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Production and Characterization of Monoclonal Antibodies against *Clostridium perfringens* Type A Enterotoxin

ANDREW P. WNEK, ROBERT J. STROUSE,† AND BRUCE A. McCLANE*

Department of Microbiology, Biochemistry, and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Received 6 June 1985/Accepted 7 August 1985

Hybridomas secreting monoclonal antibodies (MABs) specific for *Clostridium perfringens* type A enterotoxin were produced by fusion of P3X63Ag8.653 myeloma cells with spleen cells from BALB/c mice immunized with purified enterotoxin. Wells containing hybridomas secreting immunoglobulin G (IgG) antibodies against enterotoxin were specifically identified by an indirect enzyme-linked immunosorbent assay (ELISA), and 10 ELISA-positive hybridomas were selected and cloned twice by limiting dilution. All 10 hybridomas produced MABs containing immunoglobulin G1 heavy chains and kappa (κ) light chains. These hybridomas were then grown as ascitic tumors in mice, and MABs were purified from the ascites fluids with DEAE Affi-gel blue. The specificity of the MABs for enterotoxin was demonstrated by immunoblotting and ELISA. Competitive radioimmunoassay with ^{125}I-MABs suggests that these MABs recognized at least four epitopes on the enterotoxin molecule. The enterotoxin-neutralizing ability of MABs from both hybridoma culture supernatants and ascites fluids was assessed by using a ^{3}H-nucleotide-release Vero (African green monkey kidney) cell assay. Only 2 of the 10 hybridomas produced MABs which completely (>90%) neutralized the biologic activity of enterotoxin. Preincubation of ^{125}I-enterotoxin with MABs demonstrated that MAB neutralizing ability correlated with MAB-specific inhibition of specific binding of enterotoxin to intestinal brush border membranes.

Clostridium perfringens type A food poisoning is one of the most common foodborne diseases in the United States (27). The factor responsible (30) for the characteristic disease symptoms (diarrhea and abdominal cramps) of *C. perfringens* food poisoning has been shown (3, 32) to be a protein enterotoxin composed of a single polypeptide chain (M_r, 35,000). *C. perfringens* enterotoxin is produced in significant quantities only by sporulating cells (4).

C. perfringens enterotoxin possesses a unique mode of action which is distinguishable from that of other enterotoxins. The initial interaction between this enterotoxin and eucaryotic cells involves specific binding of the enterotoxin to a membrane receptor(s) (20, 23). In rabbit intestinal brush border membranes this receptor may be a protein of M_r 50,000 (35). After binding to its receptor(s), enterotoxin rapidly induces changes in plasma membrane permeability through a breakdown of the colloid-osmotic equilibrium (16). This disruption causes a size-dependent loss of intracellular material (18), which affects vital metabolic processes such as macromolecular synthesis (17), and eventually these alterations in membrane permeability are lethal (17, 24).

Monoclonal antibodies (MABs) are invaluable probes for studying structure-function relationships for toxin molecules, and MABs have been prepared against other enterotoxins (10) and clostridial toxins (12, 26, 28). We now report production and characterization of MABs against *C. perfringens* type A enterotoxin. MABs against *C. perfringens* enterotoxin should be useful for both basic enterotoxin research and applied purposes such as immunodiagnostic detection.

(A preliminary report of these experiments has been presented [A. P. Wnek, R. J. Strouse, C. S. Park, and B. A. McClane, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B127, p. 39].)

MATERIALS AND METHODS

Bacterial strains. *C. perfringens* NCTC 8239 and FD-1 were used as enterotoxin-positive and enterotoxin-negative strains, respectively (19).

Enterotoxin. Electrophoretically pure enterotoxin was prepared from *C. perfringens* NCTC 8239 by the method of Granum and Whitaker (8).

Immunization procedures. BALB/c mice were immunized with purified enterotoxin by the immunization protocol of Stahli et al. (31). BALB/c mice received an intraperitoneal injection of 200 μl containing 1 μg of enterotoxin in an adjuvant solution prepared with 100 μl of 0.85% saline and 100 μl of Freund complete adjuvant (GIBCO Laboratories). Each mouse then received two booster injections (200 μl intraperitoneally, containing 2 μg of enterotoxin in adjuvant solution prepared with 100 μl of saline and 100 μl of Freund incomplete adjuvant), which were administered at 3-week intervals. A week after the second booster, the mice were bled and tested by immunodiffusion for antibody production against enterotoxin. Three weeks after being bled, mice with detectable antibody titers received a series of four daily injections (100 μl, intraperitoneally). The initial injection in this series contained 30 μg of enterotoxin in saline, while the remaining three injections contained 20 μg of enterotoxin in saline. One day after the fourth injection, mice were sacrificed for hybridoma production.

