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Characterization of Monoclonal Antibodies That React with Unique and Cross-Reacting Determinants of Cholera Enterotoxin and Its Subunits

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Seventeen selected hybridoma cell lines that produced monoclonal antibodies against cholera enterotoxin (CT) were isolated and characterized. All of the monoclonal antibodies contained the kappa light chain; 14 were of the immunoglobulin G1 (IgG1) isotype and 3 were IgG2a. The 17 monoclonal antibodies were divided into a minimum of seven different specificity groups based on their abilities to bind to the following purified test antigens in solid-phase radioimmunoassays: CT, the A and B polypeptides of CT (CT-A and CT-B, respectively), and the heat-labile enterotoxins designated LTh and LTp from *Escherichia coli*. The binding of these antibodies to the following subunits and fragments of CT was also determined in Western blots: pentameric CT-B, monomeric CT-B, intact CT-A, and the A1 fragment of CT-A. Each of the monoclonal antibodies was tested for neutralization of CT and for precipitation with CT in immunodiffusion tests. Antigenic determinants were identified on CT that were not present either on CT-A or CT-B. One class was unique for CT and another was shared with LTh and LTp. Antibodies directed against these holotoxin-specific determinants had no neutralizing activity. Most of the monoclonal antibodies that reacted strongly with CT-A or CT-B also reacted strongly with CT holotoxin; however, one class of antibody reacted strongly with CT-A but weakly with CT. Among the monoclonal antibodies against CT-A or CT-B, some were specific for CT and others cross-reacted with LTh and LTp or with LTh only. The most potent neutralizing antibodies were against CT-B, and all of our monoclonal antibodies against CT-B had some neutralizing activity. In contrast, only some of the monoclonal antibodies against CT-A had neutralizing activity, and their specific activities were low. We found no direct correlations between the ability of monoclonal antibodies to neutralize CT and to cross-react with LTh or LTp. None of the epitopes recognized by our monoclonal anti-CT antibodies was present on CT-A and CT-B.

Cholera enterotoxin (CT) is a heat-labile enterotoxin produced by *Vibrio cholerae* (9). It has an essential role in the pathogenesis of cholera and causes secretory diarrhea by activating adenylate cyclase in the mucosal epithelium of the small intestine (13). CT is closely related to the heat-labile enterotoxin (LT) of *Escherichia coli* (34). The mode of action of LT is similar to that of CT, and LT has been implicated in the pathogenesis of secretory diarrhea in humans and in animals (13).

Both CT and LT have been purified to homogeneity (3, 9, 18, 22), and the structural genes for both toxins have been cloned (30, 35, 37). Each toxin is composed of two different polypeptide subunits designated A and B. Each holotoxin contains one A polypeptide and five copies of the B polypeptide held together by noncovalent bonds (14, 15). Comparisons of primary structures demonstrated that the A and B polypeptides from CT have extensive homology with those from LT (6, 36). Hyperimmune antisera prepared against either of the purified toxins will neutralize both toxins, and reactions of partial identity between CT and LT can be demonstrated by immunodiffusion tests or other immunochemical methods (3, 5, 18, 18a). The A subunit of CT shares antigenic determinants with the A subunit of LT, and the B subunit of CT shares determinants with the B subunit of LT (5; Holmes et al., in press). The CT toxins produced by *V. cholerae* 569B (classical biotype) and

3083-2 (El Tor biotype) give reactions of partial identity in immunodiffusion tests with antisera to 569B toxin, indicating that 569B toxin has one or more antigenic determinants that are not present in 3083-2 toxin (10). Recent studies have also documented partial antigenic cross-reactivity between the LT toxins produced by enterotoxigenic strains of *E. coli* isolated from humans (LTh) and from pigs (LTp) (4, 12, 18a, 20, 29).

Monoclonal antibodies represent a very powerful tool for dissecting and analyzing the immunochemical structure of complex proteins such as the cholera-*E. coli* family of heat-labile enterotoxins (21). Monoclonal antibodies are homogeneous populations of antibody molecules that interact with single epitopes from the array of determinants on a complex antigen such as CT or LT. In principle, a collection of monoclonal antibodies could be prepared to identify all of the epitopes on CT or LT. Such antibodies could be used to map the locations of specific epitopes, to study the role of specific structural domains of the toxin in determining its biological activities, and to develop specific immunodiagnostic tests for clinical, epidemiological or other applications.

Although monoclonal antibodies against CT have been isolated in several laboratories (24, 31, 32), the number of recognized specificities remains small, and detailed characterization of the epitopes has not yet been reported. In our studies, a variety of screening tests was used to increase the number of different antibody specificities that could be recognized and to extend significantly the results of previous studies of monoclonal anti-CT antibodies. We identified several epitopes on CT that were not previously recognized. Among determinants expressed on holotoxin but not on the isolated A or B subunits of CT, some were unique and others were shared with LT holotoxin. We also demonstrated unique and cross-reacting determinants associated with the isolated A and B subunits of CT (CT-A and CT-B, respectively). Each monoclonal antibody was also characterized with respect to its isotype, binding to CT and related antigens, neutralization of CT, and immunoprecipitation with CT.

