Radioimmunoassay for the Antigenic Determinants of Cholera Toxin and Its Components

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A radioimmunoassay procedure is described for the detection of cholera toxin and its component polypeptide chains. Cholera toxin, A subunit, B subunit, α chain, and γ chain were iodinated by the chloramine T procedure. Radiolabeling did not significantly alter the polyacrylamide electrophoretic migration patterns of the toxin or its components. Moreover, radiolabeled toxin, B subunit, and α chain preparations retained substantial ability to bind to intestinal mucosal homogenates. The minimal amount of antitoxin detectable with radiolabeled toxin was 0.04 antitoxin units/ml. Substitution of radiolabeled B subunit, A subunit, and α chain for radiolabeled toxin decreased the sensitivity of the test. Radiolabeled γ chain did not bind to the antitoxin preparation. Competitive inhibition studies, with titrated anti-choleragen serum and radiolabeled toxin or components, indicated that the minimum concentration of toxin detectable was 7.0×10^{-8} μmol/ml at a 90% inhibition level. The A subunit and α chain preparations inhibited the binding of the radiolabeled B subunit to antitoxin sites. Conversely, B subunit inhibited the binding of radiolabeled A subunit and α chain to antitoxin. The γ chain did not show any reaction with antitoxin or cross-reaction with either whole toxin or its components. These results strongly suggest that the A subunit and the α chain contain antigenic determinant(s) that are common to the B subunit. The B subunit (β chain) and the α chain of cholera toxin may therefore contain region(s) of chemical similarity.

The exotoxin produced by *Vibrio cholerae* in culture filtrates has been shown to mimic the clinical symptoms of cholera in test animals (7–9, 11) and humans (2). After colonization of the small intestine by cholera vibrios, the disease is initiated by attachment of toxin to receptors on intestinal mucosal cells (4, 21, 27). The mechanism of action appears analogous to the action of glycoprotein hormones in the subunit interrelation of adenylate cyclase stimulation, leading to increased levels of cyclic adenosine 3',5' monophosphate in these cells (1, 22). As a consequence of adenylate cyclase stimulation, there is a reversal of intestinal ion flux, causing hypersecretion of fluid and electrolytes from the intestinal mucosal cells (32), resulting in massive watery diarrhea and severe dehydration.

Cholera toxin (choleragen) is a protein with an estimated molecular weight of 84,000 (26) that consists of two subunits, denoted as A and B, that are noncovalently bound (14, 17). The A subunit is comprised of two polypeptide chains, α (A$_1$) and γ (A$_2$), covalently linked by a disulfide bridge. The B subunit is comprised of an aggregation of four to six β chains (A. Kurosky,

D. E. Markel, and J. W. Peterson, Proc. 12th Joint Conf. U.S.-Japan Cooperative Med. Sci. Prog., in press; 23, 24). Current evidence indicates that the A subunit, and specifically the α chain, is the active component responsible for increasing the levels of cyclic adenosine 3',5'-monophosphate in cell-free extracts (3, 18, 36). The B subunit is responsible for the specific binding of the toxin molecule to G$_{M1}$ (galactosyl-*N*-acetylgalactosaminyl [sialosyl] lactosylceramide) ganglioside receptors on the cell surface (4, 30, 34).

Little is known about the antigenic determinants of cholera toxin. Such systems as fat cell, ileal loop, and rabbit skin as well as many other assays relate only to the biological activity of the toxin (6). More recently, in vitro cell systems have been utilized to measure biological activity of cholera toxin and its subunits by the cyclic adenosine 3',5'-monophosphate assay (1, 3, 6, 18). These tests allow only limited study of the reaction between antitoxin with the toxin and/or its component polypeptide chains. Preliminary reports of immunological studies assumed the A and B subunits were antigenically

distinct from each other (12, 18) even though antibodies to the B subunit were found in antiserum to the A subunit. This cross-reactivity was thought to be the result of contamination of the A subunit with B subunit, although such evidence was not presented.

The purpose of this study was to develop a radioimmunoassay that would detect and characterize the antigenic determinants of cholera toxin and its component polypeptide chains. Such an assay may also facilitate studies of antitoxin (anti-choleragen toxin) specificity toward cholera toxin and its components. The sensitivity and specificity afforded by a radioimmunoassay should provide accurate quantitation of antitoxin and a better understanding of antitoxin immunity to cholera.

