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ANTIBODY-MEDIATED NEUTRALIZATION AND BINDING-REVERSAL STUDIES ON α-NEUROTOXINS FROM *MICRURUS NIGROCINCTUS NIGROCINCTUS* (CORAL SNAKE) VENOM

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A. Alape-Girón, B. G. Stiles and J. M. Gutiérrez. Antibody-mediated neutralization and binding-reversal studies on α-neurotoxins from *Micrurus nigrocinctus nigrocinctus* (coral snake) venom. *Toxicon* **34,** 369–380, 1996.—An ELISA based, non-radioactive acetylcholine receptor (AchR) binding assay was used to detect the α-neurotoxins present in *Micrurus nigrocinctus nigrocinctus* venom. Sera from horses hyperimmunized against *M. nigrocinctus* venom contain antibodies which inhibit the binding of *M. n. nigrocinctus* α-neurotoxins to AchR and reverse the binding of toxins already complexed with the receptor. This result supports the importance of using antivenom therapeutically in *M. n. nigrocinctus* envenomations even after the onset of neurological symptoms.

M. nigrocinctus antivenoms cross-reacted in an ELISA with several elapid α-neurotoxins and inhibited the binding of *Bungarus multicinctus* α-bungarotoxin and *Naja naja oxiana* neurotoxin II to AchR *in vitro*, suggesting the presence of short-chain and long-chain α-neurotoxins in *M. nigrocinctus* venom. *In vivo* neutralization experiments with *M. nigrocinctus* antivenom demonstrate that *M. nigrocinctus* venom contains short-chain α-neurotoxin(s) which share common neutralizing epitope(s) with *Naja naja oxiana* neurotoxin II.

INTRODUCTION

Snakes belonging to the genus *Micrurus* (New World coral snakes) are widely distributed from the Southeastern United States to Argentina (Campbell and Lamar, 1989). Nevertheless, *Micrurus* venoms have been poorly characterized, relative to other elapid venoms, because of the difficulty of maintaining the snakes as well as low venom yields.

Micrurus nigrocinctus nigrocinctus is the most abundant and medically important coral snake in Central America (Bolaños, 1984). Clinical and experimental studies indicate that

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its venom has several pharmacological activities (Bolaños, 1984; Gutiérrez *et al.*, 1980; Tan and Ponnudurai, 1990). The mouse LD_{50} of *M. n. nigrocinctus* venom is 0.765 mg/kg i.p. (intraperitoneally) and 2 mg/kg subcutaneously (Gutiérrez *et al.*, 1991). This venom induces paralysis and respiratory failure in animals (Jiménez-Porras, 1968) and electrophysiological changes *in vitro* that suggest the presence of postsynaptic and presynaptic toxins (Goularte *et al.*, 1995). When injected intramuscularly in mice, *M. n. nigrocinctus* venom is myonecrotic, but does not elicit edema or hemorrhage (Gutiérrez *et al.*, 1980,1986).

Therapeutic actions after *Micrurus* envenomations include administration of immunoglobulins from horses hyperimmunized against *Micrurus* venoms (Bolaños, 1984; Russell, 1983; Kitchens and Van Mierop, 1987; Coelho *et al.*, 1993). Recently, an equine *M. nigrocinctus* antivenom was reported to neutralize the PLA_2 activity, as well as the myotoxicity and lethality of this venom (Gutiérrez *et al.*, 1991; Lomonte *et al.*, 1993). This antivenom also contains antibodies which cross-react with α-neurotoxins and neurotoxic phospholipases from other elapid venoms (Alape-Girón *et al.*, 1994*a*,*b*).

Snake venom α-neurotoxins are basic, low molecular weight proteins that prevent the binding of acetylcholine to nicotinic acetylcholine receptor (AchR) at the neuromuscular endplate (Karlsson, 1979). The binding of an α-neurotoxin to AchR paralyses striated muscles, including the diaphragm, and produces death of envenomated animals by respiratory failure (Chang, 1979). The LD_{50}s of α-neurotoxins, when injected intravenously in mice, range between 0.05 and 0.15 mg/kg (Karlsson, 1979). More than 85 α-neurotoxins have been isolated from elapid venoms and their amino acid sequences elucidated. The secondary and tertiary structures of several α-neurotoxins are known from spectroscopic studies and X-ray analysis (Endo and Tamiya, 1991). According to the polypeptide chain length, α-neurotoxins are classified into two types: "short-chain" toxins (mol. wt $\approx$ 7000) consist of 60–62 amino acids and four invariant disulfide bonds, while "long-chain" toxins (mol. wt $\approx$ 8000) contain 70–74 amino acids and five disulfide bonds (Karlsson, 1979). Both toxin types bind to AchR with very high affinities (dissociation constants range from 10^{-10} to 10^{-11}). Despite structural similarities, differences exist between the two types of α-neurotoxins regarding biophysical and antigenic characteristics (Karlsson, 1979; Boquet, 1979). The short-chain and long-chain α-neurotoxins are immunologically distinct (Boquet, 1979). Cross-reactivity and cross-neutralization have been reported only among toxins within the same toxin type (Trèmeau, *et al.*, 1986; Charpentier *et al.*, 1990; Mènez, 1991).

