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Antitoxic Immunity to Cholera in Dogs Immunized Orally with Cholera Toxin

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Cholera toxin was evaluated as an oral immunogen against experimental canine cholera. Dogs were immunized orally with 100-μg doses of purified cholera toxin or comparable doses of crude toxin. Both doses caused moderate diarrhea in most nonimmune dogs. Repeated oral doses (12 doses in 54 days) gave marked protection against the diarrheal effect of oral toxin, provoked a vigorous antitoxic response in jejunal mucosa, and gave nearly complete protection against subsequent oral challenge with living virulent *Vibrio cholerae*. Protection appeared to be due largely to the antitoxic response in intestinal mucosa. The effectiveness of cholera toxin as an oral vaccine contrasts with the previously described ineffectiveness of toxoid given orally. This study provides an example of mucosal immunity due to a nonreplicating vaccine given orally and suggests that cholera toxin may be useful as a component of an oral vaccine for cholera.

Current efforts to develop an improved vaccine against cholera include attempts to immunize orally with the object of evoking a protective secretory immune response in the intestinal mucosa. Support for this approach comes from evidence that the mucosal secretory immune system is especially suited to respond to mucosally applied antigens (21). Additional support comes from the observation that volunteers given cholera show substantial and prolonged protection against rechallenge with *Vibrio cholerae*, apparently due to local intestinal immunity (1).

One approach to oral immunization is to use nonliving bacterial antigens, choosing those shown to cause a vigorous immune response in intestinal mucosa. In contrast to most proteins, locally applied cholera toxin is extremely effective in causing a specific immunoglobulin A (IgA) secretory immune response in the intestine. This has been a consistent finding in several animal species (9, 11, 12, 22) and appears to be due largely to the property of the toxin to adhere to receptors on cell membranes and to stimulate membrane-bound adenyl cyclase activity in lymphoid cells (11). On the other hand, inactivated toxoid has been poorly effective as a local antigen, except when animals were first primed by a parenteral route (12, 13). Moreover, animals and volunteers fed inactivated toxoid have shown no protection against oral challenge with living *V. cholerae* (10, 17).

Because of the superior performance of cholera toxin as a local antigen, it seemed important to know whether oral cholera toxin also provoked protection against challenge with live *V. cholerae*. Such studies would have two purposes: (i) to serve as a model for studying the extent of protection achievable by oral immunization with a protein antigen known to cause a vigorous mucosal immune response; and (ii) to encourage further study of cholera toxin, or one of its subunits, as an oral vaccine in humans. The studies in this report involved oral immunization of dogs with crude or purified cholera toxin and subsequent challenge with virulent *V. cholerae*. The results show that cholera toxin given orally caused vigorous mucosal antitoxin responses and produced substantial protection against experimental cholera.

MATERIALS AND METHODS

Animals. Dogs were healthy mongrels weighing 7 to 20 kg when challenged. Before immunization they were quarantined for 2 weeks, dewormed, and immunized against rabies and canine distemper.

Cholera toxins. Crude cholera toxin was NIH lot 001, a lyophilized culture filtrate of *V. cholerae* Ogawa B 1307 grown by the method of Craig (2). Purified cholera toxin was lot 0972; it was derived from *V. cholerae* Inaba 569B made by R. A. Finkelstein (5). The relative toxicities of these materials, expressed in rabbit skin bluing doses (BD_4; 3), was 3.2×10^3 BD_4 per mg for crude toxin and 8.5×10^7 BD_4 per mg for purified toxin (11). Both materials were provided by Carl Miller, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Oral immunization. Dogs were fasted overnight. At 10:00 a.m. 50 ml of 6% $NaHCO_3$ was given by orogastric tube followed by 100 ml of 2% Casamino Acids (Difco Laboratories, Detroit, Mich.) containing

1 g of crude toxin or 0.1 mg of pure toxin. Food was given after 2 to 5 h. Dogs were observed 24 h for diarrhea; Ringer lactate solution was given intravenously if diarrhea caused clinically evident saline depletion. Toxin was given on days 0 and 21, and daily, excluding weekends, from days 42 to 54, for a total of 12 doses. The doses of crude and pure toxin had approximately equal toxic activity and were chosen on the basis of preliminary studies, which showed these doses would cause mild or moderate diarrhea in 90% of nonimmune dogs.

