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Production and Characterization of Monoclonal Antibodies to Cholera Toxin

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Monoclonal antibodies against cholera toxin were produced to obtain highly specific antisera to cholera toxin. Fifteen hybridoma cell lines producing monoclonal antibodies specific for the determinants of cholera toxin were derived from the fusion of mouse myeloma cells and spleen cells from mice immunized with cholera toxin. The cell lines were stabilized, examined for specific antibody production, and immortalized by freezing cultured cells and tumor cells which had been grown subcutaneously in mice. All cell lines continued antibody secretion upon thawing. The antibodies produced by the hybridoma cell lines were characterized by determination of the class of light- and heavy-chain components and by determination of specificity for the cholera toxin subunit. All of the antibodies contained the κ light chain, 4 contained the μ heavy chain, and the remaining 11 contained the γ1 heavy chain. Ten of the monoclonal antibodies are specific for the B subunit of cholera toxin, and three are specific for the A subunit. The remaining two appear to react with both subunits.

Cholera toxin, produced by *Vibrio cholerae*, is one of the factors involved in eliciting the symptoms of Asiatic cholera. The precise detection of low concentrations of toxin, which has been difficult, if not impossible, is important for identifying toxin-producing strains of *V. cholerae* and for molecular and biochemical characterization of toxin production and mode of action. Many biological assays, such as the rabbit skin and ligated ileal loop (9, 10), suckling infant mouse (6), and CHO and Y-1 adrenal cell (11, 15) assays, have been used to detect toxin, but they are expensive, tedious to carry out, and may be subject to interference by cytotoxins and related substances. Immunological assays for cholera toxin, such as the radioimmunoassay (RIA) (7, 16) and reversed passive hemagglutination reaction (17), provide some advantages over biological assays, but the specificity of these assays depends on the specificity of the antiserum used. The purification of conventional antisera to cholera toxin requires an elaborate methodology and can result in the production of reagents that vary greatly among preparations, depending on the immunization and purification steps involved.

Monoclonal antibody technology provides a means of overcoming the limitations inherent in conventional antiserum production. Monoclonal antibodies can detect specifically a particular epitope of the set of antigenic determinants displayed by a complex antigen, such as cholera toxin. Because a monoclonal antibody is a single molecular species, there is no need to resort to the protocols of adsorption and purification, which are necessary to render conventionally prepared polyclonal antisera epitope specific. These antibodies are produced by hybrid cells (hybridomas) derived from the fusion of mouse myeloma cells and lymphocytes from the spleens of mice that have been immunized with a particular antigen. The resulting hybridomas display the cellular immortality of the tumor cell parent and the immunoglobulin production of the mouse B-lymphocyte lineage cell parent (20, 21). Individual cells can be isolated and cultured to produce large amounts of identical antibody specific for a single antigenic determinant.

In this paper we report the derivation and stabilization of 15 hybridoma cell lines that produce antibody specific for the determinants of cholera toxin.

(This work is from a dissertation to be submitted to the Graduate School, University of Maryland, by E.F.R. in partial fulfillment of the requirements for the Ph.D. degree in microbiology.)

MATERIALS AND METHODS

Toxin preparations. Purified cholera toxin (prepared from classical *V. cholerae* 569B, lot 118C-0217, Sigma Chemical Co., St. Louis, Mo.) was used for immunization of mice and for coating flexible microtiter plates in solid-phase RIAs. Highly purified cholera toxin, the generous gift of S. H. Richardson, Bowman-Gray

Medical School, Winston-Salem, N.C., was used to confirm antibody-producing clones for toxin specificity.

Purified A and B subunits of cholera toxin, kindly provided by Richard Finkelstein, University of Missouri, Columbia, were used in solid-phase RIAs to determine the subunit specificity of the antibodies produced.

Immunization of mice. (i) Predominantly immunoglobulin M (IgM) response. Four female BALB/cJ mice (ca. 8 weeks old; Jackson Laboratories) were intraperitoneally primed with 50 μg of alum-precipitated cholera toxin in complete Freund adjuvant. Spleen cells from three surviving mice were used in the primed cell hybridization 4 days after immunization.

(ii) Predominantly IgG response. The immunization schedule of Svennerholm et al. (28) was followed to obtain hyperimmunized female BALB/cJ mice. Briefly, 1 μg of cholera toxin in phosphate-buffered saline (PBS; pH 7.2) was injected intravenously and repeated at 10 days and every 6th day thereafter for four immunizations. The mice were rested for 4 months; they were boosted with 10 μg of alum-precipitated cholera toxin in 0.5 ml of PBS intraperitoneally 4 days before the hybridization.