The purpose of this study was three-fold: (1) determine if *M. n. nigrocinctus* venom contains α-neurotoxins; (2) evaluate the ability of therapeutic *M. nigrocinctus* antivenom to inhibit and reverse the binding of homologous α-neurotoxins to AchR; and (3) determine if *M. nigrocinctus* antivenom contains neutralizing antibodies against heterologous short-chain and/or long-chain α-neurotoxins.

MATERIALS AND METHODS

Venoms and toxins

A pool of *M. n. nigrocinctus* venom was obtained from more than 100 specimens collected in the central plateau region of Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado (San José, Costa Rica). Venom was lyophilized and stored at −20°C. Purified *Laticauda semifasciata* erabutoxin b, *Naja naja atra* α-cobrotoxin and *Naja naja oxiana* neurotoxin I (NT I) were obtained from Ventoxin (Frederick, MD, U.S.A.). *Naja naja kaouthia* α-cobratoxin, *Bungarus multicinctus* α-bungarotoxin and *Naja naja oxiana* neurotoxin II (NT II) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

M. nigrocinctus *antivenoms*

Monovalent antisera against *M. nigrocinctus* venom were produced in two horses (11 and 26) according to an immunization schedule already described (Bolaños and Cerdas, 1980). After the first immunization protocol, horses were boosted with 20 mg of venom every 60 days. Following the 9th boost, serum samples were obtained at days 28 and 50. A 10th venom injection was given at day 60, after which serum samples were also taken at days 10, 31 and 56. Five equine *M. nigrocinctus* antivenoms (batches 196, 198, 207, 221 and 232), were produced at the Instituto Clodomiro Picado between 1989 and 1993.

Preparation of acetylcholine receptor (AchR)

AchR from the electric organ of *Torpedo californica* (Pacific Biomarine, Venice, CA, U.S.A.) was purified as described (Froenher and Rafto, 1979). Briefly, frozen electric organ tissue (300 g) was minced at 4°C in a Waring blender with 250 ml of 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.5 mM sodium azide (Buffer A). The homogenate was filtered through sterile cheesecloth and the filtrate centrifuged at 26,000 ***g*** for 45 min. The pellet was extracted with the above buffer containing 1% Triton X-100 (Buffer B) for 2 hr at 4°C.

After centrifugation, the supernatant was incubated overnight at 4°C with 15 mg of *Naja naja kaouthia* α-cobratoxin covalently bound to 15 ml of packed agarose beads (Sigma). The beads were then serially washed with Buffer B, Buffer B plus 1 M NaCl, and finally Buffer A containing 0.1% Triton X-100. AchR was eluted from the beads using Buffer A containing 0.1% Triton X-100 and 1 M carbamylcholine chloride. Affinity-purified receptor was dialyzed at 4°C against Buffer A containing 0.1% Triton X-100 and stored in sterile glycerol (33% final concentration) at −70°C. All materials used to purify AchR were autoclaved or rinsed in 70% ethanol and the buffers contained 1 mM EDTA, 1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride to inhibit protease activity.

Antiserum to AchR

Guinea-pigs were each immunized i.p. with 50 μg of purified AchR in 200 μg of Ribi Adjuvant (Hamilton, MT, U.S.A.) every 2 weeks. After three injections, the animals were bled and sera pooled and frozen at −20°C.

AchR binding assays

The AchR binding assays (Stiles, 1991) were performed as follows: each well of Immulon II microtiter plates (Dynatech, McClean, VA, U.S.A.) was coated overnight at 4°C with 1 μg of purified α-neurotoxins diluted in 100 μl 0.05 M carbonate buffer (pH 9.6). Dose–response studies were done by coating wells with varying amounts of *M. n. nigrocinctus* venom (0.16–20 μg) diluted in carbonate buffer. The remaining binding sites were blocked for 30 min at 37°C with 300 μl of phosphate-buffered saline (PBS), pH 7.4, containing 1% gelatin (PBSG). After aspiration, 100 μl of AchR (50 μg/ml), diluted in PBS containing 0.1% Tween 20, and 0.1% gelatin (PBSTG), was added. Negative controls included wells coated with toxin followed by 100 μl PBSTG without AchR and wells without toxin which received 100 μl of the receptor preparation. After a 1 hr incubation at room temperature (r.t.), plates were washed three times with PBS containing 0.1% Tween 20 (PBST) and 100 μl of a 1:200 PBSTG dilution of guinea-pig anti-AchR was added per well. Following a 1 hr incubation at r.t. and four washes with PBST, 100 μl of a PBSTG dilution of goat anti-guinea pig IgG conjugated to alkaline phosphatase (Sigma) was added per well. After an additional 1 hr incubation at r.t. and five washes with PBST, 150 μl of para-nitrophenyl phosphate substrate solution (1 mg/ml) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) diluted in diethanolamine buffer, pH 9.8 was added per well. Absorbances were read at 405 nm after 30 min. The competitive assay was done by mixing varying amounts of *N. n. nigrocinctus* venom (0.16–20 μg) with 5 μg of AchR in 100 μl of PBSTG. The mixture was incubated 20 min at r.t. and applied to wells coated with *N. n. oxiana* NT II. All other steps were identical to the non-competitive assay as described before. The absorbance readings for 5 μg venom/well were within the linear portion of a dose–response curve and thus used for *in vitro* neutralization studies.

