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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*

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The use of attenuated Δ*aroA* Δ*virG Shigella flexneri* 2a strain CVD 1203 as a live vector for enterotoxigenic *Escherichia coli* (ETEC) antigens is reported. CVD 1203 alone or expressing colonization factor antigen fimbriae and CS3 fibrillae of ETEC was given to guinea pigs and mice, orogastrically (o.g.) or intranasally (i.n.). CVD 1203 given i.n. elicited high titers of antilipopolysaccharide (anti-LPS) immunoglobulin A (IgA) and was protective in guinea pigs against a homologous conjunctival challenge. Whereas a strong IgA response against colonization factor antigen CS3, and *Shigella* LPS was detected in tears and serum of guinea pigs after o.g. or i.n. immunization, the i.n. route elicited significantly higher antibody titers. A strong serum IgG response was also observed against the ETEC antigens, although no serum anti-LPS IgG response was detected. The immune response in mice followed a pattern similar to that in guinea pigs, and the difference between the responses after o.g. and i.n. administration was even more remarkable.

We recently reported the construction of Δ*aroA* Δ*virG Shigella flexneri* 2a vaccine candidate strain CVD 1203 and its ability, following oral administration, to stimulate mucosal immunoglobulin A (IgA) responses and to confer protection against *Shigella* keratoconjunctivitis in the guinea pig model (15). Orr et al. (16) have shown that nonliving *Shigella* antigens are even more immunogenic when administered intranasally (i.n.) than when administered orally. Accordingly, we investigated the immunogenicity and protective capacity of CVD 1203 administered as a live i.n. vaccine.

Enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* spp. are two of the most common bacterial enteropathogens causing traveler's diarrhea and diarrhea among infants and young children in less-developed countries. Considerable evidence indicates that immunity to ETEC is mainly based on anticolonization immunity in the small intestine (8, 9, 11, 12), involving secretory IgA directed against fimbrial colonization factors (8, 12). Volunteer studies carried out at the Center for Vaccine Development with a prototype ETEC vaccine strain, E1392-75-2A, that expresses fimbrial coli surface antigens 1 (CS1) and CS3 but not heat-labile or heat-stable enterotoxins showed that oral immunization with a single oral dose could elicit high levels of secretory IgA antifimbrial antibodies that conferred significant protection against experimental challenge (8, 9, 11).

Cloning of the genes encoding ETEC colonization factor fimbriae and their expression in suitable live vector vaccines have been proposed as one approach to develop an ETEC vaccine that might confer broad protection (9, 11). Colonization factor antigen I (CFA/I) and CS3 are two of the most common colonization factors of ETEC affecting humans, although in nature, these two antigens are never coexpressed by the same wild-type strain. We have previously described cloning of the multiple genes responsible for expression of CFA/I and CS3 (4). The operon encoding the multiple components of CFA/I was cloned in pJRD184 (giving rise to pJGX15A), and the operon encoding CS3 was cloned in pACYC184 (yielding pACCS3-1). Since these two plasmids are compatible in the same bacterial cell, it was possible to coexpress these recombinant antigens simultaneously in *Salmonella typhi* and *E. coli* (4). Herein we describe the coexpression of CFA/I and CS3 in attenuated *S. flexneri* 2a strain CVD 1203 (15) and the immune response to these fimbriae and to the *Shigella* live vector in guinea pigs and mice following orogastric (o.g.) or i.n. immunization.

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MATERIALS AND METHODS

Strains and medium. Wild-type *S. flexneri* 2457T, which was originally isolated in Japan from a patient with diarrhea and is fully virulent in volunteers (2), was grown on Trypticase soy agar with Congo red dye. Strain CVD 1203, a Δ*aroA* Δ*virG S. flexneri* 2a vaccine candidate derived from strain 2457T (15), was grown on CFA agar (5) supplemented with 10 mg of *p*-aminobenzoic acid per liter. The further derivative carrying the plasmids encoding CFA/I and CS3, strain CVD 1203(pJGX15A, pACCS3-1), was grown on *p*-aminobenzoic acid-supplemented CFA agar containing chloramphenicol (20 mg/liter) and carbenicillin (50 mg/liter). *E. coli* HS, a normal flora O9:H4 strain that lacks virulence traits and is well tolerated by volunteers in doses as high as 5×10^{10} CFU with buffer (10), was selected as the placebo control strain and was grown on Trypticase soy agar.

