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Cell-Associated Hemagglutinin-Deficient Mutant of *Vibrio cholerae*

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Cell-associated hemagglutinin-negative mutants were derived from cholera enterotoxin-negative *Vibrio cholerae* JBK70 by Tn*5* mutagenesis. One of the mutants identified, SB001, was characterized in greater detail. Its ability to colonize ilea of adult rabbits was determined by feeding approximately 10^8 *V. cholerae* to each animal. At 17 h after feeding, the numbers of viable vibrios in the ilea were determined. There was a significant, 4 log, decrease in the ability of the hemagglutinin-negative mutant to colonize ileal tissue compared with the parent strain JBK70. In addition, the higher levels of colonization attained by JBK70 and the wild-type parent of JBK70, N16961, were associated with intestinal fluid accumulation and death. Rabbits immunized orally with approximately 10^8 SB001, when challenged 3 weeks later with either homologous biotype and serotype El Tor Inaba N16961 or heterologous Classical Ogawa 395, were protected to the same extent as those animals immunized with either the challenge strain or JBK70. This was evidenced by decreases in both the number of animals showing detectable colonization and the level of colonization achieved. A hemagglutinin-negative mutant of *V. cholerae* may therefore be of potential use as a live oral vaccine against cholera.

Much effort has been devoted to the development of an effective vaccine against cholera (5). This diarrheal disease, caused by *Vibrio cholerae* O1 of either the El Tor or Classical biotype, is capable of causing severe dehydration and death in affected individuals. Oral and intravenous rehydration therapies have been very effective in decreasing the fatality rate (25), but in many less-developed countries patients may not be within reach of treatment facilities.

Parenterally administered whole-cell and toxoid vaccines have been shown to be largely ineffective at producing lasting immunity to cholera, presumably because they are incapable of stimulating an efficient intestinal immune response (3, 5). In contrast, cholera induced experimentally by feeding of virulent *V. cholerae* results in almost complete protection against reinfection (3, 18), demonstrating the desirability of oral vaccination.

Recently, attenuated live oral candidate vaccine strains have been developed. These are based on the concept of eliminating the activity of cholera toxin, which is responsible for the massive fluid outpouring characteristic of the disease. The first of these, Texas Star-SR, isolated after chemical mutagenesis (11), produces only the nontoxic binding subunit (B) of the toxin molecule but not the enzymatically active subunit (A). However, 24% of human volunteers fed this organism experienced slight diarrhea in a non-dose-dependent manner (17).

Subsequently, molecular cloning of the cholera toxin genes has allowed specific deletions in the toxin genes of pathogenic strains of *V. cholera* (15, 16, 20). When fed to volunteers, one of these, JBK70 ($A^- B^-$) was highly protective against subsequent challenge but induced mild diarrhea in 40% of vaccinees. Similarly, vaccine candidate strain CVD101 ($A^- B^+$) also caused diarrhea in some vaccinees (14). The precise reason for the reactinogenicity of these attenuated strains in humans is unknown but may be related to heavy colonization of the small intestine by these vibrios.

Early work involving attachment of *V. cholerae* to erythrocytes (RBCs) (G. L. Bales and C. E. Lankford, Bacteriol. Proc., abstr. no. M64, p. 118, 1961) and rabbit intestine (2, 6, 12) suggested that *V. cholerae* hemagglutinins (HA) may serve as possible mediators of attachment to intestinal epithelial cell surface receptors. Hanne and Finkelstein (10) have described four distinct HA. These may be divided into two main classes: cell associated and soluble. All strains of *V. cholerae* which were analyzed produced the soluble HA, whereas El Tor Biotype strains also produced a mannose-sensitive cell-associated HA (10). Studies by Finkelstein and Hanne (7) demonstrated that the purified soluble HA has the ability to agglutinate RBCs and inhibit attachment of *V. cholerae* to intestinal epithelium.

