Neutralizing Monoclonal Antibody Specific for *Naja nigricollis* Toxin α: Preparation, Characterization, and Localization of the Antigenic Binding Site†

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ABSTRACT: One homogeneous population of high-affinity monoclonal antibodies (K_D = 0.35 nM) specific for *Naja nigricollis* toxin α has been produced. It neutralizes the biological activity of the toxin under both in vivo and in vitro conditions. The molecular zone of the toxin to which the antibody binds has been precisely defined on the basis of cross-reaction experiments using five derivatives of toxin α monomodified at a single amino group and two naturally occurring homologous toxins. The epitope is located at the base of the first β-sheet loop of the toxin, involving the two positive charges at the N-terminal position and lysine-15, proline-18, and probably threonine-16. It is shown that this region is topographically distinct from the "toxic" site of toxin α. Several possibilities are offered to explain the mechanism(s) of specific neutralization.

Despite their relatively small size (60–74 residues), neurotoxins isolated from *Elapidae* and *Hydrophiidae* venoms are immunogenic polypeptides with three or four antigenic determinants simultaneously accessible to specific antibodies (Yang, 1979; Ménez et al., 1979; Boulain, 1979). Thus, their potent toxic action, which results from their ability to inhibit acetylcholine-induced depolarizations of excitable tissue (Chang, 1979), can be neutralized by specific immune serum (Boquet, 1979).

Obviously elucidation of the antigenic structure of these neurotoxins is essential for a clear comprehension of the molecular mechanism(s) associated with specific neutralization. This problem has rarely been considered. Preliminary studies only, based on cross-reaction experiments between homologous toxins, have been reported for toxin α from *Naja nigricollis* (Ménez et al., 1979; Boulain, 1979; Boquet, 1979) and erabutoxin b from *L. semifasciata* (Abe & Tamiya, 1979), using horse and rabbit antisera. The results of these studies are consistent and lead to the proposal that 10–15 residues are involved in the antigenic structure of neurotoxins and approximately 12 residues are excluded from such a structure.

In general, two major complications limit the scope of such analyses. First, the homologous proteins usually possess more than one amino acid substitution, and consequently it is difficult to estimate the specific contribution of any single residue in the antigenicity of the protein. Second, as a result of the heterogeneity of the antisera used, quantification of the degree of cross-reactivity is not easy. In order to overcome these two difficulties, it would be particularly suitable to (i) use derivatives of the protein chemically modified at a single residue, always assuming that the modification does not destroy the structure of the protein, and (ii) use a homogeneous population of antibodies specific for the protein.

In the present paper we report (i) the preparation of a monoclonal antibody population ($M\bar{\alpha}_1$) specific for toxin α from *N. nigricollis*, (ii) the binding properties between this antibody and ^{3}H-labeled toxin α, (iii) the neutralizing effect of this antibody as observed under both in vivo and in vitro conditions, and (iv) the location of the antigenic site which binds specifically to the antibody. The latter observation was based on cross-reaction experiments using recently prepared monoacetylated derivatives of toxin α (Faure et al., 1981; Faure, 1980) and two naturally occurring homologous polypeptides.

Experimental Procedures

Materials

Toxin α was purified from *Naja nigricollis* venom (Pasteur Institute, Paris) as described previously (Fryklund & Eaker, 1975). CNBr–Sephadex 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Ion-exchange resins were purchased from Bio-Rad Chemical Co., [^{14}C]acetic anhydride and tritium gas were from C.E.A. (Saclay, France), and the high-pressure liquid chromatography (HPLC) C_{18} μBondapak column was from Waters Associates (Milford, MA). Freund's adjuvant was purchased from Difco Laboratories (Detroit, MI) and Balb/c mice were from CNRS (Orléans, France). RPMI, medium, HAT mixture, fetal calf serum, and normal horse serum were purchased from GIBCO Europe (Glascow Great Britain). Poly(ethylene glycol) (PEG), M_r 1000 or 6000 (for radioimmunoassay only), was obtained from BDH Chemical Co. (Poole, Great Britain). The 96-well Linbroplates were supplied from Flow Laboratories and 2,6,10,14-tetramethylpentadecane was from Aldrich-Europe (Beerse, Belgium). Lipoluma and Lumagel were obtained from Lumac, France.