Challenge technique. Fasting dogs were challenged with 0.9×10^{11} to 2.5×10^{11} viable *V. cholerae* Ogawa 395 given by orogastric tube. At each challenge unimmunized controls and immunized dogs received identical inocula. The variation in number of viable bacteria in the inocula was within a range that does not influence the attack rate for diarrhea in unimmunized dogs (20). Preparation of the challenge inoculum and the challenge technique were as described elsewhere (15). Dogs were observed for 6 days after challenge, the results being classified as (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, decrease in skin turgor, and weakness or lethargy in some dogs); and (iv) lethal diarrhea. Diarrhea usually began less than 16 h after challenge; about 70% of deaths occurred within the first 24 h.

Bacteriology. Rectal swabs were streaked directly on thiosulfate-citrate-bile salt agar and cultured for 6 h in 1% alkaline peptone water (pH 9.0) before subculturing on thiosulfate-citrate-bile salt agar. *V. cholerae* were identified by colonial appearance and appropriate agglutination in sera specific for O group and serotype.

Antibody titrations. Sera were obtained at indicated times and stored at −40°C. Antitoxin was titrated by a mouse adrenal tumor cell assay in 96-well tissue culture plates (18). Antitoxin units were determined by comparing each specimen with a simultaneously titrated standard serum containing 4,470 antitoxin units per ml (manufactured by Swiss Serum and Vaccine Institute and provided by Carl Miller, National Institute of Allergy and Infectious Diseases). Vibriocidal antibody to *V. cholerae* Ogawa 395 was measured as described by Sack et al. (19).

ACC in jejunal lamina propria. Jejunal biopsies were obtained by laparotomy as described elsewhere (12). Cholera antitoxin-containing plasma cells (ACC) in the lamina propria were identified by a previously described fluorescent-antibody technique (13). Adjacent microscopic fields (0.33 mm in diameter) in 5-μm-thick sections were examined for ACC in the crypt region, where they were most numerous; about 25 fields in two sections were examined per specimen. ACC frequency is expressed as number per cubic millimeter in the crypt region, 1 ACC per field equalling 2,300/mm^3. The lower limit of sensitivity of this assay was about 90 ACC per mm^3.

Biochemical analyses. Serum levels of glucose, aspartate aminotransferase, and alkaline phosphatase were determined by standard techniques with automated analysis equipment.

Statistical analysis. Student's *t* test and Fisher's two-tailed exact test were used as indicated below. Percent protection against the combined incidence of severe or lethal disease was determined by comparison with concurrently challenged nonimmune controls as described previously (17).

RESULTS

Side effects of immunization. The first dose of crude or pure toxin caused diarrhea in almost all dogs (Fig. 1). Diarrhea began as early as 4 h and lasted as long as 16 h. Diarrhea was of similar severity in both immunization groups, usually being mild, but in about 9% of dogs requiring intravenous repletion of water and electrolyte losses. Subsequent doses of crude toxin caused a similar pattern of diarrhea until day 47, the 6th day of daily toxin dosing, after which severe diarrhea disappeared and mild diarrhea occurred in only a few animals. Dogs given pure toxin showed similar results except that the incidence of diarrhea from days 42 to 47 was lower.

The effect of pure toxin given orally on serum levels of glucose, aspartate aminotransferase, and alkaline phosphatase was studied in five dogs to determine whether it was absorbed in amounts sufficient to cause systemic toxicity (14). Fasting dogs were bled before oral toxin and 1 and 4 days later. Serum glucose concentrations at these intervals were 62 ± 11, 85 ± 11, and 64 ± 8 (milligrams per deciliter, mean ± standard error [SE]), respectively; aspartate aminotransferase levels were 19 ± 1.8, 13 ± 1.7, and 17 ± 2.5 (international units per milliliter, mean ± SE); and alkaline phosphatase values were 260 ± 89, 228 ± 89, and 328 ± 104 (inter-

