INFECTION AND IMMUNITY, Nov. 1983, p. 683–691
0019-9567/83/110683-09$02.00/0

Vol. 42, No. 2

Immunological Relationships Between Cholera Toxin and *Escherichia coli* Heat-Labile Enterotoxin

PETER H. GILLIGAN, JOHN C. BROWN, AND DONALD C. ROBERTSON*
Department of Microbiology, The University of Kansas, Lawrence, Kansas 66045

Received 17 January 1983/Accepted 23 August 1983

The antigenic relationships between heat-labile enterotoxin (LT) produced by a human strain of enterotoxigenic *Escherichia coli* (strain $286C_2$) and cholera toxin (CT) were examined by using antisera raised against LT and CT and specific antisera prepared against each subunit of both enterotoxins. Double immunodiffusion analysis revealed reactions of partial identity between the A subunits of LT and CT, as well as between the B subunits. Rabbit antisera raised against LT subunit A reacted with only subunit A, whereas rabbits immunized with LT subunit B produced antibodies which reacted with only subunit B. A high degree of CT neutralization was observed with antisera raised against LT. Data from neutralization assays with specific antisera to each enterotoxin showed that LT was more effectively neutralized by homologous anti-LT than CT (3.7-fold); however, anti-CT was only slightly more effective in neutralization of homologous CT compared with LT (1.9-fold). In contrast, antisera raised against the B subunit of CT (choleragenoid) exhibited significantly higher neutralization activity against CT than LT (5.8-fold); however, the amount of CT neutralized by anticholeragenoid was less (4.1-fold) than anti-CT. These results suggested that anti-CT serum contained neutralizing antibodies reactive with a shared determinant formed by interaction of the A and B subunits, whereas anti-LT and anti-choleragenoid sera did not. Sensitive solid-phase radioimmunoassays were developed to examine the affinity and degree of specificity involved in homologous and heterologous antigen-antibody interactions between LT, CT, their subunits, and specific antibodies. Only unlabeled LT competed with radiolabeled LT in polystyrene tubes coated with anti-LT, and only unlabeled CT competed with radiolabeled CT in tubes coated with anti-CT. However, when radiolabeled CT was incubated in tubes coated with anti-LT, competitive inhibition responses were observed with both unlabeled toxins. When radiolabeled LT was incubated with tubes coated with anti-CT, competitive inhibition responses were observed with both unlabeled toxins. Similar competitive inhibition responses were observed with the A subunits of LT and CT and with the B subunits using antisera specific for the subunits of each enterotoxin. Double immunodiffusion analysis and radioimmunoassay data supported the presence of unique and shared immunodeterminants in each subunit.

The pathogenesis of diarrheal disease due to *Vibrio cholerae* and enterotoxigenic strains of *Escherichia coli* (ETEC) is similar in that both bacilli must adhere to epithelial cells of the small intestine before colonization (11). Subsequent production of one or two kinds of enterotoxin ultimately causes a profuse watery diarrhea (33). Although cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) share several properties at the molecular level (4, 5, 9, 12, 21, 28, 35; D. C. Robertson, S. K. Kunkel, and P. H. Gilligan, Abstr. 17th Joint Conference on Cholera, 1979, p. 54), resistance against one disease does not necessarily confer long-lasting resistance to the other (1). Thus, it is imperative that immunological relationships between LT and CT be characterized with as many diverse approaches as possible.

The antigenic relatedness of CT and LT has been documented by numerous investigators using antisera to purified CT (2, 3, 7, 15–19, 22, 34). Precipitin and neutralization tests have been used to demonstrate that LT has antigenic determinants in common with the A and B subunits of CT (CT-A and CT-B) (2, 3). Holmgren and Svennerholm employed antisera raised against purified CT and crude preparations of LT in an enzyme-linked immunosorbent assay to show that CT and its purified subunits cross-react with LT (18). Also, their data suggested that each

enterotoxin possessed unique determinants, since individual antisera exhibited higher antibody titers against homologous toxin preparations. Other investigators have also observed that anti-CT neutralized both CT and LT; however, antisera raised against crude preparations of LT exhibited low neutralization activity against CT (15, 16, 32).

Methods for preparation of homogeneous LT have recently been described (4, 5, 23); thus, it is now possible to raise high-titered antisera which can be used to better define and characterize immunological relationships between LT and CT. Honda et al. (19) have described antisera against LT and CT which discriminate between each enterotoxin and antisera which react with both enterotoxins. Development of effective immunization regimens against both enterotoxins depends on a clear understanding of the immunological relationships between the subunits of each enterotoxin. This report describes the examination of antisera raised against LT and CT, as well as specific antisera prepared against each subunit of both enterotoxins.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. ETEC strain $286C_2$, an LT-only strain isolated in Mexico (26), was supplied by R. B. Sack, The Johns Hopkins School of Medicine, Baltimore, Md. Stock cultures were maintained on Trypticase soy agar plates or as lyophilized ampoules. The minimal salts medium containing 10 mM Tricine, 0.5% glucose, and three amino acids (methionine, lysine, and glutamic acid) and growth conditions for production of LT were as described previously (13).

Purification of *E. coli* LT. The LT produced by ETEC strain $286C_2$ was purified as described previously by Kunkel and Robertson (23). Briefly, the purification involved concentration of pH extracts by ultrafiltration, hydrophobic interaction chromatography on norleucine-Sepharose 4B, hydroxylapatite chromatography, and Bio-Gel P-150 gel filtration. The LT preparation was assessed as being 60 to 70% pure after hydroxylapatite chromatography and greater than 95% pure after the final gel filtration step.

