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THERAPEUTIC APPROACHES TO THE
TREATMENT OF BOTULISM

Annual Report

October 1, 1988

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Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5285

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Philadelphia, Pennsylvania 19107

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20030131087

89 4 03 051

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30 1986

1a REPORT SECURITY CLASSIFICATION Unclassified		1b RESTRICTIVE MARKINGS	
2a SECURITY CLASSIFICATION AUTHORITY		3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited	
2b DECLASSIFICATION/DOWNGRADING SCHEDULE		5 MONITORING ORGANIZATION REPORT NUMBER(S)	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)		5 MONITORING ORGANIZATION REPORT NUMBER(S)	
6a NAME OF PERFORMING ORGANIZATION Jefferson Medical College Thomas Jefferson University	6b OFFICE SYMBOL (if applicable)	7a NAME OF MONITORING ORGANIZATION	
6c ADDRESS (City, State, and ZIP Code) 1025 Walnut Street Philadelphia, Pa. 19107		7b ADDRESS (City, State, and ZIP Code)	
8a NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command	8b OFFICE SYMBOL (if applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER AMD17-85-C-5285	
8c ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5012		10 SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO 61102A	PROJECT NO 102BS12
		TASK NO AD	WORK UNIT ACCESSION NO 118
11 TITLE (Include Security Classification) (U) Therapeutic Approaches to the Treatment of Botulism			
12 PERSONAL AUTHOR(S) Lance L. Simpson			
13a TYPE OF REPORT Annual Report	13b TIME COVERED FROM 9/1/87 TO 8/31/88	14 DATE OF REP (Yr, Month, Day) 1988 October 1	15 PAGE COUNT 20
16 SUPPLEMENTARY NOTATION			
17 COSATI CODES		18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
06	16		
06	15		
		Botulinum neurotoxin, neuromuscular blockade, experimental therapeutics, RA 1	
		Pharmacological Antagonists, Antidotes, Antitoxins, Binding Sites, (AW)	
19 ABSTRACT (Continue on reverse if necessary and identify by block number) Work during the past year has focused on three problems: i.) defining the conditions that maximize the binding of toxin to nerve endings, ii.) evaluating the possibility that botulinum neurotoxin is a mitochondrial poison, and iii.) studying the interaction between botulinum neurotoxin and a series of monoclonal antibodies. The work on toxin binding suggests that the approach currently used by most biochemists is flawed. Their approach "maximizes" binding, but this binding does not appear to involve relevant receptors. The work on mitochondrial function indicates that botulinum neurotoxin is not a mitochondrial poison. If it does affect mitochondrial function, that effect is indirect. The work on monoclonals has shown that type E botulinum neurotoxin can be substantially detoxified by antibodies directed against both the heavy and light chains. It also shows that antigenic determinants on the toxin continue to be exposed after the toxin has bound to nerve endings. <i>Keywords:</i>			
20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS		21 ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostonian		22b TELEPHONE (include Area Code) 301-663-7325	22c OFFICE SYMBOL SGRD-RMI-S

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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1. Statement of Problem

Pharmacological methods are being sought to prevent or reverse the effects of botulinum neurotoxin. During the past year, emphasis has been placed on three problems: i.) defining the conditions that maximize the binding of toxin to nerve endings, ii.) evaluating the possibility that botulinum neurotoxin is a mitochondrial poison, and iii.) studying the interaction between botulinum neurotoxin and a series of monoclonal antibodies.

2. Conditions for Neurotoxin Binding

A. Background

The major goal of this project is to identify rational strategies for antagonizing the effects of botulinum neurotoxin. This could involve efforts aimed at preventing binding, internalization, or intracellular toxicity. The present section deals with the issue of tissue binding.

There are several laboratories that are involved in studying the binding of clostridial neurotoxins to nerve tissue. Many investigators who are biochemical in their orientation have adopted a method that "maximizes" toxin binding to synaptosomal and other membranes. This approach involves suspending tissues in an unphysiological buffer (usually Tris-HCL) that has an abnormally low pH (usually 6.0) and an abnormally low ionic strength (generally in the range of 20-40 mM). Under these conditions, labeled toxin reportedly binds to a greater extent than is observed under

more physiological conditions. The method was introduced more than a decade ago and it continues to be widely used today because it supposedly facilitates the study of ligand-receptor interactions. For a representative recent report, readers can examine Walton et al., J. Biol. Chem., 263, 2055-2063, 1988. Aside from the fact that this manuscript utilizes the binding conditions described above, it may be of further interest because it presents work funded by the U.S. Army Medical Research and Development Command.

