INFECTION AND IMMUNITY, Nov. 1984, p. 465–469 Vol. 46, No. 2
0019-9567/84/110465-05$02.00/0
Copyright © 1984, American Society for Microbiology

Oral Vaccination of Monkeys with an Invasive *Escherichia coli* K-12 Hybrid Expressing *Shigella flexneri* 2a Somatic Antigen

SAMUEL B. FORMAL,[1]* THOMAS L. HALE,[1] CHRISTINE KAPFER,[1] JOHN P. COGAN,[3] PHILLIP J. SNOY,[3] RAYMOND CHUNG,[1] MICHELE E. WINGFIELD,[1] BENNETT L. ELISBERG,[3] AND LOUIS S. BARON[2]

Departments of Bacterial Diseases[1] and Bacterial Immunology,[2] Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20307-5100, and Division of Product Quality Control, Office of Biologics, Food and Drug Administration, Bethesda, Maryland 20205[3]

Received 20 April 1984/Accepted 21 August 1984

A living oral vaccine, designed to protect against *Shigella flexneri* 2a infections, was constructed by using *Escherichia coli* K-12 as a carrier strain. The hybrid strain, designated EC104, contained both chromosomal and plasmid genes from *S. flexneri* donor strains. In addition to expressing the *S. flexneri* 2a somatic antigen, it had inherited the property of epithelial-cell invasion. After the oral administration to rhesus monkeys, EC104 was isolated from the feces for up to 3 days, but by day 4 all stool cultures were negative. The serum antibody response against *S. flexneri* 2a somatic antigen was variable, but the vaccine conferred significant protection against an oral challenge with virulent *S. flexneri* 2a.

Shigella infections continue to be a serious public health problem, especially in developing countries. The morbidity and mortality due to shigellosis in emerging nations could most certainly be reduced by installation of modern water and sewage systems coordinated with successful educational programs promoting personal hygiene. However, controlling the disease by these methods will not be possible in the forseeable future. Thus, the most likely prospect for control is immunization.

Parenterally administered killed vaccines have not been effective (8, 9), and viable *Shigella flexneri* 2a, injected subcutaneously, has failed to protect monkeys against oral challenge with homologous organisms (7). However, orally administered, living, attenuated vaccines have been shown to protect under both laboratory and field conditions (4–6, 11). These products have not come into widespread use because they do not include stable, nonreverting strains which are avirulent but which will confer immunity after the oral administration of a limited number of vaccine doses.

In the past, we have prepared two general kinds of attenuated *Shigella* vaccines. One is a spontaneously occurring mutant which lacks the ability to invade the intestinal mucosa and which was required in multiple doses to confer resistance. The other type is a *Shigella-E. coli* hybrid which can invade the mucosa but had a reduced capacity to multiply in the tissue (6). With this type of vaccine, one or two doses are sufficient to protect monkeys against experimental infection, but these strains are not considered suitable for human use because they are unstable and have residual virulence.

We have recently shown that the transfer of a 140-megadalton plasmid from a virulent strain of *S. flexneri* 2a to an avirulent *Escherichia coli* K-12 strain endows the recipient strain with the ability to invade HeLa cells (12). Further transfer of chromosomal genes encoding the *S. flexneri* somatic antigen, which has been associated with protection (5, 6, 11), allows the transconjugant *E. coli* K-12 to penetrate the intestinal epithelium in ligated rabbit ileal loops and to produce a mild inflammatory reaction in the lamina propria. Although such strains can penetrate epithelial cells, they do not evoke positive Sereny tests or fluid secretion in rabbit ileal loops. Using this experience, we have now constructed a *Shigella* vaccine strain by using an *E. coli* K-12 recipient which does not have the potential to revert to virulence. This communication concerns the ability of this hybrid vaccine to serve as an oral vaccine for protecting monkeys against experimental challenge with virulent *S. flexneri* 2a.

MATERIALS AND METHODS

Strains. Three strains were used to construct the vaccine. *E. coli* K-12 strain 395-1 served as the recipient. *S. flexneri* 2a strain 256 was used as the donor of chromosomal genes, and *S. flexneri* 1b strain M25-8A(pWR110-R64drd11) was used as the donor of pWR110, a plasmid containing genes necessary for the invasive phenotype. The pWR110 portion of the cointegrate plasmid pWR110-R64drd11 carries a Tn*5* kanamycin-resistance transposon, whereas the R64drd11 portion carries tetracycline resistance. These strains have been described previously (12), and their pertinent characteristics are listed in Table 1. *S. flexneri* 2a wild-type strain 2457T was used as the challenge strain for efficacy tests in monkeys.

