INFECTION AND IMMUNITY, Jan. 1988, p. 142–148
0019-9567/88/010142-07$02.00/0

Vol. 56, No. 1

Determinants of Immunogenicity and Mechanisms of Protection by Virulent and Mutant *Vibrio cholerae* O1 in Rabbits

NATHANIEL F. PIERCE,[1]†* WILLIAM C. CRAY, JR.,[1] JAMES B. KAPER,[2] AND JOHN J. MEKALANOS[3]

Department of Medicine, Johns Hopkins University School of Medicine, and Department of Medicine, Francis Scott Key Medical Center, Baltimore, Maryland 21224[1]; Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201[2]; and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115[3]

Received 16 July 1987/Accepted 23 September 1987

The colonizing capacities of 16 *Vibrio cholerae* strains, including nine genetically and/or phenotypically defined parent-mutant pairs, were determined in unobstructed adult rabbit small bowel. There were marked interstrain differences in colonizing capacity, which was enhanced by bacterial motility and the production of cholera holotoxin but was unrelated to production of cholera toxin B-subunit or hemolysin or to bacterial serotype or biotype. The role of colonizing capacity and other bacterial features in determining the immunizing efficiency of live *V. cholerae* was studied by determining the efficiency with which graded inocula of each strain immunized against attempted recolonization of the ileum or induction of choleralike diarrhea by the RITARD (removable intestinal tie-adult rabbit diarrhea) challenge technique using standard inocula of virulent *V. cholerae*. Mucosal colonizing capacity was the only quantitative predictor of bacterial immunizing capacity; none of the other bacterial features cited above influenced bacterial immunogenicity against either type of challenge, except as they affected colonizing capacity. Live *V. cholerae* immunized much more efficiently than Formalin-killed bacteria; the former caused marked protection after a single inoculum of 10^2 CFU, whereas the latter gave only partial protection after three inoculations of 10^{11} killed organisms. Protection induced by live bacteria was due largely to resistance to colonization and included marked inhibition of bacterial growth within the bowel lumen. These findings strongly suggest that an optimally efficient oral cholera vaccine would be composed of avirulent live *V. cholerae* selected for their capacity to colonize the small-bowel mucosa.

Enteric infection with virulent or mutant *Vibrio cholerae* can evoke marked resistance to reinfection. This resistance has been observed in volunteers and animals (2, 3, 26), is the means by which immunity is naturally acquired in cholera-endemic regions, and is the basis for efforts to develop a live oral cholera vaccine (14). However, bacterial factors responsible for the efficient induction of enteric mucosal immunity and the mechanisms by which such protection is mediated are only partly understood. There are several reasons for this, including the cost, difficulty, and limited scope of studies of induced cholera in volunteers, the lack until recently of genetically and phenotypically defined mutants of *V. cholerae*, and the lack of convenient animal models in which immunity could be induced by live *V. cholerae*. Limited studies in volunteers have suggested that colonization of the small bowel is an important determinant of the immunizing efficiency of live *V. cholerae* and that immunity induced by live bacteria is characterized by resistance to mucosal colonization (15).

In recent studies, we have shown that *V. cholerae* colonizes the unobstructed bowel of adult rabbits, that such colonization evokes marked protection against recolonization or experimental cholera induced by the RITARD (removable intestinal tie-adult rabbit diarrhea) challenge technique, and that the magnitude of a specific mucosal immune response, antitoxin, is determined by the extent to which a *V. cholerae* strain can colonize the bowel mucosa (3, 23, 26); we have also shown that production of cholera toxin enhances the mucosal colonizing capacity of *V. cholerae* (22).

* Corresponding author.
† Present address: Diarrhoeal Diseases Control Programme, World Health Organization, 1211 Geneva 27, Switzerland.

We have now used these rabbit models and a series of virulent *V. cholerae* strains, along with genetically and/or phenotypically defined mutants derived from these strains, to investigate factors that determine the immunizing capacity of live *V. cholerae* and mechanisms by which resistance to reinfection is mediated. The results reveal that (i) mucosal colonizing capacity is the major determinant of the immunizing efficiency of live *V. cholerae*; (ii) in addition to previously identified factors, mucosal colonization is enhanced by the motility of *V. cholerae*; (iii) live *V. cholerae* is a far more efficient mucosal immunogen than killed *V. cholerae*; and (iv) resistance to reinfection involves inhibition of bacterial growth within the small bowel, but not bacterial killing.

