Characterization of monoclonal antibodies against *Naja naja oxiana* neurotoxin I

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Seven monoclonal antibodies (mAbs) were developed against neurotoxin I (NT-1), a protein from central Asian cobra (*Naja naja oxiana*) venom which binds specifically to nicotinic acetylcholine receptor (AchR). All of the mAbs cross-reacted with another long-chain post-synaptic neurotoxin, *Bungarus multicinctus* α-bungarotoxin (α-BT), but not *Naja naja kaouthia* α-cobratoxin, in an enzyme-linked immunosorbent assay (e.l.i.s.a.). Short-chain post-synaptic neurotoxins like *Naja naja atra* cobrotoxin, *Laticauda semifasciata* erabutoxin b, or *N. n. oxiana* neurotoxin II did not cross-react with the NT-1 mAbs, but an antigen(s) found in *Dendroaspis polylepis*, *Acanthophis antarcticus* and *Pseudechis australis* venoms was immunoreactive. The e.l.i.s.a. readings for dithiothreitol-reduced NT-1 and NT-1 mAbs ranged from 13 to 27% of those for native toxin but reduced α-BT was not immunoreactive. Synthetic NT-1 peptides were used in epitope-mapping studies and two, non-contiguous regions (Cys^{15}–Tyr^{23} and Lys^{25}–Gly^{33} or Pro^{17}–Lys^{25} and Asp^{29}–Lys^{37}) were recognized by the NT-1 mAbs. The NT-1 mAbs individually inhibited 31–71% of α-BT binding to AchR *in vitro* and afforded a slight protective effect *in vivo* with a toxin:antibody mole ratio of 1:1.5. This report is the first to describe mAbs which recognize and protect against a heterologous, long-chain, post-synaptic neurotoxin from snake venom.

INTRODUCTION

Many snake venoms contain post-synaptic neurotoxins which specifically bind the nicotinic acetylcholine receptor (AchR) of prey and result in paralysis and death from asphyxiation (Tu, 1973). Post-synaptic neurotoxins from snake venoms are classified as either short-chain (60–62 amino acids) or long-chain (70–73 amino acids) and contain four or five disulphide bridges respectively (Tu, 1973; Dufton and Hider, 1983). Neurotoxin I (NT-1) is a long-chain toxin purified from venom of the central Asian cobra (*Naja naja oxiana*) with an isoelectric point of 9.0 and mouse LD_{50} of 0.56 mg/kg body wt. (Grishin et al., 1974). The amino acid sequence and crystal structure of NT-1 have been reported (Grishin et al., 1974; Mikhailov et al., 1990; Nickitenko et al., 1993).

The immunological properties of *N. n. oxiana* NT-1 have not been described but extensive monoclonal antibody (mAb) studies have been reported for another long-chain neurotoxin, α-bungarotoxin (α-BT), isolated from Banded Krait (*Bungarus multicinctus*) venom (Danse et al., 1986; Chuang et al., 1989; Kase et al., 1989; Pachner and Ricalton, 1989). Neutralizing epitopes, Ser^{34}–Lys^{38} (Kase et al., 1989) and Ser^{34}–Glu^{41} (Chuang et al., 1989), identified by different mAbs against α-BT are located within the central loop which intimately contacts the α-subunit of AchR with high affinity (Low, 1979; Love and Stroud, 1986; McDaniel et al., 1987; Ruan et al., 1990). Only one study describes any cross-reactivity, but not neutralization, of other long-chain neurotoxins with anti-(α-BT) mAbs (Kase et al., 1989).

Another investigation of two mAbs developed against α-cobratoxin (α-CT), a long-chain neurotoxin from *N. n. kaouthia* venom, reports neutralizing epitopes located at the base or tip of the central loop (Charpentier et al., 1990). These mAbs cross-react with four different long-chain neurotoxins but do not recognize short-chain neurotoxins. Neutralization experiments were not shown for the anti-(α-CT) mAbs and cross-reactive toxins.

Our study characterizes various mAbs developed against *N. n. oxiana* NT-1 and specifically investigates the cross-reactivity with other purified snake toxins and crude venoms, inhibition of toxin binding to purified *Torpedo californica* AchR *in vitro* and protective effects in mice. Based on epitope-mapping studies and computer analysis, the antibody contact sites located on the NT-1 molecule were compared with the peptide equivalents found within α-BT and α-CT.

MATERIALS AND METHODS

mAb production

Male ICR mice (approx. 30 g) were each injected intraperitoneally (i.p.) with 2 μg of purified NT-1 from *N. n. oxiana* venom (Natural Product Sciences, Salt Lake City, UT, U.S.A.) mixed in 200 μl of RIBI adjuvant (Hamilton, MT, U.S.A.). Mice were again injected after three weeks and serum samples obtained 48 h later from the tail vein. Antibody titres against NT-1 were detected by an e.l.i.s.a. as described below. Spleen cells from the mouse with the highest titre were fused with P3x63 Ag8.653 myelomas, and hybridomas producing IgG against NT-1 were cloned twice (Rener et al., 1985). The mAbs were purified from ascites using Protein-G (Genex Corp., Gaithersburg, MD, U.S.A.), dialysed against PBS, pH 7.4, and stored at −70 °C. The purified IgG was sub-isotyped by an e.l.i.s.a. (Hyclone, Logan, UT, U.S.A.) and protein concentrations determined with a dye-binding assay (Bradford, 1976) using bovine γ-globulin as a standard.

E.l.i.s.a.

The serum titres of mice immunized with NT-1 were determined using Immulon II microtitre plates (Dynatech, Chantilly, VA,

Abbreviations used: mAb, monoclonal antibody; NT-1, neurotoxin I; AchR, acetylcholine receptor; α-BT, α-bungarotoxin; α-CT, α-cobratoxin; PBSG, PBS containing 1% (w/v) gelatin; PBSTG, PBS containing 0.1% Tween-20 and 0.1% gelatin; PBST, PBS containing 0.1% Tween-20.

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U.S.A.) coated with a 5 μg of NT-1/ml carbonate buffer solution, pH 9.6, (100 μl/well) for 16 h at 4 °C. Wells were then blocked with PBS containing 1% (w/v) gelatin (PBSG) for 30 min at 37 °C and mouse serum, diluted 1:100 in PBS containing 0.1% Tween-20 and 0.1% gelatin (PBSTG), added (100 μl/well) for 1 h at 37 °C. After washing the plate with PBS containing 0.1% Tween-20 (PBST), a PBSTG dilution of sheep anti-(mouse IgG) conjugated to alkaline phosphatase (Sigma, St. Louis, MO, U.S.A.) was added (100 μl/well) for 1 h at 37 °C. Wells were finally washed with PBST, *p*-nitrophenyl phosphate substrate added, and absorbance readings taken at 405 nm after 30 min. Each sample was assayed in triplicate and the mean absorbance reading at 405 nm ± S.D. calculated.

