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IMMUNOENZYMATIC QUANTITATION OF ANTIBODIES TO *BOTHROPS ASPER* MYOTOXINS AFTER POLYVALENT ANTIVENOM ADMINISTRATION IN MICE

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1. Two quantitative enzyme-immunoassays (EIA) for *Bothrops asper* myotoxin and anti-myotoxin antibodies, respectively, were utilized to study their *in vivo* distribution in mice (Swiss, 18 to 20 g).

2. After polyvalent antivenom (0.4 ml) administration by the *iv* route, there was an immediate peak in plasma anti-myotoxin antibodies which declined rapidly during the first hour, and then decreased more gradually. Anti-myotoxin antibodies were detected in muscular tissue (gastrocnemius) following *iv* injection of antivenom. After *im* injection of antivenom (0.4 ml), a slow and steady increase in plasma anti-myotoxin levels was observed, with a peak at 24 h.

3. Mice that received antivenom (0.4 ml) by the *iv* or *im* route 15 min after *im* injection of *B. asper* venom (100 µg) had lower levels of plasma anti-myotoxin antibodies than controls injected with antivenom only, suggesting that at least a fraction of the antibodies combines with myotoxins *in vivo*.

4. Myotoxin was not detected in plasma at any time after venom injection by the *im* (100 µg) or *ip* (40 µg) route. Following *iv* injection of 50 µg of purified myotoxin II, all plasma samples were also negative, at a detection limit of 10 ng/ml.

5. It was demonstrated that myotoxin II binds to mouse erythrocytes *in vitro*, a fact that could partially explain its rapid *in vivo* disappearance from plasma.

6. The present results on the distribution of anti-myotoxin antibodies *in vivo* are in agreement with previous experimental studies reporting the poor neutralization of myotoxicity induced by *B. asper* venom when antivenom is injected *im*, in comparison to *iv* injection.

Key words: antivenom, snake venom, myotoxin, *Bothrops asper*.

Introduction

Myonecrosis is a typical consequence of envenomation by the snake *Bothrops asper* in Costa Rica (De Franco et al., 1983). Myotoxicity of the venom is mediated mainly by a group of basic phospholipase A_2 isoforms, including myotoxin I (Gutiérrez et al., 1984), myotoxin II (Lomonte and Gutiérrez, 1989), myotoxin III (Kaiser et al., 1990), and other isoforms which cross-react antigenically (Lomonte and Kahan, 1988). Similar toxins have been found in several other species of *Bothrops* (Lomonte et al.,

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1990b,c). The polyvalent antivenom utilized in Costa Rica contains antibodies against this group of myotoxins (Gutiérrez et al., 1984; Lomonte et al., 1991) which neutralize venom myotoxicity in preincubation-type experiments (Gutiérrez et al., 1981; Lomonte et al., 1985, 1987, 1990a). However, neutralization of myotoxicity when antivenom is administered after experimental envenomation is less efficient (Gutiérrez et al., 1981). In order to provide a basis for understanding the *in vivo* neutralizing ability of antivenom towards myotoxicity of *B. asper* venom in the mouse model, two quantitative enzyme immunoassays for myotoxin and anti-myotoxin antibodies, respectively, were developed. With these assays, the *in vivo* distributions of myotoxin and antibodies were investigated.

Material and Methods

Isolation of myotoxins and anti-myotoxin antibodies

B. asper myotoxins I and II were purified as described by Gutiérrez et al. (1986) and Lomonte and Gutiérrez (1989), respectively. Antibodies to *B. asper* myotoxins were purified from the equine polyvalent antivenom produced at the Instituto Clodomiro Picado, Universidad de Costa Rica (Bolaños and Cerdas, 1980) by affinity chromatography on a myotoxin II-Sepharose column, as described by Lomonte et al. (1990a). Purified myotoxins or anti-myotoxin antibodies were utilized as standards for the reference curves of the corresponding enzyme immunoassays. Protein concentration of standards was established by weighing lyophilized toxins or antibodies on a microbalance (Cahn Instruments, CA, USA).