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MATERIALS AND METHODS

Bacteria. All strains were from the stock culture collection in our laboratory. *V. cholerae* 569B Inaba is a highly toxinogenic strain of the classical biotype (9). *E. coli* HE12 is a hypertoxinogenic mutant of the *E. coli* K-12 substrain KL320 containing the enterotoxin plasmid pCG86 which codes for LTp (2). *E. coli* HE22 is a cured variant of HE12 that lacks plasmid pCG86 but retains the hypertoxinogenic allele *htx-2* (2). *E. coli* HE22(pTD2) contains the enterotoxin plasmid pTD2 from the *E. coli* clinical isolate Throop D and is hypertoxinogenic for LTh (29).

Cell cultures. The murine plasmacytoma cell line Sp2/O-Ag14 (33) was obtained from C. Augl, Department of Biochemistry, Uniformed Services University of the Health Sciences, and was cultured in RPMI 1640 medium (M. A. Bioproducts, Walkersville, Md.) supplemented with 10% fetal bovine serum (Reheis Chemical Co., Phoenix, Ariz.), 8-azaguanine (20 μg/ml; Sigma Chemical Co., St. Louis, Mo.), L-glutamine (2 mM; GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 U/ml; GIBCO), and streptomycin (100 μg/ml; GIBCO). HT and HAT medium contained hypoxanthine (H), aminopterin (A), and thymidine (T) as indicated and were prepared with RPMI 1640 medium containing 10% fetal bovine serum as described by Kennett (21).

Purification of antigens. CT was purified from supernatants of cultures of *V. cholerae* 569B according to published methods (27). CT-A and CT-B were isolated by gel filtration, chromatography under dissociating conditions (8). LTp from *E. coli* HE12 and LTh from *E. coli* HE22(pTD2) were purified by minor modifications of published methods (3, 18). The bacteria were grown overnight at 37°C in glucose-syncase medium modified to contain 5 g of anhydrous Na_2HPO_4 and 5 g of anhydrous K_2HPO_4 per liter (2). The bacteria were washed by centrifugation and disrupted by sonication. Particulate debris was removed from the extracts by centrifugation, and LTp or LTh was isolated by affinity chromatography on Biogel A5-M (BioRad Laboratories, Richmond, Calif.) (3), followed by elution with D-galactose, dialysis, and ion-exchange chromatography on phosphocellulose P-11 (Whatman Ltd., England) (18). Purity of the enterotoxins and the subunits of CT was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (23). LTh and LTp were isolated in the unnicked form; their A polypeptides did not dissociate into fragments A1 and A2 after reduction of disulfide bonds.

Immunization of mice. Twelve-week-old BALB/c mice (Charles River Laboratories, Inc., Wilmington, Mass.) were primed by intraperitoneal inoculation of 1 μg of CT in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) at 40 days before splenectomy (−40 days). The mice received 5-μg booster injections of CT in incomplete Freund adjuvant (Difco) intraperitoneally at −20 days and 1 μg of CT in phosphate-buffered saline intravenously at −3 days.

Isolation and cloning of hybridoma cell lines. Mice immunized with CT were sacrificed by cervical dislocation, and their spleens were removed and washed in RPMI 1640 medium. Cells were removed from the spleen by perfusion and passed through a fine-mesh sterile nylon net to obtain a homogeneous cell suspension; erythrocytes were removed by lysis with ammonium chloride in Tris buffer (28). The remaining cells were washed twice with RPMI 1640 medium and mixed in a ratio of 2:1 with Sp2/O-Ag14 plasmacytoma cells. Fusion was induced by treatment with polyethylene glycol (Carbowax 1000, Fisher Scientific Co.,

Orangeburg, N.Y.) according to published methods (11, 28). The cells were collected by centrifugation and suspended in HAT medium at a density of 3×10^6 spleen cells per ml, 100-μl samples were dispensed into individual wells in 96-well microtiter plates (Costar, Cambridge, Mass.) After incubation for 16 to 24 h at 37°C in an atmosphere of 5% CO_2, an additional 100-μl volume of HAT medium was added to each well. Subsequently, the HAT medium was changed at intervals of 3 days. After 2 weeks, the supernatants from cultures containing viable cells were screened for the presence of anti-CT antibodies by the radioimmunoassay methods described below. Cells from wells that contained anti-CT antibodies were expanded in HT medium and rescreened for production of antibodies that bound to CT, CT-A, CT-B, LTh, and LTp. Cloning of antibody-producing cells was carried out by subculturing them in HT medium at limiting dilutions. Each hybridoma cell line isolated was initially cloned with an inoculum of 10 cells per well, and subcloning was performed one or more additional times with an inoculum size of 0.5 cells per well. Cloned hybridoma lines were then expanded in HT medium and preserved by freezing in liquid nitrogen.

