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Determinants of the Immunogenicity of Live Virulent and Mutant *Vibrio cholerae* O1 in Rabbit Intestine

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Determinants of the capacity of live *Vibrio cholerae* O1 isolates to evoke specific immune responses in intestinal mucosa were studied in rabbits, using mucosal immunoglobulin A (IgA) antitoxin as the measured immune response. Antitoxin responses were evoked mostly by the primary inoculation and were dose dependent; secondary-type responses were modest and occurred only when the booster inoculum was large, i.e., 10^{10} CFU. The efficiency of mucosal immunization correlated closely with the mucosal colonizing capacity of the infecting strain and was otherwise independent of toxin genotype ($A^+ B^+$ or $A^- B^+$) or whether the strain was motile or nonmotile. Live bacteria evoked mucosal antitoxin more efficiently than did purified cholera toxin. Prior immunization with a nontoxinogenic ($A^- B^-$) *V. cholerae* strain interfered significantly with the induction of mucosal antitoxin by subsequent immunization with its fully toxinogenic ($A^+ B^+$) parent. These results demonstrate the marked efficiency with which live *V. cholerae* stimulate a specific enteric mucosal secretory IgA response. They support the view that mucosal colonization aids efficient delivery of bacterial antigens to responsive subepithelial lymphoid tissue. This might occur by transfer of colonizing bacteria through M cells into Peyer patches or by efficient delivery of secreted toxin to M cells by mucosa-associated organisms. Preexisting antibacterial immunity interferes with colonization, which may prevent efficient antigenic stimulation and which may explain the relatively weak response to booster immunization. The same process may also limit the efficacy of hybrid enteric bacterial vaccines when there is preexisting mucosal immunity to the carrier organism due to either natural exposure or prior immunization with another vaccine that uses the same carrier.

Mediators of mucosal immunity, including secretory immunoglobulin A (sIgA) antibodies, are the first line of defense against pathogens that colonize or invade mucosal surfaces; and there is much evidence that these defenses are best stimulated by mucosally applied antigens (4, 18). Results of studies in animals and volunteers suggest that live bacteria are especially efficient mucosal immunogens; one example is immunity to reinfection evoked by prior colonization or infection with *Vibrio cholerae* O1 (1, 5, 14). Such observations underlie efforts to develop oral vaccines for several enteric infections with live attenuated bacteria or avirulent genetic hybrids that produce antigens of unrelated pathogenic bacteria (3, 7, 10, 11, 15). The aim of such vaccines is to evoke mucosal immunity, including, but not necessarily restricted to, protective sIgA responses. Methods for optimal stimulation of mucosal immunity by attenuated or hybrid bacteria, however, have not been described, nor have bacterial features that determine their immunizing efficiency been determined.

One approach to this subject would be to study in experimental animals the mucosal IgA response to an antigen shared by genetically and functionally defined variants of a single bacterial species, using different immunizing regimens. We did this by measuring the mucosal IgA response to cholera toxin (CT) in rabbits fed various strains of *V. cholerae* O1, including fully virulent strains and hypovirulent mutants, some of which were developed as candidate live oral vaccines. The results, which should aid efforts to develop effective live oral bacterial vaccines, show that (i) live *V. cholerae* evoke dose-dependent primary mucosal antitoxin responses, (ii) booster responses are modest and elicited only by very large inocula, (iii) bacterial immunizing efficiency is determined predominantly by the mucosal colonizing capacity of the strain, and (iv) prior immunization with nontoxinogenic ($A^- B^-$) *V. cholerae* interferes with the efficient stimulation of a mucosal antitoxin response by toxinogenic ($A^+ B^+$) *V. cholerae*.

MATERIALS AND METHODS

***V. cholerae* O1 strains.** Eleven *V. cholerae* strains were used in this study. Six were fully toxinogenic ($A^+ B^+$ genotype). These were classical strains Ogawa 395 and Inaba 569B and El Tor strains Ogawa 3083, Ogawa RV79, Inaba N16961, and Inaba N16961-NM; the last strain was a spontaneous, nonmotile but flagellated mutant of N16961; strains 395, 569B, and N16961 were of proven virulence for volunteers. Four strains were mutants lacking gene sequences required for the production of the CT A subunit ($A^- B^+$ genotype); three of these strains were derived by recombinant DNA techniques: CVD101 and N1 (both derived from strain 395) and CVD103 (derived from strain 569B). One strain was obtained by mutagenesis with nitrosoguanidine: Texas Star-SR (derived from strain 3083). One strain was an $A^- B^-$ mutant derived by recombinant DNA techniques: NT (derived from strain 395). The preparation of mutants Texas-Star-SR, CVD101, and N1 and NT has been described

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previously (10, 12, and 15, respectively). Strain CVD103 was prepared by methods identical to those used for strain CVD101. All A^- B^+ strains produced and secreted the B subunit in amounts similar to their parent strains.

