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Procholeragenoid: A Safe and Effective Antigen for Oral Immunization Against Experimental Cholera

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The immunogenicity and safety of procholeragenoid, a minimally toxic, heat-induced aggregate of cholera toxin (CT), were studied in enterically immunized rats and dogs. Although 99% less toxic than CT, procholeragenoid was only slightly less efficient in causing jejunal anti-CT responses in rats; in contrast, choleragenoid, the nontoxic B subunit pentamer of CT, was much less effective. The immunogenicity of procholeragenoid was due almost entirely to its large-molecular-weight components (MW = 10^6 to 10^7) and was markedly reduced by preincubation with GM_1 ganglioside or treatment with Formalin to eliminate residual toxicity. These findings suggest that molecular aggregation, binding to GM_1 receptors on cell membranes, and stimulation of cellular adenylate cyclase each contributed to the effectiveness of procholeragenoid as a mucosal immunogen. In dogs, oral immunization with five 500-μg doses of procholeragenoid evoked vigorous anti-CT responses in jejunal mucosa without causing significant diarrhea. When subsequently challenged with virulent *Vibrio cholerae*, immunized dogs showed 83% protection against the development of severe or lethal diarrhea compared with non-immunized controls. These results confirm a protective role for mucosal antitoxin in experimental cholera and show that procholeragenoid is both safe and effective as an oral immunogen. Procholeragenoid, combined with other antigens of *V. cholerae*, may constitute a simple, safe, and effective oral vaccine for cholera.

Recent studies have shown that antibodies against cholera toxin (CT) and *Vibrio cholerae* lipopolysaccharide act synergistically to protect experimental animals challenged with living virulent *V. cholerae* organisms (10, 14, 22). In some of these studies, nonliving antigens were given entirely by mouth, and the observed protection was probably due to secretory immunoglobulin A antibodies elaborated in the intestinal lamina propria (14). These findings suggested that a multiantigen oral vaccine which could evoke secretory anti-CT and anti-lipopolysaccharide and perhaps secretory antibodies to other antigens of *V. cholerae*, would be much more effective than a single purified antigen and would find practical use in humans.

However, a safe and efficient method for evoking a protective mucosal anti-CT response has not been established, although CT and several nontoxic derivatives of CT have been studied as oral immunogens. CT given orally is an extremely effective mucosal immunogen (4, 12), but minimum immunizing doses also cause transient diarrhea, at least in some animals (14); whether oral CT can immunize humans without causing diarrhea has not been determined. Choleragenoid, the B subunit pentamer of CT, does not cause diarrhea, but it is poorly immunogenic when given orally or intraintestinally to animals that have not been primed enterically with CT (12, 14). Similarly, CT which has been detoxified by treatment with Formalin or glutaraldehyde is safe, but poorly immunogenic, when given by mouth to nonimmune animals or humans (7, 17, 18).

Procholeragenoid is an antigenic derivative of CT, containing both the A and B subunits, that has not been extensively studied as an oral immunogen. Finkelstein et al. first showed that heat treatment causes CT to form a stable high-molecular-weight aggregate. They termed this material "procholeragenoid" and showed that it was at least 95% less toxic than CT (2). In further studies, Fujita and Finkelstein showed that mice fed procholeragenoid were significantly protected against subsequent challenge of a

ligated intestinal loop with living *V. cholerae* (5), and similar results were obtained in rabbits by Peterson (11).

Because procholeragenoid is antigenic, but only minimally toxic, it seemed appropriate to conduct further studies of its effectiveness and safety as a mucosal immunogen. We have compared the abilities of CT, procholeragenoid, and choleragenoid to evoke mucosal anti-CT responses in rats, and we have studied procholeragenoid as an oral vaccine against experimental cholera in dogs. Our results, presented here, show that enterically administered procholeragenoid is only slightly less effective than CT in causing mucosal anti-CT responses in rats, evokes vigorous mucosal anti-CT responses in dogs without causing diarrhea, and significantly protects dogs against challenge with living *V. cholerae* organisms.