Radioimmunoassays for CT and related antigens. Solid-phase radioimmunoassay methods used previously in our laboratory (2, 16) were modified for detection of murine monoclonal anti-CT antibodies in supernatants from cultures of hybridoma cell lines. When purified CT, CT-B, LTh, or LTp was used as the test antigen, microtiter plates were first coated with ganglioside G_{M1} (Supelco Inc., Bellefonte, Pa.) Samples containing 25 μl of the test antigen at 1 μg/ml were than added to each well, and the antigens were permitted to bind to the immobilized ganglioside receptors during incubation for 18 h at room temperature. When purified CT-A was the test antigen, 25-μl samples containing CT-A at 5 μg/ml in phosphate-buffered saline with 0.01% phenol red and 0.1% sodium azide were added to each well, and the CT-A was allowed to bind directly to the wells during incubation for 18 h at room temperature. Plates sensitized with antigens by these methods could either be used immediately or stored at −70 or −20°C for use at later times with comparable results. Samples containing 25 μl of appropriately diluted supernatants from hybridoma cultures were added to the wells of antigen-coated plates and incubated overnight at 4°C. After subsequent incubations with rabbit anti-mouse immunoglobulin G (IgG) (Miles Laboratories, Inc., Elkhart, Ind.) at a dilution of 1:3,000 for 90 min at 37°C, followed by ^{125}I-labeled, affinity-purified goat anti-rabbit IgG for 90 min at 37°C, the radioactivity bound to each well was determined by gamma scintillation counting. The rabbit anti-mouse IgG antiserum used in these experiments was not heavy-chain specific, and we demonstrated in control experiments that antibodies in this serum could bind to reference samples of murine IgM myeloma proteins. Therefore, our screening assays should have been able to detect both IgG and IgM monoclonal anti-CT antibodies. For quantitative titrations of the binding activity of the monoclonal antibodies, the unit of antibody was defined as the amount required to produce 50% of the maximal response in the solid-phase radioimmunoassay with a specified test antigen.

Determination of isotypes of monoclonal antibodies. The solid-phase radioimmunoassays for monoclonal anti-CT antibodies described above were modified by using saturating concentrations of the monoclonal antibodies and substituting rabbit anti-mouse isotyping sera for the rabbit anti-mouse IgG. J. Kenny, Department of Microbiology, Uniformed Services University of the Health Sciences, provided samples of affinity-purified, heavy-chain-specific rabbit antibodies to mouse IgG1, IgG2, IgG3, and IgM prepared in his laboratory. Rabbit antisera specific for the heavy chains of IgG2a and IgG2b and for the kappa and lambda light chains of murine immunoglobulins were purchased from Boehringer Mannheim Corp., Indianapolis, Ind.

Monoclonal antibody concentration determination. Radial immunodiffusion assays (7) were used to determine the concentrations of monoclonal antibodies in concentrated or unconcentrated supernatants from hybridoma cell cultures. Rabbit anti-mouse IgG was diluted to 1:100 in 1% agarose (Sigma) and spread in thin layers on slides. A mixture of purified mouse IgG myeloma proteins for use as a standard was provided by J. Kenny. Measured volumes of samples to be tested were added to wells punched in the agar layers, and the slides were incubated for 16 h at room temperature. The concentrations of the monoclonal antibodies were calculated by comparing the diameters of the rings of precipitate surrounding the test wells with those of the standards.

Western blot analysis. Samples containing 50 μg of purified CT were boiled for 3 min in sample treatment mix containing 0.1% SDS and 5% 2-mercaptoethanol, cooled, and mixed with 10-μg samples of purified CT-B. The mixtures were subjected to electrophoresis on vertical 13% polyacrylamide slab gels containing 0.1% SDS by the method of Laemmli (23). Electrophoretic transfer of the proteins from the SDS-polyacrylamide gels to nitrocellulose paper (Schleicher & Schuell Co., Keene, N.H.) and immunological detection of the transferred proteins were performed as described by Robb et al. (32). The peroxidase-conjugated rabbit anti-mouse IgG serum used for these experiments was purchased from Miles Laboratories, Inc., Elkhart, Ind. Binding of the peroxidase-conjugated antibodies to protein bands on the nitrocellulose paper was visualized by incubating the nitrocellulose membranes with a freshly prepared solution of 0.0125% diaminobenzidine (Sigma) and 0.025% H_2O_2 (J. T. Baker Chemical Co., Phillipsburg, N. Y.) in 0.5 M NaCl in 10 mM Tris buffer at pH 7.4 (1).

