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Isolation and Characterization of a Novel Human Monoclonal Antibody That Neutralizes Tetanus Toxin[1]

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ABSTRACT

A human monoclonal antibody, designated 53-2-4, has been isolated and characterized in terms of its ability to interact with clostridial neurotoxins. In enzyme-linked immunosorbent assay assays the antibody reacted with native tetanus toxin, tetanus toxoid and the C fragment obtained from the carboxyterminus of the toxin (AA 864-1314). The antibody did not react with the B fragment of tetanus toxin (AA 1-863) or with six serotypes of botulinum neurotoxin (A to F). Both culture supernatant from the clonal line producing the antibody as well as homogeneous protein obtained by affinity purification of the antibody neutralized tetanus toxin. When tested *in vivo*, the antibody provided complete production against a supralethal injection of toxin; when tested *in vitro*, the antibody produced at least 99% inactivation of a 1×10^{-9} M solution of toxin. The exceptional neutralizing activity of the antibody was attributed to its high affinity for the toxin (4.2×10^{-10} mol/liter). Animal experiments revealed a novel phenomenon that has been labeled delayed intoxication. At the appropriate ratio of antibody to antigen, the toxin was retained in the host in a latent form. After several days the biological activity of the toxin became apparent and there was onset of nervous system poisoning. Isolated tissue experiments showed that each antibody molecule is capable of associating with two antigen molecules. The antibody has greater neutralizing activity when mixed with free toxin than when mixed with toxin already bound to plasma membrane receptors.

There have been numerous reports describing mouse and human monoclonal antibodies against tetanus toxin. However, none of these reports describes a product that has been introduced into clinical practice. Work on mouse antibodies has been aimed primarily at generating probes for structure-function analyses (Kenimer *et al.*, 1983; Sheppard *et al.*, 1984; Volk *et al.*, 1984); work on human antibodies has been generally limited to showing that human hybridomas can be stable producers of antitoxin (Zurawski *et al.*, 1978; Kozbor and Roder, 1981; Kozbor *et al.*, 1982a, b; Chiorazzi *et al.*, 1982; Gigliotti and Insel, 1982; Larrick *et al.*, 1983; Ho *et al.*, 1985; Ichimori *et al.*, 1985; Ziegler-Heitbrock *et al.*, 1986; Hirata and Sugawara, 1987; Scrivner *et al.*, 1987; Kitano *et al.*, 1988).

Although none of the antibodies studied in the past has led to the development of a therapeutic agent, there is now reason to renew the effort. The complete nucleotide sequence for tetanus toxin has recently been determined, and from this a complete amino acid sequence was deduced (Eisel *et al.*, 1986; Fairweather and Lyness, 1986). A knowledge of the primary structure of the toxin, when combined with monoclonal antibody techniques, may create unique opportunities for generating clinically useful antitoxins and toxoids. For example, it should be possible to raise antibodies that bind to critically important structural domains in the toxin molecule, thus neutralizing toxicity at very low concentrations. In addition, it should be possible to use epitope mapping and protein chemistry to identify amino acid sequences that elicit neutralizing antibodies. This in turn could serve as the basis for creating a synthetic vaccine.

In the report that follows, the initial steps have been taken that may culminate in the development of a clinically useful human antitoxin and an equally useful synthetic vaccine. A human hybridoma has been created that is stable and that produces significant quantities of a potent neutralizing antibody. Experiments have been done to characterize the antibody, to describe its interaction with antigen and to probe the effect that the antigen-antibody reaction has on the neuroparalytic actions of tetanus toxin.

Materials and Methods

Clostridial toxins and tetanus toxoid. Botulinum neurotoxin types A, B, C, D, E and F were isolated by methods that have been previously described (Sakaguchi, 1983). Tetanus toxin, the B fragment

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ABBREVIATIONS: DMEM, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; i.p., intraperitoneal.

of tetanus toxin (AA 1-863) and the C fragment of tetanus toxin (AA 864-1314) were purchased from Calbiochem (La Jolla, CA). Tetanus toxoid intended for experimental purposes (purified, 875 Lfu/ml) was obtained from the Massachusetts Public Health Biologic Labs (Jamaica Plains; MA); toxoid for human administration was obtained from Connaught Laboratories, Inc. (Swiftwater, PA).