Production of antibody-secreting hybridomas. Approximately 10^8 SP 2/0 myeloma cells (27) were fused with homogenized spleen cell suspensions prepared from the spleens of immunized mice by the method of Goldsby et al. (13). Stable hybrid cell lines were selected with Littlefield hypoxanthine-aminopterin-thymidine medium (22). The fused cells were plated in 96-well dishes with the aid of a mechanical cloning and sampling apparatus described by Goldsby and Mandell (12) at several dilutions to ensure single clones per well. Approximately 2 weeks after fusion, wells containing single and double macroscopic clones were tested for antibody production and toxin specificity by indirect solid-phase RIA. Positive clones were recloned by limiting dilution at least three times to ensure monoclonality. All of the hybridoma lines described in this paper have been immortalized by freezing cell cultures and solid tumors produced by subcutaneous injection of hybridoma cells into BALB/cJ mice. All have been successfully thawed, returned to culture, and demonstrated to continue antibody secretion.

Indirect solid-phase RIA. Cholera toxin-specific antibody was detected by indirect solid-phase RIA (26). The antigen (20 μl of 10-μg/ml cholera toxin or toxin subunit in PBS) was adsorbed to wells of flexible microtiter plates for 1 h at room temperature in a humidified chamber. The plates were washed three times with PBS-bovine serum albumin (1 g of bovine serum albumin and 0.1 g of sodium azide per liter of PBS) to remove unadsorbed antigen. Approximately 50 μl of culture supernatant or serum dilution to be tested was added to duplicate wells, incubated for 1 h at room temperature, and washed to remove unbound murine antibody. Bound antibody was detected with ^{125}I-labeled sheep antibody to murine immunoglobulins. The reagent was prepared by immunizing sheep with purified murine myeloma proteins containing the heavy chains α, γ1, γ2a, γ2b, γ3, and μ and the light chains κ and λ (Litton Bionetics, Kensington, Md.). The sheep antiserum was purified by adsorption to a mouse immunoglobulin affinity column and radioactively labeled with ^{125}I by the Chloramine-T procedure (14). The labeled sheep anti-murine immunoglobulin reagent was tested against the individual myeloma proteins and was found to bind to each. Labeled reagent (20 μl, diluted to contain approximately 20,000 cpm) was added to each well and incubated at room temperature. The plates were washed, and the wells were cut apart with a hot wire device (D. Lee Co., Sunnyvale, Calif.), after which they were counted in a gamma counter (Gamma 4000; Beckman Instruments, Inc., Fullerton, Calif.). Samples with a signal-to-noise ratio of ≥2 were considered positive.

Determination of antibody class. To determine heavy- and light-chain classes, the indirect solid-phase RIA described above was used, except that 50 μl of each of the class-specific rabbit anti-murine heavy- and light-chain antisera (concentration, 50 μg/ml; Litton Bionetics) was used to coat the plate. Culture fluids were tested for the presence of specific heavy and light chains by binding to the antiserum-coated plates. Bound antibody was detected with the sheep anti-murine immunoglobulin reagent described above. The specificity of the assay was determined with purified myeloma proteins and light-chain proteins (Litton Bionetics).

Solid-phase antibody blocking assay. To gain a measure of the degree of independence of the monoclonal antibodies produced, the solid-phase antibody blocking assay described by Oi and Herzenberg (24) was carried out. In this assay, a titration of unlabeled antibody is added to microtiter plate wells that have been coated with the antigen (cholera toxin or appropriate toxin subunit) and allowed to incubate for 1 h. A constant amount of radiolabeled antibody is added to each well and incubated for 1 h. If the unlabeled antibody interferes with the binding of the radiolabeled antibody, the antibodies probably bind to the same site or to two closely juxtaposed determinants.

Immunological cross-reactivity of a monoclonal antibody to cholera toxin with the heat-labile enterotoxin of *Escherichia coli*. A standard solid-phase competition assay (24) was used for the detection of cholera toxin. The monoclonal antibody LHR-117-233 in pooled sera from tumor-bearing mice was coated on wells of microtiter plates. A mixture of toxin-containing sample and a constant amount of ^{125}I-labeled cholera toxin (labeled by the Chloramine T procedure [14]) was incubated in each well. A reduction in counts of ^{125}I-labeled bound cholera toxin is due to the presence of unlabeled toxin taking up sites on the immobilized antibody. Culture supernatant from hypertoxigenic *E. coli* H10407 was included to determine whether the antibody used was directed against an antigenic determinant also found in the *E. coli* heat-labile toxin.

