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**CROSS REACTION OF TETANUS AND
BOTULINUM NEUROTOXINS A AND B AND
THE BOOSTING EFFECT OF BOTULINUM
NEUROTOXINS A AND B ON A PRIMARY
ANTI-TETANUS ANTIBODY RESPONSE**

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ABSTRACT

The present studies were carried out in order to investigate the cross-reaction of botulinum neurotoxins (BoNTs) with human and mouse antibodies against tetanus neurotoxin (TeNT) and determine whether injection of BoNT into a host that has been primed with TeNT would result in boosting of the response to the injected BoNT. Human antisera against TeNT obtained from 9 individuals were found to exhibit substantial cross-reaction with BoNTs A and B. We prepared antibodies (Abs) against inactivated tetanus neurotoxin (TeNT) in outbred mice and determined the binding of these Abs to active TeNT and active botulinum neurotoxins (BoNTs) A and B. Blood samples were collected before immunization (day 0) and on days 42, 82 and 125 after the first injection. The reactions of these sera with the immunizing antigen (inactivated TeNT), active TeNT, active BoNT/A and active BoNT/B

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were determined. At a fixed dilution (1:62.5 v/v), the sera contained high levels of Abs that reacted with TeNT and also with BoNTs A and B. Throughout the test period (up through day 125) and at different dilutions the cross-reactions of the antisera with BoNT/B were almost twice those with BoNT/A. The reactions of the antisera with the immunizing antigen (inactive TeNT) or with active TeNT were essentially equal throughout the dilution range tested (1:16–1:500 v/v). To determine whether injection of BoNT/A or B into a host that had been primed with TeNT resulted in boosting of the response to the priming antigen (TeNT) as well as BoNT/A or B, mice were primed with TeNT and boosted 21 days later with TeNT, BoNT/A or BoNT/B. Appropriate controls were also employed. Blood samples were collected prior to TeNT priming (day -1) and on days 21, 32, 46 and 67 after priming. In TeNT-primed mice, BoNTs A or B boosted the anti-TeNT Ab responses slightly but had no significant boosting effect on the Ab populations that bind to BoNTs A or B. It is concluded that while Abs against TeNT cross react with BoNTs and the cross reaction with BoNT/B is almost double that of BoNT/A, injection of BoNTs A or B in the presence of a prior active immunity against TeNT is not very likely to make the host mount an Ab response against the injected BoNT.

INTRODUCTION

Cross-reactions between tetanus and botulinum neurotoxins (BoNTs) have been previously reported.^[1] There is a considerable structural homology between TeNT and BoNTs. Our mapping of the Ab and T cell epitopes on the H_C domain of BoNT/A^[2–5] has shown that a number of the antigenic regions that are recognized by Abs on the H_C domain have even greater homology to the structurally equivalent regions on TeNT. Based on this homology within the antigenic regions considerable cross-reactions are expected between BoNTs A, B and TeNT. The purpose of this work was to examine (1) whether BoNTs A and B show differences in their cross-reactions with anti-TeNT Abs; and (2) whether mice that had an active immune response against TeNT and are injected with BoNT/A or BoNT/B are more likely to mount Ab responses against the injected BoNT than mice that had not been previously immunized with TeNT. It was found that prior immunization with TeNT followed by injection of BoNT/A or BoNT/B did in fact cause a slight boosting of the Ab response against TeNT but had no effect on the level of Abs that bind the BoNT itself.

MATERIALS AND METHODS

Antisera

Human sera were obtained from individuals who had been vaccinated with tetanus toxoid in the preceding 10 years. The sera were screened for

binding to TeNT and those that had high levels of anti-TeNT Abs were selected for the present studies. Mouse antisera against TeNT were raised in outbred mice by immunization with TeNT toxoid (0.714 $\mu\text{g}/\text{mouse}$) on Alum (0.89 μg) and boosted 3 weeks later with a similar dose of the antigen. Test bleed were obtained prior to immunization and thereafter every six weeks. Binding of the Abs in these antisera was determined at several dilutions with TeNT, BoNT/A and BoNT/B.