MATERIALS AND METHODS

Preparation of cholera toxin and its components. *Vibrio cholerae* (Inaba strain 569B) was grown in fermentors by the procedure of Lewis and Richardson with asparagine-glucose medium (25). Cholera toxin (lot no. 0175) was purified from cell-free supernatants by a modification of the procedure described by Finkelstein et al. (13, 16). This procedure included addition of a large excess of Al(OH)$_3$ powder (Mallinckrodt Chemical Co.) to the centrifuged culture supernatant, which had been adjusted to pH 5.5 with HCl. After overnight incubation at room temperature, the Al(OH)$_3$ was removed by centrifugation in 1-liter bottles, and the pellet was washed twice with 0.01 M ammonium formate buffer, pH 5.5. The toxin was eluted with two 1-liter volumes of tris(hydroxymethyl)aminomethane-NaCl buffer, pH 7.5 (16). The elutions were pooled and concentrated by ultrafiltration with an Amicon PM-30 membrane (Amicon Corp.). The concentrated eluate was then mixed with diethylaminoethyl-Sephadex A-50 previously equilibrated with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, containing 0.02 M NaN$_3$. The diethylaminoethyl-Sephadex was subsequently washed twice with the same buffer and centrifuged. The toxin was then eluted from the pellet with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 5.5, containing 0.02 M NaN$_3$. After concentration by ultrafiltration, toxin preparations were chromatographed on Sephadex G-75 (2.5 by 240 cm) eluted with tris(hydroxymethyl)aminomethane buffer (16) to remove choleragenoid (15). Preparations purified in this manner were packaged in 5-mg quantities, which were lyophilized and stored at 4°C.

The A and B subunits were prepared from the toxin as previously described (24). The A subunit was then resolved into α and γ chains by chromatography on a Bio-Rad P-60 column eluted with 5.2 M guanidine–0.2 M sodium formate buffer, pH 3.2, after reduction and alkylation (20).

Iodination reaction. Iodination of protein (12 to 25 µg/µl) was carried out in glass tubes (10 by 75 mm) by the chloramine T method (19). The reaction mixture consisted of protein diluted in 50 µl of 0.5 M

sodium phosphate buffer, pH 7.5, 50 µl of 0.5 M sodium phosphate buffer, pH 7.5, and 5 µl (500 µCi) of Na^{125}I (Amersham/Searle; specific activity, 11.0 to 17.3 mCi of ^{125}I/µg of I). The reaction was initiated by addition of 10 µl of chloramine T (25 µg) dissolved in 0.5 M sodium phosphate buffer, pH 7.5, and allowed to proceed for 2 min at 25°C. The reaction was stopped by addition of 50 µg of sodium metabisulfite in 20 µl of 0.15 M sodium phosphate buffer, pH 7.5. The reaction mixture was desalted on Sephadex G-25 (1 by 10 cm), preconditioned with 1 ml of bovine serum albumin, and eluted with 0.15 M sodium phosphate buffer, pH 7.5. Fractions of 1 ml were collected into glass tubes (10 by 75 mm) containing 50 µl of 2% bovine serum albumin. The radioactivity of the fractions was measured with a Nuclear-Chicago gamma scintillation counter. The peak tube representing the radiolabeled protein was selected and stored at 4°C for no longer than 1 month.

Acrylamide gel electrophoresis. Sodium dodecyl sulfate-urea (SDS-urea)-polyacrylamide gel electrophoresis was performed according to Swank and Munkres (33). After electrophoresis, the gels were cut into 2-mm slices and the radioactivity was measured. In addition, proteins were reacted with fluorescamine and similarly subjected to SDS-urea-gel electrophoresis (10). The fluorescamine-stained protein bands were made visible by illumination with ultraviolet light.

Binding to intestinal mucosal homogenate. A rabbit mucosal homogenate was prepared as described previously (29). Increasing amounts of homogenate were added to a series of glass tubes (10 by 75 mm) containing approximately 10,000 cpm of either the radiolabeled toxin or its radiolabeled components. The tubes were centrifuged at 2,500 rpm for 1 h, and the supernatant and mucosal pellet were subsequently assayed for radioactivity. The radioactivity in the pellet was expressed as a percentage of the total radioactivity.