Methods

Incorporation of tritium label into toxin α polypeptide chain was made by catalytic dehalogenation of the iodinated precursor in the presence of tritium gas as described previously (Ménez et al., 1971). Pure ^{3}H-labeled toxin α had a specific radioactivity of 45 Ci/mmol, and the percentage of counts specifically bound to an excess of acetylcholine receptor rich membrane fragments was 80%.

Polyacetylated and monoacetylated derivatives of *N. nigricollis* toxin α were prepared according to a method which will be published in detail elsewhere (Faure, 1980). In brief [^{14}C]acetic anhydride (4 μmol) reacted with an equivalent amount of native toxin α in 1.5 mL of 0.1 M sodium phosphate

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buffer, pH 7. The reaction mixture was initially fractionated by ion-exchange chromatography (Bio-Rex 70), pH 6.4, to which an ammonium acetate gradient was applied. Polyacetylated derivatives eluted first, followed by four monoacetylated fractions. Unreacted toxin α eluted finally. Each monoacetylated fraction was rechromatographed on a C_{18} μBondapak column. Five monoacetylated derivatives were pure as judged from analytical HPLC and gel electrophoresis. Native toxin α and each monoacetylated derivative were reduced, carboxymethylated, and submitted to enzymatic digestion with trypsin. Tryptic peptides were fractionated by HPLC on a C_{18} μBondapack column. Examination of the amino acid composition of the resolved tryptic peptides enabled us to localize the position of each incorporated acetyl group in the toxin. It was observed that lysine residues at positions 15, 27, 47, and 51 as well as the N-terminal positive group were selectively monoacetylated.

Production of Purified Monoclonal Antibodies. Immunization of mice was achieved by injecting subcutaneously 2, 10, and 20 μg of native toxin α at 2-week intervals in complete Freund's adjuvant (total volume 0.2 mL). One month after the last injection, 20 μg of polyacetylated toxin α in 9% NaCl (0.2 mL) was injected intraperitoneally. Four days after this fourth injection, one mouse was killed and its spleen removed. The fusion procedure was carried out according to Köhler & Milstein (1975): 10^8 spleen cells were mixed with 10^7 myeloma cells (X63 NS) in RPMI medium and centrifuged at 1000 rpm for 5 min. The pellet was slowly (1 min) resuspended at 37 °C in 1 mL of PEG–RPMI (50% w/v) with stirring and further incubated for 1 min. The cell suspension was then diluted with 20 mL of RPMI and centrifuged 5 min at 1000 rpm. The pellet was resuspended in 1.5 mL of RPMI supplemented with 20% (v/v) fetal calf serum containing 2 mM glutamine and antibiotic, for 30 min at 37 °C. The mixture was diluted with the same medium supplemented with HAT (hypoxanthine, aminopterin, and thymidine) and 10^5 macrophages, thus enabling the cells to be plated into 70 wells (1 mL/well) of Linbroplates. Antitoxin α occurrence was tested 2–3 weeks later by radiobinding assays, and positive wells were cloned into 96-well microtiter plates. Monoclonal hydridoma cells (5×10^6) producing antitoxin α were injected intraperitoneally into one Balb/c mouse prestimulated with 0.5 mL of 2,6,10,14-tetramethylpentadecane. One week later, ascitic fluid was withdrawn and centrifuged 5 min at 3000 rpm. The supernatant was stored at 4 °C for 2 days, centrifuged at 10 000 rpm for 20 min, and filtered 3 times through a toxin α–Sepharose 4B column preequilibrated in 0.2 M Tris-HCl buffer, pH 8, containing 0.5 M NaCl. The column was extensively washed with the same buffer, and bound antibodies were finally eluted by using a 0.2 M HCl–glycine buffer, pH 2. The eluate was immediatly adjusted to pH 8 with 2 M Tris-HCl buffer. Purified antibodies were concentrated with immersible CX30 ultrafilters (Millipore) and dialyzed against 0.5 M NaCl.

The immunoglobulin subclass was characterized by double diffusion in plates (Ouchterlony's method) using specific rabbit antimouse immunoglobulin subclass antisera.