Serological tests. The O-antigenic specificity of transconjugants was determined by slide agglutination tests with antiserum against group 3,4 or type II *S. flexneri* antigen. The former was prepared by absorbing rabbit anti-*S. flexneri* 2a with *S. flexneri* 2b, and the latter was prepared by absorbing anti-*S. flexneri* 2a with organisms from all of the other subgroup B serotypes.

Protection tests in monkeys. Rhesus monkeys weighing 3 to 5 kg were used. For each experiment, 40 animals, individually caged in one room, were used. Whenever they were handled for bleeding, vaccination, or challenge, they were sedated with ketamine hydrochloride (50 mg given intramuscularly). Blood was obtained before vaccination, 10 days after the last vaccine dose, and 2 weeks after challenge. During the week before vaccination, three stool specimens were obtained from each animal, and these were plated on

* Corresponding author.

TABLE 1. Characteristics of strains used to construct *E. coli-S. flexneri* 2a hybrid strain EC104

Species and strain	Presence or absence of[a]:													Resistance or susceptibility to[b]:			
	Pro	Leu	Arg	His	Nad	TnaA	MalB	Rha	Mtl	Fuc	Gal	Lac	LDC	Str	Nal	Km	Tc
E. coli K-12 395-1[c]	−	−	−	−	+	+	+	+	−	+	−	−	+	R	R	S	S
S. flexneri 2a 256[d]	+	+	+	+	−	−	−	−	+	−	+	+	−	S	S	S	S
S. flexneri 1B M24-8A(pWR110-R64drd11)[e]	+	+	+	+	−	−	−	+	+	+	−	−	−	R	R	R	R
Hybrid EC104[f]	+	−	−	+	+	+	+	+	−	+	−	−	+	R	R	R	S

[a] Abbreviations: Pro, proline; Leu, leucine; Arg, arginine; His, histidine; Nad, nicotimamide; TnaA, tryptophanase; MalB, maltose; Rha, rhamnose; Mtl, mannitol; Fuc, fucose; Gal, galactose; Lac, lactose; LDC, lysine decarboxylase.

[b] Abbreviations: Str, streptomycin; Nal, nalidixic acid; Km, kanamycin; Tc, tetracycline; R, resistant; S, susceptible.

[c] 395-1 served as a recipient strain.

[d] 256 served as a donor of chromosomal DNA.

[e] M25-8A(pWR110-R64drd11) served as a donor of the 140-megadalton *S. flexneri* plasmid (12).

[f] EC104 was originally given the laboratory designation CV61-1-2.

MacConkey agar to determine the carrier state for shigellae. *S. flexneri* 4 was isolated from one animal in the vaccine group and from three animals in the placebo group in experiment 1. No pathogens were isolated from any of the animals in experiment 2.

The vaccine strain was grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 20 μg of kanamycin per ml. *E. coli* K-12 strain 395-1 served as the placebo. The latter strain was handled like the vaccine except that kanamycin was not included in the growth medium. A 20-ml dose of vaccine or placebo was fed by stomach tube to the animals. After the last dose, the stools were cultured on MacConkey agar containing 500 μg of streptomycin per ml. The animals were challenged 2 weeks after the last dose of vaccine, by stomach tube, with virulent *S. flexneri* 2a strain 2457T suspended in 20 ml of brain heart infusion broth. The animals were observed twice daily for signs of illness for 4 days. They were considered ill if they had a diarrheal stool on 2 consecutive days or if they had one diarrheal stool with visible blood. The stools were cultured daily on MacConkey agar. Significance of protection was assessed by calculating exact probabilities for a two-by-two contingency table by the factorial method of Batson (1).