Inoculation with live *V. cholerae* or CT. The method used for enteric immunization with *V. cholerae* has been described previously (5). In brief, fasting rabbits were sedated and gastric acid was neutralized by intravenous administration of cimetidine and intragastric administration of $NaHCO_3$. The bacterial inoculum, in 15 ml of Casamino Acids (Difco Laboratories, Detroit, Mich.)–yeast extract medium, was then given through a gastric tube. After 30 min, 2 ml of tincture of opium (USP) was given intraperitoneally. Thereafter, rabbits were caged individually and allowed to eat and drink normally. Intraduodenal immunization with purified CT (Schwarz/Mann, Orangeburg, N.Y.) was performed as follows. Fasting rabbits were sedated, restrained, and anesthetized locally at the abdominal incision site. The duodenum was identified through a small laparotomy; and CT, in 5 ml of 0.01 M phosphate-buffered saline (pH 7.4) containing 0.02% gelatin, was injected intraduodenally. The wound was closed, and rabbits were returned to their cages to eat and drink normally.

Assay of mucosal colonization and jejunal antitoxin response. Viable *V. cholerae* cells that adhered to ileal epithelium were enumerated as described elsewhere (5). All bacterial counts were determined on washed, homogenized segments of distal ileum obtained 18 h after oral inoculation, when mucosal colonization was maximal (5); in all cases the oral inoculum was 10^{10} CFU. Antitoxin-containing plasma cells (ACC) were enumerated in the lamina propria of jejunal biopsy samples by a previously described fluorescent antibody technique (21). This method detects ACC evoked either by intact CT or its B subunit (14, 15). ACC counts are expressed as geometric means (×/÷ standard error) per cubic millimeter in the crypt region of the intestinal lamina propria.

RESULTS

Effect of inoculum size and number on jejunal ACC response to live *V. cholerae* or CT. In the first experiments we sought to determine how the ACC response was affected by the size of the bacterial inoculum and the number of inoculations given. Rabbits were given various numbers of viable toxinogenic *V. cholerae* Ogawa 395 cells on day 0, and some rabbits also received a booster inoculum on day 19; no rabbit developed diarrhea; jejunal biopsy samples were taken on day 24. Earlier studies showed essentially no mucosal ACC response 5 days after a single inoculum of 10^{10} CFU of strain Ogawa 395 (25). By day 24, however, dose-dependent primary ACC responses were evident (Table 1). These responses increased 3.5-fold ($P < 0.05$) when the inoculum was increased from 10^4 to 10^{10} CFU. Booster inocula given on day 19 caused modest increments in the ACC response on day 24 (2.4- to 3.2-fold), but only when the inoculum was large (10^{10} CFU); moreover, the increase was statistically significant only in rabbits that were also given a primary inoculum of 10^{10} CFU. The magnitude of the booster response was determined by the size of both the primer and booster inocula; the response was greatest when both inocula were largest, i.e., 10^{10} CFU. For comparative purposes, rabbits were also immunized intraduodenally with purified CT on days 0 and 19; jejunal ACC responses were determined on day 24. Results in Table 2 show that repeated doses of CT, up to 200 μg per dose, caused only moderate mucosal ACC responses. Larger doses of CT were not given as they often caused diarrhea.

Compared immunogenicity of various *V. cholerae* strains. In the next part of the study we evaluated the relative ability of various *V. cholerae* strains to evoke mucosal anti-CT responses in rabbit jejunum and compared this with the ability of the same strains to colonize the bowel of nonimmune rabbits. The strains studied included toxinogenic (A^+ B^+) strains, hypovirulent A^- B^+ derivatives of some of these strains, and one flagellated but nonmotile mutant of an A^+ B^+ strain. In all instances, ACC responses were determined on day 24 in rabbits inoculated with 10^{10} viable bacteria on days 0 and 19. The results (Fig. 1) show that mean ACC responses were directly and linearly related to the colonizing capacity of the tested strains. On the other hand, immunogenicity did not reflect whether a strain was of the A^+ B^+ or A^- B^+ genotype or was motile or nonmotile, except as these differences affected colonization. A^- B^+ mutants regularly colonized less efficiently than their A^+ B^+ parents (18), and the same was true for the nonmotile mutant compared with its parent. At 18 h after oral inoculation of nonimmune rabbits with 10^{10} viable *V. cholerae* N16961 isolates, washed ileum yielded $10^{7.1}$ CFU/g; similar inoculations with 10^{10} CFU of the nonmotile variant of N16961 yielded $10^{5.6}$ CFU/g.