Cell fusion and culture conditions. Hybridomas were produced according to the method of Östberg and Pursch (1983) with minor variations. A human donor was inoculated intramuscularly) with tetanus toxin, and 7 days later peripheral blood mononuclear cells from heparin-treated venous blood were isolated by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ). The myeloma partner, SPAZ-4, was maintained in log-phase growth in DMEM containing 10% selected, heat-inactivated FBS (DMEM-10% FBS). Cells were washed once in cold Hank's balanced salt solution mixed at a 1:1 ratio and pelleted. After the supernatant was removed, the cells were fused with polyethylene glycol 50% (PEG 3350, J.T. Baker, Phillipsburg, NJ) in Hank's balanced salt solution. The cells were washed once, resuspended in DMEM-20% FBS containing hypoxanthine (1×10^{-4} M), aminopterine (1×10^{-7} M), thymidine (1.6×10^{-5} M) and ouabain (1×10^{-7} M) and then dispensed in microplates at approximately 2×10^5 cells per well. Cultures were fed every 3 to 4 days with medium containing hypoxanthine and thymidine. Positive cultures identified by ELISA were cloned by limiting dilution in the presence of murine thymus cells (~2.5×10^5 per well).

ELISA assay. Antibody to tetanus toxin was detected with an ELISA method. Purified tetanus toxin, toxin fragments or toxoid were adsorbed overnight to microtiter plates at dilutions of 1/5,000 to 1/10,000 in bicarbonate buffer, pH 9.6. The plates were washed, blocked with 1% normal goat serum, incubated with test antibody to tetanus toxin, washed with phosphate-buffered saline containing 5% Triton X-100 and then incubated with peroxidase-conjugated, goat anti-human immunoglobulin (Tago, Inc., Burlingame, CA). After a final wash, enzyme was detected with *O*-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO) dissolved in a phosphate buffer, pH 5.0. Antibody isotype and light chains were identified using a panel of appropriate affinity-purified, peroxidase-conjugated goat antibodies.

Purification of antibody. Cell culture supernatants produced in DMEM-5% FBS were clarified by centrifugation and applied to a 10-ml column of protein-A-agarose (Affi-Gel, Biorad, Richmond, CA). After saturation of the column, immunoglobulin was eluted in 10-ml fractions with an HCl-glycine buffer, pH 3.5, and immediately neutralized with 1.0 ml of 1 M Tris buffer, pH 8.0. Antibody purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. Only two strongly stained bands corresponding to heavy and light chains were detected with these methods. Antibody concentration was calculated from OD_{280}, E = 14.0.

Measurement of antibody affinity. Tetanus toxin (1 mg/ml; Calbiochem La Jolla, CA) was handled as a biohazard and labeled with ^{125}I using the Bolton-Hunter reagent according to the manufacturer's recommendations (New England Nuclear, Boston, MA). Labeled toxin was separated from free label on a 1.0-ml column of Sephadex G-10. The reactants were titrated in order to determine the optimal concentration range of each for the affinity assay. First, purified MAB 53-2-4 (0.1 ml) and ^{125}I tetanus toxin (3×10^4 cpm in 0.9 ml) were individually diluted in phosphate-buffered saline containing 2% fetal bovine serum, after which the two were combined for 1 hr at 37°C followed by 1 hr at 20°C in polypropylene tubes. A 0.1-ml aliquot of 1% formalin-fixed *Staphylococcus* A cells (Immuno-Precipitin, BRL, Gaithersburg, MD) was then added and the tubes were mixed four times during incubation at room temperature for 30 min. The tubes were then centrifuged at 12,000 rpm for 10 min, and 0.5 ml of supernatant and 0.5 ml of solution containing resuspended pellet were collected for scintillation analysis. The concentration of MAB that precipitated one-half of the precipitable cpm (84.5% of total cpm was precipitable) was used in subsequent assays.

Unlabeled toxin, at varying concentrations, was added to the fixed amount of labeled toxin followed by MPA 53-2-4 at the dilution that produced half-maximal precipitation. This experiment was performed using 10-fold dilutions of toxin and then repeated at the relevant range of concentrations using 2-fold dilutions. The graph of b/f *vs.* M was generated using b peq cpm in epllet and f = cpm in the total supernatant; M was determined from the concentration of toxin (unlabeled plus labeled) based upon a molecular weight of 150,000 kD.

