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PRESYNAPTIC AND POSTSYNAPTIC NEUROTOXINS. INVESTIGATION OF THE STRUCTURES OF THE IMMUNE RECOGNITION SECTIONS

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This paper discusses literature reports over the last 12 years and the results of our own experimental investigations of the structures of the immune recognition regions of protein antigens — in particular presynaptic and postsynaptic neurotoxins from spider and snake venoms.

Of the more than 3000 species of spiders [1] and about 2340 species of snakes [2] living in the world, hundreds are poisonous. The organic parts of the venoms of these animals contain protein components with various molecular masses that consist mainly of enzymes and toxins. The roles of the majority of these enzymes remain unelucidated, while the toxins are the active principles of the venoms, which is expressed in their binding to elements of the presynaptic or postsynaptic membranes. The toxins binding to presynaptic membranes are classified into three groups: 1) those directed to the channels and neuromuscular receptors; 2) blockers of neuromuscular excitation; and 3) so-called "pure" presynaptic stimulatory toxins acting on the plasmatic membranes of nerve endings. The first group includes toxins active not only in the presynaptic compartment but also in the neurons. Some of them — for example, β-bungarotoxin — besides affecting the channels and receptors, act as proteolytic or lipolytic enzymes modifying the structures of the membranes near their specific binding sections [3]. On the other hand, the neurotoxins of the second group, blockers of neuromediator excitation, such as botulin and tetanus toxin, are highly specific. The active subunits of these toxins are directed through the plasmatic membrane to the cytoplasm of the nerve endings, where they block the release of the mediator by an as yet unexplained mechanism. The third group, of "pure" presynaptic toxins, such as latrotoxin, act on the plasmatic membranes of nerve endings, promoting the generation of interterminal signals, which in the final account leads to a massive stimulation of the release of the neuromediator [4].

Postsynaptic neurotoxins binding to nicotinic acetylcholine receptors are defined as α-neurotoxins. α-Neurotoxin molecules, which contain from 65 to 74 amino acid residues and 5 intramolecular disulfide bonds (long neurotoxins) or 60-62 residues with 4 disulfide bonds (short neurotoxins), bind specifically to nicotinic acetylcholine receptors in postsynaptic membranes and block the channel-opening function of cholinergic ligands. To determine the interrelationship between these activities requires an all-sided investigation of the structural and conformational, and also the immunochemical, features of the binding regions in the toxin and receptor molecules.

Since the determination of the primary structure of an α-neurotoxin (toxin α from the cobra *Naja nigrocollis*) [5], a fairly large number of reports have been published in which the relationship between the structure and functions of neurotoxins is considered [6-10]. On the other hand, functional investigations of the acetylcholine receptor, the α-subunit of which is responsible for binding acetylcholine [11], and of neurotoxins [12] has been completed by a determination of the binding sections in the receptor for long [13, 14] and short [15] neurotoxins, and also by the identification of the antibody-binding sections of its molecule [16]. The results of these investigations have enriched our understanding of the molecular mechanism

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Abbreviations: ACR) acetylcholine receptor; LT) α-latrotoxin; NT II) neurotoxin II; BTX) α-bungarotoxin; BSA) bovine serum albumin; PBS) 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.2; *PI*) protection index: the ratio of LD_{50} for BTX to LD_{50} for a control.

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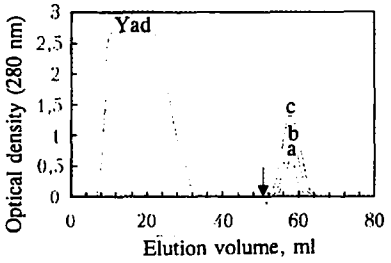

The beginning of elution is shown by an arrow

Fig. 1. Affinity chromatography of α-latrotoxin on three different antibody immunosorbents. Column 2.0 × 3.0 cm. Sorbent: *a*) antibody–cellulose; *b*) antibody–protein A–cellulose; *c*) F_{ab}–cellulose. Eluent 0.01 M HCl, pH 2.2.

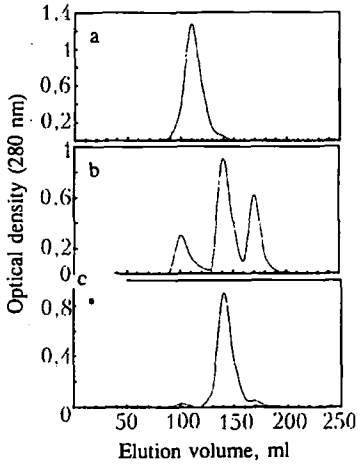

Fig. 2. Stages in the monitoring of the purification of the $F(ab)_2$ fragments of anti-LT antibodies: *a*) gel chromatography of antibodies to LT on a TSK HW-50 column; *b*) gel chromatography of a peptic hydrolysate of these antibodies under the same conditions; *c*) gel chromatography of $F(ab)_2$ fragments of anti-LT antibodies under the same conditions.

of the interaction of a toxin with a receptor. This interaction may be positively inhibited by antibodies, the specific action of which is directed to the antigenic sections of the receptor or the toxin molecule.

In addition to its fundamental significance, the fight against the intoxication of men and animals by bites of poisonous animals remains an important problem. The most effective means for it are specific sera and the vaccination of men and animals in foci of the mass distribution of poisonous animals.

In the present paper we discuss literature information and our own results on the production of highly effective sera, the study of antigen–antibody interaction, and the investigation of the structures of the antigenic determinant sections of neurotoxins with the aim of finding a new approach to obtaining synthetic peptide vaccines.