Enzyme immunoassay (EIA) for anti-myotoxin quantitation

Microplates (Linbro EIA II Plus, Flow Laboratories, VA, USA) were coated with myotoxin I (0.4 µg/well) dissolved in 0.15 M NaCl, 0.1 M Tris-HCl buffer, pH 9.0, overnight at room temperature. After washing five times with this buffer, plates were allowed to dry and were stored at 4°C. One hundred µl/well of affinity-purified antibody standards, in the range of 5 to 1250 µg/ml, or 100 µl/well of unknown samples, were incubated overnight at 4°C. Plates were washed five times with 0.12 M NaCl, 40 mM sodium phosphate, pH 7.2 (PBS) containing 0.05% Tween-20. Then, 100 µl/well of blocking solution (2% bovine serum albumin in PBS) was added for 10 min, decanted, and followed by 100 µl/well of a 1:1000 dilution (in blocking solution) of rabbit anti-horse IgG conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO, USA), for 2 h. Color was developed with 0.012% hydrogen peroxide and 2 mg/ml o-phenylenediamine in 0.1 M sodium citrate buffer, pH 5.0 (Tijssen, 1985) over a 5-10 min period. After stopping reactions with 50 µl/well of 2 M HCl, absorbances were determined on a Titertek-Uniskan II reader at 492 nm. Standards and samples were assayed at least in triplicate. Each plate included normal

mouse plasma as a negative control, and anti-myotoxin standards for the reference curve. Absorbance readings of samples were converted to concentration values of anti-myotoxin by interpolation from the reference curve.

Enzyme-immunoassay for myotoxin quantitation

A two-site EIA using monoclonal antibodies MAb-3 and MAb-4 against *B. asper* myotoxins was utilized (Lomonte and Kahan, 1988), with minor modifications. In brief, plates were coated with MAb-4 (100 µl/well, 1:250, overnight) in order to bind myotoxins from standards or unknown samples (50 µl/well, overnight, 4°C). Antigen was detected by adding biotinylated MAb-3 (50 µl/well, 1:500, 2 h), followed by an avidin-peroxidase conjugate (Sigma; 100 µl/well, 1:2000, 2 h). Color was developed as described above. Two procedures for this assay were tested: the first utilized a simultaneous addition of sample and biotinylated MAb-3, while the second utilized a sequential addition of these two reagents after washing. In the latter case, sample and biotinylated MAb-3 volumes were adjusted to 100 µl/well. Each plate included myotoxin standards for the reference curve, and normal mouse plasma as a negative control. In order to determine if plasma caused interference with myotoxin detection (Labrousse et al., 1988), myotoxin I standards (5 to 10,000 ng/ml) were assayed in parallel by EIA using either PBS or mouse plasma for dilution.

Anti-myotoxin levels in plasma after antivenom administration

Four mice (18-20 g) received 0.4 ml of polyvalent antivenom (batch 187) by *iv* injection, into the tail vein. After 0.5, 1, 3, 6, 12, 24, 48, and 72 h, blood samples were collected from the tip of the tail into heparinized capillary tubes. After centrifugation, 5 µl of each plasma sample were diluted to 1 ml (1:200) with PBS containing 2% albumin, and these solutions were assayed by EIA (N = 3). Another group of four mice (18-20 g) received 0.4 ml of antivenom by *im* injection into the thigh. Blood samples were collected at the same time periods. After centrifugation, 10 µl of each plasma sample were diluted to 1 ml (1:100), and assayed by EIA (N = 3). Normal mouse plasma was utilized as the negative control.

Anti-myotoxin levels in muscle after antivenom administration

Six groups of four mice (18-20 g) received 0.4 ml of antivenom by *iv* injection. One group of mice was sacrificed by ether inhalation 0.5, 1, 3, 6, 12, and 24 h later, respectively. The right gastrocnemius muscle of each animal was excised and homogenized in 1 ml of PBS using a tissue homogenizer (Polytron, Switzerland). Each suspension was centrifuged 4 min at 12,000 *g*, the supernatant was diluted 1:10 with PBS containing 2% albumin, and then assayed by EIA (N = 3). A normal mouse gastrocnemius treated exactly as the experimental samples was utilized as negative control. Results are reported as µg antibodies/g muscle.

