Role of Cholera Toxin in Enteric Colonization by *Vibrio cholerae* O1 in Rabbits

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The role of cholera toxin (CT) in mucosal colonization by *Vibrio cholerae* O1 was studied in rabbits by using toxinogenic *V. cholerae* and nontoxinogenic (A⁻B⁺ or A⁻B⁻) recombinant mutants derived from them. After oral inoculation, toxinogenic strains colonized intestinal mucosa significantly more efficiently than did either A⁻B⁻ or A⁻B⁺ mutants; average colonization was increased 1.5- to 30-fold with toxinogenic strains, depending on the inoculum used and the portion of intestine studied. Additionally, colonization by an A⁻B⁻ mutant was increased to the levels of its toxinogenic parent by coadministration of CT with the inoculum. We conclude that CT contributes significantly to mucosal colonization by *V. cholerae* and that this effect is not due to an interaction of the CT B subunit with its mucosal receptor. The possibility that this effect contributes to the in vivo selection of hypertoxinogenic variants of *V. cholerae* is considered.

Colonization of intestinal mucosa by *Vibrio cholerae* is a necessary step in the pathogenesis of cholera, but the mechanisms by which colonization occurs are poorly understood. Previous reports described several factors that appear to promote colonization; these include bacterial motility (6, 16), flagellar antigen (1), bacterial chemotaxis for the mucosal surface (5), elaboration of protease (mucinase) (15), and production of certain hemagglutinins (4). The possibility that cholera toxin (CT) contributes to *V. cholerae* virulence by promoting mucosal colonization, in addition to causing mucosal secretion of water and electrolytes, has been suggested (14) but not proven. We addressed this possibility by comparing the ability of toxinogenic and nontoxinogenic *V. cholerae* to colonize rabbit intestinal mucosa; the mutants used were prepared by recombinant DNA techniques and differed from their virulent parents only in lacking genes for the production of CT or its A subunit. Our results revealed that CT, but not the B subunit of CT, contributes appreciably to the ability of *V. cholerae* to colonize intestinal mucosa.

MATERIALS AND METHODS

Rabbits. Rabbits were locally supplied New Zealand White males weighing 2 to 2.5 kg (age, 9 to 11 weeks).

***V. cholerae* O1 strains.** Eight *V. cholerae* O1 strains were used. Three were wild-type toxinogenic strains of proven virulence for volunteers; these were classical biotype strains Ogawa 395 and Inaba 569B and El Tor biotype strain Inaba N16961. From these, five mutant strains lacking gene sequences required for the production of the A subunit (A⁻B⁺ genotype) or both the A and B subunits (A⁻B⁻ genotype) of CT were derived by recombinant DNA techniques. These were strains CVD 101 (A⁻B⁺), N1 (A⁻B⁺), and NT (A⁻B⁻), each derived from Ogawa 395; JBK 70 (A⁻B⁻) derived from Inaba N16961; and CVD 103 (A⁻B⁺) derived from Inaba 569B. The preparation of mutants JBK 70, CVD 101, and N1

and NT are described in references 10, 9, and 13, respectively. Strain CVD 103 was prepared by methods identical to those used for strain CVD 101. All A⁻B⁺ strains produced and secreted the B subunit in amounts similar to that of their parent strains.

Enteric colonization with *V. cholerae*. The method used for enteric colonization with *V. cholerae* is described in detail elsewhere (15). In brief, rabbits were fasted overnight and sedated. Gastric acid was neutralized by a combination of intravenous cimetidine and intragastric NaHCO₃, after which the bacterial inoculum, in 15 ml of Casamino Acids-yeast extract medium, was given by gastric intubation; in some instances, purified CT (Schwarz/Mann, Orangeburg, N.Y.) was added to the bacterial inoculum. After 30 min, 2 ml of tincture of opium USP was given intraperitoneally. Thereafter, rabbits were returned to individual cages and allowed to eat and drink.

Quantitation of mucosal colonization. The method for the quantitation of viable *V. cholerae* adherent to intestinal epithelium has also been described elsewhere (3). All bacterial counts were determined on washed, homogenized segments of the proximal jejunum or distal ileum obtained 18 h after oral inoculation, when mucosal colonization was maximal (3). Results are expressed as log₁₀ viable *V. cholerae* recovered per gram of washed intestine.