The P.I. believes that all work utilizing the abnormal paradigm may be flawed. There is no evidence to demonstrate that the enhanced binding is relevant to toxicity. In the absence of such evidence, there is the possibility that this work reveals little or nothing about authentic mechanisms of toxicity. The abnormal conditions may promote binding to an acceptor that is not linked to the processes of productive internalization and poisoning.

A number of experiments have been done to test whether there is an association between conditions that promote tissue binding and conditions that promote tissue poisoning by clostridial neurotoxins. The data strongly indicate that the two are not closely associated.

B. Methods

Experiments have involved the phrenic nerve-hemidiaphragm preparation, as described in earlier Annual

Reports. Specific details of each experiment are discussed below.

C. Results

Experiment 1. Rationale.

To determine whether abnormal conditions promote toxicity, one must first determine tissue viability in the abnormal conditions. Therefore, tissues were exposed in a multivariate paradigm that involved manipulations in pH, ionic strength and length of incubation. The conditions of pH were 5.5, 6.0, 6.5, 7.0, and 7.5. Each of these pH's was achieved in solutions that varied in ionic strength (10, 20, 40, 80, and 160 mM). Tissues were then suspended in the solutions of varying pH and ionic strength for varying lengths of time (2, 4, 8, 16, 32 and 64 minutes).

There was an additional and essential part of the assays for viability. The purpose of the work was to gauge whether conditions that maximized binding also maximized toxicity. This made it desirable to bath tissues in a way that allowed binding but did not allow steps beyond binding. Thus, tissues were incubated with toxin at 4⁰ C, in the absence of calcium and in the absence of nerve stimulation. The P.I. has reported previously that this allows clostridial toxins to bind to tissues, but it prevents internalization and poisoning.

Experiment 1. Results.

Some of the conditions described above are clearly harsh. Not surprisingly, tissue viability was a function of the severity of conditions. As a point of reference, the abnormal medium most widely used by other investigators is pH 6.0 and ionic strength of 20 mM. When tissues were incubated in this medium, at 4⁰ C, viability was a function of incubation time. Tissues kept in the medium for 2, 4, or 8 minutes, then transferred to tissue baths at 35⁰ C, with calcium and nerve stimulation, functioned almost like control tissues. When incubation was extended to 16 minutes, subsequent muscle twitch in the tissue baths was about 50% of control. When incubation was extended to 32 or 64 minutes, many tissues ceased to function.

It appeared that ionic strength was the main factor in producing decrements in function. Thus, the results described above for solutions at pH 6.0 were similar to those at pH 5.5, 6.5 and 7.5. Altering the pH had only a minimal effect on tissue function. However, progressively increasing the ionic strength promoted tissue viability.

Experiment 2. Rationale.

The results above indicate that tissues will live at least 16 minutes in medium that promotes toxin binding. Therefore, tissues were incubated with toxin for this length of time in the media of varying pH and ionic strength. The tissues were then washed and suspended in tissue baths under

physiological conditions, and paralysis times were monitored.

Experiment 2. Results.

Control tissues (i.e., those incubated in physiological medium) were exposed to botulinum neurotoxin type A at a concentration that would cause paralysis in approximately 120 minutes. When matched tissues were incubated in medium at pH 6.0 and ionic strength of 20 mM, the paralysis time was approximately 350 minutes.

This is a striking finding. Depending on the investigator quoted, the abnormal medium supposedly increases tissue binding by several-fold to as much as 20-fold. However, based on assays for toxicity (previous paragraph), the same medium is decreasing toxin potency by more than an order of magnitude.

Experiment 3. Rationale.

An effort was made to determine the generalizability of the findings above. For this purpose, three types of experiments were done: i.) several botulinum serotypes, as well as tetanus toxin, were tested at pH 6.0 and ionic strength 20 mM, ii.) with serotype A, pH was held constant and ionic strength was varied, and iii.) with serotype A, ionic strength was held constant and pH was varied.

Experiment 3. Results.

