INFECTION AND IMMUNITY, June 1977, p. 789-795
Vol. 16, No. 3

Printed in U.S.A.

Simple Method for Purifying Choleragenoid, the Natural Toxoid of *Vibrio cholerae*

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Received for publication 20 December 1976

Choleragenoid, a nontoxic aggregate of the B subunit of cholera toxin, has been purified from concentrated culture filtrates in a single step by ion-exchange chromatography on phosphocellulose or other cation-exchange resins. This procedure is far simpler than others currently used to isolate choleragenoid and yields a preparation essentially free from nucleic acid, lipopolysaccharide, toxin, and other proteins present in the crude culture filtrates. The purified choleragenoid retained the specific receptor-binding capacity of the toxin but exhibited no enterotoxic activity by either the ileal loop assay or the skin permeability assay. This purification method may therefore be superior to others currently used for obtaining choleragenoid for immunization or other purposes.

The exo-enterotoxin of *Vibrio cholerae* (molecular weight, 84,000) is known to be composed of two subunits, A and B (14). Subunit A, which actually contains two peptides, A_1 (molecular weight, 20,000) and A_2 (molecular weight, 7,500) linked by a disulfide bridge (26), is present in a single copy per toxin molecule. This subunit has been shown to promote the activation of adenylate cyclase, which is ultimately responsible for the myriad effects of cholera toxin in various mammalian cell types (20, 37). The B subunit (molecular weight, 10,000), which is present in five to six copies in the native toxin, is known to bind ganglioside GM_1, a component of most mammalian cell membranes (22, 26). This subunit is apparently responsible for attachment of the whole toxin to cells and may mediate the traversal of the lipid bilayer by the A subunit (21).

A nontoxic aggregate of the B subunit (molecular weight, 58,000) has been shown to be present in variable amounts in culture filtrates of toxinogenic *V. cholerae* and may be isolated from purified toxin after treatments with denaturants (14, 15, 17, 19). This aggregate, termed choleragenoid, blocks the action of whole toxin on mammalian cells by competing for cell surface receptors; it also contains the major antigenic determinants of the toxin (10). It has been suggested, therefore, that choleragenoid may be useful in prophylaxis against cholera.

Published methods for isolating choleragenoid in pure form are complex, involving many steps, and do not yield a product entirely free from toxin (16). Here we report a simple, single-step method for isolating pure natural choleragenoid from culture filtrates of *V. cholerae* 569B Inaba.

MATERIALS AND METHODS

Reagents. All chemicals used were supplied by commercial sources and were of analytical grade unless otherwise specified. Phosphocellulose P11 (Whatman) was purchased from Reeve Angel (Clifton, N.J.). Purified cholera toxin (lot 1071) was obtained from Robert S. Northrup (National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Md.) and prepared by R. A. Finkelstein, The University of Texas, Southwestern Medical School, Dallas, Tex., under contract for the National Institute of Allergy and Infectious Diseases (16). Choleragenoid was the generous gift of Richard A. Finkelstein.

Analytical techniques. Carbohydrate was determined as total hexose by the phenolsulfuric method (11) with glucose as a standard. Total organic and inorganic phosphate was determined in samples (dialyzed against 0.1 M $NaHCO_3$ buffer, pH 7.4) by first ashing with nitric acid (1) and then assaying for *o*-phosphate by the method of Chen et al. (7). Protein was determined by the Lowry modification of the Folin-Ciocalteau method (28) using crystalline bovine serum albumin as a standard.

Toxin assays. The passive hemagglutination inhibition assay, performed as described by Callahan et al. (5), was used to estimate the content of toxin-antigen present in crude and purified preparations and was calibrated with purified toxin (lot 1071). Because the assay does not distinguish between toxin and choleragenoid, values are reported as micrograms of "toxin-antigen" per milliliter.

