Monoclonal antibodies against the *Androctonus australis hector* scorpion neurotoxin I: characterisation and use for venom neutralisation

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Abstract A series of monoclonal antibodies (mAbs) specific for the α-neurotoxin I (Aah I) from the venom of the dangerous *Androctonus australis hector* scorpion were obtained using carrier protein-coupled toxin. Competitive RIA, receptor assays and mouse toxicity tests were performed to characterise mAbs in terms of affinity and neutralisation. Cross-reactivity studies and two-site ELISA results allowed some classification of mAbs into three groups. One mAb, 9C2, was particularly interesting since it recognised the parent toxin I with a K_D of 0.15 nM and was also reactive with toxins of the same immunological group. Its ability to neutralise the toxic effect of the parent toxin and the venom fraction has been investigated. This anti-Aah I mAb 9C2, associated with anti-Aah II mAb 4C1, provides a valuable tool to neutralise the toxicity of the venom.

Key words: Venom and toxin; Monoclonal antibody; Neutralization; Synaptosome; *Androctonus australis hector* scorpion

1. Introduction

Scorpion envenomation is an important health problem in some regions in the world [1,2]. Venoms contain toxins that act on ion channels [3,4] and that are likely to be responsible for the noxious effects observed when people are stung by scorpions. Neutralisation of these molecules is an important challenge and the use of monoclonal antibodies (mAbs) may be advantageous. Recent advances in antibody engineering methodology have enhanced the field of mAb applications [5]. Venom from *Androctonus australis hector* (Aah), a North African scorpion, contains several potent α-neurotoxins, which are deadly to mammals. These toxins bind to receptor site 3 on the rat sodium channel and inhibit inactivation [6–8]. Neurotoxins I, II and III (Aah I, Aah II, Aah III) are present in approximately equal amounts in the venom (1–2%) with slight variations depending on the geographic location of the scorpions [9]. These toxins can be classified into two different groups based on both structural and immunological properties. There is no cross-reactivity between the toxins of group 1 (Aah I and III) and group 2 (Aah II) when using specific rabbit antisera [10]. The structure-activity and structure-antigenicity relationships are well documented for the most potent toxin, Aah II [11–14] and one high-affinity mAb has been obtained [15] and expressed as a single chain antibody fragment (scFv) in *Escherichia coli* [16]. Knowledge of properties of Aah II combined with chemical peptide synthesis led to the design of non-toxic immunogens capable of inducing neutralising antibodies which protect against Aah II challenge [17].

In the current work, mAbs against Aah I were produced to study the effectiveness of complete neutralisation of the *A. australis hector* scorpion venom. We found that procedures used to obtain anti-Aah II mAbs failed for Aah I. Aah I was non-immunogenic in mice when injected in its native form in sub-lethal doses. Injection of higher quantities of a detoxified antigen or a non-toxic analogue did not improve its immunogenicity. It was possible to elicit a series of mAbs that recognise the native toxin in a liquid-phase immunoassay, after coupling Aah I to different carrier proteins. Several of these mAbs were characterised and classified into three groups. They displayed high affinity to the parent toxin and were able to neutralise Aah I. Their cross-reactivity with homologous toxins and their neutralising properties against the whole venom were also studied.

2. Materials and methods

2.1. Animals

BALB/c and C57BL/6 mice and Wistar rats were raised and housed in the conventional animal facilities in our laboratory. Animal care was in accordance with institutional guidelines.

2.2. Toxins and immunogens

Toxins Aah I, Aah II, Aah III and Aah IV were obtained from the venom of the scorpion *A. australis hector* and toxin Bot I was obtained from *Buthus occitanus tunetanus*. They were carefully purified and characterised in the laboratory [18–20]. Non-toxic analogues, $(abu)_8$Aah I, $(abu)_8$Aah II and $(abu)_8$Aah III, were synthesised as previously described [17]. For immunisation, Aah I was coupled to $(abu)_8$Aah II or BSA using 0.15% glutaraldehyde [21]. The molar ratio for Aah I:$(abu)_8$Aah II and Aah I:BSA was 4:1.

