INFECTION AND IMMUNITY, Aug. 1996, p. 3055–3061 Vol. 64, No. 8
0019-9567/96/$04.00+0

Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a Strain CVD 1205: Construction, Safety, Immunogenicity, and Potential Efficacy as a Mucosal Vaccine

FERNANDO R. NORIEGA,* GENEVIEVE LOSONSKY, CAROL LAUDERBAUGH, FANG MING LIAO, JIN YUAN WANG, AND MYRON M. LEVINE

Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21210

Received 13 September 1995/Returned for modification 29 October 1995/Accepted 9 May 1996

***Shigella flexneri* 2a strain CVD 1204, which was constructed by introducing a specific, in-frame deletion mutation in the *guaB-A* operon, was compared with Δ*aroA* strain CVD 1201. CVD 1204 was less invasive for HeLa cells than CVD 1201, whereas following invasion, the abilities of the two mutants to proliferate intracellularly were similarly impaired. The reduction in invasiveness was independent of the guanine auxotrophic phenotype and fully recovered when the chromosomal deletion mutation in CVD 1204 was repaired. Following inoculation of the conjunctival sac of guinea pigs (Serény test) at high doses (10^9 CFU per eye), both strains evoked minimal, short-lived, conjunctival inflammation, which was significantly milder with strain CVD 1204. Double mutant Δ*guaB-A* Δ*virG* (also called *icsA*) strain CVD 1205 induced, after a single intranasal dose, high mucosal immunoglobulin A antilipopolysaccharide titers, which were significantly boosted further following a second dose of vaccine given 14 days later. Upon Serény test challenge with wild-type *S. flexneri* 2a, CVD 1205-vaccinated animals were significantly protected against keratoconjunctivitis (zero of eight vaccinees versus five of seven controls, $P = 0.03$; vaccine efficacy, 100%). CVD 1205 is an attractive candidate for human clinical trials.**

Encouraged by the successful attenuation of *Salmonella* spp. by mutations in the aromatic metabolic pathway (6, 14, 28), Lindberg and coworkers (16, 17) constructed Aro auxotrophic mutants of *Shigella* spp. and demonstrated their attenuation in animal models. However, these and other Aro auxotrophic strains have exhibited some residual reactogenicity when administered in high doses to volunteers (8, 15). We attempted to enhance the attenuation conferred by *aro* mutations by introducing a specific, in-frame mutation in the virulence gene *virG* (11) (also known as *icsA* [1]), resulting in Δ*aroA* Δ*virG* strain CVD 1203 (24). This double mutant was clearly attenuated in the guinea pig model and was well tolerated when given to humans in moderate doses (circa 10^6 CFU). In contrast, at high-dose levels (10^8 to 10^9 CFU), adverse reactions occurred in a proportion of volunteers (10). Intracellular *Salmonella* spp. harboring mutations in genes encoding enzymes of the aromatic metabolic pathway are attenuated compared with the wild type as a result of their auxotrophy for paraaminobenzoic acid (PABA), a precursor of folic acid, and dehydrobenzoic acid, a precursor of the iron-binding enterochelin (24). *Shigella flexneri* does not use enterochelin (17); subsequently, Aro^- organisms of this species were thought to be attenuated because of their inability to produce folic acid (17). Folic acid is a cofactor in multiple essential biochemical reactions, including the de novo biosynthesis pathway of purines (21).

McFarland and Stocker (19), working with *Salmonella* spp. in a mouse virulence model, found that *purA* and *purB* mutations (interrupting the de novo biosynthesis of adenine nucleotides) were so highly attenuating (19) that the strains were nonprotective in animals (27) and poorly immunogenic in humans (13). In contrast, mutations in several of the genes involved in the common purine pathway (i.e., *purF*, *purG*, *purC*, *purHD*) that interrupt the biosynthesis of both purine nucleotides or mutations in *guaB* or *guaA*, which interrupt the biosynthesis of guanine nucleotides, "resulted only in reduced virulence" (19); indeed, such strains induced death in mice at doses as low as 10^2 CFU (19). Linde and coworkers (18) found that Pur^- auxotrophs of *S. flexneri* 2a and *Shigella sonnei* (derived by nonspecific mutagenesis) were not significantly attenuated; guinea pigs inoculated in their conjunctival sac with these strains developed full-blown purulent keratoconjunctivitis (positive Serény test [30]) (18).

The observations reviewed above suggest that (i) mutations affecting enzymes of the common purine pathway are mildly attenuating (18, 19), but mutations affecting cofactors in the common purine pathway (*S. flexneri* Aro^- mutants) are more attenuating (6, 14, 28); and (ii) mutations distal to the common pathway and affecting the synthesis of adenine nucleotides are overattenuating (i.e., *purA purB*) (13, 27), but distal mutations affecting the guanine nucleotide synthesis are only minimally attenuating (19). To help clarify these apparent incongruities, we sought to investigate the attenuating effect of a specific guanine auxotrophy in *Shigella* spp.

guaB and *guaA* are adjacent genes, separated by 69 bp, located at 54 min in the bacterial chromosome, that form an operon governed by a single promoter upstream of *guaB* (31). Herein we describe the construction of *guaB-A* mutant strain CVD 1204, derived from wild-type *S. flexneri* 2a by means of a specific deletion mutation that inactivates the purine metabolic pathway enzymes IMP dehydrogenase (encoded by *guaB*) and GMP synthetase (encoded by *guaA*) (21). We compared CVD 1204 with single mutant Δ*aroA S. flexneri* 2a strain CVD 1201 (24) with respect to its invasiveness for HeLa cells and ability to undergo intracellular proliferation following invasion and its propensity to evoke inflammatory reactions following inoculation of the conjunctival sac of guinea pigs. Finally, we assessed

* Corresponding author. Mailing address: Center for Vaccine Development, University of Maryland School of Medicine, 10 South Pine St., Rm. 9-30, Baltimore, MD 21201. Phone: (410) 706-2341. Fax: (410) 706-6205.