Binding of Antibodies to TeNT and to BoNTs

Human and outbred mouse (*ICR*) antibodies (Abs) against TeNT were screened with TeNT, BoNT/A and BoNT/B by a solid-phase plate RIA.^[6] Assays were done in triplicates. Active neurotoxins (1 μg) in 50 μL of 0.01 M sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl (PBS) were added to the wells of flexible polyvinyl chloride 96-well plates (Becton Dickinson and Co., California) and allowed to bind for 18 h at 4°C. After washing five times with PBS the plates were blocked for 1 h at 37°C with 1% bovine serum albumin (BSA) in PBS. Aliquots (50 μL) of anti-toxin antisera that had been prediluted with 0.1% BSA in PBS were pipetted into the appropriate wells and kept at 4°C for 20 h. The wells were then washed five times with PBS and incubated (2 h at 37°C) with 50 μL of prediluted (1:2000 v/v with 0.1% BSA/PBS) affinity-purified rabbit antibodies against the first antibody (human or mouse IgG (H + L) + IgM (Mu chain) (Accurate Chemical & Scientific Corporation, New York). After the plates were washed five times with PBS, 50 μL of ^{125}I -Protein A (2×10^5 cpm in 0.1% BSA/PBS) was applied to the wells and allowed to react for 2 h at room temperature. Finally, the plates were washed thoroughly with PBS to remove unbound radioactivity, the individual wells were cut out and transferred into separate tubes and bound radioactivity was counted in a gamma-counter (1277 Gamma Master, LKB, Finland). The results, of the triplicate analyses were expressed as mean net cpm $\pm$ SD, after correction for nonspecific binding in control wells that were coated with unrelated protein (BSA).

Determination of the Booster Effect of BoNT on a Prior TeNT Response

We tested in outbred mice whether injection of BoNT/A or B into a host that has been primed with TeNT causes any boosting of the response to the injected BoNT. All the mice were prebled prior to immunization to obtain their non-immune sera. The mice were divided into 8 groups and injected i.m. with the antigens as shown in the protocol in Table 1. Test bleeds were obtained 21 days after the first immunization and then again 10, 24, and 45 days after the second immunization.

Table 1. Determination of the Booster Effect of BoNT on a Prior TeNT Response

Mouse Group	Bleed Day	Priming Antigen Day 0	Bleed Day	Boosting Antigen Day 22	Test Bleeds on Days		
Group 1	-1	TeNT (0.714 µg/mouse) on Alum (0.89 µg)	21	TeNT (0.714 µg/mouse)	32	46	67
Group 2	-1	Same as above	21	BoNT/A toxoid (0.714 µg/mouse)	32	46	67
Group 3	-1	Same as above	21	BoNT/B toxoid (0.714 µg/mouse)	32	46	67
Group 4	-1	Same as above	21	None	32	46	67
Group 5	-1	Alum only (0.89 µg)	21	TeNT (0.714 µg)	32	46	67
Group 6	-1	Same as above	21	BoNT/A (0.714 µg)	32	46	67
Group 7	-1	Same as above	21	BoNT/B (0.714 µg)	32	46	67
Group 8	-1	Same as above	21	None	32	46	67

The above immunization protocols were tested in outbred mice in order to determine whether injection of BoNT into a host that has been primed with TeNT causes any boosting of the response to the injected BoNT. All the mice were prebled prior to immunization to obtain their non-immune sera. The mice were then injected i.m. with the antigens as shown in the protocol above. Test bleeds were obtained 21 days after the first immunization and then again 10, 24, and 45 days after the second immunization.

The antisera obtained from a given group of mice were pooled, divided into aliquots and kept frozen until use. The antisera were analyzed for their antibody titers towards TeNT, BoNT/A and BoNT/B by solid phase plate RIA as described above.

RESULTS

Binding of Outbred (ICR) Mouse Antibodies Against TeNT with TeNT, BoNT/A, and BoNT/B

Antisera against inactivated TeNT were raised in outbred (ICR) mice. Test bleeds were obtained before immunization (day 0, pre-bleed) and on days 42, 82 and 125 after the first injection. The reactions of these sera with the immunizing antigen (inactivated TeNT), active TeNT, active BoNT/A and active BoNT/B were determined. At a fixed dilution (1:62.5 v/v), the sera contained high levels of antibodies that reacted with TeNT (Fig. 1). For a given bleed the cross-reactions of the antisera with BoNT/B (day 42, 24.6%; day 82, 25.33%; day 125, 30.43%) were almost twice those with BoNT/A (day 42, 9.62%; day 82, 13.95%; day 125, 15.33%). The cross-reactions with BoNTs A and B increased with antisera concentration (Fig. 2) but the cross reaction of BoNT/B remained almost double that of BoNT/A (e.g., at antisera dilutions of 1:16 v/v the cross-reactions of BoNT/B and BoNT/A were 63.7% and 37.5%, respectively). It should be noted that the reactions of the antisera with the immunizing antigen (inactive TeNT) or with active TeNT were essentially equal throughout the dilution range tested (1:16–1:500 v/v) (Fig. 2).