ELISA. Serum antibodies against lipopolysaccharide (LPS) from *S. flexneri* 2a strain 2457T were detected by enzyme-linked immunosorbent assay (ELISA). The LPS antigen was prepared by the method of Westphal and Jann (15). The coating buffer (carbonate buffer, pH 9.6) and diethanalamine buffer (pH 9.8) were made as described by Voller et al. (13). Phosphate-buffered saline (pH 7.4) contained 8 g of NaCl, 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4, 0.1g of $MgCl_2 \cdot 6H_2O$, and 0.2 mg of KCl per liter of distilled water. Washing buffer contained 0.5 ml of Tween 20 per liter of phosphate-buffered saline. Diluting buffer contained 5 g of casein (Baker E 397-7; J. T. Baker Chemical Co., Phillipsburg, N.J.), 5 g of fatty acid and globulin-free bovine serum albumin (Sigma A-7030; Sigma Chemical Co., St. Louis, Mo.), 2 g of NaN_3, and 0.1 g of phenol red in a liter of phosphate-buffered saline. The pH of the diluting buffer was adjusted to 7.4.

A 100-μl volume of coating buffer containing 10 μg of LPS per ml was added to each well of a 96-well polystyrene microtiter plate (Costar 3590). After incubation overnight at room temperature in a humidity chamber, the plates were aspirated and washed once with diluting buffer. A volume of 150 μl of diluting buffer was added to each well, and the plates were incubated for 60 min in a humidity chamber at 37°C. The plates were aspirated and washed twice with washing buffer. Starting at a concentration of 1:20, eight twofold dilutions of sera were made with diluting buffer, and 100 μl of each dilution was placed in a well. The plates were incubated overnight at room temperature in a humidity chamber. They were then aspirated and washed four times with washing buffer. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; Kirkegaard and Perry, Gaithersburg, Md.) was diluted in diluting buffer to a concentration of 1 μg/ml; 100 μl of this solution was added to each well. The plates were incubated overnight at room temperature in a humidity chamber. They were then aspirated and washed four times with washing buffer. A 100-μl volume of diethanolamine buffer containing 1 mg of *p*-nitrophenylphosphate per ml was added to each well. The plates were then incubated for 30 min at room temperature, and 50 μl of 3 M NaOH was added to each well to stop the reaction. The plates were read by a Microelisa Auto Reader (MR500; Dynatech Laboratories, Inc., Alexandria, Va.) at 405 nm. Endpoint titers were defined as that dilution at which the absorbance fell below 0.1. This was the highest optical density observed in control wells without LPS or without monkey serum. The ELISA was reproducible to plus or minus one dilution.

Indirect hemagglutination assay. Sheep erythrocytes, sensitized with heat-extracted *S. flexneri* 2a antigen, were used in serum hemagglutination tests as previously described (3).

RESULTS

Construction and characterization of the vaccine strain. *S. flexneri* chromosomal donor strain 256 was mated with the *E. coli* K-12 strain 395-1, and the mating mixture was plated on a defined medium lacking histidine. His^+ recombinants were isolated and one (CV-5C-1-20) was chosen for further work. This strain, in addition to inheriting the His^+ trait from *S. flexneri* 2a, also expressed *Shigella* group antigen 3,4. The pWR110-R64drd11 plasmid was then transferred by mating *S. flexneri* 1b strain M25-8A(pWR110-R64drd11) with strain CV-5C-1-20 and selecting for the transfer of kanamycin resistance. One clone (CV49-4-13), which had inherited a cointegrate plasmid consisting of pWR110 and the mobilizing Tet^r plasmid pR64drd11, also gained the ability to invade HeLa cells. The mobilizing portion of the plasmid was cured from strain CV49-4-13 by plating this strain on a medium containing fusaric acid (2). A clone, CV49-4-13-4, was selected from the latter medium on the basis of sensitivity to tetracycline and retention of the capacity to invade HeLa cells. This strain had lost the ability to transfer pWR110. Strain CV49-4-13-4 was then remated with *S. flexneri* 2a strain 256 and plated on a minimal medium lacking proline. Pro^+ clones were purified, and CV61-1-2, which expressed the *Shigella* type II antigen, was chosen for the vaccine

strain. CV61-1-2 was given the serial designation EC104. The inherited markers of this strain are presented in Table 1.

EC104 agglutinated in both *S. flexneri* group factor 3,4 and in type II antisera. In absorption studies, it reduced the titer of a rabbit antiserum prepared against a wild-type, virulent *S. flexneri* 2a strain 2457T from 1:2,560 to less than 1:80. It failed to cause fluid accumulation in the rabbit ileal loop but did produce an inflammatory reaction in the mucosa. Although it invaded HeLa cells in vitro, it did not cause a positive Sereny test. It was resistant to kanamycin (due to Tn*5* carried on pWR110), streptomycin, nalidixic acid, methicillin, penicillin, erythromycin, and clindamycin; it was sensitive to tetracycline, chloramphenicol, cephalothin, ampicillin, carbenicillin, gentamycin, amikacin, tobramycin, sulfamethoxazole-trimethoprim, Gantrisin, and Furadantin.