MATERIALS AND METHODS

Animals. Rats were inbred females of the Lewis strain (LEW/Crl BR) obtained from Charles River Breeding Laboratories, Wilmington, Mass. Housing was in a conventional rodent colony. Rats weighed 125 to 150 g (7 to 8 weeks old) when first studied. Dogs were healthy mongrels of either sex weighing 9 to 20 kg when challenged. Before immunization, they were quarantined for 2 weeks, dewormed, and immunized for rabies and distemper.

Antigens. All antigens were provided in lyophile. Purified CT was NIH lot 0972, prepared by Richard Finkelstein and supplied by Robert Edelman, National Institute of Allergy and Infectious Diseases. Choleragenoid was isolated from purified CT as described previously (3) and was provided by Richard Finkelstein. Procholeragenoid was prepared by heating purified CT, derived from *V. cholerae* Inaba 569B, in Tris-EDTA buffer for 20 min at 60°C as previously described (6). For some studies, procholeragenoid was treated with 0.2% Formalin for 60 h at 30°C to virtually eliminate residual toxicity (6).

Chromatography of procholeragenoid was performed by using a Sepharose Cl 4B column (2.5 by 65 cm). Procholeragenoid (60 mg in 10 ml of Tris-EDTA buffer) was applied to a column pre-equilibrated with Tris-EDTA buffer. The column was eluted at a flow rate of 45 ml/h. Fractions of 4.5 ml were collected, and their optical density at 280 nm was measured (Fig. 1). Fractions were pooled to represent peaks I, II, and III as shown. Peak II appeared to be a shoulder of peak I, and its separation from peak I was arbitrary; peak III, however, was distinct from peaks I and II. Peaks I and II had average molecular weights of approximately 10^7 and 10^6, respectively. These were concentrated about 10-fold by ultrafiltration on Amicon PM 30 filter membranes (Amicon N.V., Netherlands); the concentrated solutions were then mixed with an equal volume of 10% lactose, for stabilization, and lyophilized. Peak III had an average molecular weight of 56,000, which corresponds to choleragenoid. It was similarly concentrated by using an Amicon UM-10 filter membrane and was lyophilized without the addition of lactose.

Immunization. Antigens were reconstituted and diluted in 0.05 M borate-buffered saline or phosphate-buffered saline, each containing 0.02% gelatin at pH 7.4. In some instances, antigens were preincubated for 30 min at room temperature with purified GM_1 ganglioside (Supelco, Inc., Bellefonte, Pa.), 60 μg/ml, before being administered.

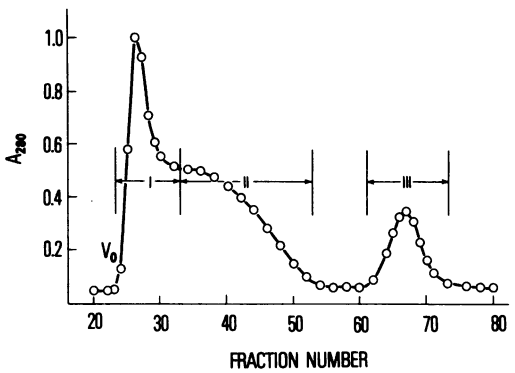

FIG. 1. Chromatography of procholeragenoid on Sepharose 4B. Procholeragenoid (60 mg) was applied to a Sepharose 4B Cl column (2.5 by 65 cm) and eluted with Tris-EDTA buffer at a flow rate of 45 ml/h. Fractions of 4.5 ml were collected, their optical density at 280 nm was measured, and they were pooled to form peaks I, II, and III as shown.

Rats were immunized by direct intraduodenal injection of antigen in a volume of 0.5 ml, using a small laparotomy as described previously (16); most studies involved two antigen doses given with a 14-day interval. Dogs were immunized by giving them antigen through an orogastric tube, also as described elsewhere (18). In brief, individually caged dogs were fasted overnight and then were given 50 ml of 6% $NaHCO_3$, followed immediately by 100 ml of 2% Casamino Acids (Difco Laboratories, Detroit, Mich.) containing 500 μg of procholeragenoid; feeding was resumed after 5 h, and dogs were observed for 24 h for diarrhea.

Assays for CT activity. Residual CT activity in procholeragenoid was measured by the rabbit skin capillary permeability assay, as described elsewhere (1). Results are expressed in 4-mm bluing doses (BD_4) per milligram of injected material. One BD_4 is that amount of CT or procholeragenoid which causes a blue lesion with a mean diameter of 4 mm 22 to 24 h after intracutaneous injection (1).