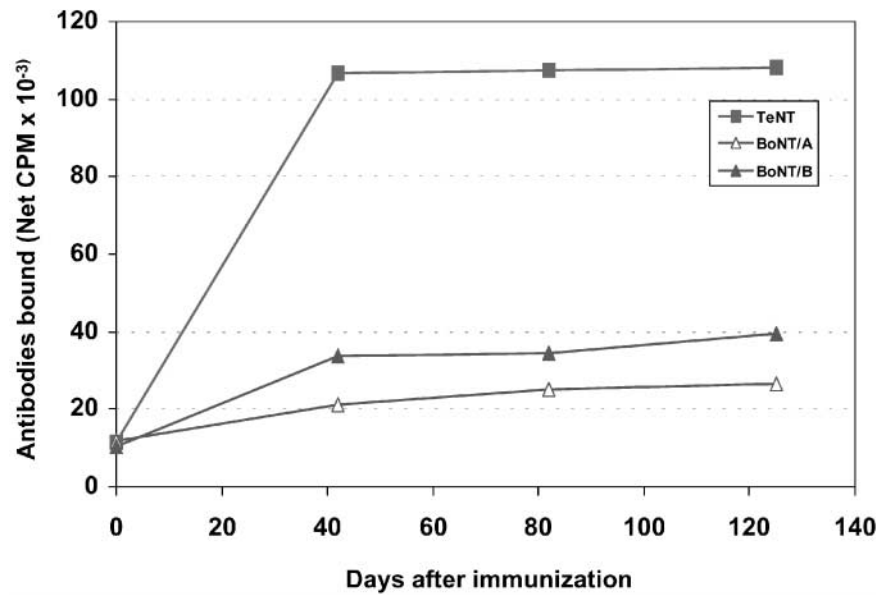


Figure 1. Binding of outbred (ICR) mouse Abs against TeNT to TeNT, BoNT/A, and BoNT/B. The antisera were diluted 1:62.5 (v/v) with 0.1% BSA in PBS and used for the binding assay as described in the text.

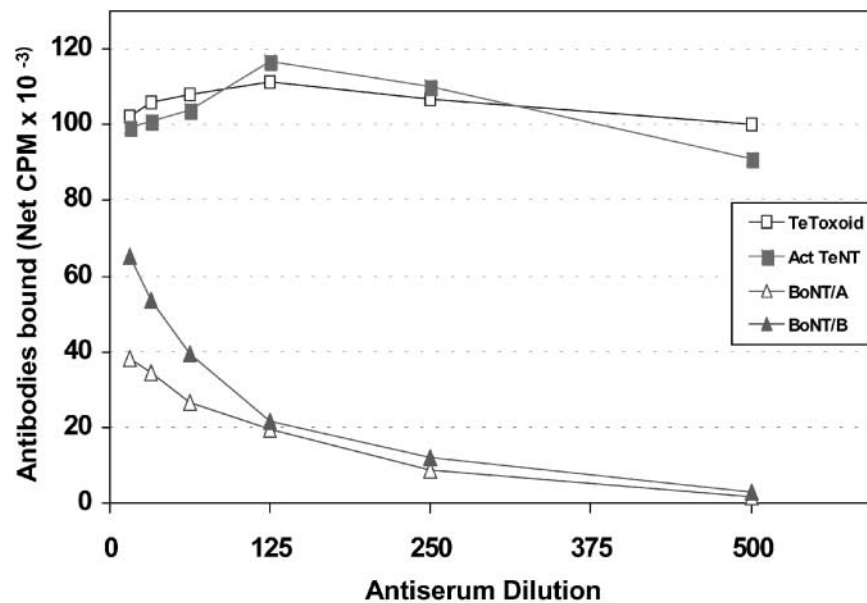


Figure 2. Binding of ICR anti-TeNT Abs (test bleed at 125 days, see Fig. 1) at dilutions 1:16–1:500 (v/v, with 0.1% BSA in PBS) to TeNT toxoid, active TeNT, active BoNT/A, and active BoNT/B.

Cross-reaction of Human Anti-TeNT Antibodies with BoNTs A and B

Human sera from 9 individuals who had active anti-TeNT immune responses were analyzed for Abs that bound to TeNT, BoNT/A and BoNT/B. (Fig. 3). The sera possessed high levels of Abs that bound to TeNT. It is important to note that these antisera showed significant cross-reactions with BoNT/A and BoNT/B (Fig. 3), despite the fact that these individuals had never been treated with either of these neurotoxins. The results clearly show that, as described above with mouse anti-TeNT Abs, human anti-TeNT Abs also cross-react with botulinum neurotoxins. The differences in the levels of cross-reactions of BoNT/A and BoNT/B were not significant. Whether the pre-existence of such Ab responses against TeNT plays any role in the development of an immune resistance to treatment with BoNTs was therefore investigated in mice.

