Construction and Characterization of Attenuated ΔaroA ΔvirG
Shigella flexneri 2a Strain CVD 1203, a Prototype Live Oral Vaccine

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We engineered an oral Shigella vaccine prototype that can invoke intestinal epithelial cells but cannot undergo extensive intracellular replication or extend to adjacent epithelial cells. Strain CVD 1203, derived from wild-type Shigella flexneri 2a by introducing deletions in chromosomal araA and invasiveness plasmid virG, was highly attenuated in the Sereny test. Two 10⁵-CFU orogastric doses (2 weeks apart) stimulated production of secretory immunoglobin A antibodies to S. flexneri 2a and protected against conjunctival sac challenge with virulent S. flexneri 2a.

The World Health Organization and the Institute of Medicine have targeted the development of Shigella vaccines as a high priority (11, 44). From experimental challenge studies with monkeys (7) and volunteers (4, 9) and from longitudinal surveillance of a large cohort of children (5), it is known that an initial clinical episode of shigellosis confers approximately 70% protection against subsequent shigellosis due to the homologous serotype. In the mid-1960s, it was shown that one can mimic wild-type infection-derived immunity against shigellosis by administering attenuated strains of Shigella as live oral vaccines (3, 4, 27–29). In particular, well-documented studies used streptomycin-dependent strains (4, 27–29). Nevertheless, the streptomycin-dependent and other early Shigella vaccines suffered from drawbacks which impeded their widespread acceptance (19, 21). Various investigators have applied the techniques of modern biotechnology in their quest to develop improved vaccines. However, so far it has proven difficult to construct a live vaccine that is vigorously immunogenic and protective yet acceptably well-tolerated.

Recognizing that Shigella spp. are largely intracellular pathogens and that mutations in the amino acid biosynthesis pathway successfully attenuate Salmonella spp. (12, 35, 39), which are also intracellular pathogens, Lindberg and coworkers (23) constructed ara mutants of Shigella spp. Shigella flexneri 2a organisms harboring ara mutations were attenuated and protective in guinea pigs and monkeys (15, 16, 23, 24, 42), but results in humans have been more variable (14, 16, 22). Nonetheless, these observations suggest that ara mutations can greatly contribute to the overall attenuation of Shigella spp., even if by themselves they do not effect sufficient attenuation.

In order to attenuate ara mutants of Shigella spp. further without losing immunogenicity, we were drawn to the plasmid gene, virG (18, 25), also known as icaA (1, 8, 41), which is involved in the intracellular dissemination of Shigella spp. and their spread to adjacent colonocytes (1, 8, 18, 25, 41). Studies by Sansonetti and coworkers with rhesus monkeys showed that S. flexneri 5 icaA::TnphoA strains with a second insertional mutation in either the phoA (coding for aerobactin, a siderophore) (36) or ompB (coding for an osmoregulatory system) (37) were attenuated and protective in diverse degrees against a homologous challenge.

Our strategy was to engineer an oral vaccine prototype capable of invading intestinal epithelial cells but incapable of extensive intracellular replication or of extension to adjacent cells. We sequentially introduced precise deletion mutations into chromosomal gene araA and plasmid gene virG in a wild-type S. flexneri 2a strain known to be virulent in volunteers (17). We then proceeded to characterize the deletion mutants in vitro, to test them for safety in the guinea pig keratoconjunctivitis test (Sereny test) (38), and finally to assess the ability of ΔaroA ΔvirG strain to protect guinea pigs against keratoconjunctivitis following orogastric immunization.

The strategy for the construction of ΔaroA is depicted in Fig. 1. PCR amplifications of the first 501 bp of the 3' end and of the last 591 bp of the 3' end of the wild-type gene were performed (Fig. 1A). The 5' end of araA was amplified with the upstream primer TAATCAGATTTAGGGAATCCCTGAC GTTA and with the downstream primer GGTTACCCTGATTGAAGGATGGCCTTCATTAGGAACCGCTACAAGTGGCGCC. The 3' end was amplified with the upstream primer AAATATTGGGGTACGGTGTAATTCTTTGCTGAAAGGCTATGCA and with the downstream primer TGTAATACTGGCTACGC GCTGCGTGGCTAAT (the EcoRI and SalI sites [underlined] were introduced for cloning). In a second PCR, the 5' and 3' segments were fused, and the resultant fusion was amplified in the same reaction (Fig. 1B). In this reaction, the indicated homologous regions (in boldface) annealed, effectively fusing the 5' and 3' segments, which at that time may have acted as their own primers and/or templates for the Taq polymerase, depending upon which strands of DNA were annealed. The ΔaroA allele was cloned in temperature-sensitive vector pIB307 (2). In a second cloning step, the sacB-neomycin resistance segment of pIB279 was transferred into the BamHI polylinker site of pIB307::ΔaroA, and the resultant plasmid was designated pFJ201. pFJ201 was electroporated into S. flexneri 2457T to achieve allelic exchange in the wild-type strain.

