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Structure-Function Analyses of Diphtheria Toxin by Use of Monoclonal Antibodies

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A large panel of hybridomas, secreting monoclonal antibodies (MAbs) specific for diphtheria toxin (DT) and prepared by immunization with either intact DT or its A or B fragment (DTA or DTB), have been isolated and characterized. The 213 MAbs were initially screened for reactivity to DT by enzyme-linked immunosorbent assay analyses and then were classified for their reactivity with DT, DTB, or DTA by solid-phase Western blot (immunoblot) analyses; 129 DTB-specific, 51 DTA-specific, and 33 non-fragment-assignable MAbs were obtained. Of the DTB MAbs, 118 recognize epitopes between residues 194 and 453, 10 recognize epitopes between residues 454 and 481, and 1 recognizes an epitope present in denatured toxin but not present in native DT located within the carboxyl-terminal receptor-binding region of DT (residues 482 to 535). Those MAbs that were the most protective in a cytotoxicity assay recognized native toxin in solution and inhibited binding of radiolabeled toxin to Vero cells to the greatest extent. A number of MAbs were able to detect epitopes that became more or less accessible when the toxin was preincubated at acidic (endosomal-mimicking) pH, suggesting that the epitopes they recognize may be important in the low-pH-induced insertion and/or translocation of DT across the endosomal membrane.

Diphtheria toxin (DT) is a potent bacterial exotoxin that inhibits protein synthesis in sensitive eukaryotic cells. The mature 535-amino-acid protein is synthesized as a single polypeptide chain that can be proteolytically cleaved to yield two distinct fragments, the amino-terminal A fragment (amino acids 1 to 193), which contains the enzymatic domain, and the carboxyl-terminal B fragment (amino acids 194 to 535), which contains the internalization and receptor-binding domains (8, 15, 19, 27, 33). DT-mediated cell killing proceeds with binding of DT to its specific receptor on the surface of toxin-sensitive cells, followed by internalization into endosomes and membrane translocation of the A fragment (after endosomal acidification) into the cytosol, and finally enzymatic inactivation of the toxin's target site, elongation factor 2 (14).

Although specific regions of the toxin have been shown to be involved in the complex series of events leading to cytotoxicity, detailed information regarding the finer structural features and critical residues composing many of the toxin's functional determinants remain poorly understood. Undoubtedly, the conformational changes which occur within the toxin molecule, leading to productive intoxication, contribute to this lack of understanding. However, recent identification of the toxin's receptor and crystallization of the toxin are major accomplishments which enrich our knowledge (7, 30). In an attempt to elucidate further the biology of the toxin, we have focused on those determinants involved in receptor binding. Our laboratory has demonstrated that a highly purified carboxyl-terminal fragment of the toxin, consisting of amino acids 482 to 535 (Fig. 1), can function as an autonomous receptor-binding domain (41).

Herein, we describe the isolation, partial characterization, and classification of 213 independently derived hybridomas which secrete monoclonal antibodies (MAbs) specific for regions within the DT molecule, including the receptor-binding region (residues 482 to 535). Additionally, we show through a series of assay systems that it is possible to identify MAbs which specifically recognize native, nonnative, or conformationally induced toxin epitopes.

MATERIALS AND METHODS

Materials and reagents. Partially purified DT was obtained from Connaught Laboratories (Willowdale, Ontario, Canada) and was further purified by anion-exchange chromatography according to published methods (35), except that 50 mM Tris-HCl, pH 7.5, was employed as the buffer system to minimize toxin aggregation (6). Purified DT was quickly frozen in liquid nitrogen and stored at −70°C for biochemical analysis. Aliquots of DT at 1 mg/ml were stored at −20°C at least 2 weeks prior to use in cytotoxicity and binding analyses in order to allow partial dissociation of the endogenous nucleotide (24, 37). Purified hydroxylamine (HA) cleavage products HA-52_{DT} and HA-49_{DT}, which correspond to HA-51_{DT} and HA-48_{DT} (28), respectively, were a generous gift from C. Villemez, The University of Wyoming, Laramie. Affinity-purified goat anti-mouse immunoglobulin G (IgG) was a gift from E. S. Vitetta, The University of Texas Southwestern Medical Center, Dallas. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril (Iodogen) was obtained from Pierce. [^{125}I]NaI (13 to 17 mCi/μg) was obtained from Amersham. L-[2,3,4,5-^{3}H]leucine (110 Ci/mmol) was obtained from ICN Radiochemicals. All reagents for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. Pansorbin cells were purchased from Calbiochem. Ultrapure urea was obtained from Boehringer Mannheim Biochemicals. All other chemicals were obtained from Sigma.

Cells and cell culture. All media and additives were purchased from Sigma, with the exception of fetal bovine serum, which was purchased from Cell Culture Laboratories. Vero cells (ATCC CCL 81) were grown on 150-cm^2

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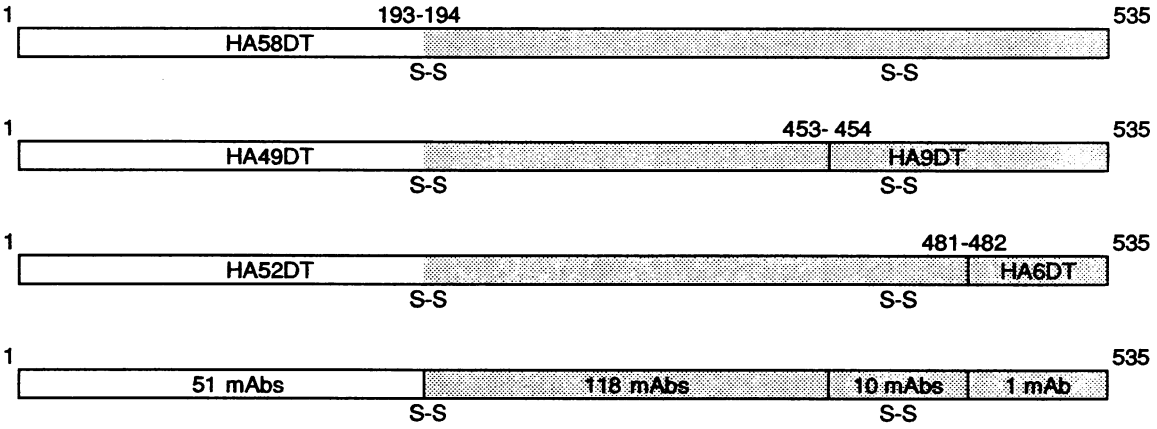