Antitoxin responses. Antitoxin-containing plasma cells (ACC) were enumerated in jejunal biopsies from rats or dogs, using a previously described fluorescent-antibody technique (16). In rats, biopsies were taken 5 days after the second injection of antigen; in dogs, they were obtained 6 to 7 days after the final antigen dose. These intervals were chosen to detect ACC when they were most numerous (16). Results are expressed as ACC per cubic millimeter in the crypt region of jejunal lamina propria. ACC were never seen in biopsies from nonimmunized animals (15, 16). ACC responses are expressed as geometric means because these reflect the logarithmic manner in which the ACC 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 (15).

Antitoxin was assayed in serum by using a previously described mouse adrenal tumor cell assay (20). Antitoxin titers were determined by comparing each sample 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 in dogs were consistently less than 1 U/ml, which was the sensitivity of the assay.

Challenge of dogs with virulent *V. cholerae* organisms. Preparation of the challenge inoculum and the challenge technique were as described elsewhere (17). Fasting dogs were inoculated with 1.0×10^{11} to 2.1×10^{11} viable *V. cholerae* Ogawa 395 organisms by orogastric tube. At each challenge, immunized dogs and an equal number of nonimmunized 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 for diarrhea in nonimmunized dogs (21).

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 accurately measured. Results of challenge were 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, decreased skin turgor, and weakness or lethargy), and (iv) lethal diarrhea. Diarrhea usually began less than 18, and often less than 8, h after challenge; about 70% of deaths occurred within the first 24 h. Challenge 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 the greatest (14).

Protection of immunized dogs was analyzed by the chi-square test, comparing the number of immunized and control dogs in three outcome categories: no diarrhea, mild diarrhea, and severe or lethal diarrhea.

RESULTS

Toxicity of procholeragenoid preparations. The toxicities of various procholeragenoid preparations and fractions, as measured by the skin capillary permeability assay, are summarized in Table 1. Procholeragenoid and its three separated peaks had residual toxicities ranging from 0.4 to 1.4% of that of purified CT. Treatment of procholeragenoid with Formalin reduced its toxicity about 1,000-fold.

TABLE 1. Relative toxicity of procholeragenoid preparations

Material	BD_4/mg ($\times 10^5$)	% Toxicity relative to CT
Cholera toxin	900	
Procholeragenoid	4.0	0.4
Peak I	4.2	0.5
Peak II	13.0	1.4
Peak III	5.7	0.6
Formalin-treated procholeragenoid	0.005	0.0006

Immunogenicity of CT, procholeragenoid preparations, and choleragenoid in rats. The relative efficacies of CT, procholeragenoid, and Formalin-treated procholeragenoid as primary enteric immunogens were studied in rats. The first studies involved intraduodenal priming with various doses of one of the antigens, followed 14 days later by a 12.5-μg intraduodenal booster dose of CT. The resultant secondary ACC responses are summarized in Fig. 2. Peak responses achieved by CT and procholeragenoid were similar, but the dose of procholeragenoid required to prime for an intermediate booster response of 1,000 ACC/mm^3 was slightly larger: 1.5 times that of CT. In contrast, Formalin-treated procholeragenoid was much less effective; at the highest dose tested (50 μg), it primed for a secondary ACC response that was only 10% of that seen in rats optimally primed with 3.2 μg of CT.

In further studies, rats were primed and challenged intraduodenally with identical, graded doses of a single antigen: CT, procholeragenoid, or choleragenoid. The order of immunizing efficiency of these materials was CT > procholeragenoid > choleragenoid (Fig. 3). Moreover, peak responses achieved by repeated immunization with choleragenoid were only 10% as great as those in CT-immunized rats. In contrast, rats given 12.5- or 50-μg doses of procholeragenoid developed ACC responses similar to those evoked by the same doses of CT.