Efficacy trials in rhesus monkeys. Two vaccine trials were conducted in monkeys. In experiment 1, two doses (10^{11} cells) of vaccine (EC104) or placebo (395-1) were fed at an interval of 1 week, and the animals were challenged 2 weeks after the last dose with 10^{11} virulent *S. flexneri* 2a (2457T). In experiment 2, the animals were fed three doses of ca. 10^{10} cells during a 1-week period (Monday, Thursday, and Monday). They were challenged 2 weeks later with 10^{10} virulent organisms.

In experiment 1, no reactions were observed in any animals after the oral administration of the first dose of 10^{11} cells. However, after the second dose, three animals in the vaccine group had reactions which ranged from mild diarrhea to frank dysentery. *S. flexneri* 4 was isolated from each of these animals. None of these animals were found to be carriers in our preliminary studies. No reactions were observed in the placebo group, nor did they occur in any animals in experiment 2. The shedding pattern of the vaccine strain was similar in both experiments (Table 2). The vaccine strain was shed no longer than 3 days and was isolated for ca. 1 day longer than was the placebo.

The serum immune response to *S. flexneri* 2a LPS was analyzed by passive hemagglutination and the ELISA technique. When the traditional criterion of a fourfold rise in titer was used as an indicator of significant immunogenicity, oral administration of three doses (8×10^9 cells per dose) of strain EC104 did not induce a dramatic immune response. Only 6 of 20 monkeys had a fourfold rise in either the ELISA or the passive hemagglutination assay (Table 3). Although both techniques indicated that 30% of the monkeys had a serum antibody response to LPS determinants expressed by the vaccine strain, the subset of responding monkeys was not identical. When all monkeys that were positive in either assay were totaled, the cumulative seroconversion was 50%.

Two animals that received three vaccine doses (8×10^9 cells per dose) developed dysentery when challenged with *S. flexneri* 2a. One of these monkeys had a fourfold postvaccination rise in serum antibody to *S. flexneri* 2a LPS. This animal also had an eightfold rise in hemagglutination titer. The other unprotected monkey showed a twofold titer rise in both assays. Of the 20 animals in the placebo group of experiment 2, 17 had a fourfold hemagglutination titer rise 2 weeks after challenge with *S. flexneri* 2a (data not shown).

The results of challenge studies with strain 2457T are presented in Table 4. Of a total of 40 animals that received two or three oral doses of vaccine EC104, 6 (15%) became ill, whereas 24 of 40 (60%) that received an *E. coli* K-12 oral placebo developed illness. The *S. flexneri* 2a challenge strain was isolated from all symptomatic animals. In the control group, 13 animals, as compared with 2 vaccinated monkeys, required antibiotic treatment for severe dysentery. Thus, significant protection against infection was afforded those animals who had received the vaccine.

TABLE 2. Shedding of EC104 (vaccine) or *E. coli* K-12 strain 395-1 (placebo)

Day postfeeding	No. of positive samples/no. tested when injected with:			
	1×10^{11} cells of:		8×10^9 cells of:	
	Vaccine strain	Placebo strain	Vaccine strain	Placebo strain
1	20/20	20/20	20/20	20/20
2	15/20	2/20	14/20	2/20
3	5/20	0/20	3/20	0/20
4	0/20	0/20	0/20	0/20
8	0/20	0/20	0/10	0/20
14	0/20	0/20	0/20	0/20

[a] Number of CFU administered.

