Antibodies Capable of Releasing Diphtheria Toxin in Response to the Low pH Found in Endosomes*

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Diphtheria toxin (DT) undergoes a rapid conformational change in response to the acidity encountered within endosomes. That transition is integral to the passage of its catalytic domain into the cytosol and thus its lethal action. The importance of this translocation mechanism led us to develop several monoclonal antibodies that bind DT at neutral pH but spontaneously release the toxin when critical epitopes denature or unfold upon lowering the pH to 4.5–5.5. Hybridomas were selected using a microtiter plate assay that measured the pH-dependent detachment of antibody from immobilized toxin. The acid-sensitive epitopes involved were on the catalytic, transmembrane, and receptor binding domains of DT. This pH-induced disruption of the binding of toxin to these monoclonal antibodies was analyzed by sedimentation velocity ultracentrifugation. Antibody combining sites were fully occupied at pH 5.5, partially bound at pH 5.0, and totally empty at pH 4.5. It was estimated that the $K_i$ for antibody-toxin binding was $\approx 1000$-fold lower at pH 5.0 than at neutral pH. This novel acid-triggered release mechanism provides a basis for delivery of antibody-bound toxin into cells accompanied by its immediate dissociation as the complex enters acidic vesicles.

Many plant and bacterial toxins utilize receptor-mediated endocytic pathways to enter cells effectively and to deliver a lethal, enzymatically active domain into the cytosol. DT,¹ in contrast to most of these protein toxins, inserts its toxic domain into the cytosol directly from endosomes soon after cellular uptake. Its translocation across the vesicular membrane depends on low pH-triggered conformational changes in one or more of its structural domains (1, 2). We took advantage of this property of DT to design antibodies that spontaneously release the toxin as it unfolds in response to endosomal acidification. Conformation-sensitive anti-DT monoclonal antibodies were selected on the basis of their ability to rapidly reline bind toxin when the complex is exposed to pH $\approx 5$ (3). The characteristics of this interaction suggest that these reagents might be useful for delivery of antibody-bound toxin into cells, resulting in the rapid dissociation of toxin upon entry of the complex into acidic endosomal compartments. Unlike conventional, covalently linked immunotoxins or genetically engineered fusion toxins, molecules delivered in this fashion should dissociate from the antibody inside the target cell in an unmodified form, thereby maintaining structural integrity and full toxic potential.

**EXPERIMENTAL PROCEDURES**

**Materials**—DT was isolated (4) from a partially purified preparation purchased from Connaught Laboratories. Toxoid produced by formalin treatment of DT was column-purified as provided by the Department of Health, Commonwealth of Massachusetts. A hybridoma cell line producing a human anti-DT monoclonal antibody designated F10 (5) was obtained from the American Type Culture Collection (Rockville, MD). These cells were injected into pristane-primed Swiss nu/nu athymic mice (Taconic, Germantown, NY) to generate ascites fluid for antibody isolation. Na$^{125}$I (17 Ci/mg) was purchased from NEN Life Science Products. Iodogen was obtained from Pierce. High resolution Sephacryl S300 and XK16 columns were purchased from Pharmacia Biotech Inc.(Uppsala, Sweden).

**Generation and Characterization of Monoclonal Antibodies—**BALB/c mice were immunized over the course of 3 months with progressively higher doses (1, 3, and 10 $\mu$g) of pure, biologically active DT emulsified in 0.25 ml of complete Freund adjuvant. Hybridomas were generated 3 days after an intravenous booster injection of 10 $\mu$g of DT in PBS and selected using Hy medium with 10% fetal calf serum plus hypoxanthine, aminopterin, and thymidine (6). Supernatants from microtiter wells with clones were screened for the ability to bind $^{125}$I-DT, $\sim 4.5$ $\mu$Ci/$\mu$g (diluted into 5% bovine serum albumin) (7), using a polystyrene glycol precipitation method (8). Antibody-positive supernatants bound $\sim 25,000$ cpm, whereas negative clones and Hy medium alone had a background of $\sim 4,000$ cpm. In a typical fusion, 35 positive antibody-producing clones were obtained from the spleen of a single animal.