Preparation of A and B subunits of *E. coli* LT and CT. CT (Schwarz/Mann, Orangeburg, N.Y.) and LT (5 mg/ml) were each dissolved in 6 M urea–0.1 M glycine (pH 3.5) and dialyzed briefly against 100 volumes of 6 M urea-0.1 M glycine (pH 3.5). The subunits were fractionated on a Bio-Gel P-60 column or a Sephadex G-75 column (1.5 by 85 cm) as described by Finkelstein et al. (10). Fractions containing 280-nm absorbance were pooled and renatured by dialysis at 4°C against decreasing concentrations of urea–0.12 M Tris-hydrochloride (pH 7.5). The samples were concentrated by using an Amicon stirred cell with a PM-10 Diaflo membrane and frozen at −20°C until use.

Preparation of antisera. New Zealand white rabbits, 3 to 5 months old, were injected intradermally at multiple sites on a shaved portion of the back and in one hind footpad with 100 μg of LT holotoxin or subunits (175 μg of subunit A or 125 μg of subunit B) dissolved in Freund complete adjuvant. After 3 weeks, the animals were bled weekly from the central ear artery until the antibody response subsided at 8 to 10 weeks. Booster doses of subunits suspended in Freund incomplete adjuvant were administered at 3-week intervals. Appropriate bleedings were pooled and stored at −20°C until use.

Neutralization assays. The neutralizing capacity of antisera against LT or CT was measured with the Y-1 adrenal cell assay (8). Twofold serial dilutions of antisera were prepared with phosphate-buffered saline (PBS)-0.1% bovine serum albumin and incubated at 37°C for 60 min with an equal volume of solution containing 10 ng of either $286C_2$ LT or CT in a final volume of 150 μl. Portions equivalent to 0.5 ng of CT or 2.0 ng of LT were added to wells seeded 2 days previously with 2×10^5 Y-1 cells in 1 ml of medium. Antitoxin titration curves were derived and used to calculate the 50% neutralization point for each antiserum reacting with homologous and heterologous enterotoxins. The 50% neutralization titer was used to determine the amount of enterotoxin (micrograms) neutralized by 1 ml of antiserum. Steroids were determined fluorometrically 18 to 21 h after toxin addition as described previously (13).

Radiolabeling procedure. Iodination of proteins was performed in glass tubes (10 by 75 mm) by the chloramine-T procedure (14). The reaction mixture contained 40 μl of protein (12.5 to 25 μg) diluted in 0.5 M sodium phosphate buffer (pH 7.5), 5 μl of chloramine-T (2.5 mg/ml) dissolved in 0.5 M sodium phosphate buffer (pH 7.5), and 5 μl (500 mCi) of $Na^{125}I$ (Amersham Corp.; carrier free). The reaction was started by the addition of $Na^{125}I$. After incubation for 2 min at room temperature, the reaction was stopped by the addition of 10 μl of sodium metabisulfite (2.5 mg/ml) dissolved in 0.5 M sodium phosphate (pH 7.5). After 1 min of incubation, 100 μg of potassium iodide (10 μl) was added, and the reaction mixture was applied to a Bio-Gel P-2 column (0.5 by 10 cm) equilibrated with PBS–0.1% bovine serum albumin. Fractions of 0.5 ml were collected in glass tubes (10 by 75 mm), and the tubes were scanned for relative amounts of radioactivity by using a Packard 5110 gamma scintillation counter. The tubes which contained radioactive protein were pooled and dialyzed extensively against PBS with several changes of dialysate. Radioactive proteins were stored at 4°C for no longer than 1 month. The specific activity (microcuries per microgram) of typical radiolabeled proteins was as follows: LT, 8.41; CT, 3.84; LT-A, 5.71; LT-B, 6.18; CT-A, 7.14; and CT-B, 3.48.

Radioimmunoassays. Polystyrene tubes (10 by 75 mm; Falcon Plastics, Oxnard, Calif.) were coated overnight at 4°C with 0.5 ml of diluted antiserum or the purified immunoglobulin G fraction of rabbit anti-LT immune serum. The antiserum or immunoglobulin G fractions were diluted such that 20 to 25% of the radiolabeled antigen was bound under the conditions noted above. The tubes were washed three times with PBS, followed by incubation with 1 ml of 0.3% bovine serum albumin for 30 min at 37°C to coat additional binding sites. After three washes with PBS, each tube contained 1 ng of radiolabeled holotoxin or purified subunits, unlabeled homologous or heterologous antigen (2 to 100 ng), and PBS to 0.5 ml. Tubes were incubated overnight at 4°C, washed three times with

PBS, and assayed for radioactivity. The immunoglobulin G fraction of rabbit anti-LT serum was prepared by ammonium sulfate precipitation followed by DEAE-Sephadex A-50 chromatography.

Immunodiffusion analysis. Immunodiffusion experiments were performed in 1% Noble agar (Difco Laboratories, Detroit, Mich.) containing 50 mM Tris-hydrochloride, 150 mM NaCl, and 0.1% sodium azide (pH 7.4) as described by Ouchterlony (29). Each well contained either 30 μl of antigen (5 to 10 μg of protein) or antiserum. Plates were incubated at room temperature for up to 4 days, with daily observation.