Effect of envenomation on plasma anti-myotoxin levels

Three mice (18-20 g) received an *im* injection of *B. asper* venom (100 µg/200 µl PBS), while three control mice received 200 µl of PBS in the thigh. After 15 min, all mice received 0.4 ml of antivenom, by the *iv* route. Plasma (1:200) levels of antibodies were determined by EIA (N = 4) 0.5, 1, and 6 h after envenomation. In another experiment, three mice that received either 100 µg of venom or PBS as described above were injected *im* into the thigh with 0.4 ml of antivenom 15 min later. Plasma (1:100) levels of antibodies were determined by EIA (N = 4) 4, 6, and 24 h after envenomation.

Detection of myotoxin in plasma after envenomation

Four mice (20-22 g) received an *im* injection of *B. asper* venom (100 µg/200 µl PBS) in the thigh. Plasma samples (1:100) were assayed for myotoxin by EIA (N = 3) 0.5, 1, 3, 6, 12, and 24 h after envenomation. Also, venom (40 µg) was administered by the *ip* route to another group of four mice (20-22 g). Plasma samples (1:50) were assayed by EIA (N = 3) 0.5, 1, 3, 6, 12, and 24 h after envenomation. In another experiment, purified myotoxin II (50 µg/250 µl PBS) was injected *iv* into four mice (20-22 g), and plasma samples (1:50) were assayed by EIA (N = 3) 0.5, 1, 3, 6, 12, and 24 h after the injection.

Binding of myotoxin II to erythrocytes in vitro

Since myotoxins were not detected in plasma after administration of venom or myotoxin II, the possibility of myotoxin binding to erythrocytes was investigated, utilizing two experimental designs. In the first, a constant amount of myotoxin II (120 ng) was mixed with increasing amounts of washed mouse erythrocytes and incubated 30 min at 37°C in the presence of PBS containing 0.4% albumin. After centrifugation, supernatants were assayed for residual toxin by EIA (N = 3). A control using PBS instead of cells was included. In the second design, a constant number of erythrocytes (40 µl of 1.7 x 10^7/µl) was mixed with increasing amounts of toxin (final amounts ranging from 1.8 to 220 ng/well), and incubated for 30 min at 37°C in the presence of PBS containing 0.4% albumin. Supernatants were assayed by EIA (N = 3). For each toxin concentration, a control containing PBS instead of cells was tested in parallel for comparison.

Results

EIA for anti-myotoxin quantitation

An example of a reference curve for anti-myotoxin quantitation using affinity-purified antibody standards is shown in Figure 1. A specific signal was usually obtained with antibody concentrations of 5 µg/ml and higher, with a linear response up to 625 µg/ml (Figure 1).

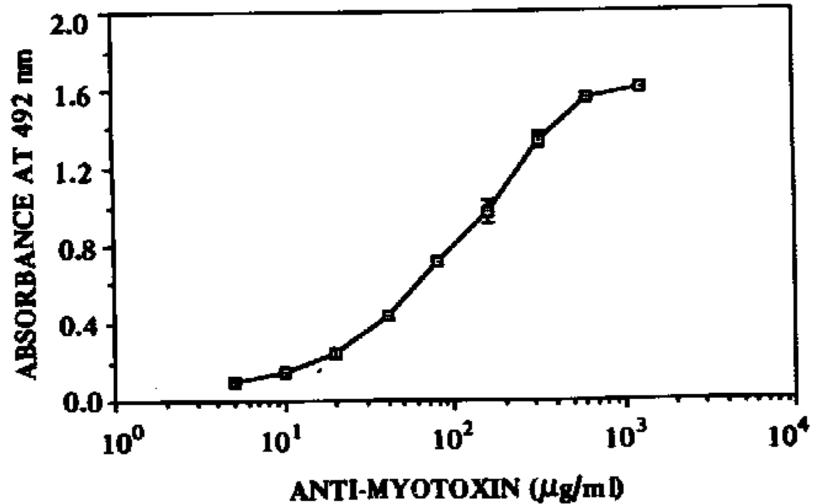

Figure 1 - Quantitative enzyme immunoassay for anti-myotoxin. A typical reference curve using affinity-purified antibodies prepared from polyvalent antivenom as standards (5-1250 μg/ml) as described in Material and Methods. Each point represents the mean ± SD (N = 3). Normal mouse plasma (negative control) gave absorbance readings of 0.030.