Fusion of spleen and myeloma cells. P3X63Ag8.653 (repository number GM 3570B) murine plasmacytoma cells were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.J., and were routinely maintained in Dulbecco minimal essential medium contain-

* Corresponding author.
† Present address: Microbiological Associates, Bethesda, MD 20816.

ing 10% fetal calf serum (M.A. BioProducts) or 10% Zetasera D (AMF Industries).

Hybridomas were produced by fusion of immune spleen cells with P3X63Ag8.653 myeloma cells as described by Fazekas de St. Groth et al. (5). Fused cells were washed with Dulbecco minimal essential medium and suspended in 80 ml of HAT medium (Dulbecco minimal essential medium supplemented with 10% NCTC 109, 10% fetal calf serum, 2 mM glutamine, and hypoxanthine-aminopterin-thymidine (HAT) prepared as described by Kennett [13]). The cell suspension was then distributed (100 μl per well) among eight 96-well microplates (Corning Glass Works) which had previously been seeded with peritoneal macrophage feeder cells (5).

Screening and cloning of hybridoma cell lines. After 2 weeks, supernatants (100 μl) from cultures containing viable cells were screened for the presence of antibodies against *C. perfringens* enterotoxin (0.5 μg per Immulon II well) by an indirect enzyme-linked immunosorbent assay (ELISA) (19). Release of *p*-nitrophenol was measured spectrophotometrically at 405 nm in an ELISA reader (Bio-Tek Instruments). Negative controls (optical density at 405 nm, <0.050) were obtained by coating the wells with (i) bovine serum albumin (0.1 to 1 μg per well) or (ii) *C. perfringens* α toxin (0.5 μg per well; Sigma Chemical Co.) in place of enterotoxin.

Cloning of hybridomas producing antibodies against enterotoxin was carried out by subculturing at limiting dilutions in HT medium (HAT medium without aminopterin) as described previously (13), except that peritoneal macrophage feeder cells were substituted for irradiated thymocyte feeders. Subcloning of ELISA-positive wells was repeated after ELISA screening, and 10 ELISA-positive cloned hybridoma lines from eight different fusions were then expanded in HT medium and preserved by freezing in liquid nitrogen. Radial immunodiffusion (10) was used to determine mouse immunoglobulin G (IgG) concentrations in hybridoma supernatants.

Production of purified MABs. Pristane-primed BALB/c mice each received an intraperitoneal injection of 1×10^7 to 2×10^7 hybridoma cells. Approximately 2 weeks later, ascites fluids were collected and cells were removed by centrifugation at 5,000 × *g* for 10 min at 4°C. The supernatant was then subjected to ultracentrifugation in a type 65 rotor (Beckman Instruments, Inc.) at 34,000 rpm for 30 min at 4°C. The middle layer (pristane-free supernatant) was collected and dialyzed overnight against 0.2 M Tris (pH 7.2). The dialyzed sample was then centrifuged (10,000 × *g* for 15 min at 4°C.), and 1-ml aliquots of the supernatant were chromatographed on DEAE Affi-gel blue (2) to purify the MABs. After purification, MABs (7.5 μg per well) were substituted for hybridoma supernatant in the ELISA described above to test for retention of immunoreactivity.

Purity of the monoclonal IgG was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (14) with 10% polyacrylamide slab gels containing 0.1% SDS.

Ranking of relative MAB affinities. The relative affinities of MABs were determined as described by Van Heyningen et al. (34), except that indirect ELISA (19) was substituted for radioimmunoassay (RIA) to determine plateau binding levels of MABs to enterotoxin (0.5 μg per Immulon II well).

Demonstration of purified MAB specificity. Two methods were used to demonstrate MAB specificity. (i) Purified MABs (7.5 μg per well) were assayed by ELISA for specificity versus several purified proteins. Wells were treated overnight at 4°C with the desired protein (0 to 1 μg of *C. perfringens* enterotoxin, BSA, or *C. perfringens* α toxin per well). (ii) *C. perfringens* NCTC 8239 (enterotoxin positive) and FD-1 (enterotoxin negative) were grown under sporulating conditions for 14 h at 37°C (19). After centrifugation to remove most spores, culture supernatants were filtered (EGWP filter, pore size, 0.22 μm; Millipore Corp.) and concentrated 10-fold by lyophilization.