Toxicity was measured by two methods. The ileal loop assay was performed essentially as described by Burrows and Musteikus (4). Starting at the appendix, 18 test loops were ligated. Each 5-cm loop received 0.5 ml of sample diluted in phosphate-

buffered saline (0.1 M sodium phosphate buffer [pH 7.4] and 0.14 M NaCl) containing 0.1% gelatin. Adjacent test loops were separated by 2-cm spacer loops. A positive control (1.5 μg of purified toxin) and a negative control (phosphate-buffered saline) were included in each animal. After 18 h, the volume-to-length ratio was determined for each loop and plotted against the total amount of toxin-antigen injected in the loops. Interpolation allowed determination of the specific toxicity in terms of the ileal loop unit. An ileal loop unit was defined as the toxic activity eliciting a response of 1 ml/cm under standard conditions.

The amount of antigen corresponding to one ileal loop unit varies with the relative proportion of choleragen to choleragenoid present in a preparation.

The skin permeability factor assay was performed as described by Craig (9). This assay was about 1,000-fold more sensitive than the ileal loop assay and could detect less than a nanogram of purified toxin.

Toxin production. *V. cholerae* 569B Inaba was used in the production of toxin and choleragenoid. The strain was maintained on meat extract agar (31). The bacteria were grown in CYE broth (30 g of Casamino Acids [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of NaCl per liter of deionized H_2O; adjusted with 5 N NaOH to pH 7.8 before autoclaving). Bacteria were inoculated to a density of 5×10^8 cells/ml and grown at 30°C under high oxygen tension for 7 h. After removal of cells by centrifugation at 10,000 × *g* for 40 min, sodium azide was added as a preservative (20 μg/ml), and the supernatant fluids were concentrated about 40-fold by ultrafiltration through a Diaflo UM-10 membrane (Amicon Corp., Lexington, Mass.) at 4°C under 20 lb/in² of nitrogen. The UM-10 retentate was dialyzed at 4°C against 10 mM sodium phosphate buffer, pH 7.4 or 8.0, and insoluble material was removed by centrifugation (20,000 × *g* for 20 min). There was almost quantitative recovery of toxin-antigen after the ultrafiltration step as measured by the passive hemagglutination inhibition assay. The amount of toxin-antigen in the concentrated solutions remained constant for several months at 4°C. However, ileal loop toxicity decreased with a half-life of about 1 month, suggesting a slow conversion of toxin to choleragenoid in the concentrated preparations at this temperature.

Chromatography on phosphocellulose. Phosphocellulose was first exposed to two cycles of alternate basic (0.1 N NaOH) and acidic (0.1 N HCl) washes. It was then resuspended in 10 mM sodium phosphate buffer, pH 7.4 or 8.0, and the pH was adjusted to that of the buffer with either NaOH or phosphoric acid. Resuspension and titration were repeated until the resin did not alter the pH of the resuspension buffer by more than 0.01 pH unit. The resin was then packed into a column and washed further with at least two column volumes of buffer before application of the sample.

Approximately 6 ml of concentrated culture supernatant, equilibrated by dialysis with the same buffer as the column, was applied for each milliliter of packed resin, and the column was then washed with initial buffer until the absorbancy at 280 nm dropped below 0.01 unit. Choleragenoid was eluted with 50 mM sodium phosphate, pH 7.4 or 8.0, or with a linear gradient of phosphate buffer. The capacity of phosphocellulose was at least 10 mg of choleragenoid per ml of packed resin.

Immunoanalyses. Antiserum to cholera toxin or choleragenoid was prepared in 8-month-old New Zealand albino rabbits. Primary immunization consisted of one subcutaneous and one intramuscular injection with 25 μg of toxin-antigen in 0.5 ml of complete Freund adjuvant. Animals were boosted 3 weeks later by subcutaneous injection of 25 μg of toxin-antigen in phosphate-buffered saline without adjuvant. Multivalent antiserum to crude ultrafiltration retentates was prepared as above by substituting 0.1 ml of the crude material in place of each 25-μg dose of purified toxin-antigen. All animals were bled 1 week after the last injection.

