Characterization of the Antigenic Determinants of Cholera Toxin Subunits

DAIEL E. MARKEL,† KELLY E. HEJTMANCIK,‡ JOHNNY W. PETERSON,†* FRANK B. MARTIN,¶ AND ALEXANDER KUROSKY

Departments of Microbiology† and Human Biological Chemistry and Genetics‡ University of Texas Medical Branch, Galveston, Texas 77550

Received for publication 15 February 1979

The antigenic specificity of purified preparations of A subunit, B subunit, α chain, and γ chain of cholera toxin was studied by double immunodiffusion and radioimmunoassay with antisera produced in rabbits and mice. Rabbits immunized with A subunit produced serum antibodies which were capable of binding radiolabeled A subunit, α chain, and B subunit. Rabbits immunized with α chain produced serum antibodies that would bind radiolabeled α chain and A subunit. Rabbits immunized with the B subunit produced serum antibodies monospecific for the B subunit. The γ chain did not elicit measurable antibodies in rabbits or mice as evidenced by radioimmunoassay or double immunodiffusion. A sensitive competitive radioimmunoassay was developed in which the B subunit could inhibit binding of radiolabeled A subunit and α chain with either antisera prepared against A subunit or α chain. Neither the A subunit nor the α chain could inhibit binding of B subunit with the antiserum prepared against B subunit. In addition, selected elution fractions obtained during A- and B-subunit purification were used to immunize groups of mice. Mice responded to immunization with the elution fractions of A subunit by producing anti-A-subunit and anti-B-subunit serum antibody responses, whereas mice immunized with elution fractions of B subunit produced only antibodies specific for the B subunit. An equimolar amount of the two resulting protein peaks was used to immunize two groups of rabbits. Rabbits immunized with A subunit, produced a serum anti-B subunit response equal to that of rabbits immunized with the B subunit. Rabbits immunized with equimolar concentrations of A and B subunits were observed to be equally protected against intestinal loop challenge with Vibrio cholerae Inaba V86. The A subunit, not the B subunit, was biologically active when tested by the S49 mouse lymphosarcoma cell test. These studies provide additional evidence supporting the hypothesis that the A subunit, specifically of α chain, of cholera toxin contains antigenic determinants in common with the B subunit.

The structural, chemical, antigenic, and biological properties of cholera toxin (choleragen) have been the subjects of intense investigations. Not only is the toxin important in the study of the pathogenesis of cholera and other adenylate cyclase-mediated diarrheal diseases, but molecular biologists have utilized it to probe cellular cyclic adenosine 5′-monophosphate-mediated events since the toxin activates adenylate cyclase in virtually every mammalian cell tested (4, 5).

 Structurally, the toxin is a protein with a molecular weight of about 84,000 (20) consisting of two noncovalently bound subunits denoted as A and B (17). The A subunit contains two polypeptide chains, α and γ, with molecular weights of approximately 24,000 and 9,700, respectively (17). There is evidence that the A subunit, and more specifically the α chain, contains the active site(s) which stimulates adenylate cyclase in cell-free extracts (8, 9, 29, 30). The A subunit has also been found to be weakly active in rabbit skin tests (3, 24, 28). The B subunit is composed of a noncovalent aggregation of four to six β chains, of which the precise number of chains is still unresolved (13, 14, 17, 19, 28). The B subunit was shown to be identical to choleragenoid, the natural toxoid of the toxin (15, 18). The B subunit contains the binding sites specific for GM1 (galactosyl-N-acetyl-galactosaminyl [sialosyl] 1-lactosyl ceramide) cell membrane receptors (12,

† Present address: Department of Microbiology and Immunology, Oral Roberts University School of Medicine, Tulsa, OK 74171.
‡ Present address: Department of Surgery, University of Texas Medical Branch, Galveston, TX 77550.
binds cyclase. It was recently reported that after the toxin binds to the ganglioside GM1 receptor, the A subunit enters the cell and in the presence of nicotinamide adenine dinucleotide ribosylates a guanosine 5'-triphosphate binding protein, the regulatory guanosine triphosphatase of adenylate cyclase, thereby inactivating it (1).

The amino acid sequence of the β chain has been elucidated (16, 18), and the first 20 N-terminal residues of the α and γ chains have been reported (17). Amino acid sequencing and amino acid analyses have thus far shown each polypeptide to be unique (16, 17, 22).

