Immunization with a synthetic robustoxin derivative lacking disulphide bridges protects against a potentially lethal challenge with funnel-web spider (*Atrax robustus*) venom

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The venom of male *Atrax robustus* spiders is potentially lethal to primates. These spiders have been responsible for a number of human deaths. Robustoxin is the lethal toxin in the venom. It is a highly cross-linked polypeptide that has 42 amino acid residues and four disulphide bridges. If these bridges are broken, the resulting polypeptide is non-toxic. Robustoxin was chemically synthesized with all of its eight cysteine residues protected with acetamidomethyl groups in order to avoid formation of disulphide bridges. The resulting derivative was co-polymerized with keyhole limpet haemocyanin. Two *Macaca fascicularis* monkeys were immunized with this conjugate. The monkeys were challenged, under anaesthesia, with a potentially lethal dose of male *A. robustus* crude venom. Both monkeys showed some minor symptoms of intoxication but recovered fully with no adverse after-effects. Immunization with the same immunogen, in the absence of keyhole limpet haemocyanin, did not protect a third monkey. The N-terminal 23 amino acid peptide derived from the sequence of robustoxin was synthesized and conjugated with ovalbumin. A fourth monkey was immunized with this conjugate. However, it was not protected against challenge. The implications of these results for the preparation of synthetic peptide vaccines are discussed.

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1. Introduction

Considerable effort has been devoted to employing synthetic peptides as vaccines (Lerner 1982; Arnon 1987; Fischer *et al* 2007). Attempts to use synthetic peptides to stimulate the production of antibodies that are protective *in vivo* have not always been successful because such peptides are often weak immunogens from the standpoint of inducing responses against the native antigen or pathogen. It is well known that the immunogenicity of antigenic determinants depends not only on continuous (sequential) epitopes, but also on polypeptide chains folding together to form discontinuous or conformational determinants consisting of amino acid residues drawn together from different parts of the sequence. Although an increasing number of reports have indicated that small peptides cannot mimic conformational epitopes of globular proteins (Benjamin *et al* 1984), many natural and synthetic peptides are known

Keywords. Monkeys; peptide immunogen; protection; spider toxin

Abbreviations used: Acm, acetamidomethyl; Boc, *t*-butoxycarbonyl; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high performance liquid chromatography; Ig, immunoglobulin; im, intramuscular; iv, intravenous; KLH, keyhole limpet haemocyanin; MTR, 4-methoxy-2,3,6-trimethylbenzene sulphonyl; ODhbt, 3,4-dihydro-4-oxobenzotriazin-3-yl esters; Opfp, pentafluorophenyl; PBS, phosphate buffered saline; sc, subcutaneous; TFA, trifluoroacetic acid

to be immunogenic in the absence of covalently coupled carrier proteins, and are of great interest in the development of vaccines against a variety of diseases, as well as tools for basic research in the field of immunology. For example, a synthetic peptide corresponding to the sequence of amino acids 141–160 of the foot-and-mouth disease virus protein VP1 contains both a B cell epitope and a helper T cell determinant, and was able to induce a virus-neutralizing antibody response in guinea-pigs (Francis *et al* 1987). A single synthetic T cell determinant of the nucleocapsid of the hepatitis B virus has also been found to stimulate specific antibody production (Milich *et al* 1987). Furthermore, it has been confirmed that the immunogenicity of peptides can be significantly enhanced by coupling them to appropriate T cell determinants derived from different proteins (Cox *et al* 1988). These results suggest that peptides themselves may have an intrinsic immunogenicity dependent on their possession of a T and a B cell determinant.

We studied the immunogenicity of the small single-chain polypeptide robustoxin (now named *δ*-atracotoxin-Ar1 [Fletcher *et al* 1997]), the sole lethal neurotoxin in the venom of the male funnel-web spider, *A. robustus*. This spider has been responsible for a number of human fatalities in Australia (Sutherland 1983). We determined the sequence of robustoxin's 42 amino acids (Sheumack *et al* 1985). Robustoxin has four disulphide bridges. It is tightly folded (Pallaghy *et al* 1997). In anaesthetized monkeys, robustoxin induced all of the characteristic neurotoxic effects of male *A. robustus* crude venom: immediate disturbances in respiration, blood pressure and heart rate, followed by severe hypotension or potential death due to respiratory and circulatory failure, together with lachrymation, salivation, generalized skeletal muscle fasciculation and a parallel increase in body temperature, and increased firing in autonomic and skeletal motor nerves (Mylecharane *et al* 1984, 1989). Immunization of monkeys with a toxoid prepared by polymerization of native robustoxin with glutaraldehyde conferred full protection against a potentially lethal challenge with male *A. robustus* crude venom (Sheumack *et al* 1991).