For inhibition experiments the adsorbed venom or toxin was incubated with serum samples diluted in PBSG for 1 hr at 37°C before the addition of AchR in order to estimate the ability of antibodies to block the binding of toxins to AchR. For reversal binding studies, a 30 min incubation at r.t. with diluted antisera was carried out after adding AchR to determine if the antibodies destabilize the AchR–toxin(s) complex(es). Subsequent steps were identical to the assay as described above. Results are expressed as a percentage, relative to the absorbances of wells incubated with PBSG instead of serum. The neutralizing activity of antivenom was expressed as an effective dilution 50% (ED_{50}), and defined as the dilution at which the binding was inhibited by 50%.

Assay for proteolytic activity

Proteolytic activity was assayed using hide powder azure (Sigma) as substrate (Riderknecht *et al.*, 1968). Briefly, 10 mg of hide powder azure was dissolved in 2.5 ml of Tris 0.05 M, pH 7.0 buffer, and incubated at 37°C for 1 hr in the presence of varying amounts of *M. n. nigrocinctus* venom (0.04–200 μg). After centrifugation, the absorbance was determined at 595 nm. Control tubes contained either *B. asper* venom and substrate or only substrate incubated with buffer.

Detection and titration of antibodies by ELISA

Immulon II microtiter plates were coated overnight at 4°C with 1 μg of *M. n. nigrocinctus* venom or purified toxin/well using carbonate buffer as diluent. The remaining binding sites were blocked with 300 μl of PBSG for 30 min at 37°C. After aspiration, 100 μl of each PBSTG diluted antivenom sample was added per well and incubated for 1 hr at 37°C. Negative controls on each plate consisted of non immune horse sera or a polyvalent *Bothrops–Crotalus–Lachesis* antivenom produced at the Instituto Clodomiro Picado. After five washes with PBST, 100 μl of PBSTG diluted goat anti-horse IgG alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories) was added per well. After a 1 hr incubation at 37°C, plates were washed five times with PBST, 150 μl of para-nitrophenyl phosphate substrate solution (1 mg/ml) diluted in diethanolamine buffer, pH 9.8 was added per well, and absorbances read at 405 nm after 30 min.

Neutralization studies

A fixed amount of either *M. n. nigrocinctus* venom, *B. multicinctus* α-bungarotoxin or *N. n. oxiana* NT II were incubated with varying amounts of *M. nigrocinctus* antivenom (batch 232) for 30 min at 37°C. Each mixture (0.5 ml), containing four LD_{50} of venom or toxin (corresponding to 3 μg of NT II and 10 μg of α-bungarotoxin) was injected i.p. into groups of four Swiss–Webster mice (16–18 g body weight). Control groups received 4 LD_{50} of venom or toxin in PBS. The time to death was recorded over a 72 hr period and neutralization was expressed as effective dose 50% (ED_{50}), defined as the volume of antivenom (μl)/μg of venom or toxin needed to protect half of the injected mice. The ED_{50} was calculated by the Spearman–Kärber method (WHO, 1981).

RESULTS

Dose–response and competition studies

The binding of AchR to varying amounts of *M. n. nigrocinctus* venom adsorbed onto microtiter wells was tested in a non-radioactive AchR binding assay. A dose–response was evident with a maximal absorbance detected in wells coated with 5 μg of venom or higher, suggesting a saturation of the available binding sites (Fig. 1). Competition for AchR