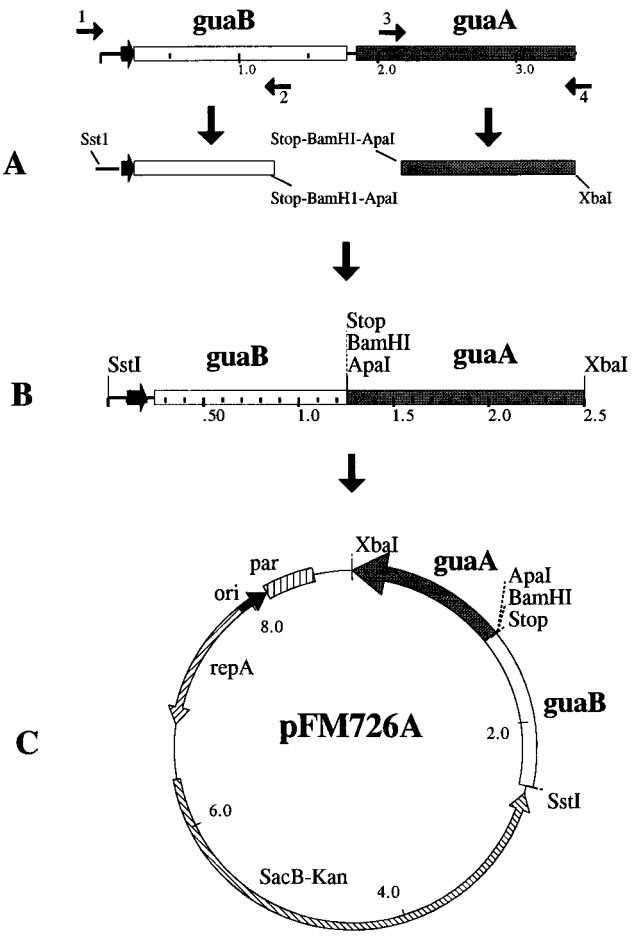

FIG. 1. (A) PCR amplification of DNA segments from wild-type *guaB* and *guaA* (*guaB-A* operon), including the promoter region (thick horizontal arrow) and the 5′ end of *guaB* and the 3′ end of *guaA*. Thin horizontal arrows indicate primers that were used to confer the cloning sites (*Sst*I and *Xba*I; primers 1 and 4) and complementing regions (with a stop signal and the additional unique restriction sites *Bam*HI and *Apa*I; primers 2 and 3). (B) Second (annealing) PCR that effectively fused the two DNA segments, forming the Δ*guaB-A* allele that is 918 bp shorter than wild-type *guaB-A*. (C) Cloning of Δ*guaB-A* in suicide plasmid pFM307A (data not shown), yielding to the deletion cassette pFM726A.

the safety, immunogenicity, and protective efficacy in guinea pigs of double mutant Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205.

MATERIALS AND METHODS

Strains and medium. Wild-type *S. flexneri* 2a strain 2457T, originally isolated in Japan, is known to cause disease in volunteers (3). M4243A is a noninvasive plasmidless strain derived from 2457T (4). Δ*aroA S. flexneri* 2a strain CVD 1201 was derived from strain 2457T (24). Strains Δ*guaB-A* CVD 1204 and Δ*guaB-A* Δ*virG* CVD 1205 are described in this work. For the tissue culture and animal experiments, *Shigella* strains were grown on Trypticase soy agar (TSA; BBL, Becton Dickinson, Cockeysville, Md.) with Congo red dye (Sigma Chemical Co., St. Louis, Mo.; TSA-CR). *Escherichia coli* HS, a nonpathogenic, smooth, human commensal organism (12), was grown on TSA. For the screening and characterization of guanine auxotrophs, clones were grown on *Shigella* minimum medium (24) with or without 10 mg of guanine per liter.

Construction of the Δ*guaB-A* deletion cassette pFM726A. In the construction of the Δ*guaB-A* deletion cassette, DNA segments that included the 5′ terminus of *guaB* and the 3′ terminus of *guaA* were amplified (from *S. flexneri* 2a strain 2457T genomic DNA) and fused by PCR, originating the Δ*guaB-A* allele (Fig. 1A and B). With the internal primers (primers 2 and 3) was introduced an in-frame stop sign upstream of two unique restriction sites that were added for the future introduction of foreign genes into the chromosomal Δ*guaB-A* allele. The external primers (primers 1 and 4) were designed to introduce unique restriction sites that were used to clone the Δ*guaB-A* allele into the temperature-sensitive, pSC101-based (2) suicide plasmid pFM307A, originating pFM726A (Fig. 1C). The same external primers were used to amplify the wild-type *guaB-A* operon (from strain 2457T), which was subsequently cloned in pGEM-T (Promega, Madison, Wis.), yielding pGEM::gua, and in pFM307A, yielding pFM215A.

Suicide cassette-driven deletion mutations and repair of the same. Deletion cassette pFM726A was used to introduce the deletion mutation into wild-type *S. flexneri* 2a strain 2457T by homologous recombination as described in previously published methods (2, 24), yielding strain CVD 1204. Plasmid FM215A was used to repair the deletion mutation by homologous recombination of the chromosomal Δ*guaB-A* allele in strain CVD 1204 for the wild-type operon contained in the suicide plasmid.

A second deletion mutation on the virulence gene *virG* was performed with a previously described suicide deletion cassette (pΔ*virG*) and methods (24), yielding strain CVD 1205. The deletion mutation corresponds to 900 bases representing amino acids 341 to 640 of the 120-kDa VirG protein. The specific engineered site for this deletion in the protein represents a highly hydrophobic, poorly antigenic portion of the molecule according to the Jameson-Wolf antigenic index (IBI Pustell sequence analysis programs).