The finding that medium that enhances toxin binding paradoxically diminishes toxin potency has been extended to botulinum serotypes B, C, D, E and F, plus tetanus toxin. In all cases, controls were titrated so that paralysis times were about 120 minutes. When tissues were incubated with toxin at pH 6.0 and ionic strength 20mM, toxicity was invariably diminished (90% or more). Thus, the dissociation between conditions that promote tissue binding and conditions that promote tissue paralysis appeared to exist for all clostridial neurotoxins (though no type G is currently available to study).

In studies on type A neurotoxin, it was clear that ionic strength was the determinant that diminished toxicity. When pH was held constant at 6.0 and ionic strength varied, the tissues incubated in 20, 40 and 80 mM solutions were much less susceptible to poisoning. Conversely, when ionic strength was held constant at 20 mM and pH was varied from 6.0 to 7.5, all tissues were less susceptible to toxin.

Experiment 4. Rationale.

Of the various ways to explain the data above, two are of immediate concern. One possibility is that lowering ionic strength lowers the affinity of toxin for its authentic receptor (i.e., increases the K_d). An alternative possibility is that binding to the authentic receptor is abolished. The diminished toxicity that is observed is due

to toxin binding to an acceptor that only poorly mediates internalization and poisoning.

A number of experiments must be done to resolve this matter, but there is one study that may be suggestive. If lowering ionic strength merely increases the apparent K_d for the authentic receptor, then it should be possible to eliminate the difference in toxin potency between normal and abnormal preparations by raising toxin concentration. At a sufficiently high concentration, the toxin should be present at levels that meet or exceed the K_d for both the normal receptor and the "ionic strength-modified" receptor.

Experiment 4. Results.

Tissues were incubated with type A toxin for 16 minutes in physiological medium in pH 6.0 and ionic strength 20 mM medium. For different sets of matched control and experimental tissues, the concentration of toxin was varied. Interestingly, as the concentration increased, the disparity between control and experimental tissues diminished. However, at no concentration did the experimental tissues paralyze faster than the controls.

Mitochondrial Studies

A. Background

In the recent past, there have been conflicting reports on whether botulinum neurotoxin is capable of altering the energetics of nerve endings. In a series of papers, Dunant and his associates (Dunant et al., J.

Physiol., 385, 677, 1987; Dunant et al., J. Neurochem., 50, 431, 1988) have argued that botulinum neurotoxin can affect the levels of ATP and CrP in synaptosomal preparations. They have also reported that, under certain circumstances, the toxin can decrease tissue levels of creatinine kinase activity. This contrasts with the findings of Sanchez-Prieto et al. (Eur. J. Biochem. 165, 615, 1987), who report that the toxin does not affect the ratio of ADP to ATP, nor does it have any obvious effect on other measures of tissue energetics. The Principal Investigator has made an attempt to resolve this conflict, studying the effects of the toxin on isolated mitochondrial preparations.

B. Methods

The techniques for studying the effects of the toxin on neuromuscular transmission have been described previously. The only new method here is the isolation of mitochondria.

Brain and liver are excised from mice and homogenized in an isotonic sucrose solution maintained at 4°C. The homogenate is centrifuged an initial time (8,000 x g) to remove cell debris, then it is spun (25,000 x g) and washed several times to obtain a fraction that is enriched in mitochondria. The pellet is essentially free of soluble enzymes (i.e., LDH).

The mitochondrial preparation is maintained at room temperature, and it is either left untreated or it is exposed to a toxin. In addition to clostridial toxins, the

mitochondria are exposed to agents known to poison ATP generation (e.g., oligomycin). The formation of ATP is measured by a standard luciferinase assay. At the beginning of the assay, AMP, ADP and other precursors are added. After two minutes a luciferinase mixture is added and the emitted light signal is monitored for 60 seconds. Known amounts of ATP are run as standards.

C. Results

Mitochondria were isolated from both mouse brain and mouse liver. As expected, a known mitochondrial poison, oligomycin (100 $\mu\text{g/ml}$) poisoned ATP synthesis. Within 2 minutes of addition of the poison to the preparation, ATP generation had fallen more than 98% when compared to untreated controls.

A similar experiment was done with tetanus toxin (1×10^{-8} M), in which the toxin was incubated with the mitochondria for 90 minutes. At 10 minute intervals, aliquots of control and toxin-treated preparations were assayed for ATP synthesis. The results indicated that there was no alteration of ATP generation.

