A monoclonal antibody which recognized the functional site of snake neurotoxins and which neutralizes all short-chain variants

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We isolated a neurotoxin-specific monoclonal antibody (Mab) which is capable of recognizing and neutralizing all short-chain toxin variants that have been tested, including those with widely divergent sequences. The epitope incorporates the three invariant residues Lys-27, Trp-29 and Lys-47 which form part of the site by which the toxins bind to the nicotinic acetylcholine receptor. To our knowledge, this is the first Mab which possesses the universal capacity of neutralizing all natural variants of a toxic protein.

Monoclonal antibody *Toxin* *Acetylcholine receptor*

1. INTRODUCTION

Antibodies are capable of neutralizing the pathogenic action of a wide range of agents such as viruses, bacteria and toxins. This is achieved by binding to critical epitopes [1,2]. In practice, however, the therapeutical importance of such properties is often limited. Amino acid substitutions at the periphery of proteins generate interspecies antigenic variations [3] and as a result antibodies raised against a given pathogenic agent often fail to recognize, and therefore to neutralize, a mutant or a variant. However, areas exist that are not or much less subject to such substitutions, for instance, the regions responsible for the common specific biological function. In principle, antibodies which are directed against such regions should be well-suited for a universal immunological therapy.

Snake bites are an important medical problem in many parts of the world [4]. Poisoning by Elapidae and Hydrophidae is often due to the neurotoxins that are present in their venoms [5]. These molecules share the property of blocking the nerve-muscle transmission by binding to the nicotinic receptor of acetylcholine (AcChR) [5]. Due to a high level of interspecies antigenic variability, no universal antivenom is available against these molecules as yet. This paper reports for the first time the preparation and characterization of a monoclonal immunoglobulin which recognizes the AcChR-binding site of neurotoxins. This site constitutes the only conserved area shared by all variants isolated from the venoms of sea-snakes and land snakes (cobras and mambas). Accordingly, the immunoglobulin is capable of inhibiting the binding to AcChR of all tested short-chain variants.

2. MATERIALS AND METHODS

Toxin α was purified from *Naja nigricollis* venom (Pasteur Institute, Paris) as described [6]. Erabutoxins b and c from *Laticauda semifasciata* were kindly given to us by Professor N. Tamiya (Sendai, Japan). Toxins I and III from the venom of *N. mossambica mossambica* as well as ^{125}I-labelled toxin I were kindly provided by Dr P.

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Bougis (Marseille, France). Cobrotoxin from the venom of *N. naja atra* was generously donated by Professor K. Hayashi (Kyoto, Japan). ^{3}H-labelled toxin α was prepared according to [7]. Poly(ethylene glycol) (PEG), M_r 6000, was from BDH (Poole, England). Lumagel and Lipoluma were obtained from Lumac, France.

Cobrotoxin and erabutoxin b (10^{-4} M in 1 M phosphate buffer, pH 9) were iodinated with ^{125}I using the chloramine T procedure [8]. The radioimmunoassays were performed by using PEG at a final concentration of 12.5% to precipitate the antigen-antibody complexes. The culture supernatants or ascitic fluids (0.1 ml) were incubated at room temperature for 2 h and then 4°C overnight with 0.1 ml (2 nM) of ^{3}H-labelled toxin α (30 Ci/mmol) in 0.05 M phosphate buffer and 0.45% NaCl, pH 7. Normal horse serum (0.025 ml) and PEG (0.5 ml) were added to the solution. After centrifugation (Sorvall HS4 rotor, 3000 rpm, 4°C, 30 min) the pellets were dissolved in 0.75 ml of 0.05 M NaOH and 10 ml Lumagel solution, and the radioactivity counted by using an Intertechnique counter. The background was less than 6% of the total radioactivity. Neutralization experiments were done under in vitro conditions. AcChR, prepared from electric organs from *Torpedo marmorata* according to [9], was incubated overnight at 4°C in the presence of radioactive toxins (protein concentrations are indicated in table 2) with various amounts of antibody in 1.5 ml Ringer buffer. The mixtures were filtered through two Millipore filters (HAWP, 0.45 μm) which were washed with 30 ml of 4°C Ringer solution. The dried filters were counted in 10 ml Lipoluma. Immunisation of Balb/c mice with toxin was achieved according to [10]. Fusion experiments were performed according to [11] between myeloma cells (NS 63) and spleen cells from the hyperimmunized Balb/c mice.