FIG. 1. Diagram of DT showing the products of hydroxylaminolysis and assignment of anti-DT MAb reactivity. Depicted are the peptides which result from HA cleavage of the 535-amino-acid toxin molecule, oriented with respect to the top DT molecule HA-58_{DT} (HA-treated, uncleaved DT). The unshaded area of the toxin represents DTA (amino acids 1 to 193), the shaded area represents DTB (amino acids 194 to 535), and the interface between the two areas represents the protease-sensitive cleavage site between the two fragments. The following abbreviations refer to the peptides resulting from HA cleavage of DT between asparaginyl-glycyl residues 453 to 454 and 481 to 482: HA52DT, amino acids 1 to 481; HA49DT, amino acids 1 to 453; HA9DT, amino acids 454 to 535; and HA6DT, amino acids 482 to 535. The toxin region encompassing amino acids 454 to 481 (HA3DT) has been isolated and is characterized in detail (40). The bottom DT molecule indicates the number of MAbs, from a total of 213, assigned by Western blot analyses to a particular toxin region; 33 MAbs were not classified in this fashion because they strictly recognize conformational toxin determinants and are unreactive with nitrocellulose-immobilized DT or DT fragments.

tissue culture flasks at 37°C (5% CO_2) in Vero medium composed of minimal essential medium with 5% fetal bovine serum and the additives penicillin (50 U/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM). Cells were passaged at confluency. Cells were not utilized beyond their 25th passage after procurement from the American Type Culture Collection (ATCC).

Myeloma SP2/0-Ag14 cells and hybridoma cell lines were grown at 37°C (5% CO_2) in hybridoma medium composed of Dulbecco's modified Eagle's medium with a high concentration of glucose, 20% fetal bovine serum, and the following additives: NCTC-109 medium for hybridomas (10%, vol/vol), insulin (0.02 U/ml), oxalacetic acid (0.1 mM), hypoxanthine (0.1 mM), thymidine (0.016 mM), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). Hybridoma cell line 1F4, secreting a mouse IgG1 which recognizes a 15-kDa integral membrane protein of *Treponema pallidum*, was a gift from M. V. Norgard, The University of Texas Southwestern Medical Center. Hybridoma cell line HY-19 P_1, secreting a mouse IgG1 which recognizes chicken IgM, was a gift from E. Humphries, West Virginia University, Morgantown.

Hybridoma production. Splenocytes from immunized BALB/c mice and the myeloma cell line SP2/0-Ag14 (5:1) were fused with polyethylene glycol 1000 (35%, wt/vol) (BDH Chemicals, Poole, England) and dimethyl sulfoxide (10%, vol/vol) (ATCC) in Dulbecco's modified Eagle's medium. After 24 h, the cells were seeded into 96-well tissue culture plates and cultured in hybridoma medium containing 0.4 μM aminopterin. Visible colonies were selected 7 to 10 days later and expanded for further testing in medium lacking aminopterin. The hybridomas that produced antibodies that bound to DT (detected by the enzyme-linked immunosorbent assay [ELISA] described below) were isolated and cloned by limiting dilution for further analysis.

Preparation of MAb-containing mouse ascitic fluids. Ascitic fluids were produced in BALB/c mice which had been primed by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethyl-pentadecane. Two weeks later, the mice were injected with 10^7 hybridoma cells which had been washed and resuspended in phosphate-buffered saline (PBS; 8.8 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 140 mM NaCl, 10 mM KCl, pH 7.4). Ascitic fluids were collected 1 to 2 weeks later by drainage of the abdominal cavity with an 18-gauge needle. Cells and fibrin clots were removed from the ascitic fluid by centrifugation. The presence of anti-DT MAbs was verified by ELISA, and the ascitic fluids were stored at −20°C until further use.

ELISA. Ninety-six-well polystyrene microtiter plates (Corning 25801) were coated with 0.25 μg of DT per well in PBS containing thimerosal (0.1%, wt/vol) for 3 h at room temperature. The wells were emptied, and the unbound sites were saturated with PBS containing Tween 20 (0.05%, vol/vol) and fetal bovine serum (1%, vol/vol) for 2 h at room temperature. After washing, MAb-containing hybridoma supernatants were added to the wells for 2 h at room temperature. The presence of antigen-bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) and the substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) containing hydrogen peroxide.

DT cytotoxicity assay. Tissue culture dishes (48 wells) were seeded with 2.5×10^4 Vero cells in each well, and cells were grown to confluency (4 to 5 days). Confluent monolayers were washed twice with PBS containing 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Binding medium (0.1 ml) (medium 199 with 50 μg of bovine serum albumin [BSA], 100 μg of gelatin, and 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.4, per ml) was added to cover the cells and was followed by the addition of 50 μl of MAb-containing mouse ascitic fluid (1:100 final dilution) in binding medium; 50 μl of DT (diluted in binding medium) in various amounts was added to the cell monolayers and incubated for 1 to 2 h at 37°C. The monolayers were washed to remove unbound proteins and were further incubated in leucine-deficient medium for 1 h at 37°C. [^{3}H]leucine was added to the cells,

which were incubated for a final hour and then washed, lysed, and counted for [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material as described previously (16, 37). All assays were done in triplicate, and variation from the mean was less than 10%. Fifty percent inhibitory concentration (IC_{50}) values were determined as the DT concentration required to inhibit 50% of Vero cell protein synthesis.

Radiolabeling of DT, HA-52$_{DT}$, and HA-49$_{DT}$. DT and DT fragments were radiolabeled by the Iodogen method (18). Fifty micrograms of protein and 0.5 mCi of carrier-free [^{125}I]NaI in a final volume of 0.1 ml in 50 mM Tris-HCl, pH 7.5, were placed in a glass tube (12 by 75 mm) that had been previously coated with Iodogen (100 μg). The tube was incubated for 5 min on ice with occasional mixing. The reaction was terminated by removing the mixture from the tube. Unincorporated radioisotope was removed by centrifugation of the solution through a 1-ml bed of Sephadex G50-150. Typically, the labeling procedure resulted in a specific radioactivity of 1×10^7 to 2×10^7 cpm/μg of protein.

