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RECEPTOR BINDING AND MEMBRANE TRANSPORT OF BOTULINUM TOXINS

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JOHN R. DANKERT

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These studies seek to better understand the mechanism of action of the botulinum toxins. Through the use of antibodies to a number of different membrane-active proteins, any cross-reactivity found between the botulinum toxin protein and other toxin molecules could point to similarities in either structure or function. This similarity could be found in any of the generalized functions of all these toxins: i) the enzymatic or toxic unit, ii) the membrane inserting or translocating domain, and iii) the receptor binding domain. As the protein sequences between these diverse proteins is not known to contain any direct analogies (and all the protein sequences have not been defined) and no direct antigenic cross-reactivities <u>during</u> the action of the toxin molecules bound to ar acting upon artificial target cells (liposomes).					
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FOREWORD

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Introduction

Antibodies to the protein molecule human complement component C9 have been shown to cross-react with a protein found in bee venom, melittin (1,9). Both of these molecules are termed "membrane active" in that they have the ability to disrupt membrane bilayers (1,2). Antibodies to one molecule that inhibit this activity that also inhibits the membrane activity of the other point to the possibility that a common mechanism of membrane perturbation exists between these molecules. Our interest in this discovery and its relationship to the study of the mechanism of action of botulinum toxin is the possibility that a common mechanism of action between botulinum toxin and other molecules could be examined. The possibility that a common "lytic" mechanism exists for a group of proteins that includes the botulinum toxin would serve to increase our understanding of the mechanism of action.

A multi-domained model for the botulinum toxins has been proposed (3). In general, the proteins are composed of a polypeptide containing a heavy (MW $\approx$ 100,000) and a light (MW $\approx$ 50,000) chain that requires proteolytic cleavage before becoming fully toxic. This toxin, like other toxin molecules, must have diverse capabilities in addition to the toxic activity of the molecule. The toxin must also recognize the correct target and then deliver the toxic activity into the target. Other proteins that must accomplish these same three objectives, it could be argued, could perform the tasks in similar ways. The complexity of the structure of the toxin

Through these studies it may be possible to better understand the mechanism of action of the botulinum toxins. Any common cross reactivities found between the botulinum toxin protein and another toxin molecule could point to similarities in any one of the following three generalized functions of these toxins: i) the enzymatic or toxic unit, ii) the membrane inserting or translocating domain, and iii) the receptor binding domain. As the protein sequences between these diverse proteins is not known to contain any direct analogies (and all the protein sequences have not been defined) and no direct antigenic cross-reactivities has been reported, we began to search for common cross-reactivities during the time the toxin molecules bound to or crossed artificial target cells (liposomes).

Materials and Methods

Materials and Supplies. All chemicals and reagents for buffers and solutions unless otherwise stated were purchased from Fisher Scientific. Dyes for the fluorescence studies from Molecular Probes, Inc. Lipids were obtained from Avanti Polar Lipids, and all electrophoretic chemicals were purchased from Serva. Botulinum toxin (Type B) was obtained from Calbiochem. Cyanogen-Bromide coupled Sepharose 4B and reagents for antibody purification were purchased from Sigma.

Lipid Vesicle Formation. Large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were formed as described in (3,4).

Acid Shock Procedure. Vesicles in 10 mM Imidazole buffer containing 150 mM NaCl, pH 7 (10 μ g lipid/mL) were subjected to a drop in pH by the addition of 10 μ L of a 10% stock solution of succinic acid at pH 3.9. After 1 min the entire mixture was diluted into 3 mL of buffer at pH 7.4.

Antibody Preparation. Polyclonal IgG from selected animal sera will be isolated as described (5) and the final IgG fractions will be dialyzed against 10mM Tris-HCl, 150mM NaCl (pH 7.3) or 10mM imidazole, 150mM NaCl (pH 7).