Chromatography of proteins. Tetanus toxin alone (500 μg) or in combination with the antibody (250 μg; 37°C; 30 min) was applied to a column of Sephacryl S-300 (1.6 × 94 cm; Pharmacia, Piscataway, NJ). The proteins were dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, and the column was equilibrated with the same buffer. The elution volume of the toxin alone and of the antibody-toxin complex was determined by monitoring absorbance at 280 nm. A calibration curve for apparent molecular weight was constructed by using thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and aldolase (158,000).

***In vivo* neutralization tests.** BALB/c male mice, 8 to 11 weeks of age, were randomized and injected i.p. with tetanus toxin, antibody or both in a total volume of 1.0 to 1.1 ml. Mice were observed for 15 days.

***In vitro* bioassay.** Phrenic nerve-hemidiaphragm preparations were excised from mice and placed either in a tissue bath or an incubation bath (see "Results"). Tissues were suspended in a physiological solution that was bubbled with 95% O_2, 5% CO_2. The solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; Na_2HPO_4, 1.0; glucose, 11. Solutions were supplemented with gelatin to diminish adsorption and nonspecific inactivation of toxin. The tissue baths were kept at 35°C, and the incubation tubes were kept at 4°C.

The parameters of phrenic nerve stimulation were 0.2 Hz square waves of 0.1 to 0.3 msec duration. Muscle twitch was recorded with a force-displacement transducer connected to a physiologic recorder. Toxin-induced paralysis of neuromuscular transmission was measured as a 90% reduction in muscle twitch amplitude evoked by nerve stimulation.

Results

Characterization of the antibody. Serial limiting dilution was used to isolate a clone producing an antibody that was strongly reactive with tetanus toxin (ELISA). This cell line was designated hu MAB 53-2-4.

Production of antibody was stable for more than 12 months in continuous culture, and it could also be obtained in serum-free media. The antibody was identified as an immunoglobulin G1 *kappa* by ELISA methods and chain specific reagents, and it had high affinity for *Staphylococcus aureus* protein A. Low-pH elution of hu MAB 53-2-4 from an antibody-saturated protein A affinity matrix yielded a homogeneous immunoglobulin when analyzed by polyacrylamide gel electrophoresis and a silver stain.

The affinity of MAB 53-2-4 for ^{125}I-tetanus toxin was determined by Scatchard analysis, as described in "Materials and Methods." The results, which are illustrated in figure 1, gave an apparent equilibrium constant of 4.2×10^{-10} mol/liter.

Antigenic determinants. The antigenic determinant recognized by hu MAB 53-2-4 was present in tetanus toxoid and in the native toxin (fig. 2*A*). Experiments with papain-derived fragments further localized the epitope to the C fragment (AA 864-1314). As determined by ELISA methods, the B fragment (AA 1-863) bound antibody at least three orders of magnitude more poorly than C fragment.

Two types of companion experiments were done. In the first, commerical antiserum was tested against the toxoid, the toxin and the B and C fragments (fig. 2*B*). All of the proteins were recognized by the antibody. In the second experiment, hu MAB

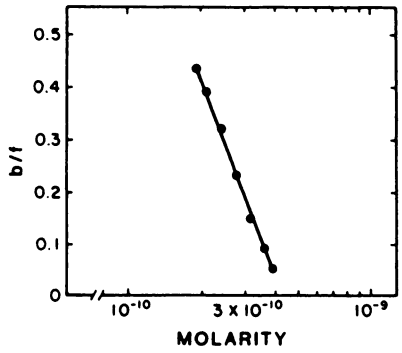

Fig. 1. Scatchard plot of the reaction between ^{125}I-labeled tetanus toxin and human monoclonal antibody 53-2-4. The equilibrium constant, as determined by regression methods, was 4.2×10^{-10} mol/liter.