Reagents. CT was purchased from Schwarz/Mann. Equine anticholeragenoid and equine anti-CT (Swiss Serum Institute reference serum; 4470 antitoxin units/ml) were kindly supplied by C. E. Miller and by R. E. Horton, National Institutes of Health, Bethesda, Md. Goat anti-CT and affinity-purified antisera to the subunits of CT (rabbit anti-subunit A and goat anti-subunit B) were generously provided by N. Ohtomo, Chemo-Therapeutic Research Institute, Kumamoto, Japan.

RESULTS

Purification of A and B subunits of *E. coli* LT and CT. Each enterotoxin incubated in 6 M urea–0.1 M glycine (pH 3.5) at 37°C for 1 h was fractionated by gel filtration. As shown in Fig. 1, CT and LT yielded almost identical profiles. Peak fractions detected by absorbance at 280 nm were pooled and dialyzed against decreasing concentrations of urea with increasing pH. The final dialysis was against TEAN buffer (4) (pH 7.5), which increased recoveries of the A subunit. The A subunit exhibited biological activity against Y-1 adrenal tumor cells at 100-fold higher concentrations than either holotoxin or stimulated adenylate cyclase in pigeon erythrocyte lysate assays at concentrations similar to both enterotoxins. The B subunits were inactive in the pigeon erythrocyte lysate assay at 1,000-fold higher concentrations relative to the A subunit but exhibited limited activity against Y-1 adrenal tumor cells at 100-fold higher levels relative to the amounts of holotoxins which exhibited a maximum response. All subunit preparations were homogeneous by sodium dodecyl sulfate gel electrophoresis (data not shown). Despite these results, there may have been trace amounts of highly immunogenic subunit B in subunit A preparations, and pooled column fractions of subunit B may have contained amounts of subunit A which were not detected by conventional protein stains.

Double immunodiffusion analysis. Serum samples from rabbits immunized with LT and its subunits and goat anti-CT sera were tested for reactivity with LT, CT, and their subunits. Typical double immunodiffusion patterns are shown in Fig. 2. Reaction of anti-CT with the subunits of each enterotoxin (Fig. 2a) showed lines of partial identity between LT-A and CT-A, as well as LT-B and CT-B. Reactions of nonidentity were formed between CT-A and LT-B (Fig. 2a) and between the A and B subunits of each enterotoxin (data not shown). Antisera raised against some purified LT-A preparations reacted with a single precipitin line against LT-A and LT-B, with a reaction of partial identity against LT-B (data not shown); however, antisera raised against more purified LT-A preparations did not react with subunit B (R. Ching and D. C. Robertson, unpublished observations). Anti-LT-B sera did not precipitate either LT-A or CT-A, but formed a single precipitin line with LT-B that showed a line of partial identity against CT-B (Fig. 2b). Anti-LT sera did not yield a precipitation reaction against either LT-A or CT-A (Fig. 2c); however, a reaction of identity was formed between LT and LT-B, and partial iden-

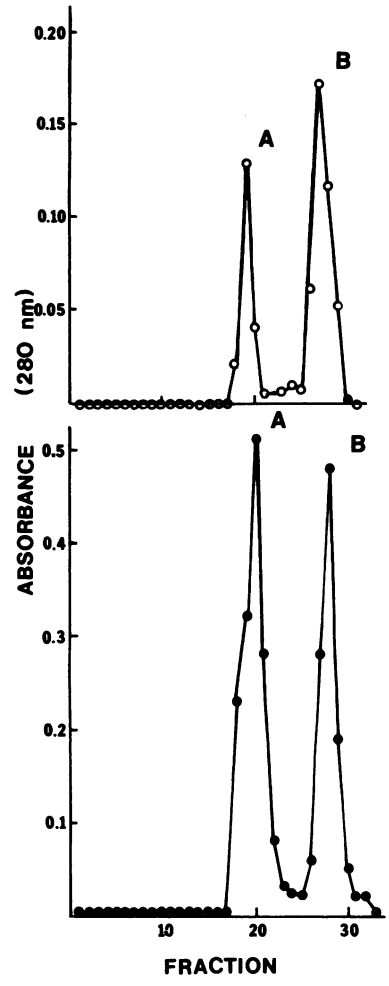

FIG. 1. Gel filtration of $286C_2$ LT and CT on a Sephadex G-75 (1.5 by 85 cm) column equilibrated with 6 M urea–0.1 M glycine (pH 3.5). Two-milliliter fractions were collected at a flow rate of 4.0 ml/h. Symbols: ○, CT; ●, $286C_2$ LT.