There is limited information about the immunological properties of the A and B subunits of the cholera toxin. Double immunodiffusion analyses have indicated that the toxin contains antigenic determinants in addition to those unique to choleracongenid (B subunit) (11, 23). These results are consistent with chemical and structural analyses of the subunits. Finklestein et al. (6) reported that rabbits immunized with purified A subunit consistently produced serum antibodies which precipitated both toxin and the B subunit. These results were interpreted as possible A subunit contamination with the B subunit. Others have reported the same findings; however, no evidence of B subunit contamination was in fact observed (24, 28). In this report we extend the immunological study of the antigenic determinants of the A and B subunits and α and γ chains by using radioimmunoassay (RIA) procedures for each component as well as the S49 mouse lymphosarcoma cell test (26).

MATERIALS AND METHODS

Cholera toxin purification. Cholera toxin (lot no. 0175) was purified from fermentors inoculated with Vibrio cholerae Inaba 569B as previously described (10, 16).

Subunit purification. The A and B subunits were purified from cholera toxin by gel filtration on a Bio-Gel P-60 column eluted with 0.2 M sodium formate buffer, pH 3.5, containing 6.2 M guanidine-hydrochloride (17). Two different filtration columns were used in this investigation. For rabbit immunization studies, 50 mg of cholera toxin was chromatographed on a column (2.5 by 110 cm), and the peak tubes were dialyzed against 0.01 M tri(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer containing 0.2 M NaCl and 0.001 M ethylenediaminetetraacetic acid, pH 7.5. For mouse and additional rabbit immunization studies, a smaller column (1 by 80 cm) was used to chromatograph 10 mg of cholera toxin. Selected elution fractions were dialyzed against the Tris-hydrochloride buffer previously described.

The α and γ chains used for immunization and RIA were purified from about 80 mg of S-[14C]carbamoylmethyl A subunit by gel filtration on a Bio-Gel P-60 column (2.5 by 110 cm) equilibrated with the formate-guanidine buffer described above. Column eluates were monitored by absorbance at 280 nm and by measurement of radioactivity (100-μl portions from each fraction).

Antiserum preparation. Four groups of five rabbits each were immunized with purified A-subunit, α-chain, γ-chain, or B-subunit preparations emulsified in Freund complete adjuvant. Each rabbit was injected by the intraperitoneal, intramuscular, and subcutaneous routes with a total of 200 μg of the respective antigenic preparation. Blood samples were taken from the rabbits every 3 weeks, and a booster dose of 200 μg was administered whenever antibody titers began to decrease, as determined by passive hemagglutination with toxin-sensitized erythrocytes (7). All sera were stored at -20°C.

Two additional groups of five rabbits each were immunized with the A and B subunits, respectively, without Freund complete adjuvant. Each rabbit was immunized intramuscularly with 8.9 × 10⁻⁴ μmol of A (28 μg) or B (50 μg) subunit as an initial injection and subsequently given a booster injection of the same equimolar amount of antigen after 4 weeks. Premunization, 4-week, and 6-week blood samples were taken from the central ear artery. At 6 weeks, the animals were challenged with live V. cholerae organisms (Inaba V86) by the ligated loop technique as previously described (2) with challenge doses ranging from 10⁶ to 10⁹ organisms per loop.

Selected fractions eluted from the ascending and descending portions of the two protein peaks from the P-60 column after application of a 10-mg sample of cholera toxin were dialyzed against the Tris-hydrochloride buffer. The protein content of each fraction was determined by the method of Lowry et al. (21). Each fraction was diluted 1:21.5 in Tris-hydrochloride buffer, and 0.5 ml of each dilution was injected by the intraperitoneal route into eleven groups of 15- to 20-g Swiss Yale mice, consisting of seven mice per group. One group of mice was injected with Tris-hydrochloride buffer, pH 7.5, as a control group. Blood samples were taken by inserting a heparinized capillary tube into the retroorbital sinus before immunization, as well as 3 and 4 weeks after immunization. Antibody titers of the mice were determined by RIA with radiolabeled A and radiolabeled B subunits.

Double immunodiffusion analysis. Plastic petri dishes (100 by 15 mm) were filled with 15 ml of 1.0% Noble agar in 0.05 M sodium barbital buffer, pH 8.6, containing 0.1% sodium azide. Each well (5-mm diameter, 2 mm deep), was filled with 50 μl of antisera or antigen preparation. All antigens were diluted to a concentration of 100 μg/ml and placed in the peripheral wells, whereas the antisera were placed in the center wells.