Probes and DNA hybridizations. A γ-^{32}P-labeled 40-bp oligonucleotide (5′-GGCGGCCTGCGCTCCTGTATGGGTCTGACCGGCTGTGGT-3′), corresponding to a deleted portion of the *guaB-A* wild-type allele, was employed to screen mutants and confirm the deletion mutation (negative probe) on the guanine-auxotrophic colonies. The same probes were used to confirm the repair of the Δ*guaB-A* deletion mutation. To confirm the deletion mutation on the invasion plasmid's *virG* allele, two probes were generated, a γ-^{32}P-labeled 40-bp oligonucleotide (5′-GGGGACAGTTGAAGCTATGACACGTACCGCTGGT GTTATT-3′; negative probe) corresponding to a deleted portion of the wild-type gene and a PCR-generated, α-^{32}P-labeled Δ*virG* (positive probe). This second probe was introduced to rule out (at the time of screening for mutants) that the clones that did not hybridize with the negative probe had lost the virulence plasmid during the process of curing the suicide plasmid. Bacterial clones to be tested were grown at 37°C overnight in a grid pattern on Luria agar plates. Colonies were transferred to no. 541 filter paper (Whatman, Maidstone, England) and blotted as described previously (5).

Immunoblottings. For the characterization of ΔVirG, whole-cell proteins were prepared by 10 min of boiling of overnight cultures resuspended in lysing buffer (0.125% Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue; Sigma). VirG was resolved by gel electrophoresis in SDS–10% polyacrylamide and transferred to nitrocellulose paper. Immunoblot identification of VirG was accomplished with a rabbit antiserum generated against a VirG peptide, Leu-55 to Thr-73 (kindly provided by Edwin V. Oaks, Walter Reed Army Institute of Research, Washington, D.C.), developed with an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Sigma).

Ipa proteins were obtained as described previously (25). Briefly, 100 ml of Penassay broth (Difco Laboratories, Detroit, Mich.) cultures were grown overnight, with shaking at 37°C, pelleted by centrifugation at 10,000 × *g*, and resuspended in 10 ml of sterile H_2O with 5 μM phenylmethylsulfonyl fluoride (Sigma). This bacterial suspension was shaken for 2 h at 37°C and pelleted by centrifugation at 16,000 × *g*. The cell-free supernatants (filtered through a 0.2-μm-pore-size membrane) were assayed with the Micro BCA protein assay reagent kit (Pierce, Rockford, Ill.), and their protein concentrations were brought to a common denominator. Serial dilutions were performed for each sample in parallel in two 96-well microtiter plates, and 150 μl of each dilution was transferred to 96-well dot blot apparatuses (Bio-Rad, Melville, N.Y.) and suctioned through 0.45-μm-pore-size nitrocellulose membranes. These membranes were exposed to either an anti-IpaB or an anti-IpaC monoclonal antibody (kindly provided by Edwin V. Oaks) (20) and developed with an anti-mouse alkaline phosphatase-conjugated antibody (Sigma).

Tissue culture assays. HeLa cell gentamicin protection assays were performed by previously described methods with slight modifications (16, 24, 26). Briefly, semiconfluent HeLa cell monolayers on 24-well plates were infected in triplicate wells with wild-type strain 2457T, plasmidless strain M4243A, Δ*aroA* strain CVD 1201, or Δ*guaB-A* strain CVD 1204 at a 50:1 ratio for 60 min, after which extracellular organisms were killed with gentamicin (Sigma; 100 μg/ml) for 30 min, washed (0 h time point), and then incubated with gentamicin (30 μg/ml) for 4 h more. Additional wells containing monolayer on coverslips were used to assess the percentage of invasion at 0 and 4 h under light microscopy after fixing with methanol and staining with Giemsa. In another set of experiments, invasion and intracellular growth were assayed by the method described above with strains 2457T, CVD 1204, and CVD 1204R (strain derived from CVD 1204 in which the Δ*guaB-A* mutation was repaired).

Immunizations. Overnight cultures of Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205 and HS strains were harvested and resuspended in phosphate-buffered saline (PBS) to an optical density at 600 nm of 0.5 (equivalent to 5×10^8 CFU/ml) and concentrated by centrifugation to the desired concentration. Randomized, nonpreconditioned guinea pigs were given intranasally 100 μl of bacterial suspension containing 10^9 CFU as described previously (23). A booster dose was administered 14 days later in the identical manner.

Sample collection and ELISA. Tears were elicited with dried *Capsicum baccatum* flakes and collected with 50-μl micropipettes (VWR Scientific, Baltimore,

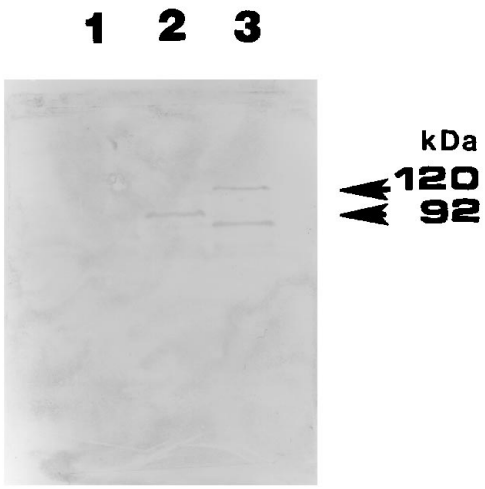

FIG. 2. Western blot identification of VirG and ΔVirG. Lanes: 1, noninvasive plasmidless *S. flexneri* 2a strain 4243A; 2, Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205; 3, wild-type *S. flexneri* 2a strain 2457T.

Md.). Blood was obtained by anterior vena cava puncture (33) from guinea pigs under anesthesia with ketamine (Parke-Davis & Co., Morris Plains, N.J.; 44 mg/kg intraperitoneally) and acepromazine maleate (Ayerst Laboratories Inc., New York, N.Y. 1.2 mg/kg intraperitoneally). Samples were collected at days 0, 14, and 28 postimmunization. Mucosal and serum IgA and serum IgG antibodies against *S. flexneri* 2a lipopolysaccharide (LPS; phenol-water extraction [32] from strain 2457T) were determined by an enzyme-linked immunosorbent assay (ELISA) using rabbit anti-guinea pig IgA alpha chain (Bethyl Laboratories, Inc., Montgomery, Tex.) and a goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) antibodies, respectively, as described previously (23). ELISA optical density titers were log transformed and compared by Student's *t* test.