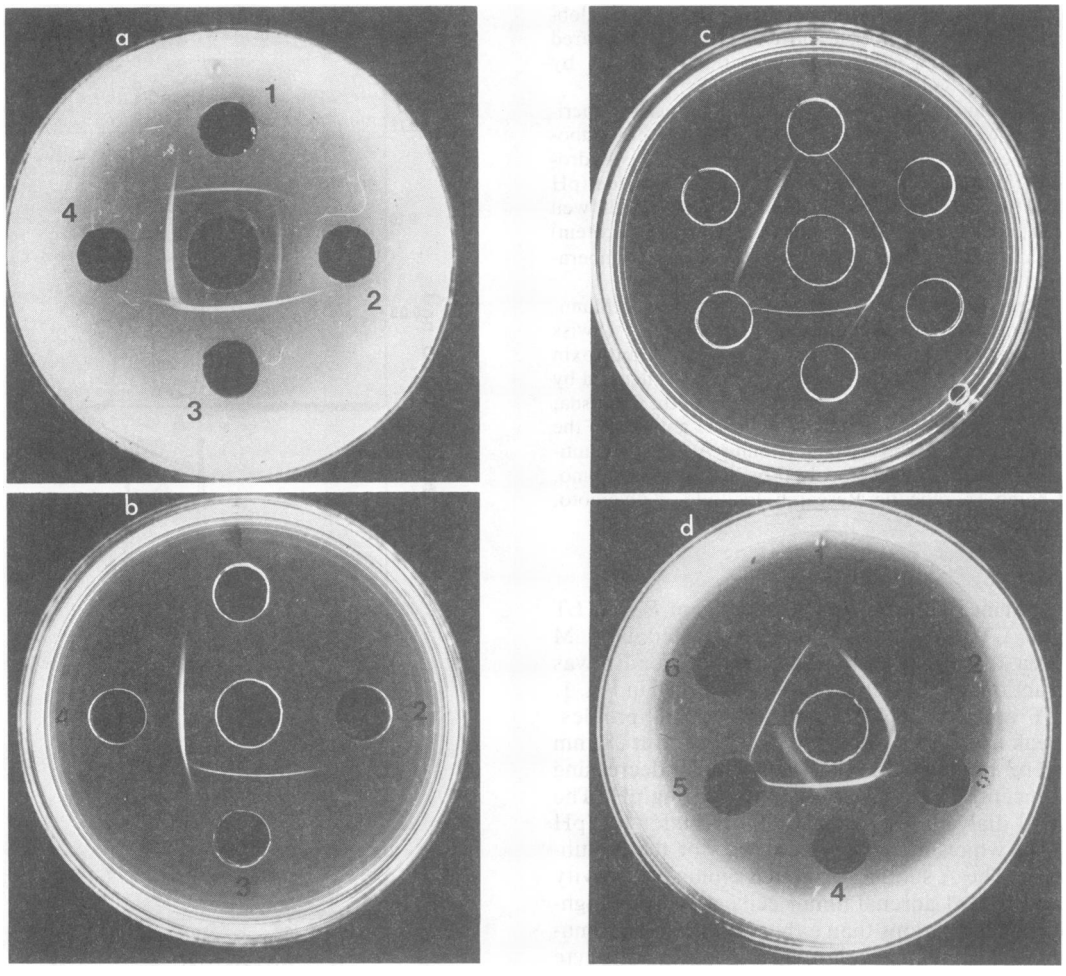

FIG. 2. Double immunodiffusion reactions of *E. coli* LT, CT, and each of their subunits with specific antisera. (a) Center, anti-CT (30 μl); 1, CT-A; 2, LT-A; 3, CT-B; 4, LT-B. (b) Center, anti-LT-B (30 μl); 1, LT-A; 2, CT-A; 3, CT-B; 4, LT-B. (c) Center, anti-286C_2 LT (30 μl); 1, LT-A; 2, LT-B; 3 and 6, LT; 4, CT-B; 5, CT-A. (d) Center, goat anticholeratoxin (30 μl); 1, LT-A; 2, LT-B; 3 and 6, LT; 4, CT-B; 5, CT-A. Outside wells contained 10 μg of antigen.

tity was detected between LT-B and CT-B, as was observed with anti-LT-B sera (Fig. 2b).

Immunodiffusion patterns with anti-CT consisted of single precipitin lines formed by each enterotoxin subunit and showed reactions of nonidentity between LT-A and LT-B, as well as between CT-A and CT-B (Fig. 2d). The double precipitin line formed with LT holotoxin is likely due to dissociation of subunit A, since the second faint precipitin line formed a line of identity with LT-A. A reaction of partial identity was observed between LT-B and CT-B, as shown by the precipitin band formed by CT-B which spurred over the line formed with LT-B. Double immunodiffusion analysis with affinity-purified antisera to CT-B and CT-A yielded identical results to those obtained when antisera raised against A and B subunits purified by acid-urea gel filtration were used (data not shown).

Neutralization assays. The results of neutralization assays with antisera raised against purified LT and choleragenoid showed that the homologous toxin was more effectively neutralized than either heterologous enterotoxin (Table 1). However, equine anti-CT neutralized homologous CT only slightly more effectively than heterologous LT. In agreement with results reported previously (7), the 50% neutralization point for 10 ng of CT was determined to be 0.13 antitoxin units of the standard Swiss Serum Institute preparation.

Antigenic relationships between the subunits of LT and CT. Information obtained from double immunodiffusion analysis was useful for charac-

terization of antibody specificity and antigenic cross-reactivity but was not particularly informative concerning affinities and degree of specific antigen-antibody interactions. Consequently, a sensitive radioimmunoassay (RIA) was developed to examine such relationships. Polystyrene tubes were coated with rabbit anti-LT serum or equine anti-CT serum. Radiolabeled homologous antigen binding was subsequently competitively inhibited through the addition of either unlabeled LT or CT. The results shown in Fig. 3A and B indicated that only unlabeled LT competed with radiolabeled LT in tubes coated with anti-LT and that only unlabeled CT competed with radiolabeled CT in plastic tubes coated with anti-CT. In contrast, when radiolabeled CT was incubated in tubes coated with anti-LT, competitive inhibition responses were observed with both unlabeled toxins. Similar results were observed when radiolabeled LT was incubated with tubes coated with anti-CT. These data were interpreted to show that each enterotoxin contains at least one individual and at least one shared determinant. The unique determinant of each enterotoxin appears to be either immunodominant or capable of eliciting an antibody population which reacts with a higher affinity toward this determinant(s).