MATERIALS AND METHODS

***V. cholerae* strains.** Sixteen *V. cholerae* O1 strains were used (Table 1). Seven were fully toxinogenic (A^+B^+ genotype and phenotype), and five of these were of proven virulence for humans or animals or both: strains 395, 3083, B36237, N16961, and 569B. Eight strains were mutants of the A^-B^+ or A^-B^- genotype derived from A^+B^+ parents. Seven of these were developed by recombinant DNA techniques, namely, CVD101, NI, CVD105, and CVD103 (all A^-B^+) and JBK70, NT, and M7922 (all A^-B^-), and one strain, Texas Star SR (A^-B^+), was developed by mutagenesis with nitrosoguanidine. One A^-B^+ strain (CVD105, derived from CVD101) was also made hemolysin negative by gene deletion. One strain was a naturally occurring, avirulent A^-B^- environmental isolate (1196-78). Strain CVD49 was a spontaneously occurring, nonmotile but flagellated mutant of N16961 (23). The preparation of mutants CVD101 (10), Texas Star SR (8), NI and NT (17), JBK70 (11),

TABLE 1. Relative ability of various *V. cholerae* strains to colonize rabbit ileum

Strain	Parent	Biotype	Serotype	Toxin genotype	Ileal colonization[a]
3083[b]		El Tor	Ogawa	A^+B^+	8.1 ± 0.5
B36237[b]		Classical	Inaba	A^+B^+	8.0 ± 0.1
N16961[b]		El Tor	Inaba	A^+B^+	7.1 ± 0.4
395[b]		Classical	Ogawa	A^+B^+	6.8 ± 0.4
CVD101	395	Classical	Ogawa	A^-B^+	6.1 ± 0.4
569B[b]		Classical	Inaba	A^+B^+	6.1 ± 0.7
CVD49	N16961	El Tor	Inaba	A^+B^+	5.6 ± 0.7
N1	395	Classical	Ogawa	A^-B^+	5.6 ± 0.3
CVD105	CVD101	Classical	Ogawa	A^-B^+	5.5 ± 0.3
JBK70	N16961	El Tor	Inaba	A^-B^-	5.4 ± 0.9
NT	395	Classical	Ogawa	A^-B^-	5.2 ± 0.2
CVD103	569B	Classical	Inaba	A^-B^+	3.5 ± 0.4
Texas Star-SR	3083	El Tor	Ogawa	A^-B^+	3.1 ± 0.9
1196-78		El Tor	Ogawa	A^-B^-	2.9 ± 0.4
RV-79		El Tor	Ogawa	A^+B^+	2.2 ± 0.2
M7922	RV-79	El Tor	Ogawa	A^-B^-	1.5 ± 0.6

[a] Mean $\log_{10}$ *V. cholerae* ± standard error recovered per gram of washed ileum 18 h after oral inoculation with 10^{10} live *V. cholerae*. Each mean reflects data from 4 to 12 rabbits.

[b] Strain with established virulence for humans, animals, or both.

CVD105 (J. B. Kaper, H. L. T. Mobley, J. M. Mickalsky, D. A. Harrington, and M. M. Levine, *in* Y. Takeda and R. B. Sack, ed., *Advances in Research on Cholera and Related Dirrhoeas*, in press), and M7922 (16) was as described. Strain CVD103 was prepared by methods identical to those used for CVD101 (Kaper et al., in press). The origin of strain 1196-78 is described elsewhere (13). All A^-B^+ strains produced and secreted B subunit in amounts similar to their fully toxinogenic parent strains. In all mutant strains except Texas Star SR (A^-B^+) and CVD49 (flagellated but nonmotile), the genetic differences between parent and mutant were defined and were essentially limited to genes encoding the production of cholera toxin, B subunit, or hemolysin.

Preparation of live *V. cholerae* for inoculation. Stock cultures were stored at −70°C in 10% skim milk or brain heart infusion broth with 15% glycerol. To prepare each inoculum, a sample was thawed, inoculated into 1% peptone-water (pH 7.4), and incubated overnight at 37°C. A 50-ml Erlenmeyer flask containing 10 ml of sterile Casamino acids-yeast extract (CAYE) medium (4) was inoculated with 0.05 ml of the overnight culture and shaken at 180 cycles per min for 4 h in a 37°C water bath. Bacteria were then centrifuged and suspended in 0.01 M phosphate-buffered saline (pH 7.4). Viable bacterial counts were determined on 3% gelatin agar. Inocula were prepared by diluting the bacterial suspension in fresh CAYE medium.