To date, a limited amount of work has been done on the mannose-sensitive HA of *V. cholerae* El Tor strains (2, 10, 13). We sought to determine the effect that HA deficiency would have on the ability of *V. cholerae* to attach to small intestine and the capacity of such strains to evoke protective immunity against challenge with pathogenic *V. cholerae*. Specific HA-negative mutants were derived from transposon Tn*5*-treated cultures of JBK70. Such mutants were analyzed for their ability to adhere to rabbit ileum and their ability to elicit a protective local immune response against subsequent oral challenge with pathogenic *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* El Tor Inaba N16961 and Classical Ogawa 395, supplied by M. Levine (Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Md.), have been previously characterized in volunteer studies (18). *V. cholerae* JBK70, which lacks the cholera toxin gene and was derived from N16961, was obtained from J. Kaper (Center for Vaccine Development). All strains were maintained in lyophilized form. *Escherichia coli* HU735 (F' [Ts] *lac*$^+$ *trp*$^+$ Tn*5*) was obtained from R. Holmes (Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Md.) and maintained in brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) with 22% glycerol. *V. cholerae* SB001, a cell-associated HA-deficient strain, was derived from JBK70 after Tn*5* mutagenesis (see below).

Media. BHI agar and broth were used routinely for *V. cholerae*. For isolation of Tn*5* inserts into JBK70, minimal

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medium was used. This contained (per liter) Difco agar, 15.0 g; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; trisodium citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glucose, 0.2%; and vitamin B1, 0.02 g. Agar and salts were autoclaved separately. $MgSO_4 \cdot 7H_2O$, glucose, and vitamin B1 were added afterward. Kanamycin (Sigma Chemical Co., St. Louis, Mo.) was used at 50 μg/ml. For isolation of HA-deficient strains, tryptic soy broth (Difco) was used. For quantitation of *V. cholerae* in feces, material was plated onto thiosulfate-citrate-bile salt agar (Difco). The API-20E system (API International S.A., Geneva, Switzerland) was used to characterize *V. cholerae* strains biochemically.

Animals. Locally supplied New Zealand White rabbits weighing 2.0 to 2.5 kg were used.

RBCs. Human RBCs were obtained from blood group O volunteers. The samples were collected in 3.8% sodium citrate, washed three times in HA assay buffer (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.9% NaCl), and suspended to an estimated concentration of 1% packed-cell volume in HA assay buffer before use.

Mutagenesis of *V. cholerae*. Tn*5* mutagenesis of *V. cholerae* JBK70 was carried out as described by Newland et al. (21). Samples (100 μl) of an overnight culture of JBK70 and HU735 (F' [Ts] *lac*$^+$ *trp*$^+$ Tn*5*) were spread on BHI plates and incubated at 30°C overnight. JBK70 transconjugants were isolated by plating onto minimal medium containing kanamycin and incubation at 30°C overnight. The cells were harvested and subcultured in BHI with kanamycin overnight. These cultures were diluted 1:100 with fresh BHI without antibiotic and incubated at 40°C for 6 to 8 h. JBK70::Tn*5* colonies were isolated on BHI agar containing kanamycin, pooled, and stored in 22% glycerol before assay for HA activity.

Isolation of HA-deficient mutants. Colonies of *V. cholerae* JBK70 harboring a Tn*5* insertion were identified by plating onto tryptic soy broth agar plates containing kanamycin. After 18 h of incubation at 37°C, individual colonies were inoculated into 1.5 ml of medium contained in a 24-well microtiter plate (Greiner, Nürtingen, Federal Republic of Germany). These plates were incubated for 18 to 20 h at 37°C with shaking. The cultures were transferred into plastic Microfuge tubes. The cells were pelleted by centrifugation at 4,000 × *g* for 10 min. The bacterial pellets were washed in 1 ml of HA assay buffer and suspended to the original volume in HA assay buffer. These bacterial suspensions were assayed for their ability to agglutinate human group O RBCs. Isolates which were negative were then plated for single colonies onto tryptic soy broth agar plates containing kanamycin. Several (n = 5 to 10) colonies were used to inoculate 10-ml cultures of tryptic soy broth (plus kanamycin) individually in a 100-ml Erlenmeyer flask. These cultures were grown for 18 h at 37°C with shaking, and the cells were tested for HA activity. Strains which remained negative were frozen at −20°C in 10% skim milk before lyophilization.