To examine relationships between the subunits of LT and CT associated with specificity and relative binding affinity, sensitive RIAs were performed with affinity-purified antisera to the A and B subunits of CT and antisera to the purified subunits of $286C_2$ LT. The data obtained were similar to those observed with holotoxins; i.e., when affinity-purified anti-CT-B was used to coat polystyrene tubes and incubated with radiolabeled CT-B, antigenic competition was observed with unlabeled CT-B and not with unlabeled LT-B (Fig. 4). Likewise, when radiolabeled LT-B was incubated in plastic tubes coated with anti-CT-B, both unlabeled LT-B and CT-B were competitive. The use of heterologous subunits as competitors in homologous antigen-binding studies again suggested that the antibodies reactive with individual determinants were of higher affinity or were present in larger amounts. Analysis of affinity-purified anti-CT-A (Fig. 5) indicated that, like the B subunits, the A subunits of each enterotoxin also contained both unique and shared determinants. When antisera raised against purified subunits of LT were substituted for the affinity-purified antisera to each subunit of CT, identical results were observed (data not shown).

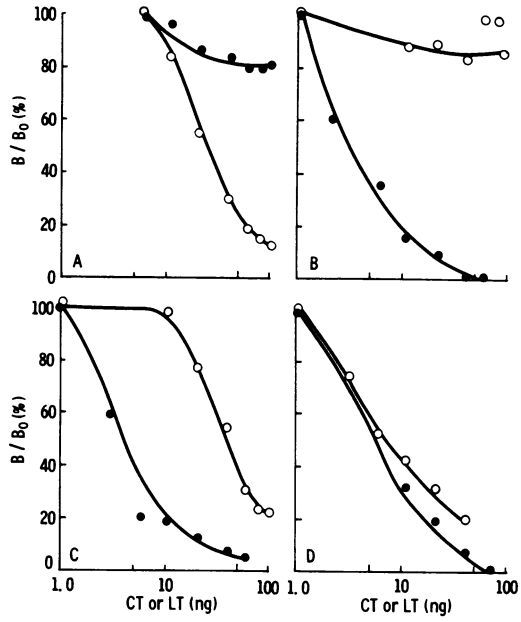

FIG. 3. Competitive inhibition studies of homologous and heterologous antigen-antibody interactions. Symbols: ○, $286C_2$ LT; ●, CT. (A) Anti-$286C_2$ LT and ^{125}I-labeled $286C_2$ LT; (B) anti-CT and ^{125}I-labeled CT; (C) anti-$286C_2$ LT and ^{125}I-labeled CT; (D) anti-CT and ^{125}I-labeled $286C_2$ LT. B/B_0, Amount of radioligand (net cpm) bound to specific antibody in the presence of unlabeled competitive inhibitor (B) divided by the amount of radioligand (net cpm) bound in the absence of unlabeled competitive inhibitor (B_0) multiplied by 100.

TABLE 1. Neutralization of purified $286C_2$ LT and CT by homologous and heterologous antisera

Antiserum	Toxin	Toxin neutralized (μg)[a]
Anti-LT	LT	239.0 ± 14
Anti-LT	CT	64.7 ± 23
Anti-CT	CT	215.0 ± 44
Anti-CT	LT	110.6 ± 22
Anticholeragenoid	CT	52.4 ± 10
Anticholeragenoid	LT	9.0 ± 2

[a] Micrograms of toxin neutralized by 1 ml of antiserum. Data are expressed as the mean ± standard error.

DISCUSSION

The availability of purified A and B subunits of both CT and LT, as well as antisera both to holotoxins and each enterotoxin subunit, permitted extensive characterization and comparison of antigenic determinants of both toxins by double immunodiffusion analysis, neutralization assays, and sensitive solid-phase RIAs. Antisera raised against LT holotoxin did not contain detectable anti-subunit A antibodies. In contrast, antisera raised against intact CT contained adequate levels of anti-subunit A antibodies to permit double immunodiffusion analysis. These results suggested that CT-A is more immunogenic relative to LT-A. Reactions of partial identity

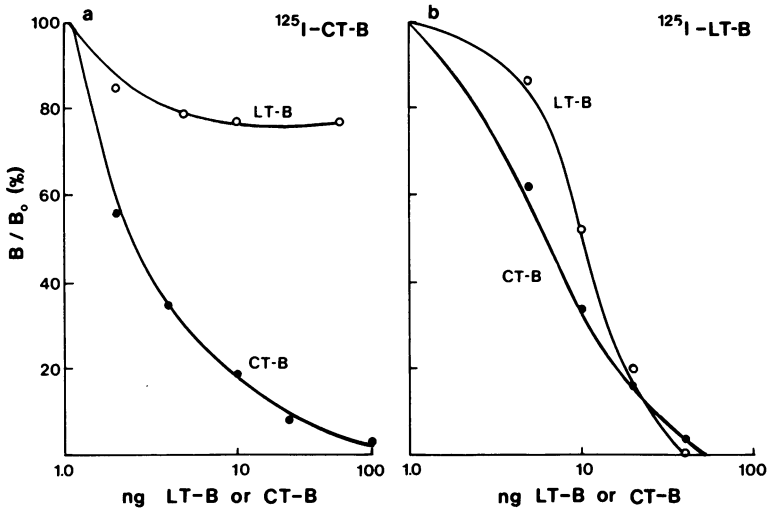

FIG. 4. Competitive inhibition studies of antigen-antibody interactions between LT-B and CT-B with affinity-purified anti-CT-B sera. Symbols: ○, *E. coli* LT-B; ●, CT-B. (a) ^{125}I-labeled CT-B; (b) ^{125}I-labeled LT-B. See the legend to Fig. 3 for definition of B/B_0.

