Biomedical Aspects of Botulism

EDITED BY

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BIOMEDICAL ASPECTS OF BOTULISM

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PREFACE

During the past five years there have been numerous developments, often in diverse and unrelated scientific disciplines, which have contributed substantially to both our understanding and awareness of botulism. The structure, actions, and effects of botulinal toxins have been described in much greater detail than ever before; methods for the laboratory identification, diagnosis, and conformation of botulism have improved considerably; *Clostridium botulinum* type G, once thought to be of little more than academic interest, has been recently associated with five sudden and unexpected human deaths; new chemotherapeutic approaches to the treatment of botulism have been explored in both laboratory animal models and in human patients; and probably the most significant of all recent developments have been the description and wide recognition of a toxicoinfectious form of botulism in infants. Thus, the time seemed appropriate to provide a forum for scientists and clinicians from all concerned disciplines to present, exchange, and discuss recent advances in our understanding of the biomedical aspects of botulism.

This volume is based on the proceedings of the first international conference devoted exclusively to the biomedical aspects of botulism. The text represents a melding of basic and applied works gathered from the disciplines of biochemistry, pharmacology, neurophysiology, microbiology, epidemiology, pathology, toxinology, and clinical medicine for the purpose of offering a complete and current reference text on botulism.

Section I provides the most complete and up-to-date information available on the structure, and structure–function relationships and oral toxicities of the various botulinal toxins. In Section II the current thinking about the cellular and subcellular effects of botulinal toxin are summarized along with the presentation of a model to account for toxin-induced blockage of transmitter release and a discussion of new approaches for dealing with and utilizing the botulinal toxins. The pre- and postsynaptic effects of botulinal toxin are examined in depth. The existence of two kinds of vesicular (quantal) transmitter release are proposed and the long-term alterations in the postsynaptic muscular membranes of botulinal toxin-poisoned preparations are examined.

Detailed evidence for the involvement of specific bacteriophages in the toxigenicity of *C. botulinum* types C and D is presented in the first two chapters of

Section III. Also included in this section is a discussion of the optimal fermentor conditions for toxin production by *C. botulinum* types A, B , and E.

Section IV provides the reader with a fascinating comparison of the many properties shared by, and the qualitative effects of, tetanus and botulinal toxins. Next the impressive use of botulinal toxin, "the most lethal substance known," to treat strabismus and thus employ this toxin to serve mankind is described. Data are presented which serve as the basis for understanding the etiology of infant botulism. The critical role of age of the host and the population of intestinal organisms in determining whether the gut of infant laboratory animal models is colonized by *C. botulinum* is convincingly presented in the last portion of this section.

Section V presents a compilation of available information on the laboratory investigation of human and animal botulism, to include infant botulism and shaker foal syndrome. The development of practical isolation and identification procedures are then reviewed in depth. The extremely thorough laboratory procedures utilized to document the association of clostridial organisms and toxins with sudden and/or unexpected human death are described in detail.

A review of selected aspects of the development of toxoids and an insight into the anticipated development of new bacterial products serve to introduce Section VI. A new generation of botulinal toxoids is initiated by the presentation of methodologies for the production, purification, and toxoiding of botulinal toxins. Recent clinical and serological data are presented from an evaluation of experimental botulinal toxoids in both laboratory animals and volunteers. An overview of approaches to prophylaxis, immunotherapy, and chemotherapy of botulism concludes this section.

Section VII begins with a comprehensive 30-year review of the epidemiologic characteristics of botulism in the United States, proceeds to a presentation of the epidemiologic and clinical findings of arctic botulism in Alaska, and continues with the detailing of botulism as it occurs in Argentina. The final chapter investigates the necropsy diagnosis of botulism in twelve patients who died unexpectedly and compares the epidemiology and pathology of these cases.

The text concludes with Section VIII in which the current trends in therapy of botulism are reviewed and two very detailed discussions on infant botulism are presented. The first chapter on infant botulism describes the pathogenesis and clinical aspects while the second chapter reviews controversies in management and treatment.

