

Antibodies Against the Light Chain of Tetanus Toxin in Human Sera

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Tetanus toxoid elicits protective antibodies against tetanus toxin in humans and animals. It has been reported that antitoxin from immunized humans contains no anti-light chain antibodies, based on immunodiffusion and quantitative precipitin analyses. We confirmed the absence of precipitating anti-light chain antibodies in tetanus immune globulin. However, the presence of antibodies against the light chain of the toxin was shown by direct binding and inhibition analyses, using enzyme-linked immunosorbent assays. Using a neutralization inhibition test, we also found that about one-fourth of the neutralizing antibodies in tetanus immune globulin are directed against the light chain. These results suggest that the light chain of tetanus toxin contains immunogenic determinants and that antibodies directed against it may have a role in the prevention of tetanus or treatment of tetanus or both.

Tetanus neurotoxin is produced by *Clostridium tetani*. This toxin is first synthesized by bacterial cells as a single polypeptide chain with a molecular weight of ca. 150,000. It is then converted to the nicked or extracellular form by endogenous proteases (7, 23). Therefore, the extracellular form of tetanus toxin consists of a heavy chain with a molecular weight of ca. 100,000 and a light chain with a molecular weight of ca. 50,000, which are held together by a disulfide linkage. The two chains may be separated under denaturing conditions by using urea or sodium dodecyl sulfate in the presence of dithiothreitol (15). Papain treatment of the toxin yields two fragments (8). Fragment C, which has a molecular weight of ca. 50,000, contains the C-terminal portion of the heavy chain; fragment B, which has a molecular weight of ca. 100,000, contains the remainder of the heavy chain along with the attached light chain.

The mechanism by which tetanus toxin exerts its pathogenic effect is not fully understood. However, it has been established that fragment C contains the receptor recognition site and is responsible for the subsequent retrograde axonal transport of the toxin molecule in neuronal cells (5, 9, 19). The biological activity of the light chain has not been defined yet. However, by analogy with cholera and diphtheria toxins, in which catalytic activity and receptor binding are on different chains or subunits (4), the toxicity of the holotoxin may reside in the nonbinding light chain. In contrast with these toxins, no enzymatic activity for tetanus toxin has been described.

Immunization with tetanus toxoid may elicit the formation of specific antibodies against different antigenic determinants (14). Because the toxicity of the toxin molecule is postulated to be on the light chain, antibodies directed against this portion of the molecule may be expected to be important for the prevention or treatment or both of human tetanus. Matsuda and co-workers (13, 14, 16) have reported that human antitoxin contains no precipitating anti-light chain antibodies. However, in this study the presence of anti-light chain antibodies in human antitoxin sera was demonstrated by using enzyme-linked immunosorbent assay (ELISA) direct binding and inhibition analyses and the ability of the light chain to adsorb toxin-neutralizing antibodies.

MATERIALS AND METHODS

Toxin, light chain, fragments B and C, and toxoid. Tetanus toxin was purchased from Massachusetts Public Health Biologic Laboratories, Boston, Mass., and was further purified on a hydroxylapatite column (12). The purified toxin was homogeneous when it was examined by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue R. The protein content was 5.85 mg/ml; the specific activity of the purified toxin was 2×10^7 mouse minimum lethal doses (MLD) per mg of protein. The light chain was obtained as a by-product during toxin purification; it was a single band at a molecular weight of about 50,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The protein content of the light chain was 1.55 mg/ml, and the toxicity, presumably due to contaminating holotoxin, was 650 MLD/mg, or less than 0.0001% that of toxin. This low level of toxicity in the light chain preparation did not interfere with our experiments or results. Another light chain preparation, which was purified by a different method (21), was a gift from J. P. Robinson, Vanderbilt University, Nashville, Tenn. Fragments B and C were gifts from Behringwerke AG. Concentrated tetanus toxoid (lot TLC-16-1; 1,450 flocculation units per ml; 1,900 flocculation units per mg of N) was purchased from Connaught Laboratories Ltd., Toronto, Canada.