The immunogenicity of each of the three chromatographically separated peaks of procholeragenoid is compared with that of whole procholeragenoid and CT in Table 2. In general, peaks I and II were similar to whole procholeragenoid with respect to priming for an ACC response, peak II being slightly more effective than peak I. In contrast, peak III was virtually ineffective for enteric priming. Table 2 also shows the effect of preincubation of these materials with GM_1 ganglioside. In every instance, priming efficiency was markedly diminished by preexposure to ganglioside.

Safety and efficacy of procholeragenoid as an oral immunogen in dogs. Dogs were immunized orally with 500-μg doses of procholeragenoid on days 0, 21, 42, 49, and 56. This regimen was based on evidence that maximum ACC responses and protection occurred in dogs given multiple oral doses of purified CT (14). Jejunal biopsies to assess ACC responses were obtained from some dogs 6 to 7 days after the final dose of antigen. Challenge with viable *V. cholerae* Ogawa 395 organisms was 21 days after the final immunization.

Mild diarrhea occurred in 6% of dogs given the first, second, or third oral dose of procholeragenoid (Table 3). This figure does not differ significantly from the previously described 4%

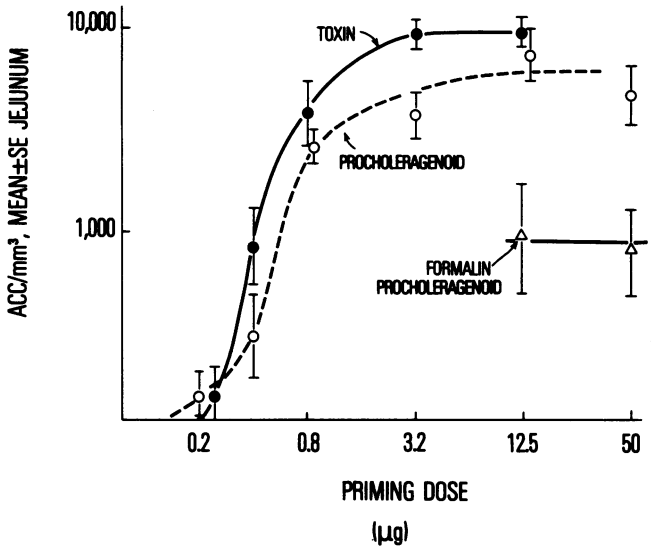

FIG. 2. Comparison of CT, procholeragenoid, and Formalin-treated procholeragenoid as primary immunogens in rat jejunum. Rats were given an intraduodenal primary immunization with CT or with one of the procholeragenoid preparations as shown. Fourteen days later, all rats were boosted intraduodenally with 12.5 μg of CT. ACC in jejunal lamina propria were assayed 5 days later. Each point represents data from at least five rats.

incidence of mild diarrhea in dogs given only the sodium bicarbonate and Casamino Acids, with no antigen (13). In all instances, diarrhea consisted of a single soft or semiliquid stool with a volume not exceeding 25 ml. Biopsies from six immunized dogs showed vigorous jejunal ACC responses. Immunized dogs were significantly protected against diarrhea when challenged with living *V. cholerae* organisms ($P = 0.03$); they showed 83% protection against severe or lethal diarrhea and 78% protection against diarrhea of any severity. In immunized dogs, the mean stool volume passed during the first 18 h after challenge was also less, i.e., 24% of the volume observed in unimmunized controls.

DISCUSSION

Enteric immunization with CT causes vigorous mucosal IgA anti-CT responses (4, 16). Previous studies have suggested that the marked effectiveness of CT as a mucosal immunogen, especially in unprimed animals, is due to its ability to bind to GM_1 receptors present in most cell membranes and its subsequent stimulation of adenylate cyclase-mediated responses (12). Specifically, it was proposed that GM_1 binding facilitates the absorption of CT or its subsequent trapping by mucosal lymphoid tissue or both, whereas adenylate cyclase stimulation in mucosal lymphoid tissue may enhance the immune response (12). Because these features of the CT molecule also account for its enterotoxic effect, it seemed unlikely that a CT derivative would be found that was safe, i.e., did not cause diarrhea when given orally, and yet had mucosal immunogenicity similar to that of CT. The relatively poor enteric immunogenicity of several nontoxic derivatives of CT, including choleragenoid and glutaraldehyde- and Formalin-treated toxoids, seemed to support this view (7, 12, 14, 17, 18).