THE EPITOPE AND ITS DETERMINATION

The antigenicity of a protein, determining the possibility of its specific binding to the active sections (paratopes) of immunoglobulins, is determined by the antigenic determinants (epitopes) of the protein molecule. An epitope is a region of a protein that is recognized by specific antibodies or receptors of definite subclasses of lymphocytes [17, 18]. In the initial stages of the development of immunochemistry antigenic sections were classified into sequential and conformational types and were

TABLE 1. Amino Acid and N-Terminal Analyses of the Peptides of a Tryptic Hydrolysate of CM-Neurotoxin II Containing the Antigenic Determinant Parts of NT II

Amino acid	Results of analysis	T_1	T_2	T_6	T_7	Total
Asx	4.06	1	1		2	4
Thr*	3.22	2	2			4
Ser*	3.11	2	1			3
Glx	5.57	4	1			5
Pro	4.12	2		1	1	4
Gly	4.44		1	2	1	4
Cys†	6.39	1	2	2	2	7
Val	1.63				2	2
Leu	1.60	1			1	2
Tyr	1.00		1			1
His	1.06	1				1
Lys	3.84	1	1	1	1	4
Arg	1.02				1	1
		15	10	6	11	42
N-Terminal		Leu	Thr	Gly	Val	

*In view of degradation during hydrolysis, the results have been corrected to the initial time of hydrolysis.
†Determined after oxidation to cysteic acid.

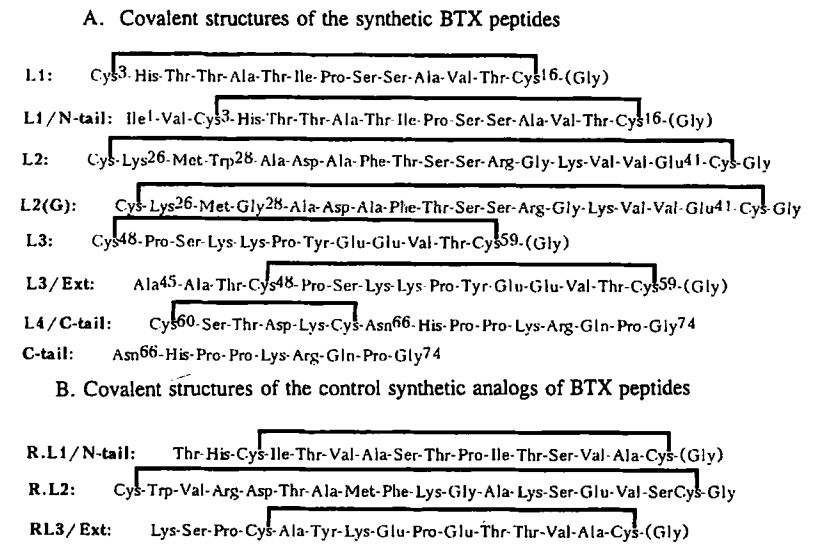

Fig. 3. Structures of the synthetic α-bungarotoxin peptides (*A*) and of three control peptides having similar amino acid compositions to the peptides L1/N-tail, L2, and L3 but with randomized amino acid sequences (*B*).

identified on the basis of amino acid sequences with no account of conformational feature or with consideration of specific conformations capable of binding with paratopes [19].

Subsequently, a classification was proposed that took into account differences between continuous and discontinuous epitopes [20]. In a continuous epitope the amino acid residues forming the components of the region are bound directly with one another by peptide bonds, while in a discontinuous epitope, also known as a topographic epitope, the amino acid residues of the region are not necessarily bound in sequence but may be present in immediate propinquity as the result of a folding of the polypeptide or of certain individual polypeptide chains [21, 22]. The protein molecule may contain both continuous epitopes (for example, myoglobin [17], hemoglobin [23, 24], and the hemagglutinin of the influenza virus [25]) and discontinuous epi-

topes (lysozyme [25]) or both types simultaneously (bovine serum albumin [26]). Sensitivity of these epitopes to conformational changes in the protein molecule has been observed repeatedly [21, 26].

Various methods taking into account structural or functional features of the molecule are used for the determination of epitopes in proteins. In the structural approach, the spatial arrangement of the atoms participating in the formation of the antigen–antibody complex is considered, and the participation of at least 15 amino acid residues in the epitope is established. The involvement of a smaller number of residues in each epitope has been proved in the functional approach. The structures of continuous and discontinuous epitopes of proteins can be investigated by surface-simulation synthesis, in which peptides minimizing (this term denotes a synthetic peptide that is a spatial analog of a discrete section of a protein molecule [17]) definite sections of the molecule are synthesized chemically. This approach was first used for determining the structure of the discontinuous epitope of lysozyme [27, 28].

For such investigations it is also possible to use an all-sided synthetic strategy that requires the synthesis of a series of mutually overlapping peptides identifying the whole sequence of the protein chain from beginning to end and determining their immunochemical activities [29]. The peptides are identical in size, and each of them overlaps with its immediate neighbor by the same number of amino acid residues (usually five). The sizes of the peptides and of the overlaps are determined in such a way that the synthetic load is optimized and the binding sections are reliably established. This method has been used for localizing the functional sections of many important biological macromolecules, such as hemoglobin, myoglobin, lysozyme, α-neurotoxins, and the acetylcholine receptor [30-34].