Expression of CFA/I and CS3 in CVD 1203. Strain CVD 1203 was electroporated in 10% glycerol–water using 0.2-cm cuvettes in a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) set at 2.5 kV and 400 Ω. After electroporation of pJGX15A, expression of CFA/I fimbriae was confirmed by agglutination and colony immunoblot with monospecific rabbit polyclonal antiserum prior to electroporation with pACCS3-1. After electroporation of pACCS3-1, expression of CS3 fibrillae was confirmed by agglutination and immunoblot with anti-CS3 rabbit antiserum. Coexpression of CFA/I and CS3 was confirmed by Western blotting (immunoblotting). Two identical sodium dodecyl sulfate (SDS)-polyacrylamide gels were electrophoresed and transferred to nitrocellulose paper in parallel: one blot was exposed to anti-CFA/I antiserum, and the other was exposed to anti-CS3 antiserum. Each antiserum was prepared by immunizing rabbits with purified fimbriae from the wild-type ETEC strain that expresses CFA/I fimbriae (strain H10407) or CS3 fibrillae (strain E9034A) (5).

o.g. and i.n. immunizations. To compare the immunogenicities of o.g. and i.n. immunizations with CVD 1203, Hartley strain guinea pigs (≥300 g) received o.g. CVD 1203 ($n = 6$), i.n. CVD 1203 ($n = 6$), or the *E. coli* HS control strain via

o.g. ($n = 6$) or i.n. ($n = 6$) inoculation, with two doses spaced 14 days apart. For studies of the immunogenicity of CVD 1203(pJGX15A, pACCS3-1) expressing CFA/I and CS3, guinea pigs received o.g. ($n = 6$) or i.n. ($n = 6$) immunization with two doses of vaccine, 14 days apart. CVD 1203 and CVD 1203(pJGX15A, pACCS3-1) were also administered by the o.g. or i.n. route to groups of mice ($n = 6$ per group) in two spaced doses given 14 days apart.

Overnight cultures were harvested by adding 10 ml of phosphate-buffered saline to each agar plate and gently resuspending the bacteria with a sterile cotton tip applicator. The bacterial suspension was brought to an optical density at 600 nm (OD_{600}) of 0.5 (equivalent to 5×10^8 CFU/ml) and concentrated by centrifugation to the desired concentration (1×10^{10} CFU/ml). For o.g. inoculation of guinea pigs, animals were fasted for 3 days before receiving 3 ml of a 2.5% sodium bicarbonate solution followed by 3 ml of bacterial suspension (10^{10} CFU/ml) via a flexible polyethylene catheter. Immediately thereafter, 1 ml of tincture of opium (10 mg of morphine) (Eli Lilly & Co., Indianapolis, Ind.) was administered intraperitoneally (3, 15). For o.g. inoculations of mice, 6-week-old BALB/c mice that were fasted for 1 day received 100 μl of sodium bicarbonate solution followed by 200 μl of bacterial suspension via a 21-gauge feeding needle. After inoculation, the animals were allowed to feed and drink ad libitum. The second immunization was given 14 days later, administered in the identical manner.

No preconditioning or anesthesia was employed for i.n. inoculations. Guinea pigs were given 100 μl of bacterial suspension i.n., whereas mice received 20 μl of the same suspension i.n.

Specimen collection. Tears were collected from guinea pigs by stimulation with flakes of *Capsicum bacatum* at days 0, 14, and 28. Blood was collected from guinea pigs by intracardiac puncture on day 28 at the time of euthanasia, whereas blood was obtained from mice (retro-orbital) on days 0, 14, and 28.

Antigens. *S. flexneri* 2a lipopolysaccharide (LPS) was prepared from strain 2457T by the hot water-phenol method (18). Purified CFA/I fimbriae from strain H10407 and CS3 fibrillae from strain E9034A were prepared as previously described (5).