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

RESULTS

Comparison of colonizing capacity of toxinogenic and nontoxinogenic *V. cholerae*. To assess relative colonizing capacity, groups of 3 to 12 rabbits were inoculated intragastrically with 10⁴, 10⁷, or 10¹⁰ CFU of each parent or mutant strain. None of the rabbits developed diarrhea. Mean counts of *V. cholerae* recovered after 18 h from washed segments of the jejunum or ileum are shown in Fig. 1 and 2. Four points deserve comment. First, the fully toxinogenic strains varied appreciably in their ability to colonize rabbit intestinal mucosa, Inaba N16961 being the most efficient and

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FIG. 1. Enteric colonization by toxinogenic and nontoxinogenic *V. cholerae*. Groups of 3 to 12 rabbits were given the indicated inocula of Ogawa 395 or one of three mutants derived from it: CVD 101 (A^-B^+), N1 (A^-B^+), or NT (A^-B^-). Mucosal colonization (CFU per gram of washed intestine) was determined 18 h later. Mean values for each group are shown, as are the combined results obtained with all three mutants.

Inaba 569B being the least efficient. Second, as previously reported (3), the level of colonization achieved by each strain was considerably greater in the ileum than in the jejunum, and the extent of colonization was generally dose dependent, increasing as the inoculum increased from 10^4 to 10^{10} CFU. Third, colonization by toxinogenic parent strains was almost always greater than that achieved by A^-B^+ or A^-B^- mutants. Finally, colonization by two A^-B^+ mutants and an A^-B^- mutant derived from the same parent (Ogawa 395) did not differ (Fig. 1).

Statistical analysis of mean differences in enteric colonization by the five parent-mutant pairs is summarized in Table 1. In the jejunum, the combined mean colonizing capacity of all A^-B^+ or A^-B^- mutants was most reduced, in comparison with the parent strains, in rabbits given the largest inoculum (mean reduction, $1.3 \log_{10}$ CFU in rabbits fed 10^{10} CFU). In contrast, ileal colonization by mutant strains was diminished to a similar extent with inocula that ranged from 10^4 to 10^{10} CFU (mean reduction, 0.9 to $1.5 \log_{10}$ CFU). These differences were statistically significant.

The possibility that CT added to an inoculum of nontoxinogenic (A^-B^-) *V. cholerae* would enhance mucosal colonization was also examined. Jejunal and ileal colonization after inoculation with 10^{10} CFU of strain NT was significantly lower than that of its toxinogenic parent, Ogawa 395 ($P < 0.005$ and $P < 0.01$ in the jejunum and ileum, respectively). However, colonization by NT was enhanced

FIG. 2. Enteric colonization by toxinogenic and nontoxinogenic *V. cholerae*. Studies were exactly as described in the legend to Fig. 1, except that parent-mutant pairs were Inaba N16961-JBK 70 (A^-B^-) and Inaba 569B-CVD 103 (A^-B^+).

in a dose-dependent manner by the coadministration of CT (Fig. 3). Thus, the addition of 200 μ g of CT to the inoculum increased jejunal and ileal colonization significantly ($P < 0.02$ and $P < 0.001$, respectively), to levels that did not differ from those of the toxinogenic parent. None of the rabbits inoculated only with *V. cholerae* or with bacteria combined with CT developed diarrhea.

DISCUSSION

We previously showed that virulent *V. cholerae* colonizes the small bowel of nonimmune adult rabbits in a dose-dependent manner, the extent of colonization being arbi-

TABLE 1. Enteric colonization by five parent-mutant pairs of *V. cholerae*

Site	Mean of differences ($\log_{10}$ CFU $\pm$ SE) in avg colonization by each parent-mutant pair with inocula of the following sizes (CFU):		
	10^4	10^7	10^{10}
Jejunum	0.19 ± 0.15 (NS)	0.43 ± 0.40 (NS)	1.3 ± 0.37 ($P < 0.02$)
Ileum	1.2 ± 0.43 ($P < 0.05$)	0.93 ± 0.30 ($P < 0.05$)	1.5 ± 0.32 ($P < 0.01$)

^a In every case, mean colonization by parent strains exceeded that of mutants; $n = 5$ pairs for each mean. P values of pairwise comparisons were determined by t test. NS, $P > 0.05$.