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Cointegrates representing a single homologous recombination were readily obtained. By using counterselection (Aro-sucrose plates at 30°C), a clone that had characteristics indicating that a double homologous recombination event had taken place (i.e., representing allelic exchange of ΔaroA for araoA in the chromosome) was identified. This clone was kanamycin sensitive and Congo red positive; it agglutinated with S. flexneri 2a antiserum and was unable to grow in Shigella minimal medium (see below). PCR of this strain confirmed that the gene product harbored a deletion; the wild-type product was 1.2 kb, whereas the product of the clone was 1.0 kb. Confirmation of the deletion was made with a 40-base synthetic oligonucleotide sequence derived from the deleted portion of the gene. The 32P-labelled probe hybridized with wild-type colonies but not with the clone. This clone was designated CVD 1201.1.

The construction of the ΔvirG deletion cassette was analogous to the construction of the ΔaroA mutation. The 5′ segment of virG was amplified with the upstream primer GGGGGAATTTCAGAACATTTTATTTC and the downstream primer TCCATGCAATTCTTATGAGATAATATTGAATT, while the 3′ segment was amplified with the upstream primer CTCCATGCAATTCTTATGAGATAATATTGAATT and the downstream primer CGGGTCGACACTGAGAATTCTCCAAACACCCATGG

The specific deletion mutation on virG in strain CVD 1201.1 was accomplished by using the suicide vector cassette pSHΔvirG as previously described (10). An antibiotic-sensitive clone representing a putative successful double homologous recombination event was confirmed by PCR, Congo red positivity, agglutination with S. flexneri 2a antiserum, and failure to hybridize with the oligonucleotide probe specific for the deleted sequence; this ΔaroA ΔvirG clone was designated CVD 1203.

Inocula of 10^7 CFU each of strains ΔaroA CVD 1201.1 and wild-type 2457T were prepared by growing the bacteria, with shaking, at 37°C in 5-ml volumes of Shigella minimal medium-broth containing (per liter) 0.4 g of NaCl, 8.4 g of K2HPO4, 3.6 g of KH2PO4, 0.8 g of (NH4)2SO4, 2.5 g of glucose, 0.05 g of nicotinic acid, 0.05 g of aspartic acid, and 0.05 g of serine, which was progressively supplemented with aromatic amino acids (50 mg each of L-tryptophan, L-tyrosine, and L-phenylalanine), ferric ammonium acetate (10 mg), and p-aminobenzoic acid (10 mg). At 72 h, growth was measured by optical density at 600 nm. In addition to supplementation with tyrosine, tryptophan, and phenylalanine, CVD 1201.1 required p-aminobenzoic acid in order to grow.

CVD 1203, like its wild-type parent, grows on enteric media (which contain sufficient p-aminobenzoic acid and aromatic amino acids) and manifests a typical acid butt-alkaline slant reaction without H2S or gas 18 to 24 h after inoculation of triple-sugar-iron-agar slants. Lipopolysaccharides (LPS) extracted (43) from strains 2457T and CVD 1203 revealed identical patterns when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining (40) and by Western immunoblotting with human antiserum to S. flexneri 2a 2457T (17). Water extracts (32) of CVD 1203 and 2457T exhibited identical single bands on Western immunoblots with monoclonal antibodies to either Ipa B (42 kDa) or Ipa C (62 kDa) (31).

The ability of the wild-type, ΔaroA, and ΔaroA ΔvirG S. flexneri 2a strains to invade and replicate within epithelial cells was assessed by the HeLa cell gentamicin protection assay (33). Although strains CVD 1201.1 and CVD 1203 efficiently invade HeLa cells in tissue culture, like their wild-type parent strain, 2457T, their intracellular growth was significantly reduced compared with that of 2457T (analysis of variance, P = 0.0025) (Fig. 2).

A classic test of the virulence of wild-type Shigella isolates and of the degree of attenuation of candidate live vaccine strains is the guinea pig keratoconjunctivitis test first described by Sereny (38). In a preliminary dose-response study with the wild-type 2457T strain, inocula of 10^6 to 10^7 CFU caused full-blown conjunctivitis in only 2 of 17 Hartley guinea pigs. Of five animals administered a 10^6-CFU dose, four animals developed severe purulent conjunctivitis, while the fifth animal manifested moderate keratoconjunctivitis characterized by hyperemia and minimal purulent exudate. All five guinea pigs given 10^8 CFU developed full-blown severe keratoconjunctivitis.