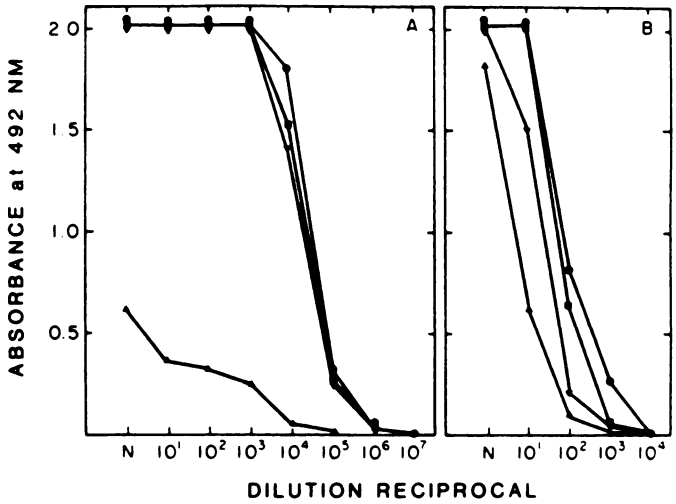

Fig. 2. Results of ELISA assays with hu MAB 53-2-4 and various tetanus antigens. Panel *A* shows the reaction of purified monoclonal antibody with: ●, native tetanus toxin; □, tetanus toxoid; ▽, fragment C; or △, fragment B. Panel *B* shows the reaction of a commerical preparation of human IgG with the same antigens displayed in Panel *A*. Note that antiserum recognizes antigens on the B fragment that are not recognized by the monoclonal antibody.

53-2-4 was tested against botulinum neurotoxins A, B, C, D, E and F. None of the proteins possessed significant reactivity with hu MAB 53-2-4 (results not illustrated).

Titration of toxin. Tetanus toxin was bioassayed in serial dilutions (10 to 0.00010 ng) that were prepared in DMEM-2% FBS. The material (1.0 ml) was administered i.p., and groups of mice (n = 10) were observed for 15 days. The LD_{50} was calculated as 2.7×10^{-2} ng.

Effect of culture supernatant on toxicity. Groups of mice (n = 10) were injected i.p. with tetanus toxin alone (10 LD_{50}; *i.e.*, 2.7×10^{-1} ng) or with tetanus toxin that had been incubated with culture supernatant from hu MAB 53-2-4 (30 min; room temperature). The results (table 1) indicate that the supernatant possessed substantial neutralizing activity. Even when diluted four orders of magnitude, the supernatant produced a detectable diminution in toxicity. When diluted three orders of magnitude, the material completely neutralized a lethal dose of tetanus toxin.

The results were further quantified by testing known amounts of antibody, either in culture supernatant or in purified form. The results of the two preparations were equivalent (table 2), and they indicated that a dose of 0.01 to 0.1 μg of antibody completely neutralized 10 LD_{50} of tetanus toxin. This

TABLE 1

Effect of hu MAB 53-2-4 in culture supernatant on the *in vivo* toxicity of tetanus toxin[a]

Group	Treatment	Mice Surviving on Day:							
		1	3	5	6	7	9	10	21
1	Diluent + tetanus toxin	10	2	1	1	1	1	1	1
2	Supernatant + tetanus toxin	10	10	10	10	10	10	10	10
3	Supernatant (1/10) + tetanus toxin	10	10	10	10	10	10	10	10
4	Supernatant ($1/10^2$) + tetanus toxin	10	10	10	10	10	10	10	10
5	Supernatant ($1/10^3$) + tetanus toxin	10	10	10	10	10	10	10	10
6	Supernatant ($1/10^4$) + tetanus toxin	10	10	10	9	6	3	0	0
7	Diluent only	10	10	10	10	10	10	10	10

[a] Mice were injected i.p. on day 0 with 10 LD_{50} tetanus toxin (0.1 ml) admixed with tissue culture supernatant containing hu MAB 53-2-4 (1.0 ml). Diluent was DMEM-2% FBS.

TABLE 2

Protection of mice from tetanus toxin by crude or purified human monoclonal antibody 53-2-4[a]

Culture Supernatant			Purified Antibody		
Group	Dose	Survival	Group	Dose	Survival
	(μg)			(μg)	
1	10	8/8	8	10	8/8
2	1.0	8/8	9	1	8/8
3	0.1	8/8	10	0.1	8/8
4	0.01	4/8	11	0.01	7/8
5	0.001	3/8	12	0.001	3/8
6	Diluent	1/8	13	Diluent	0/8

[a] Mice were divided into groups of eight. Each animal was injected i.p. with 1.1 ml of antibody mixed with 10 LD_{50} of tetanus toxin. Antibody concentrations were determined with ELISA methods calibrated with a standard of purified hu MAB 53-2-4. Survival of mice is shown for day 15 after treatment.

result was obtained in animals that were observed for an extended period of time (15 days).