A second assay was developed to examine the influence of pH on the interaction between DT and the different monoclonal antibodies. DT (100 $\mu$l at 60 $\mu$g/ml in PBS) was adsorbed to polystyrene chloroform mites wells for 18 h, and the plates were then blocked with 0.5% bovine serum albumin in PBS. Antibody or hybridoma culture medium was added to the washed wells in 100 $\mu$l of Hy medium and allowed to react for 2 h before washing the plate with PBS. Attached antibody was revealed by the subsequent addition of $\sim 3 \times 10^6$ cpm of an $^{125}$I-goat anti-mouse IgG reagent at $\sim 2 \mu$Ci/$\mu$g (7) for 1 h followed by washing with PBS and measuring radioactivity in the wells using a Beckman model 5500 gamma counter. Negative controls with Hy medium alone had a background of $\sim 100$ cpm, whereas antibody-containing samples bound $\sim 1,000–4,000$ cpm. Disruption of the binding interaction between toxin and antibody was easily measured as a function of pH, temperature, and time with this rapid assay.

To test for the effects of pH on dissociation of the complex, monoclonal antibody in 100 $\mu$l of Hy medium was bound to immobilized DT for 2 h in replicate wells, and 15 $\mu$l of 1 M sodium acetate buffer was then added to provide a final pH of 4.0, 4.5, 5.0, 5.5, or 7.0 (the control). Dissociation from the toxin was allowed to proceed for various time intervals at either 22 or 37 °C. Released antibody was then quickly washed off the plates with PBS, and the amount remaining was quantified using the $^{125}$I-goat anti-mouse IgG probe. Results were expressed as the percent bound or released compared with the final pH 7.0 control under identical conditions. This pH release assay gave better performance when carried out in Hy medium rather than PBS. Attempts to use a $^{125}$I-DT probe with immobilized antibody or in the polystyrene glycol precipitation method were not successful, because at acid pH the $^{125}$I-DT stuck nonspecifically and irreversibly to surfaces.

Selected clones were injected into pristane-primed mice, ascites fluid was collected, and the 150-kDa anti-DT monoclonal antibody was isolated by $\left(\text{NH}_4\right)_2\text{SO}_4$ precipitation and size fractionation on a high-res...
olution Sephacryl S300 column. Specificity was further assessed by testing the immunoblot reactivity of each antibody for the A versus B fragment of nicked DT that had been separated by polyacrylamide gel electrophoresis under reducing conditions (9, 10). Immunoblot analysis with cross-reactive material (CRM) mutant proteins and genetically engineered diphtheria toxin constructs, DT1–190, DT201–370, and DT201–335 was used to further characterize antibody specificity. The F10, S7A, and 1F3 antibodies recognized epitopes located on the receptor binding domain of DT, whereas 5D5 and 1D5 bound to sites on the transmembrane domain, and 5F5, 6B3, and 4B7 interacted at loci within the catalytic domain.

Binding of 125I-DT as a function of antibody concentration was measured by the polyethylene glycol precipitation assay (8). Inhibition of 125I-DT binding was used to test the reactivity of the monoclonal antibodies with synthetic polypeptides. These were made using Fastmoc chemistry performed with a Applied Biosystems Model 431 peptide synthesizer. The peptides were added at a final concentration of 0.25 mM, and their ability to block the binding of antibody with 125I-DT was compared with the displacement produced by unlabelled whole toxin.

**Analytical Ultracentrifugation Studies**—Initial binding interactions were evaluated by mixing DT and the purified monoclonal antibody in a 2:1 molar ratio in PBS, pH 7.2. A series of dilutions of the complex ranging from 87 to 1.9 μg/ml was studied, and the individual components were also examined at pH 7.2. Sedimentation velocity runs were performed at 20 °C using a Beckman Instruments Model E analytical ultracentrifuge. Sedimentation patterns were acquired with an on-line Rayleigh system (10) and converted into concentration versus radius every 20 s. The camera lens was focused at the ½ plane of the cell equipped with sapphire windows. The apparent sedimentation coefficient distribution functions were computed as described previously (11, 12). Additional sedimentation velocity experiments were performed at 37 °C using a Beckman Instruments Optima XL-A by following the absorbance at 280 nm while acquiring and averaging four flashes every 0.005 cm in the continuous mode. Both a 4-hole (60,000 rpm) and 8-hole rotor (40,000 rpm) were used.

