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STUDY OF TOXIC AND ANTIGENIC STRUCTURES
OF BOTULINUM NEUROTOXINS

Annual Report

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Amino acid composition of botulinum neurotoxin (NT) types A and B were determined. The heavy and light chains of types A and B NT were separated, purified and analyzed for amino acid compositions and partial N-terminal sequence. The single chain type B and E NT were also partially sequenced. Role of tyr, his residues and carboxyl groups in toxicity and antigenicity were determined.		

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) 78-23, Revised 1978).

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I. Structure:

Until now botulinum neurotoxin types could not be defined in molecular terms. Some of these proteins are now precisely defined in terms of amino acid compositions and partial sequence.

Amino acid composition: We have analyzed the types A and B neurotoxin (NT) and thus have completed rigorous examinations of the amino acid compositions of types A, B, E and F (Work on types E and F were completed earlier).

Type A: The NT (mol. wt. 145,000) was purified in three separate batches (two batches were purified by the same method and one batch by a different method) and analyzed separately (Table 1). A manuscript on this study submitted to Toxicon for publication will appear in 1984.

Type B: The NT (mol. wt. 152,000) was purified in three separate batches (two batches were purified by the same method and one batch by a different method) and analyzed separately (Table 2). A manuscript on this study submitted to Toxicon for publication will appear in 1984.

Summary: 1) We found that each of the three NT types (A, B, and F) purified by two methods was comparable in purity and similar in amino acid compositions. Type E was purified by one available method. None of these NT types purified by two methods (developed independently in two laboratories) was previously compared under identical examination conditions. This observation, seemingly trivial, is indeed significant because the literature on botulinum NT includes claims of purification and characterization that are irreproducible and enigmatic.

11) The immunologically distinct botulinum NT types A, B, E and F are now defined in terms of amino acid composition. This allows, for the first time, a chemical basis to compare the NT types (Table 3). Similarity between types A and E is closer than between any other two types. Five amino acids do not differ by more than 1 residue; e.g. number of residues in Type A/Type E are Thr 75/75; Pro 44/45; Tyr 71/70; His 14/15; Trp 17/16. The next best resemblance by this criteria is between types A and B; types A and F; as well as types E and C. In each pair 3 amino acids do not differ by more than 1 residue.

Heavy chain (mol. wt. 97,000) of Type A: This has been analyzed (24,48,72 hr HCl hydrolysis, cysteic acid and tryptophan determination) for its amino acid composition.

Table 1

Number of Amino Acid Residues per Type A Botulinum Neurotoxin
(Mol. Wt. = 145,000)

	<u>Batch 1</u>	<u>Batch 3</u>	<u>Batch 7</u>	<u>Mean of three</u>	<u>Best of three</u>
Aspartic acid	199	186	201	195	200
Threonine	74	76	70	73	75
Serine	80	87	78	82	79
Glutamic acid	116	112	114	114	114
Proline	39	46	43	43	44
Glycine	65	68	63	65	64
Alanine	54	52	53	53	53
Valine	71	69	65	68	70
Half-cystine	10	10	10	10	10
Methionine	22	22	22	22	22
Isoleucine	105	113	110	109	111
Leucine	106	103	114	108	104
Tyrosin	75	71	71	72	71
Phenylalanine	68	69	68	68	68
Lysine	96	100	100	99	100
Histidine	11	14	14	13	14
Arginine	44	46	43	44	43
Tryptophan	18	17	17	17	17

Each batch of neurotoxin was acid hydrolyzed for 24, 48, and 72 hr.

Maximum deviation of number of residues from mean:

<±3%	Glu, Ala, CyS, Met, Phe, Lys
>± but ≤5%	Asp, Thr, Gly, Val, Ile, Tyr, Arg
>±5%	Ser, Pro, Leu, His, Trp

(error on Durrum D-500 analyzer is ±3%)

Table 2

Number of Amino Acid Residues per Type B Botulinum Neurotoxin
(Mol. Wt. = 152,000)

	<u>Batch 1</u>	<u>Batch 2</u>	<u>Batch 3</u>	<u>Mean of three</u>	<u>Best of three</u>
Aspartic acid	204	212	213	210	212
Threonine	54	53	54	54	54
Serine	82	77	84	81	83
Glutamic acid	129	130	135	131	130
Proline	46	46	43	45	46
Glycine	61	56	62	60	61
Alanine	46	44	44	45	44
Valine	54	56	52	54	54
Half-cystine	11	11	11	11	11
Methionine	23	23	23	23	23
Isoleucine	145	144	138	142	144
Leucine	107	108	97	104	107
Tyrosin	81	81	82	81	81
Phenylalanine	77	77	78	77	77
Lysine	118	117	118	118	118
Histidine	8	7	7	7	7
Arginine	39	39	39	39	39
Tryptophan	18	17	19	18	18

Each batch of neurotoxin was acid hydrolyzed for 24, 48, and 72 hr.