Effect of prior immunization with nontoxinogenic *V. cholerae* on mucosal ACC response to toxinogenic *V. cholerae*. In the final experiment, we determined whether toxinogenic *V. cholerae* evoked mucosal ACC responses with equal efficiency in nonimmune rabbits and rabbits previously immunized with a nontoxinogenic (A^- B^-) *V. cholerae* mutant. Two groups of rabbits were studied. In the first group, two inocula of A^- B^- *V. cholerae* NT were followed by two inocula of A^+ B^+ *V. cholerae* 395; in the second group, only the last two inoculations were given. All inocula were 10^{10} CFU; and doses were given at 20-day intervals. Jejunal

TABLE 1. Ability of live *V. cholerae* Ogawa 395 to evoke mucosal antitoxin responses in rabbits

Primary inoculum[a] (log_{10} CFU)	Primary response[b]	Booster inoculum[a,c] (log_{10} CFU)	Booster response[b]	*P*
10^4	1,100 ×/÷ 1.7	10^4	1,000 ×/÷ 1.8	NS[d]
10^7	1,600 ×/÷ 1.6	10^7	1,400 ×/÷ 1.6	NS
10^{10}	3,900 ×/÷ 1.2	10^{10}	12,400 ×/÷ 1.3	<0.01
10^4	1,100 ×/÷ 1.7	10^{10}	2,600 ×/÷ 2.3	NS
10^{10}	3,900 ×/÷ 1.2	10^4	3,300 ×/÷ 1.3	NS

[a] Viable *V. cholerae* Ogawa 395 was given by mouth.
[b] On day 24 after primary inoculation. Values are geometric mean ACC/mm³ (×/÷ standard error) of jejunal lamina propria; n = 5 to 6 rabbits for each mean value.
[c] On day 19 after primary inoculation.
[d] NS, Not significant.

TABLE 2. Ability of purified CT to evoke mucosal antitoxin responses in rabbits

CT dose (μg)[a]	Response[b]
50	600 ×/÷ 2.2
100	1,100 ×/÷ 1.5
200	2,000 ×/÷ 2.5

[a] Primary and booster doses of purified CT were given intraduodenally on days 0 and 19, respectively.
[b] Values are geometric mean ACC/mm³ (×/÷ standard error) of jejunal lamina propria on day 24; n = 5 to 6 rabbits for each mean value.

biopsy samples were obtained 5 days after the final inoculation. The results (Table 3) show that ACC responses in rabbits that were initially inoculated with $A^- B^-$ *V. cholerae* isolates were 10-fold lower ($P < 0.001$) than in those inoculated only with the toxinogenic $A^+ B^+$ strains.

DISCUSSION

Results of previous studies in humans and animals (24, 25) have shown that enteric colonization or infection with virulent *V. cholerae* evokes intestinal immune responses, including sIgA antitoxin, that contribute to the substantial resistance to reinfection that follows primary infection (1, 5, 14, 22). Antitoxin evoked by oral immunization with CT or related antigens has also been shown to evoke protection against cholera or enteric challenge with CT, and such protection parallels the magnitude of the mucosal antitoxin response (2, 20). Results of this study extend these observations by using the anti-CT response in rabbit jejunal epithelium as a direct, quantitative assay of the immunogenicity of live *V. cholerae*. The anti-CT response was studied because it has been defined in experimental animals and humans (17, 24); convenient methods exist for assaying it; and *V. cholerae* with mutations or deletions in genes encoding CT synthesis are available for study. Although CT is an unusually potent mucosal immunogen (17), it is likely that the results of this study would also apply, at least qualitatively, to other bacterial antigens, and especially to proteins secreted by noninvasive organisms.