In this paper, we describe the preparation of a synthetic robustoxin derivative lacking disulphide bridges, its polymerization with glutaraldehyde in the presence of keyhole limpet haemocyanin (KLH), and the effectiveness of immunization with this immunogen in protecting monkeys against potentially lethal challenge with male *A. robustus* venom under general anaesthesia. At the time this work was performed, primates were the only animals in which the efficacy of immunization with robustoxin derivatives could be tested, because the venom of *A. robustus* appeared to have relatively little effect on non-primate mammals (Sutherland 1983).

2. Materials and methods

2.1 *Synthesis of robustoxin derivatives*

The robustoxin derivative lacking disulphide bridges and the N-terminal 23 amino acid residue (named Rs1-23) were synthesized on Pepsyn KH (polyamide–kieselguhr) resin by the solid phase 9-fluorenylmethyloxycarbonyl (Fmoc) procedure using Fmoc-amino acid pentafluorophenyl (Opfp) esters, with the exception of threonine and serine, which were coupled as Fmoc–amino acid 3,4-dihydro-4-oxobenzotriazin-3-yl esters (ODhbt) (Sheppard 1986). The side chains of Glu, Asp, Tyr, Ser and Thr amino acid residues were protected by a *t*-butyl group. The side chains of Lys and Arg amino acid residues were protected by *t*-butoxycarbonyl (Boc) and 4-methoxy-2,3,6-trimethylbenzene sulphonyl (MTR) groups, respectively. The cysteinyl residues were each protected by the acetamidomethyl (Acm) moiety. Unlike the protecting groups on the other amino acid residues, the Acm group is not removed by trifluoroacetic acid (TFA). After cleavage from the synthesis resin by treatment with TFA:water: ethanedithiol:anisole:ethylmethylsulphide in the ratios 93:7:0.95:3.10:2.97 v/v for 5 h, the crude Acm-protected peptides were purified by reverse-phase high performance liquid chromatography (HPLC) using a Waters C_{18} μBondapak column and a linear gradient of 0–70% v/v acetonitrile/water in 0.05% v/v TFA over 30 min. A typical chromatogram is shown in figure 1. The structures of the

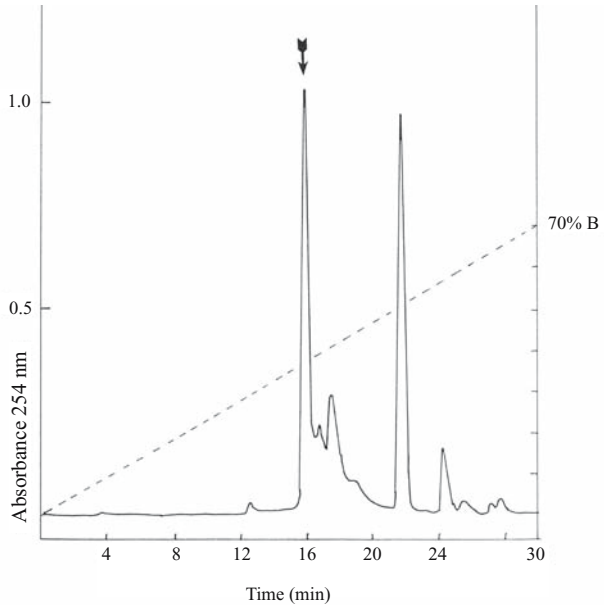

Figure 1. Reverse-phase HPLC chromatogram of crude Rs1-42 after cleavage from the synthesis resin. The peak marked with an arrow gave the correct amino acid composition after hydrolysis and analysis using the Waters Pico-tag system. Material corresponding to peaks that eluted after 20 min were scavengers. Point B corresponds to 70% acetonitrile in the solvent gradient used.

purified peptides were determined by amino acid analysis. Their sequences were confirmed by automated Edman degradation using the gas phase method.

2.2 *Polymerization and conjugation of synthetic robustoxin derivatives*

The synthetic robustoxin derivative lacking disulphide bridges was polymerized with glutaraldehyde essentially according to the method for the toxoid (denoted Rn1-42) of robustoxin (Sheumack *et al* 1991). This polymerized material will be referred to as Rs1-42. Some of the synthetic peptide (0.2 mg) was also polymerized with glutaraldehyde in the presence of 0.8 mg of KLH (Sigma Chemical Co., USA) by the same procedure as above. This produced a conjugate designated Rs1-42-KLH. Rs1-23 was not polymerized but was conjugated to ovalbumin (Sigma Chemical Co., USA) through the cysteine SH group (Liu *et al* 1979). This conjugate was named Rs1-23-Ov.