ELISA. IgA antibodies against *S. flexneri* 2a LPS, CFA/I, and CS3 in tears were measured by enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-guinea pig IgA α-chain-specific antibody (Bethyl Laboratories, Inc., Montgomery, Tex.). Specific IgG LPS and antifimbrial antibodies in sera of mice and guinea pigs were determined by ELISA using a goat anti-mouse IgG (Sigma, St. Louis, Mo.) and a goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories, Gaitherburg, Md.) conjugate, respectively. The starting dilutions of samples were 1:25 for sera and 1:40 for tears. The final dilution considered positive had an OD value that was higher than 2 standard deviations above the mean of OD values obtained from 12 unimmunized mice and 12 unimmunized guinea pigs at the starting dilution (cutoff OD value). For statistical purposes, if the starting dilution of tears or serum was below the cutoff OD value, this was reported as the next lower dilution. A postexposure titer fourfold above the mean titer of the 12 unimmunized guinea pigs or mice was considered evidence of a significant seroconversion.

Guinea pig keratoconjunctivitis test (Sereny test). To assess the protective capacity of CVD 1203 administered by the i.n. route, the guinea pigs that received i.n. immunization with CVD 1203 or HS were challenged in the conjunctival sac with 5×10^7 CFU of wild-type strain 2457T, as described elsewhere (15, 17). After challenge, guinea pigs were examined daily for 5 days and the degree of inflammatory response was graded by an observer who was unaware of what preparation the guinea pigs had received.

Statistical methods. ELISA OD titers were log transformed and compared by Student's *t* test. In the Sereny test challenge study, 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

Mucosal IgA immune response to *S. flexneri* 2a LPS after o.g. or i.n. immunizations. The mucosal IgA antibody response to *S. flexneri* 2a LPS in tears of guinea pigs is shown in Table 1. Guinea pigs immunized with CVD 1203 by the o.g. route responded only after the second dose of vaccine (Table 1). In contrast, i.n. administration of CVD 1203 elicited high titers of anti-*S. flexneri* 2a LPS IgA in tears after just a single dose, with clear-cut evidence of a booster response after the second dose (Table 1). Despite the fact that the i.n. group received a lower dose of vaccine than the o.g. group (10^9 versus 10^{10} CFU), the reciprocal anti-LPS geometric mean titers (GMTs) for the i.n. group were significantly higher after either one dose (640 versus 20, $P = 0.0007$) or two doses (5,747 versus 844, $P = 0.006$). No anti-*S. flexneri* 2a LPS IgA was detected in the tears of the control animals inoculated with *E. coli* HS (not shown).

TABLE 1. Reciprocal titers of anti-*S. flexneri* 2a LPS IgA in tears of guinea pigs after two doses of CVD 1203 given o.g. or i.n.

Route[a]	Guinea pig no.[b]	Reciprocal anti-*S. flexneri* 2a LPS titer on day:		
		0	14	28
o.g.	19	<40	<40	1,280
	20	<40	<40	320
	21	<40	<40	1,280
	22	<40	<40	2,560
	23	<40	<40	1,280
	GMT	20	20	844
i.n.	12	<40	1,280	10,240
	13	<40	1,280	10,240
	14	<40	1,280	10,240
	15	<40	320	5,120
	16	<40	80	5,120
	17	<40	1,280	1,280
	GMT	20	640[c]	5,747[d]

[a] o.g. and i.n. doses were 3×10^{10} and 3×10^9 CFU, respectively, at days 0 and 14.
[b] Guinea pig 24 died during handling at the first immunization.
[c] *t* test (two tailed), o.g. versus i.n., $P = 0.0007$.
[d] *t* test (two tailed), o.g. versus i.n., $P = 0.0067$.

Sereny test challenge of guinea pigs immunized with i.n. CVD 1203. The ability of two spaced 10^{10}-CFU doses of CVD 1203 administered o.g. to protect guinea pigs against keratoconjunctivitis following Sereny test challenge with wild-type *S. flexneri* 2a has been previously shown (15). To investigate whether two 10^9-CFU doses of CVD 1203 administered i.n. can also protect against Sereny test challenge, on day 28 the six i.n. Δ*aroA* Δ*virG* CVD 1203-vaccinated guinea pigs and the six i.n. normal flora HS placebo-vaccinated control guinea pigs were challenged in their conjunctival sacs with 7×10^7 CFU of wild-type *S. flexneri* 2a strain 2457T in 10 μl. Full-blown keratoconjunctivitis developed in all six control animals versus only one of six guinea pigs immunized with CVD 1203 ($P = 0.008$, Fisher's exact test), a protective efficacy of 83%.

