Protection against Nerve Toxicity by Monoclonal Antibodies to the Sodium Channel Blocker Tetrodotoxin

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Abstract

The sodium channel blocker, tetrodotoxin (TDT), was conjugated to keyhole limpet hemocyanin (KLH) and used to immunize BALB/c mice. Anti-TDT antibodies were detected in serum by ELISA and reached stable levels 4–5 wk after the first immunization. Spleens from immunized mice were fused with NS-1 mouse myeloma cells and ~9,329 resultant hybrids were screened by ELISA for reactivity to TDT. Two stable hybrids were isolated, subcloned, and characterized. These hybrids, termed TD13α1 and TD2C5, secreted specific anti-TDT antibodies that recognized TDT but not the related sodium channel blocker, saxitoxin (STX), as determined by competition ELISA. Both antibodies were of the IgG1 subclass with Kᵦ’s approaching 10⁷ M⁻¹. The inhibitory ability of these antibodies was tested by a competitive displacement assay for [3H]STX on rat brain membranes. Both antibodies strongly inhibited TDT binding to membranes. A nanomole of TD2C5 was able to bind ~ 1.8 nmol of TDT, whereas a comparable amount of TD13α1 bound half as much. Furthermore, TD2C5 was able to protect against TDT-induced reduction of peripheral nerve action potentials in rat tibial nerve when administered in situ. These antibodies thus represent potentially useful reagents for neurobiologic research, detection of toxin contamination and diagnosis of poisoning, and may provide protection against the toxicity of TDT in vivo.

Introduction

Tetrodotoxin (TDT),¹ a heterocyclic guanidinium compound, is one of the most lethal poisons known, with an LD₅₀ of ~ 10 μg/kg in mice (1). Death occurs through a rapidly progressive weakening of voluntary muscles, including respiratory muscles, resulting from the interruption of neuromuscular transmission in motor neurons (2) and in muscle fiber membrane (3). Responsible for ~ 100 deaths annually in Japan (4), TDT is found in animal species as diverse as newts and puffer fish (5). This widespread distribution of TDT across animal species and its apparent lack of in situ biosynthesis (6) suggested an exogenous source for this toxin, which was subsequently confirmed by the isolation of a variety of TDT-synthesizing bacteria (7).

TDT has generated considerable interest in the field of neurobiology by its ability to block sodium ion transport (8). Sodium currents can be blocked by as little as 1–10-nM concentrations of TDT in nerve and muscle (9) and the TDT-binding site is thought to lie on the external surface of membranes rather than inside the sodium channels, per se (10). A 260-kD protein has been implicated as the TDT binding site (11).

Despite the potential public health usefulness of detecting and neutralizing the toxicity of TDT, little immunologic interest has been centered on this compound. Detection of TDT has relied heavily on mouse bioassays (12) and on chemical assays (13) that have been hampered by low sensitivity or by the presence of interfering compounds. More recently, sensitive bioreceptor assays using crude brain membranes have permitted the detection of less than a nanogram of TDT per milliliter in buffer or serum solutions (14). Although sensitive, the radioreceptor assay does not lend itself to large scale screening. The availability of specific anti-TDT antibodies would permit the development of highly sensitive immunoassays that could be used to rapidly screen for the presence of TDT. To the best of our knowledge, no anti-TDT MAb have been reported. Because of the dearth of antibodies to this toxin, we sought to generate MAb that were specific for TDT. We now describe two stable, specific MAb that demonstrate an inhibitory effect against TDT in vitro and in situ.