TABLE 3. Serum antibody response against *S. flexneri* 2a LPS at day 10 after a third dose of either the EC104 vaccine or the *E. coli* 395-1 placebo

Titer rise (fold)	No. of monkeys with a positive response by:			
	ELISA for inoculum:		Indirect hemagglutination assay for inoculum:	
	Vaccine	Placebo	Vaccine	Placebo
0	3	17	11	19
2	11	2	3	1
4	4	1	5	0
8	2	0	1	0

DISCUSSION

Various attenuated *Shigella* strains have successfully immunized monkeys or human beings against shigellosis; however, these vaccines have either been insufficiently attenuated, have reverted to virulence, or have required many doses to induce immunity. To eliminate the problem of reversion to virulence, non-*Shigella* carrier strains have been used as recipients for genetic loci encoding *Shigella* virulence determinants. Previous studies have indicated that protection against shigellosis is associated with the presence of type-specific somatic antigen in the vaccine (5, 6, 11). They have also suggested that attenuated vaccine strains which have the ability to invade epithelial cells require fewer doses to induce resistance than do strains which lack this characteristic (6). Therefore, we have sought to endow carrier strains with either the ability to express *Shigella* somatic antigen or to express those antigens and to invade the colonic epithelium.

Our first attempts to construct a *Shigella* vaccine by using a non-*Shigella* carrier organism involved the transfer of genes controlling the expression of *S. flexneri* 2a somatic antigen to a strain of *E. coli*. This hybrid was not invasive, and it failed to protect volunteers against experimental challenge (10). The galactose-epimeraseless, Vi antigen-negative *Salmonella typhi* strain 21a, which protected so well against typhoid in field trials in Egypt (14), is another candidate for a carrier strain. There is no direct evidence that this strain is capable of penetrating intestinal epithelial cells and reaching the lamina propria, but the fact that three doses of vaccine induce excellent immunity indicates that this may occur. The *Shigella sonnei* 1 somatic antigen has been transferred to *Salmonella typhi* strain 21a (3). The transconjugant has undergone preliminary safety tests in

TABLE 4. Illness in monkeys after oral challenge with *S. flexneri* 2a strain 2457T

Expt.	Vaccine group[a]			Placebo group[b]			Vaccine vs control (P)[c]
	No. with diarrhea	No. with dysentery	No. ill/no. challenged	No. with diarrhea	No. with dysentery	No. ill/no. challenged	
1[d]	1	3 (1)[e]	4/20	3	11 (5)	14/20	1×10^{-3}
2[f]	0	2 (1)	2/20	2	8 (8)	10/20	6×10^{-3}

[a] Vaccine strain, EC104.
[b] Placebo strain, 395-1.
[c] Combined groups, $P = 3 \times 10^{-5}$.
[d] Two doses of 10^{11} cells each.
[e] Number requiring antibiotic treatment is in parentheses.
[f] Three doses of 8×10^{9} cells each.

volunteers, and further studies to assess efficacy are being planned.

The present study describes yet a third possible carrier strain for *Shigella* antigens. This is an *E. coli* K-12 strain that expressed O-antigen of *S. flexneri* 2a but differed from our first *E. coli* carrier in that it also possessed a nontransferable 140-megadalton *S. flexneri* plasmid which allowed it to invade epithelial cells. Strain EC104 thus possessed some of the properties that we believe an ideal *Shigella* vaccine should have; i.e., it expressed a specific *Shigella* O antigen, and it had the potential to transport this antigen into the intestinal epithelium. Furthermore, unlike vaccine strains prepared from attentuated shigellae, the *E. coli* K-12 hybrid could not revert to a virulent strain because it lacked at least three *Shigella* chromosomal regions which have been associated with pathogenicity (12). As a result, the vaccine strain caused neither a positive Sereny reaction nor fluid secretion in the rabbit ileal loop. These are characteristics of virulent shigellae.

When fed to monkeys in doses of 10^{11} cells, the vaccine strain was not detected in the feces after 3 days. This was only a day longer than for *E. coli* K-12 strain 395-1, from which the vaccine was constructed. The monkeys tolerated the three doses of 10^{10} vaccine organisms very well, and no untoward reactions were observed after the first dose of 10^{11} cells. However, intestinal reactions were observed in three monkeys after the second dose of 10^{11} vaccine organisms. Virulent *S. flexneri* 4, which was known to be carried by other animals in the experiment, was isolated from each of these animals. It is likely that these illnesses were due to the inadvertent spread of virulent organisms from carrier monkeys housed in the same room. On the other hand, the vaccine strain in some way might have activated *S. flexneri* 4 infections in undiagnosed carriers. Nonetheless, the absence of untoward reactions in most monkeys ingesting massive doses of EC104 is encouraging, so human safety tests with lower doses are planned.