Formalin-killed *V. cholerae*. Formalin-killed *V. cholerae* Ogawa 395 cells were prepared as follows. Vibrios were grown in a 2,500-ml low-form flask containing 1,000 ml of CAYE medium that was inoculated with 5.0 ml of an overnight broth culture and shaken at 100 cycles per min for 5.5 h at 37°C. Vibrios were then centrifuged, suspended in phosphate-buffered saline containing 0.025 M Formalin, and held at 22°C for 24 h and then at 4°C for 48 h. The killed vibrios were then washed with phosphate-buffered saline and stored at 4°C in phosphate-buffered saline containing 1:10,000 Merthiolate.

Rabbits. Locally supplied male New Zealand white rabbits were used. These weighed 2 to 2.5 kg (age, 9 to 11 weeks) when studies were begun.

Rabbit inoculation with live or killed *V. cholerae*. The method for oral immunization of rabbits with live or killed *V. cholerae* was as previously described (3). Briefly, fasting rabbits were sedated, and gastric acid was neutralized by intravenous cimetidine and $NaHCO_3$. The bacterial inoculum, in 15 ml of CAYE medium, was then given by gastric tube. After 30 min, 2 ml of tincture of opium (USP) was given intraperitoneally. Thereafter, rabbits were caged individually and maintained fasting, but were allowed to drink normally.

In some studies, live *V. cholerae* were inoculated into ligated segments of small intestine. The preparation of intestinal segments was as described elsewhere except that segments were 4 to 10 cm in length (21). The inoculum volume was 1.0 ml. After inoculation, the abdominal wound was closed, and rabbits were returned to their cages and given water, but not food, until they were sacrificed with intravenous pentobarbitol.

Quantitation of intestinal colonization. At the indicated interval after inoculation (usually 18 h), rabbits were killed with intravenous pentobarbitol. Pieces of proximal jejunum and distal ileum were collected, washed, and homogenized, and numbers of viable *V. cholerae* were enumerated in the homogenate as previously described (3). In one study involving inoculation of ligated intestinal segments with *V. cholerae*, all *V. cholerae* contained within a segment were enumerated as follows: the entire segment was removed unopened, any contained fluid was removed and measured, the entire segment was homogenized without being washed, the homogenate was combined with the contained fluid, and numbers of viable *V. cholerae* were determined as above.

RITARD challenge. In some studies, rabbits were challenged intraduodenally with strain 395 or B36237 by the removable intestinal tie-adult rabbit diarrhea (RITARD) technique as described by Spira et al. (24). This involved permanent obstruction of the cecal orifice with an umbilical tape tie, intraduodenal inoculation with viable *V. cholerae*, and simultaneous ileal obstruction for 2 h with a removable tie. After inoculation, rabbits were caged separately over a germicidal liquid bedding, given food and water freely, and observed for diarrhea twice daily for 5 days. When previously colonized rabbits were challenged, age-matched controls not previously colonized were given the same inoculum. In all studies, the inoculum was 10^{11} viable bacteria, which is 10,000 50% effective doses (19), 1 50% effective dose being the inoculum that causes severe or lethal diarrhea in 50% of nonimmune rabbits. Results were recorded as: no

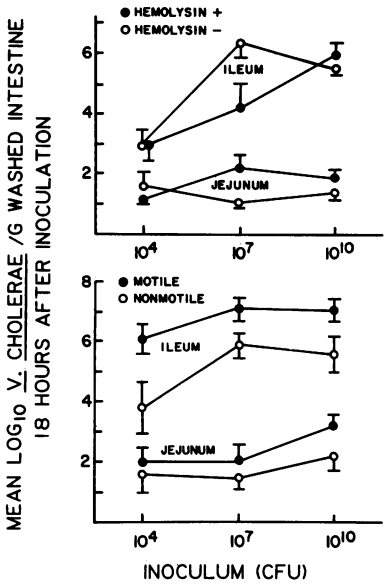

FIG. 1. Effect of hemolysin production and motility on mucosal colonization by *V. cholerae*. Nonimmune rabbits were inoculated orally with the indicated number of viable *V. cholerae* organisms. Ileal and jejunal colonization were determined after 18 h. Results shown are mean CFU per gram of washed intestine ± standard error for five to nine rabbits for each mean. Hemolysin-positive and -negative strains were CVD101 and CVD105, respectively. Motile and nonmotile strains were N16961 and CVD49, respectively.

diarrhea, mild diarrhea (minimal perirectal fecal staining), severe diarrhea (marked perirectal staining), and lethal diarrhea.