Methods

Generation of MAb to TDT: Because of its small size, TDT (Sigma Chemical Co., St. Louis, MO) was conjugated to keyhole limpet hemocyanin (KLH; Calbiochem-Behring Corp., La Jolla, CA) via formaldehyde as the coupling agent (15, 16) to render it immunogenic. Briefly, 300 μg of TDT in 1,000 μl of 1 M sodium acetate buffer, pH 7.4, was conjugated to 80 μl of KLH (53 mg/ml) in the presence of 60 μl of 37% formaldehyde. The mixture was shaken at 37°C for 3 d and dialyzed for 24 h at 4°C. After dialysis, the TDT-KLH was precipitated with 800 μl of 10% aluminum potassium sulfate and 500 μl of 1 N NaOH at 4°C. The resulting precipitate was washed six times with cold PBS. Conjugates were prepared freshly for each immunization.

Female BALB/c mice (6–8 wk old) were immunized at weekly intervals with 50 μl (50 μg) of TDT-KLH conjugate per mouse mixed with an equivalent volume of CFA (Difco Laboratories, Detroit, MI) intradermally for the first immunization, Freund’s incomplete (intra-dermally) for the second, and PBS (intraperitoneally) for all subsequent injections. Sera were collected at weekly intervals. 4 d after a final intravenous immunization in the tail vein, the spleen was removed and the cells were fused with NS-1 myeloma (17) cells in 40% polyethylene glycol-4000 (Accurate Chemical and Scientific Corp., Westbury, NY) according to Gefer et al. (18) at a ratio of three to six
spleen cells per myeloma cell in serum-free DME. The fused cells were cultured in 96-well microtiter plates (Corning Glass, Corning, NY) at a density of 0.5–1 × 10^4 cells/ml in the presence of DME-hyoxanthine-aminopterin-thymidine (HAT) selective medium (19) containing 10% fetal bovine serum (Hazelton Research Products, Inc., Lenexa, KS), 2 mM glutamine, 1× nonessential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, 50 IU/ml penicillin and 50 μg/ml streptomycin purchased from Mediatace (Washington, DC). Plates were refed with HAT medium 3 d later and subsequent feedings contained HT but no aminopterin.

**ELISA for the detection of anti-TDT antibodies.** Culture supernatants and mouse sera were screened for TDT-specific antibodies by ELISA (15, 16). Briefly, 700 μg of TDT or saxitoxin (STX) obtained from Dr. John Hewetson (U. S. Army Medical Research and Development Command, Fort Detrick, MD) were conjugated to 6 mg of BSA (Calbiochem-Behring Corp.) with 41 μl of 37% formaldehyde and incubated for 3 d at 37°C. They were then dialyzed for 3 d at 4°C against PBS. Polystyrene plates (Costar Corp., Cambridge, MA) were coated with 50 μl/well of a 10 μg/ml solution of STX-BSA or TDT-BSA and stored at 4°C until needed. The plates were blocked with 200 μl of 5% normal goat serum (Cone Biotech, Seguin, TX) in PBS for 1 h at 37°C followed by incubation with supernatants or dilutions of sera (100 μl) for an additional hour at 37°C. After the plates were washed three times with PBS containing 1% Tween-20, 15 μl of a 1:2,500 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase (Fisher Scientific, Orangeburg, NY) were then added for 1 h at 37°C. The wells were again washed and were then developed with 100 μl of 2,2-azino-di-3-ethylbenzthiazoline-6-sulfonate, 0.3% in 0.1 M citric acid buffer, pH 4.0 (ABTS; Sigma Chemical Co.). The reaction was terminated by adding 100 μl of 5% SDS in PBS and absorbance was read at 410 nm in a microplate reader (Dynatech Laboratories, Alexandria, VA).

Positive clones were transferred to 24-well plates (Costar Corp.) and subcloned by limiting dilution as rapidly as possible. Positive subclones were selected on the basis of antibody reactivity by ELISA and were resubcloned until the majority of the subclones were antibody positive. Aliquots of positive clones were cryopreserved in liquid nitrogen.