RIA. An RIA with polyvinyl chloride microtiter plates was used as previously described (10). Protein preparations were radiolabeled by the chloramine T method with ¹²⁵I (10). After the primary antibody reaction was allowed to proceed for 18 h, goat antirabbit globulin was added to precipitate the antibody bound from the unbound (free) radioactivity. A modification of this procedure was used for testing mouse
sera. Commercially prepared anti-mouse immunoglobulin (Dako-Immunoglobulins, Copenhagen, Denmark) was titrated and diluted for complete precipitation of mouse immunoglobulins and used in place of goat anti-rabbit globulin in the assay procedure. Measurements of radioactivity were based on the total counts per minute of each added radiolabeled component and the amount of radioactivity bound by antibody relative to the dilution of serum tested. Antitoxin values were based on a rabbit anti-toxin pool containing 810 antitoxin units (AU) per ml (10) (titrated by J. P. Craig) as compared with the Swiss reference antitoxin serum.

S49 mouse lymphosarcoma test. S49 mouse lymphosarcoma cells were obtained from J. R. Murphy, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Mass. The cells were maintained at 37°C and 7% CO₂ in Dulbecco-modified Eagle media supplemented with 10% irradiated fetal bovine serum, nonessential amino acids for Eagle minimum essential medium, 2 mM L-glutamine, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml as described by Ruch et al. (26). The cells were diluted in the assay to a concentration of 3 × 10⁵ cells per ml in the above medium and phosphodiesterase inhibitor, RO 20-1724 (Hoffman-La Roche, Nutley, N. J.), was added to a concentration of 10⁻⁵ M. Portions of 200 ul were dispensed into the wells of microtiter plates. A 10-μl portion of diluted sample was added to each well, and the assay plates were read after a 72-h incubation period. Biologically active cholera toxin inhibited the growth of the cells such that the phenol red pH indicator failed to turn from red to yellow. This assay detected as little as 10 pg of cholera toxin per well. The biological activity of the elution volumes of the fractionated cholera toxin was compared with a standard cholera toxin titration consisting of dilutions of toxin from 0.001 to 1,000 ng/well.

RESULTS

Subunit and polypeptide chain purification. The P-60 elution profile of the cholera toxin sample is shown in Fig. 1A. The first protein peak eluted was shown by electrophoresis to be A subunit, and the second peak was shown to be β chain (17). The two peak absorbance fractions were dialyzed against tris-hydrochloride buffer and subsequently used for rabbit immunization. Reduced and alkylated A subunit gave an elution pattern as depicted in Fig. 1B, demonstrating separation of the α and γ chains. The α chain was present in the first peak, and the γ chain was present in the second peak. Although the γ chain does not have an appreciable absorbance at 280 nm due to the absence of tryptophan (22), the eluting peak was observed by monitoring radioactivity of the S-[¹⁴C]carbamoylmethyl moiety of the protein resulting from alkylation. The peak tubes were dialyzed against the Tris-hydrochloride buffer and used in gel double immunodiffusion studies and rabbit immunizations.

Double immunodiffusion analysis. Consecutive serum samples from rabbits immunized with the A subunit were tested by gel double immunodiffusion for reaction with cholera toxin and its subunits. As illustrated in Fig. 2A, the

![Fig. 1.](http://iai.asm.org/) (A) A and B subunit purification by gel filtration of 50 mg of purified cholera toxin on a column (2.5 by 110 cm) eluted with a 0.2 M sodium formate buffer, pH 3.5, containing 5.2 M guanidine-hydrochloride. (B) Purification of α and γ chains after S-[¹⁴C]carbamomethylation of 80 mg of A subunit and gel filtration on the same column with the same buffer as described above. Each fraction was monitored by measuring absorbance at 280 nm (A₂₈₀) (-----) and radioactivity of 100-μl portions (--.--).
The serum antibody response in rabbits immunized with the B subunit produced typical Ouchterlony patterns as shown in Fig. 2B. This pattern illustrated that anti-B subunit sera were monospecific for the B subunit and shared a reaction of identity with toxin. There were no precipitin reactions between A-subunit, α-chain, or γ-chain (γ-chain data not shown) and anti-B-subunit sera.

Rabbits immunized with γ chain did not produce any detectable antibodies as tested by RIA or double gel diffusion. In addition, the γ-chain preparation was not precipitated by any of the other antisera prepared against the other preparations.