Neutralization tests. The ability of monoclonal antibodies to neutralize CT was determined in Y1 adrenal cell cultures (25). Supernatants from cultures of hybridoma cell lines were diluted serially in RPMI 1640 medium supplemented with 10% fetal bovine serum. Mixtures were prepared containing equal volumes (100 μl) of the diluted culture supernatant to be tested and a 1-ng/ml stock solution of CT in the same medium. The mixtures were preincubated for 2 h at 37°C and then added to monolayers of Y1 cells in microtiter plates. Controls included a titration of CT to demonstrate its ability to cause rounding of the Y1 cells and titrations of the monoclonal antibodies alone to demonstrate that they had no visible effects on the Y1 cells. The microtiter plates were examined after incubation at 37°C in a 5% CO_2 atmosphere for 18 h. The unit of neutralizing antibody was defined as the smallest

TABLE 1. Characteristics of monoclonal antibodies against cholera enterotoxin

Specificity group	Clone	Isotype	Conc (μg of IgG/ml)	Titers (units/μg of IgG)		Patterns of cross-reactivity by the following tests:[a]									Immunoprecipitation with CT
						Radioimmunoassays					Western blots				
				Binding[b]	Neutralization	CT	CT-A	CT-B	LTh	LTp	CT-B	CT-B5	CT-A	CT-Al	
1	17F7	IgG2a	110	190	<0.1	++	−	−	++	++	−	−	−	−	−
2	4G10	IgG1	110	100	<0.1	++	−	−	−	−	−	−	−	−	−
	42C8	IgG1	140	660	<0.1	++	−	−	−	−	−	−	−	−	−
3	4C8	IgG1	130	270	1.3	++	−	++	++	++	−	+	−	−	−
	32D3	IgG1	90	510	140	++	−	++	++	++	−	+	−	−	+
4	40D9	IgG1	60	450	56	++	−	++	++	−	+	+	−	−	+
5	4E2	IgG1	80	470	160	++	−	++	−	−	+	+	−	−	+
	15C11	IgG1	100	520	0.8	++	−	++	−	−	−	+	−	−	+
	22C6	IgG1	180	460	69	++	−	++	−	−	+	+	−	−	+
6	33D2	IgG2a	190	220	13	++	++	−	++	++	−	−	−	+	−
7A	14E7	IgG2a	100	230	1.7	++	+	−	−	−	−	−	−	−	−
	34C2	IgG1	110	380	3.0	++	+	−	−	−	−	−	−	−	−
7B	35C2	IgG1	130	140	<0.1	+	++	−	−	−	−	−	+	+	−
	40D3	IgG1	90	130	<0.1	+	++	−	−	−	−	−	+	+	−
	37G7	IgG1	120	420	<0.1	+	++	−	−	−	−	−	+	+	−
	34C11	IgG1	140	32	<0.1	+	++	−	−	−	−	−	+	+	−
7C	21B11	IgG1	100	670	<0.1	++	++	−	−	−	−	−	−	−	−

[a] −, No reaction; + and ++, positive reactions. The semiquantitative symbols + and ++ were used to indicate that for some of the monoclonal antibodies the maximal reactions in radioimmunoassays with CT and CT-A as the test antigens differed by more than threefold. In such cases, ++ indicates greater maximal binding than +. See Fig. 4B and C for examples of quantitative reactions scored as + or ++.

[b] Determined by solid-phase radioimmunoassays. CT-A was used as the test antigen for antibodies in specificity groups 7B and 7C , and CT was used as the test antigen for all other titrations.

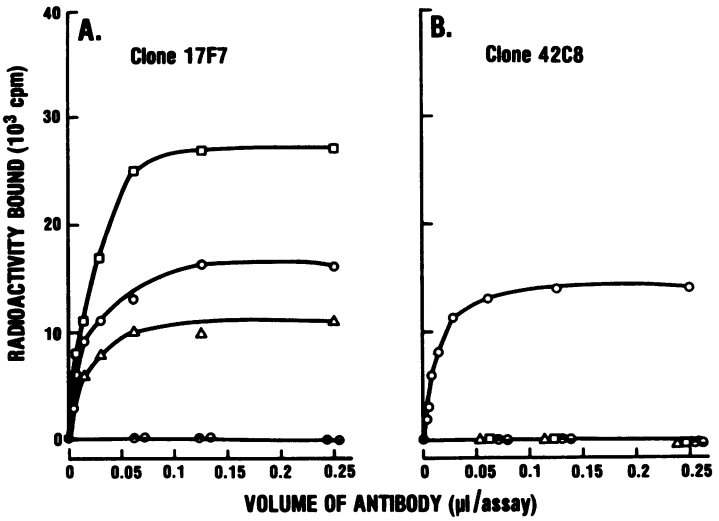

FIG. 1. Titrations of representative monoclonal antibodies with specificity for epitopes on CT holotoxin. Solid-phase radioimmunoassays were performed with each of the following test antigens: CT (○); CT-A (◒); CT-B (◓); LTh (□); and LTp (△). (A) Antibody 17F7 bound to CT, LTh, and LTp. (B) Antibody 42C8 bound only to CT.

amount required to reduce the toxicity of the 100-pg test dose of CT from 4+ to 1+ or 0.

