

Oral Immunization of Dogs with Purified Cholera Toxin, Crude Cholera Toxin, or B Subunit: Evidence for Synergistic Protection by Antitoxic and Antibacterial Mechanisms

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The immunogenicity and safety of purified cholera toxin (CT), its B subunit, and a crude culture filtrate of toxigenic *Vibrio cholerae* (CrT) were compared in dogs immunized orally and challenged with virulent *V. cholerae*. CT and CrT caused marked protection in two- or three-dose regimens. Protection due to CT occurred only with doses that caused transient, sometimes severe, diarrhea in most dogs; this protection was proportional to the peak antitoxin response in jejunal mucosa and lasted at least 15 weeks. In contrast, minimum protective doses of CrT contained much less cholera toxin, caused very mild diarrhea in only 21% of the dogs, and evoked protection that was greater than predicted from the modest jejunal antitoxin response. B subunit caused smaller jejunal antitoxin responses than did similar doses of CT and was poorly protective, the 50% protective dose being >40-fold greater than that of CT. Two observations indicated that protection due to CrT involved synergy between antibacterial and antitoxic immune responses. First, the 50% protective dose of CrT was 24-fold and >36-fold smaller than the 50% protective doses of its CT and non-CT antigenic components, respectively, when tested separately. Second, protection was greater in CrT-immunized dogs than in CT-immunized dogs for a given mucosal antitoxin response. Low doses of CrT evoked serotype-specific protection, indicating that the serotype-specific O somatic antigen contributed significantly to antibacterial protection. These results suggest that a simple, effective, nonliving oral vaccine for cholera based on combined antibacterial and antitoxic immunity can probably be achieved. However, further studies are needed to determine how a protective antitoxic response can be evoked without causing diarrhea during immunization.

There is now much evidence to support the notions that secretory immunoglobulin A (sIgA) antibody is the first line of immunological defense of the intestinal mucosa and that a mucosal IgA response is best stimulated by antigens applied to the mucosal surface (1, 14, 18, 20, 28). On this basis, it has been argued that immunization against cholera, a superficial mucosal infection, should be given orally with the aim of evoking a protective mucosal IgA response in the small intestine (15). Some progress in this direction has been made by using either living avirulent mutants of *Vibrio cholerae* or nonliving antigens derived from this organism (8, 9, 16).

Among the latter, cholera toxin (CT) has been most extensively studied. It is an especially potent mucosal immunogen in a variety of experimental animals (6, 14, 29). Small amounts applied to the intestinal mucosa reliably evoke vigorous local secretory IgA antitoxin responses, and dogs immunized by multiple oral dosing

with purified CT or with a crude, CT-rich culture filtrate (CrT) are protected against disease induced by oral challenge with live virulent *V. cholerae* (16). These observations demonstrate a protective role for mucosal antitoxin and suggest that CT or an antigen derived from it might have practical value, either alone or in combination with other antigens, as an oral vaccine.

Such a vaccine should immunize efficiently and be free of significant side effects. Although our studies show substantial protection in dogs immunized orally with CT or CrT, 12 doses were given, and a majority of the animals developed transient, sometimes severe, diarrhea after antigen feeding (16). We therefore conducted further studies to determine whether protection can be achieved with fewer doses of antigen and whether the side effect of diarrhea can be reduced or eliminated by (i) lowering the dose of CT, (ii) using the nontoxic B subunit of CT rather than the holotoxin, or (iii) using reduced doses of CT

in combination with other potentially protective antigens of *V. cholerae*. We also determined whether immunity induced by oral CT lasts beyond the immediate post-immunization period. The results of these studies are described in this report.

MATERIALS AND METHODS

Animals. The dogs used were healthy mongrels weighing 7 to 19 kg when challenged. Before immunization they were quarantined for 2 weeks, dewormed, and immunized for rabies and distemper.