FIG. 3. Effect of added CT on mucosal colonization by mutant strain NT (A^-B^-). Groups of 11 to 12 rabbits were inoculated with 10^{10} CFU of Ogawa 395, NT, or NT plus the indicated amount of purified CT. Enteric mucosal colonization was assayed after 18 h. Colonization by NT was significantly poorer than that by Ogawa 395 in both the jejunum ($P < 0.005$) and the ileum ($P < 0.01$). Moreover, mucosal colonization was greater in rabbits given NT plus 200 μ g of CT than in rabbits given NT without added CT (jejunum, $P < 0.02$; ileum, $P < 0.001$).

trarily defined as the number of viable bacteria remaining on the intestinal mucosa after it is washed by a standard method (3). With this model, we have also shown that individual strains of *V. cholerae* vary greatly in colonizing capacity and that colonization of rabbit gut generally parallels that observed in nonimmune humans fed the same strains (N. F. Pierce and W. C. Cray, Jr., unpublished observations). Although rabbits are not a natural host for *V. cholerae*, these findings suggest that *V. cholerae* colonizes rabbits and humans by mechanisms that are similar and, possibly, identical.

The present study clearly showed that CT promotes mucosal colonization by *V. cholerae*. Mutants that differed from their virulent parents only in lacking the genes that encode the synthesis of the A subunit or holotoxin colonized less efficiently than did their fully toxinogenic parents: in the ileum, mean colonization by mutants was reduced 8- to 30-fold, and in the jejunum, it was reduced 1.5- to 20-fold, depending on the inoculum size. Moreover, coadministration of CT with a nontoxinogenic mutant improved its colonizing capacity to a level similar to that of its fully toxinogenic parent. These studies are compatible with earlier ones that showed that in vivo growth of *V. cholerae* is greater for fully toxinogenic strains than for poorly toxinogenic strains (2, 7, 12).

Experimental evidence supports the notion that mucosal colonization by *V. cholerae* is a multifactorial process: bacterial motility and chemotaxis for the mucosal surface promote contact of the organism with the gut epithelium (5, 6, 12, 16); protease (mucinase) apparently disrupts the mucus gel, permitting motile bacteria to move through it (15); and

putative colonization factors, such as the soluble hemagglutinin or flagellar antigen, may mediate adherence by interaction with mucosal receptors (1, 4, 8). How CT acts to promote colonization is not certain. The present study showed that it is not by interaction of the B subunit with GM_1 , its membrane receptor, because A^-B^+ mutants colonized no more efficiently than did otherwise isogenic A^-B^- strains. On the other hand, the promotion of colonization by added or bacterially synthesized holotoxin suggests that the effect was due to a pharmacologic effect of CT on the mucosa. One possibility is that intestinal fluid secretion stimulated by CT created an improved environment for bacterial multiplication. Although diarrhea did not occur in these studies, it is possible that fluid was secreted in the small bowel and reabsorbed in the cecum or colon. Another possibility is that CT acted by altering other aspects of intestinal function, such as mucus secretion, intestinal motility, or secretion of enteric hormones, and that these changes somehow favored bacterial colonization and growth. It is also conceivable that the A subunit, rather than the whole CT molecule, is responsible, in some manner, for the observed effect of CT. Although this possibility was not excluded by the present studies, it seems unlikely as the A subunit has no recognized cell membrane receptor and alters cell function only after its penetration of the membrane is facilitated by the B subunit.

It has been shown that repeated animal passage enhances the toxinogenicity of *V. cholerae* by amplification of the toxin gene region (11). The present findings suggest this may be due, at least in part, to a colonization advantage associated with increased CT production, leading to in vivo

selection of hypertoxinogenic variants. If this is so, it seems necessary that the mechanism by which CT promotes colonization be a highly localized one, favoring the growth of individual hypertoxinogenic bacteria, rather than a generalized one, such as induced intestinal secretion, which would alter the environment for all mucosal bacteria. Exactly how CT might cause such a localized effect is unknown.

Although the recombinant mutants used were prepared as vaccine candidates, these studies demonstrated their value for precisely investigating the mechanisms of bacterial virulence. The results enhance understanding of the pathogenesis of cholera and related toxin-mediated diarrheal diseases by suggesting that small amounts of CT (or similar enterotoxins) may aid bacterial colonization and eventual multiplication to the large numbers required for the induction of diarrheal illness. By the same token, these observations may have implications for attempts to develop effective vaccines for such infections by suggesting that antitoxic immunity may act not only by preventing the diarrheagenic effect of CT, but also by blocking the role of CT in promoting mucosal colonization.

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