Immunodiffusion tests. Ouchterlony-type immunodiffusion tests in agar gels were performed as described previously (10). Samples containing 100 μl of CT at 100 μg/ml were added to the center wells, and samples containing 100 μl of monoclonal antibodies were added to the outer wells. The monoclonal antibodies in supernatants from hybridoma cell cultures were concentrated by ultrafiltration, and immunodiffusion tests were performed with antibody concentrations of 2 mg/ml unless other concentrations are indicated in the text. Plates were incubated overnight at room temperature and examined for visible immunoprecipitates.

RESULTS

A total of 2,610 cultures was prepared for isolation of hybridomas; 1,010 showed growth in HAT medium after 2 weeks of incubation, and supernatants from 646 of these contained detectable anti-CT. The cultures that produced anti-CT were expanded in HAT medium and retested; 213 remained positive for anti-CT and were preserved by freezing. Supernatants from these 213 cultures were tested in radioimmunoassays for antibodies that bound to CT, CT-A, CT-B, LTh, and LTp. These initial screening tests were performed to identify different patterns of reactivity, and 26 cultures were selected for cloning by limiting dilution. Hybridoma lines derived in this manner were considered to be clonally pure if radioimmunoassays performed with culture supernatants from a minimum of six independent subcultures gave identical patterns of cross-reactivity in radioimmunoassays with CT, CT-A, CT-B, LTh, and LTp as test antigens. On the few occasions when discrepancies were noted among the subcultures, additional subcloning and testing was performed until reproducible results were obtained with independent subcultures. A total of 17 stable clones that produced anti-CT antibodies was obtained by these methods. Each clone was expanded and preserved by freezing; all clones were viable and able to produce anti-CT antibodies after subculture from frozen stocks.

A variety of tests was performed with culture supernatants to characterize the monoclonal antibodies produced by the 17 cloned hybridoma cell lines. These results are summarized in Table 1, and representative data are illustrated in Fig. 1 through 4.

All of the monoclonal anti-CT antibodies isolated were of the IgG class: 14 were of the IgG1 subclass and 3 were IgG2a. All 17 of our monoclonal anti-CT antibodies had kappa light chains; none had lambda light chains. The concentrations of these immunoglobulins in culture supernatants varied from 90 to 190 μg/ml. The 17 monoclonal antibodies were divided into seven different major groups on the basis of their binding specificities for CT, CT-A, CT-B, LTh, and LTp as determined by radioimmunoassays.

Antibodies in specificity groups 1 and 2 (Table 1) bound to epitopes that were present only on holotoxin; they failed to bind to isolated subunits of CT in either radioimmunoassays or Western blots. These antibodies did not neutralize CT and did not immunoprecipitate with CT. Data from representative titrations in radioim-

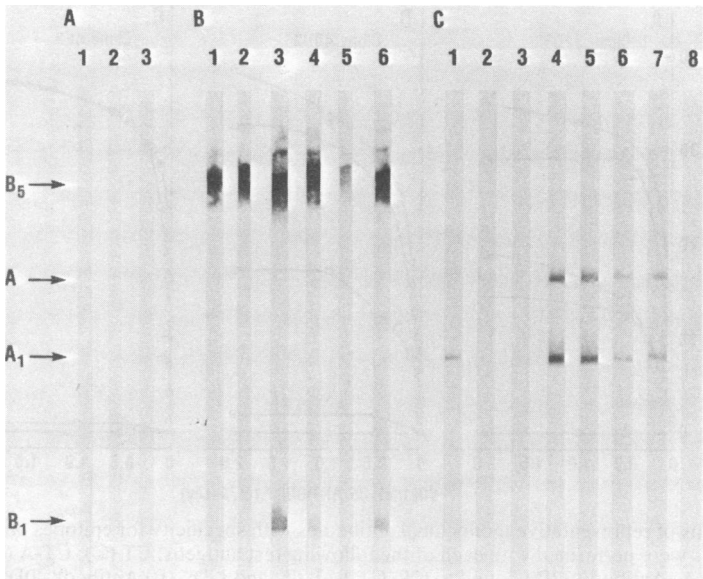

FIG. 2. Western blot analysis of binding of monoclonal anti-CT antibodies to subunits and fragments of CT. After electrophoretic transfer of the proteins to nitrocellulose, controls were stained for protein to document that transfer was successful and to determine the positions (arrows) of the bands corresponding to the pentameric CT-B (B_5), monomeric CT-B (B_1), intact CT-A (A), and the A_1 fragment of CT-A. In the experiment shown here, supernatants from hybridoma cultures were tested at a dilution of 1:10. (A) Antibodies with specificity for epitopes on CT holotoxin. Lane 1, 17F7; lane 2, 4G10; and lane 3, 42C8. (B) Antibodies with specificity for epitopes on CT-B. Lane 1, 4C8; lane 2, 32D3; lane 3, 40D9; lane 4, 4E2; lane 5, 15C11; and lane 6, 22C6. (C) Antibodies with specificity for epitopes on CT-A. Lane 1, 33D2; lane 2, 14E7; lane 3, 34C2; lane 4, 35C2; lane 5, 40D3; lane 6, 37G7; lane 7, 32C11; and lane 8, 21B11.