Similar types of studies were done with five clostridial toxin preparations, including serotypes A, B and C, as well as the heavy and light chains of type B. The proteins were added at concentrations ranging from 10^{-11} to 10^{-8} M. The duration of exposure was at least 120 minutes,

and in some cases 180 minutes. As before, oligomycin was used as a positive control.

The known mitochondrial poison markedly diminished ATP generation. Within the first collection period (10 minutes), synthesis of the nucleotide was reduced almost to zero. By contrast, none of the holotoxins or fragments had a measurable effect.

These results are at variance with those of Dunant et al. (e.g., J. Physiol. 385:677, 1987 and J. Neurochem. 50:431, 1988) but in keeping with those of Prieto et al. (Eur. J. Biochem. 165:615, 1987). Taking the findings in toto, the Principal Investigator believes that it is unlikely that botulinum neurotoxin acts directly to inhibit ATP synthesis. It might conceivably act by an indirect mechanism to achieve this, but the effect is unlikely to account for toxin-induced blockade of exocytosis.

4. Monoclonal Antibody Studies

A. Background

Monoclonal antibodies are being evaluated both as probes for SAR work and as possible therapeutic agents. During the past year, the major effort has been to evaluate monoclonal antibodies against botulinum neurotoxin type E.

B. Methods

All methods have been described previously.

C. Results

Type E neurotoxin was bioassayed on phrenic nerve-hemidiaphragms. The toxin was tested both before (unactivated) and after (activated) treatment with trypsin. The material showed a very large activation ratio. The toxicity increased about 100-fold after trypsin treatment.

The activated toxin was exposed to four monoclonal antibodies that are available. The concentration of toxin that was tested (1×10^{-11} M) produced paralysis in about 2 hours. The concentration of antibody has not been assayed yet, and therefore a presumed excess was tested. As Table 1 shows, three of the antibodies are quite effective in neutralizing the toxin.

Next, the monoclonal antibodies were mixed with toxin at various antigen:antibody ratios. As expected (based on in vivo assays), the E3 antibody did not diminish apparent toxicity at any ratio from 2:1 to 1:8. However, the other three antibodies were effective. The E14 preparation was most active, and E17 and E32 were of lesser activity (Table 1).

In the next set of experiments, the monoclonal antibodies were mixed to create a polyclonal solution. The antibodies were tested alone, in combinations of two, or as a combination of three. The results showed that the antibodies acted in an additive rather than a synergistic way to diminish toxicity (Table 1). It was interesting that the combination of E17 and E32 was no more effective than E14 acting alone. However, the combination of all three

antibodies had the greatest effect. Based on known toxicity data, one can calculate that the combination of three antibodies produced 99% or more inactivation of the toxin.

Experiments similar to those just described were done with the unactivated form of botulinum neurotoxin type E. The purpose was to determine whether the neutralizing antibodies recognized an epitope that was exposed in the trypsin-modified but not in the unmodified toxin. The results showed that when the antibody:antigen ratio with the unactivated material was the same as that with activated material, i.e., the outcome was the same.

In the next part of the work, the paradigm was changed. In the work above, the toxin was added to tissue baths at 37°C, with calcium and with nerve stimulation. This paradigm allows the toxin to bind, be internalized, and exert a poisoning effect. In the next set of experiments, tissues were incubated (60 minutes) at 4°C, with diminished calcium and no nerve stimulation. This allows the toxin to bind, but subsequent steps are arrested. After binding was complete, tissues were washed free of unbound toxin. Antibody was then incubated with tissues for an additional 60 minutes, after which the tissues were again washed and then transferred to baths at 37°C, with calcium and nerve stimulation.

The monoclonal antibodies were studied alone and in combination. The results with these antibodies were compared with those using a commercial (Lederle) polyclonal

Table 1

The Effects of Various Antibodies
on the Biological Activity of Botulinum Neurotoxin
Bound to Cholinergic Nerve Endings^a

Group	<u>Antibody</u> ^b				Paralysis Time ^c
	E14	E17	E32	PC	
1	-	-	-	-	131 ± 14
2	+	-	-	-	255 ± 22
3	-	+	-	-	161 ± 13
4	-	-	+	-	214 ± 18
5	-	-	-	+	> 300
6	+	+	+	-	> 300

^a Tissues were incubated with 1×10^{-11} M botulinum neurotoxin type E for 60 minutes at 4°C.