**Analysis of the antigenic relationship of A and B subunits.** Radiolabeled A and radiolabeled B subunits were used to titrate preimmunization as well as 3- and 6-week-post-immunization serum samples from rabbits immunized with 200 µg of A subunit emulsified in Freund complete adjuvant. A rising titer was detectable with both radiolabeled A subunit and B subunit (data not shown). Pre-immunization serum samples were incapable of binding radioactivity of the A subunit; however, 50% of the radioactivity was bound at a mean dilution of 1.2 × 10^3 at 3 weeks and 1.7 × 10^3 at 6 weeks. The same sera were titrated with radiolabeled B subunit. Preimmunization serum samples did not bind the radiolabeled B subunit; however, 50% of the radioactivity was bound at a mean dilution of 5.0 × 10^3 at 3 weeks and 2.0 × 10^4 at 6 weeks.

A more extensive survey of these anti-A-subunit sera was obtained by allowing sera to react with radiolabeled toxin, α chain, and γ chain, as well as radiolabeled A and B subunits. A typical radiolabeled binding pattern of one of these sera is shown in Fig. 3. Radiolabeled γ chain did not bind to any of the sera tested. Linear regression analysis of each line showed the slope for cholera toxin to be −0.886 (r = 0.95); the B-subunit line slope was −0.669 (r = 0.88), the A-subunit slope was −0.776 (r = 0.92), and the α-chain slope was −0.717 (r = 0.86).

When each of the sera was further studied by competitive inhibition of radiolabeled A subunit with unlabeled toxin, A subunit, B subunit, α chain, and cholera toxin all could inhibit binding of radiolabeled A subunit, indicating cross-reactivity (Fig. 4). Linear regression analysis of the resulting lines showed the A subunit to have a line slope of −0.32 (r = 0.94), the α chain to have a slope of −0.634 (r = 0.97), the toxin line to have a slope of −0.98 (r = 0.99), and the B subunit to have a line slope of −1.00 (r = 0.99). This cross-reactivity might be expected with α

**Fig. 2. Immunodiffusion patterns typical of post-immunization sera from rabbits immunized with the A subunit (A) and B subunit (B) emulsified in Freund adjuvant. The peripheral wells contain cholera toxin (CT), B subunit (B), A subunit (A), and α chain (α). The γ chain (not shown) did not react with any antisera tested.**
FIG. 3. Titration of a typical anti-A serum by RIA with radiolabeled cholera toxin (---), B subunit (-----), A subunit (——) and a chain (———). None of the anti-A sera could be titrated by radiolabeled γ chain. Percentages of radioactivity bound by antibody are expressed mathematically as logit determination for linear display.

FIG. 4. Competitive inhibition studies with increasing concentrations of cholera toxin and toxin components to compete with radiolabeled A subunit for anti-A-subunit binding sites.

to determine whether those cross-reactive sites were located on the α chain, an RIA was performed with antisera to α chain and radiolabeled α chain, A subunit, toxin, and B subunit. Only the radiolabeled α chain and A subunit were bound (data not shown). A more sensitive competitive assay was then established with radiolabeled α chain competing with unlabeled α chain, A subunit, B subunit, or toxin for antibody binding sites with anti-α-chain sera. A typical experiment is shown in Fig. 5. Substantial inhibition was obtained with the α chain and A subunit, whereas toxin and B subunit could only partially inhibit this reaction. Increasing concentrations of α chain beginning at $2.0 \times 10^{-7}$ μmol/ml inhibited all binding of the radioactive α chain in all sera tested with concentrations approaching $1.0 \times 10^{-4}$ μmol/ml. Linear regression analysis of the concentrations of α chain required for the inhibition of the radioactivity bound resulted in a line slope $-0.935$ ($r = 0.91$), with
50% inhibition values of $1.4 \times 10^{-6} \text{mol/ml}$. The higher concentrations of the A subunit approaching $3.0 \times 10^{-4} \text{mol/ml}$ did not completely inhibit the radiolabel binding. Linear regression analysis of the concentrations of the A subunit based on the inhibition of the radioactivity bound resulted in a line slope of $-1.211$ ($r = 0.92$), with a 50% inhibition value of $4.4 \times 10^{-5} \text{mol/ml}$. Concentrations of less than $1.3 \times 10^{-5} \text{mol}$ of the B subunit per ml did not inhibit the radiolabeled A chain from binding; however, increasing concentrations above $5.4 \times 10^{-4} \text{mol/ml}$ partially inhibited the reaction by 26.5%. It required concentrations above $5.4 \times 10^{-4} \text{mol}$ of cholera toxin per ml to partially inhibit radiolabel binding by 12.0%. The $\gamma$ chain was non-inhibitory in concentrations of up to $3.3 \times 10^{-3} \text{mol/ml}$.