Statistical analysis. Mucosal colonization by *V. cholerae* is expressed as the mean $\log_{10}$ (± standard error) viable *V. cholerae* recovered per g of washed, homogenized jejunum or ileum. Differences in mean values were analyzed by Student's *t* test.

The inoculum protective against recolonization was defined as the primary inoculum of live bacteria ($\log_{10}$ CFU) that reduced mucosal colonization to a mean of 10^3 CFU/g of washed ileum when rabbits were reinoculated with 10^{10} viable *V. cholerae* 19 to 22 days after primary inoculation. This was determined by linear interpolation between the smallest primary inoculum yielding mean colonization levels below 10^3 CFU/g after reinoculation and the largest yielding values greater than 10^3 CFU/g.

The 50% protective dose for RITARD challenge was defined as the primary inoculum of live bacteria ($\log_{10}$ CFU) required to evoke protection that reduced the incidence of severe or lethal diarrhea by 50% (compared with nonimmune controls) when rabbits were challenged with 10^{11} CFU of virulent *V. cholerae* O1 (10,000 50% lethal doses) 10 to 22 days after primary inoculation. When necessary, this was determined by linear interpolation between the smallest primary inoculum yielding more than 50% protection and the largest yielding less than 50% protection.

RESULTS

Mucosal colonizing capacity of virulent and mutant *V. cholerae*. In initial studies we determined the capacity of the 16 study strains of *V. cholerae* O1 to colonize unobstructed rabbit ileum. Mucosal colonization was determined 18 h after oral inoculation with 10^{10} CFU of each strain (Table 1).

The colonizing capacities of study strains varied widely, being greatest for A^+B^+ strains of proven virulence (i.e., 3083, B36237, N16961, 395, and 569B) and least for the avirulent A^-B^- environmental isolate (1196-78), an A^+B^+ strain of unknown virulence (RV-79), and an A^-B^- derivative (M7922) of the latter. As previously reported (22), A^-B^+ and A^-B^- mutants consistently colonized less well than their A^+B^+ parents. Bacterial biotype and serotype showed no consistent relation to colonizing capacity.

The possible contributions of hemolysin production and motility to mucosal colonization were studied in greater detail. Jejunal and ileal colonization by the hemolysin-negative mutant (CVD105) and the flagellated but nonmotile mutant (CVD49) was compared with that of their hemolysin-positive and motile parents (CVD101 and N16961, respectively). Colonization by the hemolysin-negative mutant did not differ from that of its hemolysin-positive parent (Fig. 1). In contrast, the nonmotile mutant colonized consistently less well than its parent, a difference that was most evident in the ileum where recovery of viable organisms averaged about 1 $\log_{10}$ less per g for the nonmotile strain; in studies with initial inocula of 10^4 and 10^{10} CFU, these differences were statistically significant ($P < 0.05$).

Relation of mucosal colonizing capacity to immunogenicity of live *V. cholerae*. The relation between mucosal colonizing capacity and immunizing efficiency of live *V. cholerae* was studied using the 16 described strains. Rabbits were immunized with various inocula of each strain; 19 to 22 days later, the extent of immunization was assessed by determining resistance to recolonization with A^+B^+ *V. cholerae* of the homologous serotype (10^{10} CFU) or by determining protection against severe or lethal diarrhea after RITARD challenge with A^+B^+ *V. cholerae* of the homologous serotype (10^{11} CFU).

(i) Protection against recolonization. For each strain, the capacity to immunize against recolonization was closely related to its ability to colonize the small bowel of nonimmune animals (Fig. 2). Thus, the inoculum required to achieve protection was least with strains that colonized well, and greatest with strains that colonized poorly. In contrast, there was no apparent relationship between immunogenicity of a strain and whether it was A^+B^+, A^-B^+, or A^-B^-, hemolysin positive or hemolysin negative, or motile or nonmotile, except as these features affected the extent of mucosal colonization by the strain.

(ii) Protection against RITARD challenge. For each strain, the capacity to immunize against a subsequent RITARD challenge was also closely related to its ability to colonize the small bowel of nonimmune rabbits (Fig. 3). Again, immunogenicity was not related to other recognized differences in the strains, such as production of toxin or hemolysin, or motility. The tendency for most A^+B^+ strains to immunize more efficiently than A^-B^+ or A^-B^- mutants appeared (with the exception of RV-79) to reflect their greater mucosal colonizing capacity.