Rabbit inoculation. To determine the ability of *V. cholerae* to colonize small intestines, we used a rabbit oral-feeding model described in detail by Cray et al. (4), with slight modifications. A log-phase culture of *V. cholerae* was sedimented at 5,000 × *g* and suspended to the original volume in phosphate-buffered saline (PBS). The A_{540} was determined (an A_{540} of 0.25 = approximately 2×10^8 *V. cholerae* per ml), and the bacteria were diluted appropriately in PBS. Each rabbit inoculum contained 1 ml of the appropriate bacterial dilution in PBS and 9 ml of BHI medium. Groups of four rabbits were fasted overnight and sedated. Gastric acidity was neutralized at time zero with intravenous cimetidine (50 mg/kg; Smith, Klein + Dauelsberg, Göttingen, Federal Republic of Germany, and 15 min later, 15 ml of 5% $NaHCO_3$ was given by intragastric intubation. At time 30 min, 15 ml $NaHCO_3$, immediately followed by the 10-ml bacterial inoculum, was given. After a further 30 min, 2 ml of tincture of opium was given intraperitoneally. Rabbits were returned to their cages and given free access to food and water.

Quantitation of intestinal colonization. At 17 h after feeding, rabbits were killed by intravenous administration of phenobarbital, and a peritoneal incision was made. The gross pathology was noted, and a 10-cm portion of the ileum, 5 cm cephaled from the mesoappendix, was removed. These segments were cut longitudinally, cleaned of solid intestinal contents on tissue paper, and then washed vigorously twice in 70 ml of cold PBS. After the excess PBS had been drained off, segments were weighed and kept in BHI broth on ice before homogenization. Tissue homogenization was done for 3 × 15 s (Kinematica GmbH, Kriens, Switzerland). Viable counts were determined by plating onto BHI agar plates and incubation overnight at 37°C. Any rare non-*V. cholerae* were easily distinguishable from the *V. cholerae* colonies.

Intragastric immunization studies. Groups of four rabbits were immunized orally with approximately 10^8 *V. cholerae* N16961, JBK70, 395, or SB001 by the above-described technique. At 3 weeks after immunization, a challenge dose of *V. cholerae* N16961 or 395 sufficient to result in a high level of colonization was given by gastric intubation as described above. The levels of intestinal colonization were determined on ileal segments 17 h after challenge as described above.

Duration of intestinal colonization. The number of *V. cholerae* in fresh fecal material was determined 1 to 9 days after intragastric inoculation as follows. BHI medium (1 ml) was added to 1 g of feces and, after disruption with a cotton-tipped swab which was then used to inoculate a thiosulfate-citrate-bile salt agar plate, 9 ml of PBS was added to the fecal suspension. A sample (100 μl) of this resultant 10^{-1} dilution was plated onto thiosulfate-citrate-bile salt agar. Any resultant *V. cholerae* colonies after 18 h at 37°C were noted and counted.

Statistics. Statistical analysis was performed by using Student's t test on geometric mean counts of *V. cholerae* recovered from the intestinal segments.

RESULTS

Isolation of HA-negative mutants. *V. cholerae* JBK70 containing Tn*5* inserts were selected on medium containing kanamycin. A total of 1,344 colonies were tested for their ability to agglutinate human RBCs, resulting in the isolation of 22 putative HA-negative mutants. All of these showed an API-20E pattern identical to that of the parent JBK70, and all were motile. One of these mutants, SB001, was studied in greater detail.

Intestinal colonization studies. The abilities of wild-type *V. cholerae* N16961, its toxin-deficient derivative JBK70, and the HA-negative mutant SB001 to colonize the small intestine were evaluated in an adult-rabbit model. The levels of colonization are shown in Fig. 1.

V. cholerae N16961 and JBK70 exhibited comparable degrees of colonization, with about 10^8 viable vibrios per g of ileum. In contrast, the HA-negative strain SB001 was limited in its ability to colonize or multiply in this model system, as evidenced by bacterial counts 4 orders of magnitude less than those of strain N16961 or JBK70 ($P < 0.01$).