EIA for myotoxin quantitation

Reference curves obtained using either myotoxin I or II as a standard gave similar results (Figure 2A). However, the curves tended to return to low absorbance values at high myotoxin concentrations (Figure 2A). It was found that this phenomenon was due to the simultaneous addition of test sample (containing myotoxin) and the biotinylated MAb-3. When the procedure was modified by adding these two reagents sequentially after washings, normal reference curves were obtained (Figure 2B). A specific signal was usually obtained with myotoxin concentrations of 10 ng/ml and higher, with a linear response up to 1250 ng/ml (Figure 2B). Mouse plasma did not interfere with myotoxin detection, giving values similar to PBS (data not shown).

Anti-myotoxin levels in plasma after antivenom administration

Figure 3 shows anti-myotoxin levels in plasma after *iv* or *im* injection of antivenom. By *iv* injection, a high initial level was achieved, which decreased rapidly between 0.5 and 1 h. After 1 h, levels decreased more slowly. Low levels of antibodies were still detectable after 72 h. By *im* injection, antibody levels increased slowly from 0.5 to 24 h, declining thereafter (Figure 3).

Anti-myotoxin levels in muscle after antivenom administration

Anti-myotoxin antibodies were detected in the supernatants of muscle homogenates rapidly after *iv* injection of antivenom (Figure 4). The change in antibody concentration observed in muscle was different from that observed in plasma.

Effect of envenomation on plasma anti-myotoxin levels

Mice experimentally envenomed by the *im* route and subsequently treated with antivenom either *iv* (Figure 5A) or *im* (Figure 5B) had significantly ($P<0.05$) lower anti-myotoxin levels at several time points than non-envenomed controls injected with antivenom.

Detection of myotoxin in plasma after envenomation

After *B. asper* venom injection by the *im* or *ip* route, myotoxins were not detected in plasma at any time point tested (data not shown). Neither was myotoxin II detected in plasma at any time when 50 µg was injected *iv* (data not shown). The reference curves included in each assay plate had a lower detection limit of at least 10-20 ng/ml.

Binding of myotoxin II to erythrocytes in vitro

When a constant amount of myotoxin II was incubated with increasing numbers of mouse erythrocytes, a proportional reduction in toxin concentration in the supernatant was evidenced by EIA (Figure 6A). Similarly, when a fixed number of erythrocytes was incubated with increasing concentrations of myotoxin II, a significant ($P<0.001$) reduction in the EIA readings was evidenced over a wide range in comparison to controls containing PBS instead of cells (Figure 6B).