Antigens. The purified CT used was National Institutes of Health lot 0972 prepared by Richard Finkelstein. The CrT used was National Institutes of Health lot 001, a lyophilized, nondialyzed culture filtrate of *V. cholerae* strain Ogawa B1307 prepared by the method of Craig (2). CT and CrT were supplied by Robert Edelman, National Institute of Allergy and Infectious Diseases. Purified B subunit of CT was prepared and provided by Richard Finkelstein (5). CrT from which CT had been selectively removed (Depl-CrT) was prepared by Robert O. Thomson, Wellcome Research Laboratories, Beckenham, Kent, England. The toxin was removed by affinity chromatography with Fab fragments of equine anti-cholera toxin aggregated with glutaraldehyde as the immunoabsorbent. Depl-CrT was CrT which, after dialysis against water, had been applied to the affinity column, washed through with 0.05 M phosphate buffer (pH 7.2), concentrated by ultrafiltration with an Amicon hollow fibre cartridge (molecular weight cut out, 5,000), and lyophilized. This yielded 262 mg of Depl-CrT per g of nondialyzed CrT. The toxicities of CrT, B subunit, and Depl-CrT were measured by the rabbit skin vascular permeability assay (3) or by an in vitro mouse adrenal tumor cell assay (22). The toxicities of these materials, expressed as the equivalent amount of CT per milligram, were as follows: CrT, 36 ng; B subunit, 25 ng; and Depl-CrT, 1.5 ng.

Oral immunization. Dogs were fasted overnight. At 10 a.m. 50 ml of 6% NaHCO₃ was given by orogastric tube, followed by 100 ml of 2% Casamino acids (Difco Laboratories, Detroit, Mich.) containing the antigen. NaHCO₃ and Casamino acids were given to minimize damage to protein antigens by acid and proteolytic enzymes in the stomach. Food was given after 5 h. The dogs were observed for 24 h for diarrhea; Ringer lactate solution was given intravenously if diarrhea caused clinically evident saline depletion. Antigen doses, up to a total of three, were given at 21-day intervals. Some dogs received 12 doses, the additional doses being given daily, except for weekends, from days 43 to 54. Each immunization regimen included 14 to 23 dogs.

Challenge technique. Unless stated otherwise, challenge was at 19 to 21 days after the final dose of antigen. The preparation of the bacterial inoculum and the challenge technique were as described previously (19). Fasting dogs were inoculated with 0.9×10^{11} to 4.8×10^{11} viable *V. cholerae* strain Ogawa 395 or *V. cholerae* strain Inaba B36237 organisms (both are classical biotypes) by orogastric tube. At each challenge immunized dogs and an equal number of unimmunized controls received identical inocula. The variation in the number of viable bacteria in the inocula was within a range that does not affect the attack rate

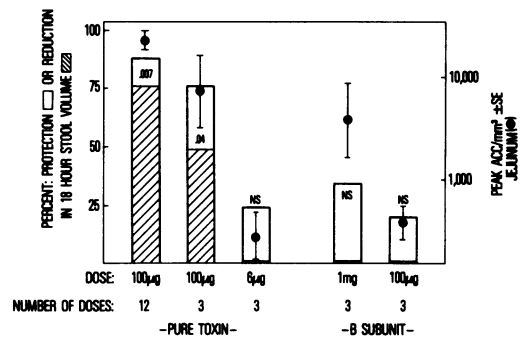


FIG. 1. Protection of dogs immunized orally with CT or B subunit. Groups of 14 to 17 dogs each were immunized with CT or B subunit as shown. ACC responses in jejunal mucosa were determined at 6 to 7 days after immunization for five to seven dogs from each group; the mean of these values is shown (●). Challenge with viable *V. cholerae* strain Ogawa 395 was at 3 weeks after immunization. Percent protection against severe or lethal diarrhea is shown by open bars and *P* values; percent reduction in first-18-h stool volume is shown by superimposed hatched bars. Both values were determined by comparison with outcomes in concurrently challenged nonimmune controls. In these studies 49% of nonimmune controls developed severe or lethal diarrhea; their mean first-18-h stool volume was 25.7 (± 4.2) ml/kg (SE). The data from dogs immunized with 12 100- μ g doses of CT have been reported previously and are included here for purposes of comparison (16).

for diarrhea in unimmunized dogs (24). In previous studies this challenge technique has caused severe or lethal diarrhea in about 50% of nonimmune control dogs (16, 17, 19, 21).