Binding Assays. Radiobinding assays were carried out by using poly(ethylene glycol) 6000 at a final concentration of 12.5% (w/v) to precipitate the antigen–antibody complexes. The Linbroplate culture supernatants (0.1–0.2 mL) were incubated at 4 °C overnight with 0.1 mL (0.6 pmol) of ^{3}H-labeled toxin α (45 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 then added to the solution which was stirred and centrifuged at 3000 rpm in a Sorvall HS4 rotor at 4 °C for 30 min. The pellets were dissolved in 0.75 mL of water and 10 mL of Lumagel solution, and the radioactivity was counted by using an Intertechnique counter. The background was less than 5% of the total radioactivity.

Saturation curves were determined by incubating at 4 °C overnight 0.2 nM purified monoclonal antibody with ^{3}H-labeled toxin α (0.05–4 nM) in 0.3 mL of 0.1 M phosphate buffer, pH 7. The complex was treated as described above. Each measurement was made in triplicate.

In competition experiments, 0.5 nM antibody was incubated overnight at 4 °C in the presence of 2 nM ^{3}H-labeled toxin α with various amounts of toxin α derivatives (from 0.15 nM to 1 μM). Each sample was treated as above. Equilibrium dissociation constants for each derivative were derived from the resulting competition curves, according to the method described (Ishikawa et al., 1977).

Neutralization by Purified Monoclonal Antibodies. Native toxin α (3 μg/mouse) was injected intraperitoneally in the presence and absence of 1 mg of purified monoclonal antibody (Table I). Mice were examined 24 h after injection.

In vitro experiments were carried out as follows: acetylcholine receptors, obtained from electric organs from *Torpedo marmorata* (0.28 nM) (Sobel et al., 1977), were incubated for 24 h at 4 °C in the presence of 0.09 nM ^{3}H-labeled toxin α with various amounts of antibody in 2 mL of Ringer's buffer. The mixtures were filtered through two Millipore filters (HAWP 0.45 μm) which were washed with 30 mL of 4 °C Ringer's solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM pH 7 sodium phosphate, 0.15 mM Triton X-100). The dried filters were counted in 10 mL of Lipoluma.

Results

Preparation of Monoclonal Antibodies. Hyperimmunization of mice necessitated injections of relatively high doses of antigen. In the case of cobra neurotoxins, the injectable amount was obviously limited. Consequently 2 μg of toxin α (approximately one LD_{50}) was first injected subcutaneously in the presence of Freund's adjuvant which delays the toxin distribution; 2 and 4 weeks later, 10 and 20 μg, respectively, were injected into survivors. A fourth and last injection was made intraperitoneally 1 month later by using 20 μg of polyacetylated toxin α. This derivative was only 20% as toxic as native toxin but was fully antigenic as demonstrated by cross-reaction experiments (Faure, 1980).

Fusion of spleen cells with *myeloma* cells leads to hybrid growth in 69 out of 70 wells containing the selective HAT medium. The cells were cloned, and four wells produced antibodies specific for toxin α as revealed by radioimmunoassay, but only one of these suitably developed. This clone was grown intraperitoneally in mice, and approximately 2 mL of ascites fluid per mouse was withdrawn. Monoclonal antibodies were purified from ascites fluid by affinity chromatography on a toxin α–Sepharose column yielding about 2 mg of pure antibody per mouse. The purified antibody population, so called $M\bar{\alpha}_1$, corresponds to mouse $Ig2_a$ isotype.

Binding of $M\bar{\alpha}_1$ to Tritium-Labeled Toxin. Figure 1 shows that the binding of ^{3}H-labeled toxin α (45 Ci/mmol) to monoclonal antibodies ($M\bar{\alpha}_1$) is saturable. There is little nonspecific binding as indicated by the low level of bound radioactivity (1–2% of total radioactivity bound) when the experiment is done in the presence of an excess of unlabeled toxin.