Preparation of cholera antitoxin. An antitoxin serum pool was prepared by immunizing four rabbits with 50-µg injections of cholera toxin (Finkelstein lot no. 0172) emulsified in Freund complete adjuvant. Serum samples obtained over a period of 6 months were pooled, distributed into aliquots, and stored at −20°C.

Radioimmunoassay procedure. A radioimmunoassay was developed using polyvinyl chloride microtiter plates. Each well contained 25 µl of 0.1 M ethylenediaminetetraacetic acid, pH 7.5, 25 µl of phosphate-buffered saline, pH 7.5, containing 0.5% normal rabbit serum, 25 µl of rabbit antitoxic serum dilution to be tested, and radiolabeled protein (approximately 10,000 cpm) dissolved in 25 µl of the same buffer. Six control wells, to establish background radioactivity, were prepared by substituting 25 µl of phosphate-buffered saline, pH 7.5, containing 0.5% normal rabbit serum for the rabbit serum to be tested. After 18 h of incubation at 4°C, each microtiter well received 50 µl of hyperimmune goat anti-rabbit globulin titrated for complete precipitation of the rabbit globulins. The microtiter plates were subsequently incubated for another 18 h at 4°C and centrifuged, and each well was washed with 150 µl of phosphate-buffered saline, pH 7.5. The wells were excised and assayed individually

for radioactivity. When the assay was used to test for cross-reactions between the unlabeled toxin or its components, each microtiter well contained 25 μl of the appropriate concentration of protein to be tested for cross-reactivity.

RESULTS

Cholera toxin antigen. The purity of cholera toxin (lot no. 0175) prepared in this laboratory has been examined previously (24). No contaminating proteins were observed in Coomassie blue-stained, SDS-urea- or acid-urea-polyacrylamide gels. Rabbits immunized with three subcutaneous 50-μg doses of cholera toxin (lot no. 0175), 4 weeks apart, produced no detectable rise in vibriocidal antibody (37) titer. The specific activity of our cholera toxin (lot no. 0175) was determined by J. P. Craig to be 25.3 limit of bluing per μg of protein compared with 26.5 limit of bluing per μg of protein for Finkelstein prepared toxin (lot no. 0172 and 0572) furnished by the National Institute of Allergy and Infectious Diseases. Polyacrylamide gel electrophoresis of our purified toxin gave identical electrophoretic patterns to the Finkelstein-prepared toxin preparations (24). The toxin preparations were judged identical by amino acid sequence analysis (A. Kurosky, D. E. Markel, and J. W. Peterson, J. Biol. Chem., in press).

Iodination of proteins. Cholera toxin and its isolated components were appropriately radiolabeled (Table 1). The specific activity for each preparation was calculated from the amount of radiolabeled protein and the percentage of radioactivity incorporated into each preparation.

Polyacrylamide gel analysis of radiolabeled preparations. Radiolabeled toxin, B subunit, A subunit, α chain, and γ chain preparations were subjected to SDS-urea-polyacrylamide gel electrophoresis. Results are expressed as the percentage of radioactivity of each 2-mm slice to total radioactivity (Fig. 1). The radiolabeled toxin dissociated into two peaks of radioactivity. The first peak corresponded to the radiolabeled A subunit preparation, and the second peak was similar to the peak for the radiolabeled B subunit preparation (β chain).

The radiolabeled A and B subunit preparations each consisted of only one radioactive peak. Reduction of radiolabeled A subunit with mercaptoethanol resulted in two radioactive peaks, consisting of the α and γ chains. Peaks of radioactivity obtained from the reduced A subunit corresponded exactly to the mobility of isolated radiolabeled α and γ chain preparations. Although not shown here, the electrophoretic migration of the reduced radiolabeled B subunit was similar to the nonreduced radiolabeled B subunit. We consistently found that the reduced B subunit migrated somewhat slower than unreduced B, possibly due to more complete protein unfolding (Kurosky et al., J. Biol. Chem., in press). Gel electrophoresis of fluores-