2.3. Immunisation

BALB/c female mice (6 weeks old) were given one subcutaneous (s.c.) injection of 40 μg of $(abu)_8$Aah II-coupled Aah I or BSA-coupled Aah I mixed with Freund's complete adjuvant on day 0, and then 40 μg mixed in Freund's incomplete adjuvant on days 15 and 30 by intraperitoneal (i.p.) injection. Ten days after the last injection, mice were bled from the retro-orbital eye socket and sera were tested for anti-Aah I activity by RIA. Mice used in fusion 1 were also given i.p. injections of 40 μg of $(abu)_8$Aah II-coupled Aah I at days 70, 105 and 148. Six months later a final boost of a mixture of 80 μg of $(abu)_8$Aah II-coupled Aah I and 4 μg of Aah I in 0.9% NaCl was given i.p. over 3 days. Mice used in fusion 2 were only given i.p. a final boost of 40 μg of BSA-coupled Aah I on day 100 over 4 days.

2.4. Production of mAbs

MAbs were produced roughly as previously described [22]. Splenocytes from immune mouse (10^8 cells) were fused with X63-F murine myeloma cells (2×10^7) using polyethylene glycol 1500. For fusion 1, hybrids were selected in HAT-RPMI 1640 medium containing 15% FCS (Boehringer Ingelheim). Antibody-secreting cells were expanded and cloned twice at limiting dilutions using mouse peritoneal macrophages as feeder cells. For fusion 2, cloning and selection were

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achieved using a methylcellulose-based medium (Clonacell-HY). After 10–14 days individual clones were picked and transferred to 96-well plates. After screening by RIA, positives clones of the two fusions were expanded and cells were grown as ascites tumours in pristane-primed mice.

Antibodies were purified on protein A-Sepharose (Pharmacia) using 0.1 M Na citrate (pH 5.5 (IgG_1), pH 4.5 (IgG_{2a}) or pH 3 (IgG_{2b})) as eluting buffer and dialysed in BBS. Purity was assessed by 12% SDS-PAGE and p*I* 3–9 IEF gel (PhastSystem, Pharmacia). The protein concentration of the IgG solutions was measured by UV spectrophotometry with $A_{278} = 1.4$ cm^2/mg. Antigen-mediated ELISA was used to determine the heavy chain isotype (Sigma ISO-2 kit).

2.5. Radiolabelling

Aah I or Aah II (0.5 nmol) was radiolabelled with iodine-125 (Amersham) by the lactoperoxidase method and purified as previously described [23]. Fully active Aah I or Aah II with a specific radioactivity of 440 or 945 Ci/mmol, respectively, was obtained. Protein A (1 nmol, Sigma) was radiolabelled by the lactoperoxidase method and purified using Sephadex G25 (Pharmacia). Specific radioactivity was 200 Ci/mmol.

2.6. Immunoassays

2.6.1. RIA. The ability of each mAb to bind to [^{125}I]Aah I was assessed. Varying dilutions of purified IgG were mixed with [^{125}I]Aah I (1×10^{-10} M) in PBS containing 0.1% BSA (final volume of 150 μl). The mixtures were incubated for 90 min at 37°C then overnight at 4°C, 0.5 ml of sheep anti-mouse precipitating antibody (UCB Bioproducts, Belgium) was added. The mixtures were incubated for 30 min at 4°C and then the immune complexes were centrifuged at $9000\times g$ for 10 min. Radioactivity was measured with a gamma counter (Packard Crystal II). All assays were performed in duplicate.

2.6.2. ELISA. For epitope mapping IgG from mAbs 2G3 and 9C2 were conjugated to ImmunoPure activated peroxidase (Pierce). Microtitre plates (Maxisorb, Nunc) were coated with 100 μl/well of one purified mAb (10 μg/ml) in PBS and incubated overnight at 4°C. Blocking solution (1% casein in PBS) was added to each well and the plates were incubated for 1 h at 37°C and then washed with 0.9% NaCl containing 0.05% Tween 20 (NT). 1 μg/ml of Aah I diluted in PBS-T was added and incubated for 2 h at 37°C. The plates were washed with NT and further incubated with a peroxidase-conjugated mAb 9C2 or 2G3. The wells were washed again with NT and peroxidase substrate solution (TMB, Kirkegaard and Perry Labs, MD) was added. After 10 min at room temperature, the reaction was stopped by addition of 1 M phosphoric acid. Absorbance at 450 nm was measured using an iEMS reader (LabSystems). The assays were carried out in duplicate.