munoassays are presented in Fig. 1, and the Western blot analysis is shown in Fig. 2. The antibody produced by clone 17F7 (specificity group 1) recognized a common epitope that was shared by CT, LTh, and LTp. In contrast, the antibodies produced by clones 4G10 and 42C8 interacted with unique epitopes of CT that were not expressed on LTh or LTp.

The monoclonal antibodies in specificity groups 3, 4, and 5 were directed against determinants of CT associated with its B subunit. All of these antibodies bound to CT holotoxin and CT-B in radioimmunoassays and to pentameric CT-B in Western blots. They neutralized CT, but their neutralizing titers per microgram of IgG varied by more than 100-fold. In contrast, their binding titers per microgram of IgG, measured in radioimmunoassays, varied by less than two-fold. All clones except 4C8 (specificity group 3) formed precipitates with CT in immunodiffusion tests, but it was necessary to concentrate the antibodies in the culture supernatants to demonstrate precipitating activity. Representative titrations in radioimmunoassays are shown in Fig. 3; both the slopes and the plateaus of the binding curves were similar in assays with CT and with CT-B as the test antigens. The antibodies in specificity group 3 defined common determinants on CT, LTh, and LTp. Antibody 40D9 (specificity group 4) detected an epitope that was present on CT and LTh but not on LTp. The epitopes recognized by the monoclonal antibodies in specificity group 5 were unique for CT. In the Western blots (Fig. 2), strong reactions were observed with pentameric CT-B, but the reactions with monomeric CT-B were either much weaker or negative. We cannot exclude the possibility that the weak reactions of antibodies 40D9, 4E2, and 22C6 with monomeric CT-B were due to aggregation of CT-B to the pentameric form at some stage of the Western blot procedure after the SDS-polyacrylamide gel electrophoresis step.

The antibodies in specificity groups 6 and 7 bound to epitopes on cholera toxin that were associated with the CT-A subunit. Antibody 33D2, representing specificity group 6, bound to a common determinant on CT, LTh, and LTp. In contrast, all of the antibodies in specificity group 7 bound to determinants that were unique for CT. Representative radioimmunoassay titrations are shown in Fig. 4. Antibodies were assigned to subgroups 7A and 7B if their reactions with CT were much greater or much less than their reactions with CT-A, respectively. In contrast, for antibody 21B11, representing sub-

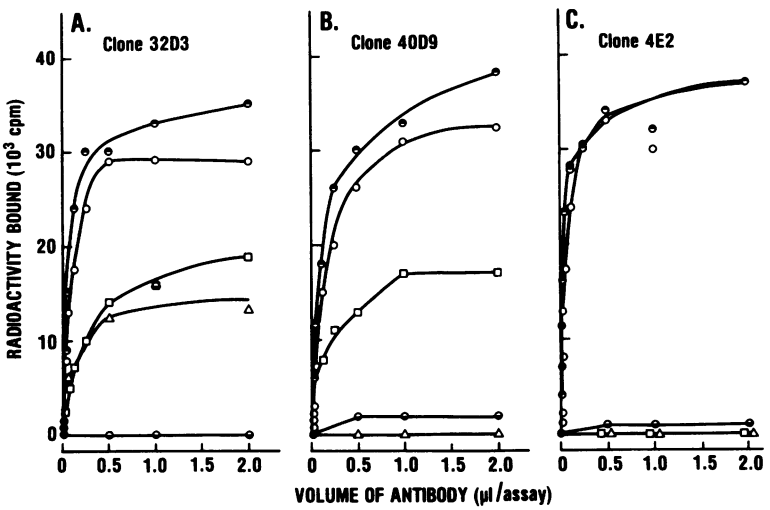

FIG. 3. Titrations of representative monoclonal antibodies with specificity for epitopes on CT-B. Solid-phase radioimmunoassays were performed with each of the following test antigens: CT (○); CT-A (◒); CT-B (◓); LTh (□); and LTp (△). (A) Antibody 32D3 bound to CT, CT-B, LTh, and LTp. (B) Antibody 40D9 bound to CT, CT-B, and LTh. (C) Antibody 4E2 bound only to CT and CT-B.

group 7C, the slopes of the binding curves were similar with CT or CT-A as the test antigen. Monoclonal antibodies in specificity groups 6 and 7A were capable of neutralizing CT, but no neutralizing activity was detected for antibodies in groups 7B and 7C. The neutralizing potency of 33D2, 14E7, and 34C2 per microgram of IgG was much less than that of the most potent monoclonal antibodies against CT-B but was slightly greater than that of antibodies 4C8 and 15C11. There was no apparent correlation between the binding titers of these antibodies in radioimmunoassays and their neutralizing titers. In the Western blot analysis, the antibodies in specificity groups 6 and 7B bound to the A1 subunit of CT, and those in group 7B also bound to the intact A subunit of CT. Antibodies in specificity groups 7A and 7C did not bind to subunits of CT in Western blots. In immunoprecipitation tests, none of the monoclonal antibodies directed against epitopes associated with CT-A were capable of precipitating with CT.