Coexpression of ETEC colonization factors CFA/I and CS3 in CVD 1203. After transformation of CVD 1203 with pJGX15A (containing the CFA/I operon) and pACCS3-1 (containing the CS3 operon), concomitant expression of both ETEC CFAs was demonstrated by agglutination with the respective specific antibodies, colony blotting, and Western blotting (Fig. 1). As previously reported with an *S. typhi* live vector (4), the CFA/I and CS3 antigens are expressed on the surface of attenuated Δ*aro S. flexneri* cells as fimbriae and fibrillae, respectively, demonstrated by immune electron microscopy (data not shown).

Mucosal immune response against *Shigella* LPS and ETEC CFA/I and CS3 after o.g. or i.n. immunizations. In the groups of guinea pigs that received two o.g. doses of CVD 1203 (pJGX15A, pACCS3-1), after the second dose a specific IgA immune response against *S. flexneri* 2a LPS and CFA/I was detected in tears, whereas no IgA anti-CS3 responses were observed (Table 2). In contrast, in guinea pigs immunized by the i.n. route, rises in IgA antibody to CS3 as well as to CFA/I and to *S. flexneri* 2a LPS were detected. Moreover, the responses in animals immunized i.n. were observed after the first dose of vaccine, with a further increase recorded following administration of the second dose (Table 2). One guinea pig (no. 8) showed a high titer of anti-LPS IgA at day 0. An error in technique or sample handling could not be ruled out, as there was not enough sample to be reassayed. The highest

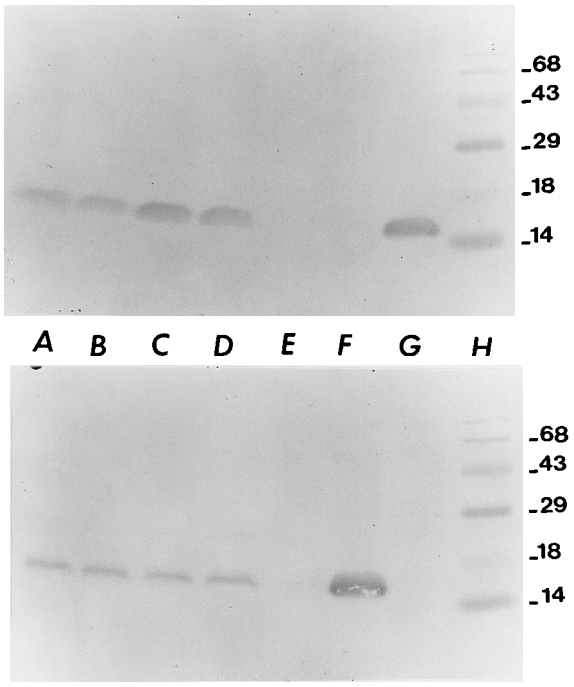

FIG. 1. Two identical SDS–10% polyacrylamide gels were electrophoresed and transferred to nitrocellulose paper in parallel. Strains: A to D, clones of Δ*aroA* Δ*virG S. flexneri* 2a CVD 1203(pJGX15A)(pACCS3-1) expressing CFA/I and CS3; E, strain CVD 1203; F, wild-type ETEC strain E9034A that expresses CS3 (5); G, wild-type ETEC strain H10407 that expresses CFA/I (5); H, prestained molecular weight markers (positions indicated in thousands on the right). The top blot was exposed to anti-CFA/I antibody, and the bottom blot was exposed to anti-CS3 antibody. Both antibodies were developed in rabbits with purified pili from the wild-type ETEC strains.

dilution tested for most of this experiment was 1:1,280; repeat assays to determine end-point titers for statistical comparison between the o.g. and i.n. groups was not possible because of the scarcity of material (tears). Likewise, there were not sufficient tears to determine the immune response against all the antigens at all time points in all the animals.