During the process of titrating the interaction between culture supernatant and toxin, a phenomenon was observed that has not previously been described. When dilute preparations of antibody were mixed with antigen and then administered to mice (*e.g.*, group 6, table 1), there were no immediate deaths nor was there immediate onset of illness. After a delay of approximately 5 days, animals developed the classic symptoms of tetanus; after an additional delay animals began to die.

Interaction between homogeneous antibody and antigen. Antibody purified from a protein A affinity column was applied to isolated phrenic nerve-hemidiaphragm preparations. At concentrations ranging from 10^{-11} to 10^{-7} M, the antibody had no observable effects on neuromuscular transmission. The addition of homogeneous toxin produced concentration-dependent blockade of transmission (fig. 3). The results are similar to those that have been reported previously (Habermann *et al.*, 1980).

Homogeneous preparations of toxin and antibody were mixed at various molar ratios (30 min; 30°C) and then applied to isolated tissues (fig. 4). The antibody proved to be very efficacious in neutralizing the toxin. At a ratio of 1:1, toxicity could be reduced by more than an order of magnitude (see fig. 3). Reduction in toxicity could be detected at an antibody:antigen ratio of 0.5:1.0.

Purified hu MAB 53-2-4 was mixed at a 10-fold molar excess with botulinum neurotoxin types A to F (3×10^{-11} M), as described above. The antibody did not delay the onset of paralysis induced by any of these toxins. The data support the ELISA findings presented above.

Tissue binding and epitope binding. Tetanus toxin (3 ×

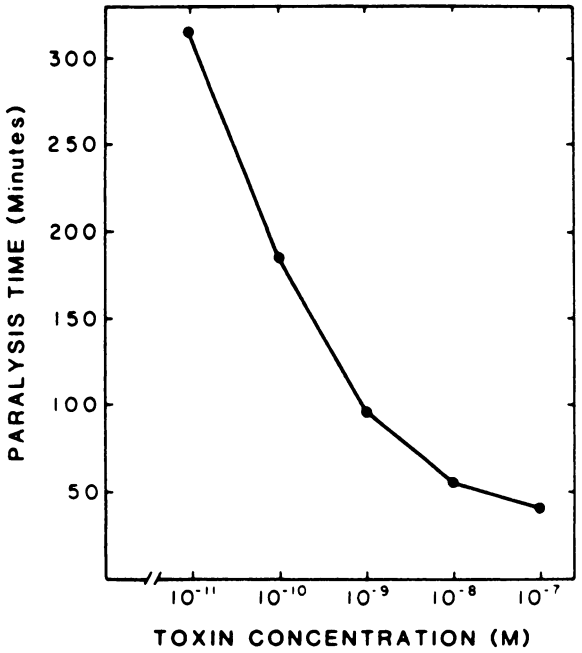

Fig. 3. Tetanus toxin was added to phrenic nerve-hemidiaphragm preparations, and the amount of time necessary for onset of paralysis was monitored. Within the concentration range of 10^{-11} to 10^{-7} M, there was dose-dependent blockade of neuromuscular transmission. Each data point is the mean of at least seven observations, and the standard error is equal to or less than 6% of the mean for the data point.

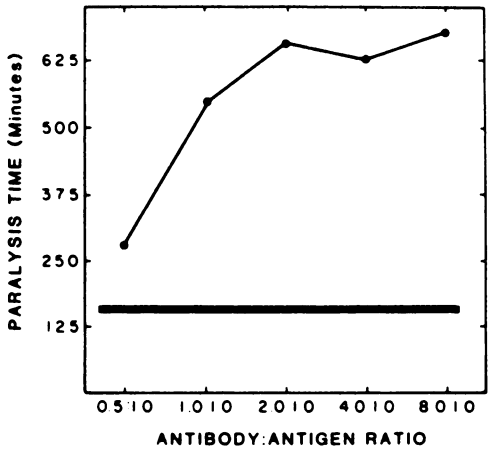

Fig. 4. Monoclonal antibody and tetanus toxin were mixed at various ratios and then applied to isolated neuromuscular junctions (see text for details). The amount of time necessary for these mixtures to produce paralysis (●) is compared to that for toxin alone (3×10^{-10} M; bar extended across graph). The data reveal that low antibody:antigen ratios cause substantial loss of toxicity. Each data point for the antibody-antigen experiment represents the mean of at least six observations; the bar for controls represents more than 30 observations.