Immunoelectrophoresis was performed on agar-coated glass slides with 0.04 M sodium barbital buffer, pH 7.4. Samples were subjected to electrophoresis for 1 h at 350 V, and antiserum was added to the central trough. After reacting at room temperature in a humid atmosphere for 30 h, the unprecipitated proteins were removed by soaking plates in phosphate-buffered saline followed by distilled H_2O. Precipitin bands were stained with a solution of 0.1% Coomassie brilliant blue (Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y.), 10% trichloroacetic acid, and 50% methanol, followed by destaining with 10% methanol in 10% acetic acid.

The amount of somatic antigen (lipopolysaccharide) present in crude and purified preparations was estimated by the vibriocidal inhibition assay of Finkelstein (12). Anti-whole-cell serum to *V. cholerae* 569B was prepared by the method of Blachman et al. (2) and had a vibriocidal titer of 50,000 U/ml. All dilutions were done in CYE broth, pH 7.4. The assay was performed by mixing 0.25 ml of diluted antiserum (10 vibriocidal U/ml) with 0.25 ml of 10-fold serially diluted toxin-antigen preparations. After incubation at 37°C for 1 h, 0.5 ml of 1:20 diluted guinea pig serum (complement) with 4×10^3 washed nongrowing 569B cells/ml was added, and incubation was continued for 1 h at 37°C. Viable counts were determined by plating on CYE agar. The dilution of antigen showing a 50% reduction in colony-forming units is the vibriocidal inhibition titer. The assay was sufficiently sensitive to detect 0.03 μg of lipopolysaccharide hexose per ml.

SDS-polyacrylamide gel electrophoresis. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed using the discontinuous gel system of Laemmli (25) in an apparatus described by Reid and Bieleski (36). The upper and lower gels were 3 and 15% in acrylamide, respectively. Proteins present in samples were completely dissociated by the addition of one-half volume of 2× SDS sample buffer without β-mercaptoethanol (25) followed by heating 2 to 3 min in boiling water. Proteins were reduced by including β-mercaptoethanol (5%) in the SDS sample buffer. Electrophoresis was carried out at constant current (15 mA) and terminated when the bromophenol blue dye marker

reached the base of the gel. Proteins were fixed and stained by the procedure described for immunoprecipitates.

Isoelectric focusing. Thin-layer polyacrylamide gel isoelectric focusing was performed on an LKB 2117 Multiphor apparatus (Stockholm, Sweden). The procedure used is described in detail by the manufacturer (LKB Application Note S-161-25). The Ampholine range used generated a linear pH gradient of 3.5 to 9.5. Standard proteins with well-characterized isoelectric points (pI) (cytochrome *c*, pI 9.3; ribonuclease, pI 8.9; sperm whale myoglobin, pI 8.2; horse myoglobin, pI 7.3; conalbumin, pI 5.9; β-lactoglobulin, pI 5.1; all purchased from Sigma Chemical Co., St. Louis, Mo.) were used to calibrate the focused position of an unknown with its apparent pI (34).

Amino acid analysis. The amino acid composition of proteins was determined according to the method of Moore et al. (30). A Beckman (Palo Alto, Calif.) model 119 automatic amino acid analyzer equipped with an Infotronics (Austin, Tex.) model $CRS_2 10$ integrator was used. Values reported for serine and threonine were determined by back-extrapolation of values from time course hydrolyses of 24, 48, and 72 h. Total cysteine was determined as cysteic acid after performic acid oxidation (29).

RESULTS

Adsorption and elution of choleragenoid from phosphocellulose. The isoelectric points (16) of cholera toxin (6.6) and choleragenoid (7.8) suggest that cation-exchange chromatography at pH's near neutrality might be useful in separating the two from one another. Figure 1 shows that this is indeed true. A 20-ml column of phosphocellulose was packed and equilibrated at room temperature (23°C) with 10 mM sodium phosphate buffer, pH 7.4. A sample of concentrated culture fluid (10 ml) was applied at a flow rate of 45 ml/h. The column was washed with two bed volumes of equilibration buffer and eluted with a linear gradient of sodium phosphate buffer.