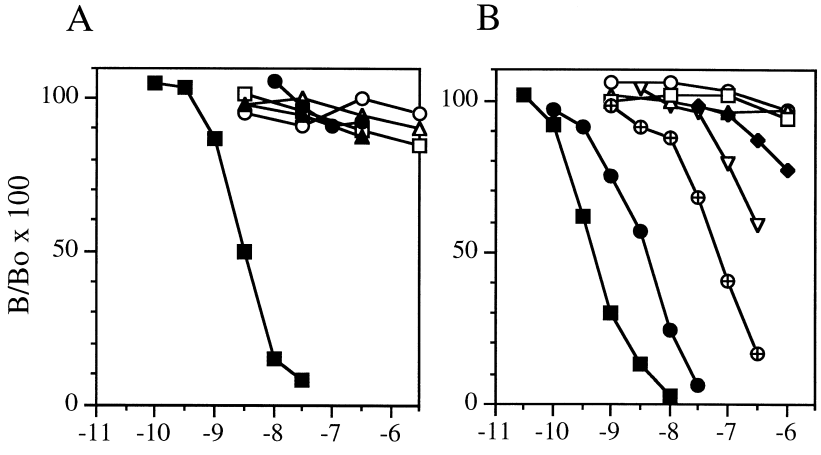
2.7. In vitro receptor assays

Synaptosomal fractions from adult rat brains were prepared as previously described [24]. The protein contents were determined using the Micro BCA protein assay reagent (Pierce). Binding assays were performed in 140 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES and 0.2% BSA at pH 7.4. Inhibition of binding of Aah I to its receptor was achieved using a mixture of [^{125}I]Aah II (1.5×10^{-10} M) and Aah I (6×10^{-8} M) incubated with different dilutions of mAbs (10^{-6}–10^{-8} M) for 90 min at 37°C. The rat brain synaptosomal preparation (1 mg/ml) was then added and incubated for 30 min at 37°C. The reaction was then stopped by centrifugation and radioactivity in the pellet was measured using a gamma counter (Packard Crystal II). Assays were performed in duplicate. [^{125}I]Aah II was used as a reference because of its high affinity ($K_D = 0.4\times10^{-10}$M) for the common receptor of α-neurotoxins. The concentration of Aah I used in the assay was determined by competitive experiments using increasing concentrations of Aah I.

The binding of [^{125}I]protein A-mAb complexes to Aah I bound to synaptosomes was determined as previously described [25].

2.8. In vivo assays

The neutralising capacity of the mAbs was tested by the intracerebroventricular (i.c.v.) or s.c. route. Aah I or venom toxic fraction, at varying concentrations equal to or higher than the LD_{50}, were preincubated for 90 min at 37°C and 30 min at 4°C with an equal volume of IgG from mAbs or from non-immune serum. Female C57BL/6 mice (groups of four) were injected i.c.v. (5 μl) or s.c. (200 μl). Surviving mice were recorded after 24 h.



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Group		
	Aah I	-KRDGYIVYPNNCVYHCVPP---CDGLCKKNGGSSGSCSFLVPSGLACWC-KDLPDNVPIKDTSRKCT
1	Aah III	-VRDGYIVNSKNCVYHCVPP---CDGLCKKNGAKSGSCGFLIPSGLACWCVA-LPDNVPIKDPSYKCHS
	Aah IV	-GRDGYIVDSKNCVYHCYPP---CDGLCKKDGAKSGSCGFLVPSGLACWC-NDLPENVPIKDPSDDCHK
2	Aah II	-VKDGYIVDDVNCTYFCGR-NAYCNEECTKLKGESGYCQWASPYGNACYCYK-LPDHVRTKGPGR-CH*
3	Bot I	-GRDAYIAQPENCVYECAQ-NSYCNDLCTKNGATSGYCQWLGKYGNACWC-KDLPDNVPIRIPG-KCHF*

Fig. 1. Cross-reactivity of anti-Aah I mAbs with Aah I and other homologous toxins assessed by competitive RIA. A: Typical curves obtained with mAb 7D3. MAbs 2G3, 10A10, 10E6 and 5B5 displayed similar results. B: Cross-reactivities of mAb 9C2 with different ligands: Aah I (■), Aah II (♦), Aah III (●), Aah IV (⊕), Bot I (▽), $(abu)_8$Aah I (□) $(abu)_8$Aah II (△), $(abu)_8$Aah III (○). B and Bo represent the reactivity measured in the presence (B) or absence (Bo) of ligand. C: Sequences of toxins from three immunological groups (review: [4]).