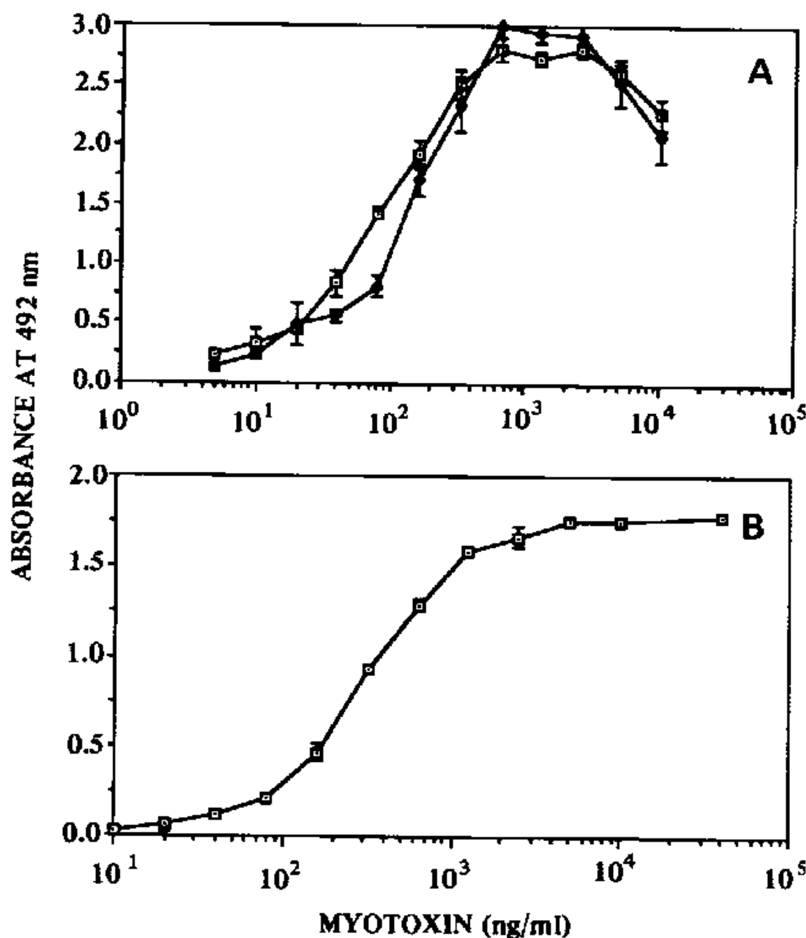

Figure 2 - Quantitative enzyme-immunoassay for myotoxin. *A*, Typical reference curve using myotoxin I (□) or myotoxin II (●) as standards (5-10,000 ng/ml) as described in Material and Methods. Standards and biotinylated MAb-3 were added simultaneously. Each point represents the mean ± SD (N = 3). Normal mouse plasma (negative control) gave absorbance readings of 0.050. *B*, Typical reference curve using myotoxin II (10-40,000 ng/ml). Standards and biotinylated MAb-3 were added sequentially, after washings. Each point represents the mean ± SD (N = 3). Normal mouse plasma (negative control) gave absorbance readings of 0.002.

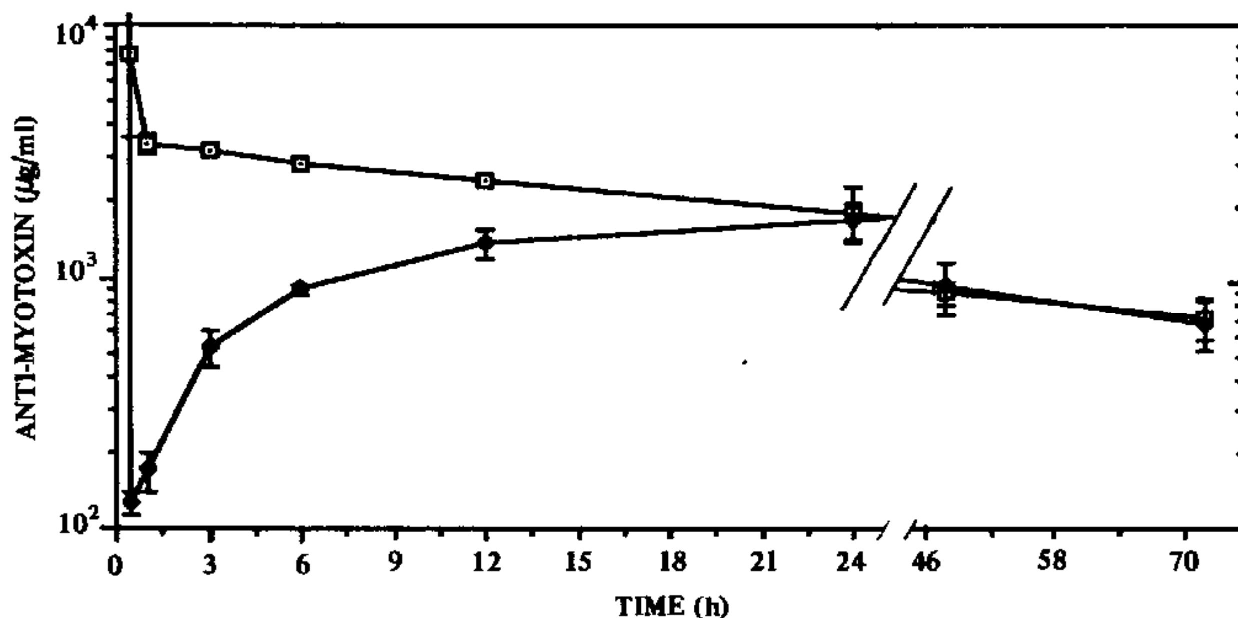

Figure 3 - Anti-myotoxin levels in mouse plasma after antivenom administration by the *iv* or *im* routes. Polyvalent antivenom (0.4 ml) was injected *iv* (□) or *im* (●) into mice (18-20 g), and plasma anti-myotoxin levels were determined by enzyme immunoassay (N = 3), as described in Material and Methods. Each point represents the mean ± SD for four mice.