Effects of Boosting with BoNTs A or B in a TeNT-Primed Host

To simplify the presentation of the large amount of binding data obtained, the Abs bound to each neurotoxin by the control group that was primed only with alum and then injected with TeNT, BoNT/A or BoNT/B were used as a reference for the correlate TeNT-primed and TeNT-, BoNT/A- or BoNT/B-boosted group (see Table 1). The results in Figs. 4–7,

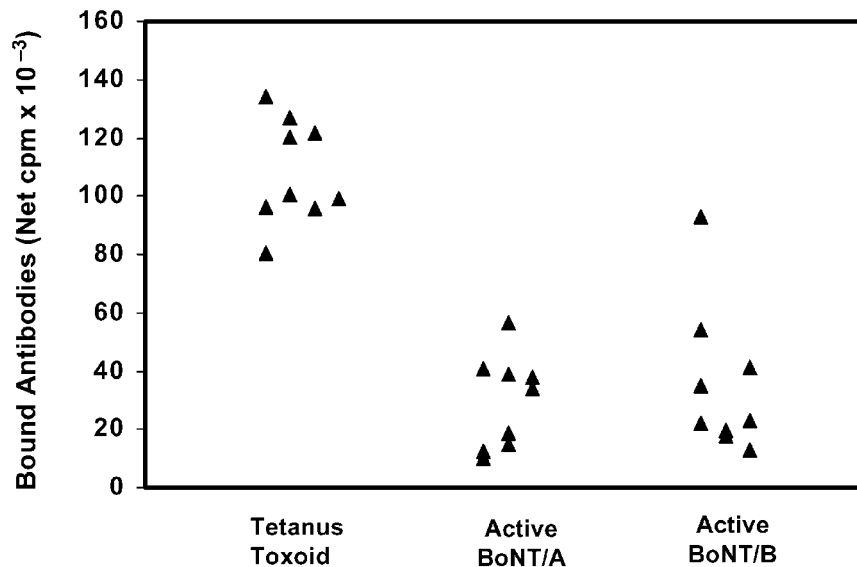


Figure 3. Binding of 9 human antisera against TeNT with tetanus toxoid, active BoNT/A, and active BoNT/B. Sera were diluted 1:64 (v/v) with 0.1% BSA in PBS and assayed by solid phase plate RIA as described in Methods.

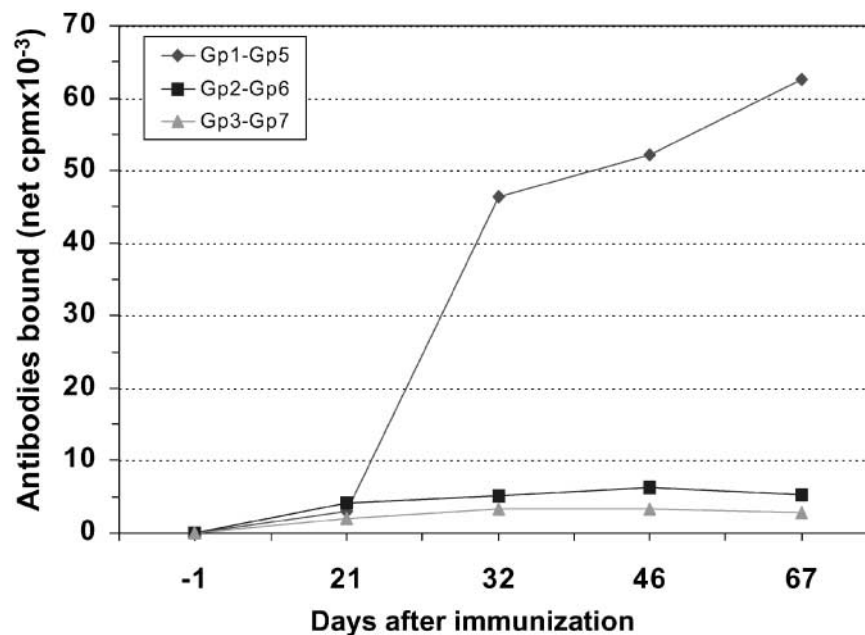


Figure 4. Amounts of Abs bound to TeNT over the course of immunization. Mice were primed with TeNT and boosted 3 weeks later with TeNT (Gp 1) BoNT/A (Gp 2) or BoNT/B (Gp 3). Test bleeds were obtained prior to immunization (day -1) and on days 21 (prior to boosting), 32, 46 and 67 after the initial immunization. Antisera were diluted 1:500 (v/v) with 0.1% BSA/PBS. Results have been corrected for binding in sera of control mice that were not primed with TeNT but received the correlate toxin (See Table 1).