The elution profile (Fig. 1) shows two peaks of absorbance at 280 nm. Peak I was unretarded in the equilibration buffer, and peak II eluted between 30 and 50 mM phosphate buffer. Fractions making up each peak were pooled and assayed for various components present in the crude sample (Table 1). Most importantly, it was found that peak II contained a high percentage of the total toxin-antigen but exhibited no detectable toxicity as tested by the ileal loop assay. As detailed in the next section, the protein present in peak II appears to be pure choleragenoid by a series of chemical, physical, and immunological criteria.

(In subsequent studies we have observed that small amounts of toxin are sometimes retained on phosphocellulose at pH 7.4 and are eluted with the choleragenoid. This problem may be eliminated without sacrificing yield by performing the chromatography at pH 8.0. We now carry out the purification at this pH, as described under Materials and Methods. We have also successfully performed the purification at this pH on carboxymethyl-cellulose or deoxyri-

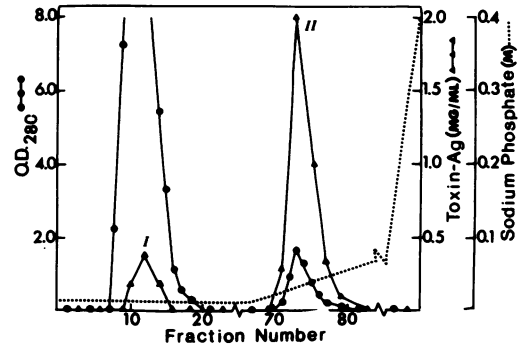

FIG. 1. *Fractionation of concentrated culture fluid by phosphocellulose chromatography.*

TABLE 1. *Analysis of phosphocellulose chromatography*

Analysis of:	Total vol (ml)	A_{280}/A_{260}[a]	Protein (mg/ml)[b]	Somatic antigen[c]	Toxin-antigen (μg/ml)[d]	Specific toxicity (ILU/μg of antigen)[e]
Sample applied[f]	10.0	1.3	4.9	10^{-5}	1,125	0.11
Peak I (fractions 8–16)	18.0	1.4	2.1	5×10^{-4}	160	3.3
Peak II (fractions 71–77)	7.2	0.5	1.0	>0.5	1,000	<0.01

[a] Units of absorbancy at 260 nm/units of absorbancy at 280 nm.

[b] Determined by the method of Lowry et al. (28) using crystalline bovine serum albumin as standard.

[c] Estimated by the vibriocidal inhibition assay (12). Values are reported as the highest dilution capable of producing a 50% inhibition of 10 U of vibriocidal antibody.

[d] Determined by the passive hemagglutination assay (5) using purified toxin (lot 1071) as standard.

[e] Measured by interpolation of dose response curve. One ileal loop unit (ILU) is equal to the dose of toxin-antigen producing a volume-to-length ratio of 1.

[f] Culture supernatant concentrated 40-fold by ultrafiltration and dialyzed against 10 mM sodium phosphate buffer, pH 7.4.

bonucleic acid [DNA]-cellulose.)

Purity and properties of the choleragenoid eluted from phosphocellulose. (i) Electrophoretic analysis. Electrophoresis on SDS-polyacrylamide gels illustrates the high degree of purity of choleragenoid obtained in this single chromatographic step. As shown in Fig. 2, peak II was composed solely of the B subunit of cholera toxin, whereas peak I contained both A and B subunits plus contaminating proteins present in the culture filtrate. In the electrophoretic system employed here, the B subunit appears as a double band with a densely staining upper region and a more diffuse lower region. This double-banded appearance has been observed by others and is probably an artifact of the system. This is indicated by the fact that both regions, when cut out and subjected to electrophoresis a second time, give rise to the same double-band pattern.

