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Production and Characterization of Monoclonal Antibodies to *Clostridium perfringens* Enterotoxin

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Four hybridoma cell lines producing monoclonal antibodies to *Clostridium perfringens* enterotoxin were established by fusion of mouse myeloma and spleen cells obtained from mice immunized with the enterotoxin and its toxoid. An enzyme-linked immunosorbent assay indicated that the two antibodies, 2-B-4 and 3-G-10, bound to those regions that were located close each other; the others, 3-B-2 and 2-H-2, bound to other independent regions on the enterotoxin. Release of ^{51}Cr from Vero cells with the enterotoxin was inhibited by either 2-B-4 or 3-G-10, both of which inhibited the binding of ^{125}I-labeled enterotoxin to the cells. Neither binding nor cytotoxicity of the enterotoxin was affected by 2-H-2; 3-B-2 only barely inhibited the binding but neutralized the enterotoxin shown by ^{51}Cr release. It seems justified to conclude that 3-B-2 blocks the toxic action after the enterotoxin has bound to Vero cells.

Clostridium perfringens enterotoxin is responsible for human diarrhea (2, 7). The enterotoxin has been purified and demonstrated to be a single protein with a molecular weight of 34,000 (7, 23, 25). It specifically binds to cells of many types including intestinal epitherial cells and Vero cells, resulting in alteration of membrane permeability, inhibition of macromolecular synthesis, morphological changes in the cells, and finally, death of the cell (10, 14–16, 27). The enterotoxin action may involve at least two steps: binding to the target cells and the ensuing cytotoxic effects (10, 14). Ca^{2+} ion is essential for the latter step (14). Various biological activities are derived from alteration of cell membrane permeability (15); however, the precise mechanism of the enterotoxin action is still unknown. The enterotoxin has been activated with trypsin (5, 6) and the sites of tryptic cleavage have been identified (20). Recently, Richardson and Granum (21) analyzed the total amino acid sequence of the enterotoxin; however, the subunits or the active site of the enterotoxin molecule has not been found.

Monoclonal antibodies (MAbs) against various bacterial toxins have been established (3, 8, 11, 19, 24). MAbs bind to a single site on an antigen molecule. For such unique specificity, MAbs have been used as a valuable tool for studying the structure and function of a variety of macromolecules (13, 17).

In this paper, we report the preparation and characterization of four distinct MAbs against *C. perfringens* enterotoxin. Using these MAbs, we have attempted to examine the antigenic structure of the enterotoxin.

MATERIALS AND METHODS

Preparation of *C. perfringens* enterotoxin and its toxoid. *C. perfringens* enterotoxin was prepared by the method of Sakaguchi et al. (23), and its biological activity was determined by the Vero cell-staining assay method (27). About 50% of Vero cells died with 100 ng of the enterotoxin per ml. It was detoxified by dialysis against 0.4% Formalin in 0.1 M phosphate buffer, pH 7.0, for 3 days.

Immunization of mice. Eight-week-old male BALB/c mice were primed intraperitoneally with 5 μg of toxoid in 0.1 ml of saline emulsified in the same volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). A secondary injection was given in the same manner after 1 week. A month after the secondary injection, the third and final injections (5 μg of toxoid and 20 μg of enterotoxin, respectively) were given at a 1-week interval. A week after the final injection, the mice were bled and cell hybridization was performed. The sera from the immunized mice reached titers of 25,600 in the passive hemagglutination test (9, 22).