3. Results

3.1. Characterisation of mAbs

Several hybridoma lines were generated by two fusions with slightly different immunogens, immunisation schedules and fusion protocols. Fusion 1 involved a long immunisation protocol using Aah I coupled to $(abu)_8$Aah II. These hybridomas were cloned twice by limiting dilution. Fusion 2 involved a short-term immunisation protocol using Aah I coupled to BSA, then methylcellulose medium was used to directly select and clone the mAbs. The mAbs from the two fusions were selected based on their capacity to recognise uncoupled native toxin in RIA. Stabilised hybridomas were expended in BALB/c mice and IgG were purified on protein A-Sepharose. Further studies were performed using mAb purified IgG. Isotyping revealed that mAbs from fusion 1 were IgG_1 and IgG_{2b} and from fusion 2 were IgG_1 and IgG_{2a}. K_D, as determined by competitive RIA, ranged from 0.15 to 3 nM, showing a high affinity of all mAbs for the native toxin (Fig. 1, Table 1).

3.2. Cross-reactivity

The possible cross-reactivity of anti-Aah I mAbs with Aah III, the toxin from the same scorpion venom that is the most similar to Aah I (79% sequence identity) was also investigated by RIA (Fig. 1). Only mAb 9C2 from fusion 2, which displayed the highest affinity for Aah I, recognised Aah III, with a K_D of 1.5 nM.

α-Neurotoxins acting on mammals have been classified into four groups on the basis of primary structure and immunological criteria [3,10]. The study of cross-reactivity of mAb 9C2 was thus continued by testing one or two toxins from each group: Aah III and Aah IV for group 1, Aah II for group 2 and Bot I for group 3 (Fig. 1). MAb 9C2 showed good immunoreactivity to the other toxins of group 1. K_D values were: 0.15 nM for Aah I, 1.5 nM for Aah III and 24 nM for Aah IV (group 1). Low reactivity was observed for toxins from group 3 (Bot I). No cross reactivity was detectable for toxins from group 2.

MAbs were also assayed in a liquid-phase RIA against the analogues of the three main toxins of the venom to determine whether the binding of mAbs to the toxins was conformation-dependent. These analogues mimic the entire sequences of toxins I, II and III but with α-aminobutyric acid instead of cysteinyl bridges. The synthetic molecules, $(abu)_8$Aah I, $(abu)_8$Aah II or $(abu)_8$Aah III, were thus devoid of their four disulphide groups. No reactivity was observed when testing these analogues (Fig. 1).

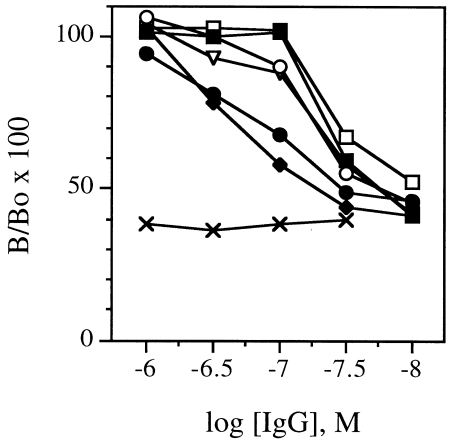

Fig. 2. Aah I neutralising capacity of anti-Aah I mAbs. Binding of Aah I (6.3×10^{-8} M) to rat brain synaptosomes, in the presence of [^{125}I]Aah II (1.5×10^{-10} M), was inhibited by preincubation with various concentrations of IgG from mAbs. Anti-Aah I mAbs inhibited the competitive binding between Aah I and [^{125}I]Aah II at the common receptor. B and Bo represent the reactivity measured in the presence (B) or absence (Bo) of mAb 9C2 (■), 2G3 (●), 10A10 (○), 10E6 (♦), 7D3 (□) and 5B5 (▽). BALB/c preimmune IgG was used as a control (X).