Reduction of choleragenoid decreases the mobility of the B subunit. This has also been observed in previous investigations (24, 26) and is probably related to the reduction of an intrachain disulfide bridge within the B subunit. Breakage of this bond results in a more extended configuration and a concomitantly reduced mobility for the SDS-protein complex. Molecular weights determined from these gel data are 9,600 and 9,000 for the reduced and unreduced B subunits, respectively.

When either the peak II protein or choleragenoid is subjected to electrophoresis in SDS without prior heating at 100°C, it migrates predominantly as a 54,000-molecular-weight band, corresponding to the undissociated, pentameric form of the B subunit (21). The B subunit eluted from phosphocellulose is, therefore, primarily in the oligomeric form, characteristic of choleragenoid prepared by other procedures (16).

Isoelectric focusing of peak II protein shows one major and one minor band with pKi's of 7.8

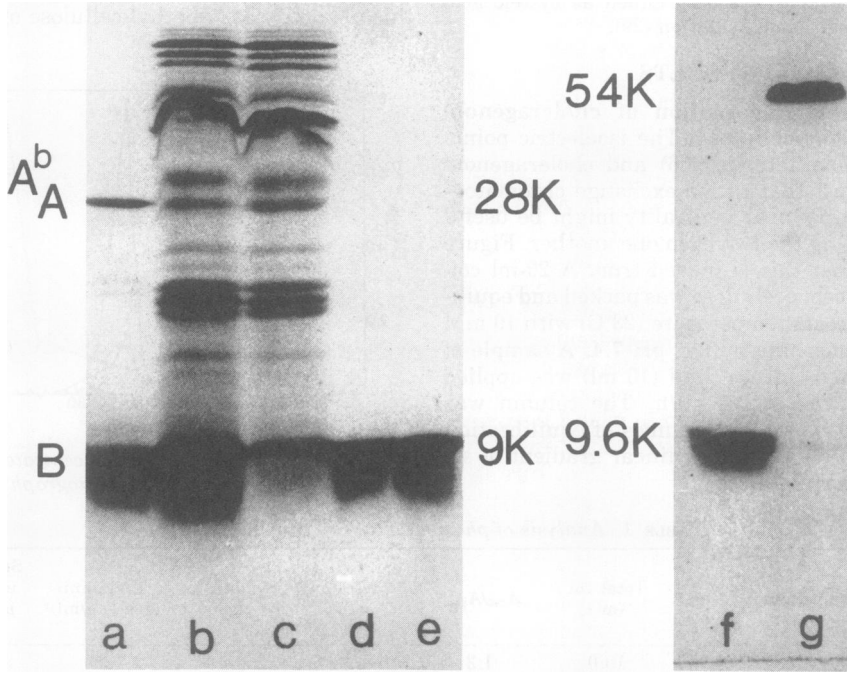

FIG. 2. *SDS-polyacrylamide gel electrophoresis. All samples except (f) and (g) were heated in SDS sample buffer in the absence of reducing agents prior to electrophoresis. Sample (f) was heated in the presence of β-mercaptoethanol. Sample (g) was neither heated nor reduced. Samples: (a) Cholera toxin purified with ganglioside affinity absorbants (method to be reported); (b) concentrated culture supernatant (we have consistently observed that the A subunit of cholera toxin migrates more slowly in crude preparations containing lipopolysaccharide. The position of the A subunit in crude preparations was determined by adding trace amounts of ^{125}I-labeled purified toxin to the samples before electrophoresis. Radioautographs of dried gels showed the A subunit to be slightly retarded [A^b], perhaps by the lipopolysaccharide present in these samples. Lipopolysaccharide causes the irregular appearance seen in the upper part of samples [b] and [c].); (c) peak I (Fig. 1); (d) peak II (Fig. 1); (e) choleragenoid purified by Finkelstein and LoSpalluto (16); (f) partially reduced peak II; (g) unheated peak II.*

and 7.6, respectively. These values are close to those for choleragenoid reported by Finkelstein and LoSpalluto (16). Each band, when cut out and subjected to electrophoresis on SDS gels, gave rise to the characteristic B-subunit pattern.