Production of hybridoma and preparation of MAb (4). Spleen cells (10^8) of mice were allowed to fuse, with the aid of 50% polyethylene glycol (molecular weight, 1,300 to 1,600; Sigma Chemical Co., St. Louis, Mo.), with myeloma cells, P3-NS1-1-Ag4-1 (NS-1) or SP2/0-Ag14 (SP2) (10^7), maintained in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 15% equine serum. After washing and centrifugation for 5 min at 200 × *g*, 0.1-ml portions of the cell mixture (5×10^5 cells) were placed in wells of a 96-well microplate. Hybrid cells were selected in the same medium containing HAT solution (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 10^{-5} M thymidine) and cloned by the soft-agar method (4). After transplantation of the cloned cells into the peritoneal cavity of the BALB/c mice primed with pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.), the ascitic fluid containing MAb was obtained. MAbs were purified by DEAE Affi-Gel blue (Bio-Rad Laboratories, Richmond, Calif.) chromatography (1). Polyclonal antibodies were purified by affinity chromatography on cyanogen bromide-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with the enterotoxin (22).

Enzyme-linked immunosorbent assay (ELISA). The wells of 96-well assay plates (Flow Laboratories, Inc., McLean, Va.) were each coated with 0.1 ml of the enterotoxin (10 μg/ml) for 3 h at 37°C. A sample, 0.1 ml of culture supernatant or of each purified antibody, was allowed to react for 2 h at 37°C. Peroxidase-conjugated rabbit antibody to mouse immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) was added. After each step, all wells of the plates were washed five times each with 0.25 ml of phosphate-buffered saline containing 0.05% Tween 20. Finally, substrate (0.08% 5-aminosalicylic acid–0.05% H_2O_2, 9:1) was added and the

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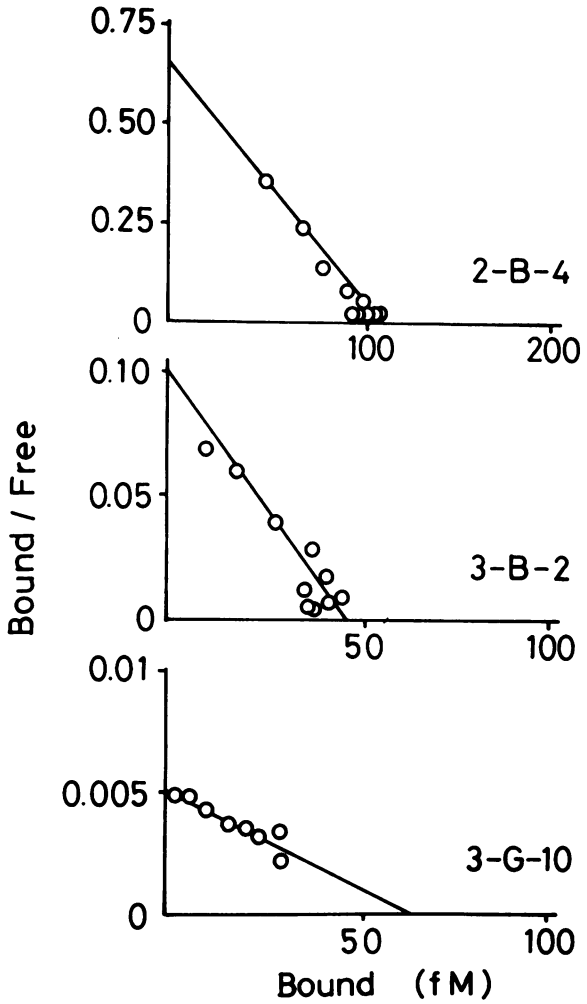

FIG. 1. Scatchard plot analyses of the binding of MAbs to ^{125}I-labeled enterotoxin. The reaction buffer (0.1 ml) and ^{125}I-labeled enterotoxin (0.1 ml, 2.9 × 10^5 cpm/μg of protein) in various concentrations were mixed with 0.1 ml of 2-B-4 (15 ng/ml), 3-B-2 (15 ng/ml), or 3-G-10 (7.5 ng/ml).

mixtures were allowed to react for 45 min at 37°C. A_{450} was determined with a microplate photometer MT-12 (Corona Electric, Tokyo, Japan).