Radiolabeled DT binding assay. For the assay of radiolabeled DT binding, Vero cells were plated as described for the cytotoxicity assay. Confluent monolayers were placed directly from the incubator onto trays of ice and washed three times with ice-cold PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Ice-cold binding medium (0.1 ml) was added to cover the cells, and then 50 μl of radioiodinated DT (100 ng/ml) and 50 μl of MAb-containing mouse ascitic fluid (1:100 final dilution) in binding medium were added. The cells were placed on ice (4 to 5 h), and unbound DT was then washed away with ice-cold PBS. The washed cells were lysed by incubation in 0.2 ml of 0.1 N NaOH for 30 to 60 min at 37°C. The cell-associated radioactivity in a 0.1-ml sample was measured. Nonspecific binding was defined by the radioactive DT that remained associated with the cells when the radiolabeled DT incubation was done in the presence of 100-fold excess of unlabeled DT. All assays were done in duplicate or triplicate, and variation from the mean was less than 10%.

Immunoprecipitation of radiolabeled DT. Antibody-containing hybridoma culture supernatants, undiluted or diluted 1:10 in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.5), were assayed for their ability to immunoprecipitate radiolabeled DT. Radiolabeled DT (100,000 cpm; approximately 10 ng) diluted into 10 μl of TBS containing 0.1 mg of BSA per ml was added to 100 μl of hybridoma supernatant. In parallel, 100 μl of goat anti-mouse IgG (0.05 mg/ml) in TBS was added to a 5-μl suspension of protein A-bearing Pansorbin cells, and both sets of tubes were incubated overnight at 4°C. The following day, the radiolabeled DT-supernatant mixture was added to the goat anti-mouse IgG-coated Pansorbin cells and allowed to incubate for 1 h at 4°C, washed with 0.5 ml of TBS, and centrifuged to pellet the Pansorbin cells, and the amount of cell-associated radioactivity was measured.

For the assays in which DT was treated with acidic pH prior to immunoprecipitation, an assay modified from the *Pseudomonas aeruginosa* exotoxin A assay described by Ogata et al. (32) was utilized. Radiolabeled DT was diluted into morpholineethanesulfonic acid buffer (5 mM morpholineethanesulfonic acid, 5 mM citric acid, 150 mM NaCl, 0.9 mM $CaCl_2$, 0.1 mg of BSA per ml, with the pH adjusted with 1.5 M Trizma base) and incubated at various pH values for 30 min at 37°C. The samples were then neutralized to pH 7.0 to 7.5 by the addition of TBS (at various pH values), and the particular hybridoma culture supernatant (1:4 final dilution) used for immunoprecipitation was then added. For the assays in which DT was heat treated, the radiolabeled toxin was treated at temperatures ranging from 37 to 56°C for 30 min at pH 7.5. The samples were then cooled to room temperature and immunoprecipitated as described above.

Preparation of DTA and DTB. Limited proteolysis of DT to generate disulfide-linked DT fragments A and B (DTA and DTB) was carried out with trypsin (13). DT, at a concentration of 10 to 20 mg/ml in 50 mM Tris-HCl, pH 7.5, was incubated with 1 μg of trypsin per 5 mg of DT at room temperature for 15 min. Proteolysis was terminated by incubation with 10 μg of soybean trypsin inhibitor per μg of trypsin at room temperature for 30 min. The nicked toxin was denatured with 0.5% sodium dodecyl sulfate (SDS) and incubated with 0.1 M dithiothreitol (DTT) at room temperature for 2 h. The preparation was applied to a Sephadex G-100 column equilibrated with 10 mM phosphate buffer–0.1% SDS–1 mM DTT, pH 7.2. Two well-separated protein peaks were obtained, containing DTB and DTA, as judged by SDS-PAGE analyses on 12% polyacrylamide gels.

HA cleavage of DT. Partial HA cleavage of the toxin was performed according to published methods (28) with modifications (41). Purified toxin, 25 mg in 50 mM Tris-HCl, pH 7.5, was concentrated to 200 μl or less in a Centricon-30 Microconcentrator (Amicon). A solution of 2 M hydroxylamine–6 M guanidine-HCl, titrated to pH 9.0 with saturated lithium hydroxide, was then added to a final volume of 1 ml. The mixture was kept at room temperature for 16 h with occasional mixing. After proteolysis, the sample was dialyzed against 50 mM Tris-HCl containing 6 M urea, pH 7.5, by using 1-kDa cutoff dialysis tubing. HA-treated DT was stored at −70°C until further use.

Western immunoblot analysis. Trypsin-treated DT (25 μg) mixed with 25 μg of untreated DT or 50 μg of HA-treated DT was loaded onto minigels containing either 12% polyacrylamide or an 8 to 25% gradient of polyacrylamide, respectively. The gels were electrophoresed at 200 V for 1 h. After electrophoresis, the resolved peptides were transferred onto nitrocellulose. Subsequently, the nitrocellulose membranes were cut into strips, which were individually incubated with MAb-containing hybridoma culture supernatant. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel) followed by horseradish peroxidase-conjugated rabbit anti-goat IgG (Cappel) and the substrate 4-chloro-1-naphthol containing hydrogen peroxide.

RESULTS

Immunization of mice with native DT proteins. Six prior reports have described a limited number of MAbs reactive with DT, some of which are capable of neutralizing toxin activity when tested with sensitive cells (1, 3, 20, 44, 46, 47). The epitopes recognized by a number of these MAbs have been directly mapped to regions of the toxin with proteolytically cleaved DT, CRM (cross-reactive material) proteins, or highly purified cyanogen bromide fragments of the toxin. The immunizing agent used in all the studies, except for one which used CRM 197 (3), was diphtheria toxoid. Toxoid rather than toxin was used because, even though mice are highly resistant to DT (8), they are killed when given large doses of toxin or even isolated fragment A. Toxoid, however, is probably not the best DT-related immunogen to use in the production of antibodies against the toxin's receptor-binding domain, because it is widely believed that chemical modification of amino acid residues of DT (through formalin

TABLE 1. Immunization[a] of BALB/c mice to generate anti-DT MAbs

Immunogen and MAbs characterized/total assayed	Diluent (μg)	Injection schedule (wk)
DTB (5)[b] and DTA (2)	CFA (100)	0
Anti-DTB[c]: 105/145	IFA (100)	2
Anti-DTA[d]: 40/145	IFA (100)	4
	IFA (100)	6
	IFA (100)	8
	IFA (100)	10
	PBS (100)	12[e]
DT (2)	CFA (5)	0
Anti-DTB[c]: 24/68	IFA (5)	3
Anti-DTA[d]: 11/68	IFA (10)	6
ND[f]: 33/68	IFA (10)	9
	IFA (20)	12
	IFA (20)	15
	IFA (50)	18
	IFA (50)	21
	IFA (100)	24
	PBS (100)	27[e]

[a] Immunizations were given intraperitoneally, except those with PBS (100 μg), which were given intravenously.
[b] Values in parentheses are numbers of animals from which actual hybridomas were prepared.
[c] Western blot with DT mixed with nicked DT—i.e., DT(+)/DTB(+)/DTA(−).
[d] Western blot with DT mixed with nicked DT—i.e., DT(+)/DTB(−)/DTA(+).
[e] Three days prior to fusion.
[f] Western blot DT fragment reactivity not accurately determinable.

treatment) not only destroys toxicity but also results in the loss of binding to sensitive cells (45).

