INFECTION AND IMMUNITY, Jan. 1989, p. 18–22
0019-9567/89/010018-05$02.00/0

Vol. 57, No. 1

Sequence Homology between Tetanus and Botulinum Toxins Detected by an Antipeptide Antibody

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Received 19 May 1988/Accepted 17 September 1988

The extent of immunological similarity between tetanus toxin and botulinum toxins A, B, C_1, and E was studied by using 10 antibodies produced against synthetic peptides representing different sequences of tetanus toxin, mouse antitetanus serum, and human Tetanus Immune Globulin. Antibodies produced against the synthetic peptides recognized tetanus toxin in an enzyme-linked immunosorbent assay and on Western blots (immunoblots) but did not appear to recognize the native protein. One of the antitetanus peptide antibodies, which was produced against a peptide from the amino terminal, cross-reacted with three of the four botulinum toxins on immunoblots. This antibody, 1, reacted strongly with botulinum toxins B and C_1 and weakly with E but did not recognize type A toxin. None of the other peptide antibodies cross-reacted with the botulinum toxins. Mouse antitetanus serum and human Tetanus Immune Globulin did not recognize any of the botulinum toxins on immunoblots. The amino-terminal region of the light chain of tetanus toxin and botulinum toxin types A, B, C_1, and E are known to have sequence homology. Our data demonstrate that for tetanus toxin and botulinum toxin types B, C_1, and E this region also has immunological homology. Type A, which has the least amount of homology with tetanus toxin in this region, does not share this immunological homology. These data also suggest that although the native structures of tetanus and botulinum toxins have relatively few common immunological determinants, the two toxins may contain short stretches of identical or very similar amino acid sequences.

Tetanus and botulinum toxins are potent neurotoxins produced by *Clostridium tetani* and *Clostridium botulinum*, respectively. These toxins are synthesized as single-polypeptide chains that are proteolytically cleaved to generate a light chain and a heavy chain linked by one disulfide bond (11). Tetanus and botulinum toxins inhibit neurotransmitter release by an undetermined mechanism of action (1, 11, 14, 18). It has been proposed that the heavy chain contains the eucaryotic cell-binding site (2, 9) and that the light chain contains the active site for toxicity; however there is little information on the functions of different regions of these toxins.

The entire amino acid sequence of tetanus toxin was recently reported (6–8) and can be compared with the partial amino acid sequences that are available for botulinum toxins (19, 20, 25; B. R. DasGupta and A. Datta, Fed. Proc. **46:** 2289, 1987). There is significant homology between tetanus toxin and some botulinum toxins at the amino terminal of both the light and heavy chains, suggesting that these toxins are derived from a common gene. In addition, it was recently reported that a monoclonal antibody against botulinum toxin type E cross-reacted with tetanus toxin as well as with botulinum toxin types B, C_1, and D (25).

We were interested in determining whether tetanus and botulinum toxins share additional regions of sequence homology as a means of identifying potentially important functional regions of the toxin. Since the majority of the botulinum toxins have not yet been sequenced, it is not yet possible to directly compare the sequences of these two toxins. We used antibodies against defined regions of tetanus toxin and polyclonal antitetanus antibodies to identify regions common to tetanus and botulinum toxins.

MATERIALS AND METHODS

Goat anti-mouse alkaline phosphatase conjugate was purchased from Bio-Rad Laboratories (Richmond, Calif.), tetanus toxoid (1,666 Lf/mg of N, 2,550 Lf/ml; 1 Lf is the amount of toxin or toxoid which gives the most rapid flocculation with 1 U of antitoxin) was obtained from Connaught Labs Ltd. (Toronto, Canada), and human Tetanus Immune Globulin (460 U/ml) was obtained from Cutter Laboratories.