TABLE 1. *Radiolabeling of cholera toxin and its components[a]*

Preparation	Amt radiolabeled (μg)	Radioactive uptake (%)	Sp act (μCi/μg)
Toxin	25.0	70.1	14.02
A subunit	23.0	76.2	19.03
B subunit	28.2	63.2	13.50
α Chain	23.0	87.9	18.60
γ Chain	12.0	60.6	25.20

[a] The toxin and each of its components were radiolabeled with 500 μCi of Na^{125}I. The radioactive uptake and protein concentration were used to calculate the specific activity of each preparation.
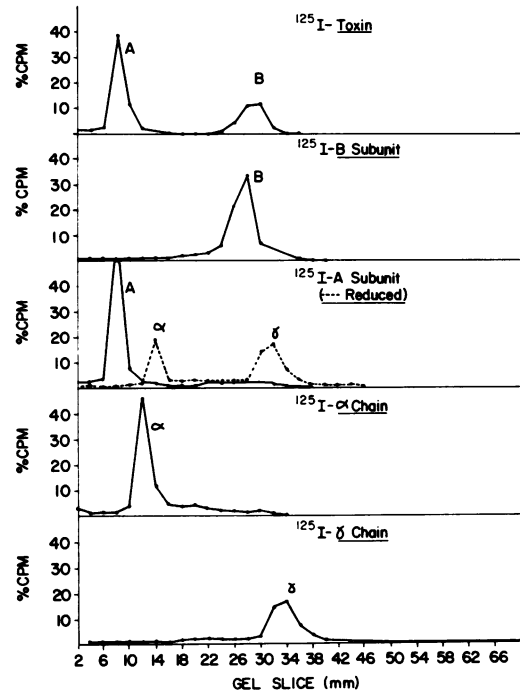

FIG. 1. *Each radiolabeled preparation was applied to an SDS-urea-polyacrylamide gel and electrophoresed overnight. Total amounts of radioactivity (counts per minute) applied to gels were: toxin, 2.2 × 10⁶; B subunit, 1.3 × 10⁶; A subunit, 0.2 × 10⁶ (reduced), 3.3 × 10⁶ (unreduced); α chain, 2.8 × 10⁶; γ chain, 2.3 × 10⁶. The radioactivity of each 2-mm gel slice was plotted in relationship to the total radioactivity added to the gel. An additional A subunit preparation was pretreated with mercaptoethanol, and those results are plotted in the same frame with the nonreduced A subunit.*

camine-reacted proteins indicated no cross-contamination between the A and B subunits.

Intestinal mucosal binding of radiolabeled preparations. An additional analysis was performed on the radiolabeled toxin and its radiolabeled components to appraise possible modification of the native toxin molecule affecting tissue binding sites. When the mucosal homogenate was increased from 16 to 63 mg of dry weight, the radiolabeled toxin, B subunit, and α chain preparations increased in binding (Table 2). With the addition of 63 mg of homogenate, approximately 60% of the radiolabeled toxin, 40% of the radiolabeled B subunit, and 28% of the radiolabeled α chain were bound. Neither radiolabeled A subunit nor γ chain bound to the homogenate.

Antitoxin titration and radioimmunoassay sensitivity. The rabbit antitoxin pool (anti-choleragen) contained 810 antitoxin units/ml (as determined by J. P. Craig) and was used to test the radiolabeled preparations for binding. As the antitoxin pool was diluted, the decrease in radioactivity indicated reduced binding for each radiolabeled preparation to the remaining antibody (Fig. 2). When the radiolabeled toxin was used for quantitation, the system could detect a minimum of 0.04 antitoxin units/ml. A concentration of 0.16 antitoxin units/ml bound 50% of the total radioactivity added to the system. The sensitivity of this test decreased to 0.60, 1.75, and 10.4 antitoxin units/ml in the case of radiolabeled B subunit, A subunit, and α chain preparations, respectively. Radiolabeled γ chain did not bind even when the antitoxin concentration approached 60 antitoxin units/ml.