Immunoblotting was performed with the following antigenic samples: (i) supernatant (100 μg of protein) from *C. perfringens* NCTC 8239; (ii) supernatant (100 μg of protein) from *C. perfringens* FD-1; and (iii) purified *C. perfringens* enterotoxin (0.2 μg of protein). After electrophoresis on 7% native gels, the separated proteins were blotted overnight onto nitrocellulose paper (Millipore) by diffusion. After blotting, the nitrocellulose sheets were washed with Tris-buffered saline (20 mM Tris hydrochloride, 500 mM NaCl [pH 7.5]) and blocked overnight at room temperature with Blotto (11) (5% nonfat dry milk, 0.01% antifoam A in Tris-buffered saline). The nitrocellulose sheets were then rewashed, cut into strips, and incubated for 2 h with MABs or control polyclonal antiserum, as indicated. Primary antibodies used in immunoblotting included (i) culture supernatant (a 1:1,000 dilution) from hybridoma VPL-1 (this hybridoma produces MABs directed against chicken neural retina protein and was kindly provided by Vance Lemmon, Department of Anatomy and Cell Biology, University of Pittsburgh School of Medicine) as a negative control, (ii) purified MABs (2 to 5 μg/ml), (iii) mouse polyclonal antiserum (PET; a 1:1,000 dilution) prepared against purified *C. perfringens* enterotoxin, (iv) mouse polyclonal antiserum (a 1:250 dilution) prepared against *C. perfringens* NCTC 8239 supernatant ($PETS^+$), or (v) mouse polyclonal antiserum (a 1:250 dilution) prepared against *C. perfringens* FD-1 supernatant ($PETS^-$). The blots were then incubated with secondary antibody (goat anti-mouse IgG conjugated with horseradish peroxidase; Kirkegaard and Perry) and developed with 4-chloro-1-naphthol as the peroxidase substrate.

Competitive RIA analysis of MAB binding. Purified MABs 2B12, 3C9, 5D6, 10G6, and 11H8 (500 μg) were ^{125}I-labeled by using lactoperoxidase beads (22), while MABs 1G7 and 14A7 (500 μg) were radioiodinated with Bolton-Hunter reagent (1). $Na^{125}I$ and Bolton-Hunter reagent were purchased from ICN Pharmaceuticals Inc., and lactoperoxidase beads were obtained from Bio-Rad Laboratories. Iodinated MABs retained immunoreactivity and specificity for enterotoxin as determined by ELISA and RIA.

Epitope diversity of MABs was examined by a labeled-unlabeled antibody RIA competition assay (28). Enterotoxin was dissolved in phosphate-buffered saline (PBS) at a concentration of 2.5 μg/ml and aliquoted (100 μl per well) into wells of Immulon II Removawell strips. After incubation overnight at 4°C, the wells were washed and blocked as described for the ELISA. Half the wells were then treated with an excess (10 μg per well) of the specified unlabeled MAB in PBS–0.05% Tween 20 for 2 h at 37°C; the remaining wells were incubated for 2 h with only PBS–0.05% Tween 20. After the wells were washed, iodinated MABs were then added to the wells. Wells preincubated with unlabeled MAB received ^{125}I-MAB (0.25 μg per well) plus a 40-fold excess of the same unlabeled MAB used for preincubation, while wells preincubated only with PBS–Tween 20 received ^{125}I-MAB alone. After ^{125}I-MAB binding, the wells were washed five times as described above for the ELISA; the wells were then separated and binding was determined with a Packard gamma counter. Inhibition with unlabeled homologous MAB provided a positive control for each ^{125}I-MAB. Nonspecific

binding of ^{125}I-MABs was corrected for by subtracting counts per minute bound to wells receiving *C. perfringens* α toxin (0.25 μg per well).

Determination of MAB neutralization abilities. The inhibition by MABs of enterotoxin-induced release of ^{3}H-labeled nucleotides from treated Vero (African green monkey kidney) cells (16, 18) was used to assay neutralizing properties of MABs. Vero cells were inoculated into 16-mm wells in tissue culture cluster dishes (CoStar) at a seeding density of 5×10^4 cells in 3 ml of medium 199 supplemented with 5% newborn calf serum. After 3 days, fresh medium was added. Confluent 6-day-old monolayers containing 6×10^5 to 8×10^5 cells per well were washed with medium 199 without serum and labeled by addition of 2 ml of medium 199 containing 2 μCi of [^{3}H]uridine (42 mCi/mmol; ICN). After 2 h (when the cells contained approximately 70% of the total cytoplasmic label in a nucleotide form [18]), the labeling medium was removed and the labeled cells were washed with medium 199 without serum.