Toxins. Tetanus toxin was purchased from the Massachusetts Public Health Biologic Laboratories and was further purified on hydroxylapatite. Briefly, toxin was dialyzed against 5.0 mM potassium phosphate (pH 6.8) and applied to a hydroxylapatite column equilibrated in the same buffer. The column was eluted with a gradient of 5 to 250 mM potassium phosphate. Tetanus toxin eluted from the column at approximately 100 mM phosphate. Botulinum toxin type E was purified as described by Schmidt and Siegel (21) and treated with trypsin to obtain the heavy and light chains. Botulinum toxin types A, B, and C_1 were purified by using this same method with a minor modification. Since type A, B, and C_1 cells generally lyse during culture, proteins from these types were acid precipitated before extraction with 0.2 M sodium phosphate.

Radioiodination of tetanus toxin. ^{125}I-labeled tetanus toxin was prepared by the procedure of Bolton and Hunter (3). Approximately 100 μg of tetanus toxin was labeled with 1 mCi of Bolton-Hunter reagent. The specific activity of ^{125}I-labeled tetanus toxin was ≈2 μCi/μg.

Production of antibodies. Peptides corresponding to dif-

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PEPTIDE	SEQUENCE	RESIDUE NUMBERS
1	P I T I N N F R Y S D P V N	1-14
2	E N L Y N R T A S L T D	450-461
3	I K I K N E D L T F I A	468-479
4	G I T E L K K L E S K I N K	840-853
5	D N E E D I D V I L K K S T	872-885
6	R V P K V S A H L E Q Y G T N E Y S	957-975
7	R D L P D K F N A Y L A N K	1017-1030
8	R E D N N I T L K L D R C	1065-1077
9	K A L N P K E I E K L	1094-1104
10	N P L R Y D T E Y Y L I P V A S S S K D	1120-1139
11	N A P S Y T N G K L N I Y Y R R L Y N G L K	1153-1174
12	Y N A P G I P L Y K K M E A V K L R D L K T Y S	1229-1252
13	T H N G Q I G N D P N R D I	1270-1283

FIG. 1. Sequence of tetanus toxin synthetic peptides. Numbering of the peptides is as described by Eisel et al. (6).

ferent regions of tetanus toxin were synthesized and coupled to bovine serum albumin (BSA) by a published procedure (16). The sequences of these peptides and their location in tetanus toxin are indicated in Fig. 1. Groups of five mice were immunized with 450 μg of BSA-peptide conjugate in complete Freund adjuvant and boosted with the same amount of peptide-BSA conjugate in incomplete Freund adjuvant 4, 8, and 12 weeks after the initial immunization. Serum was collected 1 week after each booster immunization, pooled, and stored at −20°C. Polyclonal antitetanus antibodies were produced by injecting BALB/c mice with 50 μl containing 250 μg of tetanus toxoid emulsified in an equal volume of Freund complete adjuvant. Mice were boosted at 2-month intervals; sera from 10 mice were collected and pooled after 4 to 6 boosts.

Electrophoresis. Sodium dodecyl sulfate-gel electrophoresis was performed by the method of Laemmli (15) with a 12% separating gel. Nondenaturing gels were prepared and run by the method of Davis (5).

Enzyme-linked immunosorbent assay (ELISA). Micro-dilution plates were incubated with a 5-μg/ml solution of tetanus toxin (50 μg per well) overnight at 4°C and rinsed with phosphate-buffered saline (1.0 mM KH_2PO_4, 5.0 mM Na_2HPO_4, 0.15 M NaCl, pH 7.5) containing 0.1% Brij. Sera diluted into phosphate-buffered saline–Brij were added to the plates (50 μl per well) and allowed to react for 3 h at room temperature. After the plates were washed with phosphate-buffered saline–Brij, they were incubated with a goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate for 2 h and then developed with the alkaline phosphatase substrate *p*-nitrophenyl phosphate; then the A_{405} was measured.

Immunoblotting. Proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose paper by published procedures (24). The nitrocellulose blots were blocked for 1 h in Tris-buffered saline (20 mM Tris hydrochloride, 0.5 M NaCl [pH 7.5]) containing 3% gelatin and then rinsed two times in Tris-buffered saline containing 0.05% Tween 20. After incubation for 4 h with the appropriate antibody diluted into Tris-buffered saline–Tween 20 containing 1% gelatin, the blots were rinsed twice and incubated with a goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate for 2 h. The blot was rinsed two final times with Tris-buffered saline–Tween 20 and once with Tris-buffered saline and developed by incubating with alkaline phosphatase color development solution (30 mg of nitroblue tetrazolium dissolved in 1 ml of 70% dimethylformamide and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate in 1.0 ml of dimethylformamide added to 100 ml of 0.1 M sodium carbonate buffer [pH 9.8]).