A Scatchard analysis of the data yields a straight line indicative of the antibody homogeneity. The binding affinity of ^{3}H-labeled toxin α derived from the slope of the Scatchard

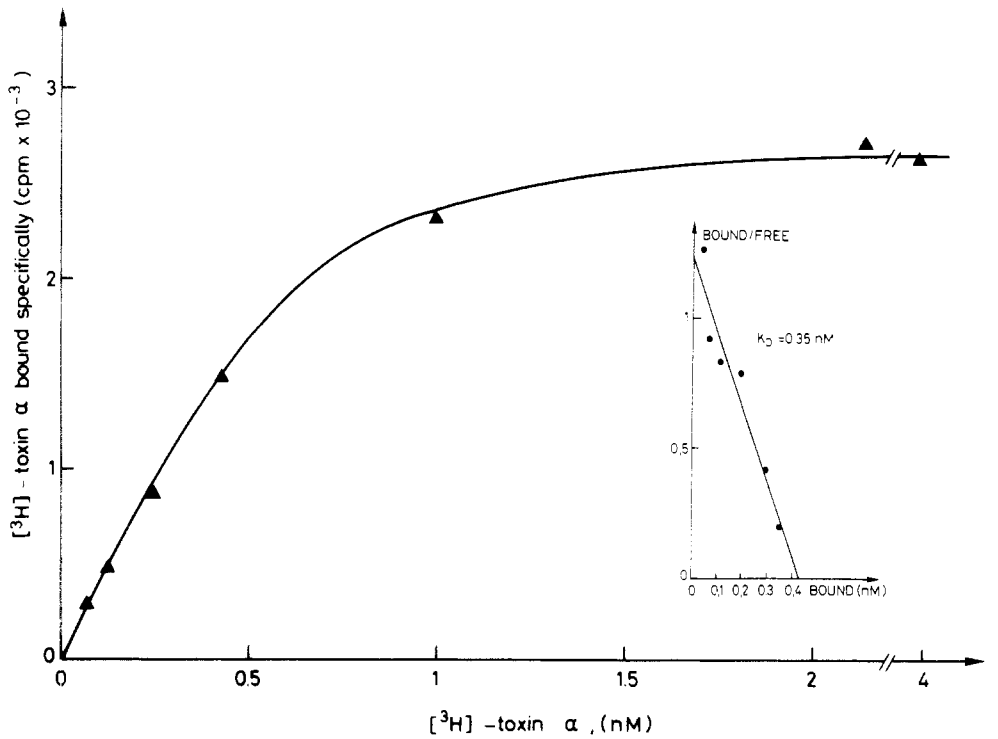

FIGURE 1: Specific binding of ^{3}H-labeled toxin α to monoclonal antibody. Antitoxin (0.2 nM) was incubated overnight at 4 °C with various concentrations of tritiated toxin. Bound radioactivity was measured as described (see Methods). The inset represents a Scatchard plot of the data obtained. The straight line was calculated by the method of least squares.

Table I: Neutralizing Effect of 1 mg (~7 nmol) of Monoclonal Antibodies $M\bar{\alpha}_1$ under in Vivo Conditions[a]

mixture injected	no. of dead mice/ no. of injected mice
3 μg of toxin α	10/10
1 mg of $M\bar{\alpha}_1$	0/8
3 μg of toxin α + 1 mg of $M\bar{\alpha}_1$	0/10

[a] 3 μg (~0.4 nmol) of native toxin α was injected intraperitoneally in the presence and absence of 1 mg of $M\bar{\alpha}_1$ into 18–20 g of Wistar-Saclay mice.

plot is high (K_D = 0.35 nM) and comparable to that observed for the binding of myoglobin to specific monoclonal antibodies (Berzofsky et al., 1980).

Neutralization of Toxin α by $M\bar{\alpha}_1$. Intraperitoneal injections made with 3 μg of toxin α, in the presence and absence of approximately 1 mg of monoclonal antibodies, revealed that the latter totally inhibits the lethal effect of the toxin (Table I). This is in agreement with the experiments made in vitro which show that at equilibrium, increasing concentrations of monoclonal antibodies inhibit the specific binding of ^{3}H-labeled toxin α to acetylcholine receptor rich membranes (Figure 2). The inhibition proceeds in a narrow range of antibody concentration, and the experimental values obtained fit a calculated curve based on the assumption that simple competition occurs between receptor and antibody molecules for toxin α with K_D values of 0.02 nM (Weber & Changeux, 1974) and 0.1 nM, respectively.