^b Antibody was added to tissues for 60 minutes at 4°C. Three monoclonal antibodies (E14, E17 and E32) and one commercial polyclonal preparation (PC) were tested.

^c Each value is the mean ± SEM of at least five observations.

Table 2

The Individual and Combined Effects of
Monoclonal Antibodies Directed Against
Botulinum Neurotoxin Type E^a

Group	Monoclonal Antibody ^b			Paralysis Time ^b
	E14	E17	E32	
1	-	-	-	127 ± 14
2	+	-	-	264 ± 21
3	-	+	-	165 ± 9
4	-	-	+	201 ± 13
5	+	+	-	300 ± 19
6	+	-	+	420 ± 16
7	-	+	+	255 ± 24
8	+	+	+	> 450

The final concentration of toxin added to tissues was 1×10^{-11} M.

Antibody was mixed with toxin at a 10-fold molar excess and allowed to incubate at 30°C for 30 minutes.

Each value is the mean ± SEM of at least five observations.

preparation. The latter has previously been shown by the author to diminish the potency of tissue-bound toxin, and this result has been reproduced here. Each of the neutralizing monoclonal antibodies also expressed an antagonistic effect. It is noteworthy that the activity of the monoclonal antibodies was almost the same irregardless of whether antibody was used prior to toxin binding or after binding. As expected, the combination of antibodies was more effective than any antibody alone.

Experiments were done to determine the binding properties that characterize the interaction between monoclonals and the toxin. For this purpose, the individual antibodies were submitted to gel permeation chromatography to determine their apparent molecular weight. Next, the toxin alone was also run for a molecular weight determination. Finally, the antibody-toxin complexes were chromatographed.

As expected, the average molecular weight of the antibodies was about 160,000 and that of the toxin was only slightly less. When the antibody and toxin were mixed prior to addition to the column, there was an interesting outcome. In theory, each antibody should have two antigen binding sites. However, the results from chromatography showed three species of antibody: i.) antibody not complexed to toxin, ii.) antibody with one associated antigen, and iii.) antibody with two antigens. The data suggest that for these particular monoclonals, the association constant for

antibody and antigen may be relatively low, i.e., the affinity may not be high.

In the next experiments, the toxin was mixed with combinations of two or three antibodies and then chromatographed. In this case there were again multiple species of antibody complexes. However, the species of antibody alone was virtually non-existent. The ratios of antibody to antigen ranged from 1 to 1 at the low end to 2 to 1 at the high end. One implication of this is that even when three antibodies were present, it was uncommon for more than two to be associated with a single antigen. This may have reflected steric hindrance.

In the last series of experiments, an attempt was made to determine whether any of the antibodies would cause "delayed intoxication" when assayed in vivo. This phenomenon was first observed with a neutralizing monoclonal against tetanus toxin. When the monoclonal was mixed with toxin at the appropriate ratio and then administered to animals, there was no immediate onset of illness. Only after 5 to 7 days did the signs of paralysis emerge.

The phenomenon of delayed intoxication was hypothesized to be due to the tight affinity between toxin and antitoxin. When the complex is injected, it stays associated for a long time, thus delaying the effects of free toxin. Only after dissociation of the complex can there be onset of symptoms.

The existence of this phenomenon was sought with antibodies E14, E17 and E32. The antibodies were mixed in

molar excess with 100 LD50 of the toxin at room temperature for 60 minutes. The mixture was then injected intravenously into mice. The time from injection to onset of symptoms was monitored in these animals and in a matched set of controls for which toxin had not been incubated with antibody. The intent was to monitor animals for up to 15 days.

The control animals developed onset of symptoms more rapidly than the experimentals, but all animals developed illness within 24 hours (Animals were not allowed to die of botulinum poisoning. When symptoms were clear, animals were euthanized with an anesthetic agent). However, no group showed delayed intoxication. This would appear to be a corollary of the earlier chromatography results. The antibody-antigen association is not tight, and thus the prospects for delayed intoxication are diminished.

5. Future Plans

The contract will terminate within several months. During the remaining time, the project will concentrate on studying toxin binding and putative drug antagonists.

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