2.3 *Collection of venom and lethality assay*

Venom of male *A. robustus* spiders for use in challenging monkeys was collected by the procedures of Sheumack *et al* (1984). The freeze-dried crude venom was stored at 4°C until used in lethality assays and challenges in monkeys. The lethalities of the crude venom, synthetic immunogens and their polymerized products were measured using newborn mice (Sheumack *et al* 1984; Sutherland 1980) weighing 1.5–1.8 g. Doses (20 μl) were injected subcutaneously (sc) into the dorsum of the mouse parallel to the spine. Each preparation was injected into a group of four mice. Each group was kept in a small box lined with cotton-wool in order to minimize temperature fluctuations.

2.4 *Antibody enzyme-linked immunosorbent assay*

An enzyme-linked immunosorbent assay (ELISA) was used to assess the production of antibodies to the various immunogens in the monkeys. Briefly, the wells of Titertek polystyrene microtitre plates (Flow Laboratories, The Netherlands) were coated with 100 μl of a solution of robustoxin (4 μg/ml) per well overnight. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% v/v of Tween 20. Non-specific binding sites were then blocked with PBS containing 2% w/v bovine serum albumin (BSA) for 2 h. After the plates were again washed, 100 μl of serially diluted monkey serum was added to each well. The plates were then incubated for 1 h at room temperature on a plate shaker. The plates were then washed as before and 100 μl of peroxidase-labelled goat anti-monkey immunoglobulin (Ig)G (Organon Teknika Corp., USA) added to each well. It was found to be important to dilute the peroxidase-labelled antibody in PBS containing 0.25 M NaCl in order to abolish the high background absorbance that otherwise interfered with the assay. The reason for the high background absorbance is unclear, but is probably due to non-specific ionic interactions between the positively charged robustoxin and the antibody. The plates were finally incubated for another hour and washed again as before. The colour reaction was developed by the addition of 100 μl per well of a solution of *o*-phenylenediamine (0.5 mg/ml) prepared in 0.1 M citrate buffer (pH 4.2) containing 0.03% hydrogen peroxide. The reaction was quenched by the addition of 4 M sulphuric acid solution. The optical density was measured at 492 nm with an ELISA reader.

2.5 *Immunization of monkeys*

Experiments were performed on five monkeys (*M. fascicularis*) weighing 2.1–3.8 kg and not gender selected. Due to the scarcity of monkeys, only five animals were available for this study. One of these monkeys had previously been immunized successfully with Rn1-42, without adjuvant or conjugation, administered sc at a dose level of 50 μg/kg at 14-day intervals for 8 weeks (i.e. five injections). This had fully protected the animal against a potentially lethal intravenous challenge, under general anaesthesia, with male *A. robustus* crude venom (50 μg/kg over 5 min), 14 weeks after the last dose of toxoid (Sheumack *et al* 1991). In the present experiments, this animal was subjected to a second similar challenge 165 weeks after the last toxoid dose. By this time, the anti-robustoxin antibody titre had declined to a very low level. The purpose of this challenge was to confirm the likely loss of Rn1-42-induced protection, and to serve as a control experiment to verify the potentially lethal action of the venom used in the anaesthetized monkey challenge experiments. The second monkey was immunized with Rs1-23-Ov administered sc at a dose of 25 μg/kg at 14-day intervals for 6 weeks (i.e. four injections). It was challenged with male *A. robustus* crude venom 124 weeks after the last injection. Another monkey was vaccinated with polymerized Rs1-42 (five sc injections, 50 μg/kg, at 14-day intervals), and was challenged 20 weeks after the last dose. The remaining two monkeys received polymerized Rs1-42-KLH using the same dosage regimen as for the monkey immunized with Rs1-42. The challenges for these two monkeys were delivered 1 week and 15 weeks, respectively, after the last immunizing dose. Venous blood samples (1–5 ml) were taken from the monkeys after the immunization regimen. The samples were allowed to coagulate, then were centrifuged at 3500 rev/min for 10 min. The serum obtained was incubated at 56°C for 30 min and stored frozen until assayed by ELISA for anti-robustoxin antibodies.