The present study shows, however, that, despite its much diminished toxicity, procholeragenoid was only slightly less efficient than CT as a mucosal immunogen in rats. It was also shown that procholeragenoid was superior to choleragenoid. In that comparison, maximum ACC responses evoked by procholeragenoid equaled those caused by CT, whereas maximum responses to choleragenoid were nearly 10-fold lower. Poorer immunogenicity of choleragenoid compared with CT has been observed previously and has been attributed to its inability to enhance the immune response by stimulation of adenylate cyclase in mucosal lymphoid cells (12).

Several properties of procholeragenoid, acting separately or in concert, might account for its efficacy as a mucosal immunogen. These include its residual CT-like activity, its high molecular weight, and possibly a preserved ability to bind to GM_1 receptors on cell membranes.

The ~1% residual CT-like toxicity of procholeragenoid could not entirely explain its antigenicity. This possibility is excluded by evidence that equivalent CT doses (i.e., equal to 1% of tested procholeragenoid doses) caused much

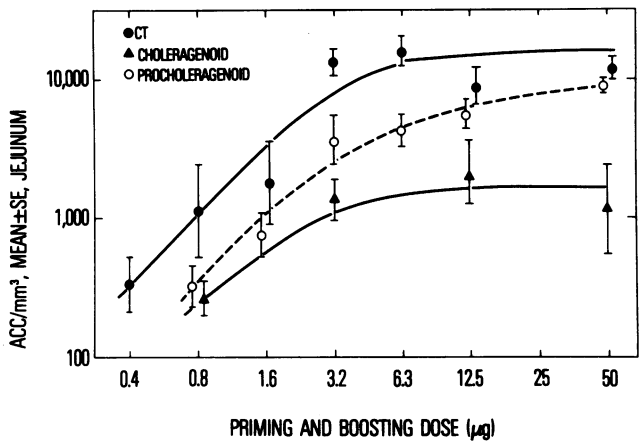

FIG. 3. Comparison of jejunal ACC responses in rats primed and boosted intraduodenally with identical doses of CT, choleragenoid, or procholeragenoid. Intraduodenal priming and boosting were done with the indicated antigens and doses, using a 14-day interval. ACC in jejunal lamina propria were assayed 5 days after intraduodenal boosting. Each point represents data from at least five rats.

smaller mucosal ACC responses (Fig. 2 and 3). It is possible, however, that residual CT-like activity had an adjuvant effect upon the response to the entire procholeragenoid molecule. This would be consistent with the observed poor mucosal immunogenicity of Formalin-treated procholeragenoid, which was essentially nontoxic. This view is also supported by evidence that CT has an adjuvant effect in parenterally immunized mice (8).

Another possibility is that the immunogenicity of procholeragenoid was partly due to its aggregated state. Aggregated proteins given parenterally cause greater systemic antibody responses, due to enhanced uptake by macrophages, than do nonaggregated proteins, and the same might be true for aggregated proteins absorbed at mucosal surfaces. Aggregation might also enhance the absorption of an intestinal antigen, via M cells in the epithelium that covers mucosal lymphoid follicles (9), thus increasing the amount delivered to mucosal lymphoid tissue. The notion that aggregation contributed to the immunogenicity of procholeragenoid is consistent with evidence that its high-molecular-weight components (peaks I and II) were most immunogenic, even though they did not possess more residual CT-like toxicity than the poorly immunogenic nonaggregated peak III.

TABLE 2. Relative immunogenicity of CT, procholeragenoid, and separated fractions of procholeragenoid

Primer dose[a]	Secondary ACC responses in rats primed with[b]:				
	CT	Procholeragenoid	Peak I	Peak II	Peak III
12.5 μg	9,240 (1.2)	7,180 (1.3)	5,050 (1.4)	8,010 (1.2)	640 (1.8)
12.5 μg plus 30 μg of GM_1	1,800 (1.5)	1,670 (1.4)	1,270 (2.3)	1,700 (1.4)	
3.2 μg	9,450 (1.2)	3,460 (1.3)	3,490 (1.3)	4,940 (1.3)	
0.8 μg	3,830 (1.4)	2,160 (1.2)	900 (1.4)	1,600 (1.8)	

[a] Intraduodenal priming was with indicated dose and material. In some instances, antigen was preincubated with GM_1 ganglioside (see the text). All rats were boosted intraduodenally on day 14 with 12.5 μg of CT; ACC were enumerated in jejunal biopsies taken 5 days later.