Competitive inhibition studies. Increasing concentrations of unlabeled toxin and its components were evaluated for inhibition of their radiolabeled analog preparations. Figure 3 depicts the cross-reactions observed when increas-
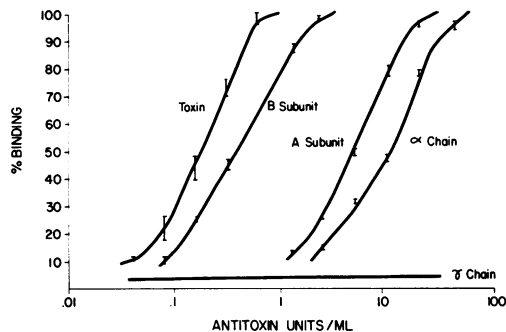
TABLE 2. *Binding of radiolabeled cholera toxin and its components[a]*

Homoge-nate (mg of dry weight)	Binding to mucosal homogenate (%)				
	Toxin	A sub-unit	B sub-unit	α Chain	γ Chain
16	49	0	30	12	0
31	53	0	35	19	0
47	55	0	38	23	0
63	60	0	40	28	0

[a] A mucosal homogenate from rabbit small intestines was prepared and used to test for the binding of the radiolabeled preparations. The homogenate was increased in concentration for each radiolabeled preparation, and the relative percentage was calculated from the total amount of radioactivity added.

FIG. 2. *An increasing concentration of rabbit antitoxin (anti-choleragen) was added in the presence of each radiolabeled preparation. The radioactivity of each radiolabeled preparation bound by the antitoxin is plotted as percentage of binding.*

ing amounts of the toxin and component polypeptide chains were allowed to compete with radiolabeled toxin (10,000 cpm/400 pg) for antitoxin sites (0.16 antitoxin units). Results obtained from each competition reaction were analyzed by linear regression and had similar slopes of -0.695, -0.756, and -0.752 for toxin, B subunit, and A subunit, respectively. The slope for the α-chain preparations was -0.937. These linear functions indicated that 50% inhibition was achieved with toxin, B subunit, A subunit, and α chain at concentrations of 2.7×10^{-7}, 2.8×10^{-6}, 4.4×10^{-4}, and 1.2×10^{-2} μmol/ml, respectively. The binding of radiolabeled toxin was completely inhibited by concentrations approaching 1.5×10^{-6}, 1.3×10^{-5}, 2.0×10^{-3}, and 4.0×10^{-2} μmol/ml of the toxin, B subunit, A subunit, and the α chain, respectively. It required approximately 10 times more B subunit, 1,000 times more A subunit, and 10,000 times more α chain to equally inhibit the binding of the radiolabeled toxin preparation on a molar basis as it did the unlabeled toxin. The γ chain did not inhibit the reaction at concentrations up to 1.55×10^{-3} μmol/ml.

The degree of inhibition observed when the toxin and its components were allowed to compete with radiolabeled B subunit (10,000 cpm/400 pg) for antitoxin sites (0.16 antitoxin units) are shown in Fig. 4. A linear regression analysis of the competitive reactions indicated comparable slopes of -0.711, -0.728, -0.691, and -0.694 for the B subunit, toxin, A subunit, and α chain, respectively. A 50% inhibition of this reaction was achieved with the toxin, B subunit, A subunit, and α chain at concentrations of 3.8×10^{-7}, 1.2×10^{-6}, 7.0×10^{-4}, and 7.7×10^{-3} μmol/ml, respectively. Complete inhibition of this reaction occurred with concentrations approaching 1.9×10^{-6}, 6.2×10^{-6}, $3.8 \times$

10^{-3}, and 4.0×10^{-2} μmol/ml of the toxin, B subunit, A subunit, and the α chain, respectively. It required approximately 5 times more B subunit, 1,000 times more A subunit, and 10,000 times more α chain to inhibit the reaction equally on a molar basis as it did the B subunit. The γ chain did not inhibit the reaction at concentrations up to 1.55×10^{-3} μmol/ml.

The concentration required for the toxin and its components to inhibit radiolabeled A subunit (10,000 cpm/290 pg) from binding to antitoxin sites (4.05 antitoxin units) are given in Fig. 5. The toxin, α chain, and B subunit were calculated to have similar line slopes of -0.667, -0.608, and -0.751, respectively. The A subunit was found to have a slope of -0.432. A 50% inhibition of this reaction occurred at a concentration of 1.6×10^{-6}, 4.4×10^{-6}, 6.9×10^{-6}, and

3.0×10^{-3} μmol/ml with the toxin, α chain, A subunit, and B subunit, respectively. The reaction was completely inhibited by concentrations approaching 9.0×10^{-6}, 3.0×10^{-5}, 1.0×10^{-4}, and 1.6×10^{-2} μmol/ml of the toxin, α chain, A subunit, and B subunit, respectively. The A subunit was almost as potent as the α chain in inhibiting this reaction. It required almost 1,000 times more B subunit to equally inhibit on a molar basis this reaction as it did the toxin. The γ chain was noninhibitory at concentrations up to 1.55×10^{-3} μmol/ml.