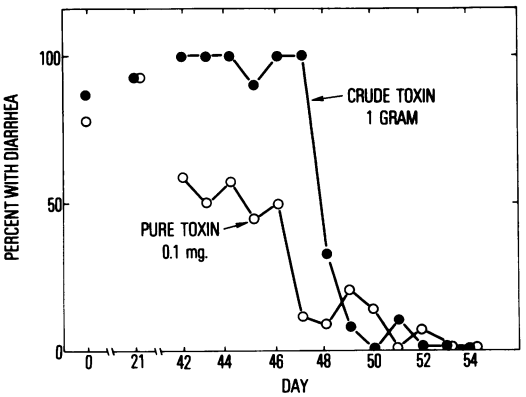

FIG. 1. *Incidence of diarrhea after each intragastric dose of crude or purified cholera toxin. Twenty dogs received crude toxin, and 15 were given purified toxin. Doses were given on days 0 and 21, and daily, except on weekends, from days 42 to 54.*

national units per milliliter, mean ± SE). The changes in serum glucose and aspartate aminotransferase were not statistically significant. Alkaline phosphatase levels in one dog, the only one developing severe diarrhea, were 117, 95, 653, and 369 U/ml on days 0, 1, 4, and 8, respectively; levels did not change appreciably in the other four dogs. Aside from diarrhea, the dogs showed no outward ill effects from oral toxin.

Systemic and mucosal immune responses. Serum antitoxin and vibriocidal antibody titers are shown in Table 1. Serum antitoxin responses were similar in recipients of crude and pure toxin, rising 15- to 40-fold 6 days after the last dose of toxin (day 60) and declining by 50 to 75% 20 days later, when challenged. In contrast, the serum vibriocidal antibody titer rose only in dogs given crude toxin.

A striking mucosal antitoxin response was seen in jejunal biopsies from both immunization groups. Whereas ACC have never been detected in jejunal lamina propria of nonimmune dogs (12), they were profuse 3 days after the last dose of toxin (Fig. 2) but had declined in number by 90% 20 days later (Table 2).

Results of challenge. Dogs were challenged with living *V. cholerae* 26 days after the last dose of toxin (day 80 after starting immunization); the outcome is shown in Table 3. Both immunization groups were highly protected. Only one of 34 immunized dogs developed severe diarrhea, whereas 17 of 35 nonimmune dogs had severe or lethal disease.

TABLE 1. *Antitoxin and vibriocidal antibody titers in serum of immunized dogs*

Immunization[a]	Titer		
	Day 0	Day 60	Day 80
Antitoxin			
Crude toxin	1.3[b]	20	11
$n = 20$	(1.1)	(1.6)	(1.3)
Pure toxin	1.0	44	9.8
$n = 14$	(1.2)	(1.5)	(1.4)
Vibriocidal antibody			
Crude toxin	2.8[c]	NT[d]	3.9[e]
$n = 10$	(0.25)		(0.10)
Pure toxin	2.7	NT	2.9[f]
$n = 10$	(0.21)		(0.23)

[a] See text for immunization schedule.
[b] Antitoxin units per milliliter, geometric mean (× SE).
[c] Log_{10} of reciprocal of endpoint dilution of serum, mean ± SE.
[d] NT, Not tested.
[e] $P < 0.001$ compared with day 0 using Student's t test.
[f] Not significantly different than day 0.

Rectal swab cultures for *V. cholerae* taken 1, 2, and 6 days after challenge showed no differences in the portion positive from either immunization group or from surviving nonimmune controls (Table 4). In all surviving dogs, colonization with *V. cholerae* was brief, terminating within 6 days.

DISCUSSION

An earlier study has shown that crude cholera toxin applied directly to jejunal mucosa of dogs causes a vigorous local antitoxic response (2). The object of the present study was to determine whether cholera toxin given orally would provoke a similar response in intestinal mucosa and protect dogs against subsequent oral challenge with virulent *V. cholerae*. The oral doses of cholera toxin were given with sodium bicarbonate and Casamino Acids to minimize their destruction in the stomach. Despite these measures, however, an unknown portion of oral toxin might still be destroyed in the stomach. Thus doses were used which caused diarrhea in most dogs, giving direct evidence that active toxin had reached the small intestine. A 12-dose immunization schedule was used to give a vigorous antigenic challenge which would favor production of a protective response. This choice was based partly on evidence that multiple enteric booster doses of purified toxoid were needed to evoke protection of dogs after parenteral priming with toxoid (17). This schedule was also designed to allow observation of the time course of the development of antitoxic protection during the period of daily dosing with toxin.

Dogs given purified toxin orally were highly protected against subsequent challenge with living *V. cholerae*. Two observations suggest that this protection was due largely or entirely to antitoxin. The first was the abrupt cessation of toxin-induced diarrhea after five daily doses of toxin (Fig. 1); because diarrhea was caused by toxin, its disappearance during immunization can only be explained by an antitoxic mechanism. The second was failure of purified toxin to evoke a vibriocidal antibody response, an observation also made in dogs given purified toxin parenterally (15); this is direct evidence that purified toxin contained insufficient bacterial somatic antigen to promote a potentially protective antibacterial immune response.