[b] Geometric mean ACC per cubic millimeter (± standard error); each mean contains data from at least five rats.

Whether procholeragenoid retains the capacity of CT to bind to GM_1 receptors on cell membranes has not been directly determined. This possibility was suggested, however, by evidence that preincubation of procholeragenoid, or its high-molecular-weight components, with GM_1 markedly reduced their mucosal immunogenicity, as was also true for CT. These results probably reflect binding of GM_1 to exposed B subunit molecules on procholeragenoid and suggest that the same interaction occurs between procholeragenoid and GM_1 in cell membranes. Such binding could contribute to the effectiveness of procholeragenoid as a mucosal immunogen, just as it appears to for CT (12).

This study provides the first demonstration of protective antitoxic immunity achieved by oral immunization of dogs without causing diarrhea during immunization. In previous studies, oral immunization with purified CT evoked marked protection, but also caused transient diarrhea in

TABLE 3. Safety and efficacy of procholeragenoid as an oral immunogen in dogs

Dogs	Observation						
	Diarrhea during immunization	ACC/MM^3, jejunum (mean ± SE)	Serum anti-CT: proportion detectable (range, U/ml)[a]	Challenge outcome (no. of dogs)[b]			First 18-h stool vol: mean, ml/kg of body wt (range)
				No diarrhea	Mild diarrhea	Severe or lethal diarrhea	
Nonimmunized	2/46 (4.3%)[c]	None[d]	0/16 (≤2.3)	7	3	6	24 (0–92)
Immunized	4/80 (5.0%)[e]	11,000 × ÷ 1.2[f]	5/16 (≤2.3–7)	14	1	1	6 (0–66)

[a] Sera were obtained on the day of challenge.

[b] Protection of immunized dogs was statistically significant: $P = 0.03$.

[c] Previously reported data from fasting dogs given 50 ml of 6% $NaHCO_3$ followed by 100 ml of 2% Casamino Acids by mouth (13).

[d] Based on previously reported studies in nonimmunized dogs (15).

[e] Episodes of mild diarrhea observed immediately after any of the five immunizations given to 16 dogs. Considering only the first three doses of procholeragenoid, three episodes occurred after 48 immunizations (6.2% incidence).

[f] Biopsies were taken 6 to 7 days after the final dose of procholeragenoid ($n = 6$).

70% of animals (13); nontoxic derivatives of CT, including choleragenoid and Formalin- or glutaraldehyde-treated toxoids, did not cause diarrhea, but they also evoked little or no protection (14, 17, 18).

The vigorous immunizing regimen used in this study was designed to enhance the possibility of demonstrating protection. Whether procholeragenoid would cause protection when fewer than five doses were given, or when individual doses were smaller than 500 μg, or both, is uncertain, but previous studies of dogs immunized orally with crude CT have shown that as few as two doses of an efficient immunogen can cause substantial protection (14).

Protection of procholeragenoid-immunized dogs was probably due entirely to antitoxin. This is likely because procholeragenoid was derived from highly purified CT which, in turn, was produced by *V. cholerae* of the opposite serotype from that used for challenge. The relative roles of antibodies to the A or B subunits of CT in this protection are, however, uncertain. Although the B subunit is the most antigenic, antibody to the A subunit has also proven highly protective in experimental animals (11). If procholeragenoid evokes a significant mucosal anti-A response, this may enhance its protective efficacy in comparison with vaccines that contain only the B subunit antigen.

The results of this study suggest that procholeragenoid would prove both safe and effective as a component of a nonliving oral cholera vaccine for humans. Such a vaccine would likely be a multiantigen product designed to stimulate enhanced (19) or synergistically protective (10, 14, 22) mucosal antibody responses, or both. Previous studies in experimental animals have shown both the importance of synergistic immunity and that anti-CT and anti-lipopolysaccharide contribute substantially to it (10, 14, 22). Which other antigens of *V. cholerae* should be included, and the optimal methods for preparing and delivering such a combined vaccine, remain to be determined.

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