Constuction of a Botulinum-Coupled Column. Cyanogen Bromide activated Sepharose 4B was prepared for coupling of the toxin according to the method prescribed by the manufacturer. Approximately 0.5 mg of toxin in the coupling buffer was mixed with 5 mLs of swollen gel. This mixture was "tumbled" for 16 hours at 4°C. Buffer containing blocking agent (0.2 M glycine) was then substituted for the original buffer, and the mixture was again tumbled for an additional 20 hours at 4°C. The gel was poured into a glass column and washed extensively with the original coupling buffer and stored at 4°C.

Botulinum Toxin Column Use. Select purified polyclonal antibodies (2 mg total protein) were applied to the column at 4°C. The column was then washed extensively with buffer (10 mM imidazole, 150 mM NaCl, pH 7). 10 mL volumes of eluting solutions were then passed over the column and collected. After each 10 mL elution, the column was re-equilibrated in the original buffer. The elution solutions were buffer containing 2 M NaCl, or 2 M KBr, or 4 M Guanidine-HCl. The elution buffers were applied sequentially in this same order. The separate collected elutions from the column were then assayed for protein by measuring the absorbance at 280 nm using an extinction coefficient of 1.4 for the antibodies. The solutions were dialyzed (4°C, 24 h) against 10 mM imidazole, 150 mM NaCl, pH 7. The pooled antibody that did not bind to the column (pass-thru fraction) was also dialyzed as described. The solutions were concentrated (when needed) to a concentration of 1 to 2 mg/mL, as determined by absorbance of the solution at 280 nm.

Fluorescence Measurements. Determination of release of the dye carboxyfluorescein (CF) from lipid vesicles was performed by forming the vesicles in the presence of a self-quenching concentration of the dye molecule (100 mM). The free dye molecules were separated from the internalized dye and liposomes by either passing the mixture over a Sephadex G-100 column (collecting the void volume fractions of the column for the vesicles) or by

repeated centrifugations (at least 3 times) with resuspension into dye-free buffer. The vesicles were then used within 4 hours for all assays of toxin activity. Measurement of dye release was performed on an Aminco Bowman Spectrophotofluorometer, adapted with a thermostatted, stirred cuvette, as described in reference (6). pH shock was performed as described above using 10% succinic acid, followed by the addition of 10 μ L of a 1 M Imidazole, pH 8 stock solution used to return the final pH to a value of 7. When the vesicles were formed in the presence of both dye and antibody molecules, the Sephadex G-100 column was replaced by a Sepharose 2B sieving column in order to insure the complete separation of free dye and antibody molecules from the liposomes containing dye and antibodies.

Immuno-blotting. The Laemmli system (7) was used to prepare the acrylamide gels for separation. Immunoblotting was performed using a Bio-Rad gel electrophoresis and transfer unit. Blots were developed using one of the various antibodies as a primary with alkaline phosphatase labeled secondary antibody used according to the manufacturer.

ELISA. Enzyme-linked immunoadsorbent assays will be performed essentially as described (8). Coating of the wells was altered in terms of length of time of incubation of antigen, and in the pH and temperature at which the incubation took place.

Results

The ability of polyclonal antibodies to human C9 to inhibit the membranolytic activity of botulinum toxin (as reported in this study previously) has been further investigated. In order to facilitate this study it would be desirable to utilize an assay of antibody recognition not involving the liposome dye release assay. A standardized method, such as that of an ELISA technique, would simplify screening of other antibodies for cross-reactivity with the botulinum toxin.