IMMUNOCHEMICAL INVESTIGATIONS OF A-LATROTOXIN

The neurotoxins of the venom of the spider *Latrodectus tredecimguttatus* have been studied widely [3, 35]. The main neurotoxin and the active principle of the venom is α-latrotoxin (LT), MM ~ 130 kDa, consisting of a single polypeptide chain with five intramolecular disulfide bonds. This neurotoxin has the isoelectric point pI 5.2, which is characteristic for weakly acidic proteins, and acts on the plasmatic membranes of the nerve endings, promoting the generation of interterminal signals and leading, in the final account, to mass stimulation of the release of the neuromediator [3, 35]. These properties determine the toxin as of the presynaptic type.

It is known that, after immunization by an antigen, T-lymphocytes do not recognize the antigen directly but recognize the fragments of it generated by antigen-presenting cells after "familiarization" and limited proteolysis of the antigen [36]. It has actually been shown that fragments of protein antigens may act as the intact antigens in the formation of a T-cell immune response capable of specifically recognizing the parental molecule [37, 38]. After this, receptors specific for the intact antigen are formed in the T-lymphocytes.

The properties of LT as a powerful antigen have been investigated by the viroimmunotest method and by passive rosette formation. The sensitivity of the viroimmunotest is greater than that of the widely used radioimmunological or immunoenzyme analyses, the label used being bacteriophage *φx174* conjugated with antitoxin antibodies. Here the membranes of lymphocytes immune to LT bind to the toxin in a degree (350-400 lysis zones) an order of magnitude greater than the control (30-40 lysis zones) [39]. An investigation of the dynamics of the formation of lymphocytes bearing specific receptors binding LT showed that the maximum number of these lymphocytes was observed on the 14th day after a single immunization by LT [40], which demonstrated the formation of immune receptors to LT.

Antitoxin monospecific serum to LT was obtained by immunizing rabbits directly in the lymph nodes because of the high neurotoxic activity of LT. In order to use them for synthesizing an antibody immunoadsorbent and for investigating the antigenic determinant regions of LT, antibodies against the toxin were isolated by affinity chromatography using LT–cellulose as immunoadsorbent. It was found that the immune adsorbent with immobilized antibodies for isolating LT directly from the whole venom had a smaller capacity than an immunosorbent with immobilized LT for isolating antitoxin antibodies. This is explained by the fact that proteins may have several antigenic determinant regions interacting with the active centers of immunoglobulins.

It has been shown that one LT molecule can bind with nine antibody molecules [41]. On the other hand, IgG contains two identical binding sections consisting of six highly exposed loops. These structures in the active centers of antibodies with hypervariable amino acid sequences are also called complementarily determinant regions (CDRs). CDRs composing the binding sections of an antigen are located at the ends of both F_{ab} fragments of an immunoglobulin and recognize the antigenic sections

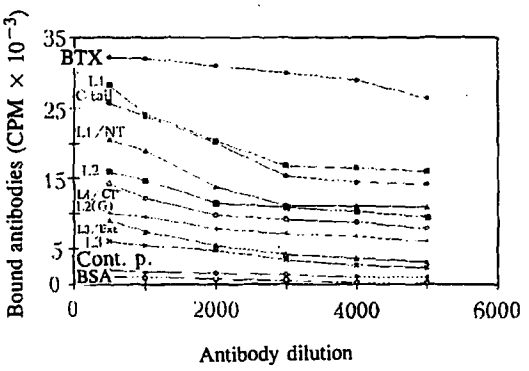

Fig 4. Binding of anti-BTX antibodies to BTX and to synthetic peptides of the toxin in various dilutions of rabbit anti-BTX antiserum, determined by double radioimmune analysis. The mean values of three experiments performed independently are given (variation not more than ±1.7%).

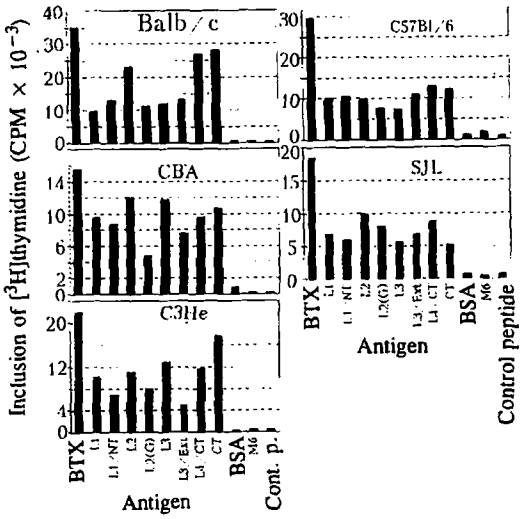

Fig. 5. Maximum proliferative responses at the optimum dose of antigens to BTX and to synthetic peptides of the toxin shown by T-lymphocytes immune against BTX.

of protein molecules [42]. The epitopic behavior of individual amino acid residues or the nature of the antigenic sections in a protein molecule can be determined only with the aid of an immunoglobulin capable of binding it specifically.

During immobilization on a solid support, the two binding sections of an antibody taking part in the binding of LT add randomly, as a consequence of which these sections may prove to be screened. In our investigations, about 20-25% of the active centers of antibodies immobilized on a cellulose sorbent proved to be capable of interacting with LT and there was no free access of LT molecules to the remainder of the active centers. To increase the capacity of a synthesized sorbent we used protein A from *Staphylococcus aureus* as a spacer between the support and the antibody molecule [43], since protein A has a fairly high affinity for some types of immunoglobulins, binding with their F_c fragments. Protein A conjugated to a cellulose matrix that has been oxidized with sodium metaperiodate to dialdehyde-cellulose binds the immunoglobulin molecule through its F_c fragment, while two active centers capable of binding LT remain free.