Hybridomas producing IgG against NT-1 were detected by e.l.i.s.a. as described above using a coating solution of 1 μg of toxin/ml of carbonate buffer followed by 100 μl of undiluted culture medium per well.

Cross-reactivity of the NT-1 mAbs (20 μg/ml of PBSTG) was assayed in microtitre plates coated with carbonate buffer dilutions of purified snake toxins (10 μg/ml; Natural Product Sciences), post-synaptic cone-snail toxins (10 μg/ml), or crude snake venoms (200 μg/ml) purchased from Sigma. The snake venoms were those from *Acanthophis antarcticus*, *Agkistrodon contortrix contortix*, *Bitis arietans*, *Bungarus multicinctus*, *Crotalus durissus terrificus*, *Dendroaspis angusticeps*, *Dendroaspis jamesonii*, *Dendroaspis polylepis*, *Dendroaspis viridis*, *Hemachatus hemachatus*, *Laticauda semifasciata*, *Micrurus fulvius*, *Naja haje haje*, *Naja melanoleuca*, *Naja naja atra*, *Naja nigricollis*, *Naja naja oxiana*, *Naja mossambica mossambica*, *Notechis scutatus scutatus*, *Pseudechis australis*, *Trimeresurus okinavensis* and *Vipera russelli*.

The e.l.i.s.a. reactivity of mAbs with reduced NT-1 and α-BT was tested by incubating 10 μg of toxin/ml of carbonate buffer with a 2000-fold molar excess of dithiothreitol for 6 h at room temperature in a closed polypropylene tube. Reduced toxin was then adsorbed overnight in microtitre wells at 4 °C and an e.l.i.s.a. done as described above.

AchR binding assay

The binding of toxin to AchR *in vitro* was assayed in microtitre plates (Stiles, 1991) coated with 100 μl solution of 20 μg of α-BT/ml of carbonate buffer. AchR plus mAb (50 μg and 100 μg/ml final concentrations respectively) were mixed in PBSTG and 100 μl added to each well for 1 h at room temperature. Receptor bound to α-BT was detected by guinea-pig anti-AchR serum, goat anti-(guinea-pig IgG) conjugate and substrate (Stiles et al., 1991). Wells were read at 405 nm and the mean value of quadruplicate samples ± S.D. was calculated. The anti-AchR serum was developed in our laboratory using Hartley guinea-pigs (female, 250–300 g). Animals were each injected subcutaneously every 2 weeks with 20 μg of purified AchR mixed with RIBI's adjuvant (Hamilton, MT, U.S.A.). After three injections, the serum contained a demonstrable anti-AchR titre as determined by e.l.i.s.a.

Protection studies in mice

Male Swiss–Webster mice (18–20 g) were each injected i.p. with 200 μl of PBS containing 12 μg of α-BT and 390 μg of mAb. Toxin and mAb were preincubated together for 30 min at room temperature before the injection. Control animals were each injected with non-reactive mAb 1E8 (390 μg), developed against *N. n. atra* cobrotoxin, pre-incubated with 12 μg of α-BT. Time to death (min) of each mouse was recorded and the mean time to death ($n = 5$ per mAb) ± S.D. was calculated.

Epitope mapping of NT-1 with synthetic peptides

Overlapping NT-1 peptides (9-mers) spanning the whole molecule were synthesized in duplicate on polyethylene pins according to the procedure of Geysen et al. (1984). Non-neurotoxin peptides were synthesized and used as negative controls. The e.l.i.s.a. for detecting antibody bound to peptides was done as previously described (Stiles and Middlebrook, 1991) with a 10 μg/ml concentration of purified mAb. A cobrotoxin mAb, 1E8, was the control for non-specific binding of mouse IgG. Data are presented as the mean absorbance (at 405 nm) of duplicate peptides.

Structural comparisons of NT-1, α-BT and α-CT

X-ray co-ordinates of α-CT (Love and Stroud, 1986) and α-BT (Betzel et al., 1991) were obtained from the Protein Data Bank (Brookhaven, NY, U.S.A.). The amino acid sequence of NT-1 was aligned with α-CT and the NT-1 molecule built using HOMOLOGY software (Biosym Technologies, San Diego, CA, U.S.A.) on an IRIS 4D35 workstation (Silicon Graphics, Mountain View, CA, U.S.A.). Dihedral torsions for the reverse turns of NT-1 were constructed employing values determined from the NT-1 crystal structure (Nickitenko et al., 1993) and the NT-1 model was refined with a molecular-dynamics simulation using DISCOVER version 2.9 software (Biosym Technologies) executed on a CRAY Y-MP. The NT-1 molecule was immersed in a 4 Å layer of water and the amino acid residues in structurally conserved β-strand regions were tethered to their initial positions with a 100 kcal/Å force constant (1 kcal = 4184 J and 1 Å = 0.1 nm). The protein force field was modelled using a consistent valence force field. Simulations were initiated with 200 cycles of minimization by using a steepest descent algorithm followed by 25 ps equilibration. The initial atomic velocities were assigned from a Gaussian distribution corresponding to a temperature of 300 K. Non-bonded interactions were smoothed to zero beyond 9 Å with a distance-dependent dielectric ($\epsilon = r$). All hydrogens were treated explicitly, employing a 1.0 fs step for integrating the equations of motion. Ensemble averages were determined from a production run of 180 ps with co-ordinates, velocities and energies saved every 100 steps for further analysis. Solvent-accessible surface areas were calculated using a 1.4 Å radius spherical probe with the INSIGHT program (Biosym Technologies).

RESULTS

Cross-reactivity of NT-1 mAbs with other toxins

Six of the seven NT-1 mAbs were subisotyped as IgG_{2b} while mAb 848 was an IgG_{2a}. Each mAb recognized NT-1 and *B. multicinctus* α-BT, another long-chain post-synaptic neurotoxin, in an e.l.i.s.a. (Table 1). None of the mAbs cross-reacted with *N. n. kaouthia* α-CT or short-chain neurotoxins. Post-synaptic conotoxins GI and MI from *Conus* sp. venom, *C. d. terrificus* crotoxin (a presynaptic neurotoxin), and non-neurotoxic cardiotoxins from *N. n. atra* and *N. n. kaouthia* venom were also not recognized by NT-1 mAbs in an e.l.i.s.a. (results not shown).