The dogs were observed in metabolic cages for 5 days after challenge. Food and water were withheld for the first 18 h so that liquid stool output could be measured accurately. Diarrhea usually began less than 18 h, and often less than 8 h, after challenge; about 70% of deaths occurred within the first 24 h. The results of challenge were classified as follows: (i) no diarrhea; (ii) mild diarrhea (one or more watery stools but no weakness, lethargy, or decrease in skin turgor); (iii) severe diarrhea (voluminous watery diarrhea, decreased skin turgor, and weakness or lethargy); and (iv) lethal diarrhea. The outcome was also expressed as the output of liquid stool (in milliliters per kilogram of body weight) during the first 18 h after challenge, when stool volume was usually greatest. This measurement was limited to 18 h because accurate stool collections were not possible after the dogs were again given food and water.

Antibody titrations. Sera were obtained at the indicated times and stored at -40°C . Antitoxin was titrated by a mouse adrenal tumor cell assay in 96-well tissue culture plates (22). Antitoxin unitage was determined by comparing each specimen with a simultaneously titrated standard serum containing 4,470 antitoxin units per ml [lot EC3(A-/67)-B, manufactured by the Swiss Serum and Vaccine Institute, Berne, Switzerland]. Preimmunization titers were consistently less than 1 U/ml, which was the sensitivity of the

TABLE 1. Prolonged protection of dogs immunized orally with CT

Challenge interval (wk) ^a	Jejunal ACC ^b	Antitoxin titer ^c	% Protection ^d	% Reduction in stool vol ^e
3	25,800 (1.2)	9.8 (1.4)	88 ($P = 0.007$)	76
15	1,070 (1.6)	<1	100 ($P = 0.04$)	87

^a Immunization was with 12 100- μ g doses. The interval is the time between the final dose of CT and challenge with *V. cholerae* strain Ogawa 395.

^b Geometric mean ACC per cubic millimeter (\times SE) in jejunal lamina propria at 6 to 7 days (3-week study) or 14 weeks (15-week study) after the final dose of CT. Each mean is based on data from six dogs.

^c Geometric mean prechallenge serum antitoxin titer in units per milliliter (\times SE).

^d Protection against the combined incidence of severe or lethal diarrhea compared with concurrently challenged nonimmune controls.

^e Reduction in mean stool volume (measured in milliliters per kilogram) during the first 18 h postchallenge compared with concurrently challenged nonimmune controls.

assay. Vibriocidal antibody to *V. cholerae* strain Ogawa 395 was measured as previously described (23).

ACC in jejunal lamina propria. Jejunal biopsies were obtained by laparotomy from five to seven dogs in each immunization group as described previously (17). Unless stated otherwise, biopsies were taken 6 to 7 days after the final antigen dose, which is when antitoxin-containing plasma cells (ACC) are most numerous in lamina propria (18). ACC in the lamina propria were identified by a previously described fluorescent antibody technique (18). Adjacent microscopic fields (0.33 mm in diameter) in sections 5 μ m thick were examined in the crypt region; about 25 fields in two sections were examined per specimen. ACC frequency is expressed as the number per cubic millimeter in the crypt region; 1 ACC per field equals 2,300/mm³. The lower limit of sensitivity of this assay was about 90 ACC/mm³. ACC were never observed in biopsies from unimmunized dogs (17). ACC responses are expressed as geometric means because these reflect the logarithmic manner in which the measured cellular response expands after immunization and because mucosal protection against challenge with CT correlates linearly with the geometric mean frequency of ACC in the lamina propria (17).

Statistical analysis. Fisher's two-tailed exact test was used to derive probability values. Percent protection against the combined incidence of severe or lethal disease and percent reduction in mean 18-h stool volume were determined by comparison with concurrently challenged nonimmune controls as described previously (21).