Enterotoxin (2.5 μg) was incubated in 2 ml of medium 199 without serum for 15 min at 37°C with 10 or 100 μg of a purified MAB. This preincubated enterotoxin was then added to a well of ^{3}H-nucleotide-labeled cells. After 30 min at 37°C, the culture supernatant was gently removed, vortexed, and centrifuged at 4°C for 3 min in an Eppendorf microcentrifuge. Radioactivity was measured in supernatant samples (0.2 ml) in a Searle Mark III liquid scintillation spectrophotometer.

Released radioactivity was calculated as described by Thelestam and Möllby (33): percent maximal release = [(toxin-induced release − spontaneous release)/(maximal release − spontaneous release)] × 100. Spontaneous release from labeled cells after 30 min at 37°C was less than 5% of maximal release. As described previously (16, 18), the maximal release of cytoplasmic label was determined after cell membrane rupture by the addition of 1 ml of 1 M citric acid and 1 ml of a 0.5% saponin buffer per well. Maximal release was 1×10^5 to 2×10^5 cpm per culture.

Effects of MAB preincubation on binding of enterotoxin to intestinal membranes. *C. perfringens* enterotoxin was radioiodinated by the method of McDonel and Demers (22) with lactoperoxidase (Bio-Rad). ^{125}I-enterotoxin retained serologic and biologic activity after radioiodination. Brush border membranes were prepared from the small intestines of female New Zealand White rabbits by the method of Sigrist et al. (29).

MABs (2 μg) were preincubated with ^{125}I-enterotoxin (0.4 μg; 75,000 cpm) in 40 μl of PBS for 15 min at 37°C. This mixture was then added to 200 μg of membranes in 0.5 ml of PBS and incubated for 1 h at 24°C. The membranes were centrifuged for 5 min in a microcentrifuge and washed twice with 0.5 ml of PBS. After recentrifugation, the pellets were counted in a Packard gamma spectrophotometer. Nonspecific binding was determined by incubating ^{125}I-enterotoxin with membranes in the presence of an excess of unlabeled enterotoxin. Specific binding was calculated by subtracting nonspecifically bound counts from the counts associated with membranes not cotreated with unlabeled enterotoxin.

Protein determination. Bovine serum albumin was used as the standard for protein determination by the method of Lowry et al. (15).

RESULTS

Ten hybridomas secreting antienterotoxin MABs from eight separate fusions were selected and stabilized in vitro. Culture supernatant from these hybridomas contained 40 to 160 μg of mouse IgG per ml. After injection into pristane-primed mice, 9 of the 10 hybridomas produced significant amounts of ascitic fluid. Only hybridoma 6H9 did not grow well in mice and gave poor yields of ascitic fluid.

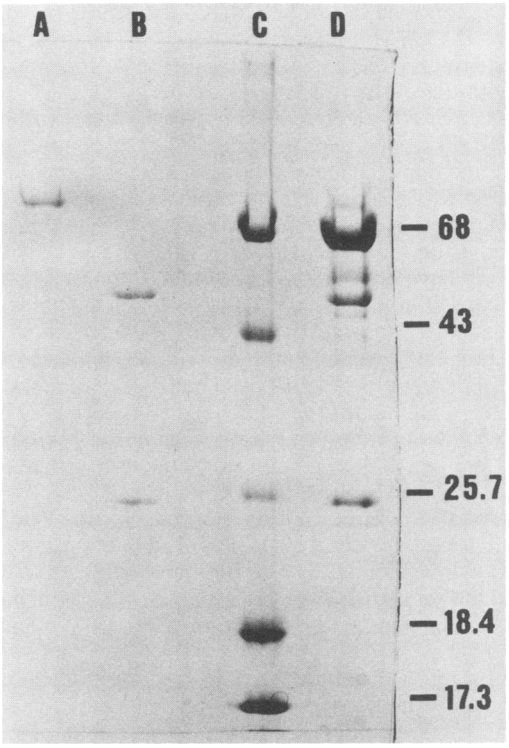

FIG. 1. SDS-polyacrylamide gel electrophoresis analysis of MAB purification by DEAE Affi-gel blue chromatography. SDS-polyacrylamide gel electrophoresis was carried out at 30 mA until the tracking dye reached the bottom of the gel; the gel was then stained with Coomassie brilliant blue. Lanes: (A) first chromatographic peak (transferrin; 2 μg of protein); B, MAB 3C9 (4 μg of protein) after purification; C, M_r markers (Bethesda Research Laboratories); D, 20 μg of protein of crude ascites fluid (from a mouse receiving 3C9 cells) before DEAE Affi-gel blue chromatography.