Using an ELISA screening method, no antibody to date generated (or purchased) has been able to provide a positive binding signal to the botulinum toxin antigen. Since the in vitro technique used to screen antibodies for any botulinum toxin inhibitory activity requires that the mixture be subjected to an acidic pulse (pH 4), it was possible that some pH-dependent expression was involved that allowed our anti-human C9 antibody to recognize an epitope on the botulinum toxin molecule. In addition, the in vitro assay also was performed in the presence of lipid vesicles (liposomes) which could play a role in altering the antigenic characteristics of the toxin molecule. To address these questions, an ELISA system was established to test the role of both pH and the presence of liposomes on the ability of antibodies to other proteins to bind to the toxin molecule. Acid-treated botulinum toxin and acid-pulsed liposomes (with or without added botulinum toxin) were used in an ELISA system as the source of antigen. Antibodies to human C8, human C9, and bee venom melittin were tested for their ability to detect the above antigens at various dilutions (serial 2-fold dilutions). Both toxin and liposome-toxin mixtures (10-20 ng toxin total per well) were exposed to acid pulses as described previously, using pH values of 6.5, 6, 5.5, 5, 4.5, 4, and 3.5 for various periods of time at 37°C. The length of time for the acid pulse was varied from 1 min to 12 min (at 3 min intervals). The samples were then used to coat the wells of standard ELISA plates for after return to pH 7. The length of time that the toxin was allowed to coat the ELISA plate wells prior to washing was varied from 1 minute to 24 hours. The ELISAs were then completed. No positive antibody binding results above background levels (data not shown) could be discerned using this ELISA technique.

The ELISA experiments just described had failed to indicate any positive binding results that could be detected above the background levels of color generated in the absence of antigen. It was possible that the concentration of antibody required to produce a positive result was such that the background level of binding obscured the effect. To address this possibility, a solid phase column bearing the botulinum toxin protein was constructed using commercially available reagents (see Materials and Methods above). The solid-phase toxin would be used to isolate those antibodies in the mixture that could recognize the toxin. The large fraction of antibody that did not bind to this column would be removed. This reduction in non-specific antibodies would serve to lower the background levels in the ELISA system, and should also enable us to repeat the in vitro liposome assay using much less protein.

Purified polyclonal rabbit antibody to human C9 and bee melittin were passed over the toxin-coupled column at 4°C. The column was washed extensively with buffer (10 mM Imidazole, 150 mM NaCl, pH 7) and any protein (antibody) that

was bound to the column was eluted using three different elution conditions. These buffers were 1) buffer containing 2 M NaCl, 2) buffer containing 2 M KBr, and 3) buffer containing 4 M Guanidine-HCl.

The eluted fractions were dialyzed as described above (Materials and Methods). It was noted that the least amount of protein eluted from the column was obtained from the KBr elution. Over 80% of the protein that bound to the column was eluted with 2 M NaCl, about 5% with 2 M KBr, and the remainder eluted with 4 M Guanidine-HCl. In addition, the pooled antibody that did not bind to the column (pass-thru fraction, >90% of the added protein) was also dialyzed as described. The solutions were concentrated (when needed) to a concentration of 1 to 2 mg/mL. Inhibition of botulinum toxin in vitro activity was assayed using the dye release assay (see Materials and Methods).

Table 1. Inhibition of the pH-dependent Membranolytic Activity of Botulinum Toxin by Polyclonal Antibodies in DPPC/DPPS Liposomes (100 μ g lipid per assay) containing Carboxyfluorescein.

<u>50 μg antibody to:</u>	<u>% Dye Release from Liposomes</u>
None	97 $\pm$ 4
Human C9	75 $\pm$ 10
Human C9, pass-thru	99 $\pm$ 6
Elution 1, 2 M NaCl	97 $\pm$ 12
Elution 2, 2 M KBr	95 $\pm$ 9
Elution 3, 4 M Guanidine-HCl	89 $\pm$ 11

From these data it can be inferred that the small inhibitory activity present in the "whole" (unfractionated) polyclonal antibody solution could not be recovered from the botulinum toxin-coupled column. This could be due to the inability of the activity of the antibody binding site to recover from the chaotropic agents (KBr and Guanidine-HCl). It could also be due to some possible combination of antibodies that are separated by this process of binding to fixed toxin and step-wise elution.