10^{-10} M) was incubated with tissues at 4°C and in the absence of nerve stimulation. After 60 min the tissues were washed free of unbound toxin and exposed to various concentrations of hu MAB 53-2-4. After an additional 60 min, the tissues were again washed and transferred to baths at 35°c. Phrenic nerves stimulated and paralysis times were monitored.

Tissues that were exposed only to tetanus toxin became paralyzed in approximately 158 min (fig. 5). Tissues that were exposed to hu MAB 53-2-4 after incubation with toxin were protected in a dose-dependent manner. However, there were two interesting features to this response. The antibody:antigen

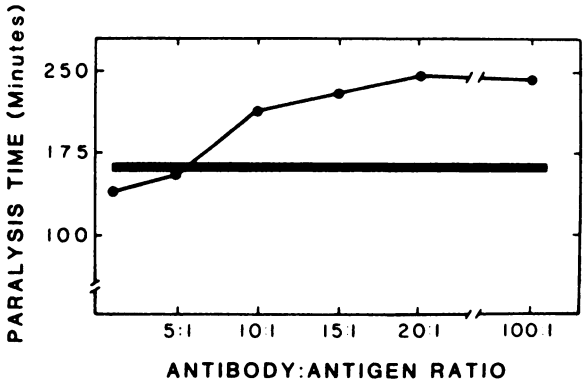

Fig. 5. Monoclonal antibody was added to tissues that had already been exposed to tetanus toxin (see text for details). The amount of time necessary for onset of paralysis was measured for tissues treated with tetanus toxin and later with antibody (●) and for tissues treated only with tetanus toxin (bar extended across graph). The results show that antibody could diminish the toxicity of tissue-bound toxin, but the magnitude of effect was not as great as that observed with free toxin (see fig. 4). Each data point for the antibody-antigen experiment represents the mean of at least five observations; the bar for controls represents more than 30 observations.

ratio that produced detectable protection was higher than that needed when the two reactants were mixed before addition to tissues. Furthermore, even a large molar excess of the antibody (*i.e.*, 100-fold) did not produce complete neutralization of toxin. By comparing figures 3 and 5, one can calculate that the extent of diminished toxicity was less than one order of magnitude.

A separate set of experiments was done to test the possibility that toxin molecules complexed to MAB 53-2-4 might bind to neuronal receptors. If this binding were to occur, occupancy of the receptor by the toxin•MAB complex should confer resistance against the free toxin. To test this possibility, tetanus toxin (1×10^{-9} M) was mixed (30 min, 30°C) with a 2-fold excess of the human antibody. This mixture was then added to tissues incubated at 4°C and without nerve stimulation. After 60 min the tissues were thoroughly washed and then incubated for an additional 60 min in a lower concentration of toxin (1×10^{-10} M). The tissues were washed again, transferred to baths at 37°C and the onset of paralysis was monitored. Three control experiments were run simultaneously: 1) tissues that were exposed to tetanus toxin (1×10^{-9} M) but not hu MAB 53-2-4 during the first incubation, 2) tissues that were exposed to hu MAB 53-2-4 but not toxin during the first incubation, and 3) tissues that were exposed to no drug during the first incubation.

Tissues that were incubated initially with 1×10^{-9} M toxin and subsequently with 1×10^{-10} M toxin paralyzed quickly (118 ± 9 min; n = 5). Tissues that were incubated only with 1×10^{-10} M toxin required a longer time to become paralyzed (185 ± 15 min; n = 5). When tissues were incubated initially with hu MAB 53-2-4 and subsequently with the lower dose of toxin, paralysis occurred in 195 ± 14 minutes (n = 8). This result indicates that the antibody must have been washed from the tissue; there was no sufficient residual antibody to neutralize the lower concentration of toxin.

For the experimental group of interest (toxin plus antibody during the initial incubation; lower toxin during the subsequent incubation) the average paralysis time was 199 ± 19 minutes (n = 10). This result is not statistically significantly different from that obtained with the low concentration of toxin alone, or that obtained with an initial incubation of antibody and a subsequent incubation with the lower concentration of toxin.

The implication is that the large molar excess of antibody-toxin complex did not bind to and occlude tissue receptors, a result that would have protected tissues from the lower dose of free toxin.