3.3. Epitope mapping

Sandwich-type ELISA and peroxidase-labelled mAbs 9C2 and 2G3 were used to establish if two mAbs could bind the Aah I toxin at the same time. Only mAbs 2G3 and 9C2 were able to bind Aah I simultaneously. Similar results were obtained when mAb 2G3 was plate-coated and mAb 9C2 peroxidase-labelled or when mAb 9C2 was plate-coated and mAb 2G3 peroxidase-labelled. The specificity of mAbs was as expected since coated 2G3 or labelled 2G3 could not bind Aah III. Polyclonal anti-Aah I or anti-Aah III IgG was used as a positive controls. The other mAbs could not bind Aah I simultaneously (data not shown).

3.4. Inhibitory effect of mAbs on binding of the toxin to its rat brain synaptosome receptor

The lethal effect of the scorpion venom is mainly due to the binding of α-neurotoxins to a common site on voltage-dependent sodium channels [6]. Aah II was used as a reference for the α-neurotoxin binding activity because of its high affinity for the channel. Specific binding of [^{125}I]Aah II was inhibited by Aah II ($IC_{50} = 0.2$ nM) or Aah I ($IC_{50} = 4.5$ nM). A competitive test, using both [^{125}I]Aah II and Aah I,

Table 1
Main characteristics of anti-Aah I monoclonal antibodies

Antigen	Clone	$K_D\times10^{-9}$ [a]		Isotype	p*I* [c]
		Aah I	Aah III		
Aah I-$(abu)_8$Aah II (fusion 1)	10A10	0.90	b	IgG_1	7.2
	7D3	1.20	b	IgG_{2b}	7.8
	2G3	1.80	b	IgG_{2b}	7.2/6.8
	5B5	2.40	b	IgG_1	
	10E6	3.00	b	IgG_{2b}	7.7–7.5
Aah I-BSA (fusion 2)	9C2	0.15	1.50	IgG_{2a}	7.6/7.4/7.2

[a] K_D was calculated as in [15,31] using the formula $K_D = 3/8[I-T]$, $T = [^{125}I]$Aah $I = 1\times10^{-10}$ M, $I = K_{0.5}$ determined from competitive RIA curves.
[b] No competition was observed at the highest concentration tested (5×10^{-6} M).
[c] p*I* was determined in p*I* 5–9 IEF gel (Phast System, Pharmacia).

was performed in order to quantify the inhibitory effect of anti-Aah I mAbs on the binding of Aah I to the brain receptor. Aah I, at a concentration which inhibited about 60% of the binding of [^{125}I]Aah II to its receptor, was incubated with increasing amounts of purified IgG from anti-Aah I mAbs. [^{125}I]Aah II and synaptosomal preparation were then added. All anti-Aah I mAbs tested were able to restore complete binding of [^{125}I]Aah II to the receptor whereas IgG from non-immune mice failed to do so (Fig. 2).

The ability of mAbs 2G3 and 9C2 to bind Aah I when the toxin was already interacting with its receptor was also tested. Aah I (10^{-6} M) was incubated with synaptosomal preparation, then [^{125}I]protein A-mAb complexes were added. Only mAb 2G3 exhibited specific binding to Aah I bound to synaptosomes (data not shown).

3.5. Neutralising effect of mAbs on the toxicity of Aah I and Aah venom

The toxicity of Aah I was measured in C57BL/6 mice by i.c.v. injection. The neutralising effect of mAbs was evaluated by preincubating various amounts of Aah I with purified IgG from each mAb and injecting the mixture i.c.v. into mice (four mice per Aah I concentration). The controls were performed in the same way but the mAb were replaced by IgG from non-immune mouse serum at the same concentration (3.2 mg/ml). In the presence of mAb 9C2, 50% of the mice injected survived an injection of 5 LD_{50} whereas all the control mice died (one LD_{50} = 12 ng of Aah I/mouse). The protective capacity of mAb 9C2 was 1500 LD_{50} per mg of purified IgG from ascitic fluid (molar ratio toxin:IgG, 0.4:1) (data not shown).