Tetanus toxin antisera. Equine tetanus antitoxin (TAT) (labeled 20,000 U/vial; lot STP 393) was purchased from Sclavo Inc., Wayne, N.J. The following three lots of tetanus immune globulin (TIG) were used: lot 32582 from Cutter Laboratories, Berkeley, Calif. (275 U/ml); lot 918254 from Parke, Davis & Co., Detroit, Mich. (290 U/ml); and lot 2702R005A from Hyland Div., Travenol Laboratories, Inc., Glendale, Calif. (480 U/ml). The TIG from Cutter Laboratories was more than 20 years old. However, recent potency testing in guinea pigs showed that it had an activity of 150 to 200 U/ml, and size analysis by high-pressure liquid chromatography indicated little degradation or aggregation. Six individual human sera with neutralization titers (2) of <0.01 , 3, 30, and >100 U/ml were also used.

Agarose double immunodiffusion. Immunodiffusion assays were performed in 1% agarose in phosphate-buffered saline (PBS) (20). Gels were photographed after overnight reaction at 4°C, stained with 2% Coomassie brilliant blue G in

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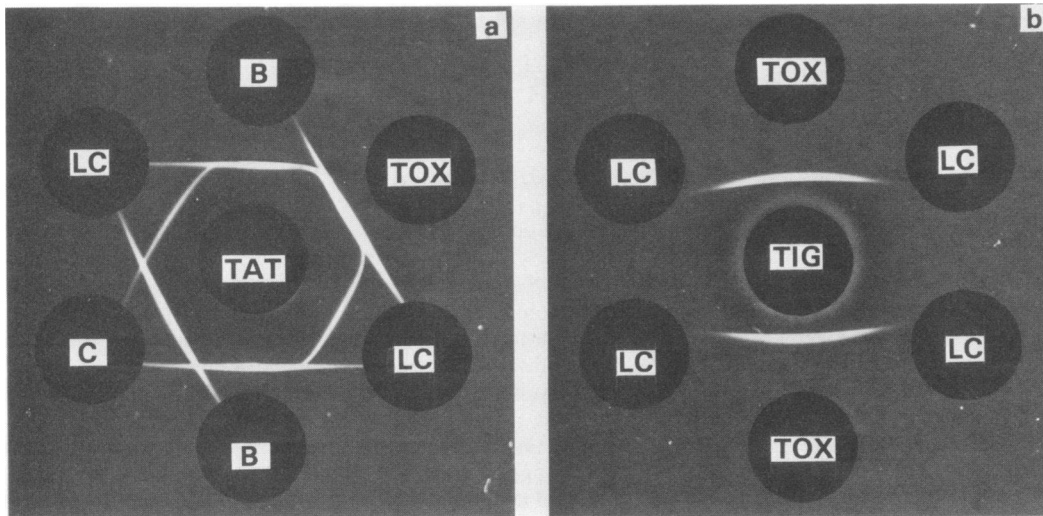


FIG. 1. Immunodiffusion analysis of the light chain. (a) The wells contained TAT (700 U/ml), tetanus toxin (TOX; 1.6 mg/ml), fragments B and C (1 mg/ml), and light chain (LC; 0.2 mg/ml). (b) The wells contained TIG (480 U/ml), tetanus toxin (TOX; 1.1 mg/ml), and light chain (LC; 0.1, 0.2, 0.4, and 1.6 mg/ml). Each well contained 15 μ l of sample.

water-methanol-acetic acid (5:4:1), and destained with the same solvent.

ELISA. The ELISA method described by Kenimer et al. (10) was used, with modifications. Individual wells of 96-well MicroELISA plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were sensitized with 50- μ l portions of dilutions of toxin or light chain in 0.2 \times PBS (pH 7.4), and the plates were incubated at 4°C overnight; the wells were aspirated, 200 μ l of PBS containing 1% bovine serum albumin was added, and the preparations were incubated at 28°C for 2 h to saturate the remaining binding sites. The wells were then washed three times with 200 μ l of PBB (PBS containing 0.1% bovine serum albumin and 0.1% Brij 35 [Sigma Chemical Co., St. Louis, Mo.]), and 50 μ l of antiserum diluted in PBB was delivered to the wells; these preparations were incubated at 28°C for 2 h. The wells were again aspirated and washed three times with PBB before 50 μ l of a 1:2,000 dilution of peroxidase anti-human immunoglobulin G conjugate (lot 5923; Miles-YEDA Ltd., Elkhart, Ind.) in PBB was added and the preparations were incubated for an additional 2 h at 28°C. The wells were again washed three times as described above, and 100- μ l portions of 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonic acid) (500 μ g/ml; Sigma) in 0.1 M citrate buffer (pH 4.2) containing 0.03% hydrogen peroxide were added to the wells. The optical density at 405 nm was determined with a model MR 580 MicroELISA auto reader (Dynatech) at several time points.