Sera from rabbits immunized with 200 $\mu$g of B subunit emulsified in Freund complete adjuvant were titrated with both radiolabeled A and B subunits. Neither the pre-immunization nor the 3- or 6-week-post-immunization sera reacted by binding radiolabeled A subunit. A mean rising titer was obtained with the radiolabeled B subunit in which 50% of the radioactivity was bound at a $5.0 \times 10^3$ dilution at 3 weeks and a $2.8 \times 10^4$ dilution at 6 weeks, whereas no detectable titer was seen with pre-immunization sera (data not shown).

Serum samples from three representative rabbits immunized with B subunit were further analyzed with radiolabeled toxin, $\alpha$ chain, and $\gamma$ chain, as well as with radiolabeled A and B subunits. The typical radiolabeled binding pattern of an anti-B-subunit serum is depicted in Fig. 6. Radiolabeled toxin and B subunit were similar in their ability to detect 50% binding of the radioactivity by these sera at higher dilutions. When titrated with radiolabeled toxin, 50% of the radioactivity was bound at dilutions of $2.9 \times 10^3$ and $2.0 \times 10^4$, with line slopes of $-0.685$ ($r = 0.79$), $-0.626$ ($r = 0.91$), and $-0.718$ ($r = 0.89$), respectively. Linear regression analysis of these sera when titrated with radiolabeled B subunit revealed line slopes of $-0.714$ ($r = 0.84$), $-0.863$ ($r = 0.85$), and $-0.772$ ($r = 0.81$), with 50% binding of the radioactivity at serum dilutions of $3.3 \times 10^3$, $5.1 \times 10^3$, and $2.2 \times 10^4$, respectively. Radiolabeled A subunit, $\alpha$ chain, or $\gamma$ chain did not react with any anti-B-subunit sera tested. The monospecificity of anti-B-subunit sera was further examined and quantitated for cross-reactivity by allowing unlabeled toxin and its polypeptide components to compete with
radiolabeled B subunit for anti-B-subunit antibody binding sites. Figure 7 shows the cross-reactive analysis of a typical anti-B-subunit serum. The anti-B-subunit serum tested was previously titrated and diluted for 50% binding of the radioactivity added of radiolabeled B subunit. Increasing concentrations of unlabeled toxin and its components were allowed to compete for anti-B-subunit antibody binding sites in the presence of radiolabeled B subunit. Figure 7 indicates that radiolabeled B subunit could be completely inhibited from binding to anti-B-subunit sera by toxin and B subunit. Concentrations of up to $1.0 \times 10^{-2}$, $3.0 \times 10^{-2}$, and $1.55 \times 10^{-3}$ μmol of A subunit, α chain, and γ chain, respectively, per ml were noninhibitory. Linear regression analysis of the percentages of radioactivity inhibited by increasing concentrations of unlabeled toxin indicated line slopes of $-0.960 \ (r = 0.91), -1.014 \ (r = 0.84)$, and $-1.128 \ (r = 0.92)$, with 50% inhibition occurring at concentrations of $2.3 \times 10^{-7}$, $3.1 \times 10^{-7}$, and $3.6 \times 10^{-7}$ μmol/ml, respectively. Similar line slopes of $-1.019 \ (r = 0.92), -0.967 \ (r = 0.91)$, and $-1.034 \ (r = 0.85)$ occurred with unlabeled B subunit, in which 50% inhibitions occurred with concentrations of $1.05 \times 10^{-7}$, $1.25 \times 10^{-7}$, and $1.9 \times 10^{-7}$ μmol of B subunit per ml, respectively.

**Mouse inoculation study with column fractions from A and B subunit purification.** Selected fractions from the ascending and descending protein peaks of the smaller Bio-Gel P-60 column used to fractionate 10 mg of cholera toxin were used for mouse inoculation (Fig. 8). This separation allowed for purification of the A subunit (fraction volumes 14 to 22) and B subunit (fraction volumes 24 to 36). Mouse groups were inoculated with proportionate amounts of antigen from the selected fractions based upon Lowry protein measurements as indicated in Table 1. Titration of all mouse sera with radiolabeled A subunit (see Fig. 9) indicated that pre-inoculation and 3-week-post-inoculation bleedings did not bind any radioactivity; however, 6-week bleedings showed geometric mean titers of

![Figure 7](image1.png)  
**Fig. 7. Competitive inhibition studies with increasing concentrations of unlabeled toxin (—) and B subunit (--) competing with radiolabeled B subunit for anti-B-subunit binding sites in three different antiserum preparations. The A-subunit, α-chain, and γ-chain preparations did not inhibit radiolabel binding.**