Serum IgA and IgG immune response against *S. flexneri* 2a LPS and ETEC CFA/I and CS3 with CVD 1203(pJGX15A, pACCS3). Evidence of serum antibody responses to the foreign antigens and to the live vector were evident following mucosal immunization by the two different routes in the two animal species. The serum IgA antibody titers against *S. flexneri* 2a LPS and CFA/I in guinea pigs immunized with CVD 1203(pJGX15A, pACCS3-1) were elevated on day 28 in all animals, with a trend (nonsignificant) towards higher titers in animals vaccinated i.n. (Table 3). The serum IgA titers to CS3 fibrillae differed more sharply, in accordance with the route of vaccine administration. Only three of six animals immunized o.g. manifested elevated titers to CS3, compared with five of five guinea pigs immunized i.n., and the GMTs were significantly different (71 versus 2,786, $P = 0.0031$) (Table 3). In mice, the effect of route of vaccine administration upon the serum IgA antibody response to the ETEC antigens expressed in CVD 1203 was even more striking (Table 4). Few animals immunized o.g. mounted modest rises in serum IgA antibodies against CFA/I or CS3 (Table 4). In contrast, significantly higher serum IgA titers were obtained against these two antigens after two i.n. immunizations (Table 4).

The serum IgG antibody response to the LPS antigen of the live vector was not detected in both guinea pigs and mice (Tables 3 and 5). However, the patterns of serum IgG antibody responses against the ETEC fimbriae (Tables 3 and 5) paralleled those of the IgA responses (Tables 3 and 4). Again, in guinea pigs and mice, the i.n. groups responded with serum IgG anti-CFA/I and anti-CS3 titers that were superior than those detected in the group that received the inoculum o.g. (Table 3 and 5).

DISCUSSION

Spurred by the report of Orr et al. (16), who observed that nonliving *Shigella* antigens were more immunogenic when administered i.n. rather than by the oral route, we undertook to characterize further the immunogenicity of attenuated *S. flex-*

TABLE 2. Reciprocal titers of anti-*S. flexneri* 2a LPS or anti-ETEC CFA/I and CS3 IgA in tears of guinea pigs after two doses of CVD 1203(pJGX15A, pACCS3-1) given o.g. or i.n.

Route[a]	Guinea pig no.[b]	Anti-LPS titer on day:			Anti-CFA/I titer on day:			Anti-CS3 titer on day:		
		0	14	28	0	14	28	0	14	28
o.g.	1	<40	<40	640	<40	<40	≥1,280	<80	<80	<80
	2	<40	<40	160	<40	<40	≥1,280	ND[c]	ND	80
	4	ND	ND	ND	<40	<40	≥1,280	ND	ND	80
	5	<40	<40	80	<40	<40	640	ND	ND	ND
	18	ND	ND	ND	<40	40	320	<80	<80	80
	GMT	20	20	201	20	23	844	40	40	67
i.n.	7	<40	≥1,280	≥1,280	<40	640	≥1,280	ND	ND	ND
	8	≥1,280	≥1,280	≥1,280	<40	160	≥1,280	ND	ND	ND
	9	<40	≥1,280	≥1,280	<40	320	≥1,280	ND	80	2,560
	10	ND	ND	ND	<40	≥1,280	≥1,280	ND	≥1,280	10,240
	11	ND	ND	ND	<40	640	≥1,280	ND	2,560	5,120
	GMT		1,280	1,280	20	485[d]	1,280[e]		640[f]	5,120[g]

[a] o.g. and i.n. doses were 3×10^{10} and 3×10^{9} CFU, respectively, at days 0 and 14.
[b] Tears from guinea pig 3 were accidentally lost. Guinea pig 6 died because of handling during the first immunization.
[c] ND, not determined.
[d] *t* test (two tailed), o.g. versus i.n., $P = 0.0004$.
[e] *t* test (two tailed), o.g. versus i.n., $P = 0.208$.
[f] *t* test (two tailed), o.g. versus i.n., $P = 0.120$.
[g] *t* test (two tailed), o.g. versus i.n., $P = 0.003$.