The results of the competitive inhibition when the toxin and its components were allowed to compete for antitoxin sites (4.05 antitoxin units) in the presence of radiolabeled α chain (10,000 cpm/300 pg) are shown in Fig. 6. The A subunit, toxin, and B subunit inhibited
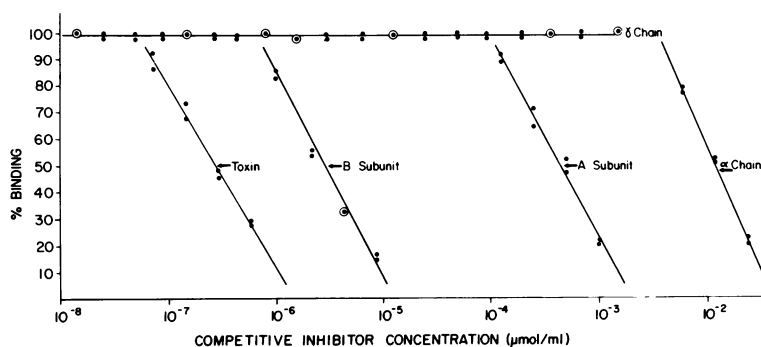
FIG. 3. *Radiolabeled cholera toxin (^{125}I label; 0.16 antitoxin unit) was allowed to compete with increasing concentrations of unlabeled toxin, B subunit, A subunit, and α chain preparations for antitoxin binding sites. Line slopes of -0.695, -0.756, -0.752, and -0.937 were calculated by linear regression analysis for the unlabeled toxin, B subunit, A subunit, and α chain preparations, respectively. The γ chain was not shown to inhibit the binding of radiolabeled toxin to antitoxin binding sites.*
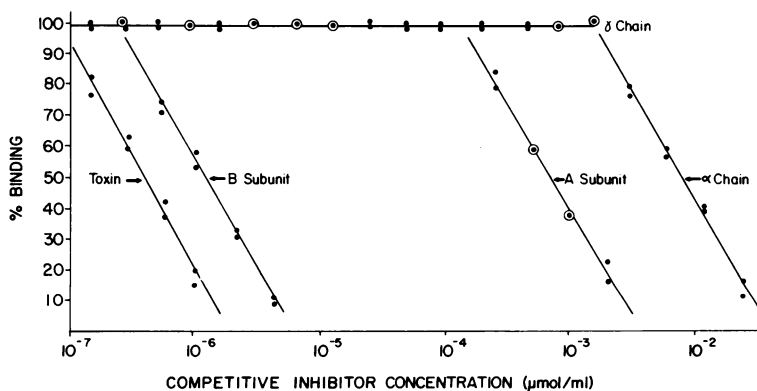
FIG. 4. *Radiolabeled B subunit (^{125}I label; 0.16 antitoxin unit) was allowed to compete with increasing concentrations of unlabeled toxin, B subunit, A subunit, and α chain preparations for antitoxin binding sites. Line slopes of -0.711, -0.728, -0.691, and -0.694 were calculated by linear regression analysis for the unlabeled B subunit, toxin, A subunit, and α chain preparations, respectively. The γ chain was not shown to inhibit the binding of radiolabeled B subunit to antitoxin binding sites.*
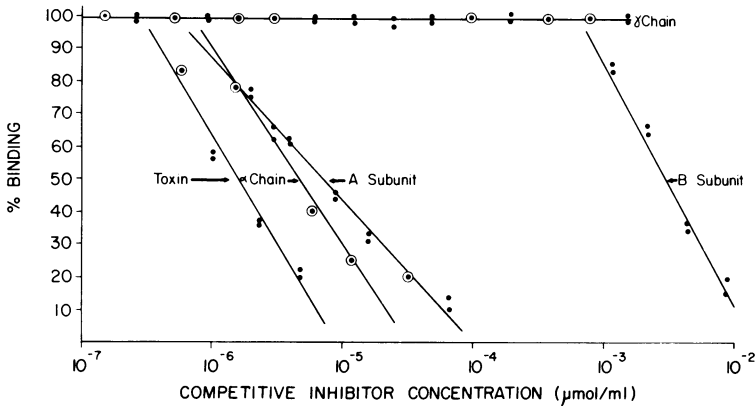
FIG. 5. *Radiolabeled A subunit (^{125}I label; 4.05 antitoxin units) was allowed to compete with increasing concentrations of unlabeled toxin, B subunit, A subunit, and α chain preparations for antitoxin binding sites. Line slopes of −0.667, −0.608, −0.751, and −0.432 were calculated by linear regression analysis for the unlabeled toxin, α chain, B subunit, and A subunit preparations, respectively. The γ chain was not shown to inhibit the binding of radiolabeled A subunit to antitoxin binding sites.*
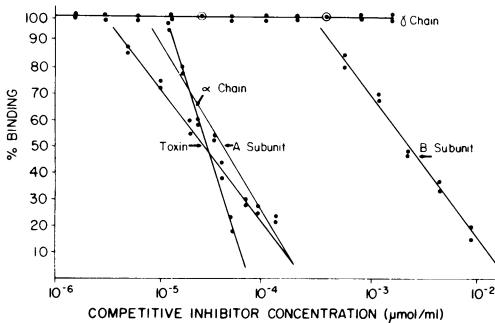