3. RESULTS AND DISCUSSION

Short-chain neurotoxins constitute a large family of structurally and functionally homologous proteins, the polypeptide chains (60–62 residues) of which differ in sequence (review [12]). To identify monoclonal antibodies that recognize conserved areas we screened antibody-secreting hybrid cells with two widely divergent short-chain neurotoxins. These are toxin α from the land snake *N. nigricollis* [13] which was the original immunogen and erabutoxin b from the sea-snake *L. semifasciata* [14]. The toxins differ by 17 amino acid residues and share little cross-reactivity toward conventional antisera [2,15]. Fifteen fusion experiments [11] were performed between myeloma cells (NS 63) and spleen cells from hyperimmunized Balb/c mice. On average, each fusion experiment led to hybrid growth in approx. 65 out of 70 wells containing the selective HAT medium. Four hybrids produced antibodies that reacted with ^{3}H-labelled toxin but not with ^{125}I-labelled erabutoxin b and one produced an antibody which reacted with both radioactive toxins. This cell line was propagated intraperitoneally in mice and gave rise to an Ig2a immunoglobulin, designated as M$\bar{\alpha}$2-3. The binding of ^{3}H-labelled toxin α (30 Ci/mmol) to M$\bar{\alpha}$2-3 is saturable and highly specific. Scatchard analysis of the binding data gave an equilibrium dissociation constant (K_d) of 9 nM.

We examined the binding capacity for M$\bar{\alpha}$2-3 of seven homologous toxins selected for their widely divergent sequences [12,16] and for their weak cross-reactivities toward polyclonal antibodies [2,15]. As shown in table 1, all toxins compete with ^{3}H-labelled toxin α for binding to the monoclonal

Table 1

Relative affinities of various homologous toxins toward M$\bar{\alpha}$2-3

Homologous short-chain neurotoxins	Apparent dissociation constants for M$\bar{\alpha}$2-3 (K_d, nM)
Naja nigricollis toxin α	9
Laticauda semifasciata erabutoxin b	2
Laticauda semifasciata erabutoxin c	2
Laticauda colubrina toxin d	12
Naja mossambica mossambica toxin I	138
Naja mossambica mossambica toxin III	1050
Naja naja atra cobrotoxin	1500

K_d values were calculated according to Ishikawa et al. [17] from competition experiments performed with ^{3}H-labelled toxin α (2 nM, 20 Ci/mmol) and M$\bar{\alpha}$2-3 (15 nM). Incubation times were 1 h at room temperature, followed by 24 h at 4°C. Toxin-antibody complexes were precipitated by poly(ethylene glycol) [10]

antibody with affinity constants ranging between 2 nM and 1.5 μM. The antibody clearly possesses the capacity for neutralizing any of the toxins. This was demonstrated in vitro by monitoring the binding inhibition to AcChR of four labelled toxins selected for their widely divergent affinities for M$\bar{\alpha}$2-3 (table 1). Since the affinities of the toxins for AcChR are very similar (unpublished) it is clear (table 2) that the inhibition capacity of M$\bar{\alpha}$2-3 follows the order erabutoxin b > toxin α > toxin I > cobrotoxin. Clearly, neutralization efficiency of M$\bar{\alpha}$2-3 correlates with the binding affinities for the toxins.

To delineate the epitope recognized by M$\bar{\alpha}$2-3 we prepared a number of derivatives of both toxin α and erabutoxin b which were chemically modified at a single amino acid residue. Five toxin α derivatives monoacetylated at the terminal amino group, Lys 15, 27, 47 and 51 together with a tryptophan derivatized toxin α have all been shown to possess a spatial structure similar to that of the unmodified toxin [19]. The relative affinities of M$\bar{\alpha}$2-3 for these derivatized toxins are presented in table 3. Acetylations at the NH_2-terminal group, or at Lys 15 or 51, do not alter the affinity from that of the native toxin while acetylations at positions 27 and 47, or Trp 29 modification induce a decrease in affinity by factors of 7, 9 and 20, respectively. Parallel studies with monoacetylated derivatives of erabutoxin b [20] indicate that Lys 27 and 47 of erabutoxin b are involved in the epitope whereas residues 15 and 51 are not. Furthermore, Lys 51 of erabutoxin b can be substituted by an asparagine (erabutoxin c [11]), without affecting the M$\bar{\alpha}$2-3-binding properties. These observations lead to the conclusion that the sites by which neurotoxins bind to M$\bar{\alpha}$2-3 contain at least the two amino groups at position 27 and 47 and the indole group at position 29.