2.6 *Challenge of anaesthetized monkeys with male* A. robustus *venom*

All challenges of monkeys with male *A. robustus* crude venom were conducted under general anaesthesia and aseptic conditions. Anaesthesia was induced with an intramuscular (im) injection of ketamine (10 mg/kg) followed by halothane (0.75–3%) in 2:1 nitrous oxide:oxygen via a face mask. An endotracheal tube was then inserted, and anaesthesia was maintained with halothane (0.25–1%) in 2:1 nitrous oxide:oxygen via the tube. The animals were placed on a thermostatically controlled heating pad maintained at 37°C, and rectal temperature was monitored. Expiratory carbon dioxide levels were measured with a Datex CD-102 capnograph. Systemic arterial blood pressure was measured by means of a pressure transducer (Statham P23a) connected to a polythene catheter passed into the aorta via a femoral artery branch. The blood pressure signal was used to trigger a Grass 7P4 tachograph so that heart rate could also be monitored. A polythene catheter in a femoral vein branch was used for the intravenous (iv) administration of male *A. robustus* venom dissolved in 0.9% w/v sodium chloride solution (30–60 ml/h). Blood pressure, heart rate and expiratory carbon dioxide levels were recorded on a Grass Model 7 polygraph. The stability of the level of anaesthesia was confirmed by observations of blood pressure and heart rate levels (taking into account any venom-induced changes), and periodic testing of pupillary, blink and withdrawal reflexes.

The various parameters being recorded were allowed to stabilize, and were then monitored for a period of at least 30 min prior to the iv infusion of the solution of male *A. robustus* venom at a dose of 50 μg/kg over a period of 5 min. Monitoring was continued for up to 210 min. The monkeys immunized with Rs1-42-KLH were little affected by the venom challenges. These monkeys were permitted to recover from the anaesthetic after removal of the catheters and suturing of the femoral incision. Xylazine (1.25 mg/kg im) was administered to all monkeys as required for sedation during the 24-h post-challenge period. None of the other monkeys would have survived the challenge; all three of them succumbed to its potentially lethal effects in 53–153 min. The unprotected monkeys recovered with the assistance of a ventilator. Two different batches of venom were used in these challenge experiments. There was no detectable difference in the lethality of these batches of venom, and they were found to contain a consistent content of robustoxin by HPLC.

3. Results

3.1 *Effects of peptide derivatives in newborn mice*

All three immunogens, Rs1-23-Ov, Rs1-42, and Rs1-42-KLH, were found to be non-toxic when tested for lethality in newborn mice.

3.2 *Effects of male* A. robustus *crude venom in a control monkey*

Monkey 1 had previously been immunized with glutaraldehyde-polymerized native robustoxin toxoid (Rn1-42), and had been fully protected against challenge with male *A. robustus* crude venom (50 μg/kg administered iv over 5 min) 14 weeks after the last dose of toxoid (Sheumack *et al* 1991). A second challenge was performed 165 weeks after the last toxoid dose, when the titre of anti-robustoxin antibodies had fallen to very low levels (table 1). As expected from the low antibody titres, there was no

Table 1. Anti-robustoxin antibody titres[a] in monkey serum samples

	Monkey number and immunogen				
	1	2	3	4	5
Weeks after start of immunization	Control	Rs1-23-Ov	Rs1-42	Rs1-42-KLH	Rs1-42-KLH
0	–	<20	<20	<20	<20
2	–	80	320	40	1280
4	–	40	160	80	5120
6	–	80	640	40	640
8	–	–	640	1280	160
Pre-challenge[b]	320[c]	<20	1280	2560	160
		(124)	(20)	(15)	(1)

[a]The antibody titre is the reciprocal of the maximum dilution which gave an absorbance value double that of a negative control.

[b]The titres immediately prior to challenge with male *A. robustus* crude venom. The intervals in weeks between the last immunizing dose and the challenge are shown in parentheses.

[c]The titre before the second challenge (165 weeks after the last immunizing dose with Rn1-42).

Table 2. Blood pressures[a] in anaesthetized monkeys challenged with male *A. robustus* crude venom

Monkey and immunogen	Pre-infusion level (mmHg)	Peak level during recording period (mmHg)	Level at end of recording period (mmHg)
1. Control (Rn1-42)	56	32 (6 min)	0 (53 min)[b]
		51 (9 min)	
2. Rs1-23-Ov	53	37 (5 min)	0 (153 min)[b]
		60 (10 min)	
3. Rs1-42	77	60 (6 min)	0 (121 min)[b]
		95 (12 min)	
4. Rs1-42-KLH	58	56 (5 min)	74 (210 min)
		66 (12 min)	
		51 (45 min)	
5. Rs1-42-KLH	95	93 (6 min)	68 (210 min)
		136 (16 min)	
		63 (165 min)	
		71 (195 min)	

[a]The values shown are individual mean systemic arterial blood pressures, before and after infusions of solutions of male *A. robustus* crude venom (50 μg/kg i.v. over 5 min) in the 5 monkeys immunized as indicated. The values in parentheses are the times after commencement of the infusions at which the various events occurred.