Maximum deviation of number of residues from mean:

<+3%	Asp, Thr, Ala, CyS, Met, Ile, Tyr, Phe, Lys, Arg
>+ but ≤5%	Ser, Glu, Pro, Val
>+5%	Gly, Leu, His, Trp

(error on Durrum D-500 analyzer is ±3%)

Table 3

Comparison of Amino Acid Composition of Botulinum Neurotoxin Types
(Number of Residues/Neurotoxin Molecule)

Mol. wt.:	<u>A</u> <u>145,000</u>	<u>B</u> <u>152,000</u>	<u>E</u> <u>147,000</u>	<u>F</u> <u>155,000</u>	<u>C⁺</u> <u>141,000</u>
Aspartic acid	200	212	240	218	225
Threonine	75	54	75	80	78
Serine	79	83	98	105	88
Glutamic acid	114	130	118	128	116
Proline	44	46	45	47	41
Glycine	64	61	58	69	59
Alanine	53	44	40	47	44
Valine	70	54	62	72	65
Half-cystine	10	11	7	9	6
Methionine	22	23	17	14	16
Isoleucine	111	144	123	128	114
Leucine	104	107	107	104	91
Tyrosin	71	81	70	86	71
Phenylalanine	68	77	62	60	70
Lysine	100	118	97	90	78
Histidine	14	7	15	13	10
Arginine	43	39	34	51	47
Tryptophan	17	18	16	23	11
Number of batches analyzed	3	3	3	3	2

⁺Type C data from Syuto and Kubo (J. Biol. Chem. 256, 3712, 1981).

Light chain (mol. wt. 53,000) of Type A: This has been analyzed (24,48,72 hr HCl hydrolysis, cysteic acid and tryptophan determination) for its amino acid composition.

Heavy chain (mol. wt. 104,000) of Type B: This has been hydrolyzed (24,48 and 72 hr HCl) and is ready for amino acid analysis.

Light chain (mol. wt. 51,000) of Type B: This has been hydrolyzed (24,48 and 72 hr HCl) and is ready for amino acid analysis.

Summary: i) Knowledge of amino acid composition will enable us to plan enzymatic and chemical fragmentation of these subunit chains (e.g. number of Lys and Arg for tryptic cleavage, Met for CNBr cleavage). ii) Quantitative comparison of the amino acid composition by the technique of Cornish-Bowden [J. Theor. Biol. 76, 369, (1979) and Methods Enzymol. 91, 60 (1983)], would allow us to see what the chances are that similarities in the composition reflect similarities in sequence. Such analysis has revealed a very intriguing sequence homology between the L- and H-chains of tetanus toxin [Taylor, C. F. et al. Biochem. J. 209, 897 (1983)].

Amino acid sequence:

Type E: The sequence determination of Type E (single chain, 150,000 mol. wt.) NT has been extended to the first 13 residues (#1-4 were determined before), see Fig. 1.