DISCUSSION

The LTs of *V. cholerae* and *E. coli* are a family of structurally homologous and antigenically cross-reacting proteins. These toxins are potent immunogens; they elicit high-titered antitoxic antibodies that can be measured by a variety of immunologic techniques (5, 9, 18). CT-B is much more immunogenic than CT-A, and most of the neutralizing activity of hyperimmune anti-CT serum is due to the antibodies against CT-B (8, 19). Although much interest has been focused on the important antigenic cross-reactions between these toxins, the immunodominant antigenic determinants of CT and LT are their unique determinants (18, 28a). Most of the antibodies in hyperimmune serum react only with the homologous antigen; therefore, CT and LT compete poorly with each other in competitive-binding radioimmunoassays. In recent studies from our laboratory, we used such competitive-binding radioimmunoassays to measure the production of CT and LT by strains of *V. cholerae* containing the enterotoxin plasmid pCG86 from *E. coli* (28a).

Studies with monoclonal antibodies provide information that is complementary to the results of classical serologic studies with CT and LT. Among 70 IgG monoclonal anti-CT antibodies characterized by Lindholm et al. (24), 61 (87%) were specific for determinants on CT-B, and 44 (63%) were specific for unique determinants of CT. These findings provided additional support for the conclusions that the unique determinants of CT are immunodominant and that CT-B is more immunogenic than CT-A. The isolation of several IgM monoclonal antibodies by Remmers et al. (31) and the greater prevalence of monoclonal antibodies specific for CT-A in the report of Robb et al. (32) most likely reflected differences in their regimens of immunization.

In previously reported studies of monoclonal anti-CT antibodies, all stable clones of hybridomas isolated were characterized. In contrast, the 17 hybridoma cell lines that we cloned and characterized were selected from among 213

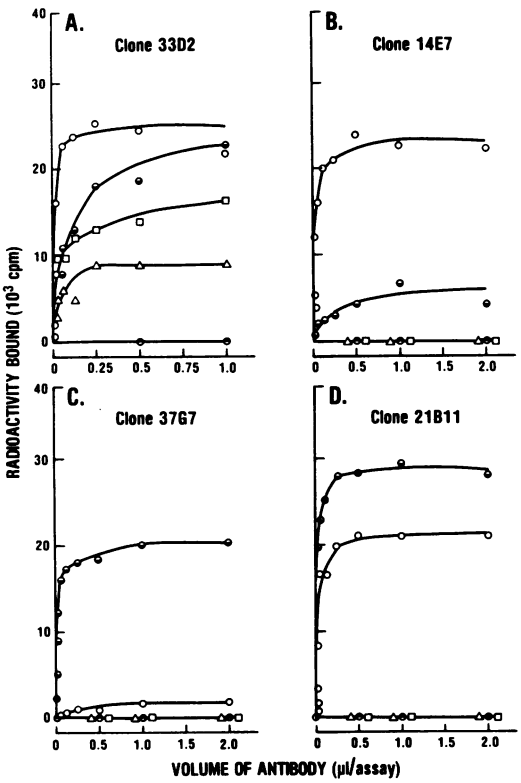

FIG. 4. Titrations of representative monoclonal antibodies with specificity for epitopes on CT-A. Solid-phase radioimmunoassays were performed with each of the following test antigens: CT (○); CT-A (◒); CT-B (◓); LTh (□); and LTp (△). (A) Antibody 33D2 bound to CT, CT-A, LTh, and LTp. (B) Reaction of antibody 14E7 with CT was much greater than with CT-A. (C) Reaction of antibody 37G7 with CT-A was much greater than with CT. (D) Titer of antibody 21B11 was similar in assays with CT and CT-A.

hybridoma cultures on the basis of preliminary screening tests. This approach facilitated our recognition of new specificity patterns but probably biased the relative frequencies with which the different specificity groups of monoclonal antibodies were represented in our collection.

Studies of monoclonal anti-CT antibodies in our laboratory and others have provided new information about the role of the CT-A and CT-B subunits in neutralization reactions. All of our monoclonal antibodies against CT-B had neutralizing activity (Table 1). In the study of Lindholm et al. (24), all of the monoclonal antibodies against CT-B which cross-reacted with LT had high neutralizing titers. We found an exception to this correlation: the antibody produced by clone 4C8 cross-reacted with both LTh and LTp but had a very low neutralizing titer (Table 1). We conclude that both unique and cross-reacting determinants on CT-B can elicit neutralizing antibody responses. Monoclonal antibodies against CT-A often have little or no neutralizing activity (24, 32). The most potent of three neutralizing monoclonal antibodies against CT-A in our collection (clone 33D2; Table 1) had only 8% of the neutralizing activity of our most potent monoclonal antibody against CT-B (clone 4E2).