Because dogs given crude toxin received the same dose of active toxin, developed similar systemic and mucosal antitoxic responses, showed a similar decline in toxin-induced diarrhea during daily dosing, and were as well protected as those given purified toxin, their protection could be explained entirely by the antitoxic

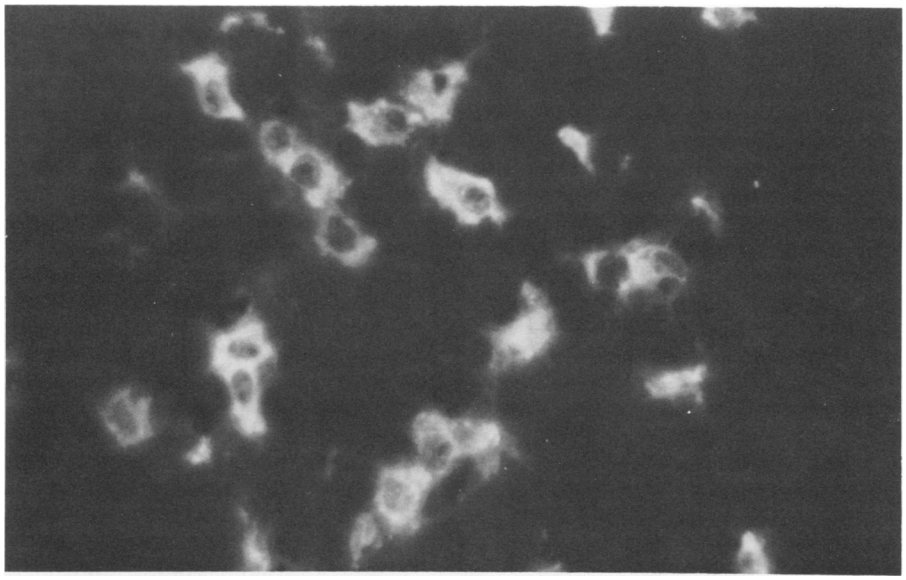

FIG. 2. *ACC in basal portion of jejunal lamina propria. Biopsy was taken 3 days after completing oral immunization with crude cholera toxin. ×640.*

TABLE 2. *ACC in jejunal lamina propria of immunized dogs*

Immunization[a]	ACC/mm³ in jejunal lamina propria		
	Day 0	Day 57	Day 77
Crude toxin	ND[b]	20,900 (1.2)[c]	2,010 (1.9)
Pure toxin	ND	25,765 (1.2)	ND

[a] See text for immunization schedule.

[b] To minimize operative procedures, we did not perform biopsies on these dogs before immunization. Biopsies from other nonimmune dogs have never revealed the presence of ACC (12).

[c] Geometric mean (× SE), n = 7 to 10 for each mean.

TABLE 3. *Protection of immunized dogs against challenge with living V. cholerae*

Immunization	No. of dogs with indicated severity of diarrhea after challenge				% Protection[a]
	None	Mild	Severe	Lethal	
Crude toxin	18	2	0	0	100
Controls	8	4	1	7	$P = 0.003$
Pure toxin	10	3	1	0	88
Controls	2	4	2	7	$P = 0.007$

[a] Protection against the combined incidence of severe or lethal diarrhea compared with concurrently challenged unimmunized control dogs. Statistical analysis was by Fisher's two-tailed exact test.

response. It remains possible, however, that potentially protective mechanisms other than antitoxin were also stimulated by crude toxin, but were masked by the high level of antitoxic immunity. Such mechanisms, presumably antibacterial in nature, might also have been overwhelmed by the very large vibrio inoculum required to cause disease in dogs. Thus, the apparent lack of effect of crude toxin immunization on fecal excretion of *V. cholerae* in challenged dogs should not be taken as firm evidence that an enteric vibriocidal mechanism had not been stimulated. It is also possible that antibacterial mechanisms act by interfering with intestinal colonization, rather than by bacterial killing, and thus may not be reflected in nonquantitative fecal cultures. If antibacterial mechanisms were provoked, their protective role might be greater against smaller bacterial inocula, such as cause naturally acquired human cholera.