Localization of the Epitope Which Binds to $M\bar{\alpha}_1$. Localization of the antigenic site was made on the basis of cross-reaction experiments using $M\bar{\alpha}_1$ as antibodies, ^{3}H-labeled toxin α as the radioactive antigen, and various derivatives of toxin. Figure 3 represents the inhibition of ^{3}H-labeled toxin α binding to $M\bar{\alpha}_1$ by native toxin α (continuous curve) as compared to that obtained (points) by (1) five derivatives of toxin α monoacetylated at Lys-15, Lys-27, Lys-47, Lys-51, and the N-terminal position, (2) *N. nigricollis* toxin α′, a toxin which

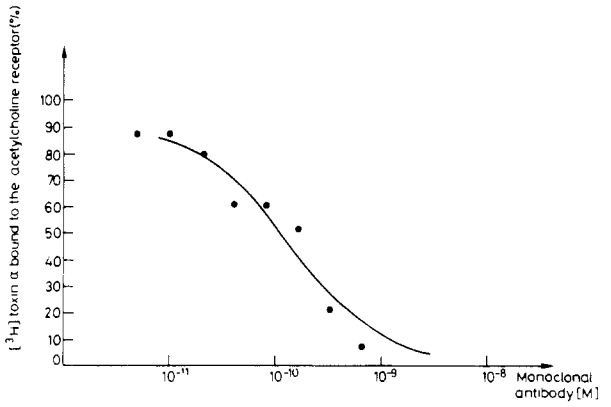

FIGURE 2: Binding of ^{3}H-labeled toxin α to the acetylcholine receptor in the presence of varying amounts of monoclonal antibody. Acetylcholine receptor rich membranes (0.28 nM) were incubated with ^{3}H-labeled toxin α (0.09 nM) together with various amounts of antibody for 24 h at 4 °C. The mixtures were filtered through Millipore filters, and the bound radioactivity was counted. Each measurement was made in triplicate. The curve was calculated by assuming that the receptor and the antibody simply compete for the toxin with affinity constants of 0.02 nM and 0.1 nM, respectively. Similar results were obtained when the period of incubation was 72 h.

differs from toxin α by two single residues at positions 1 and 2, and (3) *N.n. oxiana* toxin II which differs from *N. nigricollis* toxin α by eight residues at positions 18, 28, 30, 45, 50, 51, 56, and 59 (Grishin et al., 1973).

It is clear that native toxin α, toxin α′, [27-N^{ϵ}-monoacetyllysine]toxin α, [47-N^{ϵ}-monoacetyllysine]toxin α, and [51-N^{ϵ}-monoacetyllysine]toxin α are all approximately equally potent at competing with ^{3}H-labeled toxin α for the binding to $M\bar{\alpha}_1$. In contrast, [15-N^{ϵ}-monoacetyllysine]toxin α, [1-N^{α}-monoacetylleucine]toxin α, and *N.n. oxiana* toxin α are much weaker competitors. Binding affinities for each derivative were derived from the results shown in Figure 3 by using a method based on that described by Ishikawa et al. (1977).