TABLE 3. Reciprocal titers of anti-*S. flexneri* 2a LPS or anti-ETEC CFA/I and CS3 antibodies in guinea pig serum 28 days after o.g. or i.n. immunization with attenuated *Shigella* strain CVD 1203(pJGX15A, pACCS3-1)

Route[a]	Animal no.	Reciprocal titer[b] of:					
		IgA			IgG		
		Anti-LPS	Anti-CFA/I	Anti-CS3	Anti-LPS	Anti-CFA/I	Anti-CS3
o.g.	1	100	1,600	<25	<25	1,600	<25
	2	100	200	<25	<25	3,200	25
	3	200	200	25	<25	800	160
	4	200	800	800	<25	3,200	3,200
	5	50	400	200	25	3,200	800
	18	100	400	200	<25	1,600	800
	GMT	112.2	449	71	14	1,796	178.2
i.n.	7	400	1,600	1,600	<25	51,200	51,200
	8	100	3,200	3,200	25	25,600	12,800
	9	200	800	3,200	<25	12,800	25,600
	10	800	800	≥3,200	<25	12,800	51,200
	11	200	200	3,200	<25	12,800	25,600
	GMT	263.9[c]	919[d]	2,786[e]	14	19,401[f]	29,407[g]

[a] o.g. and i.n. doses were 3×10^{10} and 3×10^{9} CFU, respectively, at days 0 and 14.
[b] Individually tested sera from 12 unimmunized guinea pigs were consistently below the cutoff OD value for IgG and IgA at a dilution of 1:25 for the three antigens tested.
[c] *t* test (two tailed), o.g. versus i.n., $P = 0.0.078$.
[d] *t* test (two tailed), o.g. versus i.n., $P = 0.243$.
[e] *t* test (two tailed), o.g. versus i.n., $P = 0.0031$.
[f] *t* test (two tailed), o.g. versus i.n., $P = 0.00014$.
[g] *t* test (two tailed), o.g. versus i.n., $P = 0.0019$.

neri 2a strain CVD 1203 as a live *Shigella* vaccine and as a live vector vaccine administered by the i.n. route. Utilized as a potential live *Shigella* vaccine without foreign antigens, CVD 1203 elicited significantly better IgA anti-LPS responses in tears when administered by the i.n. route, even though each i.n. dose contained 1 log fewer vaccine organisms (10^9 versus 10^{10} CFU). These results with live *Shigella* vaccine corroborate those of Orr et al. (16), who used nonliving vaccine consisting of meningococcus group B proteosomes containing *Shigella sonnei* or *S. flexneri* 2a LPS. Moreover, we show that guinea pigs immunized i.n. with CVD 1203 are significantly protected against Sereny test challenge. The experience, so far, with attenuated but epithelial cell-invasive *Shigella* vaccine strains is that they are well tolerated and immunogenic at moderate dose levels but become reactogenic at higher dose levels administered in attempts to enhance immunogenicity (6). It is conceivable that in the future the concept of a common mucosal immune system (13) will allow a strategy whereby i.n. immunization of humans with attenuated *Shigella* vaccines will lead to mucosal responses in the distal intestine. If so, it may be possible to avoid gastrointestinal adverse reactions yet stimulate protective mucosal immunity by i.n. immunization with relatively low doses of live *Shigella* vaccine.

The i.n. route of administration is becoming increasingly popular in experimental animal models to elicit an immune response either in the airway mucosa or in distal mucosal sites. Studies with diverse antigens in various animal species have demonstrated that the i.n. route of administration can elicit a broad immune response, including serum, salivary, nasal, rectal, and vaginal antibodies, that is often superior to responses obtained after oral immunizations (7, 14, 16).

We also investigated whether it is possible to stimulate immune responses to foreign antigens expressed in the attenuated *Shigella* strain when this is administered i.n. While both routes of administration succeeded in eliciting secretory IgA in tears and IgA and IgG antibodies in serum, the i.n. route was superior. Obviously, considerable preclinical safety tests will have to be completed in animal models before i.n. immunization of humans can be considered. Histopathologic studies in animals have to be performed to assess the extent of invasion of these attenuated organisms in the nasal mucosa. After that, safety concerns will have to be addressed in volunteers, perhaps with otorhinolaryngologists and pathologists determining the presence and extent of any inflammatory response in the human nasal mucosa. Nevertheless, this appears to be an avenue of investigation worth exploring. Indeed, i.n. administered, live, attenuated, cold-adapted, influenza virus vaccines have been well tolerated, immunogenic, and protective in extensive clinical trials (1). As with oral immunization, the i.n. route circumvents the need for specially trained personnel and instruments, avoids discomfort, and precludes the risk of disease transmission associated with parenteral administration.