Stoichiometry of the antibody-antigen complex. Tetanus toxin alone or in combination with antibody (antibody:antigen ratio, 0.5:1.0) was applied to a Sephacryl S-300 column, and the apparent molecular weight was determined (fig. 6). The toxin alone eluted with an apparent molecular weight slightly in excess of 170,000, which is in reasonable agreement with its true molecular weight of 150,000 to 160,000 as described by sequence analysis (Eisel *et al.*, 1986; Fairweather and Lyness, 1986). The antibody-toxin complex eluted with an apparent molecular weight of slightly over 500,000.

The size of the complex suggests that each antibody molecule binds two antigen molecules. This conclusion supports the dose-response data that were obtained when the antibody-antigen complex was bioassayed *in vitro* (*e.g.*, fig. 4).

Discussion

Within a period of less than a decade substantial progress has been made in the development of methods that could lead to the introduction of clinically useful monoclonal antibodies and synthetic vaccines. This is particularly evident in the search for monoclonal antibodies that will neutralize tetanus toxin.

Several groups have reported the isolation of murine monoclonal antibodies that interact with tetanus toxin. Most of this work has been directed at structure-function analyses (Kenimer *et al.*, 1983; Sheppard *et al.*, 1984; Volk *et al.*, 1984), although some effort has been devoted to evaluating the potential of monoclonal antibodies as therapeutic agents (Ahnert-Hilger *et al.*, 1983). The collective findings have revealed that monoclonal antibodies can neutralize the toxin, though not as effectively as polyclonal antibodies. In addition, it has been shown that epitopes are located in what are believed to be the three functional domains of the toxin molecule, *i.e.*, the light chain, the aminoterminal half of the heavy chain and the carboxyterminal half of the heavy chain (Simpson, 1986).

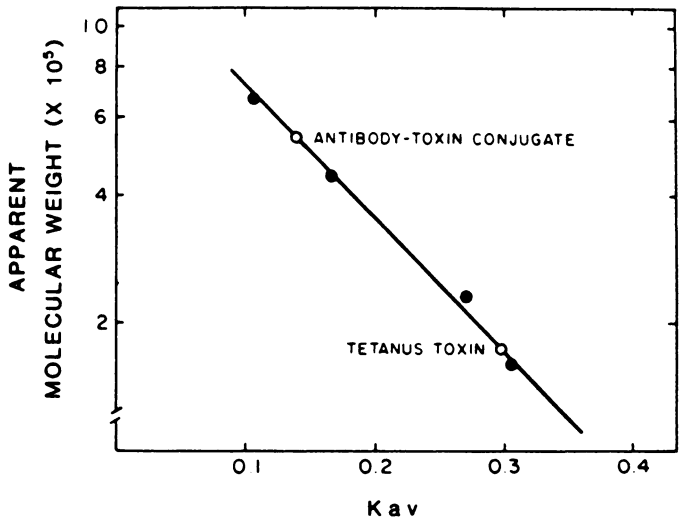

Fig. 6. The apparent molecular weights of tetanus toxin alone and of the toxin-antibody complex were determined by traditional chromatographic procecures. The results indicate that the complex is composed of one antibody molecule and two antigen molecules.

There is a more substantial literature that deals with human monoclonal antibodies, but this is focused almost entirely on the development of methods. Early efforts at generating a human hybridoma explored the use of Epstein-Barr virus-transformed cells (Zurawski *et al.*, 1978; Kozbor and Roder, 1981; Chiorazzi *et al.*, 1982; Kozbor *et al.*, 1982; Kozbor *et al.*, 1982). There have also been attempts to use human-human hybridomas (*e.g.*, Larrick *et al.*, 1983), but the most consistent success has been obtained with heterohydridomas (Ho *et al.*, 1985; Ichimori *et al.*, 1985; Gigliotti and Insel, 1982). Clones have been obtained that produce neutralizing antibody, and similarly to the results with murine clones, combinations of antibody are more efficient that individual antibodies in diminishing toxicity (Ziegler-Heitbrock *et al.*, 1986). Epitopes have been found in the heavy chain, but thus far a human monoclonal antibody that neutralizes by virtue of binding to the light chain has not been discovered.

The present study has employed a previously described and very effective method for generating heterohybridomas (Östberg and Pursch, 1983), and from this process a cell line that makes a high affinity, neutralizing antibody has been isolated. The human antibody recognizes the toxin, the toxoid and the C fragment carboxyterminal half of the heavy chain. There is evidence that the carboxyterminus of the toxin plays a dominant role in tissue targeting the toxin (Goldberg *et al.*, 1981; Simpson, 1984), so the human monoclonal antibody may recognize an epitope in or near the binding domain.