RESULTS

Immunization with CT or B subunit. Dogs that were given 12 or 3 100- μ g doses of CT developed vigorous ACC responses in jejunal mucosa and were significantly protected when challenged 3 weeks later with viable *V. cholerae* (Fig. 1). In contrast, immunization with three 6- μ g doses of CT caused ACC responses only 1 to 3% as great and was not significantly protective. The expression of protection as prevention of severe or lethal diarrhea or as reduction in the first-18-h postchallenge stool volume gave similar results. Geometric mean prechallenge serum antitoxin

titers were 9.8 (\times 1.4) and 4.1 (\times 1.4) U/ml (\times standard error [SE]) in dogs given 12 or 3 100- μ g doses of CT, respectively; antitoxin was undetectable in prechallenge sera of dogs given 3 6- μ g doses.

Protection of dogs immunized with CT (12 100- μ g doses) was also demonstrated 15 weeks after the final antigen dose (Table 1). Although prechallenge jejunal ACC responses had declined by 96% from the postimmunization peak and serum antitoxin was no longer detectable, protection against live vibrio challenge was as great as that observed 3 weeks after immunization.

B subunit was less immunogenic than CT (Fig. 1). Dogs given three 100- μ g doses of B subunit developed jejunal ACC responses that were only 5% as great as in dogs similarly immunized with CT ($P < 0.001$), and they were not significantly protected against challenge with living *V. cholerae*. Increasing B subunit immunization to three 1-mg doses evoked greater jejunal ACC responses, similar to those caused by three 100- μ g doses of CT, but the protection against severe or lethal diarrhea was still not statistically significant, and immunization had no effect upon mean stool volume during the first 18 h after challenge. Neither immunization regimen with B subunit caused detectable prechallenge serum antitoxin titers.

Immunization with CrT. Dogs given CrT in schedules ranging from 12 1-g doses to 2 63-mg doses were significantly protected against live vibrio challenge, but 1 63-mg dose or 3 16-mg doses were nonprotective (Fig. 2). The highest doses of CrT evoked vigorous jejunal ACC responses, but, in contrast to CT-immunized dogs, marked protection also occurred with doses that caused barely detectable ACC responses. Thus, 2 63-mg doses caused 90% protection against severe or lethal disease and a 77% reduction in the mean stool volume during the first 18 h after challenge, despite a peak ACC

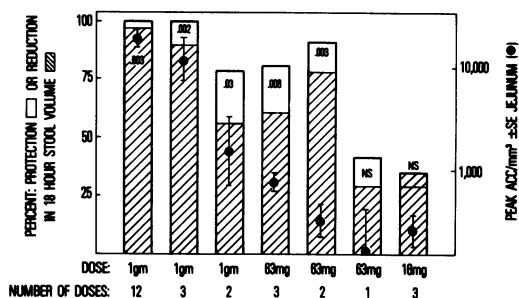


FIG. 2. Protection of dogs immunized orally with CrT. Groups of 14 to 20 dogs each were immunized with CrT as shown. ACC responses in jejunal mucosa were determined at 6 to 7 days after immunization for five to seven dogs from each group; the mean of these values is shown (●). Challenge with viable *V. cholerae* strain Ogawa 395 was at 3 weeks after immunization. Percent protection against severe or lethal diarrhea is shown by open bars and *P* values; percent reduction in first-18-h stool volume is shown by superimposed hatched bars. Both values were determined by comparison with outcomes in concurrently challenged nonimmune controls. In these studies 51% of nonimmune controls developed severe or lethal diarrhea; their mean first-18-h stool volume was 32.8 (\pm 3.9) ml/kg (SE). The data from dogs immunized with 12 1-g doses of CrT have been reported previously and are included here for purposes of comparison (16).

response only 1.3% of that caused by 12 1-g doses. Moreover, this ACC response was essentially identical to that in dogs given equivalent doses of CT (three 6- μ g doses), a regimen which was not protective against vibrio challenge (Fig. 1). After CrT immunization, prechallenge serum antitoxin titers were similar to those in dogs given comparable doses of CT. Geometric mean prechallenge serum antitoxin titers were 11 (\times 1.3), 2.7 (\times 1.4), and 1.8 (\times 1.3) U/ml (\times SE) in dogs given 12, 3, or 2 1-g doses of CrT, respec-

tively. Smaller CrT doses caused no detectable serum antitoxin responses.