For the ELISA inhibition assay, the Cutter Laboratories TIG was first incubated with a 20-fold excess of toxin or light chain at 4°C overnight. After incubation, antisera were diluted and added to the plates sensitized with toxin or light chain. PBB was used as a solvent or diluent. The rest of the procedures were the same as those used for measuring direct binding. For all of the tests performed, TIG (Cutter Laboratories) was included as a positive control, and nonsensitized wells for each serum were included as negative controls.

Inhibition of toxin neutralization in mice. The toxin neutralization assay was adapted from the method of Barile et al. (2). PBS containing 0.2% gelatin (PBSG) was used as the diluent in all cases. Portions (200 μ l) of TAT (Sclavo) or TIG

(Cutter Laboratories) at three closely spaced concentrations were preincubated with 400 μ l of light chain, fragment B, or toxoid at room temperature for 1 h. Based on the protein content, we calculated that the amounts of light chain, fragment B, and toxoid added were between 200- and 2,000-fold greater than the toxin-binding capacities of the two antisera. Toxin (4,000 MLD/ml; 200 μ l) was then added, and the preparations were incubated for another 1 h. The final concentration of toxin in each sample was approximately 1,000 MLD/ml. Twofold dilutions of the toxin-antitoxin mixtures were made, and 0.1-ml portions of each were injected subcutaneously into the left inguinal folds of mice; numbers of MLD were calculated after 96 h. The toxicity or number of MLD remaining in the toxin-antitoxin mixtures provided a quantitative estimate of the amount of antitoxin in TAT or TIG which reacted with light chain, fragment B, or toxoid. Thus, the number of MLD remaining was a direct indication of the amount of antibodies in either TIG or TAT which were directed against the added ligands.

RESULTS

The purity and identity of the toxin chains were evaluated by immunodiffusion analysis (Fig. 1a). As determined by this method, our light chain was identical to that provided by J. P. Robinson (data not shown). In contrast to the results obtained with equine antitoxin, TIG contained no detectable precipitating anti-light chain antibodies (Fig. 1a and b). Subsequent staining of the immunodiffusion plate did not reveal additional precipitin lines.

Two TIG lots (480 and 275 U/ml) and one human antiserum (>100 U/ml) were assayed by the ELISA direct binding method for the presence of antibodies which bound to toxin or light chain (Fig. 2). In each case, the ELISA value was proportional to the protective antitoxin content of the sera. All three samples appeared to have more anti-holotoxin than anti-light chain activities. Similar results were obtained with the third TIG lot (290 U/ml) and five other human sera (<0.01 to 30 U/ml) (data not shown).

The presence of antibodies against the light chain was also demonstrated by using the ELISA inhibition method. Figure 3 shows the titration curves of one lot of TIG. The TIG was

incubated with toxin or light chain to inhibit the antitoxin or anti-light chain binding activities. When the TIG was neutralized by toxin or light chain, the titration curves were shifted toward the lower dilutions because some specific antitoxin or anti-light chain antibodies were inhibited. The inhibition of anti-light chain antibodies by light chain was more clearly demonstrated when wells were sensitized with light chain (Fig. 3b). These data are in agreement with the direct binding results.

Table 1 shows the ability of selected polypeptides to adsorb the toxin-neutralizing activity in TAT or TIG. Antitoxin which had been preincubated with PBSG was more effective in neutralizing the toxin dose than was antitoxin which had been adsorbed with excess toxoid, fragment B, or light chain. Ideally, all of the toxin should have been neutralized with no excess antitoxin remaining. This was closely achieved at the highest level of each antiserum shown. At the highest level of TIG, light chain combined with sufficient antibodies such that about 24 of the initial 100 MLD remained unneutralized, whereas fragment B was capable of combining with many more antibodies. Excess toxoid inhibited all antitoxin antibodies; hence, the full amount of toxin (~100 MLD) was measured.