It is well known that conversion of toxin to toxoid is associated with conformational changes in the molecule, and it is reasonable to propose that separation of the carboxyterminus from the parent molecule is also associated with conformational changes. This means that the epitope recognized by hu MAB 53-2-4 is either a conformational determinant well preserved in spite of various manipulations of the molecule, or alternatively it is a linear determinant based on a primary sequence of amino acids. The latter possibility has clear implications for the development of a synthetic vaccine.

There is mounting evidence that the two major clostridial toxins, botulinum neurotoxin and tetanus toxin, are structurally related. Tsuzuki *et al.* (1988) have identified monoclonal antibodies against botulinum neurotoxin that also recognize tetanus toxin. In the present study, a monoclonal antibody against tetanus toxin was examined for its ability to interact with six serotypes of botulinum neurotoxin (A–F). The *in vitro* bioassay data indicated that hu MAB 53-2-4 does not neutralize heterologous toxin, and the ELISA data showed that the antibody does not bind to the various serotypes of botulinum neurotoxin.

There are three characteristics of the interaction between hu MAB 53-2-4 and tetanus toxin that warrant special attention, these being: 1) the relationship between diminished toxicity and diminished tissue binding, 2) the apparently novel phenomenon of delayed intoxication and 3) the potential for internalizing the antibody into diseased nerve cells. The antibody was very effective in diminishing toxicity when incubated with free toxin, and the stoichiometry for this effect was low; but the antibody was less effective when incubated with toxin that had already bound to nerve endings. The fact that the antibody has an effect on tissue-bound toxin is in keeping with the mechanism of tetanus toxin-induced neuromuscular blockade. There is an initial binding step at the plasma membrane, a subsequent internalization step and an eventual poisoning step that blocks

exocytosis (Simpson, 1986). When tissues are incubated with toxin at a low temperature, the binding step occurs but the later, energy-dependent steps do not (Schmitt *et al.*, 1981). The data with hu MAB 53-2-4 indicate that it can gain at least partial access to the tissue-bound toxin.

The mechanism by which the antibody diminishes toxicity of bound toxin is unclear. One possibility is that it competitively displaces a fraction of the toxin from the nerve ending. Another is that it attaches to the toxin-receptor complex, showing its transit through the subsequent steps that lead to paralysis. The former possibility is the more interesting one, because it tends to support the idea that the epitope is in or near the binding domain of the toxin molecule.

A second matter of interest relates to the findings in table 1 (see group 6). When mixed with toxin at the proper ratio, the antibody prevented the immediate onset of illness but it did not prevent the ultimate outcome of death. We have chosen to refer to this phenomenon as delayed intoxication. A likely explanation is that the antibody forms complexes with antigen and prevents the initiation of poisoning. This continues as long as the titer of antibody is high enough to ensure that any toxin molecule that dissociates from an antibody molecule will reassociate with another antibody rather than binding to the nerve ending. As antibody is cleared from the host, the probability of a toxin molecule attacking the nervous system increases. Thus, poisoning is delayed until the equilibrium relationship favors the toxin associating with vulnerable sites on or in the nerve rather than with antibodies.

The phenomenon of delayed intoxication has not been previously reported; however, if the proposed explanation for its mechanism of action is correct, one can predict that additional examples will be found.

The third matter to consider relates to therapeutics. The concept of using a neutralizing antibody as part of serotherapy is clear, but there is another and more provocative use of monoclonal antibodies. The toxin molecule possesses several functional domains that facilitate its entry into nerve cells, where it is not accessible to neutralizing antibody. There is a possibility that the toxin molecule itself could be "redesigned" to overcome the limitations of traditional antibody therapy. In keeping with the principles of a chimeric molecule, the toxin could be dissociated into two fragments, one containing the tissue-targeting and internalizing domains and the other having the poisoning domain. The latter could be replaced with a monoclonal antibody that neutralizes the parent toxin. This construct might bind to nerve endings and enter the cytosol, where it could inactivate toxin. This idea was proposed earlier (Simpson, 1981), at a time when neutralizing monoclonal antibodies against clostridial toxins were not widely available. That has now changed, and the chimeric antibody approach to the treatment of poisoning deserves to be explored.

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