**Antibody specificity and isotype.** Because of the structural similarity between TDT and another sodium channel blocker, STX, the specificity of antibody secreted by the TD2CS and TD13a anti-TDT positive cell lines was evaluated by concurrently testing supernatant from each cell line for cross-reactivity in a competition ELISA using TDT-BSA-coated wells. Supernatants to be tested (100 μl) were incubated with a 300 μl of the following: PBS, STX (200 μg/ml) or TDT (200 μg/ml), for 1 h at 37°C before addition to TDT-BSA coated wells. A test antibody was considered specific if TDT reduced the OD_{405 nm} by >75% relative to the PBS control with STX having no effect, and if the antibody demonstrated no binding reactivity in STX-BSA-coated plates.

**Antibody isotype was evaluated by direct viable immunofluorescence.** Hybrid cells were washed and incubated in the presence of either goat anti-mouse γ1, γ2a, γ2b, γ3, δ, η or η1 polyclonal anti-Ig conjugated to FITC. Cells were then washed and analyzed by a FACS (Becton Dickinson, Mountain View, CA). Isotype was further confirmed by a sandwich ELISA in which purified rabbit anti-mouse H and L chain isotypes-specific antisera (Bio-Rad Laboratories, Richmond, CA) were employed. Briefly, microtiter plates precoated with TDT-BSA were blocked for 1 h at 37°C with 5% normal goat serum. 100-μl antibodies were then added and incubated for another hour at 37°C. After washing the plate with PBS-Tween, 50 μl of the rabbit anti-mouse subclass-specific antisera were then introduced for 1 h at 37°C. The plates were again washed and 50 μl of goat anti-rabbit Ig conjugated to horseradish peroxidase added for 1 h at 37°C. The plate was again washed, developed with ABTS, and read at A_{405 nm}.

**Determination of relative binding affinity constant.** The relative binding affinity of antibody for TDT was determined by IC_{50} (20) competitive inhibition enzyme immunoassay. In this assay, dilutions of antibody-containing supernatants were tested by ELISA and the 50% binding point was estimated. The dilution yielding 50% binding (100 μl) was then incubated with different toxin concentrations (50 μl at 12 ng/ml to 50 μg/ml) in serial twofold dilutions for 1 h at 37°C followed by ELISA. To determine the IC_{50} (molar concentration of toxin giving 50% reduction in OD), the OD were converted to percent reduction of maximal OD (y). The y values were then converted to a logit according to the following formula: logit = ln y/(100 − y). Logit values were plotted against molar toxin concentration on semilog paper and the IC_{50} was determined graphically by dropping a vertical line to the x-axis from the point where the straight line crossed logit = 0. The relative affinity constant K_{a} is the reciprocal of the IC_{50}.

**Antibody purification.** Ascites fluid was harvested from pristane-primed mice that had been injected with 5 × 10^6 hybridoma cells each. The fluids were clarified by centrifugation (10,000 g, 10 min) and the presence of antibody was confirmed by ELISA. A combination of caprylic acid and saturated ammonium sulfate precipitations (21) was then used to purify both antibodies that are of the IgG subclass. Purity was checked electrophoretically by SDS-PAGE (22).

**In vitro membrane receptor binding assay.** The ability of STX and TDT to bind stoichiometrically (23) to a common receptor site was used in radioreceptor assays (14, 24) to demonstrate the protective abilities of anti-TDT MAb. Crude membrane receptors from the brains of female AXC/SH rats (from the colony maintained at the Southwest Foundation for Biomedical Research) were prepared according to Strichartz (24) and their protein content determined by the Bradford method (25) using a commercially available kit (Bio-Rad Laboratories). Aliquots were stored at −80°C until needed. Optimum incubation times and binding conditions of [3H]STX to the brain membranes were determined according to the radioreceptor studies above (14, 24). Because the TDT specificity of the anti-TDT antibodies precluded their binding to [3H]STX directly nor is suitable radiolabeled TDT readily available (26), a competitive displacement assay was developed to assess the TDT-binding capacity of the anti-TDT antibodies. Membranes (500 μg protein in 25 μl) were added to microfuge tubes and incubated under saturating conditions in the presence of [11-3H]STX with a specific activity of 63 Ci/mmol (Amersham Corp., Arlington Heights, IL) at a final concentration of 6 nM. Various amounts of radioinert TDT (0.1–10 ng) were included in the presence and absence of a constant, known, concentration of antibody for 1 h at 4°C and the amount of [3H]STX bound was measured. Two competition curves for [3H]STX binding were thus generated, with and without anti-TDT antibody, respectively, and the amount of TDT giving 50% of [3H]STX binding in the presence and absence of antibody calculated graphically on each curve. The difference between these two values represented the amount of TDT bound by antibody. Similar displacement curves were generated in the presence of radioinert STX to demonstrate the specificity of binding of anti-TDT antibodies for TDT and not STX.