Compared immunogenicity of live and Formalin-killed *V. cholerae*. The immunizing capacities of live and Formalin-killed *V. cholerae* were compared by using strain 395 for oral immunization and determining protection against attempted recolonization by the same strain. Immunization schedules and results are summarized in Table 2. The live *V. cholerae* strains were vastly more efficient as immunogens than were killed *V. cholerae*. Thus, a single inoculum of fewer than 100 live *V. cholerae* evoked marked resistance to attempted recolonization, reducing ileal colonization after challenge by more than 4 $\log_{10}$ CFU/g in comparison with nonimmunized

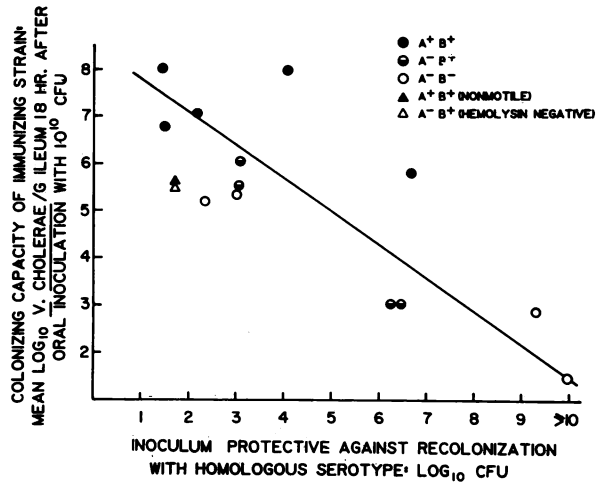

FIG. 2. Relation of mucosal colonizing capacity of *V. cholerae* to efficiency of immunization against recolonization. Strains studied were those described in Table 1, except RV-79; the ileal colonizing capacity of these strains in nonimmune rabbits is also from Table 1. To determine the protective inoculum, rabbits were immunized orally with various inocula of *V. cholerae* of the indicated phenotype. After 19 to 22 days, these rabbits were recolonized with 10^{10} CFU of *V. cholerae* of the homologous serotype, either Ogawa 395 or Inaba B36237. The protective inoculum was determined as described in Materials and Methods. The regression line was fitted by eye.

controls. In contrast, multiple large inocula of killed *V. cholerae* (10^{11} organisms given three times) were required to evoke moderate protection (i.e., to reduce postchallenge ileal colonization by 2.3 $\log_{10}$ CFU in comparison to nonimmunized controls), and immunization with fewer and smaller inocula of killed bacteria was even less effective.

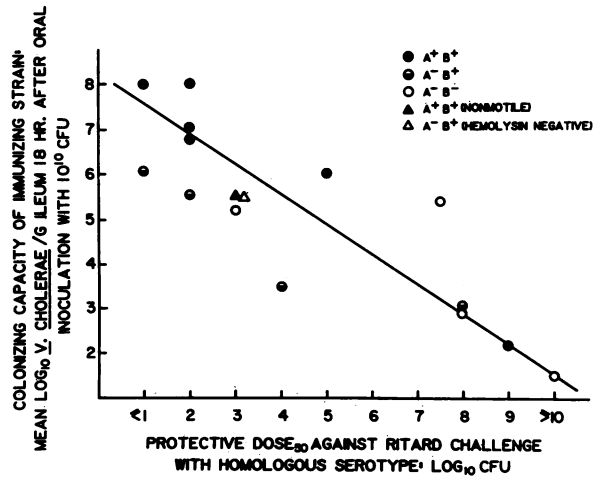

FIG. 3. Relation of mucosal colonizing capacity of *V. cholerae* to efficacy of immunization against RITARD challenge. Strains studied were those described in Table 1, which also shows their colonizing capacity for nonimmune rabbit ileum. Rabbits were immunized orally with various inocula of the indicated phenotype. RITARD challenge was performed 19 to 22 days after primary inoculation, using a challenge of 10^{11} CFU of the homologous serotype, either Ogawa 395 or Inaba B36237. The 50% protective dose was determined as described in Materials and Methods. The regression line was fitted by eye.