RESULTS

Results of immunization of mice. Sera obtained from mice whose spleens were used in the cell hybridization were tested by solid-phase RIA to determine the extent of the immune response in the immunized mice. The titer of serum from mice which received a single immunization with cholera toxin, for a predominantly IgM response, was approximately 1/1,000. The titer of serum from mice which received multiple boosts

with cholera toxin, for a predominantly IgG response, was approximately 1/10,000.

Assay for antibody to cholera toxin-secreting clones. From the hybridization with primed spleen cells, 219 wells were tested for anti-cholera toxin antibody. More than 100 of these were positive in the indirect solid-phase RIA against cholera toxin. Three of these were stabilized and immortalized. Assays confirming specificity for cholera toxin are shown in Fig. 1.

Hybridization with primed and boosted spleen cells yielded 81 wells, which were tested for anti-cholera toxin activity. All of these were positive in the indirect solid-phase RIA against cholera toxin. Subsequent cloning and expansion of cell lines led to the stabilization of 12 specific, positive clones which have been preserved as frozen cells and tumors (Fig. 2).

Production of high-titer sera by mice bearing hybridoma cell tumors. Sera from mice bearing hybridoma cell tumors were tested for anti-cholera toxin antibody. The titers of all such antitoxin antisera were ≥1/50,000, except LHR-227-62 and LHR-227-77, which were ≥1/10,000.

Determination of subunit specificity. Toxin subunit specificity (A or B) was determined for the 15 stabilized monoclonal antibodies by means of the indirect solid-phase RIA (Fig. 3). Ten were found to be B specific, and three were found to be A specific. The remaining monoclonal antibodies reacted with both A- and B-subunit preparations (Table 1).

Determination of monoclonal antibody class. All of the monoclonal antibodies described contain the κ light chain, determined by solid-phase RIA. Eleven contained the γ1 heavy chain, and four contained the μ heavy chain. The antibody class results are listed in Table 1.

Solid-phase antibody blocking assay. The 10 B-specific monoclonal antibodies were examined in the antibody blocking assay. Biosynthetically ^{3}H-labeled LHR-227-13 was blocked by five other monoclonal antibodies: LHR-227-2, LHR-227-6, LHR-227-45, LHR-227-59, and LHR-227-65. The four remaining antibodies did not block the binding of LHR-227-13.

Immunological cross-reactivity of a monoclonal antibody to cholera toxin to the heat-labile enterotoxin of *E. coli*. In the solid-phase competition assay, a crude culture filtrate of *E. coli* H10407