**In situ protection of peripheral nerve function with anti-TDT antibody.** 30 male Sprague-Dawley rats (age 40–60 d) were used. Sodium pentobarbital (Nembutal) was administered intraperitoneally (60 mg/kg body wt) and the rats were placed on an isothermal heating pad. The left rear leg and hip were shaved and the leg was immobilized on a small surgical platform. A dissection was performed to expose the sciatic nerve and tibial branch entering the gastrocnemius muscle. The skin surrounding the incision was lifted and secured with hemostats to form an enclosure for bathing the nerve in warm 0.5% NaCl that contained the various antibody and TDT concentrations. The solutions were applied and withdrawn using Pasteur pipettes.

Bipolar stimulating and recording electrodes manufactured from Teflon-insulated silver wire were positioned beneath the sciatic and tibial nerves, respectively. Single square-wave pulses (0.05 ms duration) were used to evoke action potentials that were recorded with a differential amplifier (World Precision Instruments, New Haven, CT). The tibial nerve responses were displayed and photographed using a storage oscilloscope (Tektronix, Inc., Beaverton, OR). Response amplitude was measured as the difference between the most positive and negative deflection of the compound action potential waveform. The
stimulus intensity (3–6 V) was adjusted to produce maximal compound action potential amplitude. Before any application of antibody or toxin, the action potential amplitude was recorded every 30 s for a 3-min period and the average of these six responses formed the baseline data for each preparation. Experimental treatment consisted of soaking the nerve for two 5-min periods in either antibody alone (control), TDT alone, or TDT combined with antibody. When toxin and antibody treatments were carried out the antibody was added to the toxin solution 1 h before application. After 5 min of soaking the nerve the solution was then removed and the average response amplitude was determined as described above for baseline data. The same solution was then reapplied for an additional 5 min and withdrawn; the average response amplitude was again determined. At the end of the experiment the animal was killed by an overdose of Nembutal.

Baseline and experimental responses were compared using analysis of variance (ANOVA) with a general linear model procedure (SAS Institute, Cary, NC). Data displaying an overall significance level of $P < 0.05$ were further analyzed with the Newman-Keuls multiple-range test.

**Results**

Detection of specific MAb to TDT. Sera from TDT-immunized mice frequently demonstrated antitoxin reactivity by ELISA as early as 1 wk after the first immunization (data not shown). Anti-TDT antibody titers increased with time and reached stable levels at ~4–5 wk and thereafter. After mice had received six or more injections with immunogen to ensure that their serum anti-TDT titers had reached maximal levels, their spleen mononuclear cells were fused with NS-1 mouse myeloma cells for the generation of hybridomas. Of the 9,329 clones tested, 337 (3.6%) were positive for anti-TDT antibodies by ELISA. Of these, two highly stable clones (TD2C5 and TD13a1) were isolated. These were subcloned and resubcloned until most or all of the derivative cultures were antibody positive. The specificity of TD2C5 and TD13a1 antibodies for TDT in a TDT-BSA competition ELISA is shown in Table I. The presence of 100 μg/ml TDT completely inhibited the binding of both antibodies, whereas the same amount of STX or PBS had no effect. Free TDT at a concentration of 50 ng/ml inhibited the binding of the TD2C5 and TD13a1 MAb by ~50 and 25%, respectively. In addition, neither antibody bound to an STX-BSA-coated plate (data not shown), further confirming their specificity for TDT. The most reactive sub-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Inhibition of TD2C5 anti-TDT antibody binding by TDT. The MAb, TD2C5 (4) was incubated in the presence or absence of STX (●) or TDT (○) at the concentrations indicated. The IC$_{50}$ determination of the data presented in A is depicted in B (see Methods).