The fractionation of the polyclonal antibody mixture did not produce an antibody that could inhibit the in vitro membranolytic activity of the toxin against liposomes. The fractionation was repeated using a technique that would allow for a pH-dependent expression of an epitope on the coupled toxin molecule. The above fractionation of the polyclonal antibodies was repeated using a column that was equilibrated at pH 4, using dimethylglutaric acid (DMG) as the buffering agent. This agent was chosen due to the large range of buffering capacity of this acid to allow us to use this same salt for buffering at pH 7. This would exclude some differences in binding due to different salts. The polyclonal antibody was applied to the column equilibrated at pH 4. The bound protein was eluted using the three previously defined buffer solutions (2 M NaCl, 2 M KBr, or 4 M Guanidine-HCl) in 10 mM DMG at pH 7. The eluted solution was then dialyzed into 10 mM imidazole, 150 mM NaCl, pH 7 and used in the in vitro toxin membranolytic assay as described previously. No ability to inhibit toxin activity was detected using an in vitro liposome assay

(Table 2.). It was noted that little protein (less than 0.1 μg) could be eluted from the column at pH 4 using the absorbance of the fractions at 280 nm. The fractions were used, however in the assay as shown in Table 2. It is possible that little or no antibody bound to the column at this low pH.

Table 2. Inhibition of the pH-dependent Membranolytic Activity of Botulinum Toxin by Antibody Fractions ELuted from Botulinum Toxin-Sepharose. DPPC/DPPS Liposomes (100 μg lipid per assay) containing Carboxyfluorescein.

<u>Fraction:</u>	<u>% Dye Release from Liposomes</u>
None	98 $\pm$ 6
Original anti-Human C9, 50 μg	72 $\pm$ 11
anti-Human C9, pass-thru fraction	99 $\pm$ 10
Elution 1, 2 M NaCl	97 $\pm$ 9
Elution 2, 2 M KBr	95 $\pm$ 6
Elution 3, 4 M Guanidine-HCl	102 $\pm$ 6

It would be of interest to determine if any of the antibodies used in this study had the capacity to inhibit the in vitro membranolytic activity of the toxin when the antibody was entrapped within the lumen of the liposome. Liposomes were formed in the presence of the dye carboxyfluorescein, and 0.2 mg/mL polyclonal antibody and the liposomes (containing the dye and antibody mixture) were separated from free dye and free antibodies as described in Materials and Methods. The antibody had been purified from rabbit serum as described. No inhibition of toxin pH-dependent dye release was noted using the DPPC/DPPS vesicles containing the entrapped antibodies (not shown).

Discussion

Our interest in pursuing the ability of polyclonal antibodies to proteins other than botulinum toxin to inhibit the membranolytic activity of the botulinum toxin centers upon our goal to gain a better understanding of the mechanism of action of botulinum toxin. We observed that polyclonal antibodies to human C9 or bee venom melittin are able to inhibit some of the in vitro activity of botulinum toxin. This points to the possibility that some transient, functional epitope is expressed by the toxin during the assay and this epitope is recognized by some portion of the antibodies.

The botulinum toxin molecule is able to respond to an acidic pulse in the presence of negatively-charged liposomes in such a way as to allow the leakage of small dye molecules through the membrane bilayer barrier. This implies that the toxin must interact in some way with the lipids of the vesicle at low pH values. It could be assumed, although this has not been tested by us, that this interaction renders the molecule non-toxic to a "normal" target, such as human neurons. This would be of value in designing systems that would serve to inactivate this toxin very rapidly, as in the case of accidental poisonings, spills, or from free-living pathogenic organism.

Understanding and identifying functionally-related epitopes between a variety of diverse proteins (such as membrane active toxins such as botulinum, cholera, diphtheria, etc) could be useful in gaining an understanding of common mechanisms of actions of these toxins. It is possible to use this information to select certain portions of a prototype toxin molecule for use as an antigen that could provide immunological defense to an individual to the entire "class" of toxins.

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