This volume should serve as a valuable reference to anyone disciplined in the fields of bacteriology, biochemistry, immunology, neurophysiology, pathology, pharmacology, and toxinology, as well as to both physicians and veterinarians interested in a single source for obtaining current information on the biomedical aspects of botulism.

I wish to thank all those who have contributed to this volume and to the very successful conference from which it was derived. Without the enthusiasm and responsible support of the staff of the U. S. Army Medical Research Institute of Infectious Diseases, these events would not have occurred.

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STRUCTURE AND STRUCTURE FUNCTION RELATION OF BOTULINUM NEUROTOXINS

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This presentation has an ambitious title about a protein made of nearly 1,500 amino acid residues. The report on our current work, done with the help of Andrea Nicholas and Stuart Rasmussen, is on a few amino acid residues of two kinds. The presentation will show how much or how little we know about the structure and structure-function relationship of botulinum neurotoxin 35 years after its initial purification (see refs. in 1). It is appropriate that the conference on Biomedical Aspects of Botulism is being held at Fort Detrick where the pioneering work on botulinum neurotoxin was initiated. The difficulty of studying the botulinum neurotoxin has been reduced, from those pioneering days, by a factor of six. As late as 1965 the most well characterized botulinum toxin was thought to be of mol. wt. 900,000 (2). Now we all recognize that the size of the neurotoxin is 1/6th of that huge size (2,3).

This presentation has four parts:
I. General considerations of the botulinum neurotoxin types; II. Comparison of types A and B neurotoxins and location of three 1/2 cystine residues in these two proteins; III. Study of types A and E neurotoxins by selective modification of amino groups; and IV. Discussion of activation and nicking based on some old and new data.

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I. GENERAL CONSIDERATIONS OF THE BOTULINUM NEUROTOXIN TYPES

The published information, from various laboratories, about the neurotoxin types are summarized in Fig. 1. A, B, C, D, E, and F represent antigenically distinguishable types. One to 4 days and 4-7 days represent the number of days the bacterial cultures were incubated before neurotoxin purification steps were initiated. Note that in type A the early culture has two molecular forms (4); the heavy solid line represents the single chain polypeptide. Next to it is the molecule made of two-chains, connected by a dashed line