The immune response evoked by live *V. cholerae* isolates was clearly dose dependent and was achieved mostly by the primary inoculum. The dose dependence of the anti-CT response probably reflects the very limited replication of *V. cholerae* that occurs in rabbit intestine (5); thus, the inoculum given determined the dose of antigen available for immune stimulation by determining the number of bacteria available for in vivo antigen synthesis. The lack of a substantial booster effect after reinoculation, especially with inocula that evoked significant but suboptimal priming (e.g., 10^7 CFU), differs from results obtained in studies of enteric immunization with purified CT in which distinct primary and secondary types of anti-CT responses were readily elicited (17). A partial explanation for the failure of live *V. cholerae*

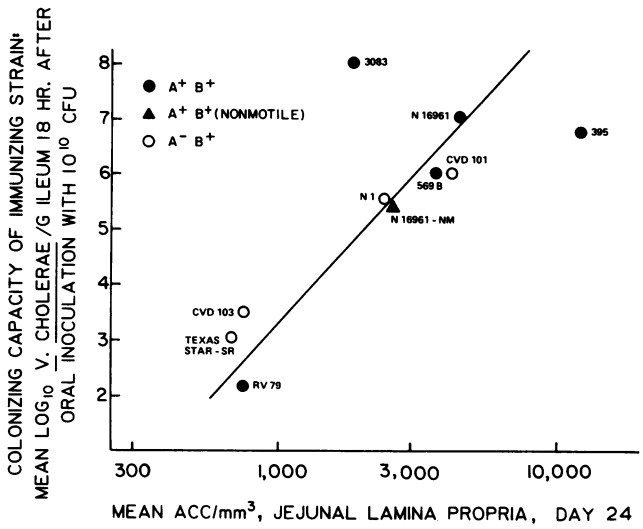

FIG. 1. Relation between intestinal colonizing capacity and jejunal ACC response evoked by virulent and mutant *V. cholerae*. Ileal colonization was determined in groups of 5 to 12 nonimmune rabbits that were inoculated once with 10^{10} *V. cholerae* isolates of the indicated toxin genotype; except as indicated, all strains were motile. Jejunal ACC responses were determined in groups of five rabbits inoculated twice, on days 0 and 19, with 10^{10} *V. cholerae* isolates of the same strains described above. Jejunal biopsy samples for ACC assay were obtained on day 24. Geometric mean ACC responses are presented.

isolates to evoke marked secondary-type responses may be that antibacterial immunity evoked by the first inoculation interfered with mucosal colonization and the efficient delivery of antigen to responsive mucosal lymphoid tissue after the second inoculation. We have shown previously that prior colonization by *V. cholerae* evokes substantial resistance to recolonization (5, 25); the same immune process may limit the access of viable bacteria or their products to absorptive M cells that overlie Peyers patches and actively transport antigens (16), including intact *V. cholerae* (16a), to underlying lymphoid tissue where mucosal sIgA responses arise (9). Modest secondary-type mucosal immune responses have also been observed after reinoculation of rabbits with another live organism, *Shigella flexneri* (13).

Immunization was also more efficient with live *V. cholerae* than with purified CT. As purified CT has been shown in other species to be an extremely effective mucosal immunogen (8, 17, 19), this observation was surprising. A possible explanation for this difference is that soluble CT injected into the bowel lumen reached responsive lymphoid tissue in Peyers patches less efficiently than did CT elaborated by bacteria colonizing the bowel mucosa. This might occur if soluble CT reached the mucosal surface less effectively than did CT elaborated by colonizing *V. cholerae*,

TABLE 3. Effect of prior immunization with nontoxinogenic $A^- B^-$ *V. cholerae* on mucosal antitoxin response to $A^+ B^+$ strain

V. cholerae strain for immunization no.[a]				Jejunal ACC response[b]
1	2	3	4	
None	None	395 ($A^+ B^+$)	395 ($A^+ B^+$)	12,400 ×/÷ 1.3
NT ($A^- B^-$)	NT ($A^- B^-$)	395 ($A^+ B^+$)	395 ($A^+ B^+$)	1,200 ×/÷ 2.1[c]

[a] Dosing interval was 20 days; each oral inoculum was 10^{10} live *V. cholerae*.
[b] Values are geometric mean ACC/mm³ (×/÷ standard error) of jejunal lamina propria, 5 days after final inoculation; n = 5 to 7 rabbits for each mean value.
[c] Significantly different than response in rabbits not initially colonized with strain NT; $P < 0.001$ by the Student t test.

which were already intimately associated with the mucosa. This seems unlikely, however, as doses of CT only twofold greater than the largest dose used in this study regularly caused diarrhea in rabbits, which is direct evidence of its interaction with the mucosa. In contrast, diarrhea was not evoked by viable *V. cholerae*, even with the largest inoculum. An alternative possibility is that live *V. cholerae* efficiently deliver CT to Peyers patch lymphoid cells via the uptake and transfer of intact vibrios through the mucosa by M cells (16a) and that this process is more efficient for intact living bacteria than for a soluble protein. Although speculative, this explanation is consistent with the experimental findings.