which were obtained on the days indicated in Table 1, show the net cpm values (corrected by the values of the correlate control group) of the amounts of Abs bound to a given neurotoxin in antisera of mice that were primed with TeNT followed by boosting with TeNT (group1), BoNT/A (group 2), or BoNT/B (group 3). Priming with TeNT followed by boosting with TeNT caused a very strong (about 50 fold at day 67) increase in the levels of Abs that bind to TeNT. However, mice boosted with BoNT/A or BoNT/B showed a small (3.5–4.5 fold) increase in the anti-TeNT Ab response but displayed no boosting of the Ab populations that bind to BoNT/A or BoNT/B (Figs. 4–7).

DISCUSSION

BoNTs have been applied, with good results, in the treatment of a variety of clinical conditions associated with involuntary muscle spasm and contractions, such as dystonia and spasticity.^[7–17] The ability of BoNTs in minute doses (less than one ng) to block acetylcholine release has been

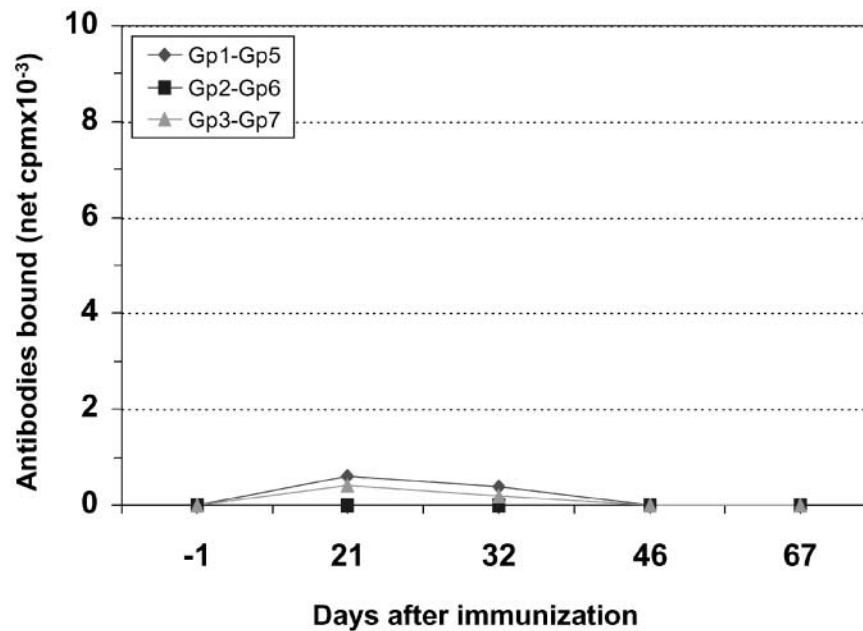


Figure 5. Antibody binding to BoNT/A over the course of immunization. Mice were primed with TeNT and boosted 3 weeks later with TeNT (Gp 1) BoNT/A (Gp 2) or BoNT/B (Gp 3). Test bleeds were obtained prior to immunization (day -1) and on days 21 (prior to boosting), 32, 46 and 67 after the initial immunization. Antisera were diluted 1:500 (v/v) with 0.1% BSA/PBS. Results have been corrected for binding in sera of control mice that were not primed with TeNT but received the correlate toxin (See Table 1).

exploited to relieve disease symptoms associated with muscle hyperactivity. Intramuscular injection of BoNTs produces a reversible partial paralysis (chemodenervation) at the neuromuscular junction. Because the therapeutic benefits obtained with the injection of BoNT are not permanent periodic injections of the neurotoxin are required. Furthermore, the treatment leads to the appearance in some patients of blocking Ab responses against the toxin, which reduce or completely prevent the patient's responsiveness to further treatment.^[18,19] Several factors might contribute to the appearance of immunoresistance in some patients and these factors are discussed below.

Toxin dose plays an important role in patients who are prone to become immunoresistant. Since the dose used is usually suboptimal for maximum Ab response, the Ab response will increase with increase of antigen dose. Therefore, increasing the BoNT dose in patients who have become immunoresistant is risky and obviously it does not resolve the problem, because it simply boosts the Ab titer. It should also be noted that antigen dose can influence the genetic control of the immune response^[20] (see below).