between LT-A and CT-A were observed with goat anti-CT; however, the precipitin line formed with LT-A was very weak. Reactions of partial identity were noted between the B subunits with antisera to both holotoxins, affinity-purified antisera to CT-B, and antisera raised against LT-B purified by acid-urea gel filtration. Our initial data suggested a cross-reaction between LT-A and LT-B, since antisera to LT-A reacted with LT-B, but antisera to LT-B did not react with LT-A. These results were similar to previous reports on the antigenic cross-reactivity of the subunits of CT (10, 25, 36). However, antisera raised against more purified LT-A preparations reacted only with subunit A in Ouchterlony analysis and did not react with subunit B in the GM_1 enzyme-linked immunosorbent assay (R. Ching and D. C. Robertson, unpublished observations). Their data indicate that there is no cross-reactivity between LT-A and LT-B and support studies in which hybridoma cell lines were isolated which secrete monoclonal antibodies reactive with CT-A but which do not react with CT-B (31). It is very difficult to rule out contamination by the strongly immunogenic subunit B in subunit A preparations; thus, if antiserum specific for the A subunit of either LT or CT is desired, it should be absorbed over an affinity column with the B subunit covalently attached.

The high degree of CT neutralization observed with antisera raised against $286C_2$ LT (Table 1) contrasts with previous data which showed that anti-CT neutralized LT, but that anti-LT did not exhibit a comparable amount of neutralization against CT (15, 16, 32). Antisera to each toxin reacted preferentially with homologous toxin antigens in RIA; however, the amount of cross-reactivity observed in immunodiffusion assays suggested that each enterotoxin expressed unique determinants in addition to at least one or more shared antigenic determinants. Similar results have been described by Holmgren and Svennerholm (18), who used antisera to purified CT and crude preparations of LT holotoxin. Honda et al. (19) also employed antisera raised against intact holotoxins and demonstrated that antisera raised against CT contained antibodies which reacted only with CT and a second population which reacted with both CT and LT. Similar results were observed with antisera raised against LT; that is, antibodies were detected which reacted only with LT and a second population was detected which reacted with both enterotoxins.

Double immunodiffusion experiments are useful in characterizing cross-reactivity of determinants on different antigens but do not indicate differences in binding affinities between antibodies and their homologous and heterologous subunits. RIAs with anti-LT, anti-CT, and radiolabeled holotoxins revealed that when polystyrene tubes were coated with anti-LT, competition of LT binding occurred only in the presence of unlabeled LT and not with unlabeled CT. Similar results were observed with tubes coated with anti-CT; that is, competition was noted only with the homologous radiolabeled enterotoxin. However, when the radiolabeled heterologous enterotoxin was incubated with either antiserum, binding competition was observed with both homologous and heterologous antigens. Similar

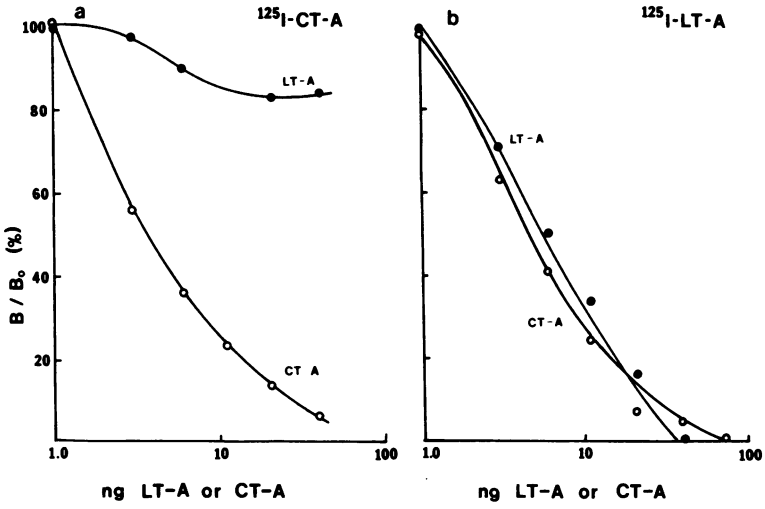

FIG. 5. Competitive inhibition studies of antigen-antibody interactions between LT-A and CT-A with affinity-purified anti-CT-A sera. Symbols: ●, LT-A; ○, CT-A. (a) ^{125}I-labeled CT-A; (b) ^{125}I-labeled LT-A. See the legend to Fig. 3 for definition of B/B_0.

data have been reported by Holmes et al. (R. K. Holmes, M. G. Bramucci, and E. M. Triddy, Abstr. 15th Joint Conference on Cholera, 1979, p. 52).

The lack of CT inhibition of LT binding and of LT inhibition of CT binding in the presence of homologous radiolabeled toxin and the presence of both LT and CT inhibition of heterologous radiolabeled toxin binding are not contradictory observations. Significant antibody affinity differences may be present in the populations reactive with unique and shared determinants of each toxin. Thus, the inability of heterologous unlabeled toxin to compete with homologous radiolabeled homologous toxin binding may be particularly evident in RIA. If the unique determinants are immunodominant, the absolute level of specific reactive antibody could significantly influence the observed results. Since the competitive inhibition assay with radiolabeled heterologous toxin depends on those antibodies which are cross-reactive, one would predict competition to occur in the presence of both heterologous and homologous toxin preparations. Therefore, the data clearly support the presence of shared determinants but suggest that the affinity and/or quantity of antibody reactive with these determinants is lower relative to the antibodies reactive with unshared toxin regions.