Since mAb 9C2 recognised Aah I and Aah III, its capacity to neutralise the Aah venom was also evaluated and compared with that of anti-Aah II mAb 4C1 [15]. The synergistic effect of the two mAbs was also investigated. For this set of experiments injections were performed by the s.c. route. Increasing amounts of Aah venom fraction (one LD_{50} = 9 μg of venom/mouse) were preincubated with mAb 9C2, mAb 4C1 or with a mixture of the two mAbs, as described in Table 2, then injected s.c. into mice. 1 mg of mAb 9C2 neutralised 45 LD_{50} of venom, 1 mg of mAb 4C1 neutralised 40 LD_{50} of venom and 1 mg of the mixture of mAbs 9C2 and 4C1 neutralised 71 LD_{50} of venom (Table 2). Thus, for an equal amount of total IgG the mixture of mAbs provides the highest venom neutralisation capacity. Furthermore, when the molar ratio 9C2/4C1 was changed, the lowest neutralisation capacity was achieved for ratios 1/4 or 4/1 (Table 2).

4. Discussion

Aah I and Aah II are present in relatively equal amounts in *A. australis hector* venom [9] and are likely to be responsible for the lethal effect of scorpion stings. Purified toxins can induce polyclonal antibodies in rabbits but these sera do not cross-react [10]. In order to better understand the mechanisms of neutralisation of the toxins, production of specific mAbs was undertaken. Using low doses of Aah II or its non-toxic analogue $(abu)_8$Aah II, it has been possible to obtain Aah II-specific mAbs [15,26]. However, the immune response induced by the toxin Aah I or its analogue $(abu)_8$Aah I was not sufficient to produce antibodies. This was only achieved after coupling the toxin to a carrier protein. Fusion 1 involved a long immunisation protocol, using Aah I coupled to $(abu)_8$Aah II. The mAbs obtained were IgG_{2b} and IgG_1, with a K_D for Aah I ranging from 0.6 to 1.5 nM. These mAbs were all strictly specific for Aah I. Fusion 2 was performed with a short immunisation protocol using Aah I coupled to BSA. The mAbs produced were IgG_{2a} (high affinity) or IgG_1 (low affinity). The mAb which displayed the highest affinity for Aah I, mAb 9C2 (K_D = 0.15 nM) was obtained from fusion 2. All these anti-Aah I mAbs failed to recognise the non-structured analogue of toxin I (abu_8Aah I). This result suggests that immunoreactivity of the mAbs is probably dependent on the native conformation of the antigen. This is already the case for the anti-Aah II mAb 4C1 (K_D = 0.8 nM) [15] which was suggested to be directed against a discontinuous epitope rather than a linear one [27].

RIA competitive experiments were performed using various homologous toxins. All mAbs from fusion 1 (2G3, 10A10, 10E6, 7D3 and 5B5) were strictly specific for Aah I (Fig. 1A). MAb 9C2 from fusion 2 also recognised toxins from the same structural and immunological group (Fig. 1B,C), Aah III (K_D = 1.5 nM) and Aah IV (K_D = 24 nM), which have 79 and 78% sequence identity with Aah I, respectively. However, the affinity of mAb 9C2 for Aah IV was 11 times less than for Aah III. This difference in immunoreactivity can be explained by examining the toxin sequences. A previous

Table 2
Neutralisation of the Aah venom fraction by varying mixtures of mAbs anti-Aah I, 9C2 and/or anti-Aah II, 4C1

	mAb 9C2 (μg)	mAb 4C1 (μg)	Aah venom (LD_{50})	Surviving mice
9C2	44	0	2	2/2
	96	0	4	0/2
4C1	0	50	2	2/2
	0	75	4	0/2
9C2+4C1	44	50	4	2/2
	44	50	6	2/2
	66	75	8	2/2
	66	75	10	2/2
9C2+4C1	0	75	4	0/4
	16	62	4	3/4
	32	50	4	4/4
	48	38	4	4/4
	64	25	4	4/4
	80	12	4	4/4
	96	0	4	0/4

Aah venom fraction was incubated with mAb 9C2 or mAb 4C1 or a mixture of mAb 9C2 and mAb 4C1 for 30 min at 37°C and then injected s.c. into C57BL/6 mice.