[b] Potential fatality due to venom.

Table 3. Heart rates[a] in anaesthetized monkeys challenged with male *A. robustus* crude venom

Monkey and immunogen	Pre-infusion level (beats/min)	Peak level during recording period (beats/min)	Level at end of recording period (beats/min)
1. Control (Rn1-42)	185	135 (6 min)	0 (53 min)[b]
		187 (9 min)	
2. Rs1-23-Ov	197	200 (4 min)	0 (153 min)[b]
3. Rs1-42	101	158 (6 min)	0 (121 min)[b]
4. Rs1-42-KLH	97	165 (12 min)	145 (210 min)
		81 (45 min)	
5. Rs1-42-KLH	151	242 (16 min)	138 (210 min)
		125 (55 min)	
		150 (150 min)	

[a]The values shown are individual heart rates, before and after infusions of solutions of male *A. robustus* crude venom (50 μg/kg i.v. over 5 min) in the 5 monkeys immunized as indicated. The values in parentheses are the times after commencement of the infusions at which the various events occurred.

[b] Potential fatality due to venom.

longer any protection against the potentially lethal effects of the venom. The effects of a potentially lethal challenge, 165 weeks after the last toxoid dose, were identical to those reported previously in non-immunized anaesthetized monkeys (Mylecharane *et al* 1989; Sheumack *et al* 1991). There were rapid-onset, marked cardiovascular disturbances (tables 2 and 3) as well as respiratory changes, salivation, lachrymation and skeletal muscle fasciculation (table 4). These effects culminated in potential lethality due to cardiac and respiratory failure 53 min after commencing infusion of the venom.

3.3 *Immunization with Rs1-23-Ov*

The monkey treated with Rs1-23-Ov (monkey 2) failed to produce any detectable anti-robustoxin antibodies in serum samples (table 1). The treatment produced no observable acute or long-term effect. When challenged with male *A. robustus* venom (50 μg/kg iv over 5 min) 124 weeks after the last dose, there was no protection against the potentially lethal effects. The characteristic neurotoxic outcomes of the venom were produced (tables 2, 3 and 4), culminating in potential lethality due to cardiovascular and respiratory

failure 153 min after commencement of infusion of the venom.

3.4 *Immunization with Rs1-42*

Anti-robustoxin antibodies were detectable in serum samples of monkeys immunized with Rs1-42. The levels of these antibodies remained stable during the subsequent 20 weeks until the challenge was undertaken (table 1). Rs1-42 produced no observable acute or long-term effects in monkeys. Challenge with the solution of male *A. robustus* venom (50 μg/kg iv over 5 min), however, proved to be potentially fatal. Figure 2 illustrates the typical rapid onset of marked cardiovascular and respiratory disturbances induced by venom infusion, which ended in potential fatality 121 min after commencing the infusion (tables 2, 3 and 4). The salivation and lachrymation produced were typical symptoms displayed by unprotected monkeys after challenge (table 4). However, skeletal muscle fasciculation appeared to be slightly delayed in onset and was less marked than that normally seen in unprotected monkeys (Sheumack *et al* 1991; and present experiments).

Table 4. Respiratory disturbances, salivation/lachrymation, and skeletal muscle fasciculation, in anaesthetized monkeys challenged with male *A. robustus* crude venom[a]

Monkey and immunogen	Parameter	Respiratory disturbances	Salivation/lachrymation	Skeletal muscle fasciculation
1. Control (Rn1-42)	Onset	5 min	32 min	18 min
	Peak	+++	+	+
2. Rs1-23-Ov	Onset	4 min	19 min	12 min
	Peak	+++	+++	+++
3. Rs1-42	Onset	5 min	8 min	31 min
	Peak	++++	+++	++
4. Rs1-42-KLH	Onset	9 min	115 min	11 min
	Peak	+	+	+++
5. Rs1-42-KLH	Onset	6 min	17 min	21 min
	Peak	+	++	++

[a]The values shown are individual onset times and peak effects for three parameters after infusions of solutions of male *A. robustus* crude venom (50 μg/kg i.v. over 5 min) in the 5 monkeys immunized as indicated. The onset times are the times after commencement of the infusions. The magnitude of the various effects were graded as follows: 0 = no effect ; + = slight ; ++ = moderate ; +++ = severe ; ++++ = very severe.