The pH-dependent dissociation was evaluated by establishing a 2:1 DT-antibody complex at a final concentration of 5 × 10⁻⁶ M in PBS, pH 7.2. This preformed complex was diluted 30-fold into 0.125 M NaCl, 0.05 M citrate buffer at pH 7.0, 5.5, 5.0, 4.5, or 4.0 and equilibrated at 37 °C for 2 h before starting the sedimentation velocity run. Dissociation of the preformed complex at pH 5.0 was studied over a concentration range of 345 to 74 μg/ml. The effect of pH on sedimentation velocity was also evaluated for antibody alone and toxin alone. Values of s* shown on the x axis of the sedimentation profiles were corrected to 20 °C.

## RESULTS AND DISCUSSION

**Selection of pH-sensitive Antibodies**—DT undergoes pivotal acid-induced conformational changes within the endosome, and this facilitates the transfer of its catalytic domain into the cytosol (13, 14). Thus it seemed feasible to elicit monoclonal antibodies directed precisely to those epitopes involved in this biologically important process. Native DT was used for the immunization of mice so that its functional sites would be preserved. Toxic effects are much less pronounced in this species (15), and greater tolerance is established with each progressive dose of the immunogen. A substantial proportion of the hybridoma clones obtained in these fusions produced anti-toxin antibodies as judged by the 125I-DT binding assay.

A solid phase assay was developed so that antibodies could be prebound to immobilized DT at neutral pH in Hy medium and then tested for release in response to the addition of an acidic buffer. This method led to the identification of 23 clones producing antibody that rapidly dissociated from the toxin at pH 4.5 and 8 clones having antibody that was sensitive to release at pH 5.0. No antibody was removed when the pH 7.0 sodium citrate buffer was added so that this control served as the standard to calculate percent of antibody bound or released.

Comparison of an antibody that was responsive to the pH change (6B3) versus one that was not (5F5) shows a clear distinction between the two types of antibody identified using this assay (Table I). Binding of some antibodies was incomplete when the immobilized DT was preexposed to acid conditions and then allowed to interact with antibody at neutral pH. This suggests that denaturation was only partially reversible for certain acid-sensitive toxin epitopes.

**Kinetics of pH-induced Complex Dissociation**—The time course of dissociation at pH 4.5 for the 6B3 antibody is presented in Fig. 1A. At 37 °C, the rate of release was faster and more complete than at 22 °C. Approximately 90% of the antibody initially bound detached from DT, and most of this change occurred within the first 5–10 min. It is known that toxin remained attached to the assay plate, since binding of monoclonal antibodies derived from different clones was completely unaffected by the same acid conditions (Table I). The interaction between toxin and the 6B3 antibody was less sensitive to disruption at pH 5.0, with only 25% having been released by 30 min in contrast to 80% dissociated within 5 min at pH 4.5 (Fig. 1B). Kinetics of release for two monoclonal antibodies (5A7 and 1F3) that did dissociate at pH 5.0, 37 °C is shown in Fig. 1C. Attachment of 5A7 to DT was even disrupted at pH levels as high as 5.5 (data not shown). Thus a substantial fraction of DT was rapidly released by these different antibodies at the pH and temperature conditions characteristic of most of the late endosomes and other acidic compartments within cells (16–18). Release of a bound antigen under these mildly acidic conditions is distinct from the reversible denaturation of an antibody at pH 2.5, as commonly deployed for elution from immunoadsorbents.

**Structural Basis of the pH-induced Dissociation**—The pH-dependent breakup of these antibody-antigen complexes appeared to be mainly based upon conformational changes within the toxin rather than the antibodies. Accordingly, the t1/2 = 1 min for this acid-triggered dissociation (Fig. 1A) is close to the t1/2 ≤ 30 s for the pH 5-induced structural transition of free toxin (19). Release of toxin from the antibody was more effective at higher temperatures (Fig. 1A). This is consistent with the report that the conformational transition of free toxin is shifted to higher pH values with increasing temperature (19). The shift to higher pH indicates that DT unfolds more at elevated temperatures.