Several observations in this study strongly support the view that the antitoxic response which protected orally immunized dogs arose in the intestinal mucosa. First, protection against toxin-induced diarrhea appeared after 5 or 6 days of daily dosing with toxin; the timing of this response corresponds almost exactly with the timing of the local secondary immune response to cholera toxin in rat or canine intestine (13, 16). Second, studies of intestinal biopsies gave direct evidence of a vigorous mucosal antitoxic response manifested by numerous antitoxin-containing cells in the lamina propria. And third, serum antitoxin titers at the time of challenge were low, averaging about 10 U/ml; earlier

TABLE 4. *Fecal excretion of V. cholerae in challenged dogs*

Immunization	Cultures positive for *V. cholerae* in surviving immunized dogs/unimmunized control dogs		
	Day 1	Day 2	Day 6
Crude toxin	19/20 (3)[a]	4/20 (3)	0/20
Controls	20/20 (3)	5/14 (2)	0/13
Pure toxin	12/15 (0)	7/15 (3)	0/15
Controls	14/15 (0)	3/9 (1)	0/8

[a] Number positive only after enrichment.

studies have shown that serum titers of this magnitude provide almost no protection against experimental canine cholera (17).

This is the first demonstration of protection against challenge with living *V. cholerae* in an intact animal after oral immunization with a nonreplicating antigen. In an earlier study, oral immunization of dogs with as many as 20 doses of an inactivated toxoid was nonprotective (17); toxoid was effective as an oral immunogen only in dogs originally primed by a parenteral injection (17). Multiple doses of toxoid given intraduodenally have also proven to be nonprotective for humans (10). The effectiveness of cholera toxin as a mucosal immunogen appears to be due to two properties of the active holotoxin which are lost during toxoiding procedures. The first is the ability of the B subunit to bind to GM_1 ganglioside receptors found in almost all mammalian cell membranes; this appears to facilitate trapping of absorbed toxin by lymphoid cells in the lamina propria, including those in Peyer's patches (11). The second is the ability to activate membrane-bound adenyl cyclase; this appears to directly enhance both the primary and secondary types of mucosal antitoxic responses (11). Apparently because of these unusual properties, cholera toxin appears vastly more effective as a mucosal immunogen than most previously studied protein antigens (11).

These observations suggest that cholera toxin may be useful as an oral vaccine for cholera, or as a component thereof. To have practical value, however, oral immunization would have to prove to be both safe and simple, as well as effective. To be safe, oral toxin should cause neither systemic toxicity nor significant diarrhea; to be simple, an immunizing schedule should require no more than two or three doses. Oral toxin appeared to be safe from systemic toxicity, evidence being failure, with one exception—to cause elevated serum levels of aspartate aminotransferase, glucose, or alkaline phosphatase; cholera toxin given parenterally in the same dose consistently causes elevations in these measurements (14); the elevation of serum alkaline phosphatase in a single dog requires determination of whether the enzyme was of hepatic or intestinal origin before concluding it was caused by absorbed toxin (14). The toxin dose used in this study was purposely chosen to be one which caused diarrhea in most dogs. Further studies, to be published elsewhere, show that crude cholera toxin given orally is still protective when the dose is reduced to one which causes virtually no diarrhea in nonimmune dogs. Moreover, this dose remains protective when the immunizing schedule is simplified to only two doses. Although additional studies are required, especially ones focused on the duration of protection, these results suggest that simple, safe, and effective oral immunization with cholera toxin may be possible.

Finally, the findings of this study give indirect support to the view that the B subunit of cholera toxin, choleragenoid, may also prove to be effective as an oral immunizing agent. This conclusion is based on evidence that the B subunit is also an effective mucosal immunogen, probably because it shares with the holotoxin the property of binding to GM_1 receptors on lymphocyte membranes (4). Because the B subunit does not activate adenyl cyclase (6), it should not cause diarrhea in vaccinees; the lack of adenyl cyclase activation may also account for the observation that the B subunit is somewhat less effective as a mucosal immunogen than holotoxin (11). Earlier studies have shown that orally administered B subunit protects mice against challenge of intestinal segments with *V. cholerae* (7). The possibility that the B subunit might be delivered as a product of a live bacterial vaccine is suggested by the recent development of an apparently nonvirulent mutant of *V. cholerae* which produces only the B subunit of cholera toxin (8). Studies comparing the efficacy of B subunit and holotoxin as oral immunogens in dogs are under way in this laboratory.

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