RESULTS

Sera from mice immunized with peptide-BSA conjugates were tested for their ability to react with tetanus toxin by ELISA and immunoblot. Of the 13 peptides, 11 elicited antibodies that reacted with tetanus toxin in the ELISA (Table 1). When these sera were tested by immunoblot at the same dilution (1/100), each antiserum reacted with the appropriate antigen (heavy or light chain) and showed no reactivity with other proteins (data not shown). Peptide 3, which contains the N-terminal region of the heavy chain of tetanus toxin, and peptide 2, which corresponds to the proteolytic cleavage site on intact tetanus toxin, elicited no antibody response; sera from these mice were not evaluated further.

The antitetanus antibodies obtained by immunization with peptide-BSA conjugates did not appear to recognize native tetanus toxin. Electrophoresis of mixtures of ^{125}I-labeled tetanus toxin with each antipeptide serum showed that all of the radioactivity migrated as uncomplexed tetanus toxin

TABLE 1. Binding of synthetic peptide antibodies to tetanus toxin[a]

Antibody	A_{405}	Antibody	A_{405}
1	0.41	10	0.27
2	0.06	11	0.33
3	0.04	12	0.25
4	0.30	13	0.43
5	0.31	Mouse antitetanus serum	0.50
6	0.43		
7	0.31	Normal mouse serum	0.05
8	0.33		
9	0.12	Blank well	0.005

[a] Synthetic peptide antibodies were tested for their reactivity with tetanus toxin with an ELISA as described in Materials and Methods. For this experiment, antisera were used at a final dilution of 1/100.

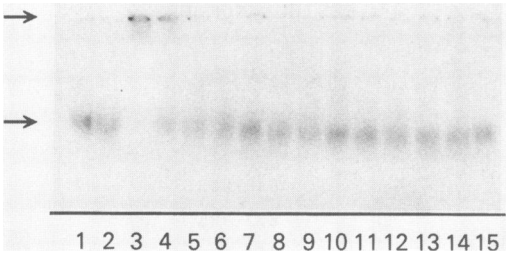

FIG. 2. Binding of synthetic peptide antibodies to native tetanus toxin. Antibodies against individual synthetic peptides or polyclonal antitetanus serum (30 μl) was mixed with ^{125}I-labeled tetanus toxin (≈10,000 cpm; 2 ng of protein) in phosphate-buffered saline containing 0.2% gelatin and incubated for 3 h at room temperature. The samples were electrophoresed on a 5% nondenaturing gel; the gel was dried and exposed to Kodak XAR-2 film for 24 h. Lanes contained ^{125}I-labeled tetanus toxin plus the following: 1, no addition; 2, normal mouse serum; 3, antitetanus mouse serum; 4, mixture of antibodies 1 through 11 (equal amounts of each antibody were mixed together and 30 μl of the mixture was incubated with the toxin); 5 through 15, antibodies 1 through 11, respectively.

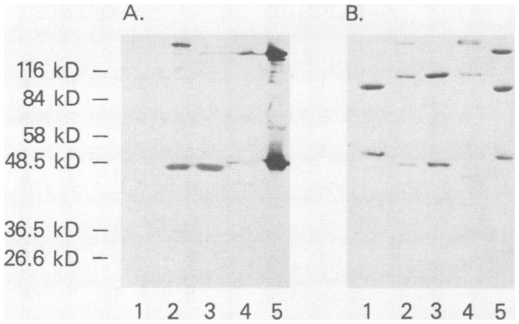

FIG. 3. Immunoblot of tetanus toxin and botulinum toxins. Tetanus toxin (4 μg) and botulinum toxin types A, B, C_1, and E (2 to 6 μg per lane) were electrophoresed on 12% gels and transferred to nitrocellulose. The blot was then immunoblotted with antibody 1 (A) or stained with amido black to visualize total protein (B). Lanes: 1, botulinum toxin type A; 2, botulinum toxin type B; 3, botulinum toxin type C_1; 4, botulinum toxin type E; 5, tetanus toxin. The two lower-molecular-weight bands in all lanes (B) are the heavy and light chains; the additional high-molecular-weight band in lanes 2, 4, and 5 represents unnicked or unreduced tetanus or botulinum toxin.