Additional evidence for the role of toxin conformation was obtained when the 6B3 antibody was bound to formalin-stabilized diphtheria toxoid at pH 7.0 but was only partially released upon changing the pH to 4.5 (Table II). Apparently, chemical cross-linking with formaldehyde stabilized the toxin epitope and prevented the pH-induced transition that allowed the 6B3 antibody to quickly detach from the same epitope on native diphtheria toxin (Table II and Fig. 1B). The observed 25% release from the toxoid might be indicative of pH-induced structural changes in the antibody that reduced binding. It is important to note that whereas 6B3 recognized its epitope on this toxoid, other monoclonal antibodies bound only to the native toxin.

Binding titrations were performed with the purified anti-toxin peptides. The peptides were added at a final concentration of 0.25 mM, and their ability to block the binding of antibody with 125I-DT was compared with the displacement produced by unlabelled whole toxin.

### Table I

| Antibody | pH change | 125I-G/M bound | Release |
|----------|-----------|----------------|---------|
| 6B3      | 7 → 7     | 3066           | 83      |
| 5F5      | 7 → 7     | 4142           | 13      |

Monoclonal antibody was bound to the immobilized DT for 2 h in replicate wells, and then 15 μl of 1 M sodium acetate buffer was added to provide a final pH of 4.0 or 4.0. Dissociation from the toxoid was allowed to proceed for 30 min at 36 °C. Released antibody was washed off the plate, and the amount remaining was quantified using a 125I-goat anti-mouse IgG probe (125I-G/M).
ies and 125I-toxin to determine if the sensitivity to acid pH could be attributed to large differences in their inherent affinity for toxin under non-acid conditions (Fig. 2). However, these data showed less than a 5-fold range in the relative binding strength of the antibodies. No consistent differences were found in the binding parameters of two antibodies, 1D5 and 5D5, that released from DT in response to low pH and a human monoclonal antibody designated F10 or the 5F5 mouse antibody, which did not respond to acid pH (Table I).

Evidence that these pH-sensitive antibodies were directed against conformational as opposed to linear determinants was obtained from their lack of reactivity with synthetic polypeptides. A large excess (0.25 mM) of five peptides comprising amino acids 241–264, 265–299, 300–339, 340–370, and 371–386 of DT failed to block the binding of 125I-DT to these monoclonal antibodies under conditions where a low level (0.01 mM) of the unlabeled whole toxin gave 68% inhibition (data not shown). A combination of all five peptides was also ineffective. The peptides were chosen because they encompassed the transmembrane region of DT where several antibodies were shown to bind by using CRM mutant proteins and genetically engineered DT domain constructs.

Sedimentation Velocity Analysis of the pH-sensitive Complexes—It was important to confirm that the acute pH dependence for antigen-antibody dissociation observed using the solid phase assay also held true for these reactants in solution. Therefore, sedimentation velocity ultracentrifugation was used to examine the dynamics of the acid-induced dissociation of a preformed complex between selected monoclonal antibodies and DT. A strong binding interaction is clearly evident from the comparison of sedimentation velocity profiles displayed by free DT (58 kDa), free 5D5 antibody (150 kDa), and a 2:1 molar mixture of these two components when each was run at 20 °C, pH 7.2 (Fig. 3). The complex formed (266 kDa) displayed a stoichiometry corresponding to an antibody molecule, with toxin bound to each of its two combining sites. The $s_{20,w}$ value for this complex was not affected when the protein concentration was varied between 87 and 1.9 mg/ml, suggesting that the association constant for antibody-antigen binding was $10^9$ M$^{-1}$ at pH 7.2.

To test the effect of pH on dissociation, antibody and DT were allowed to react at neutral pH, and the complex formed was then diluted into different buffers to yield the appropriate final pH value. Sedimentation velocity profiles obtained at 37 °C (Fig. 4A) showed the size difference between the preformed toxin-IgG-toxin complex, intact at pH 5.5, partially dissociated at pH 5.0, and then completely dissociated to yield free IgG antibody at pH 4.5. The peaks were at 8.1, 7.4, and 6.4 S at these respective pH values. Breakup of the complex had not yet begun at pH 5.5, since its size was essentially the same as found at pH 7, 37 °C (data not shown). The single broad peak at