Based on classical serologic studies, Hejtmancik et al. (17) and Markel et al. (26) proposed that CT-A and CT-B share one or more epitopes. Remmers et al. (31) isolated two hybridoma cultures that produced antibodies which reacted both with CT-A and CT-B in solid-phase radioimmunoassays, but the specificity of the cross-reactions observed in the radioimmunoassays was not confirmed by an independent method such as the Western blot technique. Robb et al. (32) identified a presumptive hybridoma clone that produced monoclonal antibodies against CT-B and CT-A, but additional subcloning demonstrated that the initial clone was impure. No monoclonal antibodies that reacted both with CT-A and CT-B were detected by Lindholm et al. (24) or in our study. We believe that the evidence for shared epitopes on CT-B and CT-A is inconclusive and that the presence of antibodies to CT-B in hyperimmune sera prepared against purified CT-A (8, 17, 26) is more likely due to the presence of highly immunogenic CT-B as a trace contaminant in the antigen.

The use of monoclonal antibodies to dissect the antigenic relationships between CT, LTh, and LTp is just beginning. Remmers et al. (31) and Lindholm et al. (24) demonstrated that some monoclonal antibodies specific for CT-A or CT-B cross-reacted with crude preparations of LTh. All five of the monoclonal antibodies against CT-A studied by Robb et al. (32) cross-reacted with purified LTh and LTp, but their monoclonal antibodies against CT-B did not react with the purified LTs.

In our studies, the LTh and LTp used as test antigens were purified from isogenic strains of *E. coli* K-12 containing enterotoxin plasmids from *E. coli* strains of human and porcine origin; this was done to ensure that any antigenic differences between the LTh and LTp toxins were not artifacts caused by differences in the bacterial strains used for toxin production. The availability of these reagents and the use of a variety of screening tests enabled us to recognize many different specificities among our monoclonal anti-CT antibodies. These included several monoclonal antibodies with new specificities, including those directed against shared and unique determinants present only on holotoxin (specificity groups 1 and 2; Table 1) and one that recognized a determinant on CT-B that was shared with LTh but not with LTp (specificity group 4; Table 1). Robb et al. (32) presented

preliminary evidence for monoclonal antibodies against CT-A that failed to react with CT holotoxin, but their attempts to clone such hybridomas were unsuccessful. Our monoclonal antibodies belongng to specificity group 7B also reacted strongly with CT-A and poorly with CT holotoxin (Table 1 and Fig. 4C). It is likely that the determinants recognized by the monoclonal antibodies in specificity group 7B are not fully exposed on CT holotoxin.

Among the six monoclonal antibodies against CT-B characterized in our studies, five were capable of forming immunoprecipitates with CT (Table 1). This result was consistent with the presence of 5B polypeptides in CT and indicated that CT was polyvalent for the antigenic determinants recognized by the precipitating antibodies. Under the conditions of our experiments, it was necessary to concentrate the monoclonal antibodies to approximately 0.5 mg of IgG per ml or greater to obtain positive results in immunodiffusion tests with CT. The monoclonal antibodies in specificity groups 1, 2, 6, and 7 (Table 1) did not give positive results in immunodiffusion tests with CT when tested at antibody concentrations of 2 μg/ml. Nevertheless, we found that mixtures of nonprecipitating monoclonal antibodies of different specificities could form immunoprecipitates with CT. It should be possible, in principle, to use mixtures of different monoclonal antibodies, all of which cross-react with CT, LTh, and LTp, as standardized reagents in rapid immunologic screening tests for LTs of the cholera-*E. coli* family. Monoclonal antibodies specific for the individual enterotoxin antigens could then be used for specific identification of enterotoxins detected by the broad specificity screening tests. Toward these ends, we have also isolated monoclonal antibodies raised against purified LTh; characterization of these anti-LTh antibodies is in progress and will be reported elsewhere (B. W. Belisle, E. M. Twiddy, and R. K. Holmes, manuscript in preparation).

Our studies have demonstrated that monoclonal anti-CT antibodies can be separated into a large number of distinct groups based on their patterns of cross-reactivity with CT, LTh, and LTp and on their biological activities. These observations provide evidence for the existence of multiple epitopes on CT holotoxin and on its A and B subunits, but they do not define the specific epitopes at the level of amino acid sequences or polypeptide conformations. The availability of a wide variety of monoclonal antibodies will facilitate future studies to define the structures of individual epitopes and to characterize with greater precision the functions of specific domains within CT at the molecular level.

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