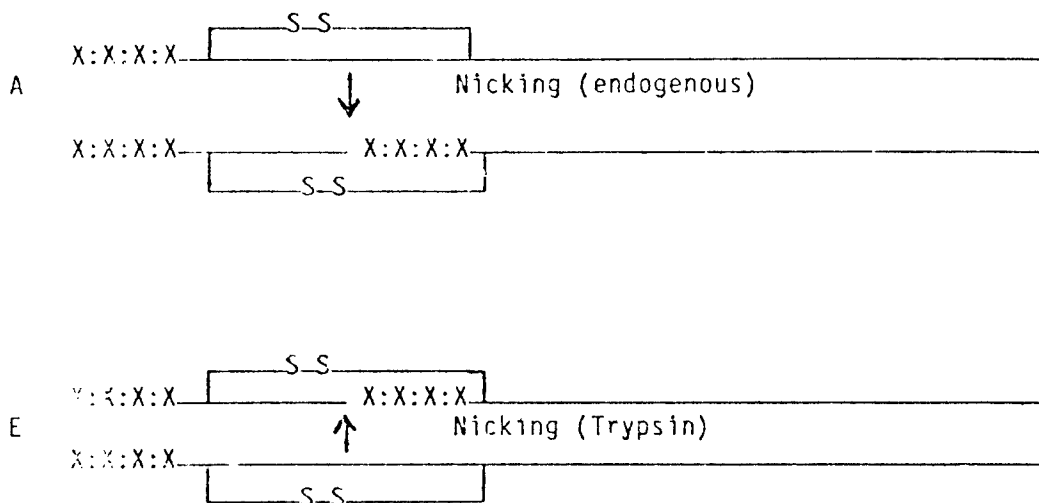
Type B: The first 12-13 residues of the N-terminal of the single chain Type B (mol. wt. 152,000) and its subunits the L- and H-chains (mol. wt. 51,000 and 104,000, respectively) are now known, see Fig. 1.

Summary: In Fig. 1 alignment of the N-terminal end of the L-chains of types A, B, and the single chain types B and E shows some homology and heterology; the H chains of types A and B are homologous only at residue #1 and 10 (Ala, Asn). This allows for the first time, at this molecular level, comparison of the immunologically distinct and pharmacologically similar NT types A, B and E. The following conclusions can be made:

- i) L chain of type B is the N-terminal of the single chain.
- ii) In the type E dichain (produced by trypsin cleavage of E single chain), its L-chain component would be at the N-terminal of the parent E single chain molecule.
- iii) L chain of type A is expected to be the N-terminal of the single chain type A.

The first amino acid sequence work was accomplished based on a collaborative study with an USAMRIID investigator Dr. James J. Schmidt. We, at Madison, separated and purified the subunits (the H- and L-chains) then Dr. Schmidt, using the automatic sequencer at the USAMRIID, sequenced the polypeptides.

Figure 1



Formation of light chain (left of the arrow) and the heavy chain (right of the arrow) from the single chain type A and E neurotoxins. The model is the same for type B. Hatched portions are targets of amino acid sequence.

PARTIAL SEQUENCE OF BOTULINUM NEUROTOXIN TYPES

	1	5	10	15
A Light chain	Pro	Phe-Val-Asn-Lys-Gln	Phe-Asn-Tyr-Lys-Asp-Pro-Val	Asn-Gly-
B Light chain	Pro	Val-Thr-Ile-Asn-Asn-Phe-Asn-Tyr-Asn-Asp-Pro-Ile-		
B Single chain	Pro	Val-Thr-Ile-Asn-Asn-Phe-Asn-Tyr-Asn-Asp-Pro-Ile-		
E Single chain	Pro	-----Lys-Ile-Asn-Ser-Pne-Asn-Tyr-Asn-Asp- ? ? -Asn-		
	1	5	10	
A Heavy chain	Ala	Leu-Asn-Asp-Leu-Cys-Ile-Lys-Val	Asn	
B Heavy chain	Ala	Pro-Gly-Ile-Cys-Ile-Asp-Val-Asp	Asn	Glu-Asp-

Note: the homologous sequences are marked within the blocks.

Separation and isolation of subunit chains: We have developed dependable ion exchange chromatographic procedures to isolate the heavy and light chains of type B and E NT. The isolated chains appear pure in SDS-polyacrylamide gel electrophoresis. Two major problems that had to be solved were: i) Type E NT (single chain) is first converted to the dichain form by cleaving a single peptide bond with trypsin. During this step secondary cleavages produce, in minor quantity, fragments other than the H and L chains; these had to be removed. ii) Type B NT is isolated as a mixture of dichain (made of the H- and L-chains) and its precursor, the single chain molecule. A method had to be developed to isolate the two subunit chains from the single chain molecule.