In order to increase the probability of producing antibodies to native toxin determinants, including anti-receptor-binding domain MAbs, we utilized DTB possessing an intact receptor-binding domain or an approach which allowed us to immunize with native DT. Additionally, some animals were immunized with DTA for the purpose of having anti-DTA antibodies for toxin structure-function studies involving this fragment.

Table 1 summarizes the immunization protocols utilized. Highly purified preparations of DTB (100 μg), DTA (100 μg), or DT (5 μg) in complete Freund's adjuvant (CFA; GIBCO) were injected intraperitoneally into BALB/c mice. At 2-week intervals, 100 μg of DTB or DTA in incomplete Freund's adjuvant (IFA; GIBCO) was injected intraperitoneally into the respective animals. At 3-week intervals, mice given native DT were injected intraperitoneally with increasing amounts of DT. Typically, the mice receiving DT immunization required 9 to 10 immunizations of increasing doses before they could neutralize and survive a 100-μg intravenous injection of DT. Animals having high-DT-reactive titers detected by ELISA or radioimmunoprecipitation analyses were immunized intravenously with 100 μg of the respective protein, and 3 days after the final injection, the spleen from the selected animal was removed and used for hybridoma preparation. The 213 anti-DT-secreting hybridomas produced, as described in Materials and Methods, were identified and isolated by ELISA analysis with plates coated with intact DT.

Characterization of DT-recognizing MAbs employing enzymatically or biochemically cleaved DT proteins. In order to define the sites of reactivity of the anti-DT MAbs within DT, trypsin- and HA-cleaved toxin was utilized. Limited trypsin digestion, which cleaves DT into fragments A and B, and MAb-facilitated Western blotting analyses of the electrophoretically separated fragments were performed as follows. A sample of trypsin-treated (nicked) DT was mixed with an equal amount of untreated DT and analyzed by electrophoresis on a 12% polyacrylamide gel, and the resolved peptides were transferred to nitrocellulose, which was cut into strips and probed with DT-specific MAbs (Fig. 2). The bound antibodies were detected by using the sequential combination of horseradish peroxidase-conjugated goat anti-mouse and rabbit anti-goat IgGs and the substrate 4-chloro-1-naphthol, to which hydrogen peroxide was added (Fig. 2). This analysis resulted in the identification of three types of antibody reactivity (Fig. 2 and Table 1). MAb 60D5 (Fig. 2, lane 2) and MAb 83B8 (Fig. 2, lane 3) represent examples of the DTB-reactive (129 out of 213) and DTA-reactive (51 out of 213) MAbs identified, respectively. These MAbs recognized intact DT immobilized onto nitrocellulose and in all cases recognized only one of the trypsin-generated fragments, and never both DTB and DTA. MAb 79G8 (Fig. 2, lane 1) is representative of the anti-DT MAbs identified which could not be accurately assigned reactivity to either the isolated B or the isolated A fragment (33 out of 213). These latter MAbs were weakly reactive or nonreactive with toxin preparations immobilized onto nitrocellulose, even though they had been identified as being reactive with intact DT by an ELISA, indicating that they recognize conformationally dependent determinants which become modified after denaturing SDS-PAGE and immobilization of toxin onto nitrocellulose. Interestingly, all of these latter MAbs were generated with intact DT as the immunogen (Table 1).

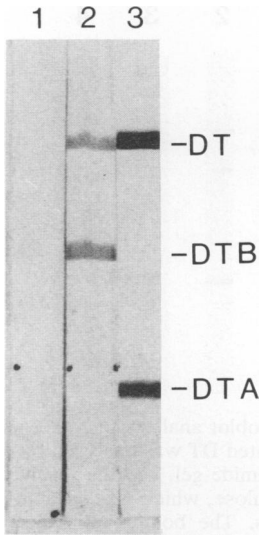

FIG. 2. Immunoblot analysis of MAbs with intact and trypsin-treated DT (see text for details). The major products detected are DT, DTB, and DTA. Lanes 1 through 3 show immunoblots assayed with MAbs 79G8, 60D5, and 83B8, respectively.

Partial HA treatment of the toxin, which cleaves DT at two carboxyl-terminal sites (DT amino acids 453 and 454 and 481 and 482; Fig. 1) (28, 41), and electrophoretic separation of the resultant fragments followed by MAb-facilitated Western blotting analysis resulted in the identification of four types of reactivity (Fig. 3). (i) MAb 60D5 (Fig. 3, lane 3) is representative of 169 of the 213 MAbs tested, having reactivity to HA-58_{DT} (HA-treated but uncleaved DT), HA-52_{DT}

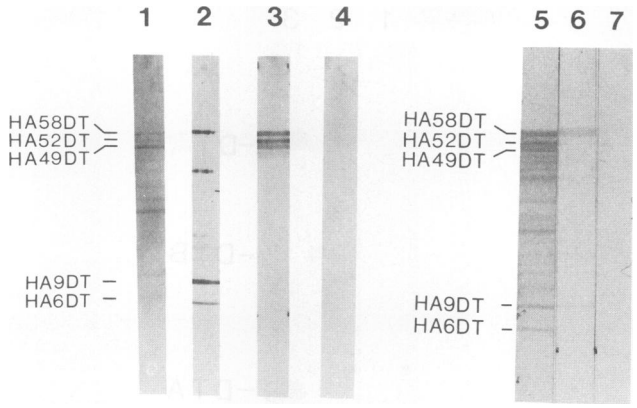

FIG. 3. Immunoblot analysis of MAbs with HA-treated DT. A sample of HA-treated DT was analyzed by electrophoresis on an 8 to 25% polyacrylamide gel, and the resolved peptides were transferred to nitrocellulose, which was cut into strips and probed with DT-specific MAbs. The bound antibodies were detected as described in the text (Fig. 2). Lanes 1 and 5, blot of HA-treated DT stained with amido black; the major bands (along with their corresponding DT amino acids in parentheses) are HA58DT (1 to 535), HA52DT (1 to 481), HA49DT (1 to 453), HA9DT (454 to 535), and HA6DT (482 to 535). Lanes 2 to 4 and 6 and 7 show immunoblots assayed with the following MAbs: 2, 43A4; 3, 60D5; 4, 1F4 (anti-*T. pallidum*); 6, 27A3; and 7, 79G8.