Since anti-286C_2 LT sera did not contain demonstrable subunit A reactivity, RIAs were performed with either affinity-purified antibodies to CT-A and CT-B or antisera raised against the purified subunits of LT. Data obtained with each of the subunit preparations were similar to those observed with the holotoxins. For example, only unlabeled CT-B exhibited a competitive inhibition response with ^{125}I-labeled CT-B when incubated with tubes coated with anti-CT-B, whereas both unlabeled subunits competed with ^{125}I-labeled LT-B. RIAs with affinity-purified anti-CT-A led to similar results, as did experiments with anti-LT-B raised against LT-B purified by acid-urea gel filtration. These data were interpreted to show that the A and B subunits of LT and CT contain an immunodominant unique antigenic determinant(s) and at least one minor shared antigenic determinant. LT-B and CT-B have been shown to share about 80% sequence homology (6, 27). The one or more regions which involve variation in the amino acid sequences of LT-B and CT-B, which may form the unique immunodeterminants, must therefore be localized to a relatively small region of the B subunit.

These observations underline the necessity for the careful assessment of individual toxin assay procedures and suggest interesting possibilities concerning the presence of shared determinants formed by the interaction of A and B subunits in the individual holotoxins. Anticholeragenoid serum is essentially an anti-CT-B serum, whereas anti-CT serum contains antibodies reactive with both CT-A and CT-B. Interestingly, anti-CT serum effectively neutralized both CT and LT, whereas anticholeragenoid did not. Significantly, the anti-LT serum examined in the present study did not contain detectable amounts of anti-LT-A, and this antiserum was equal to anticholeragenoid serum in the ability to neutralize CT. Earlier investigations (18) have shown that anti-CT-A is not a particularly strong neutralizing antibody population; furthermore, recent studies with monoclonal antibodies pre-

pared against individual CT subunits suggest that anti-CT-A antibodies are not neutralizing (24). Although the extent of anti-A activity in an individual antiserum may be the explanation for the enhanced neutralizing activity within an antiserum, the present data and those of other investigators suggest that anti-CT contains neutralizing antibodies which recognize determinants formed by interaction of CT-A and CT-B. These antibodies can therefore react with similar determinants present on LT. There remains the possibility, then, that such determinants may be more accessible on CT than LT and would allow a relatively more pronounced antibody response.

The immunization potential of the shared and unique antigenic determinants of CT and LT remains to be established in studies with human volunteers. However, the antigenic heterogeneity of LTs produced by human and porcine strains of ETEC may decrease effectiveness against one or both organisms. Significant antigenic differences have been observed between human and porcine LTs and possibly within both human LTs and porcine LTs (12, 20; Holmes et al., Abstr. 17th Joint Conference on Cholera, 1981, p. 43; P. H. Gilligan, J. C. Brown, and D. C. Robertson, manuscript in preparation). Rappaport and Bonde demonstrated synergistic protection against experimental cholera using cholera toxoid and a killed ETEC whole-cell vaccine (30). Antitoxic immunity due to cholera toxoid and the ETEC vaccine was probably enhanced by anti-LT antibodies directed toward one or more antigenic determinants shared with CT. Whatever the mechanisms involved in synergistic protection by antigens present in vaccines, antitoxin immunity will likely make an important contribution to protection and resistance.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-12357 to D.C.R. under the U.S.-Japan Cooperative Medical Program, administered by the National Institute of Allergy and Infectious Diseases, and Public Health Service research grant AI-16220 to J.C.B. from the National Institutes of Health. J.C.B. is a recipient of Public Health Service Research Career Development Award 1K04 AI-00427 from the National Institute of Allergy and Infectious Diseases.

We thank Nobuya Ohtomo, Chemo-Therapeutic Research Institute, Kumamoto, Japan, for his generous gift of affinity-purified antisera to the subunits of CT and anti-CT and Leonora Valencia for her excellent technical assistance.