Inoculation of rabbits with live *V. cholerae* followed treatment with cimetidine, a drug known to have immunoenhancing effects (6). Although there is no reason to believe that cimetidine would alter the relative immunogenicity of different strains of *V. cholerae*, it is possible that it could affect the comparison of antitoxin responses to live *V. cholerae* with those evoked by intraduodenal injection of CT given without cimetidine pretreatment. Results of other studies from this laboratory (data not shown), however, suggest this did not occur, as antitoxin responses were similar in rabbits given CT intraduodenally without cimetidine or intragastrically after treatment with cimetidine. Moreover, studies demonstrating an in vivo effect of cimetidine on antibody production involved daily treatment for several weeks, whereas only two doses were given in the present study (6).

The major determinant of immunogenicity of live *V. cholerae* isolates was their ability to colonize bowel mucosa, which is in accord with our unpublished findings that the efficiency of immunization against either recolonization or experimental cholera in rabbits is also closely correlated with the colonizing capacity of the immunizing strain. Although the mechanism of this effect was not determined, it was apparently not due to different capacities for intraluminal bacterial growth, as the inocula used (10^{10} CFU) were such that little, if any, bacterial multiplication occurred in vivo (5). On the other hand, it is likely that colonization enhanced immunogenicity by bringing bacteria (and their products) into close contact with mucosal sites of antigen absorption, by prolonging the presence of vibrios (and their products) at the mucosal surface, or by both mechanisms.

In contrast with colonization, the synthesis of holotoxin (rather than the B subunit) and motility were not important determinants of bacterial immunogenicity. Although each type of mutant immunized less well than its parent, the reduction was modest and in proportion to its reduced capacity to colonize bowel mucosa. The similar efficacy of bacterically synthesized B subunit and holotoxin for stimulation of mucosal anti-CT responses does not agree with previous reports that purified B subunit is a significantly less effective immunogen than CT (8, 17, 19), which is probably due to the inability of B subunit to stimulate cyclic AMP in mucosal lymphoid cells. Failure to observe this difference after immunization with live bacteria is unexplained, but may be due to other immune-enhancing properties of *V. cholerae*, such as its lipopolysaccharide, which might act as adjuvants for the response to the B subunit (23). These might be especially effective when immune stimulation is by intact bacteria transported into Peyers patches.

Prior immunization with A^- B^- strains of *V. cholerae* markedly affected the ability of fully toxinogenic strains to evoke a vigorous mucosal anti-CT response. This experiment was designed as a model for mucosal immunization with hybrid bacteria that express antigens of unrelated pathogens, an approach that is being used in the development of a variety of new vaccines. In this instance, the A^+ B^+ strain represented such a hybrid, possessing genes and producing a secreted protein that were lacking in the A^- B^- derivative. The results support the general conclusion that preexisting mucosal immunity to a carrier bacteria can substantially interfere with its ability to stimulate a mucosal sIgA response to the carried antigen; the mechanism of this effect likely involves interference with mucosal colonization by the carrier strain, with such interference being mediated by previously stimulated mucosal immune mechanisms. To our knowledge, this is the first report of studies that directly assess this aspect of mucosal immunization with hybrid bacteria. The results appear to be relevant to efforts that are now under way to develop hybrid vaccines for enteric bacterial infections with carrier strains such as the live oral typhoid vaccine, Ty21a (3, 7).

The findings of this study should have relevance for current efforts to develop live oral bacterial vaccines for enteric infections, such as those caused by shigella, salmonella, enterotoxinogenic *Escherichia coli*, and *V. cholerae*. They confirm that live bacteria may be highly immunogenic and substantially more efficient than purified antigen for evoking a specific mucosal immune response. They support the view that candidate vaccine strains should be those known to colonize bowel mucosa efficiently. They also suggest that such vaccines should be given in large inocula, unless marked replication occurs in vivo. If booster inocula are given, these should probably also be large so that the antigen-excluding effects of previously stimulated mucosal immunity may be at least partly overcome. Although results of this study suggest that the benefit of booster immunization is modest, results of an earlier study showed that protection against recolonization is longer lasting in rabbits immunized twice, rather than once, with live *V. cholerae* (5). Finally, this study reveals that hybrid bacterial vaccines may be of reduced efficacy in the presence of preexisting immunity to the carrier strain, as could result from natural encounter with the carrier or by prior use of the same carrier to deliver a different antigen.

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