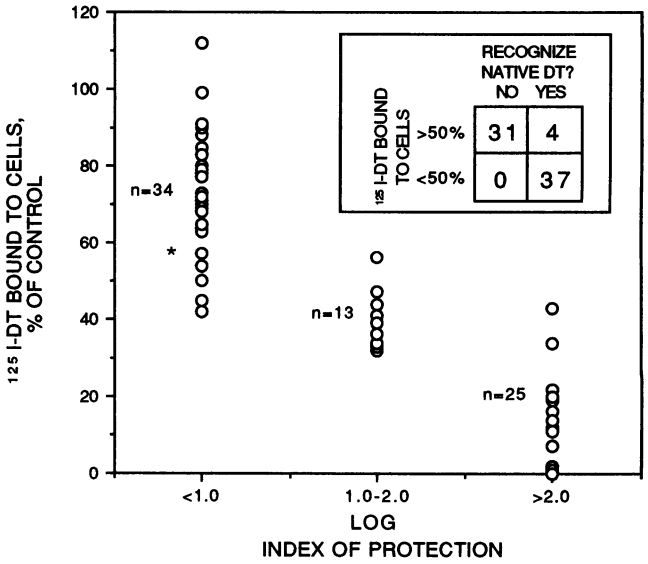

FIG. 4. MAb effects upon cytotoxicity, receptor binding, and immunoprecipitation of DT. (*x* Axis) MAbs classified according to their neutralizing capability (see text for details). The results are expressed as the percentage of control protein synthesis in the absence of DT and the presence of the irrelevant control MAb HY-19 P_1. *n* indicates the number of MAbs within each group. Of the 34 MAbs that failed to inhibit cytotoxicity significantly (<1.0 log protective effect), 7 were anti-DTA, 19 were anti-DTB, and 8 were non-fragment assignable; of the 13 MAbs that inhibited cytotoxicity (1.0 to 2.0 logs protective effect), 1 was anti-DTA, 5 were anti-DTB, and 12 were non-fragment assignable; and, of the 25 MAbs that inhibited cytotoxicity strongly (>2.0 logs protective effect), 3 were anti-DTA, 4 were anti-DTB and 13 were non-fragment assignable. (*y* Axis) Prevention of toxin binding to receptors by MAbs which neutralize cytotoxicity. Anti-DT MAbs were tested in the radiolabeled DT binding assay, and the capacity of the members of each group to inhibit specific DT binding was assessed (see text for details). The results are expressed as the percentage of specific binding of radiolabeled DT in the presence of the MAb. *, MAb 43A4. (Insert) Prevention of toxin binding to receptors by MAbs which recognize native toxin. An immunoprecipitation assay was used to determine which MAbs recognized toxin in solution, and the results were compared with those obtained from the Vero cell binding analyses described in text. The immunoprecipitation of radiolabeled DT assay was performed with duplicate samples of MAb-containing hybridoma supernatant as described in Materials and Methods. MAb-containing supernatants capable of precipitating more than twice the radiolabeled toxin precipitated by the irrelevant control MAb 1F4 were considered capable of recognizing native toxin.

(DT amino acids 1 to 481), and HA-49_{DT} (DT amino acids 1 to 453), but not HA-9_{DT} (DT amino acids 454 to 535) or HA-6_{DT} (DT amino acids 482 to 535), indicating that these MAbs recognized epitopes retained within DT amino acids 1 to 453 (Fig. 1). The MAbs within this group included 51 anti-DTA MAbs and 118 anti-DTB MAbs; the latter ones, therefore, map more specifically to determinants located within amino acids 194 to 453 of DT (Fig. 1). (ii) MAb 79G8 (Fig. 3, lane 7) represents the 33 MAbs which are weakly reactive or nonreactive with HA-cleaved toxin preparations immobilized onto nitrocellulose. Interestingly, these MAbs could also not be assigned reactivity to the trypsin-generated fragments of DT (Fig. 2), a finding consistent with the hypothesis that they recognize conformational determinants of the toxin. (iii) MAb 27A3 (Fig. 3, lane 6) represents the 10 antibodies having reactivity to HA-58_{DT}, HA-52_{DT}, and HA-9_{DT} but not to HA-49_{DT} or HA-6_{DT}, indicating that these MAbs recognize epitopes retained within amino acids 454 to 481 of DT (HA-3_{DT}; Fig. 1). (iv) A single MAb, 43A4 (Fig. 3, lane 2), had reactivity to HA-58_{DT}, HA-9_{DT}, and HA-6_{DT}, but not to HA-52_{DT} or HA-49_{DT}, indicating that it recognizes an epitope located within the carboxyl-terminal receptor-binding domain (amino acids 482 to 535 [HA-6_{DT}], Fig. 1).

Effect of MAbs upon cytotoxicity, receptor binding, and immunoprecipitation of DT. To identify DT-neutralizing MAbs, a Vero cell cytotoxicity assay was employed. MAb preparations were obtained from 72 (28 anti-DTB, 11 anti-DTA, and 33 Western blot-negative) hybridoma cell lines. The effect on protein synthesis of Vero cells after coincubation with MAb-containing ascitic fluid for 2 h at 37°C with increasing amounts of DT and anti-DT MAb was compared with that of DT and an irrelevant control MAb, HY-19 P_1 (Fig. 4). These cells were subsequently assayed for [^{3}H]leucine incorporation into acid-precipitable material. By comparing the neutralizing capacity at the IC_{50} with that of the control, the antibodies could be separated into three distinct groups: (i) those that failed to inhibit cytotoxicity significantly, i.e., less than 1.0 log protective effect above the IC_{50} of a control MAb; (ii) those that inhibited cytotoxicity, i.e., 1.0 to 2.0 logs protective effect; and (iii) those that inhibited cytotoxicity strongly, i.e., greater than 2.0 logs protective effect (Fig. 4, *x* axis). The same MAb preparations were employed to determine specifically which MAbs were capable of preventing the binding of radiolabeled toxin to the Vero cell receptor (Fig. 4, *y* axis). Briefly, for binding analysis, MAb-containing ascitic fluid in binding medium was coincubated with 100 ng of radiolabeled DT (in the absence or presence of 100-fold excess unlabeled DT) per ml on Vero cell monolayers for 4 h at 4°C. The cells were subsequently washed to remove unbound DT, lysed, and measured for their cell-associated radioactivity (Fig. 4, *y* axis). As seen from Fig. 4, a strong correlation between

neutralization of cytotoxicity and inhibition of specific toxin binding to the cell surface was observed. Interestingly, those MAbs which have been classified as recognizing conformational toxin determinants, by virtue of their incapacity to recognize toxin or toxin cleavage products immobilized onto nitrocellulose, had the greatest ability to neutralize toxin and prevent toxin binding to receptors. Specifically, 25 of the 33 MAbs with non-fragment-assignable reactivity (Fig. 4, *x* axis) neutralized cytotoxicity in a significant manner (i.e., 1.0 log or greater protective effect); all of the non-fragment-assignable MAbs were derived from mice immunized with intact toxin (Table 1). Furthermore, a strong correlation between those antibodies which recognize native toxin by immunoprecipitation and those that prevent binding of toxin to the Vero cell receptor (Fig. 4, insert) was found.