To examine further the cross-reactivity of NT-1 mAbs with other antigens, 22 different snake venoms were assayed by an e.l.i.s.a. but only five venoms resulted in positive signals (Table

Table 1 E.l.i.s.a. cross-reactivity of NT-1 mAbs with long- and short-chain post-synaptic neurotoxins purified from snake venoms

Cross reactivity values represent the mean of three absorbance readings ± S.D. of various purified toxins (10 μg/ml) with NT-1 mAbs (20 μg/ml). Long-chain toxins were *N. n. oxiana* NT-1, *B. multicinctus* α-BT and *N. n. kaouthia* α-CT. The short-chain toxins were *N. N. atra* cobrotoxin, *L. semifasciata* erabutoxin b and *N. n. oxiana* neurotoxin II.

	Toxin cross-reactivity (mean absorbance reading ± S.D.)					
mAb	NT-1	α-BT	α-CT	Cobrotoxin	Erabutoxin b	Neurotoxin II
835	1.249 ± 0.094	0.913 ± 0.051	0.025 ± 0.007	0.021 ± 0.004	0.015 ± 0.010	0.035 ± 0.013
840	1.333 ± 0.065	0.941 ± 0.088	0.036 ± 0.021	0.024 ± 0.018	0.006 ± 0.003	0.037 ± 0.022
841	1.310 ± 0.053	0.973 ± 0.020	0.024 ± 0.012	0.020 ± 0.010	0.015 ± 0.008	0.038 ± 0.009
847	1.244 ± 0.041	0.951 ± 0.021	0.023 ± 0.006	0.018 ± 0.003	0.013 ± 0.002	0.041 ± 0.003
848	1.540 ± 0.050	1.535 ± 0.068	0.021 ± 0.008	0.001 ± 0.001	0.000 ± 0.000	0.009 ± 0.000
849	1.322 ± 0.038	0.849 ± 0.025	0.009 ± 0.006	0.020 ± 0.005	0.013 ± 0.005	0.029 ± 0.004
850	1.453 ± 0.061	1.219 ± 0.065	0.037 ± 0.007	0.040 ± 0.011	0.061 ± 0.007	0.061 ± 0.008

Table 2 E.l.i.s.a. cross-reactivity of NT-1 mAbs with various snake venoms

Cross-reactivities are expressed as the mean of three absorbance readings ± S.D. using various snake venoms (200 μg/ml) and NT-1 mAbs (20 μg/ml).

	Snake venom cross-reactivity (mean absorbance reading ± S.D.)					
mAb	*N. n. oxiana*	*B. multicinctus*	*D. polylepis*	*A. antarcticus*	*P. australis*	No antigen
835	0.393 ± 0.015	0.596 ± 0.035	1.511 ± 0.030	1.175 ± 0.104	0.236 ± 0.008	0.055 ± 0.009
840	0.410 ± 0.022	0.574 ± 0.018	1.180 ± 0.012	1.160 ± 0.050	0.222 ± 0.009	0.062 ± 0.014
841	0.487 ± 0.008	0.777 ± 0.035	1.612 ± 0.055	1.430 ± 0.043	0.274 ± 0.009	0.057 ± 0.014
847	0.550 ± 0.028	0.811 ± 0.020	1.345 ± 0.031	1.499 ± 0.074	0.287 ± 0.006	0.047 ± 0.006
848	0.480 ± 0.017	1.680 ± 0.046	1.104 ± 0.066	1.955 ± 0.056	0.110 ± 0.014	0.026 ± 0.009
849	0.384 ± 0.029	0.732 ± 0.017	2.041 ± 0.052	1.051 ± 0.077	0.222 ± 0.019	0.000 ± 0.000
850	0.414 ± 0.022	0.886 ± 0.018	1.124 ± 0.029	1.546 ± 0.052	0.240 ± 0.006	0.023 ± 0.013

(a)

NT-1
ITCYKTPIPITSET**CAPGQNLCYTKTWCDAWCGSRGK**VIELGCAATCPTVESYQDIKCCSTDDCNPHPKQKRP

α-BT
IVCHTTATIPSSAVT**CPPGENLCYRKMWCDAFCSSRGK**VVELGCAATCPSKKPYEEVTCCSTDKCNHPPKRQPG

α-CT
IRCFITPDITSKD**CPNGHVCYTKTWCDAFCSIRGK**RVDLGCAATCPTVKTGVDIQCCSTDNCNPFPTRKRP

(b)

NT-1	CAPGQNLCY	PGQNLCYTK	KTWCDAWCG	DAWCGSRGK
α-BT	CPPGENLCY	PGENLCYRK	KMWCDAFCS	DAFCSSRGK
α-CT	CPNG HVCY	NG HVCYTK	KTWCDAFCS	DAFCSIRGK

Figure 1 Comparison of amino acid sequences and immunoreactive peptides of NT-1 with α-BT and α-CT

(**a**) Comparison of the amino acid sequences of NT-1 (Grishin et al., 1974), α-BT (Mebs et al., 1972) and α-CT (Karlsson et al., 1972). The immunoreactive region of NT-1 and analogous regions of α-BT and α-CT are shown in bold. Variation from the NT-1 amino acids are underlined within α-BT and α-CT. (**b**) Comparison of the immunoreactive NT-1 peptides with analogous peptides of α-BT and α-CT. Variations from the NT-1 amino acids are underlined within α-BT and α-CT.

2). As expected, *N. n. oxiana* and *B. multicinctus* venoms were recognized by NT-1 mAbs as they contain NT-1 and α-BT respectively. The venoms of *A. antarcticus*, *D. polylepis* and *P. australis*, all of which contain long-chain post-synaptic neurotoxins or non-toxic analogues (Strydom, 1972; Kim and Tamiya, 1981; Takasaki, 1989; Sheumack et al., 1990), also had an antigen(s) that cross-reacted with the NT-1 mAbs.