Serény test. Overnight TSA-CR cultures were brought to the desired concentration of bacteria in PBS by optical density at 600 nm. Hartley guinea pigs were inoculated in the conjunctival sac with 10^9 CFU of wild-type strain 2457T or attenuated strain CVD 1201, CVD 1204, or CVD 1205 in 10 μl of PBS for the safety studies or with 3×10^7 CFU of strain 2457T for the protection studies. Guinea pigs were examined daily for 5 days, and their inflammatory responses were graded. The individual examining the guinea pigs each day and scoring the results was blinded as to what each animal had received and which eye had been inoculated. The statistical significance in the degree of the inflammatory response was calculated by a nonparametric sum of ranks (Mann-Whitney test). The overall frequencies of occurrence of inflammation of any severity in the vaccine and control groups were compared by Fisher's exact test.

RESULTS

Construction of a deletion mutation in the *guaB-A* operon of wild-type *S. flexneri* 2a strain 2457T. Δ*guaB-A* mutants were screened by detecting clones that failed to hybridize with a γ-^{32}P-labeled 40-bp oligonucleotide probe representing a sequence within the deleted portion. The Δ*guaB-A* allele was amplified by PCR from those probe-negative clones, yielding a 2.3-kbp product versus a 3.2-kbp product for the wild-type *guaB-A* (data not shown). The Δ*guaB-A S. flexneri* 2a clones were not able to grow in *Shigella* minimum medium (24) unless supplemented with 10 mg of guanine/liter. One Δ*guaB-A S. flexneri* 2a clone was arbitrarily selected and named CVD 1204 after confirming that the *Shigella* invasion plasmid was still present. The deletion mutation in strain CVD 1204 was repaired by virtue of homologous recombination with the proficient *guaB-A* operon in pFM215A, by use of the same methods that yielded CVD 1204. The resulting strain, named CVD 1204R, is able to grow in minimum medium without guanine supplementation.

Supplementation of *guaB-A*. Strain CVD 1204 was transformed by electroporation with pGEM::gua, pFM215A, and pGEM-T (as a negative control). The guanine auxotrophy was effectively supplemented by pGEM::gua and pFM215A, since CVD 1204 transformed with these plasmids was able to grow in *Shigella* minimum medium without guanine supplementation.

Construction of Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205. After homologous recombination with suicide deletion cassette pΔ*virG* (24), Δ*virG* mutants were screened by negative probing with a γ-^{32}P-labeled 40-bp oligonucleotide and positive probing with a α-^{32}P-labeled PCR-amplified Δ*virG* probe. The deletion mutation in *virG* was confirmed genotypically by PCR amplification of the deleted allele (yielding 2.5 kbp for Δ*virG* versus 3.5 kbp for wild-type *virG*) (data not shown).

Expression of invasion-related proteins. The expression levels of IpaB and IpaC by strains CVD 1204, CVD 1204 (pFM15A), CVD 1204R, CVD 1201, 2457T, and M4243A (a plasmidless strain used as a negative control) were compared on serial dilutions of Ipa-enriched supernatants (25) with the same protein concentration. Equivalent concentrations of IpaB and IpaC were detected by dot blotting these supernatants in all strains, with the exception of the negative control strain M4243A. Positive dot blots were detected with total protein concentrations as low as 14 ng/ml for IpaB and 7 ng/ml for IpaC.

Phenotypically, the deletion mutation was confirmed by the expression of a truncated VirG protein visualized by Western immunoblotting, which showed a predicted 90-kDa band versus a 120-kDa band for wild-type VirG (Fig. 2). Interestingly, VirG2, the soluble 80-kDa fraction of VirG, was not derived from ΔVirG (Fig. 2).

Invasion and intracellular growth in tissue cultures. In three different experiments, wild-type *S. flexneri* 2a strain 2457T efficiently invaded HeLa cells and replicated in them more than 30-fold (five doublings) in a 4-h period (Table 1). Occasionally, we detected a few CFU of the noninvasive plas-

TABLE 1. Invasion and intracellular growth in HeLa cells

Strain	Genotype	CR[a]	Invasion (%)[b]	Intracellular CFU (SD)[c]	
				0 h	4 h
2457T	Wild type	+	24	$2.14\ (0.4) \times 10^4$	$6.83\ (1.5) \times 10^5$
M4243A	Plasmidless	−	0	$6\ (5.7) \times 10^1$	0
CVD 1201	Δ*aroA*	+	28	$2.3\ (1) \times 10^4$	$3.68\ (0.7) \times 10^{5d}$
CVD 1204	Δ*guaB-A*	+	0.8	$2.82\ (1) \times 10^{2e}$	$1.84\ (0.3) \times 10^{3f}$

[a] Uptake of Congo red dye.
[b] Percentage of invaded HeLa cells determined in more than 400 HeLa cells observed.
[c] Mean (standard deviation) of three wells per group from a single representative experiment.
[d] CVD 1201 versus 2457T at 4 h, $P = 0.011$.
[e] CVD 1204 versus CVD 1201 at 0 h, $P = 0.007$.
[f] CVD 1204 versus CVD 1201 at 4 h, $P = 0.003$.