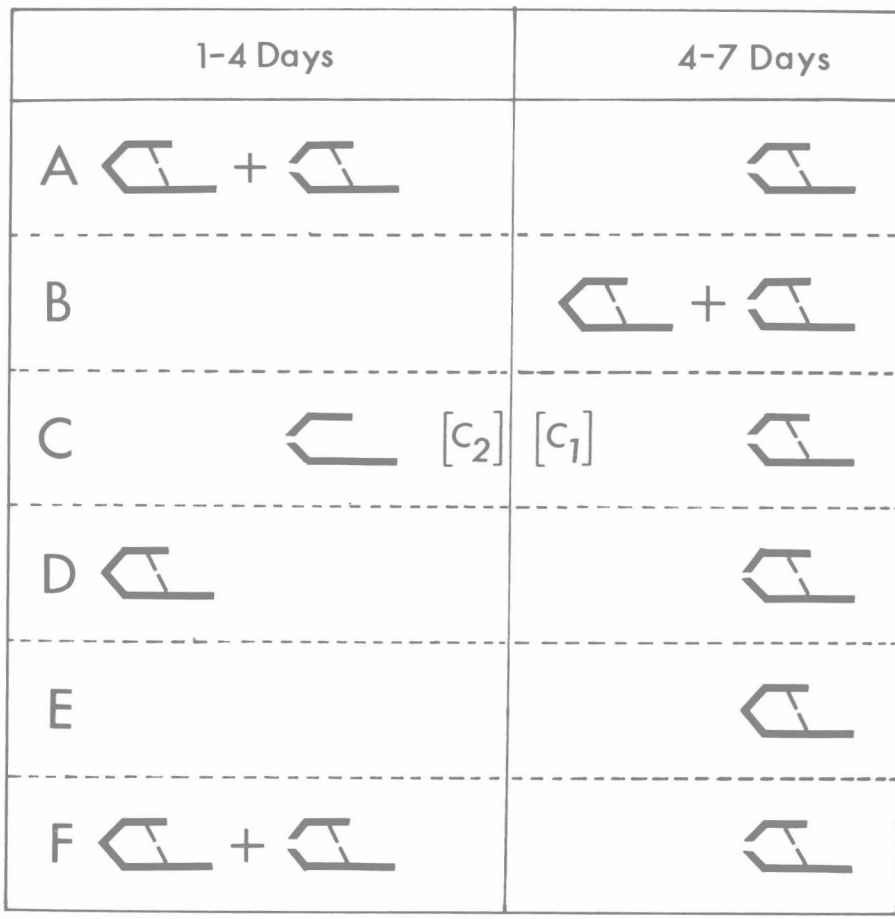

Fig. 1. The neurotoxin polypeptide chains derived from the bacterial cultures.

which represents a disulfide bond. In the late culture only the two-chain structure is found (2,3). Exactly the same situation was found in type F cultures, i.e., two forms of neurotoxins in the early culture and only one form in the late culture (4,5). Types C (6,7) and D (8) in the late culture were found to have only the two-chain structure. Three-day-old cultures of type D yields single chain neurotoxin (9). All of these suggest that a proteolytic enzyme present in the culture clips a peptide bond of the single chain molecule, thus producing a dichain molecule. Type E cultures always yield single chain neurotoxin, the culture apparently lacks the enzyme that converts the single chain to the dichain form (2,3). The case of type B is a surprise. Studies in our (10) and Dr. Sakaguchi's (11) laboratories have independently demonstrated that late cultures contain mixtures of single and dichain neurotoxins. Presence of the dichain molecule suggests that the enzyme was present to produce it, but why is conversion of single chain to dichain incomplete? We don't know. Another important point in this illustration is that in each case the two chains are connected by a disulfide bond. The exception is C_2. The two chains are not connected by a disulfide bond (7). The structure with a disulfide bond in the 4 to 7 days column is C_1 (6).

The generalized structure of the neurotoxins, with some other features is presented in Fig. 2. Molecular weights of the two forms (i.e. the single chain and the dichain molecules) of any particular type are indistinguishable, they are ∿150,000 (2,9). The smaller chain is of mol. wt. ∿50,000, it is called light chain or fragment I. The bigger chain is of mol. wt. ∿100,000, it is called the heavy chain or fragment II. The two chains are connected by at least one disulfide bond (2,9), the exception is the type C_2 (7).

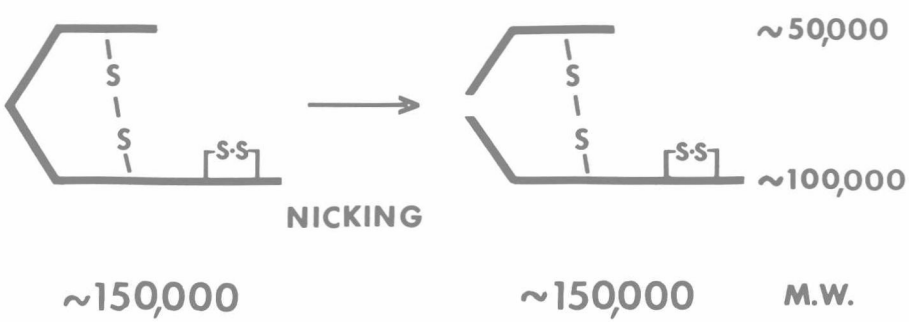

Fig. 2. Generalized structure of botulinum neurotoxin.

Another disulfide loop at one end of the heavy chain was found in types A and B (12), other types have not been examined yet. Size of type A neurotoxin, in terms of Stokes radius is 48Å; the technique used was gel filtration (13). For type E, a similar size, 90-100 Å diameter, was found by electron microscopy (14). The 26% helix, 32% of β-sheet and 44% of turns for type B were calculated by myself (following the technique in ref. 15) based on the amino acid composition (16). The Q_{10} of 2.36 and activation energy for neurotoxin-induced muscle paralysis of 1.55×10^4 cal/mol were determined by Simpson (17).