Binding of NT-1 mAbs to dithiothreitol-reduced toxins

E.l.i.s.a. readings for the mAbs against dithiothreitol-reduced NT-1 were considerably less than those for native toxin. Relative to native NT-1, the mean values for mAbs 835, 840, 841, 847, 849 and 850 with reduced NT-1 ranged from 22 to 27%. Although mAb 848 yielded the highest absorbance readings for native NT-

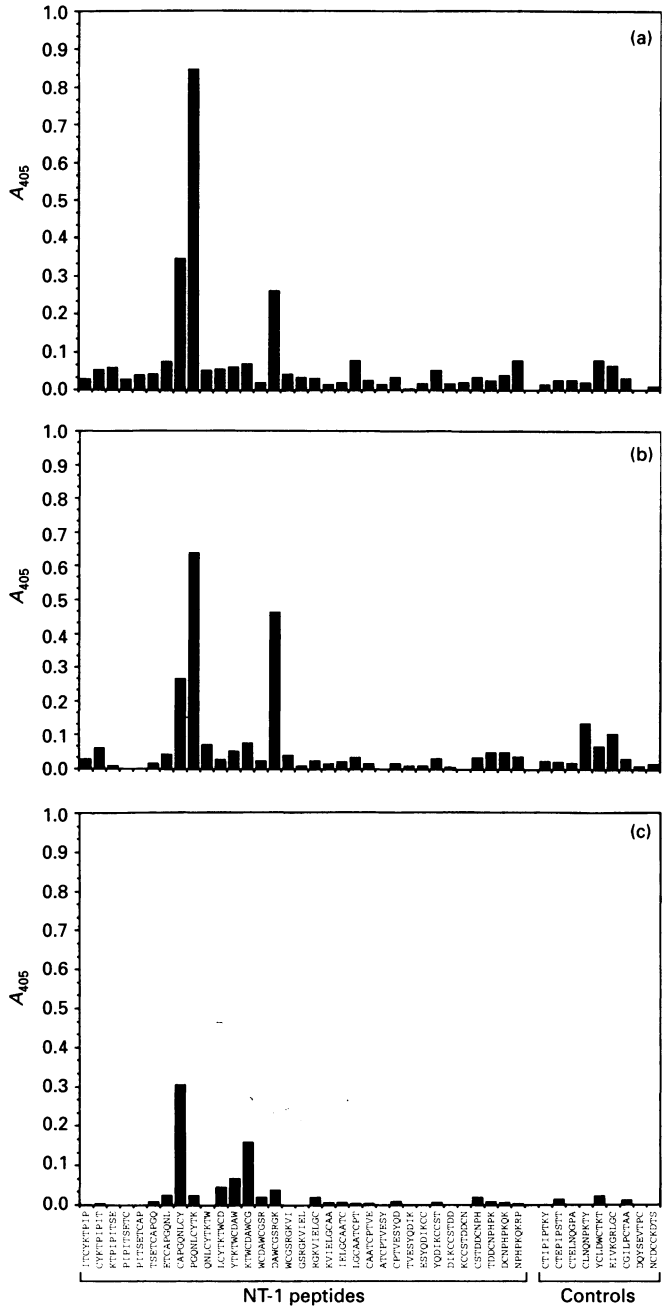

Figure 2 Epitope mapping of NT-1 using 9-mer peptides spanning the molecule, moving two amino acids at a time from the N-terminus

The last nine peptides are nonsense peptides used as negative controls. All peptides were synthesized in duplicate and the results represent the mean absorbance reading at 405 nm. The concentration of individually tested mAbs was 10 μg/ml. (**a**) Actual readings for the mAb 847 which are similar to the binding pattern for mAb 840 or 849. (**b**) Actual readings for mAb 850 which are similar to the binding pattern for mAb 835 or 841. (**c**) Binding pattern for mAb 848.

1, results for reduced toxin were only 13% of those observed for native toxin. The e.l.i.s.a. results with mouse pre-immune serum and reduced NT-1 were less than 1% of the readings for native toxin recognized by any NT-1 mAb. Reduced α-BT did not cross-react with the NT-1 mAbs.

Table 3 Inhibition of α-BT binding to AchR *in vitro* and neutralization of α-BT *in vivo* using NT-1 mAbs

Mean absorbance readings at 405 nm ± S.D. were calculated from quadruplicate samples in the AchR-binding assay. Values are for a final concentration of mAb (100 μg/ml) and AchR (50 μg/ml) mixture added to wells previously coated with a 20 μg/ml concentration of α-BT. The percentage inhibition of α-BT binding to AchR was calculated from the mean reading of control mAb 1E8 used as 0% inhibition. mAbs 835 and 850 were not tested in either *in vivo* or *in vitro* neutralization assays.

mAb	A_{405} (Mean reading ± S.D.)	Inhibition of binding (%)	Time to death (min)*
840	0.497 ± 0.035	40	38.2 ± 2.3
841	0.566 ± 0.042	31	39.2 ± 6.6
847	0.400 ± 0.039	51	49.8 ± 3.3
848	0.242 ± 0.040	71	75.0 ± 14.1
849	0.422 ± 0.040	49	41.3 ± 3.0
1E8 (control)	0.824 ± 0.072	–	29.8 ± 2.2
None	0.913 ± 0.048	–	–

*Five mice per group were each injected i.p. with a preincubated mixture of 12 μg of α-BT and 390 μg of mAb (1:1.5 mole ratio respectively).

Epitope mapping with NT-1 peptides

As the NT-1 mAbs reacted with dithiothreitol-reduced NT-1, epitope mapping was initiated with overlapping peptide regions (9-mers) spanning the NT-1 molecule. The amino acid sequences of NT-1, α-BT and α-CT are shown in Figure 1(a). Sequence homology of NT-1 with α-BT and α-CT is 58% and 62% respectively. Epitope mapping resulted in two distinct patterns of mAb binding to NT-1 peptides located within the central loop (Cys^{15}–Cys^{43}). Peptides Cys^{15}–Tyr^{23} and Pro^{17}–Lys^{25} were recognized by mAbs 840, 847 and 849 (Figure 2a). There was also a reduced, yet definite, binding to a non-contiguous peptide, Asp^{29}–Lys^{37}, by the same mAbs. Identical peptides were recognized by mAbs 835, 841 and 850 (Figure 2b), but the absorbance readings for Asp^{29}–Lys^{37} were relatively higher than those for mAbs 840, 847 or 849. Although Pro^{17}–Lys^{25} was the most reactive NT-1 peptide, readings for Gln^{19}–Trp^{27} were near background and thus emphasize the importance of Pro^{17} and/or Gly^{18} in the binding of these mAbs.