DISCUSSION

The roles that light chain and antibodies directed against light chain play in the mechanism of toxin action and in immune protection are important for a better understanding of the structure-function relationships of tetanus toxin and for the prevention and treatment of human tetanus.

Tetanus toxin has multiple immunogenic determinants, as reported by previous workers (6, 14, 27); this is supported by the diversity of monoclonal antibodies described thus far (1, 10, 17, 24). The results of amino acid analysis, protease digestion (25), and monoclonal antibody experiments (26) further suggest that there may be some sequence homology between the heavy and light chains of tetanus toxin. Toxoid is able to elicit protective antitoxin antibodies in humans and animals which may be used for passive immunization or treatment of human tetanus. It has been reported that human antitetanus sera contain no anti-light chain antibody when they are assayed by immunodiffusion methods (13, 16) and quantitative precipitation techniques (14). We were also not able to detect human antibodies directed against the light

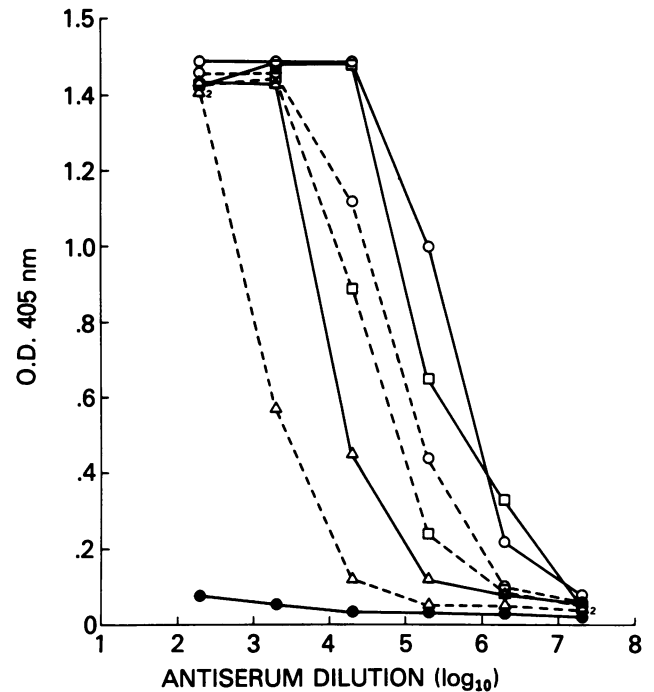


FIG. 2. Measurement of antitoxin and anti-light chain antibodies of human antitetanus sera by ELISA. Solid lines, Wells sensitized with toxin (5 µg/ml); dashed lines, wells sensitized with light chain (10 µg/ml). Symbols: ○, TIG (480 U/ml); □, TIG (275 U/ml); △, individual antitoxin serum (>100 U/ml); ●, toxin or light chain controls (wells not sensitized). O.D. 405 nm, Optical density at 405 nm.

chain by immunodiffusion. However, our ELISA and inhibition-of-neutralization data suggest that human TIG, as well as individual human sera, contains protective anti-light chain antibodies.

Five individual antisera and three lots of TIG showed both antitoxin and anti-light chain titers in ELISA direct binding analyses. The sixth serum (<0.01 U/ml) showed the lowest antitoxin activity; the anti-light chain antibody titer was not tested. Although the parallel titration curves in Fig. 2 suggest that ELISA values can be used to compare antitoxin and anti-light chain antibodies, this could not be done directly in our experiments because of the unknown efficiencies with which these antibodies coated the ELISA wells. However, useful results can be obtained in ELISA inhibition experiments. Figure 3 demonstrates that more of the antitoxin in TIG was inhibited by toxin than by light chain. When wells were sensitized with light chain, the inhibitory effects of light chain on antibody binding were shown more clearly (Fig. 3b).

The results of ELISA direct binding and inhibition experiments complemented each other and were in agreement with the in vivo neutralization inhibition data.