Assessment of immunogenicity is particularly important in the case of living vaccines. Antigenic characteristics may vary with each lot of vaccine organisms, so a readily quantifiable parameter of antigenicity is desirable. The most relevant way to quantitate the mucosal immune response would involve measurement of IgA levels in intestinal secretions. However, we have not successfully intubated the monkey duodenum, so collection of intestinal secretions was not attempted. In the past, both serum and coproantibody against *Shigella* somatic antigens have been measured after vaccination of monkeys with attenuated *Shigella* vaccines. Increases in coproantibody were not detected by indirect hemagglutination assays, but fourfold rises in serum antibody were often demonstrated after oral vaccination (3, 5). However, the current EC104 *E. coli* hybrid elicited a fourfold rise in hemagglutination titer in only a small proportion of the monkeys vaccinated.

A more sensitive ELISA test was also used to test pre- and postvaccination serum. The ELISA procedure was adaptable to monkey serum because monkey immunoglobulin cross-reacts strongly with goat anti-human IgG (J. B. Robbins, personal communication). Unfortunately, only 30% of the vaccinated animals exhibited fourfold rises in serum IgG recognizing *S. flexneri* 2a somatic antigen. No change was observed in postvaccination serum when goat anti-IgM or IgA was used in the ELISA test (data not shown).

Even though the serum antibody response to EC104 LPS was modest when compared with that elicited by *Shigella* hybrid vaccines, protection was comparable to that evoked by those less attenuated organisms (4–6). Only six monkeys vaccinated with EC104 became ill after challenge with *S. flexneri* 2a, and only two of these had progressive disease requiring antibiotic treatment. In contrast, a total of 24 unvaccinated monkeys became ill, and 13 of those required treatment. This level of protection against a substantial *Shigella* challenge suggests that, if safety tests are successful, field trials of an *E. coli*-based *Shigella* vaccine can reasonably be proposed.

LITERATURE CITED

1. **Batson, H. C.** 1956. An introduction to statistics in the medical sciences, p. 48. Burgess Publishing Co., Minneapolis.
2. **Bochner, B. R., H.-C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
3. **Formal, S. B., L. S. Baron, D. J. Kopecko, O. Washington, C. Powell, and C. A. Life.** 1981. Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form I antigen genes into the *galE Salmonella typhi* Ty21a typhoid vaccine strain. Infect. Immun. **34**:746–750.
4. **Formal, S. B., T. H. Kent, S. Austin, and E. H. LaBrec.** 1966. Fluorescent-antibody and histological study of vaccinated and control monkeys challenged with *Shigella flexneri*. J. Bacteriol. **91**:2368–2376.
5. **Formal, S. B., T. H. Kent, H. C. May, A. Palmer, S. Falkow, and E. H. LaBrec.** 1966. Protection of monkeys against experimental shigellosis with a living attenuated oral polyvalent dysentery vaccine. J. Bacteriol. **92**:17–22.
6. **Formal, S. B., E. H. LaBrec, A. Palmer, and S. Falkow.** 1965. Protection of monkeys against experimental shigellosis with attenuated vaccines. J. Bacteriol. **90**:63–68.
7. **Formal, S. B., R. M. Maenza, S. Austin, and E. H. LaBrec.** 1967. Failure of parenteral vaccines to protect monkeys against experimental shigellosis. Proc. Soc. Exp. Biol. Med. **125**:347–349.
8. **Hardy, A. V., T. DeCapito, and S. Halbert.** 1948. Studies on acute diarrheal disease. XIX. Immunization in shigellosis. Public Health Rep. **63**:685–688.
9. **Higgins, A. R., T. M. Floyd, and M. A. Kader.** 1955. Studies in

shigellosis. III. A controlled evaluation of a monovalent *Shigella* vaccine in a highly endemic environment. Am. J. Trop. Med. Hyg. **4**:281–288.

10. **Levine, M. M., W. E. Woodward, S. B. Formal, P. Gemski, Jr., 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.
11. **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.
12. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
13. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme-linked immunosorbent assay, p. 359–371. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
14. **Wahdan, M. H., C. Serie, R. Germanier, A. Lackany, Y. Cerisier, N. Guerin, S. Sallam, P. Geoffroy, A. Sadek El Tantawi, and P. Guesry.** 1980. A controlled field trial of a live oral typhoid vaccine. Bull. W.H.O. **58**:469–474.
15. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5**:83–91.