Protection by CrT or its antigenic components against challenge with *V. cholerae* strain Ogawa or *V. cholerae* strain Inaba. Protection due to the lowest effective dose of CrT was compared with that due to its toxin (CT) and nontoxin (Depl-CrT) antigenic components. Whereas two 63-mg doses of CrT were highly protective, neither three 2.6-fold-greater doses of CT nor three doses of Depl-CrT, each derived from a 16-fold-greater amount of CrT, caused significant protection (Table 2). The lowest CrT doses which protected against challenge with *V. cholerae* strain Ogawa 395 (the serotype used to make CrT) did not protect against challenge with *V. cholerae* strain Inaba B36237 (Table 2). None of these immunization groups had detectable prechallenge serum antitoxin titers. Among dogs given Depl-CrT, mean serum vibriocidal antibody titers rose from 2.9 \pm 0.2 (preimmunization) to 3.5 \pm 0.2 (prechallenge), expressed as 1/log₁₀ of the endpoint serum dilution \pm SE (*P* < 0.02 by the *t* test).

Relative protective efficacy of CT, B subunit, CrT, and Depl-CrT. An analysis of the preceding results showed that equivalent doses of CT or B subunit, in three-dose regimens, were most protective when given as CrT and least protective when given as B subunit (Fig. 3). Based on this figure, the estimated 50% protective dose (PD₅₀) for each material against severe or lethal disease, expressed in terms of its CT or B subunit content, was: CrT, 0.95 μ g (i.e., the amount of CT in 26 mg of CrT); CT, 25 μ g; and B subunit, >1000 μ g. Thus, the protective efficacy of CT was more than 40-fold greater than that of B subunit, and the efficacy of CrT, expressed in terms of its CT content, was 24-fold greater than that of CT.

TABLE 2. Protection of dogs immunized with CT, CrT, or Depl-CrT when challenged with Ogawa or Inaba serotypes of *V. cholerae*

Antigen	Dose (mg) (no. of doses)	Equivalent dose of CT (μ g)	Jejunal ACC ^a	<i>V. cholerae</i> challenge strain	% Protection ^b	% Reduction in stool vol ^c
CT	0.006 (3)	6	250 (1.8)	Ogawa 395	24 (NS) ^d	0
CrT	63 (2)	2.3	280 (1.5)	Ogawa 395	90 (<i>P</i> = 0.002)	77
Depl-CrT	250 (3) ^e	0.4	140 (1.7)	Ogawa 395	44 (NS)	0
CrT	63 (3)	2.3	210 (1.4)	Inaba B36237	44 (NS)	0

^a Geometric mean AAC per cubic millimeter (\times SE) in jejunal biopsies taken at 6 to 7 days after the final antigen dose. Each mean is based on data from 5-7 dogs.

^b Protection against the combined incidence of severe or lethal diarrhea compared with nonimmune controls. Among controls challenged with *V. cholerae* strain Ogawa 395 or *V. cholerae* strain Inaba B36237, 46 and 50%, respectively, developed severe or lethal diarrhea.

^c Reduction in mean stool volume during the first 18 h after challenge compared with nonimmune controls. The mean stool output of controls challenged with *V. cholerae* strain Ogawa 395 or *V. cholerae* strain Inaba B36237 was 27.1 (\pm 4.9) and 27.4 (\pm 8.8) ml/kg (SE), respectively.

^d NS, Not significant.

^e A total of 250 mg of dialyzed Depl-CrT was derived from 950 mg of nondialyzed CrT.