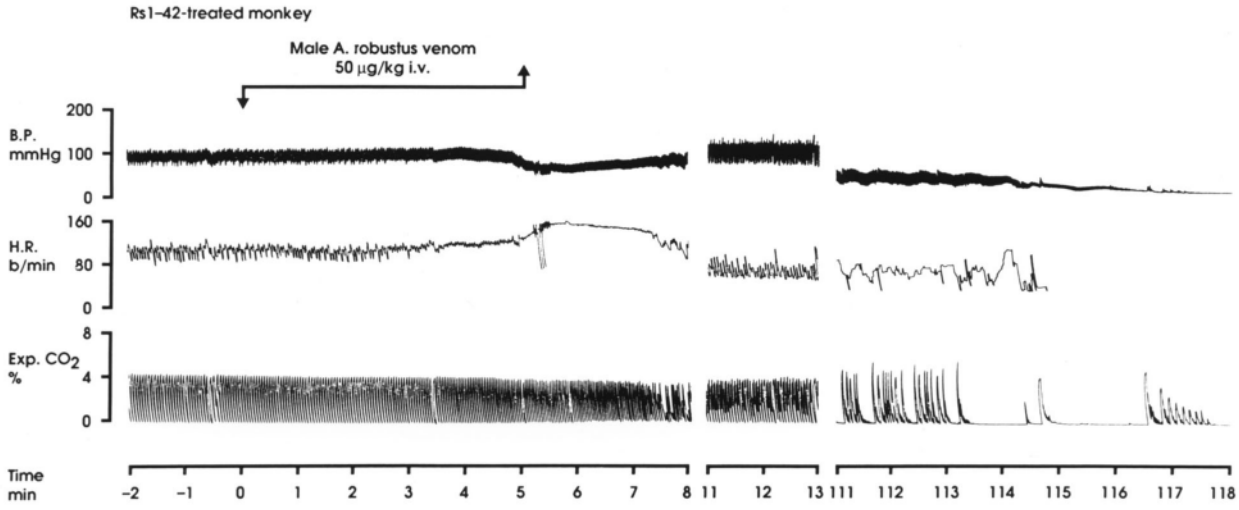

Figure 2. Traces from monitoring the anaesthetized monkey 3 immunized with polymerized Rs1-42 showing systemic arterial blood pressure (BP), heart rate in beats/min (HR), and expiratory CO_2 (Exp. CO_2), before and after the i.v. infusion of 50 μg/kg of male *A. robustus* crude venom.

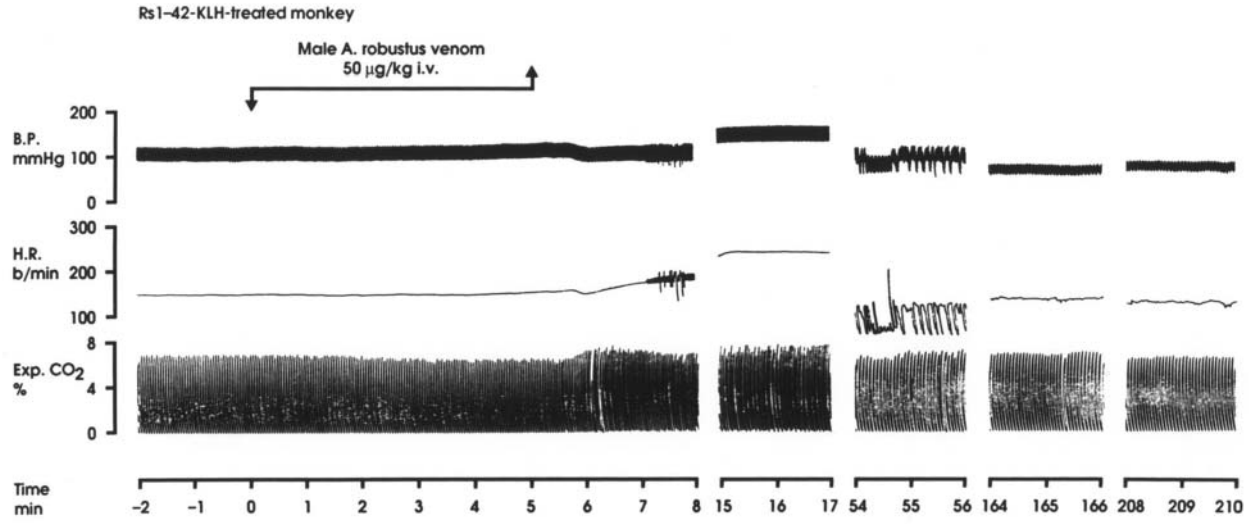

Figure 3. Traces from monitoring the anaesthetized monkey 5 immunized with Rs1-42-KLH and challenged and monitored as in figure 2.