study has shown that three residues found in Aah I and Aah III but not in Aah IV, namely V17Y, D51E and K61D, are very important for determining biological function and explaining the differences in toxicity between Aah I, Aah III and Aah IV [20]. Furthermore, this study suggested that the change of the doublet RK (Aah I) or YK (Aah III) at positions 60–61 to DD (Aah IV) is significant since changing the charge may affect the biological properties of the toxin. Chemical modification of R60 or Y60 was also shown to dramatically decrease the functional properties of the Aah I or Aah III toxins respectively [12,13]. It is possible that one (or more) of these three residues (V17, D51, K61) is part of the epitope on Aah I recognised by mAb 9C2. Since mAb 9C2 decreased the toxicity of Aah I and the venom (see below), K61 is likely to be involved. However, exact determination of the conformational epitope of 9C2 requires further investigation [28,29]. In addition, mAb 9C2 showed low recognition of Bot I from group 3 which shares 57% sequence identity with Aah I (K_D could not be determined but was higher than 250 nM), but no cross-reactivity was observed with Aah II (44% sequence identity) from group 2.

A competition method (ELISA sandwich) was used to establish whether several mAbs could bind to the antigen simultaneously. Only mAb 2G3 and mAb 9C2 could bind to Aah I at the same time. Furthermore, mAbs 2G3 and 9C2 competed with all other mAbs for antigen binding. These results allowed the classification of the mAbs into three groups. MAb 2G3 and mAb 9C2 were in two different groups and their epitopes did not overlap each other, but they did overlap with epitopes of mAbs from the third group (10A10, 10E6, 7D3, 5B5). In addition, only mAb 2G3 was able to bind Aah I already interacting with its receptor on rat brain synaptosomes.

All mAbs were able to inhibit the binding of Aah I to its receptor on rat brain synaptosomes with varying efficacy: 9C2 or 7D3 > 10A10 or 5B5 > 10E6 or 2G3. As already suggested for anti-Aah II mAb 4C1 [15], one possible mechanism by which antibodies could neutralise the toxin likely arises from the close location of the antigenic and pharmacological sites. Binding of a mAb on the toxin in a region more or less close to the pharmacological site could prevent the simultaneous binding of the toxin to synaptosomes by totally or partially masking this site. MAbs 9C2 and 2G3 may bind the toxin at the same time as their binding sites are probably completely independent and located in different regions of the molecule. MAb 2G3 was shown to bind to Aah I bound to its receptor. The mAb 2G3 site must then not overlap with the pharmacological site on Aah I. However, mAb 2G3 was also shown to inhibit the binding of Aah I to its receptor, although with a lower efficacy than mAb 9C2. The mAb 2G3 site may be more distal than the mAb 9C2 site from the pharmacological site, thus having a smaller steric hindrance.

The potency of mAb 9C2 to neutralise Aah I toxin or venom was assessed using mouse strain C57BL/6. The neutralising capacity of mAb 9C2 was 1500 LD_{50} (18 μg) of Aah I per mg of purified IgG when injected i.c.v. (one $LD_{50} = 12$ ng of Aah I/mouse). This result is comparable to that for anti-Aah II mAb 4C1 [15] whose neutralising capacity was at least 32 000 LD_{50} (16 μg) of Aah II per mg of purified IgG in the same conditions (one $LD_{50} = 0.5$ ng of Aah II/mouse). To test the capacity of the mAb to neutralise the venom toxic fraction we chose the s.c. route (one $LD_{50} = 9$ μg/mouse). Equal amounts of venom were neutralised by anti-Aah I mAb 9C2 as by anti-Aah II mAb 4C1 (40 LD_{50} of venom by 1 mg of purified IgG). This capacity was doubled when 1 mg of the mixture of mAbs 9C2 and 4C1 was used (71 LD_{50} of venom per mg of purified IgG). The appropriate dose of commercial polyclonal antivenom is usually calculated as sufficient to neutralise 50–100 LD_{50} [30]. This work shows that it is possible to neutralise efficiently the venom toxicity by using two highly specific mAbs directed against one toxin in each immunological group.

A series of anti-Aah I mAbs have been produced and partially characterised with respect to affinity, neutralisation properties and epitope mapping. The complete description of the functional epitopes requires further protein engineering or chemical modification of the toxin. These mAbs, presented as IgG, Fabs or scFv, should be a good tool for neutralisation and pharmacokinetic studies of envenomation.