Effect of oral immunization on intraintestinal growth of *V. cholerae*. The possibility that oral immunization with live *V. cholerae* evokes protective mechanisms that inhibit growth of *V. cholerae* within the small bowel was studied by comparing growth of *V. cholerae* in ligated intestinal segments of immunized and control rabbits. Rabbits were immunized orally with 10^{10} CFU of strain 395 on day 0 and again after 14 to 20 days. Intestinal segments were prepared 6 to 18 days after the second immunization and inoculated with 10^3 or 10^8 *V. cholerae* 395 (A^+B^+) or its A^-B^- derivative, strain NT. Groups of rabbits were sacrificed after 0.5, 4, 8, or 16 h to determine total counts of viable *V. cholerae* within each segment and the volume of fluid in the segment, if any.

Recovery of viable *V. cholerae* from intestinal segments is summarized in Fig. 4, and the accumulation of fluid in the segments is shown in Table 3. These results show that small inocula of strain 395 (i.e., 10^3 CFU) grew well in unimmunized rabbits and poorly in immunized animals, the differences in growth being greatest when bacterial counts were determined at least 4 h after inoculation. Little or no fluid accumulated in these segments during the 16 h of observation. In contrast, large inocula of strain 395 (i.e., 10^8 CFU) grew modestly in nonimmune rabbits, whereas viable counts remained essentially unchanged in immunized animals. Substantial amounts of fluid accumulated after 8 h in segments in nonimmune rabbits, and much smaller amounts were recovered from segments of immunized animals. In similar experiments using strain NT for challenge, bacterial counts after 16 h were virtually identical to those in immune and nonimmune rabbits challenged with strain 395. However, less intestinal secretion was observed; fluid was present only in segments in nonimmunized rabbits challenged with 10^8 CFU, and, moreover, the amount present was 80% less than in rabbits similarly challenged with strain 395.

DISCUSSION

Enteric colonization with live, virulent *V. cholerae* O1 evokes immunity to reinfection in humans (2) and animals (3, 26); in rabbits this includes resistance to recolonization and protection against choleralike diarrhea after RITARD challenge (3, 26). Such protection is probably mediated by intestinal secretory antibodies induced by the primary inoculation and directed against a variety of bacterial antigens (6). In studies on the immunogenicity of live *V. cholerae*, the

TABLE 2. Resistance to recolonization in rabbits immunized orally with live or killed *V. cholerae*

Oral immunization strain (CFU)	No. of inoculations	Ileal colonization[a]	*P*[b]	
			Nonimmunized rabbits	Immunized rabbits
None		6.8 ± 0.4		<0.001
Live 395 (4×10^1)	1	2.5 ± 0.2	<0.001	
Killed 395 (10^6)	1	7.6 ± 0.4	NS	<0.001
Killed 395 (10^{11})	1	5.6 ± 0.7	<0.001	<0.001
Killed 395 (10^{11})	3[c]	4.5 ± 1.6	<0.001	

[a] Mean $\log_{10}$ (± standard error) *V. cholerae* recovered per gram of washed intestine 18 h after oral inoculation with 10^{10} live *V. cholerae* 395 organisms. Colonization was on day 19 after single oral immunization or day 35 after repeated oral immunizations. Each mean represents data from four to six rabbits.

[b] *P* values: nonimmunized rabbits, compared with colonization in nonimmunized rabbits; immunized rabbits, compared with colonization in rabbits immunized with live strain 395. NS, $P > 0.05$.

[c] Oral immunization given on days 0, 7, and 14.

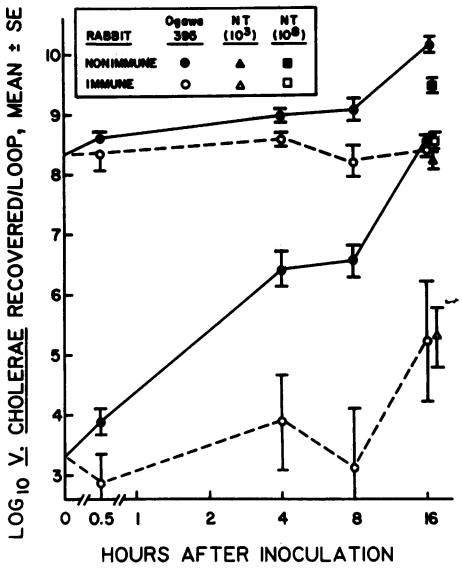

FIG. 4. Effect of oral immunization on intraintestinal growth of *V. cholerae*. The experimental design was described in the text. The figure compares total bacterial recovery from ligated intestinal segments of immune and nonimmune rabbits inoculated with 10^3 or 10^8 CFU of *V. cholerae* 395 (A^+B^+) or NT (A^-B^-) and assayed after the indicated interval. Rabbit immunization was with viable *V. cholerae* 395, by mouth. Each mean represents data from four or five rabbits.