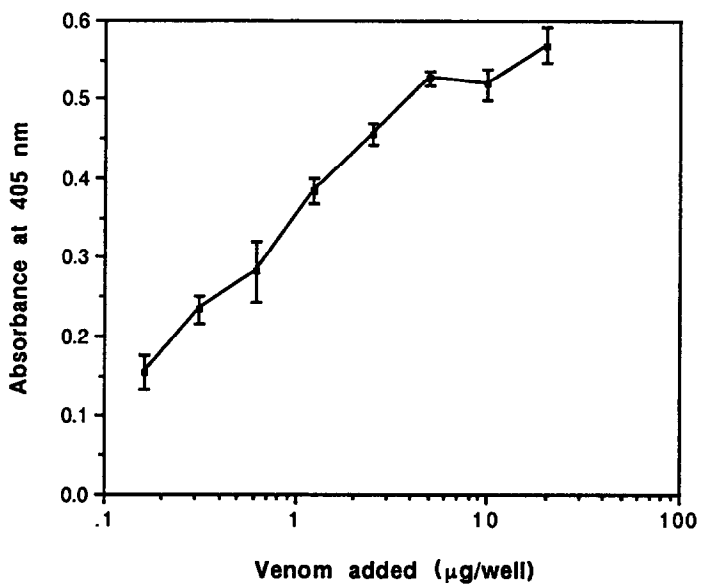

Fig. 1. Binding of *M. n. nigrocinctus* α-neurotoxins to AchR. Varying amounts of venom (0.16–20 μg) were adsorbed onto microtiter wells followed by the addition of AchR (5 μg/well), guinea-pig anti-AchR serum, anti-guinea-pig alkaline phosphatase conjugate, and substrate. Control wells containing either PBSG plus receptor or venom without receptor gave absorbance readings lower than 0.15. Each point represents the mean $\pm$ standard deviation (S.D.) of four determinations.

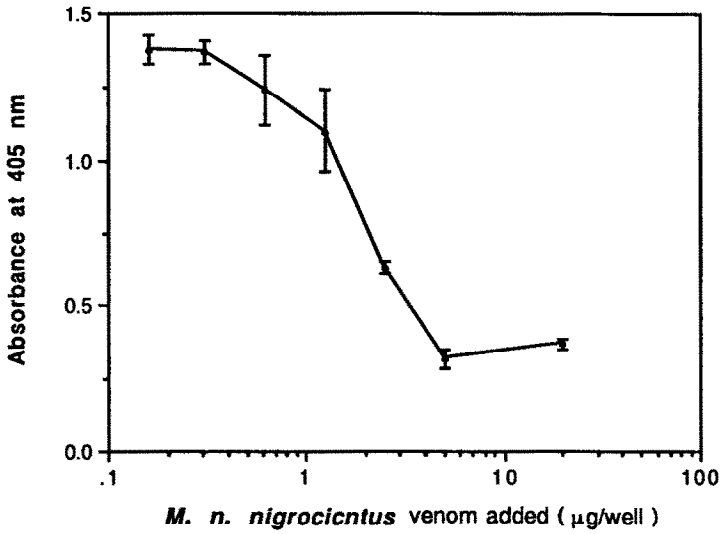

Fig. 2. Binding of *Naja naja oxiana* NT II to AchR in the presence of varying amounts *M. n. nigrocinctus* venom.
Microtiter wells were coated with 1 µg of NT II. AchR (5 µg/well) and varying amounts of *M. n. nigrocinctus* venom (0.16–20 µg) were co-incubated 20 min before addition to the wells. Bound receptor was detected with guinea-pig anti-AchR serum, anti-guinea-pig alkaline phosphatase conjugate, and substrate. Control wells containing either PBSG plus receptor or venom without receptor gave absorbance readings lower than 0.12. Each point represents the mean ± S.D. of four determinations.

binding between *N. n. oxiana* neurotoxin II, a short-chain postsynaptic neurotoxin, and varying amounts of *M. n. nigrocinctus* venom also showed a dose–response (Fig. 2). Proteolytic activity was not detected with the amounts of venom used in the AchR assay (data not shown), suggesting that inhibition was not due to proteolytic degradation of AchR during the assay.

Inhibition of the binding of M. n. nigrocinctus *α-neurotoxins to AchR by specific antibodies*

The ability of sera from horses hyperimmunized against *M. nigrocinctus* venom to block homologous α-neurotoxins binding to AchR was tested in the non-radioactive AchR binding assay. Serum samples were obtained from two horses at day 31 after the 9th venom injection. A concentration-dependent inhibition of the binding of *M. n. nigrocinctus* α-neurotoxins to AchR by antiserum was observed in preincubation experiments (Fig. 3). The antiserum from horse 26 was more potent ($ED_{50} = 3548$) than serum from horse 11 ($ED_{50} = 955$) for inhibiting the binding of *M. n. nigrocinctus* venom to AchR.

Hyperimmune sera contained antibodies which prevented the binding of *M. n. nigrocinctus* α-neurotoxin(s) to AchR *in vitro*, and also reversed the binding of toxin(s) already complexed with the receptor. A concentration-dependent dissociation of the toxin(s)–AchR complex(es) was observed in the presence of antiserum (Fig. 4). Although sera from horses 26 and 11 reversed the binding of *M. n. nigrocinctus* α-neurotoxin(s) to AchR, reversal levels were not higher than 43%, even at the lowest dilution of antivenom tested (1:100).