Glutaraldehyde in a concentration of 0.0075% was used to stabilize a protein A–antibody complex. In the isolation of LT from the whole venom, the capacity of this immunosorbent proved to be 1.5-2.0 times higher than that of an immunosorbent obtained by the traditional immobilization of antibodies. However, a marked decrease in capacity was observed

TABLE 2. Binding of Anti-BTX Antibodies to BTX and Its Synthetic Peptides

Antigen	^{125}I-Antibodies bound by antisera (CPM)*			
	№233	№235	№236	rabbit antiserum
BTX	36480	34746	38015	44120
L1	14675	15220	18715	16768
L1/N-tail	13040	14706	16420	14487
L2	5204	6500	8590	10952
L2(G)	1879	2361	3340	4953
L3	2419	3380	2570	3682
L3/Ext	1796	1830	2340	4952
L4/C-tail	5105	7256	7374	9122
C-tail	16481	11588	12256	17468
Negative controls				
R.L1/N-tail	1132	755	962	1551
R.L2	875	1092	1324	1108
R.L3/Ext	962	867	1011	1487
BSA	895	1121	1157	985

*The results are the mean maximum values of three experiments performed independently (observed variation not more than ±1.9%) and have not been corrected for nonspecific binding.

TABLE 3. Binding of Anti-BTX Antibodies from Three Lines of Mice to BTX and Its Synthetic Peptides

Antigen	Bound ^{125}I-antibodies (CPM)*		
	C57BL/6 (H-2^b)	Balb/c (H-2^d)	SJL (H-2^s)
BTX	42517	68898	52109
L1	18511	27605	23520
L1/N-tail	15678	23673	16585
L2	6357	9937	8016
L2(G)	3033	6344	6430
L3	3985	5191	2599
L3/Ext	4867	7470	5618
L4/C-tail	9774	14856	8426
C-tail	19321	28472	22060
Negative controls			
R.L1/N-tail	2165	1958	2584
R.L2	1951	2195	2476
R.L3/Ext	2979	1422	2619
BSA	2250	901	600

*The results are the mean maximum values of three experiments performed independently (the observed variability did not exceed ±1.5%) and have not been corrected for nonspecific binding.

on repeated use, owing to the partial elution of the antitoxin antibodies. In view of this, we synthesized an immunosorbent in which the active binding centers of the antibodies are used in a highly effective manner.

It is known that the F_{ab} fragments of antibodies in the free state have sulfhydryl groups located on opposite sides of the active binding center [44], and the use of the reaction of these groups with the iodide ion on an adsorbent permits the addition of an F_{ab} fragment to the support in strictly oriented fashion. In the isolation of LT by this immunosorbent, practically all its active centers (90-95%) reacted with the LT. Figure 1 shows profiles of the isolation of LT from the venom of the karakurt spider by affinity chromatography. The highest capacity was observed for an immunosorbent with F_{ab} fragments immobilized in oriented fashion.

In the synthesis of this immunosorbent we used hydrolysis of the immunoglobulin molecule with pepsin and purification of the active $F(ab)_2$ fragments of the antibodies by means of adsorbents with immobilized pepsin and protein A, respectively [45]. These processes were monitored by gel permeation chromatography.

TABLE 4. Binding of Antipeptide Antibodies to the Immunizing Peptide and to BTX

Antigen	Bound antibodies (net CPM)*					
	Balb/c			SJL		
	number of mice	bound to the peptide	bound to BTX	number of mice	bound to the peptide	bound to BTX
L1	10	58942	16432	8	77639	2128
L1/N-tail	8	95793	8460	9	62183	1964
L2	9	88619	12831	9	65676	10735
L3	9	49601	6200	10	38654	1074
L3/Ext	10	35217	1447	8	43704	3361
L4/C-tail	8	63909	4170	10	73795	3802
C-tail	8	43306	5149	9	44393	6062

*Antisera were obtained against each peptide in Balb/c and SJL and were equivalent to a mixture of sera obtained on the 87th day after immunization. For the RIA the antisera were diluted 1:500 (v/v) with PBS. The results were corrected for the nonspecific binding of each antiserum to a negative control, their levels being identical with those shown in Tables 2 and 3.

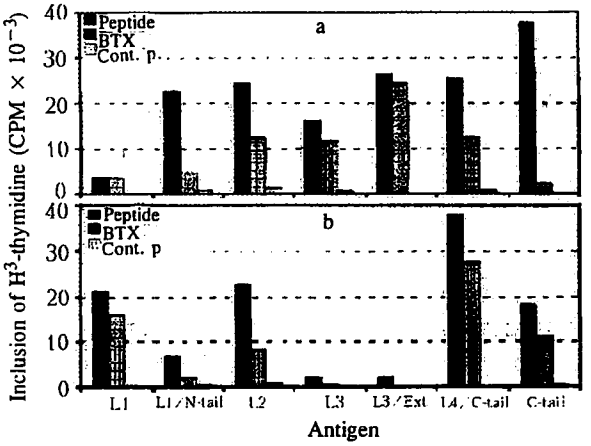

Fig. 6. Maximum proliferative responses at the optimum dose of antigen to the corresponding peptide and to BTX shown by the T-lymphocytes immune to each peptide of the toxin. T-Cells were obtained from mice of the Balb/c (*a*) and SJL (*b*) lines.