When we examined the MAbs (e.g., MAb 27A3) which recognized HA-9_{DT} but not HA-6_{DT}, and therefore recognize toxin epitopes retained within amino acids 454 to 481, all were found to be nonneutralizing and incapable of significantly inhibiting toxin binding. Additionally, these MAbs were incapable of recognizing native toxin in solution. Consistent with our observation, the recent X-ray crystallography of DT suggests that this region of the toxin, β-strands RB6 and RB7 (7), is not exposed prior to proteolytic cleavage of the toxin and separation into its A and B fragments.

MAb 43A4, the only MAb which recognizes an epitope within the receptor-binding region (amino acids 482 to 535; HA-6_{DT}), was included in the analyses and was likewise found to belong to the first group of MAbs—those which poorly neutralized the toxin's cytotoxic effects. MAb 43A4 also had only a marginal effect on prevention of toxin binding to receptor, inhibiting only 45% of radiolabeled toxin binding (Fig. 4, *) compared with an irrelevant control MAb, and did not recognize native toxin in solution. MAb 43A4, characterized by epitope-mapping analysis with synthetic peptides, recognizes a determinant within residues 506 to 514 (40). This region of HA-6_{DT} is exposed within the published crystal structure (7), suggesting that MAb 43A4 recognizes an epitope present only in denatured toxin.

Reports that polyclonal anti-DTA antibodies are nonneutralizing or poorly neutralizing have led to the theory that the majority of DTA is not exposed within the native toxin (11, 35, 39). Zucker and Murphy (46), on the other hand, have described several toxin-neutralizing anti-DTA MAbs, suggesting that antigenic determinants located on DTA are accessible to antibody. It was of interest, therefore, to determine whether any of our anti-DTA MAbs were also neutralizing. All 51 anti-DTA MAbs were tested for their ability to immunoprecipitate native DT. Six MAbs (all obtained from mice immunized with intact DT) were able to immunoprecipitate radiolabeled toxin, and four of these six anti-DTA antibodies (for example, MAb 83B8) were indeed identified as being DT neutralizing. Surprisingly, these anti-DTA MAbs also prevented binding of toxin to the Vero cell receptor, suggesting that the DTA determinants they recognize, although remote in sequence, may be in close structural proximity to the receptor-binding site within DTB. Our laboratory has previously postulated the existence of such sites of DTA and DTB interaction, including those sites involved in ATP binding, in which the adenine-binding subsite is located on DTA and the polyphosphate-binding subsite is located on DTB (38). Additionally, the crystal structure of the toxin supports the existence of DTB-DTA interaction sites, particularly near the enzymatic-site cleft (7).

TABLE 2. Characterization of native DT-recognizing MAbs with chemically truncated DT

MAb group (no. of MAbs identified/group)	Immunoprecipitation of radiolabeled DT/HA-52_{DT}/HA-49_{DT}	Western immunoblot of HA-treated DT/HA-52_{DT}/HA-49_{DT}/HA-9_{DT}/HA-6_{DT}	Prevention of DT binding[a]
Group 1 (27)	+/+/+	+/+/+/−/−	23/24
Group 2 (18)	+/−/−	−/−/−/−/−	12/14
Group 3 (3)	W[b]/+/+	+/+/+/−/−	1/2
Group 4 (2)	+/+/−	+/W/W/−/−	2/2
Group 5 (1)	+/+/−	−/−/−/−/−	1/1

[a] Number of MAbs that prevent binding/total number of MAbs tested.
[b] W, weak or negative reactivity.

Characterization of native toxin structures employing binding domain-deleted DT and DT-recognizing MAbs. The larger purified products resulting from HA cleavage of the toxin, HA-52_{DT} and HA-49_{DT}, have been demonstrated to retain their various domain-associated functions, with the exception of receptor binding. In fact, few differences between higher-order structural changes in intact toxin and those in HA-52_{DT} and HA-49_{DT} are detectable when conventional biochemical techniques are used (28). Therefore, we sought to determine whether more subtle changes in toxin structure, due to removal of the toxin's receptor-binding domain, might be detectable with any of our MAbs. Employment of the sensitive immunoprecipitation assay previously described had allowed us to identify systematically a large panel of MAbs that recognized native toxin structures. This system was used for more extensive characterization of structures retained within receptor-binding domain-deleted forms of the toxin.

Fifty-one of the 213 MAbs recognized native DT, as determined by immunoprecipitation of radiolabeled DT. These immunoprecipitating antibodies have been further assigned to five groups on the basis of two criteria: (i) their reactivity with toxin and with highly purified HA-52_{DT} and HA-49_{DT} in solution and (ii) their reactivity with the same proteins immobilized onto nitrocellulose (Table 2).

Twenty-seven group 1 MAbs were characterized. These antibodies, for example, MAb 60D5, recognized surface-accessible epitopes common to all three proteins whether in solution or immobilized onto nitrocellulose, suggesting that these MAbs recognize linear surface-exposed epitopes of the toxin. Continuing assessment of their reactivity with small peptide fragments of the toxin may confirm this possibility. Four anti-DTA MAbs were included in this group.

Eighteen group 2 MAbs, for example, MAb 79G8, were characterized. When tested with soluble toxin, these antibodies strongly recognized intact toxin but failed to react with either of the receptor-binding domain-lacking proteins. When tested with immobilized toxin, the antibodies failed to recognize or only weakly recognized DT or the HA-treated DT products, indicating that the antibodies in this group recognize toxin epitopes which are conformationally dependent on the presence of DT amino acids 482 to 535.

