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CAPACITY OF ANTIBODIES TO SYNTHETIC PEPTIDES OF α-BUNGAROTOXIN FOR RECOGNIZING CONFORMATIONAL SECTIONS OF THE NEUROTOXIN MOLECULE

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The capacity of antibodies to various synthetic peptides of α-bungarotoxin for binding peptides of the neurotoxin and recognizing the corresponding regions of the α-bungarotoxin molecule has been investigated. It has been established that individual conformational sections of the α-bungarotoxin molecule are specifically recognized by antibodies to synthetic epitopes of the neurotoxin.

It is known that the antigenic sections of a protein exist in the form of discrete regions of its native molecule, and various approaches are used for their investigation [1—3]. The most informative among them are an all-sided synthetic strategy [4] and surface-stimulation synthesis [5] which enable reliable results to be obtained on the antigenic structure of proteins (detailed information on the principles of the all-sided synthetic strategy and of surface-stimulation synthesis and also on mimicking peptides may be obtained from the given references [4] and [5]. These methods permit the synthesis of peptides that reflect the whole polypeptide chain of a protein molecule from the C-terminal to the N-terminal amino acid or that reflect the most exposed and superficial sections of the protein molecule. Afterwards the capacity of antibodies to the intact protein for binding the synthetic peptides and, conversely, the capacity of antibodies to the synthetic peptides for recognizing the corresponding sections of the molecule under investigation are studied. Such immunochemical screening provides the possibility of drawing preliminary conclusions for the subsequent accurate determination of the antigenic structure of the given protein.

Using these methods, we have previously determined the immunodominant antibodies and the T-cell antigenic sections of α-BT [6,7] by means of the synthesis of all the highly exposed and superficial sections of the molecule of α-BT — one of the main neurotoxins of the venom of the snake *Bungarus multicinctus* — with a very high affinity ($K_d = 10^{-11}$M) of binding with the acetylcholine receptor in a postsynaptic membrane. The primary structures of the peptides that we synthesized are given in Fig. 1. We have established [6, 7] that the immunodominant antigenic sections of the α-BT molecule are located in the first loop structure, consisting of amino acid residues 3—16, the second loop structure (amino acid residues 26—41), and the C-terminal part of the molecule, consisting of amino acid residues 66—74.

The task of the present work was an investigation of the recognition of the corresponding sections of the neurotoxin molecule by antibodies to synthetic α-BT peptides. In view of the fact that the peptides were intended for the synthetic reflection of all the conformationally expressed sections of α-BT, among which the first, second, third, and fourth loop structures of the neurotoxin are represented respectively by the peptides L1, L1/N-tail, L2, L3, L3/Ext, and L4/C-tail, we obtained [6, 7] their loop monomeric forms by creating disulfide bonds between the cysteine residues located at the two ends of the molecules of the corresponding peptides.

It is known that to obtain antibodies to low-molecular-mass compounds or peptides that are fragments of a protein molecule they are usually bound chemically to a high-molecular-mass support. For many years this was considered the generally accepted procedure used in practice [8]. But the discovery of the fact that the biosynthesis of antibodies can be

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TABLE 1. Binding of Antibodies to α-BT Peptides with each Peptide and with α-BT

Peptide	Binding of the antibody (net cpm)					
	Balb / c			SJL		
	Number of mice	Binding to the peptide	Binding to α-BT	Number of mice	Binding to the peptide	Binding to α-BT
L1	10	58942	16432	8	77639	2128
L1/NT	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/CT	8	63909	4170	10	73795	3802
C-tail	8	43306	5149	9	44393	6062