Conversion of the single chain molecule to the dichain form is called nicking. Nicking can be achieved with trypsin. The dichain molecules produced naturally, i.e. in the bacterial culture, and produced artificially, i.e. with trypsin, are indistinguishable (2).

Separation of the light and heavy chain of types B and C_1 neurotoxins after reduction of the disulfide bond have been reported (18,19). In the case of C_2 the two chains were purified from the culture as separate entities (7). The individual chains are not toxic; but when the two chains are brought together, the combination becomes toxic (7). This means that *in vivo* toxicity depends on the cooperative action of the complementary chains. The separated chains of type B and C_1 neurotoxin are also nontoxic; the reconstituted neurotoxins, after combining the constituent chains, are toxic (18,19). The separated H and L chains of type B (18), C_1 (19) and C_2 (7) were found to be distinct antigens.

II. COMPARISON OF TYPES A AND B NEUROTOXINS

So far we have looked at the neurotoxin types at a gross level. Let us now see what we can find at a higher resolution. For more precise comparison two neurotoxin types, i.e. A and B and their respective heavy and light chains, were studied. The two neurotoxins were mixed and then subjected to coelectrophoresis in polyacrylamide gels containing SDS. The results are presented in Fig. 3. The light chain of type A is of mol. wt. 53,000 and thus slightly larger than type B. The heavy chain of type B is of mol. wt. 104,000 and slightly larger than the heavy chain of type A. The technique used to detect these small differences has been published (20). The lengths of the straight lines are drawn according to the scale of mol. wt. The vertical arrow is the site of nicking. When A and B polypeptide chains are

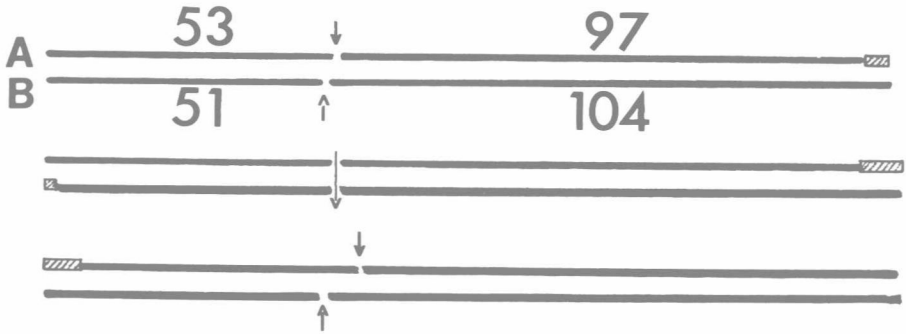

Fig. 3. Comparison of the polypeptide chains of types A and B neurotoxins.

paired in three different ways, at least three implications become apparent: i) In the top pair when the left ends of the two are aligned a small portion of type A, indicated by the hatched area, at the right, appears to be missing and the site of nicking on the two types appears at different positions. ii) In the middle pair when the nicking sites of the two are aligned we see that portions of type A and B are missing - represented by the hatched areas. iii) In the bottom pair, alignment at the right end shows that portion of type A is missing and the sites of nicking on the two types are different. The conjectural missing portions in these proteins could originate at the translational step and/or result from post-translational modification due to proteolytic degradation.

Let me now present some new and unpublished structural studies based on chemical cleavage of these two neurotoxin types. Half-cystine residues were chosen as sites of cleavage because there are only a few of them; 7 in type A and 11 in type B (see ref. 16 and 17 in 2). Hence the expected number of fragments would be 8 in A and 12 in B. The reaction mechanism (21) is presented in Fig. 4. The protein is incubated with the reagent 2-nitro-5-thiocyanobenzoate at pH 8.0 for 15 min at 37°C. This step converts the cysteine residues to S-cyanocysteine. In the next step at pH 9 for 12 hr at 37°C, cleavage of the amino peptide bond of the S-cyanocysteine residue is obtained. The newly formed N-terminal is blocked by a thiazolidine ring. The reaction products are then analyzed by polyacrylamide gel electrophoresis in the presence of SDS, to separate the fragments and to determine the mol. wt. of the fragments (22).