ETEC and *Shigella* spp. are both human pathogens, and vaccines developed to protect against these organisms will have to demonstrate their efficacy in clinical studies. We have promulgated a live vector vaccine approach to develop an ETEC vaccine that can confer broad-spectrum protection (9, 11, 12). This involves expressing the most prevalent ETEC colonization factor fimbrial antigens and heat-labile enterotoxin toxoid (B subunit) in a suitable bacterial live vector vaccine (9, 11, 12). Ultimately, such a vaccine will have to include CFA/I, CFA/II (CS1-3), and CFA/IV (CS4-6) fimbrial antigens (11). The use of attenuated *Shigella* strains holds particular attraction for use as a live vector in this situation, as we believe that it is possible to prepare a multivalent vaccine that will provide broad-spectrum protection against the most important *Shigella* serotypes and the majority of ETEC strains. As a step towards that ultimate goal, we have shown that it is possible to coexpress in attenuated *Shigella* cells two fimbrial antigens that are never found together in the same bacterium in nature. Moreover, the expressed ETEC fimbrial antigens were successfully delivered to the mucosal immune system of guinea pigs, leading to prom-

TABLE 4. Reciprocal titers of serum anti-CFA/I and anti-CS3 IgA in mouse serum 28 days after o.g. or i.n. immunization with attenuated *Shigella* strain CVD 1203(pJGX15A, pACCS3-1)

Route	Animal no.	Reciprocal titer[a]	
		Anti-CFA/I	Anti-CS3
o.g.	A2-1	<25	<25
	A2-2	<25	<25
	A2-3	<25	<25
	B2-1	25	50
	B2-2	<25	<25
	B2-3	<25	<25
	GMT	14.03	15.75
i.n.	A1-1	100	200
	A1-2	200	200
	A1-3	200	100
	B1-1	100	200
	B1-2	100	200
	B1-3	200	50
	GMT	141.03[b]	141.42[c]

[a] Preimmunization sera from each of the 12 mice gave readings consistently below the cutoff OD value for IgA at a dilution of 1:25 for the two antigens tested.
[b] *t* test (two tailed), o.g. versus i.n., $P = 0.0000006$.
[c] *t* test (two tailed), o.g. versus i.n., $P = 0.00006$.

TABLE 5. GMTs of IgG antibodies against *S. flexneri* 2a LPS or ETEC CFA/I and CS3 in murine serum following o.g. or nasal immunizations

Strain	Route (n = 6)	GMT								
		Anti-*S. flexneri* 2a LPS on day:			Anti-CFA/I on day:			Anti-CS3 on day:		
		0	14	28	0	14	28	0	14	28
CVD 1203(pJGX15A, pACCS3)	o.g.	12.5	14	15.7	12.5	14	28	12.5	19.8	35.4
	i.n.	12.5	12.5	22.3	12.5	89.1[a]	2,015.9[b]	12.5	14.9[c]	3,200[d]
CVD 1203	o.g.	12.5	12.5	12.5	—[e]	—	12.5	—	—	12.5
	i.n.	12.5	14	25	—	—	12.5	—	—	12.5

[a] t test (two tailed), o.g. versus i.n. at 14 days, $P = 0.00007$.
[b] t test (two tailed), o.g. versus i.n. at 28 days, $P = 0.0002$.
[c] t test (two tailed), o.g. versus i.n. at 14 days, $P = 0.476$.
[d] t test (two tailed), o.g. versus i.n. at 28 days, $P = 0.0003$.
[e] —, not determined.

inent antibody responses to the foreign antigens and to the live vector (Tables 1 and 3 to 6). On the basis of these encouraging preliminary results, we are proceeding to clone and express other ETEC fimbrial antigens in other epidemiologically important serotypes of attenuated *Shigella* species such as *S. sonnei* and *S. dysenteriae* 1 in progressing towards a multivalent *Shigella*-ETEC hybrid vaccine.

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