TABLE 2. Serény test in guinea pigs of vaccine candidate strains CVD 1201, CVD 1204, and CVD 1205

Strain	Genotype	Total no. of animals	No. of animals with degree of inflammation[a] at:														
			24 h					48 h					72 h				
			0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
2457T	Wild type	8	0	0	2	2	4	0	0	0	0	8	0	0	0	0	8
CVD 1201[b,c]	Δ*aroA*	16	3	7	4	2	0	10	6	0	0	0	16	0	0	0	0
CVD 1204[d]	Δ*guaB-A*	16	5	10	1	0	0	16	0	0	0	0	16	0	0	0	0
CVD 1205	Δ*guaB-A* Δ*virG*	16	11	1	3	1	0	15	1	0	0	0	16	0	0	0	0

[a] Inflammation degrees: 0, normal; 1, palpebral edema; 2, palpebral edema with conjunctival hyperemia only; 3, conjunctival hyperemia plus slight exudate; 4, full-blown purulent keratoconjunctivitis.
[b] CVD 1201 versus CVD 1204 at 24 h, $P = 0.1002$ by Wilcoxon or Mann-Whitney test and $P = 0.6851$ by Fisher's exact test.
[c] CVD 1201 versus CVD 1204 at 48 h, $P = 0.0612$ by Wilcoxon or Mann-Whitney test and $P = 0.0176$ by Fisher's exact test.
[d] CVD 1204 versus CVD 1205 at 24 h, $P = 0.148$ by Wilcoxon or Mann-Whitney test and $P = 0.0378$ by Fisher's exact test.

midless strain M4243A, but none at 4 h, indicating that those organisms were probably extracellular. As previously reported (24), Δ*aroA* strain CVD 1201 was able to invade the tissue culture monolayer as efficiently as its wild-type parent. However, consistently fewer intracellular generations (i.e., 15-fold, four doublings) were detected between 0 and 4 h (Table 1). Interestingly, single-mutant strain Δ*guaB-A* CVD 1204 was significantly less invasive for HeLa cells than its wild-type parent, strain 2457T, or its Δ*aroA* CVD 1201 strain counterpart ($P = 0.007$) and showed a more striking reduction in intracellular growth (12-fold, 2.5 doublings in 4 h).

In subsequent experiments, we compared the HeLa cell invasion of CVD 1204 with that of its *guaB-A*-supplemented derivatives (transformed with pGEM::gua or pFM15A) or its *guaB-A*-repaired derivative (CVD 1204R). Although the guanine auxotrophy was efficiently complemented by the plasmids carrying the proficient *guaB-A* operon (pGEM::gua and pFM15A), the recovery of the full invasive phenotype was not obtained until the proficient *guaB-A* operon was exchanged for Δ*guaB-A* in CVD 1204, efficiently repairing the chromosomal deletion mutation in this strain (Table 2). The intracellular CVD 1204R CFU found at 0 and 4 h were equivalent to those obtained with the wild-type parent strain 2457T but significantly higher than those of CVD 1204 at 0 h ($P = 0.0014$) and 4 h ($P = 0.0008$), demonstrating the restitution of the wild-type phenotype in CVD 1204R (data not shown).

Comparative safety of vaccine candidate strains CVD 1201, CVD 1204, and CVD 1205. To compare the attenuating effect of the Δ*guaB-A* deletion mutation with that of Δ*aroA* and to assess the safety of the double-mutant strain Δ*guaB-A* Δ*virG* CVD 1205, a randomized, comparative, keratoconjunctivitis safety test (Serény test) was performed with guinea pigs and a blinded observer. An initial, mild, self-limited inflammatory response has been reported previously with Δ*aroA S. flexneri* 2a strains (24). Twenty-four hours after a high-dose ($>10^9$ CFU per eye) inoculation, the frequency and severity of the initial inflammatory response were not significantly different between the Δ*aroA* strain CVD 1201 and the Δ*guaB-A* strain CVD 1204, although this mild inflammation resolved faster in the animals inoculated with CVD 1204 (Table 2). At 48 h, 6 of 16 animals inoculated with CVD 1201 but none of the animals inoculated with CVD 1204 had residual signs of inflammation ($P = 0.018$) (Table 2). Similarly, the double-mutant strain Δ*guaB-A* Δ*virG* CVD 1205 induced a mild, self-limited, inflammatory response in fewer animals than the group challenged with the single-mutant strain CVD 1204 (11 of 16 versus 5 of 16; $P = 0.04$). At 72 h postinoculation, the (blinded) observer grading the inflammatory response in the guinea pigs could not distinguish the inoculated eye from the noninoculated one in any of the animals that received the attenuated mutant CVD 1201, CVD 1204, or CVD 1205, while all animals that received wild-type strain 2457T had full-blown purulent keratoconjunctivitis (Table 2).

Immune response against Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205. Sixteen Hartley guinea pigs were randomly allocated to receive two intranasal immunizations, two weeks apart, of 10^{10} CFU of CVD 1205 or control strain *E. coli* HS. A strong anti-*S. flexneri* 2a LPS mucosal IgA response was detected after a single dose of CVD 1205 (i.e., 54-fold rise in geometric mean titers [GMT]) (Table 3). Furthermore, the second dose of CVD 1205, given at day 14, markedly boosted the IgA anti-LPS titers (11-fold over the primary response at day 14; 609-fold over day 0 or HS controls) (Table 3).

The serum antibody response was more delayed, since no serum IgG or IgA anti-*Shigella* LPS was detected after the first immunization. However, after the second immunization, those animals immunized with CVD 1205 had specific anti-*S. flexneri* 2a LPS IgA (i.e., 78-fold rise in GMT) and IgG (i.e., 60-fold rise in GMT) titers that were highly significant with respect to

TABLE 3. Mucosal IgA immune response against *S. flexneri* 2a LPS following intranasal immunization with strain CVD 1205 or placebo

Animal no.	Strain	IgA titer on:		
		Day 0	Day 14	Day 28
3	CVD 1205[a]	40	1,280	10,240
4	CVD 1205	20	1,280	20,480
5	CVD 1205	20	320	5,120
7	CVD 1205	20	1,280	10,240
8	CVD 1205	20	5,120	20,480
11	CVD 1205	20	1,280	20,480
14	CVD 1205	20	640	10,240
16	CVD 1205	20	1,280	20,480
GMT		21.81	1,173.76[b]	13,279.64[c]
1	HS[d]	20	20	20
2	HS	20	20	20
6	HS	20	20	20
9	HS	20	20	20
10	HS	20	20	20
12	HS	40	20	20
13	HS	20	20	20
13	HS	20	20	20
GMT		21.81	20	20

[a] Δ*guaB-A* Δ*virG S. flexneri* 2a administered intranasally at days 0 and 14.
[b] CVD 1205 versus HS at 14 days, $P = 0.0000008$ by *t* test.
[c] CVD 1205 versus HS at 28 days, $P = 0.000000002$ by *t* test.
[d] *E. coli* HS (control) administered intranasally at days 0 and 14.