response to one of these antigens, cholera toxin, was shown to have two major determinants: inoculum size and colonizing capacity of the immunizing strain (23). In the present studies we used the same rabbit models of infection and a well-characterized collection of *V. cholerae* strains, including several genetically and/or phenotypically defined parent-mutant pairs, to identify features of live *V. cholerae* that determine the organism's ability to immunize against a live bacterial challenge, and to explore the mechanisms of such protection. On the basis of earlier studies (23, 26), particular emphasis was given to the role of mucosal colonization in determining bacterial immunogenicity and resistance to recolonization as a means of defense against reinfection.

Virulent and mutant strains of *V. cholerae* differed markedly in their capacity to colonize rabbit intestine, with virulent strains colonizing most efficiently. The latter observation agrees with evidence that mucosal colonization is a critical event in the pathogenesis of cholera and suggests that mechanisms of colonization in rabbits and humans are similar. The bacterial factors that control colonization are, however, not entirely clear. We have previously shown that colonization is enhanced by production of cholera toxin (but not its B subunit; 22), and the present study accords with earlier evidence (7) that colonization is also aided by bacterial motility (as distinguished from the presence of a flagellum), but not by production of hemolysin nor, probably, by bacterial biotype or serotype. However, these findings do not explain the marked differences in colonizing capacity observed (Table 1), in which all strains except CVD49 were motile and one of the poorest colonizers, RV-79, was also toxinogenic. A possible explanation for these findings is that another factor (or factors) plays a major role in mucosal colonization and is required before cholera toxin and bacterial motility can play a contributing role; it has been proposed that a soluble hemagglutinin may be a major colonization factor of *V. cholerae*, but this is not firmly established

TABLE 3. Fluid secretion in intestinal segments of immunized and control rabbits challenged with *V. cholerae* strain 395 or NT

Time (h) after inoculation	Challenge strain	Fluid recovered from gut segment[a] (mean ml/cm) after inoculation with:			
		10^3 CFU[b]		10^8 CFU[b]	
		Control	Immune[c]	Control	Immune[c]
4	395	0	0	0.1	0
8	395	0.2	0.1	1.1	0.4
16	395	0.1	0	1.9	0.1
16	NT	0	0	0.4	0

[a] n = 6 to 12 intestinal segments for each mean (two segments per rabbit).
[b] Challenge inoculum in gut segments of control or immunized rabbits.
[c] Immunization was with 10^{10} *V. cholerae* 395 orally on days 0 and 14. Challenge was on day 28.

(5). Recently, evidence has been presented for a pilus colonization factor for *V. cholerae* O1, the expression of which is coordinated with that of cholera toxin (R. K. Taylor, V. L. Miller, D. B. Furlong, and J. J. Mekalanos, Proc. Natl. Acad. Sci. USA, in press).

The ability of different live *V. cholerae* strains to immunize rabbits against recolonization or RITARD challenge was determined largely by their ability to colonize the nonimmune small intestine; for strains that colonized best, immunization was extremely efficient, with a single inoculum of less than 100 live bacteria evoking significant protection against either type of challenge. In contrast, other bacterial features, including motility and the production of hemolysin, cholera toxin, or B subunit, did not independently affect immunizing efficiency against either type of challenge; i.e., these factors appeared to enhance immunogenicity only to the extent that they improved the colonizing capacity of a particular strain. There are at least two nonexclusive explanations for these observations. First, protection may be due to secretory antibodies that prevent colonization by binding to and blocking specific colonization factors on the organism; thus, strains that colonize best because they produce these factors abundantly could also evoke specific (and protective) immune responses to these antigens most efficiently. This possibility cannot be supported or excluded until colonization factors are identified that largely explain the sort of interstrain differences in colonizing capacity seen in this study. Second, efficient mucosal colonization may aid the delivery of a variety of bacterial antigens (other than colonization factors) to mucosal lymphoid tissue and thus promote more vigorous local immune responses to these antigens. This possibility is directly supported by evidence that the magnitude of a specific mucosal immune response (i.e., antitoxin) induced by live A^-B^+ or A^+B^+ *V. cholerae* is directly related to the colonizing capacity of individual strains (23). A third possibility, that certain strains multiply more extensively than others within rabbit bowel, thus greatly increasing the effective inoculum, appears to be excluded by evidence that virulent *V. cholerae* multiply only modestly within unobstructed rabbit intestine (3).