clones of these hybridomas were chosen and characterized further.

Antibody isotype and affinity. The antibody isotype of the above two clones was determined and both were of the IgG1k subclass. By IC$_{50}$ analysis, the anti-TDT MAb possessed the following K$_{D}$'s: TD2C5, 0.5 × 10^{-7} M$^{-1}$ and TD13a1, 0.2 × 10^{-7} M$^{-1}$. The titration-inhibition data for one of these antibodies, TD2C5, is illustrated in Fig. 1 A. A predetermined dilution of TD2C5 giving ~50% binding in a TDT-BSA ELISA was mixed with concentrations of TDT ranging from 10^{-4} to 10^{-8} M. The reduction in TD2C5 binding in the presence of free TDT is depicted in Fig. 1 A. The binding of TD2C5 was inhibited by ~40% by as little as a 10^{-7} M solution of TDT, whereas a 1,000-fold higher concentration of STX had no effect, again demonstrating the specific binding of TD2C5 to TDT and not STX. The IC$_{50}$ analysis of the TD2C5 antibody is displayed in Fig. 1 B and was generated from the data presented in Fig. 1 A, yielding an K$_{D}$ value of 2 × 10^{-7} M. The K$_{D}$ is therefore the reciprocal of the IC$_{50}$ or 0.5 × 10^{-7} M$^{-1}$. Similar analyses were used for TD13a1 (data not shown).

**Inhibitory effect of anti-TDT antibodies in vitro.** Using the brain receptor displacement assay, both anti-TDT antibodies demonstrated the ability to bind radioinert TDT and prevent its binding to the receptors resulting in increased binding of $[^3$H]STX to these receptors. TD2C5 (1.2 μg) was able to displace ~5 ng of TDT at the 50% inhibition point (Fig. 2). In terms of molar capacity, 1 nmol of TD2C5 bound 1.8 nmol of TDT, which approached the theoretical capacity of 2 nmol of antigen bound by 1 nmol of an IgG antibody. The high specificity of this antibody was also manifested by the inability of even a 10-fold higher (13 μg) amount of antibody to bind STX (Fig. 2). 1 nmol of the antibody TD13a1, by similar analysis, bound 0.87 nmol of TDT (data not shown).

In situ protection against TDT-induced nerve toxicity by TD2C5. The results of these experiments are summarized in Table II. The amplitudes of the action potentials generated in rat tibial nerve were recorded in the presence of 5.5 μM TD2C5 alone (control), in the presence of 10 μM TDT alone (TDT), and in the presence of 10 μM TDT plus 5.5 μM TD2C5 (Exp 1), 10 μM TDT plus 2.78 μM TD2C5 (Exp 2) or 10 μM TDT plus 0.5 μM TD2C5 (Exp 3). Baseline recordings were made, before addition of any compound and effects of
the test agents were measured after two 5-min exposure periods. When antibody was applied alone there was no significant change in response amplitude. However, application of the toxin alone reduced the compound action potential to 37% of baseline after 5 min and to 22% after an additional 5 min. Addition of the two highest concentrations of TD2C5 (Exp 1 and 2, Table II) markedly counteracted the diminution in response amplitude caused by TDT, whereas the lowest dose (Exp 3) afforded little protection against TDT action. Typical effects of TDT on action potentials in the presence of 5.5 mM TD2C5 alone (control), 10 mM TDT alone (TDT), and 10 mM TDT plus 5.5 mM TD2C5 (TD2C5) are depicted in Fig. 3. Addition of TD2C5 completely prevented the toxic effect of TDT on action potentials. The small differences in waveform shape shown in the three baseline traces were due to slight variations in electrode placement that normally occur from one recording preparation to another and do not reflect any significant difference in the nerve response.