Like with all antigens the appearance of Ab responses is closely linked to duration of treatment and frequency of immunization. In cervical dystonia

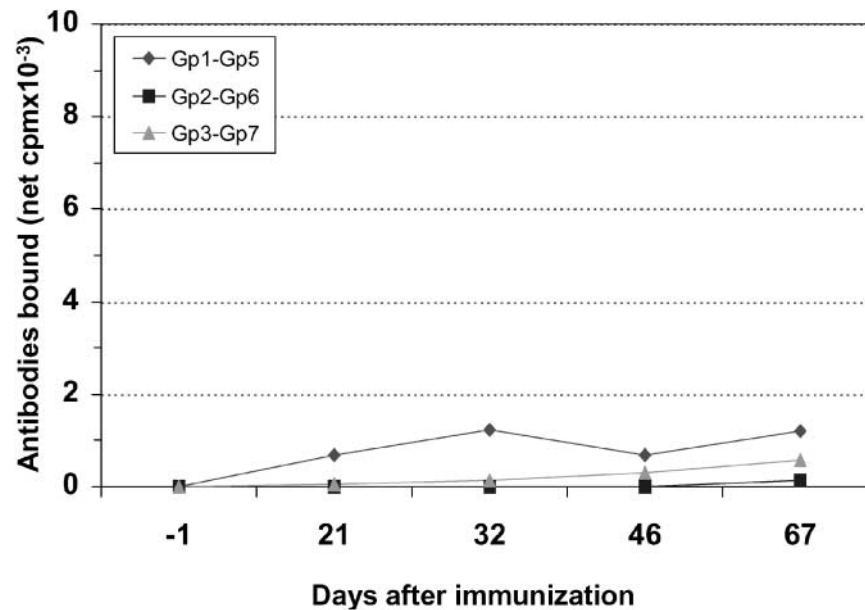


Figure 6. Antibody binding to BoNT/B over the course of immunization. Mice were primed with TeNT and boosted 3 weeks later with TeNT (Gp 1) BoNT/A (Gp 2) or BoNT/B (Gp 3). Test bleeds were obtained prior to immunization (day -1) and on days 21 (prior to boosting), 32, 46 and 67 after the initial immunization. Antisera were diluted 1:500 (v/v) with 0.1% BSA/PBS. Results have been corrected for binding in sera of control mice that were not primed with TeNT but received the correlate toxin (see Table 1).

patients treated with BoNT, it has been found that the appearance of blocking Abs seems to correlate with the cumulative toxin dose, the time interval between injections and the total protein load.^[21]

Genetic predisposition or major histocompatibility (MHC) susceptibility may play a role in those patients who develop immunoresistance early in the course of treatment even after a relatively low dose of BoNT. We have shown that immune responses to a protein antigen are under genetic control and that the responses to the various epitopes on the antigen are each under separate genetic control.^[22] Studies have not been done to determine the relationship, if any, of immunoresistance due to blocking Abs and the MHC of the patient. These studies are presently in progress in our laboratory.

When a patient becomes immunoresistant to one BoNT, the therapeutic benefit can be recaptured by using another BoNT serotype. Although the patients usually respond to the alternate serotype, some eventually lose their response because they mount blocking Abs against the second BoNT. For example, when BoNT/A was used in patients with focal dystonia some patients mounted Ab responses against BoNT/A and became unresponsive to further treatment with BoNT/A but showed improvement that was sustained for three additional injections of BoNT/F.^[23] In another study,^[24]