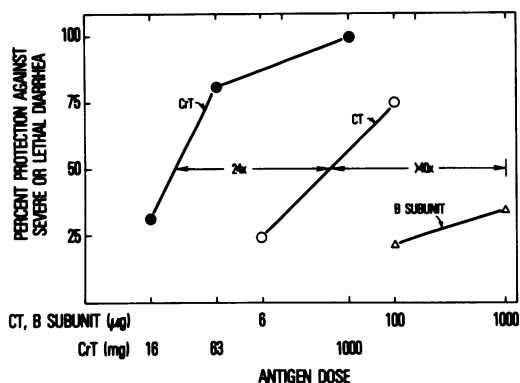


FIG. 3. Comparison of protection of dogs immunized orally with CrT, CT, or B subunit. All data are from groups of dogs immunized with three doses of antigen and challenged with *V. cholerae* strain Ogawa 395 after 3 weeks. Antigen doses are plotted so that doses of CT and B subunit are compared with doses of CrT containing similar amounts of CT. The fold differences in estimated PD_{50} s for CrT and B subunit, relative to CT, are shown by horizontal arrows.

In an additional analysis, using data from Fig. 3 and Table 2, we compared the PD_{50} s of CrT and Depl-CrT. Expressed as the actual amounts of CrT given or used to prepare Depl-CrT, the PD_{50} s were: CrT, 26 mg; and Depl-CrT, >950 mg. Thus, the protective efficacy of CrT was more than 36-fold greater than that of an equivalent dose of Depl-CrT.

Relationship of protection to peak mucosal ACC response. Protection by CT, CrT, and B subunit were also analyzed with respect to the peak mucosal ACC responses evoked by different immunization regimens (Fig. 4). Protection induced by CT appeared to correlate linearly with the mean ACC response achieved, as has been noted previously (17); substantial protection occurred only with a mean peak ACC response of >7,000/mm³. The results in CrT-immunized dogs differed in that the curve relating protection to peak ACC response rose more steeply. Thus, significant protection was observed with mean peak ACC responses as low as 280/mm³, i.e., 4% of the lowest mean response associated with similar protection in CT-immunized dogs. It also appeared that the slope of the line relating ACC response to protection was less steep in dogs immunized with B subunit than in those given CT.

Incidence of diarrhea during immunization with various antigens. Some dogs developed transient diarrhea after oral immunization. This usually began within 7 h and always ended within 22 h. Table 3 shows the incidence and severity of such diarrhea according to the dose of specific antigen used. Although the results shown are those after the first dose, the inci-

dence was similar or only slightly reduced after a second or third dose. Diarrhea frequently followed oral doses of CT or CrT, the incidence and severity being dose related. In contrast, B subunit and Depl-CrT caused little or no diarrhea. Antigen doses which caused diarrhea in a majority of dogs were also protective against *V. cholerae* challenge, whereas those that caused little or no diarrhea were nonprotective. The only exception involved dogs immunized with two or three 63-mg doses of CrT; very mild diarrhea occurred in only 21% of these animals, but highly significant protection against vibrio challenge was observed.

DISCUSSION

Our purpose in this study was to determine whether a simple, safe, and effective means of oral immunization against experimental canine cholera could be developed by using CT or its B subunit, alone or in combination with other antigens of *V. cholerae*. We also sought to better define the duration of protection caused by oral immunization with a single purified protein antigen, and we employed CT for this purpose.

With CT as an oral immunogen, protection was achieved with a 3-dose immunization series and lasted at least 15 weeks after a 12-dose series. However, the doses used in these studies caused transient, but sometimes severe, diarrhea in a majority of dogs. A 16-fold reduction in CT dose nearly eliminated the side effect of diarrhea but evoked poor jejunal ACC responses and was nonprotective. These results give further evidence that CT is a highly effective mucosal immunogen (16, 27) but also suggest that

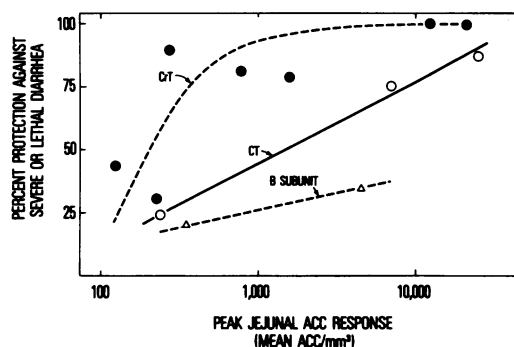


FIG. 4. Protection of dogs relative to jejunal ACC responses achieved by oral immunization with CrT, CT, or B subunit. Data from all immunization schedules, irrespective of size of dose or number of doses given, are included, provided that challenge was with *V. cholerae* strain Ogawa 395 at 3 weeks after immunization. Mean jejunal ACC responses are from biopsies of five to seven dogs in each group taken 6 to 7 days after completion of immunization. Lines were drawn by sight to best apparent fit.