FIG. 6. *Radiolabeled α chain (^{125}I label; 4.05 antitoxin units) was allowed to compete with increasing concentrations of unlabeled toxin, A subunit, B subunit, and α chain preparations for antitoxin binding sites. Line slopes of −0.649, −0.516, −0.540, and −1.235 were calculated by linear regression analysis for the unlabeled A subunit, toxin, B subunit, and α chain preparations, respectively. The γ chain was not shown to inhibit the binding of radiolabeled α chain to antitoxin binding sites.*

this reaction with linear slope functions of −0.649, −0.516, and −0.540. The α chain inhibited at a slope of −1.235. The reaction was inhibited by 50% at concentrations of 2.6×10^{-5}, 2.8×10^{-5}, 3.9×10^{-5}, and 2.3×10^{-3} μmol/ml by the toxin, α chain, A subunit, and the B subunit, respectively. This reaction was completely inhibited with concentrations approaching 6.8×10^{-5}, 2.8×10^{-4}, 2.6×10^{-4}, and 1.9×10^{-2} μmol/ml of the α chain, toxin, A subunit, and the B subunit, respectively. It required approximately 100 times more B subunit on a molar basis to inhibit this reaction as it did the toxin.

The γ chain was noninhibitory at concentrations up to 1.55×10^{-3} μmol/ml.

DISCUSSION

Since the discovery and purification of cholera toxin, efforts have been made to develop toxoids from cholera toxin by treatment with formalaldehyde or glutaraldehyde. The hypothesis was that toxoid immunization might elicit protective immunity. Glutaraldehyde toxoid preparations have been studied in animal models, and significant protection was achieved against experimental cholera infections (28, 31). However, field trial immunization studies, in Bangladesh, to evaluate protective antitoxic immunity with these toxoids have been rather disappointing (5). The demand has increased for a test capable of comparing the antigenicity of these toxoids and characterizing the antibodies elicited by each.

The specificity of the immune response has been used to develop a sensitive radioimmunoassay procedure to detect and characterize antitoxic immunity in rabbits immunized with cholera toxin. The toxin and its components were purified in this laboratory and radiolabeled by the chloramine T method. The guanidine-prepared β chains and the A subunit for these experiments were determined to be chemically pure by N-terminal sequence analysis, SDS-urea- and acid-urea-gel electrophoresis stained with Coomassie blue (24), and SDS-urea-gel electrophoresis of radiolabeled and fluorescamine-reacted toxin and its components. The radiolabeled gel studies showed that there was no significant, detectable damage to the

proteins due to the radiolabeling procedure. Gel electrophoresis of fluorescamine-reacted A and B subunits failed to detect any cross-contamination of the subunits even when the gels were loaded with 50 μg of protein. It should be also pointed out that after removal of the guanidine by dialysis, the β chain reaggregated to form a B subunit capable of binding to intestinal mucosal homogenates (Kurosky et al., Proc. 12th Joint Conf. U.S.-Japan Cooperative Med. Sci. Prog., in press).