As can be seen from Fig. 2*a*, 20 mg of anti-LT antibodies was eluted in the form of a single peak while, under the same conditions, a peptic hydrolysate of antitoxin antibodies was separated into three fractions — IgG, $F(ab)_2$, and F_c fragments (Fig. 2*b*). To obtain the $F(ab)_2$ fragments, the antibodies were hydrolyzed with pepsin immobilized on cellulose, and the resulting antibody hydrolysate was subjected to affinity chromatography on the protein A–cellulose adsorbent, on which the free immunoglobulins and the F_c fragments were retained. Gel permeation chromatography of the active antibody fragments obtained in this way showed the practical absence of fractions belonging to the active antibody molecules and their F_c fragments (Fig. 2*c*). The pepsin–cellulose and protein A–cellulose affinity adsorbents can be used repeatedly without substantial loss of activity for the rapid preparation of the active fragments of antibodies to any antigen.

IMMUNOCHEMICAL INVESTIGATIONS OF A-NEUROTOXINS

Interesting investigations have been carried out with α-neurotoxins from the venoms of snakes of the Elapidae and Hydrophiidae families [2, 5, 6, 9]. These neurotoxins, of which both short and long types, with MM 6500 and ~8000 Da, respectively, are known, possess a postsynaptic action and have been isolated from the venoms of numerous species of snakes.

TABLE 5. Protection Parameters of BTX and Its Synthetic Peptides

Antigen	Protection parameters for			
	Balb/c		SJL	
	LD_{50}, μg BTX/mouse	protection index, *PI*	LD_{50}, μg BTX/mouse	protection index, *PI*
Without antigen	3.20	1.00	3.60	1.00
L1	10.27	3.21	8.86	2.46
L1/N-tail	8.36	2.61	7.86	2.18
L2	10.27	3.21	9.76	2.71
L3	7.86	2.46	7.94	2.21
L3/Ext	8.36	2.61	8.57	2.38
L4/C-tail	8.36	2.61	8.64	2.40
C-tail	10.27	3.21	8.86	2.46
BTX	31.00	9.69	26.50	7.36
Mixture of L1, L2, and C-tail	14.63	4.57	n.d.	n.d.
Multipeptide conjugate of L1, L2, and C-tail	57.80	18.10	n.d.	n.d.

Investigations of the sections of α-neurotoxins and of the acetylcholine receptor that take part in mutual binding have shown that α-neurotoxins have three main regions of binding with the receptor, and these are located in the first, second, and third ring structures of the molecule [46], while from three to five toxin-binding regions are known for the ACRs from various species. A universal binding section is also known in the ACRs for long and short neurotoxins. It is remarkable that this region (the segment Ala^{122}–Ile–Phe–Lys–Ser–Tyr–Gly–Glu–Ile–Ile–Val–Thr–His–Phe–Pro–Phe–Asp^{138} in the human ACR) is also the section responsible for binding acetylcholine and contains the immunodominant antigenic section of the receptor. On the basis of the facts given above; the differences in their affinities for this region (α-neurotoxin > antibody > acetylcholine) may ensure at the molecular level the ability of the toxins and the antireceptor or antitoxin antibodies to inhibit with a high efficiency the binding of acetylcholine or a neurotoxin with the ACR. For the successful achievement of the binding of a neurotoxin, which is shown by a strong neurotoxic effect, the investigation of the structures of the antigenic determinant sections of neurotoxins is fundamental.

The postsynaptic neurotoxin II (NT II) (MM 6786 Da) — one of the main toxins in the venom of the Central Asian cobra *Naja naja oxiana* — blocks the action of the cholinergic ligand of acetylcholine by binding with the ACR. The amino acid residues of NT II present in closest contact with the ACR have been investigated with the aid of fluorescently labeled derivatives of the neurotoxin [47, 48].

In view of the fact that NT II is not such a powerful antigen as LT (its molecule has a molecular mass 19 times smaller than LT), to increase its immunogenicity it was conjugated with a suspension of cellulose that had been oxidized with sodium metaperiodate (SOC). It possesses a high immunostimulating activity in relation to the antigen introduced into the composition of a chemical conjugate [49]. The amount of specific antibodies to NT II (determined by the immunoadsorbent method) in the antiserum obtained by the use of the NT II–SOC conjugate was 2-2.5 times higher than in the serum obtained by immunization with the intact toxin. These antibodies, purified by affinity chromatography on the NT II–cellulose immunoadsorbent, were used to obtain ordinary active F_{ab} fragments with the aid of which an orientedly immobilized F_{ab}-antibody immunosorbent was synthesized.

Exhaustive information has been obtained on the spatial structure of NT II, starting from its primary structure and the results of comparative investigations of NT II and erubatoxin *b* [2, 47, 50]. The profile of its binding to the acetylcholine receptor from the electric organ of *Torpedo* has been studied with the aid of various photoaffinity derivatives of the toxin [51, 52]. The results of an investigation of the structure of the antigenic sections of NT II determining the immunochemical properties of the toxin explain the interrelationship between the sections binding the neurotoxin with the α subunit of the receptor and the epitopes of the NT II molecule binding to the antibodies.