![Figure 8](image2.png)  
**Fig. 8. Elution of 10 mg of cholera toxin on a small Bio-Gel P-60 column (1 by 80 cm) monitored by absorbance at 280 nm (A280). Fractions chosen for immunization of mice and for testing biological activity on S-49 mouse lymphosarcoma cells are the larger circles.**

**Table 1. Mouse immunization study**

<table>
<thead>
<tr>
<th>Elution fraction no.</th>
<th>Protein determination (μg/ml)</th>
<th>Injection (μg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>19.6</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>137.8</td>
<td>3.2</td>
</tr>
<tr>
<td>18</td>
<td>346.0</td>
<td>8.1</td>
</tr>
<tr>
<td>20</td>
<td>184.0</td>
<td>4.3</td>
</tr>
<tr>
<td>21</td>
<td>114.7</td>
<td>2.7</td>
</tr>
<tr>
<td>23</td>
<td>22.1</td>
<td>0.5</td>
</tr>
<tr>
<td>25</td>
<td>114.7</td>
<td>2.7</td>
</tr>
<tr>
<td>28</td>
<td>623.6</td>
<td>14.7</td>
</tr>
<tr>
<td>31</td>
<td>1,063.1</td>
<td>25.0</td>
</tr>
<tr>
<td>33</td>
<td>484.8</td>
<td>11.4</td>
</tr>
<tr>
<td>35</td>
<td>45.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* The elution volumes selected from the Bio-Gel P-60 column used to fractionate 10 mg of cholera toxin were measured for protein content by the Lowry method. Each eluate was used to immunize a group of seven mice intraperitoneally with a proportionate amount of protein in respect to the protein concentrations of their respective eluate.

* Each mouse received a 0.5-ml intraperitoneal injection with the respective amount of antigen.
2.6 and 6.4 and 4.0 AU/ml which were limited to mice inoculated with elution volumes 16, 18, and 20, respectively. Titrations of all these sera with radiolabeled B subunit are also shown in Fig. 9. Although pre-immunization sera did not bind any radiolabeled B subunit, an increase in serum anti-B-subunit titer was seen between 3- and 6-week-post-inoculation bleedings in all mouse groups. The highest geometric mean antitoxin titer of 29.8 AU/ml was found with the mouse group inoculated with elution volume 28. The height of antibody responses in each mouse group at 6 weeks closely followed the respective proportionate amount of protein antigen administered (see Table 1). However, an important observation, illustrated in Fig. 9, was that radiolabeled B subunit not only detected antibody responses in mouse groups immunized with B-subunit eluates (elution volumes 25 to 35), but also detected antibody responses in mouse groups immunized with A-subunit eluates (elution volumes 14 to 21).

Serological responses of rabbits immunized with A and B subunits. Pre-immunization, 4-week, and 6-week serum samples from rabbits immunized with $8.92 \times 10^{-4}$ μmol of either A or B subunit (elution volume 18 or 31, respectively) in which a booster immunization, consisting of the same respective amount of antigen per rabbit, was given at 4 weeks, were each titrated with radiolabeled A and B subunits. No antibody titers were detectable in any pre-immunization sera with either radiolabeled subunit. However, rabbits immunized with A subunit, when titrated with radiolabeled A subunit, produced a geometric mean antibody titer of 2.1 AU/ml at 4 weeks. A subsequent booster immunization at 4 weeks produced a much higher anamnestic-type antibody response with a geometric mean titer of 2,528 AU/ml (Fig. 10). Rabbits immunized with B subunit did not produce detectable antibody responses when titrated with radiolabeled A subunit. In comparison, when each of the antisera was titrated with radiolabeled B subunit, geometric mean antibody titers of 1.8 for the A rabbits and 10.8 AU/ml for the B subunit were noted at 4 weeks. After a subsequent booster immunization at 4 weeks, these titers increased to 64.2 and 112.4 AU/ml at 6 weeks, respectively.

Rabbit intestinal loop challenge. Rabbit groups immunized with equimolar concentrations of either A or B subunit eluted from the Bio-Gel P-60 column were challenged with Vibrio cholerae Inaba V-86 organisms by the intestinal loop method (Fig. 11). The mean loop responses for rabbits immunized previously with the A subunit were slightly lower than those of rabbits receiving an equal molar concentration of B subunit as immunogen, indicating less fluid accumulation in the loops or better protection. However, these responses were not significantly different when compared by the analysis of variance (F test).