Three group 3 MAbs were characterized. The antibodies in this group recognized DT and the truncated DT peptides that were denatured and immobilized onto nitrocellulose. In contrast to their recognition of denatured and immobilized toxin, they recognized intact soluble toxin weakly, if at all, but reacted with soluble HA-52_{DT} and HA-49_{DT}, suggesting that these MAbs recognized determinants normally seques-

TABLE 3. Interaction of MAbs with DT pretreated with acidic pH or high temperature

MAb (1:4 dilution)	Radiolabeled DT immunoprecipitated (% of MAb 60D5 control) after treatment[a]							
	pH				Temp (°C)			
	7.5	5.5	4.5	3.5	37	42	50	56
60D5 (group 1)	100[b]	100	99	99	100	93	93	36
79G8 (group 2)	124	102	100	90	104	97	93	33
83B8 (group 3)	19	16	16	20	14	14	14	18
51G10 (group 4)	107	66	87	107	106	99	92	24
79E3 (group 4)	105	50	80	104	108	101	101	29
85G7 (group 5)	38	36	38	38	59	58	52	22
43A4 (anti-HA-6_{DT})[c]	3	3	6	14	7	5	5	5
22A11 (anti-HA-3_{DT})[d]	5	7	7	31	7	7	7	9
27A3 (anti-HA-3_{DT})	7	5	5	44	7	7	7	9
1F4 (anti-*T. pallidum*)	5	5	7	7	7	5	5	7

[a] Radiolabeled DT was treated at the indicated pH or heat-treated at the indicated temperatures prior to immunoprecipitation as described in Materials and Methods.
[b] 69,000 cpm was set as 100%; all data were compared with this value.
[c] HA-6_{DT} = amino acid residues 482 to 535 (Fig. 1).
[d] HA-3_{DT} = amino acid residues 454 to 481 (Fig. 1).

tered within the toxin molecule which become accessible after a conformational change. One of the group 3 MAbs, 83B8, an anti-DTA antibody, neutralized DT cytotoxicity and prevented toxin binding to the receptor. Although the prevention of toxin binding seen with the native DT-recognizing MAbs (Table 2) could be due to nonspecific action such as steric interference, the finding that 83B8 prevented toxin binding even though it reacted only weakly with soluble intact DT may also be explained by the ability of 83B8 to recognize a conformationally induced DT epitope involved in a process concomitant with or leading to toxin binding.

Two group 4 MAbs were characterized. These anti-DTB MAbs, 51G10 and 79E3, recognize an epitope common to all three immobilized proteins; in solution, however, this epitope no longer remains accessible within the truncated toxin when amino acids 454 to 535 are removed, suggesting that the epitope(s) becomes conformationally sequestered within the HA-49_{DT} protein but remains accessible in HA-52_{DT}.

Finally, one group 5 MAb was characterized. This antibody, 85G7, is similar to those of group 4, except that the epitope recognized is completely destroyed upon denaturation and immobilization of the toxin. The integrity of the epitope with which MAb 85G7 reacts appears to be dependent upon the presence of amino acids 454 to 481 of the toxin.

Characterization of low-pH-inducible toxin epitopes employing DT-recognizing MAbs. Although the series of steps which occur during the cytotoxic process are well characterized (9), the conformational changes induced within the toxin molecule which accompany these steps remain poorly understood. Studies involving exposure of DT to low pH have determined that incubation of the toxin at a pH similar to that found in the endosomal environment exposes normally sequestered hydrophobic regions within the toxin (4, 42). Low-pH exposure of DT has also been used to induce the insertion of the toxin into lipid bilayers (5, 12) and is necessary for DT to gain entry into the cytosol (23, 25, 26). Because the anti-DT MAbs we had produced in this study were generated against either functionally active intact toxin or fragments thereof, the likelihood existed that we could identify antibodies with specificity for pH-modifiable, conformationally induced epitopes of DT. Therefore, we examined the interaction of MAbs with toxin treated at various pH levels.

The ability of the MAbs to recognize in solution toxin which had been preincubated at various pH levels for 30 min and then neutralized to pH 7.5 was assessed by immunoprecipitation of radiolabeled DT. The relative amount of radioactive DT precipitated by each MAb-containing preparation at the pH levels examined was expressed as the percentage of the total amount of radioactive DT, incubated at pH 7.5, precipitated by MAb 60D5. MAb 60D5 was used because it precipitated roughly equivalent amounts of DT at all pH levels examined (Table 3). MAb 1F4 (anti-*T. pallidum*) was used as an irrelevant control.

As seen from Table 3 (pH columns) none of the MAb preparations representing group 1, 2, 3, or 5 (described in Table 2) recognized toxin after its treatment with low pH in a significantly different manner from toxin incubated at neutral pH. The two group 4 MAbs, 51G10 and 79E3, on the other hand, had unique profiles when tested with pH-treated toxin; the DT epitope(s) that the group 4 MAbs recognized became less accessible to antibody after treatment of the toxin at pH 4.5 and 5.5 but was equivalently accessible to antibody when toxin was treated at pH 7.5 or 3.5. The pH range of 4.5 to 5.5 is similar to that which exists in the endosomal environment, suggesting that the toxin region(s) which the group 4 MAbs recognizes may actually become buried in the toxin during conformational changes such as those purported to occur during translocation or activation.

Also examined were MAb 43A4, which recognizes an epitope within DT amino acids 506 to 514 (40), and MAbs 22A11 and 27A3, which recognize an epitope(s) within DT amino acids 454 to 481 (Fig. 3). The epitopes recognized by these three MAbs are not exposed on DT at neutral pH (Table 3). When these MAbs were tested to detect conformational changes in DT induced by acidic pH, no change in the binding to DT was detected at a pH equivalent to or higher than 4.5. At pH 3.5, however, an increase in reactivity was observed. This interaction was most significant for the last two MAbs, where 31 and 44% of the control binding was observed, respectively. Less dramatic was the binding

of MAb 43A4 to pH 3.5-treated DT, in which 14% of the control binding occurred. Interestingly, these three MAbs are similar to the MAbs directed against *P. aeruginosa* exotoxin A (PE) (32), which lacked reactivity for PE in solution at neutral pH but recognized soluble PE that had been preincubated at acidic pH (pH 3.5 or lower). Ogata et al. (32) proposed that the hidden epitopes that the anti-PE MAbs recognize, exposed by low-pH treatment, may correspond to regions of PE which are biologically relevant for its activity during endocytosis.

When these MAbs were analyzed with toxin that was heat treated at temperatures from 37 to 50°C, all of the preparations recognized toxin in a temperature-independent manner (Table 3, temperature columns). In contrast, all of these MAbs, when assayed with toxin that was heat treated at 56°C, a temperature at which DT rapidly coagulates (34), demonstrated binding that was either unaltered or reduced but not increased. This finding strengthens the notion that the pH-induced changes in toxin structure detected by the antibodies may reflect a specific conformational change necessary for toxin function rather than mere exposure of cryptic toxin epitopes because of its denaturation.