Discussion

Knowledge of the distribution of venom components and their corresponding antibodies *in vivo* both in clinical cases and in animal models is important for the design of adequate serotherapeutic protocols (Ismail et al., 1983; Ho et al., 1990). In the present study we determined the distribution of anti-*B. asper* myotoxin antibodies in mice after the administration of a commercially available polyvalent antivenom. Intravenous administration of antivenom resulted in a high initial level of antibodies in plasma, which decreased sharply during the first h, due to equilibration of antibodies between vascular and extravascular compartments (Bellanti and Robbins, 1980; Ismail et al.,

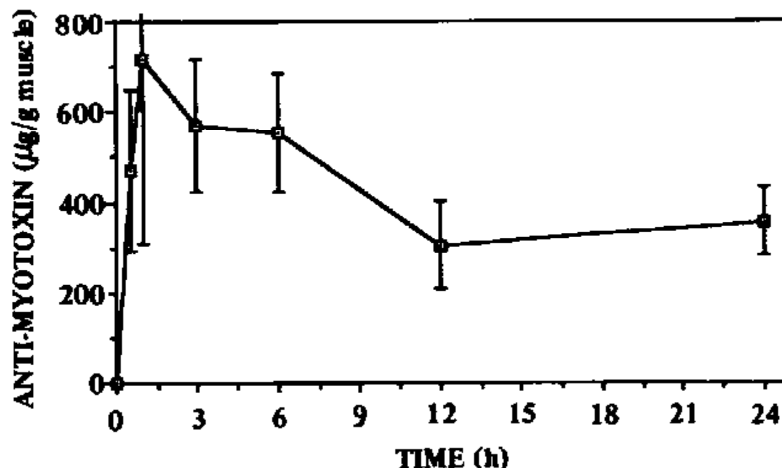

Figure 4 - Anti-myotoxin levels in skeletal muscle of mice after *iv* antivenom administration. Polyvalent antivenom (0.4 ml) was injected *iv* into mice (18-20 g), and the anti-myotoxin content of the supernatant of a homogenized suspension of the whole gastrocnemius muscle was determined by enzyme immunoassay (N = 3). Results are reported as µg anti-myotoxin/g wet muscle. Each point represents the mean ± SD for four mice.

1983; Maung-Maung-Thwin et al., 1988). Plasma antibody levels decreased more gradually after 1 h and were still detectable after three days in the absence of envenomation. Similar results have been described by Bober and Ownby (1988a) studying the distribution of rabbit antibodies against myotoxin *a* from *Crotalus viridis viridis* in mice.