(Fig. 2). In contrast, anti-tetanus toxoid sera bound to tetanus toxin to form a more slowly migrating complex. The antipeptide sera were also not able to immunoprecipitate ^{125}I-labeled tetanus toxin. The ability of the antipeptide antibodies to recognize native tetanus toxin was also assessed with a dot blot assay. For this experiment, tetanus toxin incubated with or without 0.1% sodium dodecyl sulfate for 10 min was applied directly to nitrocellulose paper before immunoblotting with antipeptide antibodies and anti-tetanus toxin serum. The antipeptide antibodies recognized only sodium dodecyl sulfate-treated tetanus toxin and not untreated toxin (data not shown).

The 10 antipeptide antibodies with good reactivity to tetanus toxin were tested for cross-reactivity with botulinum toxin types A, B, C_1, and E by immunoblot analysis. Of the 10 anti-tetanus peptide antibodies tested, only antibody 1 cross-reacted with botulinum toxin. Antibody 1 reacted with botulinum toxin types B, C_1, and E on immunoblots but did not recognize type A (Fig. 3). The reactivity of antibody 1 with types B and C_1 was approximately equivalent to its reactivity with comparable amounts of tetanus toxin, whereas reactivity with type E toxin was significantly less. The immunological comparison of tetanus and botulinum toxins was extended by testing the cross-reactivity of polyclonal anti-tetanus toxin serum from mice and humans with botulinum toxins with both immunoblots and ELISAs. Neither polyclonal antitetanus serum generated in BALB/c mice nor human Tetanus Immune Globulin reacted with any of the botulinum toxins either by immunoblot or ELISA (data not shown).

DISCUSSION

We have found that an antibody raised against a synthetic peptide corresponding to the amino-terminal end of the tetanus toxin light chain recognized several types of botulinum toxin. The sequence of peptide 1 and the corresponding regions of tetanus toxin and botulinum toxins A, B, C_1, and E are shown in Fig. 4. The extent of cross-reactivity of antibody 1 with the different botulinum toxins correlated well with the number of residues in common with tetanus. Botulinum toxin type E (which shares 12 out of 14 residues with tetanus toxin) and botulinum toxin type B (which shares 9 out of 14 amino acids with tetanus toxin at this site) were recognized most strongly by antibody 1. Botulinum toxin type C_1, which shares 8 out of 14 amino acids with tetanus toxin, was much less cross-reactive, and botulinum toxin type A, which only has 6 out of 14 amino acids identical with tetanus, was not recognized at all.

The light chains of tetanus and botulinum toxins are postulated to act intracellularly to inhibit neurotransmitter release by a similar mechanism of action (10, 17). Our results showing that the toxins have immunologically related regions are further evidence to support this hypothesis. The observed conservation in the amino terminal of the toxin light chains may indicate an essential role for this region in the intoxication process. Using a monoclonal antibody against botulinum type E, Tsuzuki et al. demonstrated immunological homology between botulinum toxin types E, B, C_1, D and tetanus toxin (25). The pattern of cross-reactivity observed in this study was somewhat different from that seen with antibody 1. Whereas antibody 1 reacted strongly with botulinum toxin types B and C_1 and weakly with type E, the monoclonal antibody against botulinum toxin type E reacted strongly with types C_1 and E and weakly with type B. Thus, the two antibodies may be directed toward two distinct epitopes on the light chain of tetanus and botulinum toxins.