In the subsequent safety test, 40 guinea pigs were randomized to one of three groups to be inoculated with 10^9 CFU of either the ΔaroA deletion mutant strain CVD 1201.1 (16 animals), ΔaroA ΔvirG strain CVD 1203 (16 animals), or wild-type 2457T (8 animals). The observer who examined the coded guinea pigs each day and scored the ophthalmologic findings was unaware of the group to which any animal belonged or of what eye had been inoculated. Keratoconjunctivitis was graded as follows: normal eye, 0; conjunctival erythema only (i.e., mild conjunctivitis), 1; conjunctival erythema and minimal purulent exudate (i.e., moderate conjunctivitis), 2; and conjunctival edema and overt, copious, purulent exudate (full-blown, severe conjunctivitis), 3. A clear-cut hierarchy of responses was evident, as summarized in Table 1, in which inflammatory responses are presented by day of observation. By 24 h after inoculation, all right guinea pigs that received the wild-type strain developed keratoconjunctivitis, which was severe in six; by 48 h, all these animals manifested
full-blown, severe keratoconjunctivitis. The 16 guinea pigs that received the ΔaroA CVD 1201.1 strain showed a significantly different pattern. Only one animal manifested severe conjunctivitis, which was evident only on day 1. At 24 h, 11 other animals in this group manifested mild (n = 7) or moderate (n = 4) keratoconjunctivitis, but this inflammatory response was short lived, as no animals showed ophthalmologic pathology by 72 h. Recipients of the double mutant strain CVD 1203 exhibited significantly milder inflammatory responses than recipients of the ΔaroA mutant. No recipients of CVD 1203 manifested either moderate or severe keratoconjunctivitis at any time. Four animals inoculated with CVD 1203 exhibited mild conjunctivitis at 24 h that was no longer evident at the 48-h observation point (Table 1).

In order to assess the ability of the CVD 1203 vaccine candidate to elicit a protective immune response, 33 guinea pigs were randomly allocated to receive orogastrically 10⁷ CFU of CVD 1203 or control strain E. coli HS (6). A second immunization was given 15 days later. Tears were collected from 10 guinea pigs (5 immunized with CVD 1203 and 5 immunized with E. coli HS) on days 7, 14, and 21 after the first orogastric dose to measure secretory immunoglobulin A (SIgA) antibodies against S. flexneri 2a LPS by enzyme-linked immunosorbent assay (17). Immunization with CVD 1203 clearly stimulated production of SIgA antibodies to S. flexneri 2a LPS in tears (Table 2).

On the 28th day after the first immunization, 16 vaccinated guinea pigs and 17 control guinea pigs were challenged with 5 × 10⁶ CFU of the wild-type 2457T strain in 10 μl. The results observed during the 5 days after the challenge are shown in Table 1. Full-blown keratoconjunctivitis developed in 16 of 17 control animals orogastrically vaccinated with the placebo (a 94% attack rate), in contrast to only 3 of 16 guinea pigs immunized with two spaced orogastric doses of CVD 1203 (a 19% attack rate) (vaccine efficacy = 80%, P < 0.0001) (Table 3).

We hypothesized that two precise deletion mutations, one in a chromosomal gene (aroA) and the other in an invasion plasmid gene (virG), might function in concert to yield a well-tolerated yet immunogenic live oral vaccine candidate from fully virulent S. flexneri 2a strain 2457T. The guinea pig keratoconjunctivitis test demonstrated that the ΔaroA mutant was significantly attenuated compared with the wild-type parent strain (Table 1) and that the ΔaroA ΔvirG double mutant was significantly further attenuated compared with the ΔaroA strain. An early, self-limited inflammatory response observed in the eyes of guinea pigs inoculated with large doses (10⁶ CFU) of the deletion mutants allowed us to distinguish

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**FIG. 2.** Fold rise (mean ± 2 standard deviations) in CFU over baseline (CFU at 0 h) of intracellular wild-type S. flexneri 2a strain 2457T (○) or its attenuated derivatives ΔaroA CVD 1201.1 (■) and ΔaroA ΔvirG CVD 1203 (▲).

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**TABLE 1.** Sereny test of vaccine candidate S. flexneri 2a strains ΔaroA CVD 1201.1 and ΔaroA ΔvirG CVD 1203

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>n²</th>
<th>24 h²/³</th>
<th>48 h</th>
<th>72 h</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>2457T</td>
<td>Wild type</td>
<td>8</td>
<td>0 0 2 6</td>
<td>0 0 0 8</td>
<td>0 0 0 8</td>
</tr>
<tr>
<td>CVD 1201.1</td>
<td>ΔaroA</td>
<td>16</td>
<td>4 7 4 1</td>
<td>12 3 1 0</td>
<td>16 0 0 0</td>
</tr>
<tr>
<td>CVD 1203</td>
<td>ΔaroA ΔvirG</td>
<td>16</td>
<td>11 4 1 0</td>
<td>16 0 0 0</td>
<td>16 0 0 0</td>
</tr>
</tbody>
</table>

a Number of guinea pigs inoculated.