(ii) Biological properties. Because the ileal loop assay is inherently a rather insensitive method of measuring cholera toxin activity, we have employed the rabbit skin test, which can detect amounts of cholera toxin as low as 0.2 ng. Samples as high as 50 μg of protein from peak II (Fig. 1) produced no erythema, induration, or bluing reactions in rabbit skin. In contrast, choleragenoid purified by other methods has been reported to have a skin reactive dose of 0.2 μg (13); we have confirmed this.

Peak II was also shown to inhibit the action of cholera toxin in the ileal loop assay. When a mixture of 4 μg of protein from peak II was mixed with 1.5 μg of purified cholera toxin, an 80% reduction of the intestinal response was observed. Therefore, choleragenoid present in peak II is active in binding membrane receptors and thus blocks the action of the toxin (10). Removal of choleragenoid by phosphocellulose chromatography undoubtedly explains the increase in specific toxicity of peak I over the concentrated culture fluid (Table 1).

(iii) Immunological analyses. Immunoelectrophoresis at pH 7.4 was used to analyze the material fractionated by phosphocellulose chromatography (Fig. 3). The concentrated culture fluid shows two precipitin bands against rabbit antitoxin. As expected, that corresponding to the more cationic species was present in peak II from phosphocellulose, whereas the other was contained in peak I. This is consistent with the separation of toxic antigen in peak I from nontoxic choleragenoid (peak II). Rabbit antiserum prepared against the concentrated culture fluid forms multiple bands against this crude material but only one band against peak II. Likewise, antiserum against peak II forms two bands against the concentrated culture fluid, corresponding to toxin and choleragenoid. Anti-peak II serum forms only one precipitin band against either peak II or purified choleragenoid. Only a normal level of background vibriocidal activity was present in antiserum to peak II (vibriocidal titer less than 5), indicating very little contamination with 569B somatic antigen. Clearly, a high degree of immunological purity of choleragenoid has been achieved in the single chromatographic step.

(iv) Chemical analyses. Chemical analyses were performed on choleragenoid isolated by phosphocellulose chromatography. Less than 1% of the dry weight of choleragenoid present in peak II was carbohydrate. No phosphate could be detected (less than 0.3% dry weight), indicating the absence of phospholipid in the preparation. These analyses agree well with the very low level of somatic antigen (lipopolysaccharide) present in phosphocellulose-purified choleragenoid when estimated by the highly sensitive vibriocidal inhibition assay (Table 1).

Amino acid analysis of choleragenoid purified by phosphocellulose chromatography agreed closely with that reported by Lai et al. (26) for the purified B subunit of cholera toxin. The only difference found between the amino