Since the conformation of NT II is stabilized by four intramolecular disulfide bonds, it was reduced with dithiothreitol, carboxymethylated, and hydrolyzed with trypsin. We then studied the binding of the resulting tryptic peptides to antitoxin antibodies and, after this, isolated them by affinity chromatography with the help of an immune adsorbent having orientedly immobilized F_{ab} fragments of antibodies to NT II. The isolated peptides were identified by analyzing their amino acid compositions and determining their N-terminal residues. Table 1 gives the results, which show that the amino acid residues

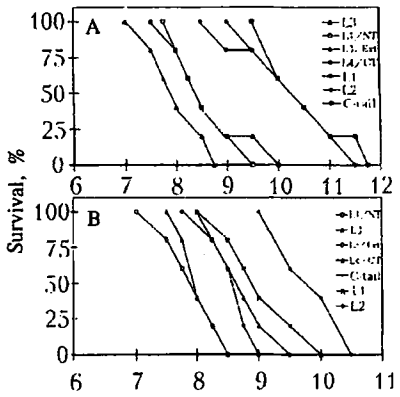

Fig. 7. Survival curves after the neurotoxic action of BTX on Balb/c (*A*) and SJL (*B*) mice immunized with synthetic BTX peptides. Each point of a curve represents the percentage survival of five mice for the given amount of BTX.

of the antigenic determinants of NT II are located in the first loop of the molecule (Leu^1–Glu–Cys–His–Asn–Gln–Gln–Ser–Ser–Gln–Pro–Pro–Thr–Thr–Lys^{15}), in the transitional part between this loop and the second large loop (Thr^{16}–Cys–Ser–Gly–Glu–Thr–Asn–Cys–Tyr–Lys^{25}), and in the third loop (Gly^{39}–Cys–Gly–Cys–Pro–Lys^{44} and Val^{45}–Lys–Pro–Gly–Val–Asn–Leu–Asn–Cys–Cys–Arg^{55}) of the neurotoxin molecule. It must be mentioned that the binding of small amounts of the peptide Gly^{33}–Thr–Ile–Ile–Glu–Arg^{38} shows that the second loop of NT II also contains amino acid residues participating in binding with the antitoxin antibodies. These results correlate well with those that we obtained in a study of the binding of antitoxin antibodies to NT II by means of the immunoadsorption method. At the same time, the possibility was shown of the simultaneous binding of the NT II molecule to four molecules of antibody. Thus it has been established that the amino acid residues in the antigenic regions of NT II are present in three loops and in the globular part of its molecule.

A deeper study of the structures of the antigenic sections of toxins appeared of interest, but when this work was begun there was no detailed information on the spatial and structural features of the toxins described above and we therefore rested our choice on the molecule of α-bungarotoxin (BTX) from the venom of the snake *Bungarus multicinctus*, which consists of 74 amino acid residues in a single polypeptide chain. The complete primary structure of BTX has been established and its conformational features have been investigated in detail in the crystalline state [9, 53] and in solution [54, 55]. We may note that our investigations were carried out in order to identify the region of immune recognition and not with the aim of an absolutely accurate determination of the boundaries of the epitopes, for which we used different investigation strategies [17, 18, 56].

Starting from the amino acid sequence and conformational structure of BTX, established by x-ray structural analysis [9, 53] using the fluoren-9-ylmethoxycarbonyl solid-phase method, we synthesized peptides mimicking all the loops of the molecule and those exposed on its surface [57]. In addition, for a control, we synthesized randomized peptides (i.e., peptide analogs having the same amino acid compositions as those mimicking the BTX peptides but with a different sequence of the residues, as determined with the aid of the Quick Basic, Microsoft, computer program). The structures of the synthesized BTX peptides and their control analogs are presented in Fig. 3.

The peptides were purified by column chromatography and HPLC to a homogeneous state, and for the peptides reflecting the terminal sections of the BTX molecule we obtained monomeric cyclic structures by forming disulfide bonds between the terminal cysteine residues. The synthesized BTX peptides were investigated for specific recognition by antibodies and for the stimulation of T-lymphocytes obtained by immunization with the intact BTX molecule [37]. We also investigated the capacity of antibodies and T-lymphocytes immune to the synthetic peptides for recognizing specific conformational sections in the parental BTX molecule [37].

Antibodies against BTX were obtained by immunizing rabbits and mice with sublethal amounts of the toxin. The binding of the antitoxin antibodies to BTX and the recognition of the regions on the surface of its molecule represented by these

synthetic peptides were determined by double antibody radioimmune analysis, with anti-BTX antisera in dilutions of from 1:500 to 1:5000

The results, which are shown in Fig. 4, demonstrated that even in a dilution of 1:5000 considerable amounts of the antitoxin antibodies were bound by the peptides L1, C-tail, L1/N-tail, and L2. The other peptides [L4/C-tail, L2(G), L3/Ext, and L3] bound smaller amounts of the antibodies. The results of the binding of mouse anti-BTX antibodies to the synthetic BTX peptides are presented in Table 2. The following synthetic peptides showed a high binding activity: L1, L1/N-tail, C-tail, L2, and L4/C-tail. The amounts of antibodies bound to the peptides L3, L3/Ext, and L2(G) were very low or only slightly higher than for the control peptides and other negative controls (Table 2).

The results presented in Fig. 4 and Table 2 permit the following conclusion: in the BTX molecule precisely the same immunodominant antigenic regions are recognized by antibodies from different immune hosts. The greatest binding with antibodies was shown by the regions of BTX represented by the synthetic peptides L1, L2, and C-tail. It must be mentioned that the inclusion of the aminoterminal residues Ile-1 and Val-2 in the first loop of BTX (i.e., the L1/N-tail peptide) did not enhance the binding of peptide L1 (compare the binding of peptides L1 and L1/N-tail to antitoxin antibodies, Table 2); consequently, the N-terminal amino acid residues of BTX, Ile-1, and Val-2 do not form part of the antigenic sections of the toxin. At the same time, the addition of the fourth loop of BTX (amino acid residues 60-65) to the carboxy-terminal region of the toxin molecule (i.e., the L4/C-tail peptide) gave no additional binding activity whatever in comparison with the activity of the C-tail peptide (Table 2). Replacement of one of the invariant amino acid residues found in the molecules of the long and the short neurotoxins [2, 9] — Trp-28 in peptide L2 — by a glycine residue [i.e., the peptide L2(G)] led to a great diminution in the antibody-binding activity of peptide L2. This indicates that amino acid residue Trp-28 of BTX, which is one of the contact residues in binding to the ACR [2] (i.e., it participates in the neurotoxic activity of BTX) is also a component part of the antigenic determinant of the BTX molecule.