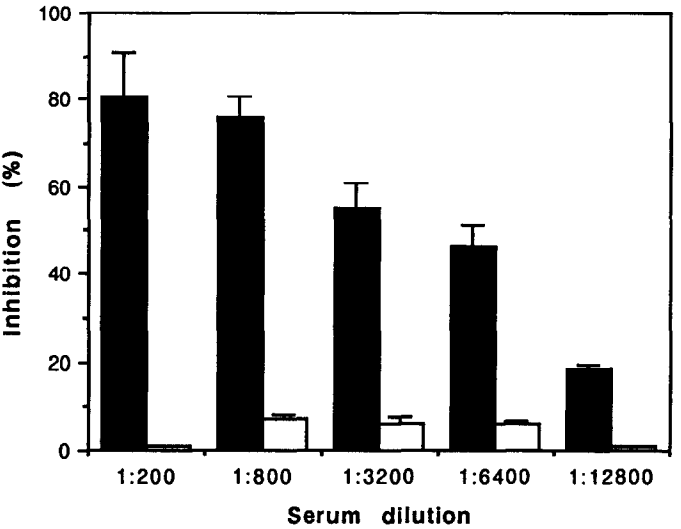

Fig. 3. Inhibition of the binding of *M. n. nigrocinctus* α-neurotoxins to AchR by specific antibodies. Microtiter wells were coated with 5 μg of *M. n. nigrocinctus* venom followed by incubation with dilutions of a hyperimmune serum (black bars), sample taken from horse 26 at day 31 after the 10th venom injection. A non immune serum was used as control (white bars). After a 1 hr incubation at 37°C, AchR, guinea-pig anti-AchR serum, conjugate and substrate were sequentially added, and the absorbances recorded at 405 nm 30 min later. Results are expressed as a percentage of inhibition relative to the absorbances given by wells preincubated with PBSG instead of serum. Each bar represents the mean ± S.D. of three determinations.

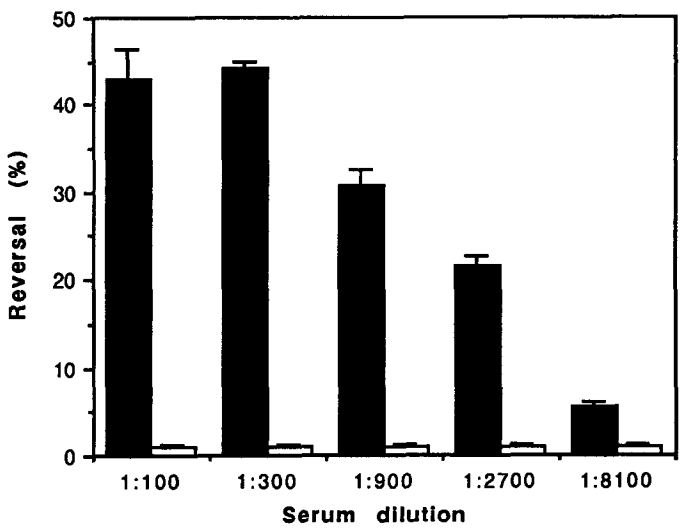

Fig. 4. Reversal of the binding of *M. n. nigrocinctus* α-neurotoxins to AchR by specific antibodies. Microtiter wells were coated with 5 μg of *M. n. nigrocinctus* venom and incubated with AchR (5 μg/well). After removing unbound receptor, wells received dilutions of a hyperimmune serum (black bars), sample taken from horse 26 at day 31 after the 10th venom injection. A non immune serum was used as control (white bars). After a 30 min incubation at room temperature, guinea-pig anti-AchR, serum, conjugate and substrate were sequentially added and the absorbances recorded at 405 nm 30 min later. Results are expressed as percentage of reversal, relative to the absorbance of wells incubated in parallel with PBSG instead of serum. Each bar represents the mean S.D. ± of three determinations.