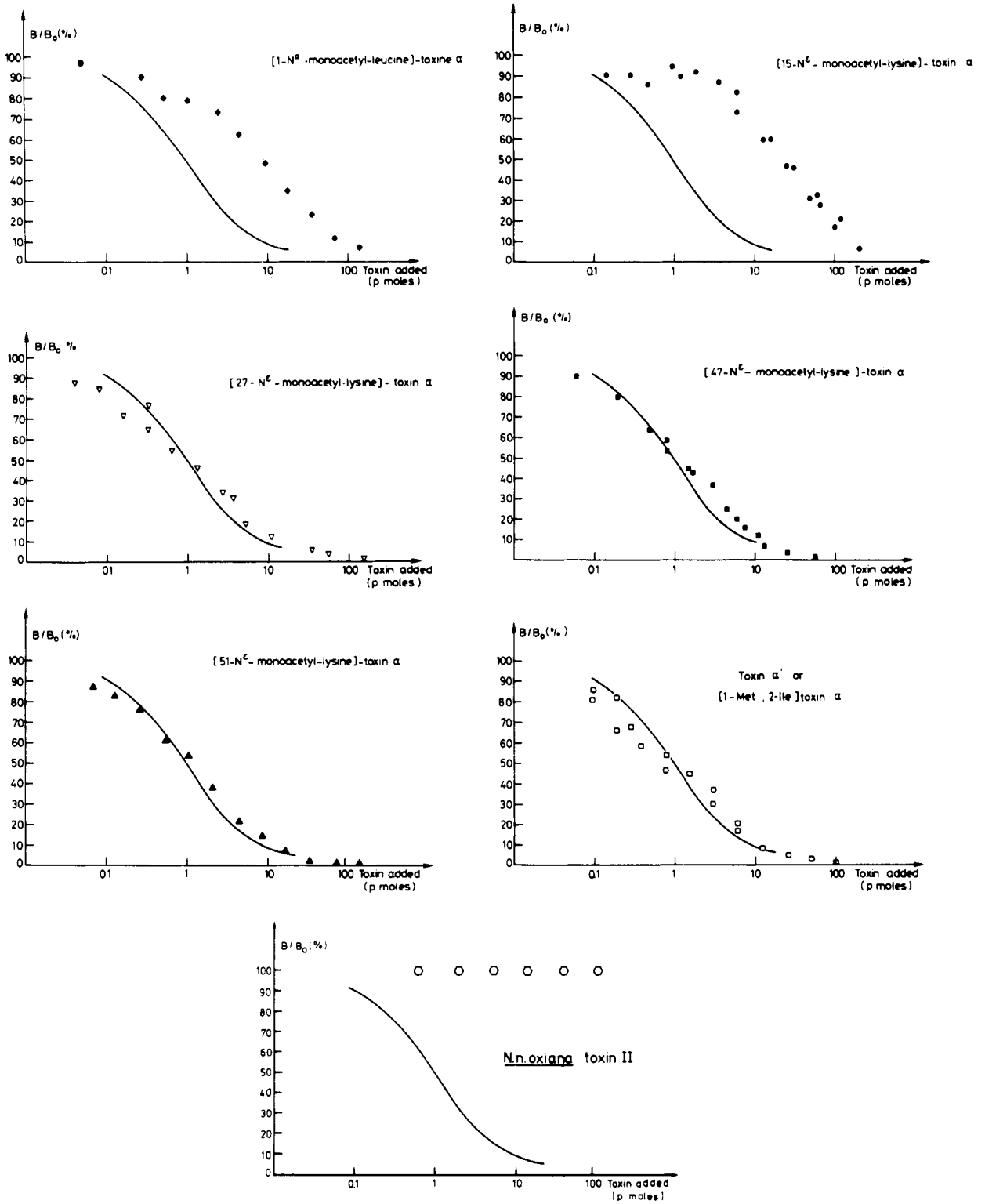

FIGURE 3: Binding of ^{3}H-labeled toxin α to the monoclonal antibody in the presence of varying amounts of a range of toxin α derivatives. Concentrations of tritiated toxin and antibody were 2 and 0.5 nM, respectively. In all cases the continuous curve corresponds to the inhibition observed in the presence of *native* toxin α.

Knowing that the binding affinity of toxin α for M$\bar{\alpha}_1$ is 0.35 nM (see above), it was found that the derivatives of toxin α monoacetylated at Lys-27, Lys-47, and Lys-51 and toxin α all have binding affinities close to 0.5 nM while those mono-modified at the N-terminal position and Lys-15 have affinities equal to 4.5 nM and 11.5 nM, respectively. The binding affinity of *N.n. oxiana* toxin α was not determined.

Discussion

In the present study we prepared a monoclonal antibody (M$\bar{\alpha}_1$) specific for toxin α, a well-characterized polypeptide present in *Naja nigricollis* venom, using the recently described technique of somatic cell fusion (Köhler & Milstein, 1975). Since toxin α is toxic to mammals, the immunization procedure has of necessity been adapted: a partially detoxified derivative of toxin α (polyacetylated toxin α) was used for the last injection into hyperimmune mice, prior to removal of the spleen cells. However, the monoclonal antibody resulting from this method did not exhibit a higher affinity for the acetylated derivatives when compared to that of the native toxin (Figure

3), implying that the latter was the actual antigen in the course of the immunization process. The high binding affinity (K_D = 0.35 nM) of tritium-labeled toxin α to M$\bar{\alpha}_1$ is in agreement with this assumption.