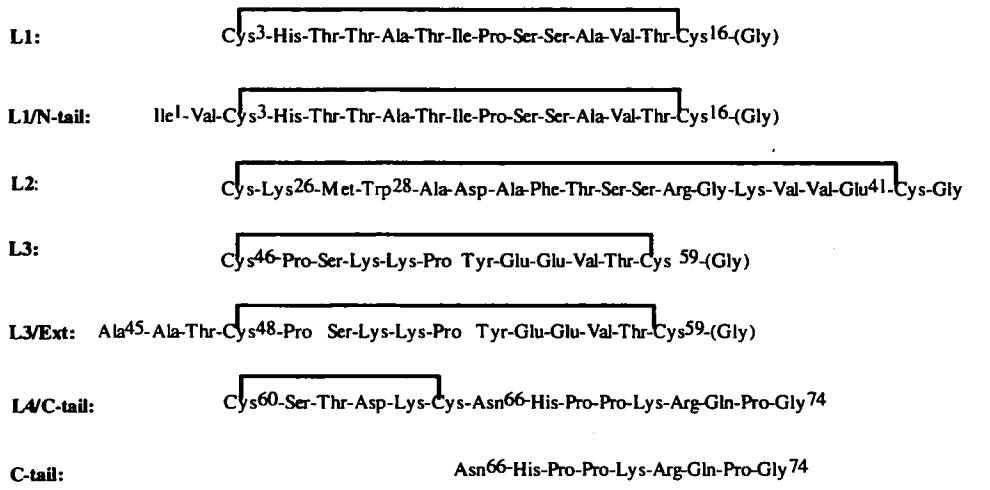

Fig. 1. Primary structures of the α-BT peptides synthesized.

initiated by peptides consisting of six or more amino acid residues in the free form (i.e., without chemical binding to any high-molecular-mass support) [9, 10] permitted us to use the synthesized peptides as immunogens in the free form.

Antibodies to each of the synthesized peptides were obtained after the immunization of Balb/c and SJL mice, and the binding of the antipeptide antibodies with the immunizing peptide and the recognition by these antibodies of the sections in the α-BT molecule corresponding to this peptide were investigated. For this we used radioimmune assay (RIA), and the results of this are generalized in Table 1.

As we see, in both lines of mice the synthetic peptides led to the evocation of a fairly strong immune response which was expressed in the binding of the antibodies with each peptide (RIA results from 35217 to 95793 net cpm) In spite of this, not all the antipeptide antibodies exhibited a capacity for recognizing the corresponding sections of the neurotoxin molecule. In both lines, antibodies to L2 and C-tail proved to be capable of strongly recognizing those sections of the α-BT molecule that these peptide mimicked. However, the binding with α-BT of the antibodies to the other peptides depended on the structural features of each peptide and of the immune host from which these antibodies had been obtained.

We have established previously [6, 7] that the specificity of antibodies to α-BT peptides is directed to the spatial disposition of each of the amino acid residues composing the antigenic section of α-BT, and it may be expected that for each determinant section the integrity of the loop structure of each peptide is very important. We decided to investigate these prerequisites by obtaining α-BT peptides in linear form and obtaining antibodies to the linear peptides, after which we were faced with the question of whether antibodies against the antigenic sections in linear form could recognize sections of the native

TABLE 2. Binding of Antibodies to Linear α-BT Peptides with the Peptides and with α-BT

Linear peptide	Bound antibodies (net cpm)					
	Balb / c			SJL		
	Number of mice	Binding to the peptide	Binding to α-BT	Number of mice	Binding to the peptide	Binding to α-BT
L1	8	64832	3134	10	61301	2359
L1/NT	8	85210	1386	10	71711	1344
L2	10	88381	2899	8	66513	4215
L3	9	47286	974	9	82154	435
L3/Ext	9	92329	854	9	91315	759
L4/CT	10	54811	3345	9	73265	2113

neurotoxin molecule having a loop structure. To obtain answers to these questions we obtained carboxymethylated derivatives of the peptides L1, L1/N-tail, L2, L3, L3/Ext, and L4/C-tail, and in the Balb/c and SJL lines obtained antibodies to all the linear peptides of α-BT. We then investigated the binding of these antibodies with each acyclic peptide and with α-BT by means of RIA: the results of this are given in Table 2.

As can be seen from Table 2, strong immune responses to all the peptides of the neurotoxin (net cpm from 47286 to 92329) were obtained in both lines of mice. By analyzing the results on the recognition of individual conformational domains of α-BT by antibodies to loop and acyclic peptides of the toxin, it was established that antibodies to the loop sections specifically recognized the integrity of the corresponding loop structures of the native neurotoxin molecule, while this was not observed in the case of antibodies to the linear peptides (compare the results on the binding of α-BT in Tables 1 and 2).

Thus, the use for immunization in the free form of α-BT peptides obtained by the method of surface-stimulation synthesis [5] led to the appearance of a strong antibody immune response and these antibodies bound strongly with the peptides. In spite of this, in an individually taken immune host the binding of the antipeptide antibodies with the intact α-BT molecule varied according to the specific features of each peptide. The reason for this is, most probably, a difference in the spatial orientation of discrete domains of the α-BT molecule.

