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Neutralizing monoclonal antibody specific for α-bungarotoxin:

Preparation and characterization of the antibody, and localization of antigenic region of α-bungarotoxin

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We prepared an α-bungarotoxin-specific monoclonal antibody that neutralizes the biological activity of the toxin in vivo. The antigenic determinant combining specifically with this antibody was determined on the basis of cross-reaction experiments using three other long neurotoxins and peptide fragments of α-bungarotoxin. The antigenic determinant was located on the peptide fragment containing S34-S35-R36-G37-K38, which forms a part of the expected site that binds to the acetylcholine receptor proteins

Monoclonal antibody; Bungarotoxin, α-; Antigenic determinant

1. INTRODUCTION

Neurotoxins isolated from the venom of *Elapidae* and *Hydrophiidae* families are immunogenic polypeptides with three or four antigenic determinants simultaneously accessible to specific antibodies [1,2]. Because the neurotoxicity can be neutralized by specific antibody, work on the identification of these antigenic determinants and elucidation of the molecular mechanism of this specific neutralization are practically important.

α-Bungarotoxin (αBTx) isolated from the venom of *Bungarus multicinctus* is a long α-neurotoxin. The primary structure of α-BTx was determined [3–5]. The crystalline and solution structures have also been extensively well defined [6–8], so that α-BTx is a good model to investigate the interaction between the antigenic determinant and the antibody. Recently, Chuang and Chang reported the antigenic determinants of α-BTx using polyclonal antibodies and peptide fragments [9]. However, in general, due to the heterogeneity of polyclonal antibodies, quantitative evaluation of the degree of cross-reactivity is not easy. So, it would be particularly suitable to use a monoclonal antibody [10]. In the present study, a monoclonal antibody specific for α-BTx was prepared. The antigenic determinant specific to this antibody (MaB-2311) was specified on the basis of the comparative cross-reactivity to homologous neurotoxins and peptide fragments obtained from limited proteolysis of α-BTx. The antigenic determinant was located on a peptide fragment containing S34-S35-R36-G37-K38 which forms a part of the expected site that binds to the acetylcholine receptor proteins.

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Abbreviations: αBTx, α-bungarotoxin; Tx B, toxin B; Tx III, toxin III; Ls III, *Laticauda semifasciata* III; MaB, monoclonal antibody

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2. MATERIALS AND METHODS

2.1. *Materials*

Lyophilized venom of *Bungarus multicinctus* was purchased from Miami Serpentarium Laboratories. ^{125}I-labeled α-BTx (1.8 mCi/mg) was obtained from ICN Radiochemicals. Male BALB/c and ddY mice were purchased from Shisuoka Agriculture Cooperative Association for Laboratory Animals. α-BTx and toxin B (Tx B) were purified from the venoms of *Bungarus multicinctus* and *Naja naja*, respectively. Toxin III (Tx III) (*Naja naja siamensis*) and Ls III (*Laticauda semifasciata*) were kind gifts from Professors R.C. Hider (University of London) and N. Tamiya (Tohoku University), respectively.

2.2. *Preparation of monoclonal antibody*

Immunization of BALB/c mice (8-week-old) was achieved by subcutaneous injection of 2, 4, 8, and 10 μg of α-BTx (LD_{50}: about 5 μg/mouse) at intervals of 2, 3, and 5 weeks in incomplete Freund's adjuvant (0.1 ml). Two weeks after the last injection, 5 μg of α-bungarotoxin in physiological saline (0.2 ml) was injected intraperitoneally. Four days after the fifth injection, the spleens of two mice were removed. Spleen cells were combined with myeloma cells (NSI/1. Ag 4.1) in a ratio of 1:4 and fused with poly(ethylene glycol) according to the method of Galfré and Milstein [11], using RPMI-1640 medium. Fused cells were plated in HAT medium (2×10^5 cells/well) into 460 wells (0.2 ml/well) of 96-well microtiter plates. Three weeks later, culture supernatants were screened for binding α-BTx by an antigen-solid-phase enzyme-linked immunoassay. Bound antibodies were detected by addition of the immunoglobulin G fraction of peroxidase-conjugated goat anti-mouse immunoglobulins (Cappel). From 9 positive wells, two representative wells were selected and cloned three times. One of the two lines, designated MaB-2311 was chosen for amplification in ascitic fluid. Monoclonal hybridoma cells (1×10^7) were injected intraperitoneally into one BALB/c mouse prestimulated with 0.5 ml of 2,6,10,15-tetramethylpentadecane. One week after inoculation, the mouse was killed and the ascitic fluid was removed and centrifuged ($250 \times g$) for 10 min at 4°C. The cell precipitate was suspended and injected into 10 mice for the next inoculation, which was the same as described above. Sequential inoculations were repeated several times.