The mouse protection test demonstrated that anti-light chain antibodies of TIG were neutralizing. Table 1 summarizes the evidence for the presence of neutralizing anti-fragment B, anti-light chain, and anti-toxoid antibodies in both TAT and TIG. Our results are only semiquantitative because of the twofold dilutions used, but they unequivocally show the presence of neutralizing antibodies directed against the light chain. Toxoid adsorbed virtually all of the antitoxin in both TAT and TIG. Fragment B combined with

TABLE 1. Inhibition of the toxin-neutralizing ability of (equine) TAT and (human) TIG by preincubation with excess toxoid, fragment B, and light chain

Antitoxin	Concn (U/ml) ^a	No. of MLD/0.1 ml after incubation with: ^b			
		PBSG	Toxoid	Fragment B	Light chain
TAT	0.058	16	NT ^c	64	32
	0.064	2	NT	64	16
	0.070	0	120	32	8
TIG	0.068	8	NT	64	32
	0.078	4	NT	64	24
	0.085	1	100	64	12

^a The numbers of protective units per milliliter for TAT and TIG were calculated on the basis of the numbers of units in the products claimed by the manufacturers.

^b The antitoxins were preincubated for 1 h with the following quantities of antigen (or PBSG): toxoid, 5 flocculation units per ml (~10 µg/ml); fragment B, 10 µg/ml; light chain, 15.5 µg/ml. Tetanus toxin was then added to a final concentration of 0.13 µg/ml (~1,000 MLD/ml), and after an additional 1 h of incubation, the remaining toxicity was determined by injection into mice.

^c NT, Not tested.

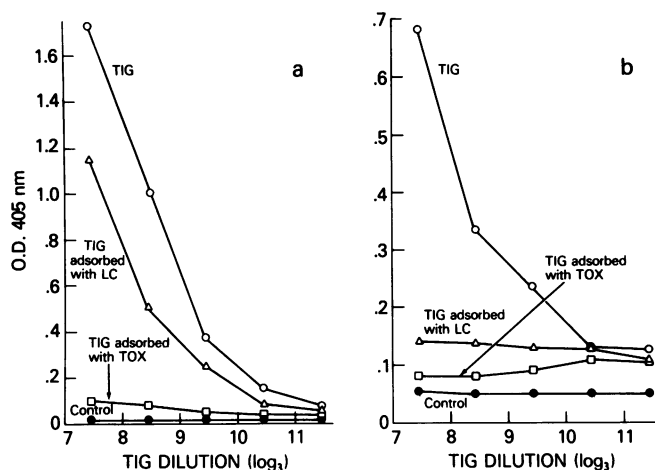


FIG. 3. Inhibition curves of TIG specific for toxin and light chain, as determined by an ELISA. (a) Wells sensitized with toxin (5 µg/ml). (b) Wells sensitized with light chain (5 µg/ml). Symbols: O, TIG (275 U/ml), not absorbed; □, TIG absorbed with a 20-fold excess of toxin; △, TIG absorbed with a 20-fold excess of light chain; ●, toxin or light chain controls (wells not sensitized). O.D. 405 nm, Optical density at 405 nm; LC, light chain; Tox, tetanus toxin.

more neutralizing antibodies than light chain, but less than toxoid. The control (PBSG) showed the lowest level of MLD because all of the protective antibodies were available to neutralize the 100 MLD of toxin added.

We estimate that about 75% of the neutralizing antibodies in human TIG are directed against fragment B (consisting of the light chain and one-half of the heavy chain) and that about 25% of the total neutralizing antibodies are directed against the light chain of the toxin. These proportions are somewhat in accord with the quantity of protein represented by each antigen. Helting and Nau (6) have shown that both fragment B and fragment C can induce the formation of protective antibodies in mice and guinea pigs. The inability to detect the precipitation reaction with human anti-light chain antibodies may reflect a limited clonal response to epitopes on the light chain, thus preventing the formation of an insoluble complex. Even mixtures of several mouse monoclonal antibodies may be unable to form a visible precipitin line when they are reacted with toxoid (18).

Numerous mouse monoclonal antibodies which react with light chain have been described (1, 10, 26), and at least two of these are capable of neutralizing toxicity (10). This is not surprising if indeed the light chain is the active chain of the toxin molecule. No human monoclonal antibodies which are specific for light chain have been described yet, although most monoclonal antibodies have not been tested for this property (3, 11). However, light chain-specific human T-cell clones have been described (22).

The light chain of tetanus toxin contains immunogenic determinants and appears to be an important component of toxoids. The antibodies directed against it may play a role in the prevention and treatment of tetanus.

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