It is important to note that in both Balb/c and SJL the peptides L2 and C-tail stimulated a powerful biosynthesis of antipeptide antibodies, and these antibodies on recognizing the α-BT molecule also exhibited a dominating characteristic, which is in agreement with information on the antigenic structure of α-BT [7]. These results also correlate well with those on the structure of α-BT in the crystalline state [11] and in solution [12], which show that the second loop domain in α-BT, comprising amino acid residues 26—41 (in our work, this corresponds to peptide L2) and the C-terminal section of the neurotoxin molecule (residues 66—74) have a higher degree of protrusion than the other sections of the α-BT molecule.

In contrast to this, depending on which line of animals they were obtained from, antibodies to the other peptides had, on binding with the toxin, a feature distinguishing them from one another, as can be observed in the binding of the antibodies with peptides L1, L1/tail, L3, and L3/Ext from the Balb/c and SJL lines (see Table 1). Thus, the results obtained show that, in addition to the molecular features of the binding of α-BT by antibodies and the conformational disposition of an individual section of the α-BT molecule used for immunization, in order to stimulate a strong antibody immune response capable of recognizing and strongly binding the intact neurotoxin molecule, factors introduced on the part of the immune hosts (Balb/c and SJL) having different genetic origins may also have great importance.

EXPERIMENTAL

α-BT from the venom of the snake *Bungarus multicinctus* was obtained from Miami Serpentarium Laboratories (Punta Gora, FL., USA), and its homogeneity was established by reversed-phase high-performance liquid chromatography (HPLC) with a linear gradient of from 0 to 90% of acetonitrile in 0.1% trifluoroacetic acid, using a Vydac 218TP54 analytical column (5 μ,

C_{18}, 4.6 × 250 mm). All the α-BT peptides were synthesized [6] by the solid-phase method using derivatives of *L*-amino acids protected with a N-α-fluoren-9-ylmethoxycarbonyl grouping and purified to homogeneity by reversed-phase HPLC on a Vydac 218TP54 column (5 μ, C_{18}, 4.6 × 250 mm). Peptides L1, L2, L3, L3/Ext, and L4/C-tail were purified in the presence of β-mercaptoethanol. The carboxymethylation of the cysteine-containing peptides was achieved by the Crestfield method [13].

Antibodies to the Peptides. Mouse antisera to the peptides were obtained from two lines of animals: Balb/c and SJL (National Cancer Institute and Jackson Laboratory, Bar Harbor, ME, USA). The animals were kept in a vivarium for two weeks, and several days before immunization blood was taken and the serum was separated. Immunization was conducted in the spinal region in multipoint fashion with 50 μg of the peptide concerned, dissolved in 25 μl of 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.2 (PBS) and emulsified with an equal volume of complete Freund adjuvant. Reimmunization was conducted three times with the same amount of peptide every two weeks (here, incomplete Freund adjuvant was used in accordance with the NIH requirements for animal immunization) and then every three weeks. The level of antibodies in the antiserum was monitored by solid-phase RIA. The antibodies used in the present work were obtained 87 days after the beginning of immunization,

Solid-phase Radioimmune Assay. The binding of the antipeptide antibodies with the peptides used for immunization and with α-BT was carried out by solid-phase RIA. A synthetic peptide (2.5 μg/50 μl of PBS) and α-BT (0.5 μg/50 μl of PBS) were adsorbed (4°C, 24 h) in the wells of 96-well polyvinyl plates (Falcon, Lincoln Park, NY, USA) and, after the nonbound antigen had been washed out with PBS, the capacity of the support that had remained free was blocked (37°C, 1 h) with 100 μl of 1% BSA/PBS and five times with PBS. The corresponding antisera after dilution 1:500 (v:v, in 50 μl of 0.1% BSA/PBS) were added to the wells and incubated at 4°C for 16 h and, after washing, 50 μl of rabbit antibodies against mouse IgG and IgM (Accurate Chemical and Scientific Co., Westbury, NY, USA) in a dilution of 1:2000 (v:v, in 0.1% BSA/PBS) was added and incubation was carried out at 37°C for 2 h. Protein A from *S. aureus* was labeled with I^{125} by a procedure using chloramine T [14]. The immune complex obtained was revealed with an excess of I^{125}-labeled protein A (200,000 cpm/50 μl of 0.1% BSA/PBS) at room temperature for 2 h and was then washed copiously with PBS, and all the wells of the plates were dried and the material was transferred quantitatively into individual test-tubes for measuring the incorporated radioactivity in a gamma-radiation counter (1277 Gamma Master, LKB). Three experiments were performed in parallel and the values for nonspecific binding obtained from control wells coated only with BSA or with the use of normal mouse serum were deducted from the values obtained from the experimental wells containing the α-BT peptide.

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