The mAb 848 was again easily distinguished from the others since it recognized different peptides, Cys^{15}–Tyr^{23} and Lys^{25}–Gly^{33} (Figure 2c). E.l.i.s.a. readings of mAb 848 against either recognized peptides or reduced NT-1 were relatively lower than those of the other mAbs and contrasts with the high reactivity of mAb 848 with native NT-1 or α-BT (Table 1). Immunoreactive peptides of NT-1, and the peptide equivalent regions of α-BT and α-CT, are aligned in Figure 1(b).

Non-specific binding of mouse IgG or anti-(mouse IgG) conjugate was tested with the NT-1 peptides using a mAb against *N. n. atra* cobrotoxin which did not cross-react with native NT-1 in an e.l.i.s.a. (results not shown). The cobrotoxin mAb did not recognize the NT-1 peptides as shown by absorbance readings of $\leqslant 0.010$. Additionally, the NT-1 mAbs did not react with overlapping α-BT peptides spanning the molecule.

Inhibition of α-BT binding to AchR *in vitro* by NT-1 mAbs

Since the NT-1 mAbs cross-reacted strongly with α-BT, we tested the effectiveness of the mAbs at inhibiting α-BT binding to AchR *in vitro* (Table 3). A 100 μg/ml concentration of individually tested NT-1 mAbs, especially 848, effectively blocked the binding of α-BT to AchR *in vitro*. Inhibition of NT-1 binding

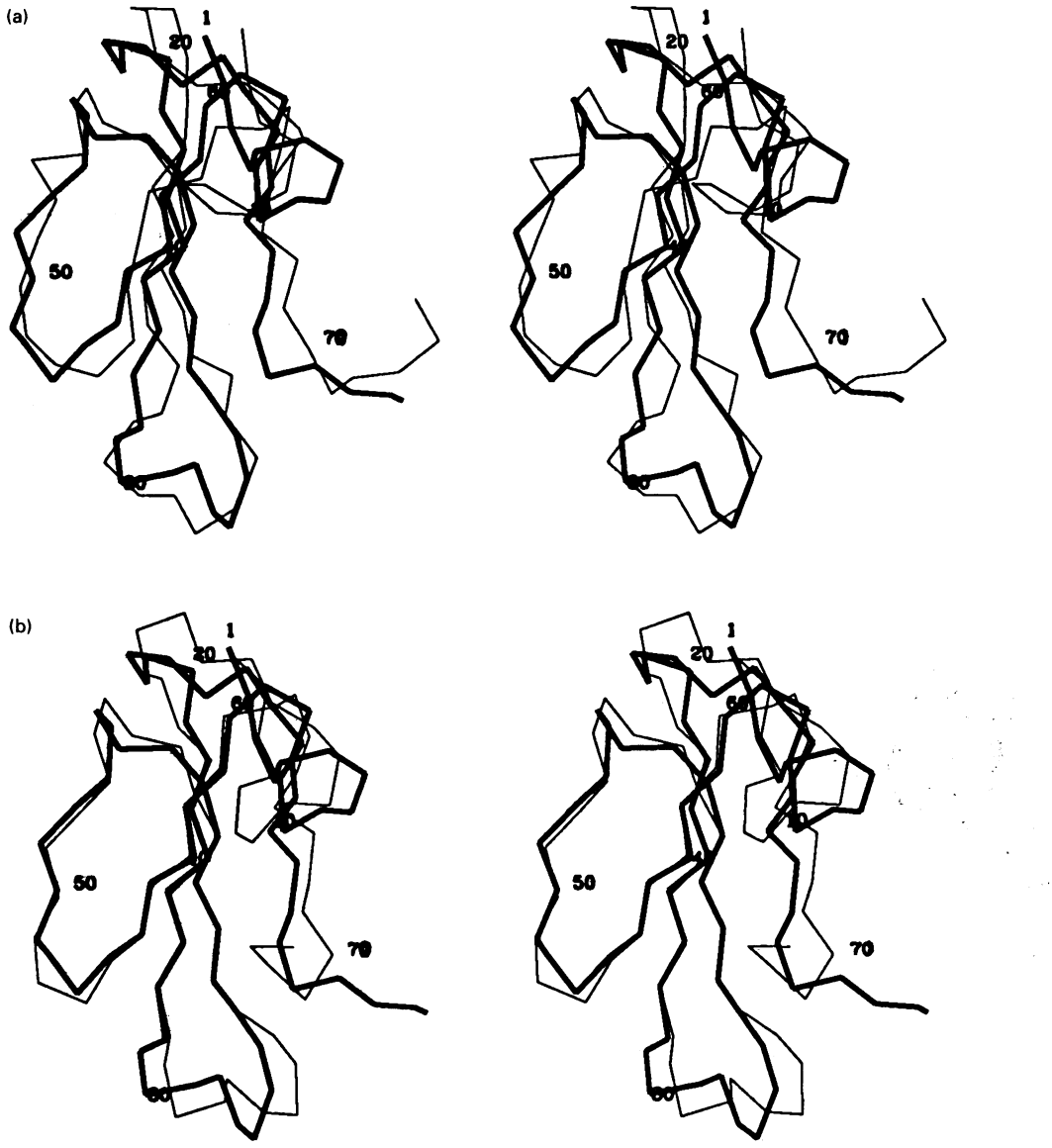

Figure 3 Superimposed stereoviews of α-BT (a) and α-CT (b) X-ray crystal structures with the modelled NT-1 (thick line and residue numbers)

to AchR *in vitro* could not be determined because the toxin consistently yields low readings in the binding assay (Stiles, 1993).

In vivo neutralization of α-BT with NT-1 mAbs

Although *in vitro* binding of α-BT to AchR was greatly diminished using individually tested NT-1 mAbs, *in vivo* protection studies resulted in only a maximal 2.5-fold increase in time to death with a toxin/mAb molar ratio of 1:1.5 (Table 3). Neutralization of NT-1 in a mouse lethal assay was not attempted because of the high LD_{50} (560 μg/kg; Grishin et al., 1974), in contrast with α-BT (150 μg/kg; Mebs et al., 1972), and difficulty in obtaining favourable NT-1:mAb molar ratios.