DISCUSSION
In a previous publication, we reported an RIA
with each of the radiolabeled proteins. Interestingly, the antisera produced against A subunit precipitated radiolabeled A subunit, α chain, and B subunit; however, the antisera to B subunit would only precipitate the radiolabeled B subunit. These observations paralleled what we had observed in the Ouchterlony tests. We had expected that this methodology would have detected some precipitation of the radiolabeled A subunit with B-subunit antisera. Even if low dilutions of B subunit antisera (1:10) were tested, no radioactivity was precipitated with either the radiolabeled A subunit or radiolabeled α chain.

More extensive studies of these antisera were performed to evaluate and define possible antigenic cross-reactivity between the A and B subunits. A competitive RIA system was developed for each antiserum in which radiolabeled components competed with unlabeled components for antibody binding sites. When radiolabeled A subunit was used to compete with each of the other unlabeled components for antibody binding sites to the A subunit, the B subunit was capable of inhibiting the binding by 50% of the radiolabeled A subunit at concentrations (in micromoles per milliliter) 1/100 of the control A subunit. At higher concentrations the B subunit was able to completely inhibit radiolabeled A-

for the antigenic determinants of cholera toxin and suggested that the A and B subunits composing cholera toxin shared some antigenic determinants (10). This hypothesis was based on the observation that unlabeled A subunit competed with radiolabeled B subunit for antitoxin binding sites. Similarly, unlabeled B subunit competed with radiolabeled A subunit for antitoxin binding sites. Experiments presented here further suggest that the A subunit and, more specifically, the α chain possess antigenic determinants that are common to the B subunit. Antisera produced against each of the purified toxin components were initially tested in Ouchterlony double-immunodiffusion plates. Results of this test indicated that antiserum prepared against purified A subunit not only precipitated the α chain, but also precipitated the B subunit, and antisera prepared against B subunit only precipitated B subunit.

Based on the assumptions that the amount of contaminating B subunit is negligible and that the sensitivity of the Ouchterlony test might not detect this contamination, RIA methods were developed for the measurement of A subunit, B subunit, and α chain in our purified preparations. Each of the above antisera were first titrated
subunit binding. The slopes of the B-subunit line and toxin line were similar, suggesting similar antigenic binding sites competing with radiolabeled A subunit. It was surprising to observe that the A subunit was capable of inhibiting radiolabel binding at concentrations from $10^{-3}$ to $10^{-7}$ μmol/ml in the test. This observation was thought to be due to the fact that at higher concentrations the A subunit aggregates, thereby burying many of the antigenic sites. As the A subunit is diluted, the aggregated subunits disperse, exposing more antigenic sites to bind antibody. This aggregation has been observed when testing different dilutions of A subunit by Ouchterlony (data not shown). The higher-concentration samples precipitate as a broad diffuse band composed of several lines, whereas diluted samples precipitate as much sharper lines.

When radiolabeled α chain was used to compete with each of the other unlabeled components for antibody binding to the α chain, complete inhibition of radiolabel binding was found with increasing concentrations of α chain. Unlabeled B subunit was capable of inhibiting binding of about 27% of the radioactivity. The α chain antiserum neither precipitated the B subunit in the Ouchterlony experiments nor bound radiolabeled B subunit, thus exemplifying the sensitivity of this competitive assay. The inability of unlabeled toxin to inhibit more than 15% of the radiolabel indicates that the α-chain antigenic sites are buried within the toxin molecule and are unavailable for antibody binding. This is substantiated by the fact that anti-α-chain sera do not neutralize cholera toxin when analyzed by the permeability factor neutralization test (25).

Contrary to what would have been expected in the competitive RIA with B-subunit antiserum and radiolabeled B subunit, only the toxin and B subunit inhibited the radiolabel binding. The B subunit antiserum was monospecific for only the B subunit. The results of these conflicting competitive inhibition assays suggested the possibility that the α chain and β chain may contain regions of chemical similarity. Perhaps the antibodies raised against the A subunit and α chain were more directed to amino acid sequence, whereas antibodies to the B subunit were against structural conformation. This might explain why the antiserum to A subunit and α chain precipitate B subunit, but antibodies against the B subunit precipitate only the B subunit. One other possibility was that the B subunit in the competitive assays was binding to the radiolabels such that the radiolabels were unable to bind antibody, thereby producing erroneous competitive results. This was ruled out as a possibility by performing an assay with radiolabeled α chain or A subunit with unlabeled B subunit followed by the addition of B-subunit antiserum. None of the radioactivity was precipitated (data not shown). The possibility that the A-subunit and α-chain preparations were contaminated with B subunit was still a concern since perhaps the amount of contamination was so small that we were unable to detect it by this assay. To continue characterization of the immunological and functional properties of the molecule, additional antigenic and biological experiments were performed.