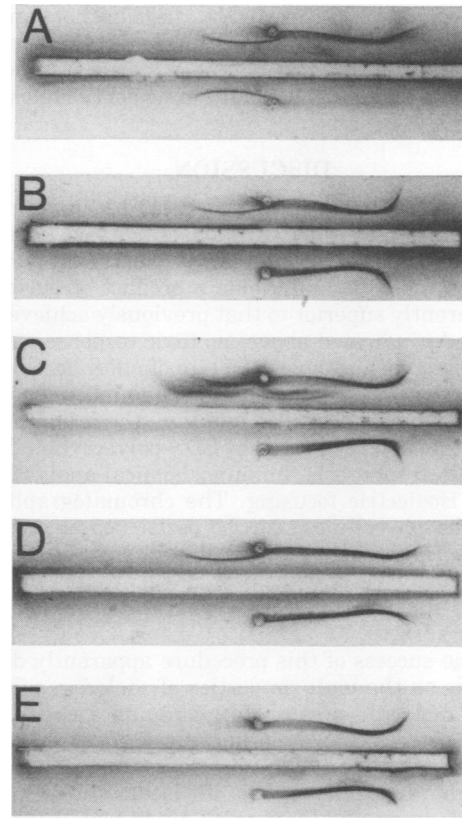

FIG. 3. *Immunoelectrophoresis. Slide A: anti-toxin; top well, concentrated culture supernatant; bottom well, peak I (Fig. 1). Slide B: anti-cholera toxin; top well, concentrated culture supernatant; bottom well, peak II (Fig. 1). Slide C: anti-concentrated culture supernatant; top well, concentrated culture supernatant; bottom well, peak II. Slide D: anti-peak II; top well, concentrated culture supernatant; bottom well, peak II. Slide E: anti-peak II; top well, choleragenoid purified by Finkelstein and LoSpalluto (16); bottom well, peak II. The cathode is on the right.*

acid composition reported by these authors and our own was the presence of one less residue per B subunit of the three amino acids asparagine, alanine, and leucine in our amino acid composition.

DISCUSSION

The single-step procedure described here for isolating and purifying choleragenoid from culture fluids is far simpler than existing, published methods and yields a product of purity apparently superior to that previously achieved (16). As indicated above, no toxic response was detected with samples of 50 μg of phosphocellulose-purified choleragenoid in the highly sensitive rabbit skin test. In addition, the protein is homogeneous as judged by SDS-polyacrylamide gel electrophoresis, immunochemical analyses, and isoelectric focusing. The chromatography on phosphocellulose can be performed at room temperature using dilute protein samples and can easily be scaled up or down. It therefore lends itself to large-scale purification of nontoxic antigen.

The success of this procedure apparently depends on the ionic properties of choleragenoid. The molecule presumably contains a cationic surface or facet which permits it to adsorb to negatively charged resins even at pH 8.0, which is slightly above its isoelectric point. Whole toxin and most or all other contaminating macromolecules in culture fluids are sufficiently anionic at pH 8 that they do not adsorb and thus are not retained on the column.

The ionic properties probably also account for the fact that choleragenoid binds strongly to DNA. We have observed (unpublished data) that choleragenoid, but not cholera toxin, coprecipitates with DNA when the latter is removed from solution by precipitation with lysozyme. We have also found that choleragenoid and cholera toxin chromatograph on columns of DNA-cellulose exactly as they do on phosphocellulose. This interaction of choleragenoid with DNA present in culture filtrates may well produce artifacts in immunodiffusion systems performed in low-ionic-strength buffers.

Choleragenoid purified on phosphocellulose may have significant advantages over immunogens currently available. Choleragenoid is known to be almost as immunogenic as whole toxin (18), and that purified on phosphocellulose appears to be free from traces of toxin and lipopolysaccharide that frequently contaminate preparations produced by other methods (8, 16, 23, 27, 35). Thus, it could probably be used as an immunogen without further purification and without fear of reactivation, which is sometimes seen with formalinized toxoids (6). Also the fact that phosphocellulose-purified choleragenoid retains activity in binding to membrane receptors for the toxin suggests that it might be useful as an immunogen in the natural site of infection. Evidence for the importance of local immunity in cholera is ever increasing (3, 32, 33). Fixation of toxin or choleragenoid to the intestinal mucosa may well be a necessary event in eliciting a strong local immune response.

The major limitation with the purification method described here is the variability in the ratio of choleragenoid to cholera toxin found in otherwise identical cultures of *V. cholerae*. Finkelstein, and LoSpalluto have also noted this variability, but have found that the amount of choleragenoid is nearly always greater than the amount of toxin (16). Experiments are under way to determine growth conditions that will optimize production of nontoxic antigen. We are also seeking methods to convert the toxin present in crude preparations to choleragenoid by an easy, reproducible procedure.

ACKNOWLEDGMENTS

One of us (J. J. Mekalanos) is a predoctoral trainee supported by Public Health Service grant GM07104 from the National Institute of General Medical Sciences. This investigation was supported by Public Health Service grants AI07877 and AI08024 from the National Institute of Allergy and Infectious Diseases. We wish to thank Patricia D. Bernard for her skilled technical assistance in performing the amino acid analyses.

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