DISCUSSION

MAbs from 213 hybridoma cell lines, representing the largest collection of independently derived anti-DT MAbs reported by one laboratory, have been characterized for their sites of reactivity within the toxin molecule and for their potential to inhibit DT-mediated cytotoxicity, as well as for their potential to delineate further the steps involved in the cytotoxic process. Through hyperimmunization of BALB/c mice with toxin A or B fragments, or low-dose immunization with intact native toxin followed by toxin challenge at doses known to be lethal for naive mice, we have developed immunization and prefusion regimens allowing for the selection of animals having high-anti-DT titers. This preselection strategy has been combined with solid- and solution-phase assays in order to identify MAb-secreting hybridomas directed against both native and conformationally sequestered toxin determinants.

A strong correlation has been observed between those MAbs which recognize native toxin in solution (via immunoprecipitation analyses) and those which are most effective at protecting Vero cells from DT-mediated cytotoxicity (Fig. 4).

Although we have characterized many toxin-neutralizing MAbs, only one MAb, 43A4, has reactivity to a determinant within amino acids 482 to 535, the region that we have previously shown to contain the receptor-binding site (41). This MAb, however, was found to be nonneutralizing because it recognizes an epitope (within residues 506 to 514) present in denatured but not in native toxin (40). Our experience is consistent with that of Zucker and Murphy (46), who also noted the absence of efficiently neutralizing MAbs directed against this region of the toxin despite strong evidence from the X-ray crystallographic structure of DT that the receptor-binding region is part of an exposed area (β-strands RB8, RB9, and RB10) (7). Thus, the reason for the low incidence of antibodies directed against the receptor-binding region, at least for DT-immunized BALB/c mice, is not clear. Our results suggest that determinants carried within the receptor-binding region of intact toxin or of isolated DTB, however, may not be among the major target determinants for the neutralizing humoral response to toxin. Indeed, the conclusion that HA-6_{DT} (Fig. 1, amino acids 482 to 535) is not an immunodominant region of the toxin is supported by the finding that the antibody response to the HA-6_{DT} region (monitored with test samples of sera from toxin-immunized mice analyzed by Western blot of HA-treated DT) was detected only after multiple injections of mice with the toxin (data not shown).

The difficulty in obtaining anti-receptor-binding domain antibodies may simply reflect the nature of the toxin's receptor-binding site. Continuing characterization of the DT receptor (29–31) should eventually address the possibility that a natural ligand, the one whose receptor is usurped by DT to gain access to the cell cytosol, may have homology to a toxin domain involved in receptor binding. The toxin's receptor-binding site, therefore, may possibly be recognized by the immunized host as a self-determinant and be unavailable for immunological recognition.

Like Zucker and Murphy (46), we were surprised to isolate a number of hybridomas secreting anti-DTA MAbs that immunoprecipitate native toxin and neutralize DT cytotoxicity and which also inhibit binding of the toxin to Vero cell DT receptors. That these MAbs recognize a secondary binding site on the toxin molecule, as suggested by Zucker and coworkers, remains to be directly demonstrated. In light of the recent toxin crystallization data, it is more likely that they recognize a DTA epitope(s) in close spatial proximity to the primary receptor-binding site located within the final 54 DTB amino acids of the native toxin molecule.

To our knowledge, ours is the first report of anti-DT MAbs which selectively recognize native toxin conformational determinants (toxin in solution) yet fail to recognize intact or cleaved toxin preparations under solid-phase conditions (toxin denatured and immobilized onto nitrocellulose). Why previous investigators have not reported similar MAbs, which we demonstrate are highly toxin neutralizing (Fig. 4), is unclear; perhaps the primary screening techniques failed to select these particular MAb-secreting hybridomas. Antibodies which recognize only native protein structures, however, have been described in numerous other systems, including anti-peptide antibodies to the binding domain of *Bordetella pertussis* toxin (43) and MAbs to the B subunit of the type IIb heat-labile enterotoxin of *Escherichia coli* (36). We offer the suggestion that diphtheria toxoid, the immunizing agent used in previous studies, lacked the conformational determinants present in native toxin needed to elicit this type of antibody.

Systematic classification of anti-DT MAbs which recognize native versus nonnative structures has allowed us to examine induced conformational changes in the toxin. Through the use of MAbs which recognize native toxin structures and/or receptor-binding domain-deleted toxin molecules, we have identified those MAbs which recognize differences in the overall structure of the latter proteins (Table 2, groups 2 to 5), even though only minor differences in the structural properties of these toxins are observed with conventional biochemical or biophysical techniques (28). Because these receptor-binding domain-deleted toxins retain their cytotoxic potential after conjugation to a cell-binding moiety (28), some practical applications of these particular MAbs may be made in the affinity isolation and purification of the modified toxins and in evaluation of their structural integrity after conjugation to a particular binding moiety.

Employing MAbs which recognize toxin treated at various pH values, we have identified antibodies with specificity for DT epitopes induced upon low-pH treatment. In light of ongoing speculation that the hydrophobic nature of the toxin at low pH somehow mimics the behavior of toxin during an

"unfolding-refolding" cycle accompanying its translocation from the endosome into the cytosol (21, 22), we propose that the MAbs we have characterized with low-pH-treated toxin may actually recognize distinct conformations of toxin occurring during its translocation. MAbs whose reactivity we have mapped to the highly cationic region of the toxin (Fig. 1, amino acids 454 to 481), for example, MAb 27A3 (Fig. 3), are of particular interest because they recognize determinants on the carboxyl-terminal side of the hydrophobic region of DTB described by Falmagne et al. (17) as a membrane-penetrating segment. Interestingly, these MAbs recognize toxin in solution only after low-pH treatment (Table 3), suggesting that the induced conformational determinants they recognize are similar to those promoting insertion of the toxin into membranes upon exposure to the acidic environment of the endosome. The general region of the toxin that these MAbs recognize has been indirectly associated with translocation of DTA because toxins lacking this region (e.g., CRM 45 and MspSA) do not make as efficient immuno- or hybrid toxins as does intact toxin (2, 10). However, it is possible that the acidic pH affects residues on DT remote from the epitope recognized by a particular MAb and that the resultant conformational change exposes the MAb-detectable epitopes. We hope to utilize these MAbs to characterize more extensively the conformationally induced DT epitope(s) that they recognize and to monitor changes occurring within the DT molecule during its translocation.

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