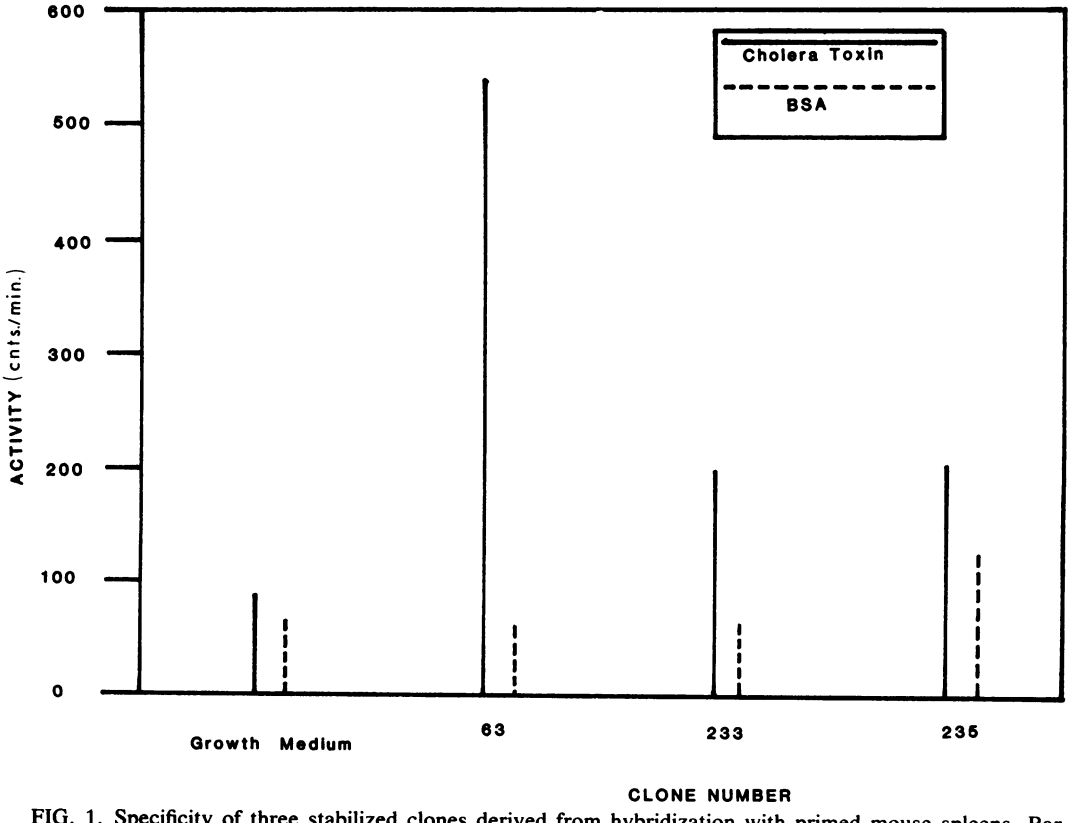

FIG. 1. Specificity of three stabilized clones derived from hybridization with primed mouse spleens. Bar height indicates activity of bound ^{125}I-labeled sheep anti-mouse immunoglobulin. (I) Activity against cholera toxin-coated plates; (⋮) activity against plates coated with bovine serum albumin (BSA), the unrelated antigen.