Monoclonal antibody MaB-2311 was partially purified from ascites fluid supernatant (10 mice) by ammonium sulfate (33% saturation) and Q Sepharose column chromatography and purification was completed by using an α-BTx-conjugated Affi-gel 10 (Bio-Rad) column (2 ml). The yield of the purified antibody was 1–1.5 mg/ml of ascitic fluid. Purity of MaB-2311 was assessed by SDS-polyacrylamide gel electrophoresis employing 10% gels. The class of MaB-2311 was determined to be IgG_1.

2.3. *Binding assay*

Radiobinding assays were performed according to the procedure of Boulain et al. [10], with minor modifications. Dissociation constant was determined by incubation at 4°C for 18 h of 3 nM monoclonal antibody with ^{125}I-labeled α-bungarotoxin (0.8–4 nM) in 0.2 ml of 50 mM sodium phosphate buffer (pH 7) containing 0.45% NaCl and bovine serum albumin (2 mg/ml). After incubation, 0.04 ml of bovine γ-globulin fraction 2 (25 mg/ml, Miles Laboratories Inc.) was added to the reaction mixture, then, 0.4 ml of 20% (w/v) poly(ethylene glycol) 6000 (BDH Chemicals) was added to precipitate the immunocomplexes. The reaction mixture was stirred and centrifuged ($1500 \times g$) at 4°C for 30 min, and the radioactivity of the pellets was then counted.

In cross-reaction experiments, 6 nM monoclonal antibody was incubated with 6.5 nM ^{125}I-labeled α-bungarotoxin and various amounts of long neurotoxins or peptide fragments from α-bungarotoxin. Each sample was treated as described above.

2.4. *Fragmentation of α-bungarotoxin*

Enzymatic hydrolysis of reduced and carboxymethylated α-bungarotoxin with lysyl endopeptidase was carried out by the method of Ohta et al. [4]. The peptide fragments were separated by reverse-phase high-performance liquid chromatography on a TSK-GEL ODS-120T (Toyo Soda) column (4.6 mm × 15 cm) with a linear gradient from 0 to 30% acetonitrile containing 0.1% (v/v) trifluoroacetic acid.

3. RESULTS

3.1. *Neutralization of α-BTx by MaB-2311*

The effect of monoclonal antibody MaB-2311 on the lethality of α-BTx was studied in vivo. 10 μg of α-BTx, corresponding to a 100% lethal dose, was intraperitoneally injected into mice in the presence or absence of MaB-2311, which corresponds to a 100% lethal dose. The mean time until death in the absence of MaB-2311 was 37 min. However, when we injected mixed solutions of α-BTx and MaB-2311 into two groups of mice with molar ratios (α-BTx/MaB-2311) of 1:1 (group A) and 1:5 (group B), the mean times before death for both groups were significantly prolonged to 160 and 500 min, respectively, showing that MaB-2311 actually neutralizes the lethal effects of α-BTx. When we compared the mice in the two groups, the mice in group A were already dead 200 min after injection, while the mice in group B only showed the initial clinical symptoms of α-BTx poisoning. Thus, the effect of MaB-2311 on the α-BTx-injected mice does not only prolong the mean time until death but also delays the whole process leading to death.

3.2. *Localization of the antigenic determinant recognized by MaB-2311*

Localization of the antigenic determinant was studied on the basis of cross-reaction experiments using MaB-2311 as antibody, ^{125}I-labeled α-BTx as the radioactive antigen and four kinds of unlabeled long neurotoxins – α-BTx, Tx B, Tx III, and Ls III – as competitors. The primary structures of these long neurotoxins are shown in table 1. Inhibition of