^{51}Cr release assay. ^{51}Cr release from Vero cells was determined by the method reported before (10). A suspension of Vero cells (2.0 × 10^6/ml) was incubated with 10 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) per ml. The labeled cells were washed three times in phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} and diluted to 5.0 × 10^5 cells per ml. The enterotoxin (0.5 ml) was added to the labeled cell suspension (0.5 ml). The mixtures were incubated for 1 h at 37°C and then centrifuged for 5 min at 200 × *g* at 4°C. The supernatant (0.6 ml) was transferred to a polystyrene tube, and radioactivity was determined with an NaI well type of scintillation counter. The specific radioactivity released was calculated by subtracting the spontaneous release from the total count. The spontaneous release was measured by the same procedure in the absence of enterotoxin and MAb. The maximal radioactivity of the labeled cells was determined with samples ruptured with 0.5% saponin.

Competition between MAbs for binding to the enterotoxin. Epitope specificities of peroxidase-conjugated MAbs were determined by ELISA. Each MAb was conjugated with peroxidase (Toyobo, Osaka, Japan) by the method reported by Nakane and Kawaoi (18). After the conjugated MAbs were mixed with various amounts of unconjugated homologous or heterologous MAbs, ELISA was carried out as described above.

Inhibition of binding of enterotoxin to Vero cells. Preparation of ^{125}I-labeled enterotoxin and the binding assay were performed as reported elsewhere, with slight modifications (10). ^{125}I-labeled enterotoxin was incubated with or without MAb for 30 min at 37°C. With 0.1 ml of a Vero cell suspension containing 10^5 cells, an equal volume of the mixture was mixed in a well of a 96-well Millititer GV plate (Millipore Corp., Bedford, Mass.). The plate was incubated for 45 min at 37°C. The reaction mixture was filtered by suction. The filter membrane was rinsed five times with 0.25 ml of phosphate-buffered saline containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}. The filters were punched out and each disk was put into a polystyrene tube. The radioactivity was determined with an NaI well type of scintillation counter. The amount of labeled enterotoxin bound was determined by subtracting the count associated with the cells treated in the presence of the native enterotoxin (100 μg/ml) from the total count.

Determination of affinity of MAb for the enterotoxin. To determine the affinity for the enterotoxin, an assay for the binding of MAb to ^{125}I-labeled enterotoxin was performed. In this assay, 50 mM Tris hydrochloride buffer, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 5 mM KI, 0.1% bovine serum albumin, 0.1% Nonidet P-40, and 0.02% NaN_3 was used as the reaction buffer. MAb (0.1 ml) at an appropriate concentration was mixed with ^{125}I-labeled enterotoxin (0.1 ml) in various concentrations and the reaction buffer (0.1 ml) in a polystyrene tube. The mixtures were incubated for 30 min at 37°C and then overnight at 4°C. Then, 0.1 ml of rabbit anti-mouse immunoglobulin G (30 μg/ml), isolated by the method of Steinbach and Audran (26), was added to each tube followed by incubation overnight at room temperature. Then, 0.1 ml of a 10% Cowan I cell suspension was added to the mixture, and the resulting mixture was incubated for 20 min at room temperature. After centrifugation for 10 min at 1,000 × *g*, the radioactivity of the pellet was determined with an NaI well type of scintillation counter. Nonspecific counts were determined by the same procedure with the reaction buffer without MAb.

Other methods. Isoelectric focusing was performed by the method of Vesterberg (28). Agar gel diffusion tests were carried out to determine MAb for the immunoglobulin class and subclass with rabbit anti-mouse immunoglobulin G (Nordic Immunology, Tilburg, The Netherlands). The light chains of MAbs were examined by ELISA with anti-mouse kappa and lambda chains (Bionetics Laboratory Products, Kensington, Md.). The passive hemagglutination test was performed in the same way as described previously (22) with formalized sheep erythrocytes coupled with the enterotoxin. Protein contents were determined by the method of Lowry et al. (12).