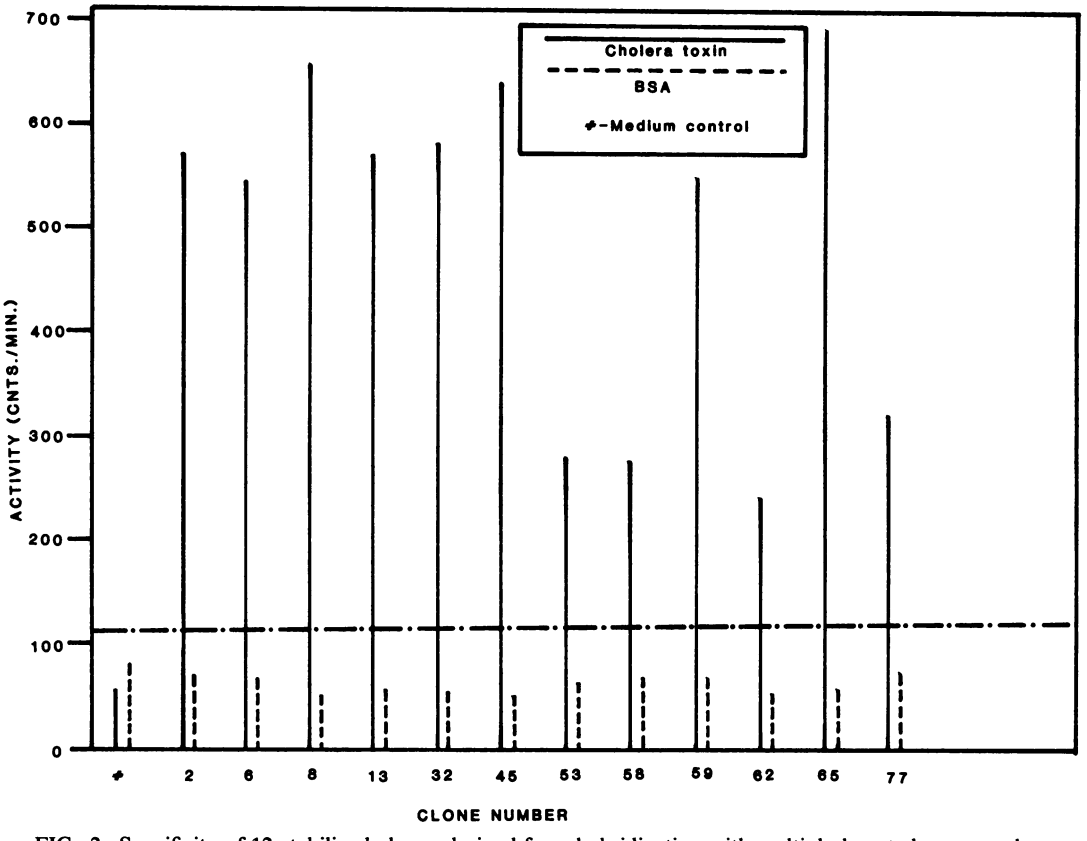

FIG. 2. Specificity of 12 stabilized clones derived from hybridization with multiply boosted mouse spleens. The horizontal line indicates a signal-to-noise ratio of 2. See legend to Fig. 1 for symbols. BSA, Bovine serum albumin.

inhibited the binding of ^{125}I-labeled cholera toxin to immobilized LHR-117-233 by >50%. Culture filtrate from a nontoxigenic strain of *E. coli*, WP-2, did not interfere with the binding of labeled cholera toxin. This result indicates that LHR-117-233, a cholera toxin B-subunit-specific antibody, recognizes a determinant also carried by the closely related *E. coli* heat-labile enterotoxin.

DISCUSSION

The monoclonal antibodies described in this report are of two classes: IgG and IgM. The IgG class antibodies are characteristically well suited to affinity column purification of both antibodies and antigens, since the antigen-antibody complex can be disassociated by gentle means. Thus, these antibodies, the toxin, and even the individual subunits can be readily purified by affinity column techniques. The IgM class antibodies are useful as binding assay reagents in enzyme-linked immunosorbent assay or RIA because of their pentameric structure and consequent high avidity for antigen. The results of the cell hybridizations described here demonstrate that it is possible to tailor monoclonal antibodies of a desired class. If spleens from mice primed with the antigen 3 to 4 days before the hybridization are used, predominantly IgM monoclonal antibodies can be expected, since the primary immune response is predominantly IgM antibodies. If, however, spleens from mice multiply boosted with antigen are used, predominantly IgG class monoclonal antibodies can be expected, since the secondary immune response is overwhelmingly IgG class antibody. The results of the two hybridizations described here are consistent with these observations. All of the monoclonal antibodies derived from primed spleens were of the IgM class, and all but one of the monoclonal antibodies derived from boosted spleens were of the IgG class.

Polyclonal antisera to cholera toxin and its purified subunits have been prepared (16). However, the B-subunit-specific and A-subunit-specific antisera cross-react. It is suggested that, rather than contamination of the A- and B-