Table 1

Primary structures of α-bungarotoxin (α-BTx), toxin B (Tx B), toxin III (Tx III) and Ls III

```
                ----+----1----+----2----+----3----+----4----+----5----+----6----+----7----+
α-BTx   : IVCHTTAT SPISAVTCPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVTCCSTDKCNPHPKQRPG
Tx B    : IRCF  ITP DITSKDCPNGH VCYTKTWCDGFCSSRGKRVDLGCAATCPTVRTGVDIQCCSTDDCDPFPTRKRP
Tx III  : IRCF  ITP DITSKDCPNGH VCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQCCSTDNCNPFPTRKRP
LS III  : RECY  LNP HDT QTCPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIKCCSADKCNTYP
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the binding of ^{125}I-labeled α-BTx to MaB-2311 by unlabeled α-BTx, Tx B, Tx III, and Ls III is shown in fig.1. Inhibition capacity of MaB-2311 followed the order of α-BTx > Tx B > Ls III with IC_{50} of 8 nM, 100 nM and 3 μM, respectively. However, in spite of the high homology between the primary structures of Tx B and Tx III, the latter did not show any competition. Both toxins contain 71 amino acid residues and only 5 amino acid residues of G32, S34, R53, D66 and D67 in Tx B are replaced by A32, I34, K53, N66 and N67 in Tx III. The primary structures of these long neurotoxins were compared [12,13] on the basis of the present results of the cross-reactivity experiments. The amino acid sequence common to α-BTx, Tx B, and Ls III was found to be C33-S34-S35-R36-G37-K38. However, in Tx III, S35 is replaced by I35. Thus, we concluded that the antigenic determinant recognized specifically by MaB-2311 is localized in the amino acid sequence around S35. This sequence is located on the top of the central loop and is expected to be involved in the receptor binding. It is also to be noted that there is a disulfide bridge between C29 and C33 that may give a specific conformation around this segment.

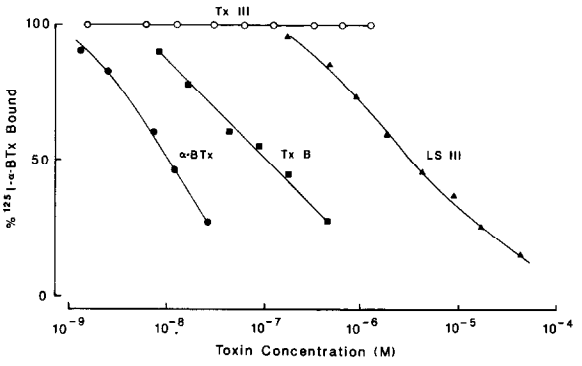

Fig.1. Binding of ^{125}I-labeled α-bungarotoxin (α-BTx) to monoclonal antibody MaB-2311 in the presence of varying amounts of unlabeled α-bungarotoxin (α-BTx), toxin B (Tx B), toxin III (Tx III) or Ls III. In these experiments, 6 nM monoclonal antibody was incubated with 6.5 nM ^{125}I-labeled α-bungarotoxin and varying amounts of long neurotoxins.

3.3. *Cross-reactivity of peptide fragments of α-BTx*

In order to elucidate the effect of the disulfide bridge on the binding of α-BTx to MaB-2311, we studied the cross-reactivities of the reduced and carboxymethylated α-BTx (RCM α-BTx) and its peptide fragments. The peptide fragments shown in table 2 were prepared by the limited proteolysis of RCM α-BTx using lysylendopeptidase after the method by Ohta et al. [4]. Cross-reactivities of RCM α-BTx and the peptide fragments were studied in a similar manner as used for long neurotoxins. RCM α-BTx and the peptide fragment L-5 showed similar inhibition of binding between ^{125}I-labeled α-BTx and antibody, and their IC_{50} values were found to be 80 μM and 160 μM, respectively, while, no competition was found with other five peptide fragments (fig.2). Since RCM α-BTx and L-5 showed cross-reactivity, the disulfide bridge between C29 and C33 is not essential for the bind-

Table 2

Probable sequences of peptides from the proteolytic digestion of α-bungarotoxin with lysylendopeptidase

Peptide	Amino acid sequence
L-1 (71–74)	Q-R-P-G
L-2 (65–70)	C-N-P-H-P-K
L3- (52–64)	K-P-Y-E-E-V-T-C-C-S-T-D-K
L-4 (39–51)	V-V-E-L-G-C-A-A-T-C-P-S-K
L-5 (27–38)	M-W-C-D-A-F-C-S-S-R-G-K
L-6 (1–26)	I-V-C-H-T-T-A-T-S-P-I-S-A-V-T-C-P-P-G-E-N-L-C-Y-R-K

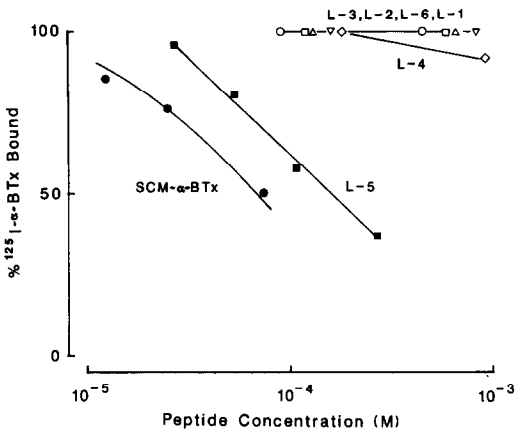

Fig.2. Binding of ^{125}I-labeled α-bungarotoxin to monoclonal antibody MaB-2311 in the presence of varying amounts of reduced and carboxymethylated α-bungarotoxin and its peptide fragments. The sequence of each peptide fragment is shown in table 2.