### Discussion

In this paper, we have described two anti-TDT MAb of high specificity for TDT, which to our knowledge represent the first reported MAb to TDT. Production of these MAb is highly stable, as evidenced by their continuous elaboration for over a year in culture. These MAb were generated from mice immunized with TDT-KLH conjugate in a manner as previously described for STX-KLH (27). This TDT conjugate was highly immunogenic and stimulated the production of anti-TDT antibodies in serum as early as 1 wk after the first immunization. Spleens from all immunized mice were successfully fused with murine myeloma cells so that all fusions yielded anti-TDT positive hybridomas. The overall percentage of positive hybrids in our experiments compared favorably with those obtained (3.6%) with the STX-KLH fusions (27).

Further testing of these positive hybrids yielded only two stable antibodies specific for TDT. These hybrids produced highly specific MAb, TD13a.1 and TD2C5, which did not cross-react with the closely related toxin, STX, even when the toxin was present at a final concentration of 100 μg/ml TDT. On the other hand, was easily detectable at a 1,000-fold lower concentration by either antibody. These MAb were both of the IgG1 subclass and possessed high affinities for TDT, with the TD2C5 MAb having the higher affinity. The differences in affinity were apparent in the [3H]STX radioreceptor displacement assay, in which 1 nmol of TD2C5 bound nearly 2 nmol
of TDT, whereas TD13a.1 was only able to bind 0.87 nmol. Both antibodies thus had sufficient affinity to compete for TDT binding against STX on brain membranes with a $K_d$ ~ 100-fold greater than either antibody (14). The specificity for TDT and not STX of the TD2C5 MAb was also evident in the $[^3H]$STX displacement assay. Concentrations of TD2C5 10-fold higher than those used to bind TDT had no significant effect on STX displacement of $[^3H]$STX binding. This specificity of TD2C5 might prove useful in neurobiologic studies to elucidate and differentiate the binding site of TDT from STX on nerve or muscle.

The protective ability of the TD2C5 MAb was further demonstrated by its ability to inhibit TDT-induced toxicity in rat tibial nerve. TD2C5 prevented the diminution in action potential caused by a twofold greater mol cataract of TDT that correlated with the antibody-binding capacity for TDT observed in the radioreceptor assay. Although further documentation would be needed, the observations above suggest that the radioreceptor assay may serve as a screen for predicting the in situ protective effects of particular MAb. The TD13a.1 MAb was not tested on rat nerve but based on its strong performance in the receptor assay, it is reasonable to speculate that it, too, would protect against TDT toxicity in situ.

The capacity of these MAb to interfere with TDT binding in vitro and of TD2C5 to inhibit TDT-induced toxicity in situ would strongly support the possibility that these MAb would protect against the lethal effects of TDT in vivo. In addition, the strong affinity of both MAb for TDT might allow these MAb to be ideal vaccine candidates (28). They could be used to generate specific antiidiotypic reagents as potential vaccines for induction of a protective immune response against TDT as has been demonstrated in other systems (29). Such an antiidiotypic vaccine would obviate the potentially toxic nature of TDT conjugates as immunogens. The same conjugation methods used in this study, have tended to be unstable when used for other toxins, resulting in their potential release (30).

A further use for these antibodies could be for public health testing for the presence of contaminating TDT in seafood. They could also serve to screen patients suspected of TDT intoxication and aid in the diagnosis of poisoning.

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