Each of the other peptides used in this study represented tetanus toxin sequences located on the C-terminal end of the heavy chain or fragment C portion of the molecule. Antibodies generated against these regions did not recognize any of the botulinum toxins, implying that at the epitopes recognized by the peptide antibodies, tetanus and botulinum do not share significant homology. Fragment C has been demonstrated to bind to gangliosides found in neural tissue (see reference 10 for a review). The C-terminal end of the heavy chain of botulinum toxin has also been reported to bind to gangliosides (13, 22); however botulinum toxin and tetanus toxin do not appear to compete for the same binding sites in vivo. The lack of detectable cross-reactivity in the C terminal of the two toxins shown here suggests that tetanus and botulinum toxins are not very homologous in this region; this is consistent with the differences in binding reported for these toxins.

Neither human nor mouse anti-tetanus toxin serum cross-

PEPTIDE 1	ClAcG	G	P	I	T	I	N	N	F	R	Y	S	D	P	V	N						
TETANUS TOXIN[a]			P	I	T	I	N	N	F	R	Y	S	D	P	V	N	N	D	T	I	I	M	M	E	P
BOTULINUM A[b]			P	F	V	N	K	Q	F	N	Y	K	D	P	V	N	G	V	D						
BOTULINUM B[b]			P	V	T	I	N	N	F	N	Y	N	D	P	I	D	N	N	N	I	I	M	M	E	P
BOTULINUM C_1[c]			P	I	T	I	N	N	F	N	Y	S	D	P	V	D	N	K	N	I	L	Y			
BOTULINUM E[b]			P	-	K	I	N	S	F	N	Y	N	D	P	V	N	D	R	T	I	L	Y	I		

FIG. 4. Sequence homology between tetanus toxin and botulinum toxin types A, B, C_1, and E. Peptide 1 was synthesized with a *N*-chloroacetyl-glycine-glycine as an end group as previously described (14). Previously published data are from the following sources: tetanus toxin (6); botulinum toxin types A, B, and E (20, 21; DasGupta and Datta, Fed. Proc. **46**:2289, 1987); and botulinum toxin type C_1 (25).

reacted with botulinum toxin on Western blots (immunoblots) or in an ELISA. Both of these preparations of immunoglobulin are derived from pools of individuals that have been hyperimmunized with tetanus toxoid and would reasonably be expected to contain antibodies against most or all of the epitopes present on tetanus toxin. The failure of both human Tetanus Immune Globulin and mouse antitetanus serum to react with botulinum toxins suggests that the native forms of tetanus and botulinum toxins have very few or no surface antigenic determinants in common. This confirms the lack of significant cross-reactivity between tetanus and botulinum toxins (23). In agreement with this, Tsuzuki et al. (25) reported that out of 306 monoclonal antibodies generated against a native form of the light chain of botulinum type E toxin, only one cross-reacted with tetanus toxin and other botulinum toxin types. Antibody 1 reacted with tetanus toxin in an ELISA and on Western blots but did not bind to tetanus toxin in solution. These results suggest that antibody 1 does not react with the native conformation but that adsorption of tetanus toxin to ELISA plates or denaturation in SDS can lead to a conformational change that exposes the sequence represented by peptide 1. Our results suggest that homology between tetanus and botulinum toxins is present primarily as short contiguous amino acid segments that are either not accessible or not immunogenic in the native molecule. This is similar to the homology that has been demonstrated for cholera and pertussis toxins. Antibodies against the native forms of pertussis toxin have not been shown to cross-react with cholera toxin. However, an antibody made against a synthetic peptide corresponding to amino acids 6 through 17 of the A subunit of pertussis toxin did recognize cholera toxin (4).

We have demonstrated that an antibody generated against a synthetic peptide representing the amino-terminal sequence of the light chain of tetanus toxin cross-reacted with three out of four types of botulinum toxin tested. This sequence homology was not recognized as cross-reactive by antibodies raised against native tetanus toxin. Antibodies against synthetic peptides may be a generally useful technique for identifying regions of sequence homology of these and other proteins and for understanding their mechanism of action.

ACKNOWLEDGMENTS

We thank James Schmidt for supplying the botulinum toxins, Judy Regan for synthesizing peptides 1 and 2, and Frank Robey and Wolfgang Lindner for synthesizing peptides 3 to 13.

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