TABLE 4. Serum immune response against *S. flexneri* 2a LPS after immunization with vaccine candidate strain CVD 1205 or placebo

Animal no.	Strain	IgA titer on:		IgG titer on:	
		Day 0	Day 28	Day 0	Day 28
3	CVD 1205[a]	12.5	800	12.5	1,600
4	CVD 1205	12.5	400	12.5	400
5	CVD 1205	12.5	800	12.5	200
7	CVD 1205	12.5	1,600	12.5	800
8	CVD 1205	12.5	1,600	12.5	800
11	CVD 1205	12.5	800	12.5	1,600
14	CVD 1205	12.5	1,600	12.5	800
16	CVD 1205	12.5	400	12.5	800
GMT		12.5	975.21[b]	12.5	724.58[c]
1	HS[d]	12.5	12.5	12.5	12.5
2	HS	12.5	12.5	12.5	12.5
6	HS	12.5	12.5	12.5	12.5
9	HS	12.5	12.5	12.5	12.5
10	HS	12.5	12.5	12.5	12.5
12	HS	12.5	12.5	12.5	12.5
13	HS	12.5	12.5	12.5	12.5
13	HS	12.5	12.5	12.5	12.5
GMT		12.5	12.5	12.5	12.5

[a] Δ*guaB-A* Δ*virG S. flexneri* 2a administered intranasally at days 0 and 14.
[b] CVD 1205 versus HS, $P = 0.0000003$ by *t* test.
[c] CVD 1205 versus HS, $P = 0.000003$ by *t* test.
[d] *E. coli* HS (control) administered intranasally at days 0 and 14.

those obtained at day 0 in the same guinea pigs or at days 0 and 28 in the strain HS controls (Table 4).

Protection of guinea pigs against wild-type challenge. On day 28 after the first immunization, the 16 guinea pigs that had received CVD 1205 or placebo were challenged with 3×10^7 CFU of wild-type *S. flexneri* 2a strain 2457T in 10 μl of PBS. Full-blown purulent keratoconjunctivitis developed in five of seven control animals vaccinated with placebo (71% attack rate) versus none of the eight guinea pigs immunized with two, spaced intranasal doses of CVD 1205 (vaccine efficacy = 100%, $P = 0.0069$) (Table 4).

DISCUSSION

Aro$^-$ *S. flexneri* strains such as Δ*aroD S. flexneri* Y strain SFL 124 (15), Δ*aroD S. flexneri* 2a strain SFL 1070 (8), and Δ*aroA S. flexneri* 2a strains CVD 1201 and CVD 1203 (24), are auxotrophic for aromatic amino acids and PABA. Other Aro$^-$ members of the *Enterobacteriaceae*, such as Δ*aroA Salmonella typhimurium* SL3261 (6) and Δ*aroC* Δ*aroD Salmonella typhi* CVD 908 (7), in addition, are auxotrophic for dehydroxybenzoic acid, a precursor of the Fe^{2+}-binding enterochelin. Since aromatic amino acids are readily found in the intracellular environment of the mammalian host cell, the PABA auxotrophy is thought to be responsible for the attenuation of the *Shigella* strains (17). PABA is a precursor of tetrahydrofolic acid (THFA), which as its further derivative N^{10}-formyl-THFA acts as a cofactor in the common purine metabolic pathway in the reactions from 5′-phosphoribosyl-1-glycinamide (GAR) to 5′-phosphoribosyl-1-formylglycinamide (FGAR) (Fig. 3) and from 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR) to 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole (FAIC AR) (Fig. 3) and as 5,10-methylene-THFA in the pyrimidine metabolic pathway in reactions from dUMP to dTMP (reaction mediated by *thyA*) (21). In addition, downstream of the metabolic reaction from GAR to FGAR, another pathway emerges for the formation of thiamine pyrophosphate (Fig. 3), another essential cofactor. Therefore, mutations in the aromatic pathway interrupt other pathways, which may explain why *aro* mutants may be more attenuated than strains with mutations in the de novo common purine pathway. Consequently, if we assume that the mutations in the common purine metabolic pathway reported in *Salmonella* (19) and *Shigella* (18) spp. are stable, their failure to severely handicap the growth of these organisms may be a reflection of their ability to use preformed purines via salvage pathways (Fig. 3) (21). However, McFarland and Stocker's (19) experiments show that mutations in the terminal steps of the purine biosynthesis pathway, at a point beyond the hypoxanthine salvage pathway (Fig.