TABLE 3. Incidence of diarrhea during immunization with various antigens

Oral antigen	Dose (mg)	No. of dogs	% With diarrhea ^a			Best observed % protection ^b
			Mild	Moderate	Severe	
CT	0.1	49	25	31	13	88 ($P = 0.007$)
	0.006	17	6	0	0	
B subunit	1	17	0	0	0	34
	0.1	16	13	0	0	20
CrT	1,000	52	19	46	10	100 ($P = 0.002$)
	63	52	21	0	0	90 ($P = 0.002$)
	16	18	11	0	0	31
Depl-CrT	250	23	0	0	0	44
Control ^c		46	4	0	0	

^a Results are those observed after the first dose of antigen. Severe diarrhea caused clinical evidence of saline depletion and required intravenous replacement of water and electrolytes; moderate diarrhea was several loose stools with no evidence of saline depletion; mild diarrhea was a single loose stool with an average volume of about 25 ml.

^b Best protection against the combined incidence of severe or lethal diarrhea in any immunization group that was given the indicated antigen dose one or more times and challenged at 3 weeks after the final dose. P values indicate statistically significant protection; all other values were not statistically significant.

^c Dogs were inoculated orally with 50 ml of 6% NaHCO₃ and 100 ml of 2% Casamino acids but no antigen.

immunization with only CT is probably impractical because the side effect of diarrhea may be unavoidable at the doses required for protection.

Attempts to immunize with the nontoxic B subunit of CT and thereby avoid the side effect of diarrhea were also disappointing. Although B subunit did not cause diarrhea, it was inferior to CT as an immunogen in at least two respects. First, when equal doses were given, B subunit evoked much smaller mucosal ACC responses. Second, protection associated with a given ACC response caused by B subunit appeared to be poorer than that associated with a comparable ACC response evoked by CT. In combination, these factors caused the PD₅₀ of B subunit to exceed that of CT by more than 40-fold.

Poorer mucosal ACC responses to B subunit than to CT have also been observed in enterically immunized rats and attributed to the inability of B subunit to stimulate adenylate cyclase activity in mucosal lymphoid cells (14); adenylate cyclase stimulation modulates lymphoid cell function and probably accounts, in part, for the marked efficiency of CT as a mucosal immunogen (14). The apparently poorer protection in B subunit- than in CT-immunized dogs, despite similar mucosal ACC responses, is unexplained. Possibly, antibody to A subunit plays a protective role in CT-immunized dogs; previous studies have shown that parenteral immunization with A subunit protects rabbits against challenge with *V. cholerae* (13). Alternatively, mucosal antitoxin evoked by CT may be more avid, and thus more protective, than that caused by B subunit.

Although these results do not exclude a practical role for B subunit as an oral immunogen, they suggest that large doses may be required to evoke protective antitoxin responses, especially in unprimed individuals. The oral dose of B subunit required for enteric priming in humans is not known, but studies in adults primed by natural exposure to *V. cholerae* show that a detectable enteric sIgA antitoxin booster response occurs after an oral dose of 500 µg of B subunit (26). It is not known, however, whether such responses are protective.

Of the antigens tested, only CrT caused substantial protection while carrying only a modest risk of diarrhea during immunization. This was probably because the PD₅₀ for CrT, expressed on the basis of CT content, was only 4% of that for purified CT. Thus, in contrast to the high incidence of diarrhea in dogs immunized with CT, the lowest protective dose of CrT caused very mild diarrhea, usually a single small loose stool, in only 21% of the dogs. The incidence of diarrhea might have been even lower if hypertonic NaHCO₃, equivalent to 230 ml of an isotonic sodium solution, had not been given with CrT. It is likely that the observed diarrhea, with an average volume of 25 ml, was not due to CrT-induced intestinal secretion but simply reflected impaired absorption, caused by CrT, of a portion of the concurrently administered oral sodium solution. Although diarrhea is an undesirable, and probably unacceptable, side effect of oral immunization, these results suggest that protective doses of CrT might be as safe as those of a recently developed *V. cholerae* mutant.