3.5 *Immunization with Rs1-42-KLH*

Anti-robustoxin antibodies were detected in serum samples from monkey 5 (table 1) within 2 weeks of commencing immunization with Rs1-42-KLH, but the titres decreased substantially as the immunization schedule progressed. The other animal (monkey 4) did not produce high levels of specific antibodies until after the fourth dose of immunogen. These differences in antibody titres over time between monkeys 4 and 5 are attributed to individual responses expected in a natural system, where genetic traits may play a part. If a large number of animals could have been used, then such variations could have been viewed in a proper statistical context. Rs1-42-KLH produced no observable acute or long-term effects in the two monkeys immunized with it, other than small localized inflammatory lesions at some of the injection sites. The lesions became fibrosed within 2 weeks of injection. The fibroses then regressed and virtually disappeared over the succeeding 4 weeks. Despite the early differences in their specific antibody levels, both monkeys were fully protected from the potentially lethal effects of challenge with the solution of male *A. robustus* crude venom (50 μg/kg iv infused over 5 min).

The challenges produced some cardiovascular and respiratory disturbances, but these were only minor compared to those in the three unprotected monkeys (monkeys 1, 2 and 3; control, Rs1-23-Ov and Rs1-42 immunizations, respectively). The traces in figure 3 illustrate the typical minor cardiovascular and respiratory effects of the venom in the monkey immunized with Rs1-42-KLH. The changes are summarized in tables 2, 3 and 4. The onset of these effects in all of the monkeys tested occurred at comparable times, but the changes in the protected monkeys were smaller than those in the unprotected monkeys. At the end of the monitoring period (210 min), blood pressure and heart rates were similar to the pre-infusion levels and respiration was normal. Both of the protected monkeys recovered fully and uneventfully. Salivation and lachrymation were much less marked in animals immunized with Rs1-42-KLH than in monkeys 2 and 3 (table 4). In one monkey, the onset of salivation was substantially delayed, and there was no lachrymation. Skeletal muscle fasciculation in the protected monkeys, however, was similar in onset and severity to that observed in the unprotected animals (table 4).

4. Discussion

The results obtained in the two monkeys immunized with Rs1-42-KLH indicated the feasibility of using a synthetic peptide derivative to immunize primates against the effects of male *A. robustus* venom. When challenged with a potentially lethal dose of male *A. robustus* venom, the monkeys immunized with Rs1-42-KLH did show some minor symptoms typical of both the venom and of robustoxin, as previously described (Duncan *et al* 1980; Mylecharane *et al* 1989). However, the effects in these monkeys were much less severe in both magnitude and duration, and the monkeys recovered fully and uneventfully. It would seem reasonable to postulate that venom infused iv over a 5 min period is not immediately and completely neutralized, hence some minor effects would be observed. Nevertheless, the neutralization of the venom is clearly sufficient to protect the animals from the potentially lethal effects, which are usually manifested within 5 h. Neutralization of the venom is also adequately long-lasting for the venom to be metabolized and

rendered non-toxic, because the monkeys did not exhibit any evidence of subsequent toxicity. The present results show that protection persists for at least 15 weeks after completion of immunization with Rs1-42-KLH.

Our results suggest that co-polymerization with KLH is essential for the preparation of a protective immunogen using the synthetic derivative lacking disulphide bridges, because the immunogen (Rs1-42) produced by its glutaraldehyde polymerization in the absence of KLH had no protective effect. We conclude that this derivative incorporates a protective B cell epitope, but no T cell epitope. There was no correlation between specific antibody titre and protective immunity. However, there seemed to be a better correlation between the protected status of the animals and the inverse of the time between immunization and challenge. One possibility is that antibody class is important for protection. The carrier KLH moiety might have contributed one or more helper T cell epitope(s) responsible for stimulating activation of the cellular response leading to protective immunity. Harris and Markl (1999) have reviewed the molecular basis of action of KLH and its use as a carrier protein for peptides in vaccines by provision of T cell epitopes. Several groups have recognized the desirability of including a T cell epitope as part of a synthetic vaccine (Francis *et al* 1987; Milich *et al* 1987; Borras-Cuesta *et al* 1987; Good *et al* 1987). Francis *et al* (1988) have shown that the use of single peptides containing known B cell epitopes and T helper cell epitopes from homologous or heterologous proteins can overcome genetic restriction, which was responsible for the low immunogenicity of some peptides in some animals. Similarly, Leclerc *et al* (1987) produced a vaccine by co-polymerization of a streptococcal peptide containing both B and T cell epitopes, with a peptide representing a B cell epitope from hepatitis B virus. The T cell epitope conferred immunogenicity on the virus B cell epitope and also worked as a carrier molecule. Similar conclusions were reached by Enomoto *et al* (1990) after comparison of the antibody responses towards synthetic peptide immunogens from αs1-casein. These authors found that antibodies raised against peptides bind to the parent protein only when the peptides contain both B cell and T helper cell determinants. The formation, by a hapten-conjugate, of an antigen bridge which physically links the cooperating T and B cells was postulated by Mitchinson (1971).