II. Structure-function relationship:

- i) Studies on the role of tyrosine residues and carboxyl groups in the antigenicity and toxicity have progressed. Specific modification of tyrosine residues with p-nitrobenzenesulfonylfluoride detoxified type E without damaging its serological reactivity. Carbodiimide modification of carboxyl groups of types A and E did not change their serological reactivity; while the toxicity (quantification is preliminary) of type A was slightly damaged, type E was significantly detoxified. This preliminary data indicates that toxicity of type A is more refractory to the effect of modification of carboxyl groups than type E. Similar observations were made following modification of lysine and histidine residues of types A and E NT.
- ii) First phase of the studies on the role of histidine residues in the toxicity and antigenicity of types A and E NT was completed. Histidine residues of type E NT that are critical for toxicity are not important for serological activity and immunogenicity. In type A NT the situation is somewhat different; modification of histidine residues did not completely detoxify the NT, but produced some damage in its serological reactivity. The completely detoxified type E NT was used as an immunogen. Rabbits immunized with the toxoid (second generation immunogen, i.e., pure NT detoxified by selective modification of one kind of amino acid residues) produced antiserum that neutralized the toxin.

Publications from Research Supported by Contract DAMD17-80-C-0100

1. DasGupta, B. R. and Rasmussen, S. Role of amino groups in the structure and biological activity of botulinum neurotoxin type A and E. *Biochem. Biophys. Res. Commun.* 101, 1209 (1981).
2. DasGupta, B. R. "Structure and structure function relation of botulinum neurotoxins" in *Biomedical Aspects of Botulism* (ed. G. E. Lewis). pp. 1-19, Academic Press, N.Y. (1981).
3. DasGupta, B. R. "Microbial food toxicants: *Clostridium botulinum* toxins," in *CRC Handbook of Foodborne Diseases of Biological Origin* (ed. M. Recheigl, Jr.). pp. 25-55. CRC Press, Inc., Boca Raton, Fla. (1983).
4. DasGupta, B. R. and Rasmussen, S. Purification and amino acid composition of *C. botulinum* type E neurotoxin. *Toxicon* 21, 535 (1983).
5. DasGupta, B. R. and Rasmussen, S. Amino acid composition of type F botulinum neurotoxins. *Toxicon* 21, 566 (1983).
6. Simpson, L. L. and DasGupta, B. R. Botulinum neurotoxin type E: Studies on mechanism of action and on structure-activity relationships. *J. Pharmacol. Exptl. Therap.* 224, 135 (1983).
7. Sellin, L. C., Kauffman, J. A. and DasGupta, B. R. Comparison of the neuromuscular effect of botulinum neurotoxin types A and E. *Medical Biol.* 61, 120 (1983).
8. Sellin, L. C., Thesleff, S. and DasGupta, B. R. Different effects of types A and B botulinum toxin on transmitter release at the neuromuscular junction. *Acta Physiol. Scand.* 119, 127 (1983).
9. DasGupta, B. R. and Rasmussen, S. Role of histidine residues in the biological activities of botulinum neurotoxin. Abstract for American Soc. Microbiol. Annual Meeting p. 28 (1983).
10. Schmidt, J. J., Sathyamoorthy, V., Rasmussen, S. and Das Gupta, B. R. Partial sequence of botulinum neurotoxin. *Fed. Proc.* 42, 1811 (1983).
11. DasGupta, B. R. and Woody, M. A. Amino acid composition of *Clostridium botulinum* type B neurotoxin. *Toxicon*, 22, 312 (1984).
12. DasGupta, B. R. and Sathyamoorthy, V. Purification and amino acid composition of type A botulinum neurotoxin. *Toxicon*, 22, 415 (1984).
13. DasGupta, B. R. and Rasmussen, S. Effects of diethylpyrocarbonate on the biological activities of botulinum neurotoxin types A and E. *Arch. Biochem. and Biophys.* 232, 172 (1984).
14. Schmidt, J. J., Sathyamoorthy, V. and DasGupta, B. R. Partial amino acid sequence of the heavy and light chains of type A botulinum neurotoxin. *Biochem. Biophys. Res. Commun.* 119, 900 (1984).

15. Schmidt, J. J., Sathyamoorthy, V. and DasGupta, B. R. Partial sequence of botulinum neurotoxin type B. Fed. Proc. 43, 1954 (1984).
16. Sathyamoorthy, V., Schmidt, J. J. and DasGupta, B. R. Partial sequence of botulinum neurotoxin type E; comparison with types A and B. Fed. Proc. 43, 1954 (1984).

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