Structural comparisons of NT-1, α-BT and α-CT

To help explain the cross-reactivity of NT-1 mAbs with other purified long-chain neurotoxins, we compared the X-ray crystal structures of α-BT (Love and Stroud, 1986) and α-CT (Walkinshaw et al., 1980) to the NT-1 molecule modelled from α-CT crystal co-ordinates (Figure 3). The antibody contact sites on NT-1 were found within the central loop and consisted of two peptides located in different hairpin-looped regions, residues 1–18 and 19–43, separated by approx. 30 Å. The NT-1 residues 1–18 contained an irregular reverse turn of five amino acids connected to an antiparallel β-strand, followed by the antibody-binding site found within Cys^{15}–Lys^{25}. The NT-1 peptide region, Ala^{16}–Gln^{19}, and analogous site on α-BT (Figure 3a) had a type-II β-turn which differed from the type-I β-turn found at the comparable α-CT site (Figure 3b). Based on epitope mapping of the NT-1 molecule, the Cys^{15}–Asn^{20} region appeared to be solvent accessible (283 Å) and important for the binding of NT-1 mAbs. Both α-BT (Cys^{16}–Asn^{21}) and α-CT (Cys^{14}–Val^{19}) had solvent-accessible regions quite similar to that of Cys^{15}–Asn^{20} within NT-1. The conformation of Cys^{15}–Asn^{20} in native NT-1 resulted in similar main-chain dihedral angles found in the

analogous α-BT peptide region, but significant angle differences occurred within the equivalent α-CT site. In addition to conformational dissimilarities, the Cys^{14}–Val^{19} region of α-CT also had a different electrostatic charge distribution relative to the corresponding peptides of NT-1 and α-BT. The Leu^{21}–Lys^{25} site within the immunoreactive Cys^{15}–Lys^{25} region of NT-1 had a solvent-accessible surface area of 105 Å and probably played a minor role in antibody recognition as it was imbedded within the molecule as a β-strand surrounded by two other β-strands immobilized by disulphide bonds.

The other mAb contact site on NT-1 was Lys^{25}–Lys^{37}, which was exclusively located within the second hairpin loop (amino acids 19–43) which contained a type-I β-turn (Cys^{28}–Trp^{31}) and a five-residue turn (Trp^{31}–Arg^{35}). The surface solvent-accessibilities of Lys^{25}–Gly^{33} and Asp^{29}–Lys^{37} in NT-1 were 495 and 6380 Å respectively. Analogous regions of α-BT and α-CT had very similar surface solvent-accessibilities.

DISCUSSION

All of the mAbs against *N. n. oxiana* NT-1 cross-reacted in an e.l.i.s.a. with another long-chain post-synaptic neurotoxin, *B. multicinctus* α-BT, but the closely related *N. n. kaouthia* α-CT failed to react with any NT-1 mAb. Although NT-1 shared more primary sequence similarity with α-CT (62%), than α-BT (58%), the immunoreactive peptides of NT-1 were less homologous with the analogous regions of α-CT (65%) versus α-BT (74%). The inability of NT-1 mAbs to recognize native α-CT might have been due to diverse amino acid sequences and conformations among snake venom, long-chain, post-synaptic neurotoxins (Goas et al., 1992). A previous study classifies α-BT and α-CT as two distinct subclasses within the family of long-chain neurotoxins, based on predicted secondary structure (Dufton and Hider, 1983). Further comparisons of the crystal structures also point out obvious structural differences between α-BT and α-CT (Love and Stroud, 1986).

Epitope-mapping studies with NT-1 mAbs identified four immunoreactive peptides; Cys^{15}–Tyr^{23}, Pro^{17}–Lys^{25}, Lys^{25}–Gly^{33} and Asp^{29}–Lys^{37}. Each NT-1 mAb recognized two, non-contiguous peptides of NT-1 located within the central loop, which is reportedly important in the binding of snake venom post-synaptic neurotoxins to AchR (Dufton and Hider, 1983; Love and Stroud, 1986; McDaniel et al., 1987; Ruan et al., 1990). To examine further the cross-reactivity of NT-1 mAbs with α-BT, and the non-reactivity of α-CT, computer models of the three toxins were generated using X-ray crystallography co-ordinates of α-BT and α-CT (Love and Stroud, 1986; Betzel et al., 1991). Unfortunately, the crystal co-ordinates for NT-1 were not available for our study, although the three-dimensional structure was recently published (Nickitenko et al., 1993). Crystal co-ordinates of α-CT and the dihedral torsion values for reverse turns from NT-1 (Nickitenko et al., 1993) were used to construct the three-dimensional structure of NT-1. The NT-1 molecule was not built from α-BT co-ordinates since we did not want to bias the results with a cross-reactive neurotoxin. However, reactivity of the NT-1 mAbs with native α-BT probably indicated more conformational similarity between NT-1 and α-BT, as compared with that of NT-1 and α-CT. Computer analysis of NT-1, α-BT and α-CT revealed conformational differences centred within the immunoreactive Cys^{15}–Lys^{25} region of NT-1 and the analogous site on α-CT. The same peptide region on cross-reactive α-BT was more conformationally similar to that found in NT-1.

Besides the effects of toxin conformation on antibody recognition, comparing the primary sequence of each reactive NT-1 peptide with the analogous peptides from α-BT and α-CT revealed significant differences that could explain the cross-reactivity pattern of NT-1 mAbs with whole toxin and synthesized toxin peptides. The Cys^{15}–Tyr^{23} and Pro^{17}–Lys^{25} regions of NT-1 differed from the α-BT equivalents since the α-BT peptides contained a Pro and Glu replacement for Ala and Gln respectively. These non-conservative substitutions in synthesized 9-mers, or a dithiothreitol-reduced version of α-BT, probably changed the appearance of this antibody contact site relative to native α-BT so that it was not recognized by the NT-1 mAbs.

The α-CT peptide equivalents of Cys^{15}–Tyr^{23} and Pro^{17}–Lys^{25} from NT-1 contained an Asn, instead of a Pro. Additionally, a six-amino-acid region of α-CT located on the C-terminus of Pro^{17}–Lys^{25} was shifted one amino acid towards the N-terminus, relative to NT-1. Both of these changes in α-CT, along with the observed conformational differences, were apparently sufficient to disrupt the interaction of NT-1 mAbs with native α-CT. The Cys^{15}–Lys^{25} region of NT-1 contained a C-terminal region (Leu^{21}–Lys^{25}) imbedded within a β-strand and immobilized by a disulphide bond. The peptides used for epitope mapping were tethered on polyethylene pins via the C-terminus and the most strongly recognized Cys^{15}–Lys^{25} region of NT-1 might mimic the peptide conformation found in the native toxin.