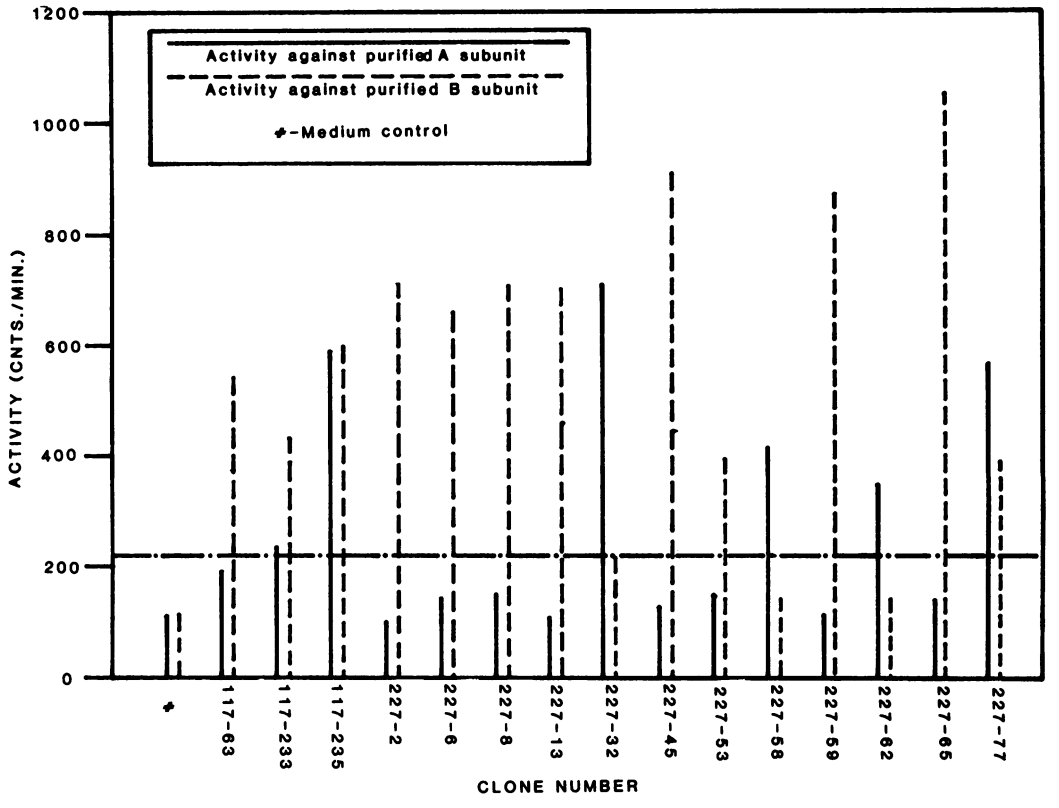

FIG. 3. Subunit specificity of monoclonal antibodies prepared against cholera toxin. (I) Activity against the purified A subunit; (i) activity against the purified B subunit. The horizontal line represents a signal-to-noise ratio of 2.

subunit preparations, shared antigenic determinants on the two subunits are responsible for cross-reactivity. An advantage of using monoclonal antibodies is that A- and B-subunit specificities are not a problem if the antibodies are specific for unshared determinants. The monoclonal antibodies produced were demonstrated to be reactive against at least one of the purified toxin subunits, as well as the holotoxin. Of the 15 monoclonal antibodies produced, 10 are specific for the B subunit and 3 are specific for the A subunit of cholera toxin. The remaining two appear to react with both subunits. These will be examined further to determine whether they are directed against similar or shared determinants on the A and B subunits. The subunit-specific antibodies, produced without the adsorption protocols necessary to render polyclonal antisera specific, should prove useful in the detection and purification of toxin subunits.

Monoclonal antibody technology is beginning to find wide application in the field of microbiology. Clearly, monoclonal antibodies have excellent potential for diagnostic and therapeutic use. Monoclonal antibodies against the antigens of streptococci and *Neisseria gonorrhoeae* have already been described (23, 25). In this report, monoclonal antibodies against cholera toxin are characterized which can serve as tools in the detection of antigenic determinants of cholera toxin.

As isolations of *V. cholerae* from the environment in areas nonendemic for the disease continue, it is becoming apparent that the organism is an autochthonous inhabitant of many estuarine and other aquatic systems (8, 18; J. V. Lee, D. J. Bashford, T. J. Donovan, A. L. Furniss, and P. A. West, J. Appl. Bacteriol., in press). In light of the Louisiana outbreak of cholera in 1978 (1–5), it is important to determine the presence of potentially pathogenic toxin-producing vibrios. To detect cholera toxin or related molecules, a mixture of several appropriate monoclonal antibodies can be employed in a precisely defined, standardized polyclonal antiserum to gain the advantages of multiple-determinant detection, thereby permitting the detection of related molecules. Subsequent analysis with individual monoclonal antibodies should permit the detection of the extent of antigenic relatedness

TABLE 1. Summary of monoclonal antibody subunit specificity and heavy-chain class

Monoclonal antibody no.	Toxin subunit specificity[a]	Heavy-chain class
LHR-117-63	B	M
LHR-117-233	B	M
LHR-117-235	A and B	M
LHR-227-2	B	G1
LHR-227-6	B	G1
LHR-227-8	B	G1
LHR-227-13	B	G1
LHR-227-32	A	M
LHR-227-45	B	G1
LHR-227-53	B	G1
LHR-227-58	A	G1
LHR-227-59	B	G1
LHR-227-62	A	G1
LHR-227-65	B	G1
LHR-227-77	A and B	G1

[a] Subunits A and B of cholera toxin (see text).

to the toxin of *V. cholerae* 569B.

Molecular and genetic studies have shown that possession of the O1 serological antigen is not correlated with possession of toxin genes (19). Thus, non-O1 and O1 strains of *V. cholerae* can be potentially toxigenic and should be examined for toxin production. Furthermore, it is possible that vibrios, including various strains of *V. cholerae*, may produce functionally and biochemically related toxin molecules. Since monoclonal antibodies detect specific antigenic determinants, the battery of monoclonal antibodies reported here will be helpful in evaluating the antigenic relatedness of toxins elaborated by various clinical and environmental vibrio strains. Applications of this set of monoclonal antibodies to cholera toxin are being investigated in our laboratory.

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