Dis. **171:**371–375.
39. **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.

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