RESULTS AND DISCUSSION

After hybridization with primed spleen cells, 192 wells of SP2 spleen cells and 152 wells of NS-1 spleen cell were tested for production of anti-enterotoxin antibody. Positive wells of hybridomas from SP2 and NS-1 were 9 and 7, respectively. After subsequent cloning, four distinct cell

lines secreting MAb were established. Two hybrid cells producing 2-B-4 and 2-H-2 were derived from SP2 myeloma and the other two, 3-B-2 and 3-G-10, were derived from NS-1. All MAbs, being nearly the same as that of polyclonal antibody in ELISA titer, were precipitated with anti-mouse immunoglobulin G1. Their light chains were all kappa chains. The pIs of MAbs were 6.36 (2-H-2), 6.40 (3-B-2), 6.32 (3-G-10), and 5.88 (2-B-4). Affinities for the enterotoxin of MAbs were determined (Fig. 1). Scatchard plot analysis found that each MAb bound ^{125}I-labeled enterotoxin in a uniform mode. The association constants (K_a) of 2-B-4, 3-B-2, and 3-G-10 were 1.91×10^9, 7.29×10^8, and 2.43×10^7 M^{-1}, respectively. The affinity of 2-H-2 was too low to determine the K_a ($<10^7$ M^{-1}).

The specificities of MAbs for the epitopes on the enterotoxin molecule were examined by ELISA, using peroxidase-conjugated MAb. The decreased ELISA titers with unconjugated MAbs from different cell lines indicate competition for an antigenic region on the enterotoxin (Fig. 2). Since the binding of conjugated 2-H-2 and 3-B-2 was inhibited with only homologous MAb, it is clear that these MAbs bind to different regions. The binding of conjugated 2-B-4 to the enterotoxin was inhibited with homologous, unlabeled 2-B-4 and also with heterologous, unlabeled 3-G-10 (Fig. 2), and that of conjugated 3-G-10 was inhibited with either unlabeled 2-B-4 or 3-G-10 (data not shown). Inhibition of the binding of either conjugated 2-B-4 or 3-G-10 with unconjugated 2-B-4 was higher than that with unconjugated 3-G-10. These results may indicate that 2-B-4 and 3-G-10 recognize those epitopes that are located close to each other on the enterotoxin molecule and the affinity of 2-B-4 is stronger than that of 3-G-10. In fact, we found that the affinity of 2-B-4 for the enterotoxin ($K_a = 1.91 \times 10^9$ M^{-1}) was stronger than that of 3-G-10 ($K_a = 2.43 \times 10^7$ M^{-1}).

Three MAbs, 2-B-4, 3-B-2, and 3-G-10, neutralized the cytotoxicity of the enterotoxin (Fig. 3). Of four MAbs, 2-B-4 was the most effective neutralizer; 3-B-2 neutralized at a lower concentration than 3-G-10. Neutralization with 3-B-2, however, was incomplete at even a high concentration, while that with 3-G-10 was complete. 2-H-2 did not neutralize cytotoxicity.

Since the binding of ^{125}I-labeled enterotoxin to Vero cells was inhibited by them, 2-B-4 and 3-G-10 may neutralize the cytotoxicity by occupying the binding region on the enterotoxin molecule (Fig. 4). One or two molecules of 2-B-4 per enterotoxin molecule completely neutralized the enter-