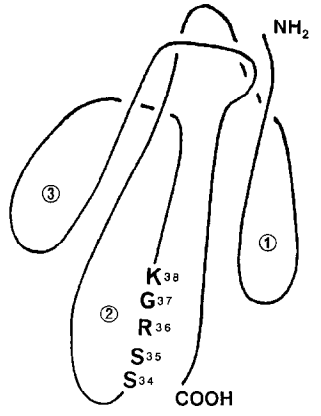

Fig.3. Location of the antigenic determinant recognized by MaB-2311 on the schematic structure of α-bungarotoxin elucidated by X-ray and NMR analyses [6–8].

ing to MaB-2311. Thus, we reached the conclusion that the antigenic determinant recognized by MaB-2311 is S34-S35-R36-G37-K38.

4. DISCUSSIONS

This is the first report on the preparation of a monoclonal antibody specific for α-BTx. From the present study, monoclonal antibody MaB-2311 was found to neutralize the toxicity of α-BTx and other long neurotoxins by specific binding to the antigenic determinant of S34-S35-R36-G37-K38. Using polyclonal antibodies and peptide fragments, Chuang and Chang recently reported that four antigenic determinants were located on α-BTx [9]. One of the antigenic determinants was located around residues 34–41. The antigenic determinant elucudated in the present study was restricted to a narrower range, to residues 34–38. Moreover, MaB-2311 was found to bind to long neurotoxins with the sequence of S34-S35-R36-G37-K38 but not to those with the sequence of S34-I35-R36-G37-K38. This means that S35 is mainly responsible for the binding specified by MaB-2311. The crystalline and solution structures of long neurotoxins elucidated by X-ray and NMR analysis are composed of three polypeptide loops that are shown in fig.3 [6–8]. A three-stranded antiparallel-pleated β-sheet structure is formed by the central and the third loops, and is expected to be common to all neurotoxins. The antigenic determinant is located around the top of the central loop (fig.3). On the basis of the sequence homology and chemical modification studies, the side chains of the amino acid residues located on one surface of the three-stranded antiparallel-pleated β-sheet structure are suggested to be essential for neurotoxicity [12,13]. These include K27, W29, D31, R36, and K47. Since R36 is involved in the region of the antigenic determinant recognized by MaB-2311, neutralization of toxicity by MaB-2311 is induced by the direct binding of MaB-2311 to the toxic region. However, it should be noted that the side chain of S35 is located on the opposite surface of this β-sheet structure. Thus, MaB-2311 may bind to the region from the back of the toxic surface and induce a conformational change that inhibits the binding of toxins to the receptor proteins.

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REFERENCES

[1] Chang, C.C. and Yang, C.C. (1969) J. Immunol. 102, 1437–1444.
[2] Menez, A., Boulain, J.C. and Fromageot, P. (1979) Toxicon 17, suppl. 1, 123.
[3] Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. and Lee, C.Y. (1972) Hoppe-Seylers Z. Physiol. Chem. 353, 243–262.

[4] Ohta, M., Ohta, K., Nishitani, H. and Hayashi, K. (1987) FEBS Lett. 222, 79–82.
[5] Kosen, P.A., Finer-Moore, J., McCarthy, M. and Basus, V.J. (1988) Biochemistry 27, 2775–2781.
[6] Inagaki, F., Hider, R.C., Hodges, S.J. and Drake, A.F. (1985) J. Mol. Biol. 183, 575–590.
[7] Basus, V.J., Billeter, M., Love, R.A., Stroud, R.M. and Kuntz, I.D. (1988) Biochemistry 27, 2763–2771.
[8] Stroud, R.M. (1982) Proc. 2nd SUNYA Conversion Discipline Biomolecular Stereodynamics (Sarma, R.H. ed.) vol. 11, p. 55, Academic Press, New York.
[9] Chuang, L.Y. and Chang, C.C. (1988) Biochem. Biophys. Res. Commun. 155, 870–876.
[10] Boulain, J.C., Menez, A., Couderc, J., Faure, P., Liacopoulos, P. and Fromageot, P. (1982) Biochemistry 21, 2910–2915.
[11] Galfré, G. and Milstein, C. (1981) Methods Enzymol. 73, 3–46.
[12] Dufton, M.J. and Hider, R.C. (1983) CRC Crit. Rev. Biochem. 14, 113–171.
[13] Endo, T. and Tamiya, N. (1987) Pharmacol. Ther. 34, 403–451.