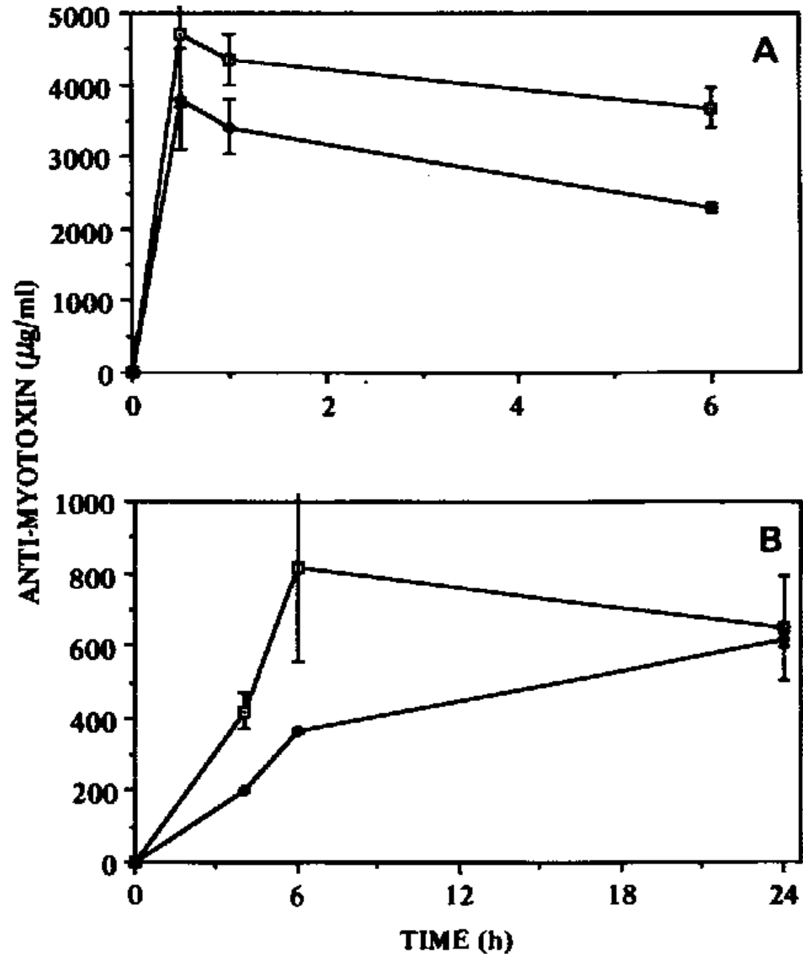

Figure 5 - Effect of envenomation on plasma anti-myotoxin levels in mice. *A*, Antivenom (0.4 ml) was administered *iv* 15 min after *im* injection of 100 µg of *B. asper* venom into mice (18-20 g) (●). Control animals (□) received phosphate-buffered saline instead of venom. Plasma anti-myotoxin levels were determined by enzyme immunoassay (N = 4). Each point represents the mean ± SD for three mice. Differences between means were statistically significant at 1 and 6 h ($P<0.05$, Student *t*-test). *B*, Same as above, except that antivenom was administered *im*. (●) Envenomed mice, (□) control mice. Differences between means were statistically significant at 4 and 6 h ($P<0.05$, Student *t*-test).

In general, pharmacokinetic studies of antivenoms are performed utilizing the *iv* route. However, since *im* antivenom injection is often utilized as an immediate therapeutic procedure in the field, we also tested this route. As expected, antibodies reached the circulation slowly, increasing until 24 h after injection, and then decreasing gradually. Only a low level of antibodies was detected in plasma at 0.5, 1, and 3 h, in comparison to the *iv* route. These findings are in agreement with previous experimental studies reporting the poor neutralization of myotoxicity induced by *B. asper* venom when antivenom is injected *im*, in comparison to *iv* injection (Gutiérrez et al., 1981). Since venom (Gutiérrez et al., 1980) and myotoxins (Gutiérrez et al., 1984; Lomonte and Gutiérrez, 1989) act rapidly in the induction of myonecrosis, antibodies must reach affected tissues quickly if this effect is to be prevented. The observations of Gutiérrez et al. (1981) can be explained by the faster and higher level of plasma anti-myotoxin antibodies, as observed in the present study, equilibrating rapidly with tissues. In this regard, we investigated the concentration of anti-myotoxin in muscular tissue following *iv* injection of antivenom.

Anti-myotoxin antibodies had reached the tissue as early as 0.5 h after injection. The muscle antibody curve profile was not parallel to that observed in plasma, suggesting that the antibodies detected were not only due to the contribution by blood irrigating the tissue, but were also present in the extravascular compartment of muscle. This is also supported by the fact that the declining phase of plasma antibodies was concomitant to the increase in muscle antibodies observed at 0.5 and 1 h. Labrousse et al. (1988) detected antibodies to *Vipera ammodytes* crude venom in muscular tissue of rabbits after antivenom administration.

Another point investigated here was the effect of envenomation on anti-myotoxin levels. A significant reduction of plasma antibodies was observed at several time periods in *B. asper*-envenomed animals in comparison to saline-treated controls when antivenom was administered *iv* or *im*. Similar findings have been previously described by Bober and Ownby (1988b) in the case of anti-myotoxin *a* antibodies. This suggests that at least a fraction of the antibodies injected 15 min after *im* envenomation combines with myotoxins *in vivo*. However, other possible explanations for the differences observed, such as venom-induced hemodynamic alterations, cannot be excluded.

The second objective of this study was to determine myotoxin levels *in vivo*. During the development of an