Mice immunized with different eluted fractions obtained from gel filtration of the A and B subunits elicited serum antibody titers which were titrated by their respective radiolabel. The A-subunit fraction antiserum, when titrated with radiolabeled A subunit, showed a peak antitoxin titer corresponding to the peak protein fraction of the A subunit (fraction number 18). The peak antitoxin titer response of the B-subunit fractions was with fraction number 28 rather than the protein peak fraction number 31. Either this may represent the animal's peak immunological response to an antigen at concentrations greater than 14.7 μg, or possibly this may be simply due to dilution error in the experiment.

Each of the A subunit mice antiserum was then titrated with radiolabeled B subunit, and again we observed cross-reactivity. Interestingly, though there was a shift of the peak B-antitoxin titer response from the peak A-subunit protein fraction number 18 to fraction number 21 which was on the descending portion of the elution curve. These findings were suggestive of a possible β chain complex, possibly a dimer, with a molecular weight of about 22,000 which might have eluted at this particular fraction; however, when this fraction was tested in a competitive RIA with B-subunit antiserum and radiolabeled B subunit, no competition was observed. Thus, the data would argue against the presence of β-chain dimers. Interestingly, all of the fractions under the A-subunit curve were able to elicit this cross-reactive antibody. If contamination of the A subunit with B subunit was a viable explanation of this observed cross-reactivity, one would expect that the competitive RIA with B subunit would demonstrate its presence or the peak antibody response would be on ascending portion of the A-subunit protein elution peak and not on the descending portion since the molecular weight of the combined polypeptide chains would be greater than the molecular weight of the A subunit.

The antiseras prepared against the B-subunit fraction failed to cross-react with the radiola-
beled A subunit. Even when the sera were tested by competitive RIA, no cross-reactivity with the A subunit was demonstrated.

Biological activity of the different elution fractions as assayed by the mouse lymphosarcoma cell system indicated that the A subunit by itself does have biological activity. Biological activity of the A subunit in different systems has been observed by others (3, 23, 26). The ability of the A subunit to elicit biological activity was about 1/10,000 on a molar level to that of intact toxin. This makes the A subunit a much less effective agent of biological activity in a whole cell system than intact toxin. The B subunit, on the other hand, did not have biological activity which was demonstrable in this system.

Rabbits immunized with equimolar concentrations of A and B subunits responded by producing serum antibodies with similar specificities to that of the mice. We were able to detect a rising titer to both the A and B subunits in rabbits immunized with the A subunit. Furthermore, at 6 weeks after immunization the A-subunit-immunized rabbits possessed an anti-B-subunit response comparable to those rabbits immunized with B subunit at 6 weeks after immunization. Antibodies specific for the A subunit were not detected in rabbits immunized with the B subunit by using radiolabeled A subunit in the RIA. It was interesting that rabbits immunized with the A subunit, in comparison with rabbits immunized with an equimolar concentration of B subunit, were similarly protected during challenge, even the titrations by RIA indicated a titer which was comparable to that obtained in rabbits immunized with the B subunit. This might indicate that antibody directed against the B subunit determinants may be the antibodies responsible for toxin neutralization in vivo. This is a plausible explanation because recently we have shown that antisera specific for the B subunit effectively neutralize the skin permeability of the toxin. In contrast, antisera to the α chain of the A subunit and to the A subunit (absorbed with B subunit) react with the A subunit but do not neutralize skin permeability activity of the toxin (26).

In this report we present three additional competitive RIAs for studying the antigenic characteristics of cholera toxin. We have provided further evidence for the possible antigenic relatedness between the A and B subunits, specifically between the α chain and the B subunit. We have been unable to detect B-subunit contamination in the A-subunit or α-chain preparations. In fact, all fractions of the A subunit obtained by gel filtration of cholera toxin in the presence of guanidine elicited antibodies which were able to bind radiolabeled B subunit in RIA. In addition we have also reported that rabbits immunized with A subunit were equally protected when challenged with live vibrios as those immunized with equimolar amounts of B subunit.

In biological testing, the A subunit but not B subunit, α chain, or γ chain, was found to have biological activity when tested in the mouse lymphosarcoma cell system. Lastly, the γ chain was nonantigenic and nonreactive in all immunological testing.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant AI 06818 and research contract N01 AI 02101, both from the National Institute of Allergy and Infectious Diseases. Additional support was provided by the James W. McLaughlin Fellowship Fund and the Burkitt Foundation.

LITERATURE CITED

sequence of the three chains of cholera enterotoxin.