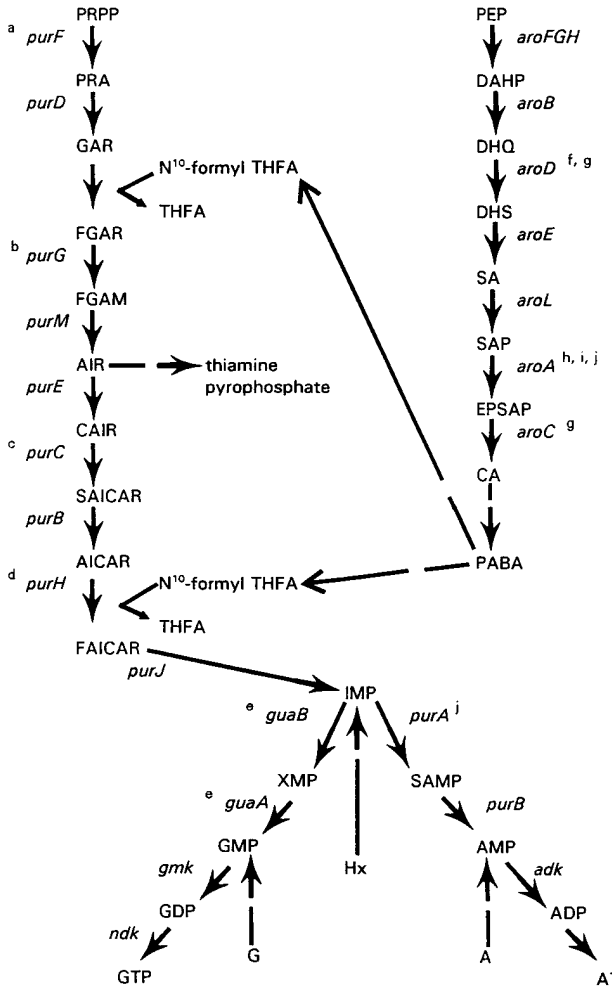

FIG. 3. Purine de novo biosynthesis pathway and contribution of the aromatic metabolic pathway. Interrupted arrows illustrate pathways in which the individual steps are not represented. Enzymes are represented by their genes. Superscript letters represent selected published strains with a mutation of the gene involved in that reaction. Strains represented are as follows: a, *purF1741*::Tn*10 Salmonella dublin* SL5437 (19); b, *purG876*::Tn*10 Salmonella dublin* SL5436 (19); c, *purC882*::Tn*10 Salmonella dublin* SL5435 (19); d, *purH887*::Tn*10 Salmonella dublin* SL2975 (19); e, Δ*guaB-A S. flexneri* 2a CVD 1204 and Δ*guaB-A* Δ*virG* CVD 1205 (this work); f, *aroD25*::Tn*10 S. flexneri* Y SFL114 (16), SFL124 (9), and *S. flexneri* 2a 1070 (8); g, Δ*aroC* Δ*aroD S. typhi* CVD908 (7); h, *hisG46* DEL407 *aroA554*::Tn*10 Salmonella typhimurium* SL3261 (6); i, Δ*aroA S. flexneri* 2a CVD1201 and Δ*aroA* Δ*virG* CVD1203 (24); j, Δ*aroA* Δ*hisG* Δ*purA Salmonella typhi* 541Ty (13). Abbreviations: PRPP, 5-phosphoribosyl-α-1-pyrophosphate; PRA, 5-phosphoribosylamine; FGAM, 5′-phosphoribosyl-*N*-formylglycinamidine; AIR, 5′-phosphoribosyl-5-aminoimidazole; CAIR, 5′-phosphoribosyl-5-aminoimidazole-4-carboxylic acid; SAICAR, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole; Hx, hypoxanthine; G, guanine; A, adenine.

3), hyperattenuate if they block the de novo synthesis of adenine nucleotides but confer only a minimal attenuation if they block the synthesis of guanine nucleotides (19).

In an attempt to explore this seemingly incongruent set of results, we performed a specific in-frame deletion mutation in the *guaB-A* operon. As predicted, the only requirement for growth of Δ*guaB-A S. flexneri* 2a in minimal medium was guanine. Interestingly, our results with strain CVD 1204 indicate that Δ*guaB-A* strains are effectively attenuated at a level at least comparable to that of the Δ*aroA* strain. Nevertheless, in contrast to the experience with the Δ*purB* or Δ*purA* strains of *Salmonella* spp. in mice (19), Δ*guaB-A* Δ*virG S. flexneri* 2a strain CVD 1205 was able to elicit high antibody titers in mucosa (Table 3) and serum (Table 4) that were protective against challenge with wild-type organisms in our guinea pig animal model.

It is evident that the attenuation observed in the Δ*guaB-A* strain (CVD 1204) may be due to both a 100-fold reduction in invasion and a severe limitation in its intracellular growth. But, as opposed to the *aro* mutants, in which their reduced intracellular growth is explained by the lack of PABA in the mammalian intracellular environment, the phenotype of the *guaB-A* mutants is more complex to justify. Guanine nucleotides are thought to be readily available in the eukaryotic host cell. However, bacteria cannot scavenge them directly because they have to be dephosphorylated and internalized as nucleosides (21), which may limit their intrabacterial availability to be used in the formation of guanine nucleotides via the salvage pathway. At this time, we do not have a complete explanation for the decrease in the invasion of cells in tissue culture exhibited by the Δ*guaB-A S. flexneri* 2a strain CVD 1204. Since guanine auxotrophy was reverted by plasmids bearing the proficient *guaB-A* operon but it was necessary to repair the deletion mutation in CVD 1204 to reestablish its full invasive potential, it is tempting to conclude that the deletion mutation in *guaB-A* may be interfering with the correct expression of a factor related to invasion. Since the moieties that have been directly associated with invasion in *Shigella* spp. are encoded in the invasiveness plasmid (reviewed in reference 29), it is tempting to suggest that a polar effect caused by the deletion in *guaB-A* may be acting on a chromosomal regulator that subsequently acts on the transcription of genes in the invasiveness plasmid of *Shigella* spp. Several of such chromosomal regulators have been described previously (29). However, given that we have demonstrated levels of expression of IpaB and IpaC in strain CVD 1204 and of ΔVirG in strain CVD 1205 that are equivalent to those found in wild-type organisms (strain 2457T), we consider this an unlikely possibility. Nonetheless, we continue to investigate these findings. In this regard, with the *guaB-A* allele as a probe, we will proceed to identify and sequence the flanking regions of the *guaB-A* operon in *Shigella* spp. by use of a *S. flexneri* 2a genomic library (4). With this, we may be able to identify possible genes upon which a polar effect is taking place.

We believe that the combination of the Δ*guaB-A* and Δ*virG* mutations in *Shigella* spp. will bring us close to a suitable balance between immunogenicity and minimal reactogenicity. We should be able to increase the clinical acceptability of Δ*guaB-A* Δ*virG* strains of attenuated *Shigella* spp. even further by inactivating the genes encoding two newly described enterotoxins, *Shigella* enterotoxin 1 (found almost exclusively in *S. flexneri* 2a strains) (22) and *Shigella* enterotoxin 2 (found in all serotypes). We consider it likely that these enterotoxins play a role in the pathogenesis of the watery diarrhea observed in the early stages of shigellosis and as an adverse reaction encountered in volunteers who receive high doses of *aro* and *aro virG* mutants. Properly planned clinical trials with such vaccine candidates will be able to corroborate or refute this hypothesis and thereby provide a rationale for inactivating *Shigella* enterotoxin expression as a means of enhancing the clinical acceptability of candidate *Shigella* vaccine strains.