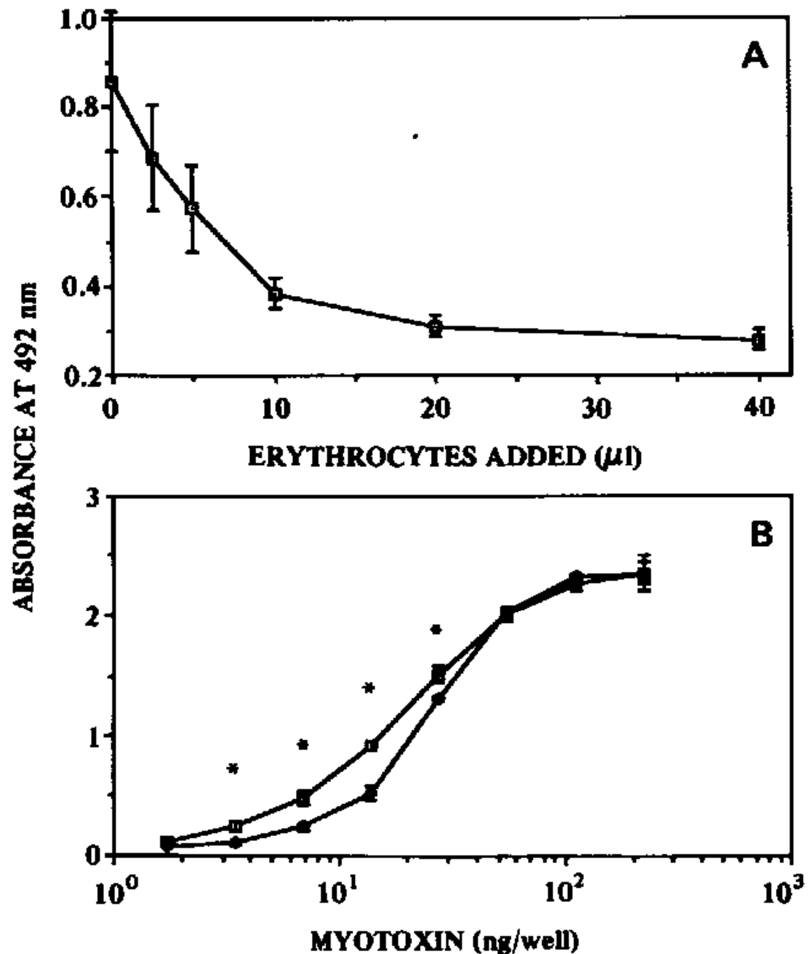

Figure 6 - Binding of myotoxin II to washed mouse erythrocytes *in vitro*. *A*, A fixed amount of myotoxin II (120 ng) was incubated with increasing numbers of washed erythrocytes (1.1×10^7/µl) for 30 min at 37°C, in the presence of phosphate-buffered saline containing 0.4% bovine serum albumin. Supernatants were assayed for residual myotoxin by enzyme immunoassay. Each point represents the mean ± SD (N = 3). *B*, A constant number of washed erythrocytes (40 µl of a 1.7×10^7 cell/µl suspension) was added to solutions containing different amounts of myotoxin II (final amounts ranging from 1.6 to 220 ng/well), and incubated as described above. Supernatants were assayed by enzyme immunoassay. Each point represents the mean ± SD (N = 4). (□) Myotoxin plus saline, (●) myotoxin plus erythrocytes. Differences between means were highly significant (P<0.0001, Student *t*-test) at the myotoxin dilutions indicated by an asterisk.

EIA, an interesting observation was the decrease of absorbance values at high toxin concentrations (Figure 2A). After modifying our procedure according to the observations of Nomura et al. (1983), i.e., performing a two-step instead of a single-step incubation of toxin and second monoclonal antibody, normal reference curves were established. Unexpectedly, when venom was injected *im* or *ip* myotoxin was not detected in plasma. Even when 50 µg of purified myotoxin II was injected *iv* the toxin was not detected in plasma after 0.5 h. We recently extended these observations to samples obtained 10 min after *iv* injection of 75 µg myotoxin II (data not shown). Also, the possibility that heparin (contained in capillaries utilized for blood samples) may interfere with myotoxin detection was excluded. A previous study on the distribution of ^{125}I-labelled myotoxin I in mice (Moreno and Gutiérrez, 1988) described high levels of radioactivity in whole blood samples, as well as in several organs after *iv* injection. The present results demonstrated that mouse erythrocytes bind myotoxin II *in vitro*. Binding of ^{3}H-labelled myotoxin II to erythrocytes and other cell types *in vitro* has also been observed by Gené et al. (J.A. Gené, J.M. Gutiérrez, B. Lomonte and M. Thelestam, unpublished results). Taken together, these observations suggest that binding of myotoxins to erythrocytes (and possibly other cell types) may be responsible for their rapid *in vivo* disappearance from plasma.

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