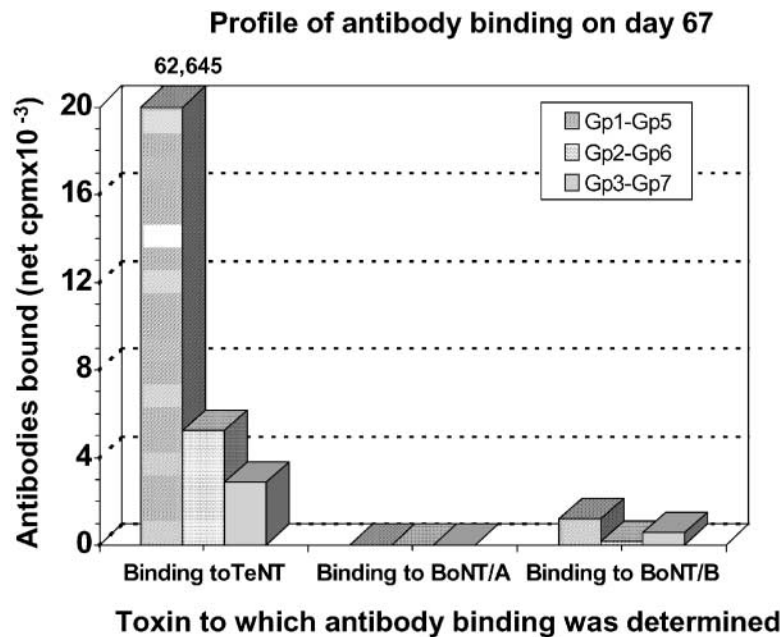


Figure 7. Antibody binding to TeNT, BoNT/A, or BoNT/B of sera obtained on day 67 after the initial injection. Groups are as described in Figs. 4–6 and in Table 1. Antisera were diluted 1:500 (v/v) with 0.1% BSA/PBS. Note the level Ab binding of Group 1 to TeNT was 62,645 cpm. Ab binding to BoNT/A was 0. Also little or no Abs were bound to BoNT/B in Groups 1, 2, and 3 (values were 1189, 147, 567 cpm respectively).

patients who had mounted anti-BoNT/A Ab responses and reached complete non-responsiveness to BoNT/A therapy were treated with BoNT/B. Although all patients initially improved with the BoNT/B about a third of the patients eventually lost their response and developed evidence of blocking Abs.

Another major factor that contributes to changes in the immunogenic properties of a protein is the quality of the antigen. The following are very important: (a) Intrinsic factors (e.g., size, shape, disulfide cross-links, subunits, etc.); (b) Presence of foreign proteins might enhance the immune response (foreign proteins might act as adjuvants); (c) Presence of denatured antigen; (d) Presence of polymerized antigen; (e) Changes of the covalent structure (e.g., cleavage of disulfide bonds. Göschel et al.^[18] studied the effects of Dysport® and BOTOX® (both BoNT/A) in dystonia patients and found that the presence of toxoid in the material increased the chance of Ab production. Also, impurities in the botulinum neurotoxin might act as adjuvants and stimulate antibody formation. In a recent study, Jankovic et al.^[21] compared the immunogenicity of the original BoNT/A preparations (BOTOX®, prior to December 1997) in patients treated for cervical dystonia (n = 130) with current BOTOX® (after January 1998) (n = 119). While the

frequency of blocking Abs with the original BOTOX[®] was 9.5%, they found no evidence for immunoresistance in the patients treated with the current BOTOX[®] and proposed that the latter decreased the probability of Ab responses by a factor of 6. This may be a reflection of dose (original BOTOX[®] contained 25 ng of neurotoxin complex/100 units while the current BOTOX[®] contains 5 ng/100 units) and/or of the quality of the antigen (current BOTOX[®] is about half the molecular size of the old BOTOX[®]).

It has been well established that, in a set of homologous proteins, the regions of immune recognition occur at structurally equivalent locations.^[22,25–27] The primary structures of TeNT,^[28–29] BoNT/A^[30–32] and BoNT/B^[33,34] show extensive homology. Four cysteine residues are conserved in BoNT/A and TeNT.^[32] The structural identity appears to increase in the epitope regions that have been localized on BoNT/A (See Fig. 8 and Table 2). Region 947–967 in TeNT is recognized by human peripheral blood lymphocytes.^[35] This region of TeNT is homologous to BoNT/A region 938–958 within peptide 939–957, which is recognized by SJL and BALB/c T cells.^[4] Region 916–932 of TeNT [equivalent to BoNT/A region 907–923 within the overlap of peptides 897–915 and 911–929 recognized by SJL T cells^[4]] is also recognized by human T cells.^[36] These similarities in T-cell recognition regions indicate that the clostridial toxins, BoNTs A and B and TeNT, share some of immunological features, along with a number of structural and functional similarities.

It is therefore to be expected that these proteins would exhibit considerable immunological cross-reactivity.^[22] In the present work this cross-reactivity between anti-TeNT Abs and BoNTs A and B has been confirmed with both mouse and human antisera against TeNT. In view of this cross-reactivity, it was important to determine whether the appearance of blocking Abs in some patients receiving BoNT therapy correlated in any way with the presence of a prior anti-TeNT Abs. Often a primary immune response to a protein antigen may be boosted by injection with a closely related protein. This phenomenon, termed ‘original antigenic sin’^[37,38] was originally observed on vaccination or infection with an influenza virus strain. When this was followed by injection or infection with a second strain of a closely related influenza virus the Abs that were boosted by the second virus were in fact directed against the first virus. In the present work, we have found that in TeNT-primed mice both BoNTs A and B did in fact stimulate a small but significant (between 3.5–4.5 fold) boosting of the anti-TeNT Ab response. This was much smaller than the boosting effect of TeNT itself (over 50 fold). However there was no increase in the level of Abs that cross-reacted with BoNT/A or BoNT/B. Thus BoNTs A and B are unable to boost Ab responses against themselves in animals that had been primed with TeNT.