The site by which neurotoxins bind to AcChR has been previously elucidated [12,19,21]. It comprises invariant residues which are mainly distributed on the second and third loops of the toxins and are orientated to the same side of the β-pleated sheet formed by three long and adjacent loops. The three antigenically critical residues 27, 29 and 47 form part of the AcChR-binding site (fig.1). This is illustrated in table 3 where it is

Table 2

Neutralization in vitro of various short-chain neurotoxins by M$\bar{\alpha}$2-3

	Receptor concentration (nM) (1)	Labelled toxin concentration (nM) (2)	Concentration of M$\bar{\alpha}$2-3 at 50% inhibition (μM) (3)
^{125}I-labelled erabutoxin b	0.3	1.1	0.013
^{3}H-labelled toxin α	0.9	1.8	0.250
^{125}I-labelled toxin I	0.1	0.3	1.6
^{125}I-labelled cobrotoxin	0.1	0.1	12

AcChR (column 1) was incubated overnight with the radioactive toxins (column 2) in the presence of various amounts of antibody. The concentration of antibody which inhibits 50% of the binding of each toxin to AcChR is indicated in column 3. Specific activities of ^{3}H-labelled toxin α, ^{125}I-labelled erabutoxin b, ^{125}I-labelled toxin I (kindly provided by P. Bougis) and ^{125}I-labelled cobrotoxin were equal to 30, 120, 670 and 450 Ci/mmol, respectively. The values are means of four independent determinations

Table 3

Relative affinities of various monoderivatized toxins toward M$\bar{\alpha}$2-3 or AcChR

Derivatives of two short-chain neurotoxins	Apparent dissociation constants (K_d)	
	M$\bar{\alpha}$2-3 (nM)	AcChR (pM)
Naja nigricollis toxin α	9	20(b)
(1-N^{α}-acetylleucine) toxin α	9	30(b)
(15-N^{ϵ}-acetyllysine) toxin α	5	90(b)
(27-N^{ϵ}-acetyllysine) toxin α	61	290(b)
(47-N^{ϵ}-acetyllysine) toxin α	85	520(b)
(51-N^{ϵ}-acetyllysine) toxin α	5	90(b)
(29-C_2-NPS tryptophan) toxin α (a)	172	590(b)
Laticauda semifasciata erabutoxin b	2	120
(15-N^{ϵ}-acetyllysine) erabutoxin b	2	nd
(27-N^{ϵ}-acetyllysine) erabutoxin b	15	nd
(47-N^{ϵ}-acetyllysine) erabutoxin b	60	4000(c)
(51-N^{ϵ}-acetyllysine) erabutoxin b	2	nd

K_d values were calculated as indicated in table 1. (a) NPS indicates that a nitrophenyl group has been incorporated on the C_2 position of the indole side chain by a thioether bond. (b) After Faure et al. [19]. (c) After Ishikawa et al. [17]

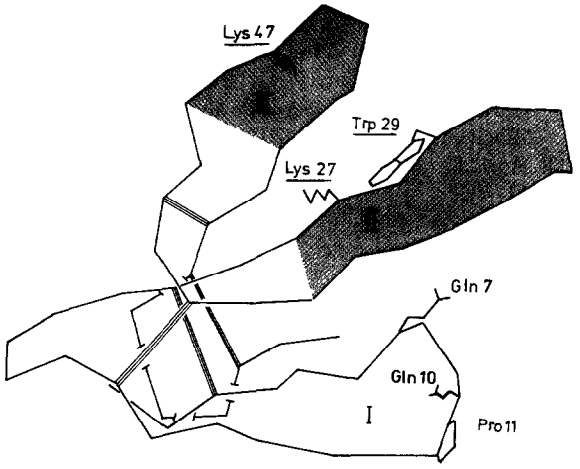

Fig.1. Amino acid residues incorporated in the epitope recognized by M$\bar{\alpha}$2-3. The spatial structure is based on X-ray data of erabutoxin b [23,24] according to Kimball et al. [22]. The three underlined residues at position 27, 29 and 47 are common to both the epitope and the AcChR-binding site. The residues incorporated in the AcChR-binding site [12,19,21] are mainly localized on the shaded loops II and III.

reported that their selective derivatizations always induce a decrease in toxin-binding affinity for AcChR [19]. It is clear therefore that the toxin sites recognized by AcChR and M$\bar{\alpha}$2-3 overlap.

The AcChR-binding site and the epitope, however, are not identical in the strict sense. This is indicated, in particular, by the large affinity differences for M$\bar{\alpha}$2-3 and AcChR of toxin α or erabutoxin b (table 3) and by the lack of cross-reactivity (not shown) of long-chain neurotoxins [25] which are high-affinity competitors of short-chain neurotoxins for AcChR [12,17]. To clarify the differences between the two sites we delineated more precisely the epitope by determining the correlation of cross-reactivity with the seven homologous toxins listed in table 1 and amino acid differences in the primary structures [12,16]. This analysis which will be published in detail elsewhere indicates the critical role of residues 7, 10 and 11 which all occupy positions on the first loop of the toxins and are orientated to the same side of the sheet as residues 27, 29 and 47. We conclude, therefore, that the binding sites of M$\bar{\alpha}$2-3 and AcChR are located on the same face of the β-sheet, overlap within loops II and III and differ from each other in that the epitope extends to the first loop of the molecule.