b The dose-response grading system is described in the text. The degrees of inflammatory response among the three groups of animals were compared by a non-parametric sum of ranks (Mann-Whitney) test. The proportions of animals in each group exhibiting any inflammatory response were compared by Fisher's exact test.

c For 2457T versus CVD 1201.1, P = 0.0009 (Wilcoxon and Mann-Whitney tests); the difference is not significant by Fisher's exact test.

d For 2457T versus CVD 1203, P = 0.0001 (Wilcoxon and Mann-Whitney tests) and P = 0.0018 (Fisher's exact test).

e For CVD 1201.1 versus CVD 1203, P = 0.0174 (Wilcoxon and Mann-Whitney tests) and P = 0.032 (Fisher's exact test).

f For 2457T versus CVD 1201.1 (or CVD 1203), P < 0.0001 (Fisher's exact test).
between the ΔaroA and the ΔaroA ΔvirG strains. This short-lived inflammatory reaction observed at 24 h may be elicited by the initial invasive event or by limited intracellular growth of these organisms in the corneal and conjunctival epithelial cells, or both. The observation that immunization with the ΔaroA ΔvirG double mutant resulted in significantly milder inflammation suggests that the curtailed intercellular spread of this strain was responsible for the lesser epithelial damage.

It is hoped that the virG deletion mutation will further attenuate the vaccine strain for humans beyond the level of attenuation that Karnell et al. (13, 14) have reported for a derivative of 2457T mutated in aroD alone. Although CVD 1203 appears promising for animal models, the ultimate test of the innocuity and immunogenicity of a candidate vaccine resides with human clinical trials. The minimal inflammation evident in the conjunctiva at 24 h after inoculation with 10⁻⁸ CFU of CVD 1203, albeit mild and short lived, suggests that humans might experience transient adverse reactions during the first 24 h after ingestion of large doses of this vaccine candidate.

Live oral Shigella vaccine candidates must be protective besides being well tolerated. Conjunctival sac challenge of guinea pigs immunized orogastrically with two spaced doses of CVD 1203 demonstrated that the vaccine elicited significant protection against purulent keratoconjunctivitis. This can be construed as the result of successful functioning of the common mucosal immune system, whereby intestinal immunization protects against mucosal invasion of a distal site (in this instance the conjunctiva) (26). The detection of specific S-IgA antibodies in tears of immunized guinea pigs supports this contention that S-IgA mucosal antibodies protected the corneal and conjunctival epithelia from invasion by wild-type S. flexneri 2a. The guinea pig model has also proven useful for testing orally administered inactivated vaccines (34).

The suicide plasmids that we constructed can be used to introduce aroA and virG deletion mutations into strains of other serotypes of Shigella. In this manner, it may be possible to prepare a multivalent Shigella vaccine consisting of a collection of aroA virG mutants of the most important Shigella serotypes.

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REFERENCES


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TABLE 2. Reciprocal titers of S-IgA antibodies against S. flexneri 2a LPS in guinea pig tears following orogastric immunization[a]

<table>
<thead>
<tr>
<th>Strain and animal no.</th>
<th>Reciprocal titer[b] on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>CVD 1203</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>GMT[d]</td>
<td>23</td>
</tr>
</tbody>
</table>

E. coli HS

| No. of guinea pigs with degree of inflammation[d] |
|------------------------|-------------|
| 0 | 1 | 2 | 3 |
| 1 | 0 | 0 | 0 | 3 |

[a] Attenuated ΔaroA ΔvirG S. flexneri 2a vaccine (CVD 1203) or placebo (E. coli HS) was administered on days 0 and 14.
[b] Preimmunization titer not done.
[c] ND, not done.
[d] GMT, reciprocal geometric mean titer.

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TABLE 3. Protection against conjunctival challenge with wild-type S. flexneri 2a[a] (Sereny test) after orogastric immunization with vaccine or placebo

<table>
<thead>
<tr>
<th>Immunizing strain[b]</th>
<th>Characteristics</th>
<th>No. of guinea pigs with degree of inflammation[d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HS</td>
<td>Normal flora</td>
<td>17</td>
</tr>
<tr>
<td>CVD 1203</td>
<td>ΔaroA ΔvirG</td>
<td>16</td>
</tr>
</tbody>
</table>

[a] 5 × 10⁸ CFU of 2457T was injected into the conjunctival sac.
[b] Two doses (1 × 10⁸ to 5 × 10⁸ CFU of CVD 1203 [attenuated S. flexneri 2a] or E. coli HS [placebo]) were administered 14 days apart.
[c] Number of guinea pigs inoculated.
[d] The dose-response grading system is described in the text. Results were cumulative for 5 days of follow-up.
[e] Vaccine efficacy, 89%; Fisher’s exact test, P < 0.0001.