Since we were interested in eventually radioiodinating MABs, the MABs in ascitic fluids were purified by DEAE–Affi-gel blue chromatography. Representative purification results for MAB 3C9 are shown in Fig. 1; DEAE Affi-gel blue chromatography yielded MABs of >95% purity as determined by densitometric scanning. Purified MABs were tested for retention of immunoreactivity with enterotoxin by ELISA (Table 1). MABs from eight hybridomas retained high immunoreactivity after purification; however, hybridomas 7C6 and 13B2 produced MABs which had low immunoreactivity after the purification procedure. These results indicate that hybridomas 1G7, 2B12, 3C9, 5D6, 10G6, 11H8, and 14A7 produced large amounts of MABs with high immunoreactivity after purification, and MABs from these seven hybridomas were used extensively throughout these studies; MABs from hybridomas 6H9, 7C6, and 13B2 were used only as noted.

A ranking of the relative affinities of the purified MABs for enterotoxin is also shown in Table 1. The MABs can be classified into three categories: high affinity (relative affinity of ≤0.2 μg, including MABs 3C9, 5D6, and 11H8), moderate affinity (relative affinity of ≥0.2 μg but ≤1 μg, including MABs 1G7, 2B12 and 10G6), and low affinity (relative

TABLE 1. Comparative ELISA reactivities of MABs after purification

MAB[a]	ELISA reactivity[b] (OD_{405})	Relative affinity[c]
1G7	0.597 ± 0.073	1.10
2B12	0.771 ± 0.063	0.50
3C9	0.683 ± 0.053	0.03
5D6	0.789 ± 0.103	0.15
6H9	0.454 ± 0.030	2.10
7C6	0.253 ± 0.025	4.10
10G6	0.533 ± 0.056	0.80
11H8	0.710 ± 0.095	0.06
13B2	0.307 ± 0.137	5.20
14A7	0.494 ± 0.97	1.90

[a] The antibody subtype was kappa IgG1 for all MABs.
[b] Results presented represent specific ELISA values (MAB ELISA values corrected for background binding of 7.5 μg of nonimmune mouse IgG per well). OD_{405}, Optical density at 405 nm. Values shown represent mean ± standard error of the mean. Results shown were obtained from at least four test samples.
[c] Defined (34) as the antibody concentration at which approximately 50% of plateau binding occurs.

affinity of ≥1 μg, including MABs 6H9, 7C6, 13B2, and 14A7) for enterotoxin. The low immunoreactivities of MABs 6H9, 7C6, and 13B2 may be at least partially due to their low affinity for enterotoxin.

All 10 hybridomas produced MABs containing IgG1 heavy chains and kappa (κ) light chains. Identical subtyping results were obtained whether hybridoma culture supernatants or purified MABs were used as the antibody source for the subtyping ELISA.

To demonstrate specificity of the MABs for *C. perfringens* enterotoxin, both ELISA and immunoblotting techniques were used. All 10 purified MABs and hybridoma supernatants showed ELISA specificity for enterotoxin (data not shown) versus two control proteins (*C. perfringens* α toxin and bovine serum albumin). Immunoblotting results are shown in Fig. 2 for purified MAB 3C9, again as a representative MAB. MAB 3C9 reacted with purified enterotoxin and a comigrating band in culture supernatant fluid from a sporulating enterotoxin-positive strain of *C. perfringens*, but did not react with culture supernatant from a sporulating enterotoxin-negative strain of *C. perfringens*. Polyclonal antiserum from a mouse immunized with enterotoxin reacted with a comigrating supernatant band from the enterotoxin-positive strain, as did MAB 3C9. Neither MAB 3C9 nor mouse polyclonal antienterotoxin reacted on immunoblots with bovine serum albumin, ovalbumin, urease, lactalbumin, or carbonic anhydrase (data not shown). MAB VPL-1, which is directed against chicken neural retina protein, did not react with enterotoxin or *C. perfringens* culture supernatants. These immunoblotting results indicate that MAB 3C9 reacted specifically with enterotoxin. Identical immunoblotting results were obtained with all other purified MABs or hybridoma culture supernatants (data not shown).

Competitive RIA analysis (Table 2) was used for a preliminary study of MAB epitope diversity. The seven purified MABs which could be obtained in large amounts while also retaining high immunoreactivity were radioiodinated. MABs 2B12, 3C9, 5D6, 10G6, and 11H8 retained ELISA immunoreactivity after radioiodination by lactoperoxidase; MABs 1G7 and 14A7 lost immunoreactivity when radioiodinated by the lactoperoxidase technique and were ^{125}I-labeled by the Bolton-Hunter procedure (1). Note (Table 2) that all ^{125}I-MABs were significantly (>60%) inhibited by unlabeled homologous MAB. These results suggested that MABs 1G7 and 10G6 and MABs 2B12, 3C9, and 5D6 appear to recognize the same, or closely adjacent, epitopes. Therefore, the seven MABs tested appear to recognize at least four distinct epitopes on the enterotoxin molecule.