In an investigation of the T-cell immune response to BTX and a comparison of the T-cell regions of the toxin with those sections that were recognized by antibodies in the same lines, we used mice of the following lines: Balb/c ($H\text{-}2^d$), CBA ($H\text{-}2^k$), C3He ($H-2^k$), C57Bl/6 ($H-2^b$), and SJL ($H-2^s$). Profiles of the recognition of the toxin peptides by T-cells immune to BTX in these lines are shown in Fig. 5. The binding of the peptides to anti-BTX antibodies that was obtained in three of these lines was determined by quantitative radioimmunoadsorbent titration with ^{125}I-labeled antibodies in the presence of various amounts of peptide adsorbents. The results of these experiments are generalized in Table 3. A comparison of the results given in Fig. 5 and Table 3 showed that at the T-cell level the $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes were distinguished by a high immune response to BTX, while $H\text{-}2^s$ and $H\text{-}2^k$ gave a moderate response. In a concrete line a definite region was strongly recognized both by antibodies and by T-cells. Regions were also observed that were recognized preferentially only by antibodies or only by T-cells. These results for BTX are in harmony with the antibody and T-cell recognition of other antigenic protein molecules [58, 59].

In the following stage we studied the recognition by antipeptide antibodies of the corresponding regions of the BTX molecule, for which mice of the Balb/c and SJL lines were immunized with each of the synthetic BTX peptides in the free form (i.e., in the absence of binding with any substrate). The results of the binding of these antibodies to the peptides used for immunization and with BTX are given in Table 4. It can be seen from Table 4 that in both lines each of the peptides caused an intensive formation of antibodies to the peptide being immunized. In spite of this, the capacity of the antipeptide antibodies for recognizing the regions that they represent in the BTX molecule varied with the peptide, which is explained by the surface-exposure demands for the recognition by antibodies of a discrete region in the intact protein molecule [17, 18]. This property did not depend on whether the region represented by the synthetic peptide was or was not dominant in the binding of anti-BTX antibodies. In both lines, some peptides stimulated the formation of antibodies (i.e., antibodies to the L2 and C-tail regions) strongly recognizing those regions of BTX that they represented. Antibodies against a definite peptide differed in their capacity for recognizing intact BTX, which depended on the line in which they had been obtained (compare the binding of antibodies from two lines to BTX in comparison with L1, L1/N-tail, L3 or L3/Ext, Table 4).

It is well known that after immunization the antigen is not recognized immediately but requires "processing" with antigen-presenting cells in association with glycoproteins of the second class [60]. We investigated the capacity of T-cells immune to each of the peptides that we had synthesized for binding an antigen and recognizing the corresponding region in the BTX molecule. The results obtained are given in Fig. 6. They show that in the Balb/c line a strong response is observed to all the peptides in the BTX molecule with the exception of peptide L1. In spite of this, the capacities of T-cells immune to the peptides for recognizing regions in the BTX molecule differed. Attention must be directed to the fact that, on the one hand, the T-cells specific to the C-tail peptide showed the strongest response to the C-tail peptide itself while, on the other hand, they did not show or showed to only a small degree a capacity for recognizing the region represented by this peptide in the BTX

molecule, which can be explained by the orientation of the C-terminal section in the spatial conformation of the native BTX molecule [9]. In the Balb/c line, recognition of the regions of BTX was observed in the following sequence: L3/Ext > L2 > L4/C-tail > L3 > L1/N-tail > L1 > C-tail (Fig. 6*a*). In SJL the degree of recognition of BTX changed thus: L4/C-tail > L1 > C-tail > L2 > L1/N-tail (Fig. 6*b*).

PROTECTION AGAINST A NEUROTOXIN WITH SYNTHETIC PEPTIDES

By using synthetic peptides mimicking discrete regions of BTX we have determined the antigenic sections located in the first (amino acid residues 3-16) and second (amino acid residues 26-41) cyclic structures, and also in the C-terminal (amino acid residues 66-74) part of the BTX molecule that are recognized by various antitoxin antibodies and T-cells. In spite of this, the capacity of the antipeptide T-cells for recognizing the toxin directly did not correlate with the capacity of the antibodies against the peptides for recognizing the BTX molecule. In view of this, it was of interest to investigate the protector activity of each peptide from the neurotoxic action of BTX.

We started from the following considerations: on using as immunogen a peptide representing an immunodominant region of the protein molecule, this peptide should stimulate immune responses and should promote the recognition of this region in the intact molecule. This is necessary if from the antipeptide immune response the possibility of some neutralizing action or other in relation to the parental molecule is expected.

The protector properties of synthetic BTX peptides were studied on mice of the Balb/c and SJL lines (60 animals for each of the peptides). After immunization and five boosters, the corresponding titers of the antipeptide antibodies were determined by the solid-phase radioimmune method. To determine the toxic effects of different amounts of BTX we used groups of five mice immunized by BTX peptides. The number of mice surviving was taken as a function of the action of a definite amount of BTX.

Figure 7 shows curves of the survival, after the toxic action of BTX, of Balb/c and SJL mice immunized with various BTX peptides.