Rs1-23-Ov was also tested as a putative vaccine because the hydrophilicity analysis of robustoxin, determined according to the method of Hopp and Woods (1983), indicated that the region consisting of the chain of amino acids 1–23 should be exposed on the surface of robustoxin. Also, this part of the robustoxin sequence is largely conserved in the two funnel-web spider neurotoxins lethal to primates and characterized so far: robustoxin and versutoxin from *Hadronyche* (formerly also called *Atrax*) *versuta* (Brown *et al* 1988). No protection was observed when a monkey immunized with Rs1-23-Ov was challenged with venom. However, the 1–23 peptide was conjugated to ovalbumin through the SH group. Pratt (1978) reported that the efficacy of KLH and ovalbumin as carrier proteins and the immune response that they elicit are difficult to compare since they vary depending upon the individual and the species. Rs1-23 may not have included a B cell epitope. Also, monkey 2, immunized with Rs1-23-Ov, was challenged 124 weeks after immunization, compared with 15 weeks and 1 week for monkeys 4 and 5 (table 1). This may have affected the ability of monkey 2 to withstand challenge. In view of the results with the other monkeys, it would be interesting to see whether the same peptide co-polymerized with KLH had any protective effect.

The toxic effects of the challenges with venom in the three unprotected animals (those immunized with Rs1-23-Ov and Rs1-42, and the control monkey) were identical to those reported previously in monkeys (Duncan *et al* 1980; Mylecharane *et al* 1989; Sheumack *et al* 1991), i.e. potential fatality due to respiratory and circulatory failure, together with lachrymation, salivation, generalized skeletal muscle fasciculation, and a parallel increase in body temperature. The potentially lethal effects in the control monkey confirmed the eventual disappearance of the protective effect of immunization with Rn1-42 described by Sheumack *et al* (1991). The potentially lethal challenge was given 165 weeks after completion of the immunization regimen.

Many reports on B cell recognition of globular protein immunogens, with conformations dictated by disulphide bridging and folding, have demonstrated that antibodies are generated, which are directed predominantly against conformational (discontinuous) epitopes and against regions with certain structural features such as hydrophilicity (Hopp and Woods 1983), mobility (Tainer *et al* 1984) and accessibility (Novotny *et al* 1987). Our results with the synthetic robustoxin derivative demonstrate that protection was obtained in two animals without reliance on the disulphide bridges of the native toxin. We speculate that the immune response to a peptide may be influenced by its primary structure as well as by its conformation. The peptide derivative used in these experiments had all of the eight cysteine residues blocked by Acm groups. It was non-lethal, and may have a three-dimensional structure different from that of the native conformation of the parent toxin.

Anti-robustoxin antibodies prepared using our synthetic immunogen could be purified by affinity chromatography against robustoxin. Such antibodies have the potential to be the basis of a more specific and more purified antivenom than the antiserum currently available for treatment of humans envenomated by *A. robustus* spiders. This antiserum is prepared from rabbit serum following a prolonged immunization schedule with male *A. robustus* crude venom

(Sutherland 1980). We have previously used affinity chromatography successfully with native robustoxin bound to the solid phase to isolate an endogenous antitoxin from the sera of rabbits that had not been immunized (Sheumack *et al* 1991).

Our results may also have implications for genetically engineered polypeptide immunogens, which are often produced by cell culture in forms lacking disulphide bridges or with unnatural bridging. Many of these have proved difficult to fold into the native conformation by chemical means. If results similar to ours were obtained for a range of other immunogens, it may be possible to propose that, in some cases, it may only be necessary to immunize with forms of these immunogens lacking disulphide bridges in order to obtain a protective immune response. Such forms are not expected to be pathogenic.

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