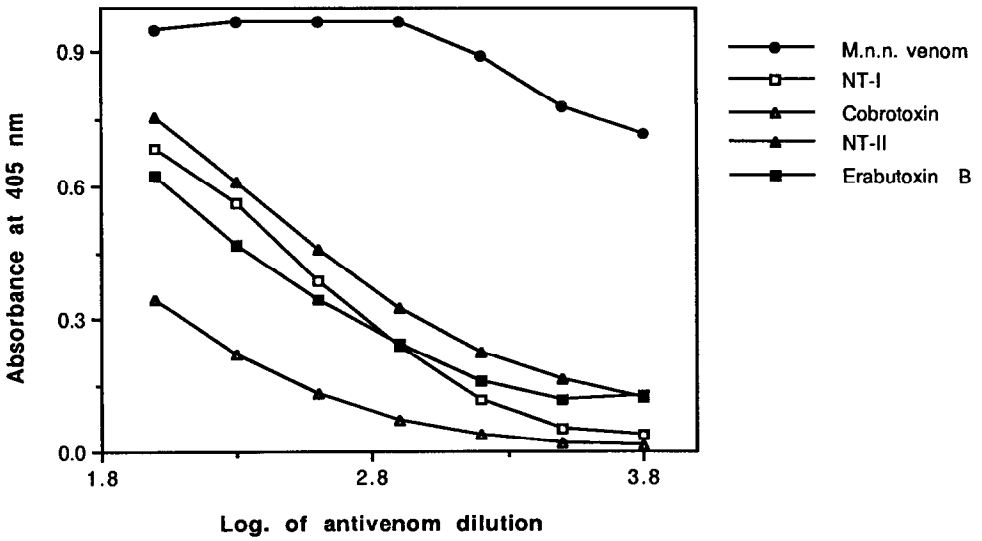

Fig. 5. Titrations of a *M. n. nigrocinctus* equine antivenom against heterologous α-neurotoxins. Microtiter wells were each coated with either 1 μg of *M. n. nigrocinctus* venom or 1 μg of the indicated toxin, and then incubated with different antivenom (batch 207) dilutions. Conjugate and then substrate were added and the absorbance readings at 405 nm recorded 30 min later. Each point represents the mean of four determinations. Standard deviations were less than 10% and a polyvalent *Bothrops–Crotalus–Lachesis* antivenom gave readings lower than 0.1 for all toxins at all tested dilutions.

Antibodies in M. nigrocinctus *antivenoms cross-react with heterologous α-neurotoxins*

Antivenom contained antibodies which recognize three short-chain α-neurotoxins (*N. n. oxiana* NT II, *Laticauda semifasciata* erabutoxin b and *N. n. atra* cobrotoxin) and one long-chain toxin (*N. n. oxiana* NT I), as evidenced by ELISA (Fig. 5). It was previously shown that this antivenom also recognizes in an ELISA two long-chain toxins: *B. multicinctus* α-bungarotoxin and *N. n. kaouthia* α-cobratoxin, as well as a short-chain toxin, *N. nigricollis* toxin α (Alape-Girón *et al.*, 1994*a*,*b*).

Table 1. ELISA reactivity of different toxins with equine *M. nigrocinctus* antivenom batches

	Antivenom batch				
	196	198	207	221	232
M. n. nigrocinctus venom	0.79 ± 0.03*	0.98 ± 0.03	0.92 ± 0.01	1.07 ± 0.03	1.13 ± 0.04
Toxins:					
NT II (*N.n.o*)	0.37 ± 0.02	0.49 ± 0.02	0.51 ± 0.02	0.44 ± 0.01	0.53 ± 0.02
Erabutoxin B (*L.s*)	0.42 ± 0.01	0.31 ± 0.01	0.25 ± 0.01	0.21 ± 0.01	0.19 ± 0.00
α-Cobratoxin (*N.n.a.*)	0.11 ± 0.01	0.12 ± 0.01	0.22 ± 0.01	0.16 ± 0.01	0.17 ± 0.00
NT I (*N.n.o*)	0.38 ± 0.02	0.31 ± 0.01	0.44 ± 0.02	0.44 ± 0.02	0.44 ± 0.02
α-cobratoxin (*N.n.k*)	0.27 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01	0.31 ± 0.01
α-Bungarotoxin (*B.m*)	0.33 ± 0.01	0.17 ± 0.01	0.27 ± 0.02	0.26 ± 0.01	0.34 ± 0.02
Cardiotoxin (*N.n.a*)	(—)†	(—)	(—)	(—)	(—)
Cardiotoxin I (*N.n.k.*)	(—)	(—)	(—)	(—)	(—)

*Results correspond to the absorbance values recorded at 405 nm (mean ± S.D; $n = 4$). All samples were diluted 1/200. A polyvalent *Bothrops*, *Crotalus*, *Lachesis* antivenom used as control gave absorbances < 0.05 for all toxins.

†Absorbance < 0.1.