The ability of purified MABs to inhibit the biologic activity of enterotoxin is shown in Table 3. MABs belonged to two distinct classes, based on neutralizing abilities. MABs 2B12 and 3C9 completely neutralized (>95% neutralization) enterotoxin when preincubated for 15 min at equimolar ratios with enterotoxin. The remaining eight purified MABs did not significantly inhibit (<15% neutralization) enterotoxin biologic activity at equimolar ratios (or even if a 10-fold antibody excess was added [data not shown]). Neutralization studies conducted by preincubating enterotoxin with hybridoma culture supernatants showed a complete correlation with the purified MAB studies; only culture supernatants from hybridomas 2B12 and 3C9 showed neutralization (>95% neutralization with undiluted culture supernatant). The remaining supernatants had no significant (<15% neutralization) neutralizing ability during purification. Control results presented in Table 3 with mouse poly-

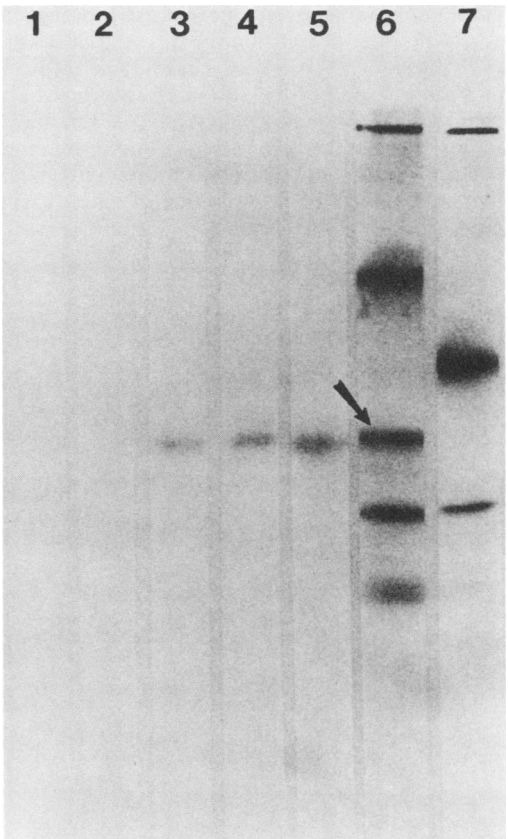

FIG. 2. Immunoblot determination of MAB specificity. Lanes: 1, VPL-1 supernatant reacted with *C. perfringens* NCTC 8239 supernatant; 2, purified MAB 3C9 reacted with *C. perfringens* FD-1 supernatant; 3, purified MAB 3C9 reacted with *C. perfringens* NCTC 8239 supernatant; 4, PET reacted with purified enterotoxin; 5, PET reacted with *C. perfringens* NCTC 8239 supernatant; 6, $PETS^+$ reacted with *C. perfringens* NCTC 8239 supernatant; 7, $PETS^-$ reacted with *C. perfringens* FD-1 supernatant.

TABLE 2. Competitive RIA analysis of MAB epitope diversity

MAB	% inhibition of specific binding for ^{125}I-MAB[a]:						
	1G7	2B12	3C9	5D6	10G6	11H8	14A7
1G7	**99.3**	<1	<1	6.7	77.3	<1	8.3
2B12	<1	**91.6**	94.2	95.8	<1	<1	<1
3C9	<1	94.4	**86.8**	94.5	<1	<1	3.6
5D6	2.8	69.1	100	**93.9**	<1	<1	27.2
10G6	100	<1	<1	1.7	**77.2**	<1	4.5
11H8	<1	2.5	<1	10.9	<1	**64.1**	13.8
14A7	7.7	1.3	<1	10.5	<1	<1	**89.5**

[a] Standard error of the mean was less than 10% for all values. Results shown were obtained from at least four test samples. Boldface numbers show inhibition by homologous antibody.

clonal antienterotoxin, myeloma culture supernatant, and VPL-1 supernatant indicate the appropriateness of the biologic activity assay.