As demonstrated several years ago, the toxic effect of a cobra neurotoxin, the induced peripheral paralysis of respiratory muscles, can be inhibited by a specific antiserum obtained from an immunized animal. In that case three or four antibody molecules simultaneously bind to the surface of one toxin molecule (Yang, 1979; Ménez et al., 1979; Boulain, 1979). The present study has shown that a single specific monoclonal antibody produces qualitatively the same effect. The results of experiments made under in vitro conditions are entirely consistent with this observation. Indeed the binding of ^{3}H-labeled toxin α to the nicotinic acetylcholine receptor, the well-known physiological target of cobra neurotoxins, is totally inhibited by a slight excess of monoclonal antibody. The mechanism of this inhibition is simply explained on the basis of a competition between the receptor and antibody for the toxin. In order to understand at a molecular level the manner by which M$\bar{\alpha}_1$ neutralizes the toxin–receptor interaction, it was essential to determine the molecular region to which M$\bar{\alpha}_1$ binds and to compare this structure with that to which the receptor binds.

The epitope of toxin α specific for M$\bar{\alpha}_1$ has been approximatively localized on the basis of cross-reaction experiments using toxin α derivatives modified at a single lysine residue. It should be stressed that evidence is now being accumulated which indicates that the overall structural characteristics of these compounds are similar to those of native toxin α (Faure, 1980). This being the case, the change in cross-reactivities as observed for the monoacetylated N-terminal and lysine-15 derivatives only is a result of the neutralization of the positive charges at either position 1 or 15 and not due to indirect conformational modifications. That these two derivatives remain fully biologically active (Faure, 1980) strongly strengthens this argument. Thus it is concluded that these two positive charges which are separated by 10.4 Å (Kimball et al., 1979) are involved in the antigenic structure to which M$\bar{\alpha}_1$ binds. In contrast the positive charges of lysine residues at positions 27, 47, and 51 are clearly outside the epitope, as demonstrated by their native toxin-like cross-reactivities.

Examination of a model based on the X-ray structure of an homologous toxin (Kimball et al., 1979) reveals that the epitope thus defined is at the base of the first β-sheet loop of the toxin (Figure 4A). In order to obtain a more precise description of the antigenic site, we examined the cross-reactivities of two homologous toxins with appropriate substitutions. *N. nigricollis* toxin α' (Fryklund & Eaker, 1975), the sequence of which differs from that of toxin α only by two single residues at positions 1 and 2 (Leu and Glu in the latter are replaced by Met and Ile in the former), exhibits a toxin α like cross-reactivity. This result demonstrates that neither of the two side chains of residues 1 and 2 is involved in the epitope to which M$\bar{\alpha}_1$ binds, in spite of their proximity (~4–5 Å) to the N-terminal positive charge. *N.n. oxiana* toxin II has a sequence which differs from that of *N. nigricollis* toxin α by eight residues, but seven of them, that is, residues at positions 28, 30, 45, 59, 51, 56, and 59, are clearly far from the base of the first β-sheet loop (Kimball et al., 1979). Since this toxin possesses an overall structure similar to that of *N. nigricollis* toxin α (Bystrov et al., 1978), we conclude that the lack of cross-reaction observed for this homologous polypeptide is due to the substitution occurring at position 18 only (Pro → Ser), indicating the antigenic importance of this residue.

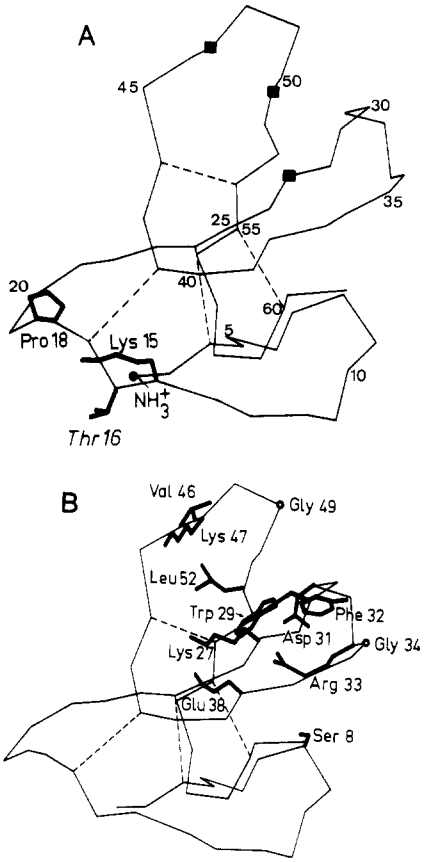

FIGURE 4: (A) Amino acids associated with the antigenic site of toxin α specific for the binding with M$\bar{\alpha}_1$. Selective acetylation of any of the three lysine residues at positions 27, 47, and 51 (black squares) does not alter the binding of toxin α with M$\bar{\alpha}_1$. (B) The residues believed to be directly responsible for neurotoxicity. The backbone configuration is based on the data of Kimball et al. (1979).