The observation that live *V. cholerae* are vastly more immunogenic than Formalin-killed vibrios is also consistent with the view that live bacteria are especially efficient at delivering protective antigens to responsive mucosal lymphoid tissue, although other explanations for these results are also possible. This interpretation is directly supported by evidence that live *V. cholerae* are readily translocated from the bowel lumen to submucosal lymphoid tissue by epithelial

M cells that overlie Peyer's patches, whereas under similar circumstances uptake of killed *V. cholerae* by M cells is not detected (18). Live *V. cholerae* organisms have also been shown in several studies to be more immunogenic in volunteers than killed vibrios given orally (2, 14, 15).

Protection evoked by live bacteria was largely antibacterial, with little or no contribution by antitoxin, and was manifest as marked resistance to mucosal colonization. This conclusion is supported by evidence that colonization was markedly reduced in rabbits immunized with live *V. cholerae* and that, in relation to their colonizing capacities, A^-B^- strains of *V. cholerae* immunized as efficiently against RITARD challenge (in which toxin-induced diarrhea occurs) as did A^-B^+ or A^+B^+ strains. Although small inocula of toxinogenic *V. cholerae* evoke detectable mucosal antitoxin responses (23), and under some conditions antitoxin and antibacterial immunity can act in concert to produce enhanced protection against choleralike illness (20, 25), the results in this study suggest that mucosal colonization in immunized rabbits was never sufficient for appreciable amounts of cholera toxin to be produced, and thus antitoxin played no significant protective role. It remains possible, however, that under circumstances in which resistance to mucosal colonization is less marked, antitoxin may contribute appreciably to protection against choleralike disease.

On the basis of studies in ligated segments of small bowel, resistance to mucosal colonization in immunized rabbits appeared to be due in part to suppression of bacterial growth in the bowel lumen, at the mucosal surface, or both. Thus, small inocula failed to multiply for at least 8 h in immune rabbits, whereas viable bacterial counts increased more than 1,000-fold in nonimmune animals. It seems likely that this process acted in concert with impaired mucosal adherence of bacteria to account for the even larger differences in bacterial counts observed in unobstructed intestine; in immune rabbits, poorly adherent bacteria would be swept out of the small bowel by peristalsis, whereas in nonimmune animals bacteria would adhere to and multiply on the mucosa. Failure of bacterial counts in ligated gut segments to decline with time when inocula were large suggests that growth in immune rabbits is not controlled by a bactericidal effect. More likely, this growth inhibition reflects such "nonspecific" effects of antibacterial antibodies as immobilization and possibly aggregation. However, if aggregation occurred as has been reported previously (12), the aggregates were probably small, since viable bacterial counts showed little or no decline 30 min after inoculation. If aggregates were large, e.g., composed of 10 or more bacteria, and aggregation occurred rapidly, a more marked decline in bacterial counts should have been seen.

These findings have important implications for current efforts to develop effective vaccines for cholera and other enteric infections, especially those due to noninvasive organisms. First, they show that mucosal colonizing capacity is the major determinant of the immunogenicity of live *V. cholerae*, and second, they reveal that live *V. cholerae* strains that colonize the small bowel efficiently are vastly superior to killed *V. cholerae* as oral immunogens. Taken together, these findings strongly suggest that the most effective oral cholera vaccine would be composed of live *V. cholerae* that colonize the bowel efficiently without causing untoward side effects. Immunization by strains with less than optimal colonizing capacity may also be protective, but for maximum efficacy this would likely require larger or repeated inoculations, or both. Killed *V. cholerae*, although immunogenic, are likely to be least effective and to require very large, repeated doses to evoke even partial protection. In this regard, these studies accord with volunteer studies showing that live virulent or mutant *V. cholerae* immunize more efficiently than nonliving vaccines composed of killed bacteria, with or without the addition of purified B-subunit or procholeragenoid (1, 2, 9).

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI18818, AI19547, AI19716, and AI18045 from the National Institute of Allergy and Infectious Diseases and by a grant from the Global Diarrhoeal Diseases Control Programme of the World Health Organization. Research facilities were provided by the Gerontology Research Center, National Institute on Aging, under its Guest Scientist Program.

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