Binding studies with intestinal mucosal homogenates were performed on the radiolabeled toxin and its components to estimate possible protein modification as a result of the radiolabeling process. The binding of the radiolabeled toxin and the B subunit to mucosal homogenates demonstrated that these radiolabeled preparations retained substantial ability to bind to tissue. As previously reported, some damage to the tissue binding sites on the whole molecule was expected (29); however, the binding of the radiolabeled α chain was unexpected. Additional experiments (unpublished data) indicated that 50% of the radiolabeled toxin can be inhibited from binding to the mucosal homogenate by preincubation with unlabeled α chain at a concentration of 5.2×10^{-1} μmol/ml. Since the radiolabeled α chain was shown to be pure by a number of criteria, the binding of the α chain appears to be an inherent property of the peptide and not due to contamination with the B subunit or β chains. Neither the radiolabeled A subunit nor the γ chain preparations bound to the mucosal homogenate. This indicates that the α chain may not participate in the initial binding of the native molecule to the membrane due to possibly steric hindrance by the γ chain moiety. However, the α chain may bind secondarily to the membrane, subsequent to conformational changes in the toxin after binding of the β chains and the stimulation of adenylate cyclase.

In this radioimmunoassay procedure, the radiolabeled toxin or its radiolabeled components were used to quantitate antitoxin. It is significant that the immune response in rabbits immunized with toxin consisted of antibodies elicited by the antigenic determinants located in both the A and B subunits. This was illustrated by the binding of the radiolabeled A and B subunits to the antitoxin preparation. With radiolabeled toxin, the test system could detect a minimum of 0.04 antitoxin units/ml. The radiolabeled γ chain did not bind to the antitoxin, and we have never observed unlabeled γ chain to inhibit the reaction between antitoxin and radiolabeled toxin or its components. It appears that the α chain moiety possesses all the observed antigenic determinants of the A subunit, since the γ chain was found to be consistently noninhibitory in this study. This would suggest that the γ chain determinants may be nonimmunogenic or sequestered within the toxin molecule. The latter conclusion may also be valid since, after reduction of the radiolabeled toxin and subsequent analysis on SDS-urea-polyacrylamide gel electrophoresis, there was no significant increase in the radioactivity in the region where γ chain coelectrophoresed with β chains (data not shown). However, when the A subunit was iodinated, the γ chain was shown to be substantially radiolabeled, as can be seen after reduction (Fig. 1).

Competitive inhibition studies with this radioimmunoassay indicate that antigenic similarities may exist between A subunit (probably in the α chain exclusively) and the B subunit components of the toxin. Increasing concentrations of B subunit competitively inhibited the binding of the radiolabeled A subunit and the α chain preparations to antitoxin binding sites. Conversely, the unlabeled A subunit and α chain preparations inhibited the radiolabeled B subunit from binding to its antitoxin sites. It is well to point out here that Finkelstein et al. (12) noted that antiserum from rabbits immunized with the A subunit precipitated both the A and B subunits but was interpreted as A subunit contamination with the highly immunogenic B subunit. Similarly, van Heyningen observed cross-reactivity between the A and B subunits, which he also attributed to possible contamination (35). Although the present report does not provide conclusive evidence for antigenic similarity between the A and B subunits of cholera toxin, it does present data that strongly suggest such a relationship. Additional studies of this phenomenon with antiserum to purified α, β, and γ chains are in progress.

In summary, we have developed a radioimmunoassay capable of defining the antigenic structure of cholera toxin and its components. This assay may be useful in detecting differences between antibodies elicited by toxoid immunization and antibodies to cholera toxin after natural infection. The sensitivity of this assay should allow the detection of secretory antibody (immunoglobulin A) and help to evaluate its role in antitoxic immunity. Results from future data achieved with this radioimmunoassay should expand the knowledge of antitoxic immunity.

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