TABLE 1. Reactions noted in rabbits after oral administration of various *V. cholerae* strains[a]

V. cholerae strain administered	Phenotype	Animals with intestinal fluid accumulation (%)[b]	Deaths (%)[c]
N16961	Parent wild-type Inaba, El Tor	83	43
JBK70	Enterotoxin deficient	25	20
SB001	Enterotoxin deficient, HA deficient	0	0

[a] Rabbits (minimum of 12 animals per strain) were orally fed doses of bacteria ranging from 5×10^7 to 5×10^8.
[b] Fluid accumulation was determined by examining the intestines of sacrified or dead animals. Percentage refers to the total number of animals per experimental group.
[c] Death always occurred within 20 h postfeeding. Percentage refers to the total number of animals per experimental group.

The high levels of colonization achieved by strains N16961 and JBK70 were associated with pronounced intestinal fluid accumulation, in some instances resulting in death. These effects were absent in rabbits fed strain SB001 (Table 1). It is important to note that fluid accumulation and death were the result of infection with strain JBK70 even though this strain does not synthesize classical cholera toxin.

Carriage of *V. cholerae*. Fecal shedding of *V. cholerae* after intragastric feeding was monitored for 9 days. Wild-type strain N16961 and toxin-negative JBK70 could be detected in the feces of 50% of the rabbits at levels up to 6.6×10^3 vibrios per g of feces for up to 5 days postfeeding. The HA-deficient strain SB001 was found at levels of up to 3×10^3 per g of feces in 50% of the rabbits up to 24 h postfeeding, but after 48 h it could not be detected.

Immunization against intestinal colonization. The ability of the HA-negative strain SB001 to prevent colonization by wild-type *V. cholerae* when administered as an oral immunogen was compared with those of strains N16961 and JBK70 (Table 2). All three strains were equally effective against a homologous serotype challenge strain (N16961, Inaba, El Tor). A single immunizing dose reduced the number of colonizing challenge bacteria more than 6 logs from a mean of 2.5×10^8 per g of ileum to less than 10^2 per g. In approximately 60% of the immunized animals, challenge organisms were unable to establish themselves in the ileum, whereas 100% of control animals were colonized. Similar results were obtained when a heterologous challenge strain (395, Ogawa, Classical) was used. The mean number of bacterial counts per g of ileum was decreased from 2×10^4 in the control groups to 3.5×10^1 to 1.6×10^2 for the immunized groups. Colonization was reduced from 100% of the control animals to between 33 and 50% in the immunized groups. It is important to note that there were no deaths nor evidence of fluid accumulation in immunized animals colonized after challenge.

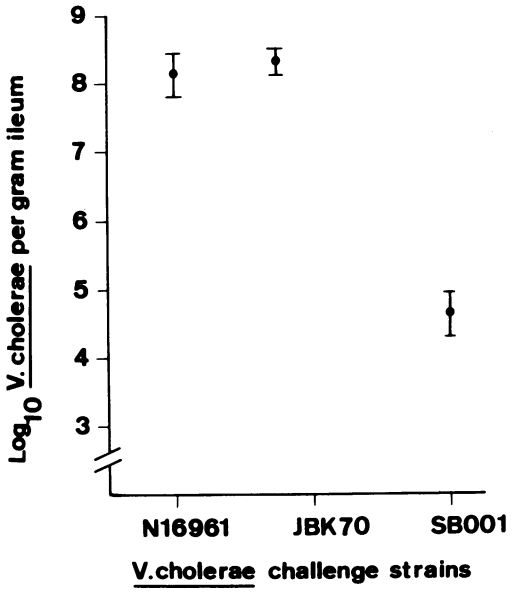

FIG. 1. Colonization of rabbit ileum 17 h after oral administration of 5×10^8 *V. cholerae* N16961, JBK70, or SB001. Each point represents the mean number of *V. cholerae* per g of ileum, with the standard error of the mean, for groups of 8 to 11 animals per test strain.

DISCUSSION

Colonization of the small intestine by *V. cholerae* is an essential first step in the infectious process which leads to cholera (5). This process appears to be multifactorial, involving motility (9, 24), flagellar antigens (1), chemotaxis (8), protease production (23), soluble HA (7), and cholera enterotoxin (22). The aim of the present study was twofold: (i) to determine what role cell-associated HA plays in colonization and (ii) to evaluate the vaccine potential of an HA-negative mutant strain administered orally. Any strain of *V. cholerae* producing active enterotoxin would be unsuitable for consideration as a live oral vaccine. Therefore, we used *V. cholerae* JBK70, a strain in which the cholera toxin genes have been deleted (15), from which to isolate mutants of the desired phenotype.