It should be stressed that the hydroxyl group of Thr-16 is localized in between the positive charges at lysine-15 and the N terminal (Kimball et al., 1979). As a result, we wish to suggest that this residue also belongs to the epitope. Thus the latter appears to involve the two positive charges at positions 1 and 15, the proline residue at position 18 and probably the threonine residue at position 16. Recent data obtained from cross-reaction studies of various homologous "short" neurotoxins with horse antiserum prepared against *N. nigricollis* toxin α indicated that residues 15, 16, and 18 are involved in the antigenic structure of toxin α (Ménez et al., 1979; Boulain, 1979). Therefore, the conclusions drawn from experiments made with complete antiserum entirely support those emerging from the present study.

The amino acids believed to be directly involved in binding to the acetylcholine receptor (Low, 1979; Tamiya et al., 1980; Ménez et al., 1982) are indicated in Figure 4B. They are mostly distributed on the second and third β-sheet loops of the molecule, and except for Ser-8 and Val-46, they are oriented approximately in the same direction toward the viewer. As such the acetylcholine receptor binding site and the antigenic determinant shown in Figure 4A do not overlap. As a result, at least two possibilities can be offered to explain the inhibition of the toxin biological activity by the monoclonal antibody: (i) For steric reasons, the toxin molecule cannot be bound simultaneously to the receptor and antibody molecules. This is possible if the toxin binding site of the receptor is simply embedded in a narrow cavity of the postsynaptic membrane. (ii) The toxin bound to the antibody molecule adopts a conformation which is not recognized by the receptor. Immu-

noglobulin-induced conformational changes in proteins are certainly well established (Celada & Strom, 1972). Work designed to obtain information on these points is currently in progress.

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Dual Effects of Pyridoxal 5′-Phosphate on Glucocorticoid–Receptor Complexes†

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ABSTRACT: The ability of pyridoxal 5′-phosphate to inhibit DNA–cellulose binding of activated glucocorticoid–receptor complexes is pH and protein concentration dependent. At the tested pHs, all of the inhibitory activity of pyridoxal 5′-phosphate appears to be due to its ability to form a Schiff base. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (100 mM) is unable to prevent or reverse the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding, while the same concentration of lysine is partially effective. Pyridoxal 5′-phosphate does not alter the elution profile of glucocorticoid–receptor complexes as ascertained by diethylaminoethyl (DEAE)-cellulose or DEAE-Sephadex chromatography. This observation permitted the use of these resins in detecting the previously unreported stimulation of glucocorticoid–receptor complex activation by pyridoxal 5′-phosphate. This stimulation is specific for pyridoxal 5′-phosphate and appears to be mediated via a Schiff base formation. Additionally, glucocorticoid–receptor complexes activated by pyridoxal 5′-phosphate treatment at low temperatures do not differ in size from thermally activated complexes. Thus, in vitro, pyridoxal 5′-phosphate can exert both a stimulatory effect on activation as well as an inhibitory effect on the binding of activated complexes to DNA–cellulose.

Glucocorticoid–receptor complexes undergo a two-step process in order to bind to nuclei, chromatin, or purified DNA (Baxter et al., 1972; Milgrom et al., 1973; Simons et al., 1976). The first step, termed "activation", is presumed to involve a conformational change resulting in a more positively charged molecular species with an increased affinity for DNA and other polyanions (Milgrom et al., 1973; Kalimi et al., 1975). This conformational change is reflected in the observed shift in the elution of glucocorticoid–receptor complexes from DEAE-Sephadex and DEAE-cellulose columns at lower salt concentrations (Parchman & Litwack, 1977; Sakaue & Thompson, 1977). The second step, termed "translocation", involves the movement and subsequent binding of the activated complex to a nuclear acceptor site. Thus, the receptor-mediated biological response will be observed only when activation is followed by translocation.

Endogenous inhibitors of either the activation or the

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