NT-1 peptides located within the Lys^{25}–Lys^{37} region were weakly recognized by NT-1 mAbs. Binding of six different NT-1 mAbs to the Asp^{29}–Lys^{37} peptide critically depended on the N-terminal Asp and/or Ala. Peptides within the Lys^{25}–Lys^{37} region were relatively mobile when compared with the same disulphide-bridged (Cys^{26}–Cys^{30}) region located on native NT-1. Variations between the Lys^{25}–Lys^{37} sequence of NT-1 and equivalent α-BT or α-CT peptides were relatively conservative, but the changes might be enough to prevent binding of NT-1 mAbs to native α-CT or the α-BT peptides.

The NT-1 mAbs strongly recognized native NT-1 and α-BT but there were distinct differences between epitopes on both toxin molecules, as determined by e.l.i.s.a. results after dithiothreitol reduction of the toxins. These results suggest that the NT-1 mAbs bind optimally to a conformational epitope. Additionally, each of the antibodies recognized two non-contiguous NT-1 peptides that probably represent contact sites of a conformational epitope, which is much more common than relatively simple, linear epitopes (Geysen et al., 1986; Van Regenmortel, 1989, 1992).

The two non-contiguous regions of NT-1 recognized by the NT-1 mAbs are approx. 30 Å apart. This corresponds to a reported 30 Å × 20 Å interface between an antibody Fab and lysozyme epitope consisting of two non-contiguous peptides separated by a minimum of 11 Å (Amit et al., 1986). Other investigators describe a 26 Å × 19 Å interface between a different antibody and lysozyme epitope containing three non-contiguous, but spatially adjacent, contact sites separated by a minimum of 6 Å (Sheriff et al., 1987). Obviously, there are diverse sizes among the hypervariable regions of different antibodies which accommodate various-sized antigens and this is accomplished via the addition or deletion of amino acids within the hypervariable region (Poljak, 1975). Because of the five disulphides and extensive β-region interactions among long-chain neurotoxins, it seems unlikely that an NT-1 mAb could induce the two distant, immunoreactive peptides into closer contact with each other. Based on epitope mapping results with synthesized NT-1 peptides, the Cys^{15}–Lys^{25} region was probably the predominant contact site for the NT-1 mAbs while the Lys^{25}–Lys^{37} region provided a weaker, yet significant, secondary contact site.

In addition to the cross-reactivity of NT-1 mAbs with purified long-chain neurotoxins, three short-chain neurotoxins were also

tested by e.l.i.s.a. and found to be non-reactive. The immunoreactive regions of NT-1 share less than 40% sequence homology with the equivalent sites found in the non-reactive, short-chain neurotoxins. Although these neurotoxins specifically compete for the same receptor and have a similar LD_{50}, there is little amino acid sequence homology and a lack of antibody cross-reactivity between the short- and long-chain toxins from snake venoms (Boquet et al., 1973; Tremeau et al., 1986; Charpentier et al., 1990). One mAb against the short-chain, *N. nigricollis* toxin α reportedly neutralizes various short-chain, post-synaptic neurotoxins in an *in vitro* receptor-binding assay but none of the long-chain neurotoxins were cross-reactive (Tremeau et al., 1986). A 'universally protective' mAb has never been described for long-chain, post-synaptic neurotoxins, which is probably due to the conformational differences among these snake toxins (Dufton and Hider, 1983; Love and Stroud, 1986; Goas et al., 1992). In our study, NT-1 mAbs cross-reacted and individually inhibited 31–71% of α-BT binding to AchR *in vitro*. Because of the apparent weak binding of NT-1 to AchR in a non-radioactive receptor assay (Stiles, 1993), a characteristic which coincides with the low lethal potency (Grishin et al., 1974), we could not accurately measure the inhibition of NT-1 binding to AchR *in vitro*. The toxicity of snake venom, post-synaptic neurotoxins generally correlates with the K_d of toxin binding to AchR *in vitro* (Ishikawa et al., 1977).

Since the various NT-1 mAbs protected against α-BT *in vitro*, neutralization studies were done in mice using individually tested NT-1 mAbs preincubated with α-BT. An α-BT/antibody molar ratio of 1:1.5 resulted in a $\leqslant$ 2.5-fold increase for the time to death relative to control animals. A higher amount of NT-1 mAb would probably be more efficacious since increased times to death of 4.3- and 13.5-fold were previously reported for an α-BT/mAb molar ratio of 1:1 and 1:5 respectively (Kase et al., 1989).

Another study with *N. n. atra* cobrotoxin and various mAbs also reported prolonged times to death in mice administered toxin plus mAb (Stiles et al., 1991). There were good correlations between *in vitro* and *in vivo* neutralization assays using cobrotoxin and the mAbs. We observed a similar correlation with the NT-1 mAbs, especially mAb 848, against α-BT. Although absolute protection *in vivo* against a post-synaptic neurotoxin by one mAb is reportedly rare, a cocktail of antibodies recognizing different epitopes would probably be more effective since three to four antibodies can concomitantly bind to snake venom post-synaptic neurotoxins (Chang and Yang, 1969; Menez et al., 1979).

Previous studies with mAbs against α-BT reported that Ser^{34}–Lys^{38} and Ser^{34}–Glu^{41} represent neutralizing epitopes on the central loop (Chuang et al., 1989; Kase et al., 1989). In theory, antibodies which bind to a receptor-binding region of a snake post-synaptic neurotoxin with a higher affinity than the toxin–AchR complex ($K_d \sim 1 \times 10^{-9}$–$10^{-11}$ M) should have neutralizing potential (Weber and Changeux, 1974). Our study revealed that six of the seven NT-1 mAbs bound appreciably to Asp^{29}–Lys^{37}, but the most immunoreactive NT-1 peptides were Cys^{15}–Tyr^{23} or Pro^{17}–Lys^{25}, which are located at the surface-exposed N-terminal region of the central loop.

α-CT can also be neutralized by either of two different mAbs which bind to the base or tip of the central loop (Charpentier et al., 1990). Both mAbs against α-CT recognize NT-1 and α-BT, but the apparent binding K_d is at least 640- and 1900-fold greater respectively, than that for homologous toxin in a competitive binding assay using radiolabelled toxin. Our studies were done with a less sensitive, non-radioactive e.l.i.s.a. which did not detect any binding of the NT-1 mAbs with α-CT.