The parameters of the protective action of each peptide in both lines are generalized in Table 5. It may be concluded from these results that the greatest protective effect from the toxic action of BTX is possessed by peptides L1, L2, and C-tail (*PI* for Balb/c, 3.2; for SJL, 2.5-2.7), which were previously determined as peptides containing immunodominant antigenic sections of BTX in binding with antitoxin antibodies [37]. The protection shown by the other peptides was also fairly considerable (*PI* for Bal/c, 2.5-2.6; for SJL, 2.2-2/4).

Since peptides L1, L2, and C-tail proved to be fairly protective (threefold increase in LD_{50} for BTX as compared with a control), it appeared of interest to study the protective properties of these peptides on immunization with all three of them simultaneously. The immunization of Balb/c mice with an equimolar mixture of the peptides enabled protection to be increased by a factor of 4.6 in comparison with a control. This showed that antibodies against the discrete regions of the toxin molecule represented by the immunodominant peptides L1, L2, and C-tail are more effective in the neutralization of the toxic action of BTX than antibodies directed against any one of the regions. The protector property of an equimolar mixture of these peptides was interrelated with the titer of the fraction of antipeptide antibodies interacting with BTX. However, they were moderate, and did not increase appreciably after intensive immunization. We investigated the protector capacity of a chemical multiepitopic peptide conjugate.

With this aim, peptides L1, L2, and C-tail were conjugated chemically to ovalbumin. The conjugate, containing three immunodominant regions of BTX, promoted the formation of high titers of antibodies strongly interacting with BTX. Although the amount of peptides immunized in the composition of the conjugate was far smaller than the immunizing amount of free peptides, an antiserum with a high antibody content was obtained. This result can be explained by the fact that the antibody response formed by an antigen is not only a function of the amount of antigen but depends largely on its presentation to the immune system [36], as was in fact observed during our experiments. In this case the conjugate promoted the formation of a strong immune response, showing a high degree of recognition of the BTX molecule. Consequently, the BTX epitopes are presented more effectively on a multivalent carrier as compared with each epitope in the free state. The triepitopic chemical conjugate of peptides L1, L2, and C-tail exhibited a very high protective efficacy in relation to BTX (*PI* = 18.1) which is almost double the protection obtained with native BTX (*PI* = 9.7) (Table 5) [61].

CONCLUSION

The immunochemical properties of some representatives of presynaptic and postsynaptic neurotoxins have been investigated by the methods of protein chemistry with biochemical and immunochemical approaches. Highly specific antisera to these neurotoxins have been obtained. It has been shown that LT may contain up to nine antigenic determinant sections, while NT II has four such regions. It has been established that the antigenic sections of NT II are located in three loops and the globular part of the molecule.

Peptides mimicking all the cyclic and exposed regions of BTX have been obtained by means of fluoren-9-ylmethoxycarbonyl solid-phase peptide synthesis. These peptides and their control analogs have been used for investigating profiles of antibody and T-lymphocyte recognition after immunization with BTX. The capacities of the antipeptide antibodies and of T-cells for recognizing an immunizing peptide and the parental BTX molecule have been determined.

We have established that the immunodominant antigenic sections of BTX are located in the first (amino acid residues 3-16) and second (amino acid residues 26-41) loops and also in the carboxy-terminal part (amino acid residues 66-74) of its molecule. Profiles of T-cell recognitions of these peptides varied according to the haplotype of the animal used for immunization, which corresponds to Ir genetic control of the immune responses to individual antigenic regions [62]. Molecular specificities of antibodies and T-lymphocytes have been compared for three lines of mice (C57Bl/6, Balb/c, and SJL). In a definite line regions have been found that are recognized predominantly by antibodies or by T-cells. On use as antigens in the free form, the peptides synthesized evoked a strong antibody response. Antibodies against peptide L2 showed the highest capacity for recognizing the corresponding region in the BTX molecule. The immune response of the antipeptide T-cells to the immunizing peptide was not interrelated with the immunodominance of a definite region in the case of immune T-cells to BTX.

The capacity of the synthetic peptides for stimulating protection immunity against the neurotoxic action of BTX has been determined. Immunization of mice of the Balb/c and SJL lines with each of the peptides created a definite protection. The highest protector capacity was found for the peptides L1, L2, and C-tail, immunization with which enabled the animals to withstand a three times greater dose of BTX than in the control. It is important to note that the three most effective peptides (L1, L2, and C-tail) also contain immunodominant antigenic sections for binding with antibodies to BTX. Immunization with an equimolar mixture of these peptides led to even higher protection (4.6 times higher than the control: i.e., the protective index $PI = 4.6$). On use as an immunogen, a chemical ovalbumin conjugate containing all three peptides L1, L2, and C-tail simultaneously led to the appearance of exceptionally high protection ($PI = 18.1$) and, thus, was almost twice as effective as protection using BTX ($PI = 9.7$). Consequently, a multiepitopic chemical conjugate of peptides L1, L2, and C-tail may serve as an effective vaccine against the neurotoxic action of BTX.

It has been shown that in homologous groups of proteins the antigenic sections are frequently present in structurally equivalent regions [63]. Since BTX is a representative of a broad family of long and short neurotoxins and cytotoxins [2] having considerable homology in their amino acid sequences and also in their spatial structures, it may be assumed that the antigenic sections of BTX can be extrapolated to structurally equivalent sections of the molecules of other α-neurotoxins and cytotoxins with the aim of obtaining synthetic peptide vaccines against them.

In view of freer migration and the greatly increasing probability of the distribution of various pathogens as a result of the geopolitical changes that have taken place in recent years, the development of methods of obtaining artificial peptide vaccines of a new generation with the aid of synthetic and immunochemical approaches is extremely important for our region.

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