ACKNOWLEDGMENTS

This work was supported in part by World Health Organization grant VRD/V27/181/21 (principal investigator, F. R. Noriega) and by grant RO1-AI-29471 from the National Institute of Allergy and Infectious Diseases (principal investigator, M. M. Levine).

We thank Shevon Kaintuck and Dave Maneval for providing us with their technical expertise.

REFERENCES

1. **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.
2. **Blomfield, I. C., V. Vaughn, R. F. Rest, and B. I. Eisenstein.** 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. **5:**1447–1457.
3. **Du Pont, 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.
4. **Fasano, A., F. R. Noriega, D. R. Maneval, Jr., 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.
5. **Gicquelais, K. G., M. M. Baldini, J. Martinez, L. Maggi, W. C. Martin, V. Prado, J. B. Kaper, and M. M. Levine.** 1990. Practical and economical method for using biotinylated DNA probes with bacterial colony blots to identify diarrhea-causing *Escherichia coli*. J. Clin. Microbiol. **28:**2485–2490.
6. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
7. **Hone, D. M., A. M. Harris, S. Chatfield, G. Dougan, and M. M. Levine.** 1991. Construction of genetically-defined double *aro* mutants of *Salmonella typhi*. Vaccine **9:**810–816.
8. **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.
9. **Kärnell, A., B. A. D. Stocker, S. Katakura, F. P. Reinholt, and A. A. Lindberg.** 1992. Live oral auxotrophic *Shigella flexneri* SFL124 vaccine with a deleted *aroD* gene: characterization and monkey protection studies. Vaccine **10:**389–394.
10. **Kotloff, K., F. R. Noriega, G. Losonsky, J. Nataro, I. Burrows, and M. M. Levine.** Unpublished data.
11. **Lett, M.-C., C. Sasakawa, N. Nobuhico, T. Sakai, S. Makino, M. Yamada, K. Komatsu, and M. Yoshikawa.** 1989. *virG*, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the *virG* protein and determination of the complete coding sequence. J. Bacteriol. **171:**353–359.
12. **Levine, M. M., E. J. Bergquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are not invasive. Lancet **i:**1119–1122.
13. **Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. Stocker.** 1987. Safety, infectivity, immunogenicity, and in vivo stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. J. Clin. Invest. **79:**888–902.
14. **Levine, M. M., D. M. Hone, B. A. D. Stocker, and M. Cadoz.** 1990. New and improved vaccines against typhoid fever, p. 269–287. *In* G. C. Woodrow and M. M. Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.
15. **Li, A., A. Karnell, P. Huan, P. Cam, N. Minh, L. Tram, N. Quy, D. Trach, K. Karisson, and A. Lindberg.** 1993. Safety and immunogenicity of the live oral auxotrophic *Shigella flexneri* SFL 124 in adult Vietnamese volunteers. Vaccine **11:**180–189.
16. **Lindberg, A., A. Karnell, T. Pal, H. Sweiha, K. Hultenby, and B. Stocker.** 1990. Construction of an auxotrophic *Shigella flexneri* strain for use as a live vaccine. Microb. Pathog. **8:**433–440.
17. **Lindberg, A. A., A. Karnell, and B. A. D. Stocker.** 1990. Aromatic-dependent *Shigella* strains as live oral vaccines, p. 677–687. *In* G. C. Woodrow and M. M. Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.
18. **Linde, K., V. Dentchev, V. Bondarenko, S. Marinova, B. Randhagen, M. Bratoyeva, Y. Tsvetanov, and Y. Romanova.** 1990. Live *Shigella flexneri* 2a

and *Shigella sonnei* I vaccine candidate strains with two attenuating markers. I. Construction of vaccine candidate strains with retained invasiveness but reduced intracellular multiplication. Vaccine **8:**25–29.
19. **McFarland, W. C., and B. A. D. Stocker.** 1987. Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of *Salmonella dublin* and of two strains of *Salmonella typhimurium*. Microb. Pathog. **3:**129–141.
20. **Mills, J. A., J. M. Buysse, and E. V. Oaks.** 1988. Shigella flexneri invasion plasmid antigens B and C: epitope location and characterization with monoclonal antibodies. Infect. Immun. **56:**2933–2941.
21. **Neuhard, J., and P. Nygaard.** 1987. Purines and pyrimidines, p. 445–473. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Noriega, F. R., F. M. Liao, S. B. Formal, A. Fasano, and M. M. Levine.** 1995. Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. J. Infect. Dis. **172:**1408–1410.
23. **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.
24. **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 S. flexneri* 2a strain CVD 1203, a prototype live oral vaccine. Infect. Immun. **62:**5168–5172.
25. **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.
26. **Oaks, E. V., M. E. Wingfield, and S. B. Formal.** 1985. Plaque formation by virulent *Shigella flexneri*. Infect. Immun. **48:**124–129.
27. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–423.
28. **Robertson, J. A., A. A. Lindberg, S. Hoiseth, and B. A. D. Stocker.** 1983. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infect. Immun. **41:**742–750.
29. **Sasakawa, C.** 1995. Molecular basis of pathogenicity of *Shigella*. Rev. Med. Microbiol. **6:**257–266.
30. **Sereny, B.** 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. **4:**367–376.
31. **Tesfa-Selase, F., and W. T. Drabble.** 1992. Regulation of the *gua* operon of *Escherichia coli* by the DnaA protein. Mol. Gen. Genet. **231:**256–264.
32. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further application of procedures. Methods Carbohydr. Chem. **5:**83–91.
33. **Whorton, J. A.** 1977. A technique for repeated collection of blood from the guinea pig. Lab. Anim. Sci. **24:**522–523.

Editor: A. O'Brien