The cross-reactivity of the NT-1 mAbs with *A. antarcticus*, *D. polylepis* and *P. australis* venoms, all of which contain long-chain, post-synaptic neurotoxins or non-toxic homologues (Strydom, 1972; Kim and Tamiya, 1981; Takasaki, 1989; Sheumack et al., 1990), also suggested that the NT-1 mAbs recognize other long-chain, post-synaptic neurotoxins. *A. antarcticus* toxin Aa b and acanthophin d, *P. australis* Pa ID, and three different long-chain toxins from *D. polylepis* venom share approx. 60% amino-acid sequence homology with NT-1, which compared favourably with the 58% sequence similarity of cross-reactive α-BT. Other toxins or non-toxic homologues found in these venoms, but not described in the literature, could obviously cross-react with the NT-1 mAbs. Non-reactive venoms containing long-chain neurotoxins yielded negative results because these toxins share little antigenic similarity with NT-1 at the epitopes recognized by the NT-1 mAbs and/or sub-detectable concentrations of these toxins were present. The uniformly higher e.l.i.s.a. readings of NT-1 mAbs with *B. multicinctus* venom, relative to *N. n. oxiana* venom, were probably due to the higher concentrations of α-BT in *B. multicinctus* venom (Mebs et al., 1972) versus the NT-1 concentrations in *N. n. oxiana* venom (Grishin et al., 1974).

This study is the first to describe a cross-protective effect afforded by mAbs against a snake venom long-chain, post-synaptic neurotoxin. Each of the NT-1 mAbs optimally recognized two non-contiguous peptides found on NT-1 that were located within the central loop which intimately contacts AchR. As shown by e.l.i.s.a. results with various snake venoms, the NT-1 mAbs also cross-react with other antigens that are possibly long-chain post-synaptic neurotoxins.

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REFERENCES

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V. and Poljak, R. J. (1986) Science **233**, 747–753

Betzel, C., Lange, G., Pal, G. P., Wilson, K. S., Maelicke, A. and Saenger, W. (1991) J. Biol. Chem. **266**, 21530–21536

Boquet, P., Poilleux, G., Dumarey, C., Izard, Y. and Ronsseray, A.-M. (1973) Toxicon **11**, 333–340

Bradford, M. (1976) Anal. Biochem. **72**, 248–254

Chang, C. C. and Yang (1969) J. Immunol. **102**, 1437–1444

Charpentier, I., Pillet, L., Karlsson, E., Couderc, J. and Menez, A. (1990) J. Mol. Recognit. **3**, 74–81

Chuang, L. Y., Lin, S. R., Chang, S. F. and Chang, C. C. (1989) Toxicon **27**, 211–219

Danse, J. M., Toussaint, J. L. and Kempf, J. (1986) Toxicon **24**, 141–151

Dufton, M. J. and Hider, R. C. (1983) CRC Crit. Rev. Biochem. **14**, 113–171

Geysen, H. M., Meloen, R. H. and Barteling, S. J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3998–4002

Geysen, H. M., Rodda, S. J. and Mason, T. J. (1986) Mol. Immunol. **23**, 709–715

Goas, R. L., LaPlante, R. R., Mikou, A., Delsuc, M.-A., Guittet, E., Robin, M., Charpentier, I. and Lallemand, J.-Y. (1992) Biochemistry **31**, 4867–4875

Grishin, E. V., Sukhikh, A. P., Slobodyan, L. N. and Ovchinnikov, Y. A. (1974) FEBS Lett. **45**, 118–121

Ishikawa, Y., Menez, A., Hori, H., Yoshida, H. and Tamiya, N. (1977) Toxicon **15**, 477–488

Karlsson, E., Eaker, D., Ponterius, G. and Arnberg, H. (1972) Biochim. Biophys. Acta **257**, 235–248

Kase, R., Kitagawa, H., Hayashi, K., Tanoue, K. and Inagaki, F. (1989) FEBS Lett. **254**, 106–110

Kim, H. and Tamiya, N. (1981) Biochem. J. **193**, 899–906

Love, R. A. and Stroud, R. M. (1986) Protein Eng. **1**, 37–46

Low, B. W. (1979) Handbook of Experimental Pharmacology **52**, 213–257

McDaniel, C. S., Manshouri, T. and Atassi, M. Z. (1987) J. Protein Chem. **6**, 455–461

Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. and Lee, C.-Y. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 243–262

Menez, A., Boulain, J. C. and Fromageot, P. (1979) Toxicon **17**, (Suppl. 1), 123
Mikhailov, A. M., Nickitenko, A. V., Trakhanov, S. D., Vainshtein, B. K. and Chetverina, E. V. (1990) FEBS Lett. **269**, 255–257
Nickitenko, A. V., Michailov, A. M., Betzel, C. and Wilson, K. S. (1993) FEBS Lett. **320**, 111–117
Pachner, A. R. and Ricalton, N. (1989) Toxicon **27**, 1263–1268
Poljak, R. J. (1975) Adv. Immunol. **21**, 1–33
Rener, J. C., Brown, B. B. and Nardone, R. M. (1985) J. Tissue Cult. Methods **9**, 175–177
Ruan, K.-H., Spurlino, J., Quiocho, F. A. and Atassi, M. Z. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6156–6160
Sheriff, S., Silverton, E. W., Padlan, E. A., Cohen, G. H., Smith-Gill, S. J., Finzel, B. C. and Davies, D. R. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8075–8079
Sheumack, D., Spence, I., Tyler, M. and Howden, M. E. H. (1990) Comp. Biochem. Physiol. **95**, 45–50
Stiles, B. G. (1991) Toxicon **29**, 503–510
Stiles, B. G. (1993) Toxicon **31**, 825–834
Stiles, B. G. and Middlebrook, J. L. (1991) J. Protein Chem. **10**, 193–204
Stiles, B. G., Lidgerding, B. C., Sexton, F. W. and Guest, S. B. (1991) Toxicon **29**, 1195–1204
Strydom, D. J. (1972) J. Biol. Chem. **247**, 4029–4042
Takasaki, C. (1989) J. Biochem. (Tokyo) **106**, 11–16
Tremeau, O., Boulain, J.-C., Couderc, J., Fromageot, P. and Menez, A. (1986) FEBS Lett. **208**, 236–240
Tu, A. T. (1973) Annu. Rev. Biochem. **42**, 235–258
Van Regenmortel, M. H. V. (1989) Immunol. Today **10**, 266–272
Van Regenmortel, M. H. V. (1992) in Structure of Antigens (Van Regenmortel, M. H. V., ed.), vol. 1, pp. 1–28, CRC Press, Boca Raton, FL
Walkinshaw, M. D., Saenger, W. and Maelicke, A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2400–2404
Weber, M. and Changeux, J.-P. (1974) J. Mol. Pharmacol. **10**, 15–34

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