LITERATURE CITED

1. **Boseman-Finkelstein, M., and R. A. Finkelstein.** 1982. Protection in rabbits induced by the Texas star-SR attenuated $A^- B^+$ mutant candidate live oral cholera vaccine. Infect. Immun. **36**:221–226.
2. **Clements, J. D., and R. A. Finkelstein.** 1978. Immunological cross-reactivity between heat-labile enterotoxins of *Escherichia coli* and subunits of *Vibrio cholerae* enterotoxin. Infect. Immun. **21**:1036–1039.
3. **Clements, J. D., and R. A. Finkelstein.** 1978. Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **22**:709–713.
4. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24**:760–769.
5. **Clements, J. D., R. J. Yancey, and R. A. Finkelstein.** 1980. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli*. Infect. Immun. **29**:91–97.
6. **Dallas, W. S., and S. Falkow.** 1979. The molecular nature of heat-labile enterotoxin (LT) of *Escherichia coli*. Nature (London) **277**:406–407.
7. **Donta, S. T.** 1974. Neutralization of cholera enterotoxin-induced steroidogenesis by specific antibody. J. Infect. Dis. **129**:284–288.
8. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183**:334–335.
9. **Field, M.** 1979. Mechanisms of action of cholera and *Escherichia coli* enterotoxins. Am. J. Clin. Nutr. **32**:189–196.
10. **Finkelstein, R. A., M. Boseman, S. H. Neoh, M. LaRue, and R. Delaney.** 1974. Dissociation and recombination of the subunits of the cholera enterotoxin (choleragen). J. Immunol. **113**:145–150.
11. **Freter, R.** 1978. Association of enterotoxigenic bacteria with the mucosa of the small intestine: mechanisms and pathogenic implications, p. 155–170. *In* O. Ouchterlony and J. Holmgren (ed.), Cholera and related diarrheas. S. Karger, Basel.
12. **Geary, S. J., B. A. Marchlewicz, and R. A. Finkelstein.** 1982. Comparison of heat-labile enterotoxins from porcine and human strains of *Escherichia coli*. Infect. Immun. **36**:215–220.
13. **Gilligan, P. H., and D. C. Robertson.** 1979. Nutritional requirements for synthesis of heat-labile enterotoxin by enterotoxigenic strains of *Escherichia coli*. Infect. Immun. **23**:99–107.
14. **Greenwood, F. W., and W. H. Hunter.** 1963. The preparation of ^{131}I-labelled human growth hormone of high specific activity. Biochemistry **89**:114–123.
15. **Gyles, C. L.** 1974. Immunological study of the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. Infect. Immun. **9**:564–570.
16. **Gyles, C. L.** 1974. Relationships among heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. **129**:277–283.
17. **Holmgren, J., O. Soderlind, and T. Wadstrom.** 1973. Cross-reactivity between heat-labile enterotoxins of *Vibrio cholerae* and *Escherichia coli* in neutralization tests in rabbit ileum and skin. Acta. Pathol. Microbiol. Scand. Sect. B **81**:757–762.
18. **Holmgren, J., and A.-M. Svennerholm.** 1979. Immunological cross-reactivity between *Escherichia coli* heat-labile enterotoxin and cholera toxin A and B subunits. Curr. Microbiol. **2**:55–58.
19. **Honda, T., Y. Takeda, and T. Miwatani.** 1981. Isolation of special antibodies which react only with homologous enterotoxins from *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. Infect. Immun. **34**:333–336.
20. **Honda, T., T. Tsuji, Y. Takeda, and T. Miwatani.** 1981. Immunological nonidentity of heat-labile enterotoxins from human and porcine enterotoxigenic *Escherichia coli*. Infect. Immun. **34**:337–340.
21. **Kantor, H. S.** 1975. Enterotoxins of *Escherichia coli* and *Vibrio cholerae*: tools for the molecular biologist. J. Infect. Dis. **131**:S22–S32.
22. **Klipstein, F. A., and R. F. Engert.** 1977. Immunological interrelationships between cholera toxin and heat-labile and heat-stable enterotoxins of coliform bacteria. Infect. Immun. **18**:110–117.

23. **Kunkel, S. L., and D. C. Robertson.** 1979. Purification and chemical characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. Infect. Immun. **25**:586–596.
24. **Lindholm, L., J. Holmgren, M. Wikstrom, U. Karlsson, K. Andersson, and N. Lycke.** 1983. Monoclonal antibodies to cholera toxin with special reference to cross-reactions with *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **40**:570–576.
25. **Markel, D. E., K. E. Hejtmancik, J. W. Peterson, F. B. Martin, and A. Kurosky.** 1979. Characterization of the antigenic determinants of cholera toxin subunits. Infect. Immun. **25**:615–626.
26. **Morris, G. K., M. H. Merson, D. A. Sack, J. G. Wells, W. T. Martin, W. E. Dewitt, J. C. Feeley, R. B. Sack, and D. M. Bessudo.** 1976. Laboratory investigation of diarrhea in travelers to Mexico: evaluation of methods for detecting enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **3**:486–495.
27. **Moseley, S. L., and S. Falkow.** 1980. Nucleotide sequence homology between the heat-labile enterotoxin gene of *Escherichia coli* and *Vibrio cholerae* deoxyribonucleic acid. J. Bacteriol. **144**:444–446.
28. **Moss, J. M., J. C. Osborne, P. H. Fishman, S. Nakaya, and D. C. Robertson.** 1981. *Escherichia coli* heat-labile enterotoxin: ganglioside specificity and ADP-ribosyltransferase activity. J. Biol. Chem. **256**:12861–12865.
29. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta. Pathol. Microbiol. Scand. **26**:507–515.
30. **Rappaport, R. S., and G. Bonde.** 1981. Development of a vaccine against experimental cholera and *Escherichia coli* diarrheal disease. Infect. Immun. **32**:534–541.
31. **Robb, M., J. C. Nichols, S. K. Whoriskey, and J. R. Murphy.** 1982. Isolation of hybridoma cell lines and characterization of monoclonal antibodies against cholera enterotoxin and its subunits. Infect. Immun. **38**:267–272.
32. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **29**:333–351.
33. **Sack, R. B.** 1978. Pathogenesis and pathophysiology of diarrheal diseases caused by *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, p. 53–63. *In* O. Ouchterlony and J. Holmgren (ed.), Cholera and related diarrheas. S. Karger, Basel.
34. **Smith, N. W., and R. B. Sack.** 1973. Immunologic cross-reactions of enterotoxins from *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. **127**:164–170.
35. **Takeda, Y., T. Honda, S. Taga, and T. Miwatani.** 1981. In vitro formation of hybrid toxins between subunits of *Escherichia coli* heat-labile enterotoxin and those of cholera enterotoxin. Infect. Immun. **34**:341–346.
36. **Van Heyningen, S.** 1976. The subunits of cholera toxin: structure stoichiometry, and function. J. Infect. Dis. **135**(Suppl.):5–13.