It should be noted that the present studies were done in mice. We used outbred mice rather than selected mouse haplotypes in order to obtain results

869-887 (peptide H _c 2)	Te	IDVILKKSTILNLDINNDI
	A	YIKNIINTSILNLRYESNH
	B	YNSEILNNIILNLRYSKDN
883-901 (peptide H _c 3)	Te	INNDIISDISGFNSSVITY
	A	YESNHLIDLSRYASKINIG
	B	YKDNNLIDLSGYGAKVEVY
939-957 (peptide H _c 7)	Te	NNFTVSFWLRVPKVSASHL
	A	ENFSTSFWIRIPKYFNSIS
	B	LD ~~F~~ SVSFWIRIPNIRMMVY
995-1013 (peptide H _c 11)	Te	RQITFR-DLPDKFNAYLANK
	A	QRVVFYKYSQMINISDYI-NR
	B	KS ~~V~~ FFEYNIRE ~~D~~ ISEYI-NR
1051-1069 (peptide H _c 15)	Te	NNITLKLDRCNNNNQYVSI
	A	NNIMFKLDGCRDTHRYIWI
	B	GEIIFKLDGDI ~~D~~ R ~~T~~ QFIWM
1093-1111 (peptide H _c 18)	Te	SITFLRDFWGNPLRYDTEY
	A	NSGILKDFWGDY ~~L~~ QYDKPY
	B	YSEY ~~L~~ KDFWGNPLMYNKEY
1177-1195 (peptide H _c 24)	Te	GDFIKLYVSYNNNEHIVGYP
	A	NDRVYINVVVKNEYRL AT
	B	EDYIYLDFFNLNQEWRV YT
1275-1296 (peptide H _c 31)	Te	DKII-LGCDWYFVPTDEGWTND
	A	SRT--LGC ~~S~~ WE ~~F~~ IPVDDGWG ERPL
	B	PYNLKLGCNWQFIPKDEGWT E

Figure 8. Alignments of the BoNT/A peptides that are recognized by anti-BoNT/A Abs of ICR mice (Refs. 2-5) with the corresponding regions on BoNT/B and on TeNT. The sequences of TeNT, BoNT/A, and BoNT/B were obtained from Refs. 4-6, respectively.

Table 2. Sequence Identity of Botulinum Neurotoxins B and A Relative to Tetanus Neurotoxin

Neurotoxin	% Identity Relative to Whole TeNT	% Identity Within the Epitope Regions
BoNT/B	39	59
BoNT/A	32	54

that will be general and less likely to be skewed by the genetic characteristics of the host. Although humans are outbred this does not rule out the possibility that in some patients receiving BoNT treatment and who express certain HLA alleles, a prior anti-TeNT Ab response might act as a priming antigen for an anti-BoNT Ab response. We have commenced studies on cervical dystonia patients to determine the linkage, if any, of the appearance of blocking Abs with MHC of the host and whether a prior TeNT response and the expression of certain HLA alleles in the patient is more likely to influence the outcome of BoNT treatment.

CONCLUSIONS

The following conclusions can be made from the present findings: (1) Antibodies against one of the neurotoxins TeNT, BoNT/A or BoNT/B cross-react with the other two neurotoxins. This is to be expected from their structural homology, particularly within the antibody and T cell recognition regions (as mapped on BoNT/A, Refs. 2–5). (2) With mouse antisera against TeNT, the cross-reaction of BoNT/B is higher than that of BoNT/A. (3) With human antisera against TeNT, no significant differences were found in the cross-reactions of BoNT/A and BoNT/B. (4) When BoNT/A or BoNT/B is given to a host that has a prior active Ab response against TeNT, it causes a small increase in the level of Abs that bind to TeNT but neither seems to act as a significant booster of the Ab response that binds to the BoNTs.

ABBREVIATIONS

Ab, antibody; BSA, bovine serum albumin; BoNT, botulinum neurotoxin; BoNT/A and BoNT/B, botulinum neurotoxins A and B; TeNT, tetanus neurotoxin; H_C, The C-terminal domain (residues 855–1296) of the heavy (H) chain of BoNT; PBS, 0.01 M sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl.

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