Five batches of *M. nigrocinctus* antivenom were run in parallel to determine interbatch variability in antibody cross-reactivity with heterologous α-neurotoxins. All samples were diluted 1:200 and absorbance readings taken as an estimate of antibody concentration

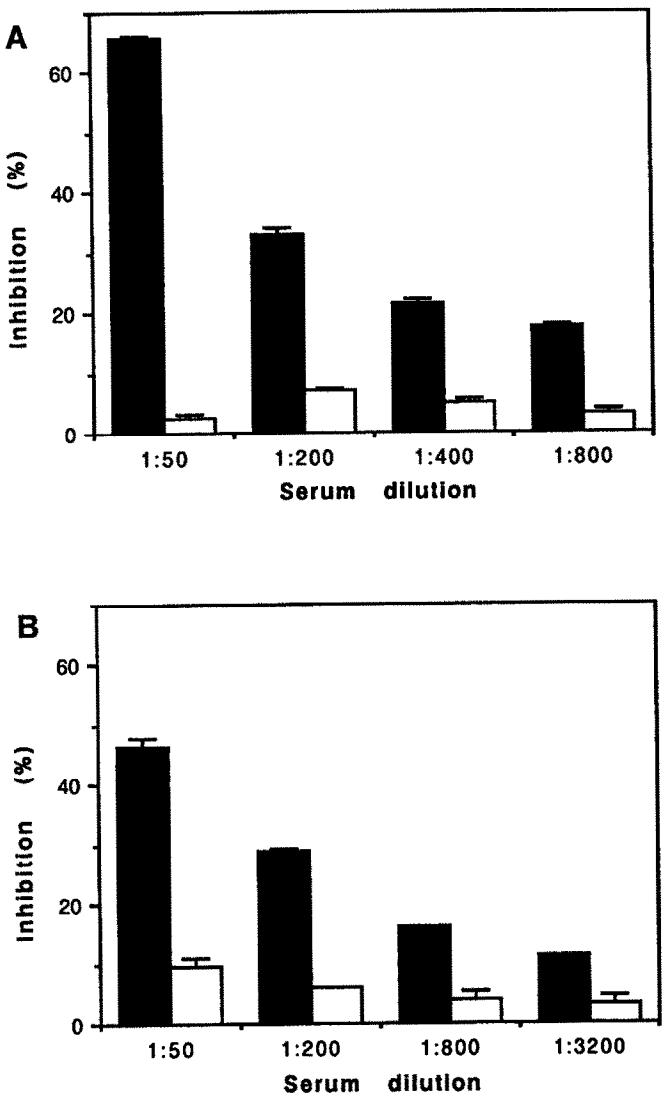

Fig. 6. Inhibition of the binding of heterologous α-neurotoxins to AchR by horse antisera to *M. n. nigrocinctus* venom.

Naja naja oxiana NT II (A) or *Bungarus multicinctus* α-bungarotoxin (B) were adsorbed onto microtiter wells (1 μg/well). Dilutions of *M. n. nigrocinctus* antivenom were then added (black bars). Samples from horse 26 (A) or horse 11 (B) were used. Samples were taken at day 31 after the 10th venom injection. A non immune serum was used as a control (white bars). After a 1 hr incubation at 37°C, AchR, guinea-pig anti-AchR serum, conjugate and substrate were sequentially added and the absorbances recorded at 405 nm 30 min later. Results are expressed as percentage of inhibition, relative to the absorbances of wells incubated with PBSG instead of serum. Each bar represents the mean ± S.D. of three determinations.

against six different α-neurotoxins (Table 1). Cardiotoxins from *N. n. kaouthia* and *N. n. atra* venoms were also tested, but did not cross-react with *M. nigrocinctus* antivenom. *N. n. oxiana* NT I and II gave the highest absorbance readings among the toxins tested (Table 1).

Ten sera from two horses hyperimmunized against *M. nigrocinctus* venom had antibodies that cross-react in ELISA with several α-neurotoxins. Serum samples were taken at different times following venom injections, and run in parallel to determine if there were variations in antibody cross-reactivity during immunization (data not shown). An interesting difference was observed between both sera: serum from horse 11 showed higher reactivity with *N. n. oxiana* NT I and *B. multicinctus* α-bungarotoxin than with *N. n. oxiana* NT II, whereas sera from horse 26 showed higher reactivity with *N. n. oxiana* neurotoxin II relative to the long chain toxins.

Inhibition of heterologous α-neurotoxins binding to AchR by M. nigrocinctus *antivenoms*

The ability of *M. nigrocinctus* antivenoms to inhibit the AchR binding of heterologous α-neurotoxins was tested *in vitro* using the non-radioactive AchR binding assay. Serum from horse 26 inhibited the binding of *N. n. oxiana* NT II to AchR in a concentration-dependent manner (Fig. 6A) with an ED_{50} of 100. Serum from horse 11 also inhibited α-bungarotoxin binding to AchR, but even at the lowest dilution tested (1:50) the inhibition was not higher than 50% (Fig. 6B). Although sera from both horses inhibited the binding of heterologous α-neurotoxins to AchR, they did not reverse the binding of these toxins already complexed with AchR (data not shown).