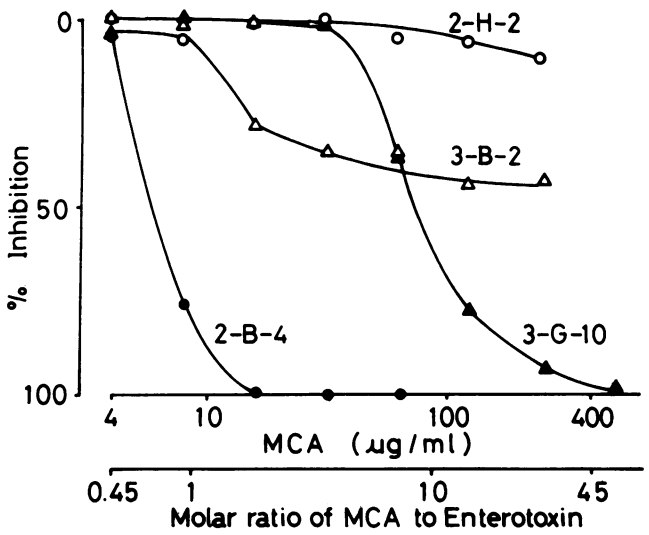

FIG. 3. Neutralizing activities of MAbs (MCA) to enterotoxin. The enterotoxin (2 μg/ml) was incubated with each MAb in various concentrations for 30 min at 37°C. The mixture (0.5 ml) was added to ^{51}Cr-labeled Vero cells (2.5×10^5/0.5 ml). The radioactivity of ^{51}Cr released was determined as described under Materials and Methods.

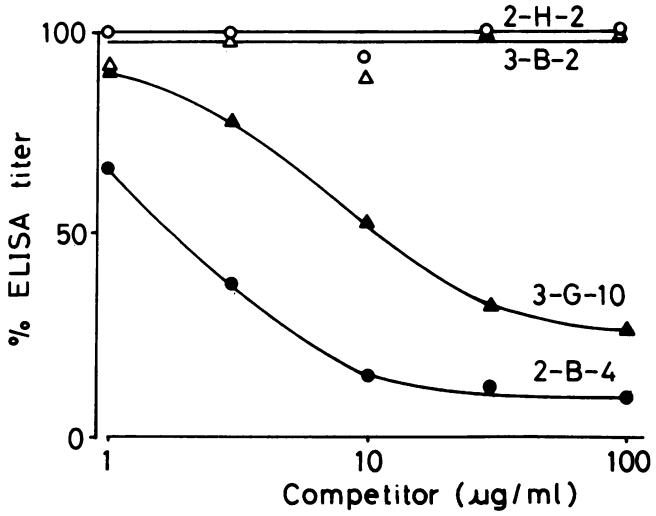

FIG. 2. Inhibition of the binding of peroxidase-conjugated 2-B-4 with unconjugated homologous and heterologous MAbs. Unconjugated MAb (0.15 ml) in various concentrations were each mixed with peroxidase-conjugated 2-B-4 (0.15 ml). The mixture (0.1 ml) was placed in a well of a microtiter plate coated with 10 μg of enterotoxin per ml. The A_{450} with no competitor was 1.285.

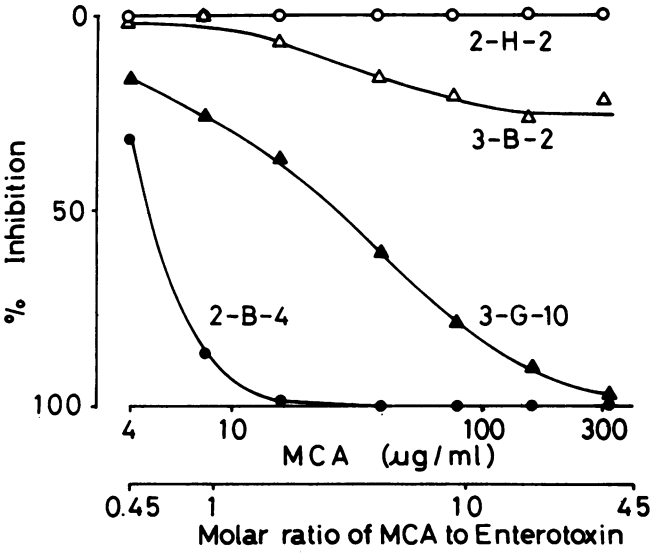

FIG. 4. Binding of the enterotoxin to Vero cells in the presence of MAb (MCA). ^{125}I-labeled enterotoxin (4 μg/ml, 0.25 ml) and various amounts of MAb (0.25 ml) were mixed, and the mixtures were incubated for 30 min at 37°C. The mixtures (0.1 ml) were each incubated with 10^5 Vero cells (0.1 ml) for 45 min at 37°C. The radioactivity of ^{125}I-labeled enterotoxin specifically bound without antibody was 2.5×10^4 cpm.