The monoclonal antibody described here recognizes part of the functional site of the short-chain neurotoxins and possesses accordingly the capacity for both recognizing and neutralizing all homologous molecules that have been tested. This property is of potential importance since it should allow the detection and/or neutralization of such a molecule without knowledge of its actual origin, i.e. the nature of snake responsible for the bite. Four different toxin classes are responsible for the lethal effect of most Elapidae and Hydrophidae venoms [5]. These are short-chain neurotoxins, long-chain neurotoxins, cardiotoxins and phospholipases, all of which present extensive interspecies antigenic variations (review [2]). In principle, therefore, a cocktail of four antibodies each directed to the conserved area of one of the four specific toxins should be sufficient for detecting and/or neutralizing any venom of Elapidae or Hydrophidae. M$\bar{\alpha}$2-3 constitutes the first monoclonal immunoglobulin of this type.

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REFERENCES

[1] Alouf, J.E. (1985) Ann. Inst. Pasteur/Microbiol. 136, 309-321.
[2] Boquet, P. (1979) in: Snake Venoms (Lee, C.Y. ed.) Handb. Exp. Pharm., vol.52, pp.751-824, Springer, Berlin.
[3] Norrby, E. (1985) in: Vaccines 85, pp.387-394, Cold Spring Harbor Laboratory.
[4] WHO Offset Publication (1981) no.58, 1-44.
[5] Chang, C.C. (1979) in: Snake Venoms (Lee, C.Y. ed.) Handb. Exp. Pharm., vol.52, pp.309-376, Springer, Berlin.
[6] Fryklund, L. and Eaker, D. (1975) Biochemistry 14, 2865-2871.
[7] Ménez, A., Morgat, J.-L., Fromageot, P., Ronsseray, A.M., Boquet, P. and Changeux, J.-P. (1971) FEBS Lett. 17, 333-335.

[8] Hunter, W.M. (1974) Br. Med. Bull. 30, 18–23.
[9] Saitoh, T., Oswald, R., Wennogle, L.D. and Changeux, J.-P. (1980) FEBS Lett. 116, 30–36.
[10] Boulain, J.-C., Ménez, A., Couderc, J., Faure, P., Liacopoulos, P. and Fromageot, P. (1982) Biochemistry 21, 2910–2915.
[11] Kohler, G. and Milstein, C. (1975) Nature 256, 495–497.
[12] Dufton, M.J. and Hider, R.C. (1983) CRC Crit. Rev. Biochem. 14, 113–171.
[13] Eaker, D. and Porath, J. (1967) 7th Int. Congr. Biochem., Tokyo 1967, Col. VIII-3, Abstr. III, p. 499.
[14] Nishida, S., Kokubun, Y. and Tamiya, N. (1985) Biochem. J. 226, 879–880.
[15] Abe, T. and Tamiya, N. (1979) Toxicon 17, 571–582.
[16] Tamiya, N. and Yagi, T. (1985) J. Biochem. 98, 289–303.
[17] Ishikawa, Y., Ménez, A., Hori, H., Yoshida, H. and Tamiya, N. (1977) Toxicon 15, 477–488.
[18] Weber, M. and Changeux, J.-P. (1974) Mol. Pharmacol. 10, 1–14.
[19] Faure, G., Boulain, J.-C., Bouet, F., Montenay-Garestier, T., Fromageot, P. and Ménez, A. (1983) Biochemistry 22, 2068–2076.
[20] Hori, H. and Tamiya, N. (1976) Biochem. J. 153, 217–222.
[21] Ménez, A., Boulain, J.-C., Bouet, F., Couderc, J., Faure, G., Rousselet, A., Trémeau, O., Gatineau, E. and Fromageot, P. (1984) J. Physiol. (Paris) 79, 196–206.
[22] Kimball, M.R., Sato, A., Richardson, J.S., Rosen, L.S. and Low, B.W. (1979) Biochem. Biophys. Res. Commun. 88, 950–959.
[23] Low, B.W., Preston, H.S., Sato, A., Rosen, L.S., Seral, J.E., Rudko, A. and Richardson, J.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2991–2994.
[24] Tsernoglou, D. and Petsko, G.A. (1976) FEBS Lett. 68, 1–4.
[25] Walkinshaw, M., Saenger, W. and Maelicke, A. (1980) Proc. Natl. Acad. Sci. USA 77, 2400–2404.