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References

[1] Goyffon, M., Vachon, M. and Broglio, N. (1982) Toxicon 20, 337–344.
[2] Ismail, M. (1995) Toxicon 33, 825–858.
[3] Rochat, H., Bernard, P. and Couraud, F. (1979) Adv. Cytopharmacol. 3, 325–334.
[4] Martin, M.-F. and Couraud, F. (1995) in: Handbook of Neurotoxicology (Chang, L.W. and Dyer, R.S., Eds.), pp. 683–716, Marcel Dekker, New York.
[5] Hayden, M.S., Gilliland, L.K. and Ledbetter, J.A. (1997) Curr. Opin. Immunol. 9, 201–212.
[6] Catterall, W.A. (1977) J. Biol. Chem. 252, 8660–8668.
[7] Jover, E., Martin-Moutot, N., Couraud, F. and Rochat, H. (1980) Biochemistry 19, 463–467.
[8] Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. (1998) J. Toxicol. Toxins Rev. 17, 131–159.
[9] El Ayeb, M. and Rochat, H. (1985) Toxicon 23, 755–760.
[10] Delori, P., Van Rietschoten, J. and Rochat, H. (1981) Toxicon 19, 393–407.
[11] Fontecilla-Camps, J.C., Habersetzer-Rochat, C. and Rochat, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7443–7447.
[12] Kharrat, R., Darbon, H., Rochat, H. and Granier, C. (1989) Eur. J. Biochem. 181, 381–390.
[13] Kharrat, R., Darbon, H., Granier, C. and Rochat, H. (1990) Toxicon 28, 509–523.
[14] Granier, C., Novotny, J., Fontecilla-Camps, J.C., Fourquet, P., El Ayeb, M. and Bahraoui, E. (1989) Mol. Immunol. 26, 503–513.
[15] Bahraoui, E., Pichon, J., Muller, J.M., Darbon, H., El Ayeb, M., Granier, C., Marvaldi, J. and Rochat, H. (1988) J. Immunol. 141, 214–220.
[16] Mousli, M., Devaux, C., Rochat, H., Goyffon, M. and Billiald, P. (1999) FEBS Lett. 442, 183–188.
[17] Zenouaki, I., Kharrat, R., Sabatier, J.M., Devaux, C., Karoui, H., Van Rietschoten, J., El Ayeb, M. and Rochat, H. (1997) Vaccine 15, 187–194.
[18] Miranda, F., Kopeyan, C., Rochat, C., Rochat, H. and Lissitzky, S. (1970) Eur. J. Biochem. 16, 514–523.
[19] Martin, M.F. and Rochat, H. (1986) Toxicon 24, 1131–1139.
[20] Mansuelle, P., Martin, M.F., Rochat, H. and Granier, C. (1992) Nat. Toxins 1, 61–69.
[21] Briand, J.P., Muller, S. and Van Regenmortel, M.H. (1985) J. Immunol. Methods 78, 59–69.
[22] Galfre, G. and Milstein, C. (1981) Methods Enzymol. 73, 3–46.
[23] Rochat, H., Tessier, M., Miranda, F. and Lissitzky, S. (1977) Anal. Biochem. 82, 532–548.

[24] Jover, E., Couraud, F. and Rochat, H. (1980) Biochem. Biophys. Res. Commun. 95, 1607–1614.
[25] El Ayeb, M., Bahraoui, E.M., Granier, C. and Rochat, H. (1986) Biochemistry 25, 6671–6678.
[26] Devaux, C., Clot-Faybesse, O., Juin, M., Mabrouk, K., Sabatier, J.M. and Rochat, H. (1997) FEBS Lett. 412, 456–460.
[27] Van Regenmortel, M.H. (1992) FEMS Microbiol. Lett. 79, 483–487.
[28] Laver, W.G., Air, G.M., Webster, R.G. and Smith-Gill, S.J. (1990) Cell 61, 553–556.
[29] Morris, G.E. (1996) in: Methods in Molecular Biology (Morris, G.E., Ed.), Vol. 66, Humana Press, Totowa, NJ.
[30] Belghith, M., Boussarsar, M., Haguida, H., Besbes, L., Elatrous, S., Touzi, N., Boujdaria, R., Bchir, A., Nouira, S., Bouchoucha, S. and Abroug, F. (1999) Clin. Toxicol. 37, 51–57.
[31] Muller, R. (1980) J. Immunol. Methods 34, 345–352.