To determine whether any of the MABs were directed against epitopes at or near the receptor-binding region of the enterotoxin, the ability of purified MABs to block enterotoxin binding to intestinal brush border membranes was assessed (Table 4). When preincubated for 15 min with radiolabeled enterotoxin, only MABs 2B12 and 3C9 strongly blocked (>85% inhibition) specific binding of ^{125}I-enterotoxin. The remaining MABs had little significant (<20% inhibition) effect on enterotoxin binding. Similar results were obtained if hybridoma supernatants were substituted for purified MABs (data not shown). Mouse polyclonal antienterotoxin strongly inhibited enterotoxin binding (>95% inhibition), while normal mouse serum or VPL-1 supernatant had no effect on ^{125}I-enterotoxin binding, indicating a requirement for antibodies directed specifically against enterotoxin to obtain inhibition of enterotoxin binding in this assay.

TABLE 3. Effect of MABs on biologic activity[a] *of C. perfringers* entertoxin

MAB[b] or control	% neutralization[c]
Neutralizing MABs	
2B12	99.0 ± 1.9
3C9	97.1 ± 4.9
Nonneutralizing MABs	
1G7	5.4 ± 2.6
5D6	2.5 ± 2.5
6H9	8.8 ± 1.5
7C6	4.2 ± 3.5
10G6	11.7 ± 4.4
11H8	13.4 ± 2.5
13B2	9.5 ± 5.3
14A7	10.9 ± 1.7
Controls (μl/2 ml)	
Mouse polyclonal antienterotoxin (25)	95.8 ± 3.8
Myeloma P3X63Ag8.653 supernatant (2,000)	3.8 ± 2.0
Monoclonal VPL-1 supernatant (2,000 μl, 350 μg of IgG)	1.2 ± 0.9

[a] As determined by ^{3}H-nucleotide release (16,18).
[b] Concentration of purified MABs was 5 μg/2 ml of incubation medium.
[c] Percent neutralization = 1 − (percentage of maximal release after MAB preincubation/percentage of maximal release without MAB incubation) × 100. Percent neutralization values represent the mean ± standard error of the mean from at least four test samples.

DISCUSSION

This report describes the production and characterization of MABs specific for *C. perfringens* type A enterotoxin. Some MABs completely neutralized enterotoxin biologic activity as measured by a membrane permeability alteration assay. Since the neutralizing MABs were shown to be highly specific for enterotoxin, this strongly suggests that enterotoxin (rather than a highly active minor contaminant, such as α toxin, possibly present in the enterotoxin preparation) is the permeability-altering factor in purified enterotoxin preparations. This important observation supports previous proposals (16–18) that the primary action of *C. perfringens* enterotoxin involves induction of plasma membrane permeability changes, leading to changes in intracellular ion and metabolic precursor levels.

It was also observed that MABs which blocked specific binding of enterotoxin also neutralized enterotoxin biologic activity. Interestingly, nonspecific binding levels (data not shown) were not affected by preincubation of enterotoxin with neutralizing MABs, suggesting that nonspecific binding is of little importance for obtaining the biologic action of enterotoxin. Collectively, these results support earlier studies (23) which suggested that specific binding of enterotoxin is required for expression of enterotoxin action.

MABs are recognized as valuable probes for identifying structure-function relationships for toxin molecules. Relatively little is known about the *C. perfringens* enterotoxin

TABLE 4. Effect of MABs on ^{125}I-enterotoxin binding to brush border membranes

MAB	% specific binding[a]
Neutralizing MABs	
2B12	13.4 ± 4.3
3C9	4.2 ± 2.0
Nonneutralizing MABs	
1G7	109.9 ± 12.2
5D6	81.9 ± 9.9
6H9	ND[b]
7C6	134.3 ± 9.6
10G6	104.9 ± 6.1
11H8	112.9 ± 10.0
13B2	106.3 ± 5.6
14A7	109.8 ± 5.1

[a] Percent specific binding = (specific binding after MAB preincubation/specific binding without MAB preincubation) × 100. Values represent the mean ± standard error of the mean from at least four test samples.
[b] ND, Not determined.

molecule; it appears to be a single polypeptide chain of about M_r 35,000 (3, 32), and the amino acid sequence of enterotoxin has recently been determined (3, 25). There is some evidence suggesting that enterotoxin can be proteolytically-activated with trypsin (9) or Clostripain (L. K. Duffy, A. Kurosky, and B. A. McClane, unpublished observation). Precise assignments of locations such as biologic activity, specific binding, or epitope sites on the enterotoxin molecule have not been reported. The present MAB studies begin to approach this problem.

Competitive RIA analysis with MABs suggested that *C. perfringens* enterotoxin contains at least four epitopes. Considering its size (M_r 35,000), additional epitopes probably are present on the enterotoxin. Experiments are in progress to generate a wider diversity of MAB epitope specificities; these additional MABs will be used with competitive RIA and epitope mapping studies to better define the upper limit of enterotoxin epitopes.