Using a mutant strain derived from JBK70, termed SB001, we were able to document the important role played by El Tor cell-associated HA in the colonization of adult-rabbit

TABLE 2. Prevention of small-bowel colonization by *V. cholerae* after oral immunization

Immunizing strain[a]	Challenge with *V. cholerae* N16961 (Inaba El Tor)[b]		Challenge with *V. cholerae* 395 (Ogawa Classical)[b]	
	Mean CFU/g (range) of ileum	No. of rabbits colonized/total (%)	Mean CFU/g (range) of ileum	No. of rabbits colonized/total (%)
None	2.5×10^8 (6.8×10^7–5.5×10^8)	4/4 (100)	2.1×10^4 (2.2×10^2–2.4×10^6)	4/4 (100)
N16961	5.7×10^1 (<25–7.5×10^3)	3/7 (43)	ND[d]	
395	ND[d]		3.5×10^1 (<25–8.2×10^1)	1/3 (33)
JBK70	3.9×10^1 (<25–5.8×10^2)	2/7 (29)	1.6×10^2 (<25–7.1×10^3)	1/3 (33)
SB001	9.7×10^1 (<25–2.1×10^3)	3/8 (38)	5.3×10^1 (<25–3.4×10^2)	2/4 (50)

[a] Rabbits were orally immunized on day zero with approximately 10^8 bacteria.
[b] Rabbits were challenged orally on day 21 with 5×10^8 *V. cholerae* N16961 or 3×10^8 *V. cholerae* 395.
[c] The limit of detection was 25 CFU/g of tissue.
[d] ND, Not done.

ileum. Strain SB001 displayed markedly reduced capacity to adhere to rabbit ileal tissue as compared with a wild-type strain of *V. cholerae* and its cholera toxin-negative derivative. This limited colonization correlated with an absence of severe adverse reactions, such as intestinal fluid accumulation and death, seen in animals fed either a toxinogenic or a nontoxinogenic strain of *V. cholerae*. The precise cause of these reactions after oral inoculation cannot be stated with certainty. However, roughly 10% of human volunteers fed a heat-labile and heat-stable enterotoxin-deficient strain of *E. coli* with an unimpaired ability to colonize small-intestinal mucosa developed mild diarrhea (19). Therefore, high numbers of bacteria multiplying in the usually sterile ileal environment may disrupt the local balance to induce fluid outpouring.

The current study points to only a limited role for cholera toxin in the initial stages of the infectious process, since the toxin-negative strain JBK70 colonized as well as its toxinogenic parent. This is in contrast to prior results reported by Pierce and co-workers (22), who noted that the degree of colonization by a nontoxinogenic strain of *V. cholerae* was significantly less than that seen when purified toxin was simultaneously administered with the bacterial challenge. This difference was far more pronounced at challenge doses several orders of magnitude less than those currently used and may account for the divergent findings.

Of critical importance was the fact that rabbits immunized with the HA-negative strain were refractive to colonization upon subsequent challenge with virulent strains of *V. cholerae*. Protection extended to both serotypes and biotypes of *V. cholerae*. Furthermore, the protective capacity of strain SB001 was comparable to those of both the wild-type strain N16961 and its toxin-negative derivative JBK70. Therefore, the mutation responsible for the HA-negative phenotype does not appear to decrease the immunizing potential of SB001 substantially, at least at the doses used by us.

Attempts to use enterotoxin-negative strains of enteric pathogens derived from genetic manipulation as live oral vaccines have been plagued by an unacceptable rate of adverse reactions in human volunteers (17). This problem may be circumvented by the finding that SB001 can evoke a protective immune response even though it possesses a limited capacity to colonize the ileum. We are in the process of attempting to clone the cell-associated HA with the goal of deleting the relevant gene(s) to create an attenuated strain suitable for evaluation in human volunteers.

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