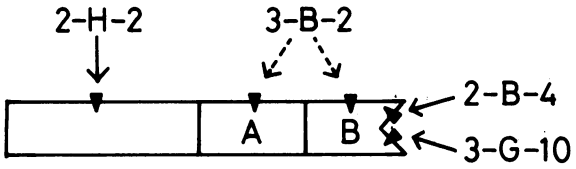

FIG. 5. Possible locations of the epitopes on *C. perfringens* enterotoxin. Antibodies 2-B-4 and 3-G-10 may occupy the receptor-binding region. 3-B-2 may bind the active site on the enterotoxin or another site located close to the binding site. The antigenic site that 2-H-2 recognizes is not associated with the enterotoxin action. A, Proposed active site; B, binding site.

otoxin in both binding and cytotoxicity. It seems that one molecule may completely occupy the binding region of the enterotoxin. As the amount of 3-G-10 was increased, the binding was more strongly inhibited until it was completely inhibited with 20 to 40 molecules per enterotoxin molecule; inhibition of cytotoxicity in response to the dose of 3-G-10 was steeper. The higher affinity of 2-B-4 (K_a = 1.91 × 10^9 M^{-1}) than that of 3-G-10 (K_a = 2.43 × 10^7 M^{-1}) might explain the fact that 10-fold more 3-G-10 than 2-B-4 was required to inhibit binding. When inhibition of the binding of the enterotoxin to Vero cells by 2-B-4 and 3-G-10 was higher than 50%, neutralization of the cytotoxicity became apparent. This observation may indicate that there is a certain threshold of binding inhibition (about 50%) for appearance of neutralization of cytotoxicity.

It seems that 3-B-2 neutralizes the cytotoxicity differently than 2-B-4 and 3-G-10 do. At a lower concentration, 3-B-2 neutralized the cytotoxicity of the enterotoxin more effectively than did 3-G-10 (Fig. 3). 3-B-2 with affinity comparable to that of 2-B-4 did not neutralize completely at any concentration, while 3-G-10 did so completely. When 100 μg of 3-B-2 per ml was incubated with an equal volume of the enterotoxin (1 μg/ml), 82.9% inhibition of ^{51}Cr release was observed (data not shown). More work is necessary to explain why 3-B-2 only incompletely blocks the cytotoxicity.

Inhibition of the binding of the enterotoxin by 3-B-2 never exceeded 25% (Fig. 4). It seems that inhibition of the binding by 3-B-2 may not be the basis for neutralization of the cytotoxicity. Granum (5) has suggested that the enterotoxin molecule consists of at least two different functional portions: the binding and active sites. 3-B-2 may directly bind to such an active site of the enterotoxin and neutralize it. Alternatively, 3-B-2, recognizing the nearby region to the binding site, may prevent the second step of the action of the enterotoxin by changing its conformation.

2-H-2 inhibited neither the binding nor the cytotoxicity (Fig. 3 and 4). It may recognize the region of the enterotoxin responsible for neither the binding nor the cytotoxicity. Alternatively, since the affinity of 2-H-2 for the enterotoxin was too low to determine the K_a (<10^7 M^{-1}), it may be liberated from the site recognized when the enterotoxin binds to Vero cells.

Binding to a single site on an antigen molecule, MAb is useful for studying the relationship between structure and function of the enterotoxin. From the present results, a possible orientation of the epitopes on the enterotoxin molecule is presented in Fig. 5. MAbs can be produced against several different sites of the enterotoxin: the binding site for the target cells, the active site, and other sites. To verify the speculation as illustrated in Fig. 5, we are now continuing to prepare more MAbs to study the structure and function of the entertoxin.

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