That strain, which produces B subunit, but no A subunit, of CT and has been proposed as a live oral vaccine strain, causes mild diarrhea in about 19% of volunteers (9; M. M. Levine, R. E. Black, M. L. Clements, C. R. Young, T. Honda, and R. A. Finkelstein, manuscript in preparation).

Two observations indicated that the protection of dogs immunized orally with CrT was due to the combined, apparently synergistic, immunizing effects of CT and other bacterial antigens. First, the PD_{50} for CrT was 24- and more than 36-fold lower than the PD_{50} s for its toxin (CT) and nontoxin (Depl-CrT) antigen components, respectively, when used separately. Second, greater protection was seen in CrT-immunized dogs than in CT-immunized dogs for a given mucosal ACC response, indicating that immune responses other than antitoxin contributed to the protection of those given CrT. Evidence for protective synergy between antitoxin and antibacterial or antilipopolysaccharide antibodies has also been observed in rabbits immunized parenterally with cholera toxin or toxin and killed *V. cholerae* or its lipopolysaccharide (12, 25).

One antigen responsible for the greater protective efficacy of CrT appeared to be the serotype-specific O somatic antigen associated with *V. cholerae* lipopolysaccharide. Indirect evidence for this was the failure of the lowest CrT doses which protected against challenge with the homologous (Ogawa) serotype to protect against the heterologous (Inaba) serotype of *V. cholerae*. Serotype-specific protection has also been observed in humans immunized parenterally with killed *V. cholerae* of a single serotype (11).

The protection of dogs challenged shortly after oral immunization with CT or CrT was probably due to secretory antibodies produced by plasma cells in the intestinal lamina propria. Evidence that this, rather than serum, was the source of protective antitoxin has been reviewed previously (16, 17). It is likely that oral CrT also evoked secretory antibodies to other bacterial antigens, but these were not measured in this study; however, significantly increased vibriocidal antibodies were measured in serum, indicating that bacterial antigens were absorbed in immunogenic amounts. Whether preformed secretory antibodies also mediated the protection observed several months after oral immunization is less certain. In this study, protection was undiminished after 3.5 months despite a 96% decline in the mucosal ACC response of CT-immunized dogs, and in an earlier study, protection persisted when mucosal ACC responses had become undetectable (17). Although this and other studies show that oral immunization can cause lasting mucosal protection in animals and

humans (10, 17, 21), the immune mechanisms which mediate such protection are not certain (17).

The results of this study may have practical importance for efforts to develop a nonliving oral vaccine for cholera. They support the view that oral vaccines containing at least two protective antigens will be much more efficient at evoking protection than will those containing a single purified antigen (25). They also identify at least two specific antigens that stimulate such synergistically protective immune responses: CT and the serotype-specific somatic O antigen. Although immunization with CT may cause diarrhea, the incidence of this side effect was much diminished in a polyvalent vaccine (CrT), because smaller amounts of CT were required. It is possible that other immunizing combinations, not tested in this study, may cause synergistically protective antitoxin responses without the risk of diarrhea. These include (i) reduced amounts of CT in combination with increased amounts of other bacterial antigens, (ii) the combination of bacterial antigens with large doses of B subunit (7), and (iii) the combination of bacterial antigens with an altered form of CT which has reduced toxicity but retained immunogenicity. With respect to the latter possibility, we have shown, and will report elsewhere, that heat-aggregated CT (procholeragenoid) (4) is highly immunogenic and minimally toxic in the intestine. Dogs immunized with this antigen alone experience no diarrhea during immunization but develop vigorous mucosal ACC responses and are highly protected when challenged with viable *V. cholerae*. This suggests that heat-aggregated CT combined with other antigens of *V. cholerae* may provide a safe and effective polyvalent oral vaccine for cholera.

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