**TABLE II**

| Binding to epitope on: | pH change | 125I-G/M bound | Release |
|-----------------------|-----------|----------------|---------|
| Formalin toxoid       | 7 → 7     | 3453           | 73%     |
|                       | 7 → 4.5   | 2606           | 25%     |
| Native toxin          | 7 → 7     | 3078           | 10%     |
|                       | 7 → 4.5   | 621            | 80%     |
pH 5.0 is indicative of a sedimentation reaction boundary for a system in rapidly reversible equilibrium, consistent with the presence of a complex of low affinity (20, 21). All three components, free DT, anti-DT, and complex, are present but not resolved into separate peaks because of the instantaneous re-establishment of equilibrium between them during sedimentation. The peak position in the sedimentation velocity profiles at pH 4.5 or 4.0 was the same as that for antibody alone, indicating that dissociation of the toxin was complete at these pH values. At neutral pH, DT alone gave a discrete pattern, having a peak value at 4.0 S (Fig. 3), but under acid conditions, no free toxin could be detected either with or without antibody. This is probably due to aggregation or adsorption, owing to the hydrophobic nature of the toxin at acidic pH values (2).

Dissociation of the partial complex at pH 5.0 was further evaluated by concentration-dependence (Fig. 4B). The shift to the left of the peak position of the sedimentation velocity patterns as a function of decreasing concentration indicated that the stability of the complex was weak at this pH. Judging from the concentrations used, the association constant at pH 5 was $-10^{6} \text{ M}^{-1}$, which is approximately 1000-fold lower than the minimum value estimated by a dilution series at pH 7.2. When exposed to pH 4.5, the altered epitope was even less tightly bound by the antibody, since no interaction was detected at these concentrations (Fig. 4A).

The analytical ultracentrifugation studies shown were carried out using the 5D5 antibody that recognizes an epitope in the transmembrane region of the B fragment (1). However, very similar results were obtained with the 4B7 antibody, which interacts with the catalytic A fragment of this molecule. In fact, comparable pH-induced transitions were seen by sedimentation when the genetically engineered catalytic domain (amino acids 1–190) or translocation domain (amino acids 201–370) of DT were tested with 4B7 and 5D5, respectively (data not shown). However, since each domain is only ~20 kDa, the absolute differences between complexed and free antibody were smaller than observed when whole toxin was used (Fig. 3).

Conclusions—This study demonstrates that anti-DT monoclonal antibodies can be selected for their capacity to release bound toxin upon exposure to a moderately low pH. Dissociation was based upon pH-induced conformational changes in epitopes located on the toxin. Different antibodies were reactive with acid-sensitive epitopes located on the catalytic, transmembrane, and receptor binding domains of DT. The diverse pH optima for DT release, differential formalin toxoid reactivity, and individual DT binding affinities measured for the antibodies suggest that they are all distinct antibody-epitope combinations. These anti-DT monoclonal antibodies

**FIG. 2.** Binding of $^{125}$I-toxin as a function of antibody concentration. Two acid-sensitive antibodies 1D5 (○) and 5D5 (●) were compared with two acid-insensitive antibodies F10 (□) and 5F5 (▲). Reactions were carried out at $25 \degree C$ in 100 μl of PBS, pH 7.2, and then precipitated using polyethylene glycol (9).

**FIG. 3.** Sedimentation velocity ($g(s^*)$) profiles obtained at $20 \degree C$, pH 7.2, for diphtheria toxin alone, the 5D5 monoclonal antibody alone, and the complex formed by a 2:1 molar mixture of these two components. $s^*$, sedimentation coefficient.

**FIG. 4.** Sedimentation velocity ($g(s^*)$) profiles showing the dissociation of the preformed toxin-antitoxin 2:1 complex. A, dissociation of complex at $1.3 \times 10^{-6} \text{ M}$ as a function of pH at 37 °C. B, dissociation of complex as a function of concentration at pH 5.0 and 37 °C. (1), 345 μg/ml; (2), 222 μg/ml; and (3), 74 μg/ml. A.U., absorbance units.
were developed so that they could be used as part of a novel bispecific antibody delivery system. The accompanying paper shows that this acid-triggered release mechanism functions inside cells, allowing the antibody-bound toxin to dissociate to a fully active form once the pH within the endosomes drops to a critical level.

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