Only 2 of our 10 MABs inhibited specific binding of enterotoxin to intestinal membranes. Since several high-affinity MABs did not inhibit binding, this indicates that specific binding of enterotoxin to its membrane receptor involves a defined region of the enterotoxin molecule. MABs 2B12 and 3C9 should be useful probes for identifying the region(s) of enterotoxin associated with binding. Similarly, these MAB studies also demonstrate that high- or moderate-affinity MABs to several epitopes do not neutralize enterotoxin action, suggesting that biologic action of enterotoxin is also confined to a defined region(s) of the enterotoxin. These results are similar to MAB studies (6) with *C. perfringens* θ toxin and streptolysin O, which suggested that nonneutralizing epitopes are commonly encountered on membrane-active toxins (as well as other toxins [10]).

An important question not resolved by the present data concerns whether the binding and biologic activity sites on the enterotoxin molecule are the same. To show that these sites are not overlapping, it is necessary to demonstrate antibody-mediated neutralization of enterotoxin without affecting specific binding of enterotoxin to sensitive membranes. None of the MABs produced in this study had such activity, indicating that either such MABs cannot be produced (implying that biologic and binding activity occur at the same site on enterotoxin) or insufficient numbers of hybridomas were produced to obtain such a MAB. Resolution of this issue is critical to understanding enterotoxin action and will elucidate such questions as whether binding leads directly to biologic action in susceptible cells and what the molecular action of enterotoxin entails.

To better clarify this important relationship between enterotoxin binding and biologic action, two approaches appear useful. First, additional fusions are being carried out to produce greater MAB diversity. Second, experiments have been performed to determine whether polyclonal anti-enterotoxin could inhibit biologic activity of prebound enterotoxin. Similar studies (6) with MABs against *C. perfringens* θ toxin have suggested that this membrane-active toxin has two sites (a lytic and a binding site). Enterotoxin was bound to ^{3}H-nucleotide-labeled Vero cells for 5 min, unbound enterotoxin was washed away, and fresh medium containing neutralizing concentrations (25 μl/2 ml, as determined previously in Table 3) of polyclonal rabbit anti-enterotoxin was added to these cells. If the polyclonal antiserum neutralized enterotoxin already bound to its receptor, this would suggest that the binding and biologic sites were not the same. However, no inhibition of biologic activity (measured by either ^{3}H-nucleotide release or prevention of enterotoxin-induced morphologic alterations) was observed. This indicates (i) that the binding-biologic sites on enterotoxin are overlapping, (ii) that binding to the membrane receptor sterically limits access of polyclonal antibodies to a separate biologic activity site on enterotoxin, (iii) that the biologic activity site is not readily accessible to antibodies or is poorly antigenic, or (iv) that enterotoxin becomes inaccessible to antibodies immediately after its binding, perhaps owing to membrane insertion (21).

It is interesting that MAB 5D6 competes with MABs 2B12 and 3C9 by competitive RIA but, unlike these MABs, has no significant effect on enterotoxin binding or biologic activity. Since MAB 5D6 has a higher relative affinity for enterotoxin than does MAB 2B12 and a 10-fold increase in MAB 5D6 concentration still does not neutralize enterotoxin biologic activity, it appears that these results cannot be explained simply as differences in affinity. One possible explanation for these results involves the recognition by MAB 5D6 of an epitope located near, but distinct from, the binding region of enterotoxin recognized by MABs 2B12 and 3C9. In this situation, prebound MAB 5D6 sterically hinders access of MABs 2B12 and 3C9 to their epitopes on enterotoxin; however, the enterotoxin epitope recognized by MAB 5D6 is not close enough to the receptor-binding region of enterotoxin to also block binding and biologic activity. Precise definition of the relationship between epitopes recognized by MAB 5D6 versus MABs 2B12 and 3C9 should be further resolved by future epitope mapping studies.

MABs produced in this study appear to have specificity for enterotoxin with respect to other *C. perfringens* supernatant proteins. However, it is important to note that Frieben and Duncan (6, 7) have reported that some enterotoxin-positive and -negative strains of *C. perfringens* produce spore coat proteins which serologically cross-react with enterotoxin. Since our preparative scheme for immunoblotting-ELISA determination of MAB specificity involved the use of both centrifugation and filtration to remove spores, no cross-reactive spore coat proteins were observed in either the enterotoxin-positive or -negative *C. perfringens* culture supernatants. Our MABs should prove useful for investigating epitope relationships between enterotoxin and spore coat proteins, particularly the putative precursor-product relationship (21).

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