Neutralization studies

The ability of a *M. nigrocinctus* antivenom (batch 232) to neutralize the lethal effect of *M. n. nigrocinctus* venom, *B. multicinctus* α-bungarotoxin and *N. n. oxiana* neurotoxin II was determined in mice. Four LD_{50}s of either venom or the purified toxins caused respiratory failure and death of the animals within 2 hr. Antivenom effectively neutralized the lethal effects of *M. n. nigrocinctus* venom (ED_{50} of 5 μl antivenom/μg venom) and *N. n. oxiana* NT II (ED_{50} of 240 μl antivenom/μg toxin). Mice died at lower antivenom/NT II doses, although a concentration-dependent delay in time to death was observed (data not shown). In contrast, neither a neutralizing effect nor a delay in time to death was observed when antivenom was tested against α-bungarotoxin, even at an antivenom/toxin ratio of 96 μl antivenom/μg of toxin.

DISCUSSION

Various *in vitro* methods employing radiolabeled snake venom postsynaptic neurotoxins have been used to detect binding to AchR, and study the effects of antitoxins (Charpentier *et al.*, 1990; Boulain and Ménez, 1982; Boulain *et al.*, 1982; Pachner and Ricalton, 1989). In this study, an ELISA-based, non-radioactive assay was used to detect *M. n. nigrocinctus* venom components which bind to AchR. Although it is well known that *Micrurus* venoms are neurotoxic *in vivo*, this is the first report of AchR binding activity found in one of these venoms. Competition between *M. n. nigrocinctus* venom and *N. n. oxiana* NT II for binding to AchR was observed, thus indicating that *M. n. nigrocinctus* venom contains α-neurotoxin(s) which specifically bind to AchR.

Several *M. nigrocinctus* antivenom batches and serum samples from horses hyperimmunized against crude venom cross-reacted with various elapid short- and long-chain α-neurotoxins. Antisera from horses hyperimmunized against crude *M. nigrocinctus* venom not only cross-reacted with heterologous α-neurotoxins, but also inhibited the binding of a heterologous short-chain toxin (*N. n. oxiana* NT II) and a long-chain toxin (α-bungarotoxin) to AchR *in vitro*. These results suggest the presence of both α-neurotoxin types in *M. nigrocinctus* venom. We have recently isolated four proteins which bind to AchR from *M. n. nigrocinctus* venom; the N-terminal sequences of two revealed identity with short-chain α-neurotoxins whereas two others share N-terminal sequence identity with long-chain toxins (Alape-Girón *et al.*, 1995). In addition, *M. nigrocinctus* antivenom also neutralized the lethal effect of *N. n. oxiana* NT II in mice, demonstrating that *M. n. nigrocinctus* venom contains short chain α-neurotoxin(s) which share common neutralizing epitope(s) with *N. n. oxiana* NT II.

Since *M. n. nigrocinctus* venom also has presynaptic effects (Goularte *et al.*, 1995), the question then arises as to the relative importance of α-neurotoxins in lethality. In the case of *N. n. siamensis* antivenoms, *in vivo* neutralization correlates well to ELISA titers against α-cobratoxin (Rungsiwongse and Ratanabanangkoon, 1991). If this were the case in *M. nigrocinctus* antivenoms, an ELISA against α-neurotoxin(s) isolated from this venom could also be useful to estimate antivenom potency.

Two different horses hyperimmunized against *M. nigrocinctus* venom had different antibody responses against short- and long-chain α-neurotoxins. This result emphasizes the importance of using several animals to prepare therapeutic antisera against snake venoms, in order to assure an effective response against various toxin types. Antisera from both horses readily inhibited the binding of homologous α-neurotoxin(s) to AchR and also reversed the binding of toxin already complexed with receptor. This result supports the importance of using antivenom therapeutically even after the onset of neurological symptoms. Two neutralizing epitopes have been described in *N. nigricollis* α-toxin: one overlaps the AchR binding site and the other is topographically unrelated to the binding domain, since it remains accessible after the toxin is bound to AchR (Ménez *et al.*, 1992). Similar epitopes might also be present on *M. n. nigrocinctus* α-neurotoxin(s).

In the preparation of antivenoms, experiments have to be carried out at different time points during the immunization schedule to evaluate the development of an antibody response, as well as during different stages of the plasma fractionation process. Antivenom potency is determined by venom neutralization in a mouse lethality assay. An assessment of the ability to inhibit and reverse the binding of α-neurotoxin(s) to AchR, using this non-radioactive AchR binding assay, may offer an alternative to estimate the potency of *M. nigrocinctus* antivenoms. Since elapid venoms usually contain several α-neurotoxin isoforms (Endo and Tamiya, 1991), the use of crude venom in the assay would be desirable for this purpose. A correlation study between the *in vivo* and *in vitro* neutralizing capabilities of several *M. nigrocinctus* antivenom preparations is currently underway in our laboratories.

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