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Toxicokinetic and toxicodynamic analyses of *Androctonus australis hector* venom in rats: Optimization of antivenom therapy

D. Hammoudi-Triki [a,b,c,1], J. Lefort [d], C. Rougeot [e], A. Robbe-Vincent [a,1], C. Bon [a,1], F. Laraba-Djebari [b,c], V. Choumet [a,*,1]

[a] *Unité des Venins, Institut Pasteur, Paris, France*
[b] *Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie "Houari Boumédienne" Bab Ezzouar, Alger, Algérie, France*
[c] *Laboratoire de Recherche et de Développement sur les Venins, Institut Pasteur d'Algérie, Algérie, France*
[d] *Unité de Pharmacologie cellulaire, Institut Pasteur, Paris, France*
[e] *Laboratoire de Recherche et Développement en Pharmacologie des Régulations Neuro-endocrines, Institut Pasteur, Paris, France*

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Abstract

This paper reports the simultaneous determination of toxicokinetic and toxicodynamic properties of *Androctonus australis hector* venom, in the absence and presence of antivenom ($F(ab')_2$ and Fab), in envenomed rats. After subcutaneous injection of the venom, toxins showed a complete absorption phase from the site of injection associated with a distribution into a large extravascular compartment. The injection of Fab and $F(ab')_2$ induced the neutralization of venom antigens in the blood compartment, as well as the redistribution of venom components from the extravascular compartment to the blood compartment. Interestingly, $F(ab')_2$ and Fab showed distinct efficiencies depending on their route of injection. $F(ab')_2$ induced a faster venom neutralization and redistribution than Fab when injected intravenously. Fab was more effective than $F(ab')_2$ by the intramuscular route. The hemodynamic effects of Aah venom were further investigated. Changes in mean arterial pressure and heart rate were observed in parallel with an upper airway obstruction. Fab was more effective than $F(ab')_2$ for preventing early symptoms of envenomation, whatever their route of administration. Intraperitoneal injection of $F(ab')_2$ and Fab was similar for the prevention of the delayed symptoms, even after a late administration. Fab was more effective than $F(ab')_2$ in the inhibition of airway resistance, independent of the route and time of administration. These results show that the treatment for scorpion stings might be improved by the intravascular injection of a mixture of Fab and $F(ab')_2$. If antivenom cannot be administered intravenously, Fab might be an alternative as they are more effective than $F(ab')_2$ when injected intramuscularly.

Keywords: Scorpion venom; Antivenom therapy; Toxicokinetics; Toxicodynamics; Antibody fragments

Introduction

Scorpion stings are a serious problem in many parts of the world. In 2001, about 50,000 people sought medical attention due to scorpion stings in Algeria, and about 150 deaths were reported, mostly children. The yellow scorpion *Androctonus australis hector* (Aah) was responsible for most of these cases. The clinical symptoms observed after scorpion stings have mainly been attributed to the pharmacological properties of the 7 kDa venom toxins that affect the Na^+ channels at the excitable and non-excitable cell surface (Martin Eauclaire et al., 1999). Envenomations cause various symptoms such as pain, sweating, fever and hypertension (Ismail, 1995; Krifi et al., 1998; Ghalim et al., 2000). In the more severe cases, these symptoms are associated with pulmonary edema and myocardial damage (Ismail, 1995).

Antivenom immunotherapy is the only specific treatment for scorpion stings. Although widely used in several countries as Mexico, Saudi Arabia and Tunisia (Dehesa-Davila, 1989;

* Corresponding author. Unité de Biochimie et de Biologie Moléculaire des Insectes, 28 rue du Dr. Roux, Institut Pasteur, 75724 Paris cedex 15, France. Fax: +33140613471.
E-mail address: vchoumet@pasteur.fr (V. Choumet).
[1] Present address: Muséum National d'Histoire Naturelle, Département: Régulations, Développement et Diversité moléculaire, Unité de Chimie et de Biochimie des Substances naturelles (UMS 0502 MNHN-UMR 5154 CNRS), France.

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Ismail et al., 1994; De-Rezende et al., 1998; Krifi et al., 1998), antivenoms are still administered empirically and consequently their efficacy is controversial, particularly in the case of mild or moderate scorpion envenomations (Abroug et al., 1999). A previous epidemiological and clinical evaluation of scorpion stings in Algeria demonstrated the limitation of the current antivenom treatment and concluded that an optimization of this treatment is necessary (Hammoudi-Triki et al., 2004). Based on experimental pharmacokinetic studies, the optimal efficacy of antivenom immunotherapy was shown to require the intravenous injection of a dose of $F(ab')_2$ antivenom, adapted to the severity of envenomation, as soon as possible after the sting (Krifi et al., 2001, 2005). However, an accurate determination of the chronology of appearance of the pathophysiological effects of scorpion venoms and the evaluation of their prevention or treatment after antivenom administration is also required to provide clues for improving antivenom immunotherapeutic treatment of scorpion stings. For this purpose, we determined the toxicokinetic properties of the Aah toxic fraction (Aah FtoxG-50) in experimentally envenomed and anesthetized rats. We then investigated the effects of two antibody fragments ($F(ab')_2$ and Fab) and of their administration routes (intravenous and intramuscular) on the toxicokinetic properties of the Aah venom. We also measured cardiopulmonary function (mean artery pressure, heart rate and airway resistance) in rats before and after antivenom immunotherapy.

Methods

Preparation of venom toxic fraction. Lyophilized crude Aah venom was supplied by the Research and Development Laboratory on venoms of the Pasteur Institute of Algeria. Aah venom toxic fraction (FtoxG-50) was purified from the venom by gel filtration on Sephadex G50. The toxic fraction represents a very small fraction of the venom proteins (5%). Its purity was tested for by SDS-PAGE electrophoresis, and its lethal potency was determined as described by Laraba-Djebari and Hammoudi (1998). The toxins contained in the FtoxG50 account for the almost totality of the venom toxicity.

Purification of specific antibodies: $F(ab')_2$ and Fab anti-FtoxG-50. $F(ab')_2$ and Fab specific fragments were obtained from horse IgG prepared against Aah venom by enzymatic hydrolysis with pepsin and papain, respectively. To obtain $F(ab')_2$ and Fab specific for Aah FtoxG-50, a further step of purification was performed by affinity chromatography on CNBr-activated Sepharose 4B coupled with the toxic venom fraction. The homogeneity, the immunoreactivity and the specific activity were analyzed by SDS-PAGE and ELISA respectively. Neutralizing potency was determined in mice by preincubating 1 h at 37 °C antibody fragments with Aah venom (Laraba-Djebari and Hammoudi-Triki, 1999). Both fragments were shown to have similar neutralizing efficiencies.

Preparation of peroxidase-labeled anti-FtoxG-50 F(ab')2. High affinity $F(ab')_2$ was conjugated to peroxidase (Boehringer Mannheim, GmbH) by the method described by Avrameas and Ternynck (1969, 1971). After labeling, the antibodies were tested by ELISA and kept at −20 °C in 50% glycerol.

ELISA determination of A. australis hector venom concentration. Microtitration plates were coated by incubation for 2 h at 37 °C with 100 µl of 100 mM sodium bicarbonate pH 9.5 containing 5 $\mu g/ml^{-1}$ of specific $F(ab')_2$. The plates were washed six times with PBS containing 0.1% Tween 20. Non-specific binding was saturated by a 1 h incubation at 37 °C with 100 µl per well of PBS–Tween containing 5% skimmed milk. The plates were again washed 6 times and then incubated with dilutions of venom or sera for 1 h at 37 °C. After washing, the conjugate solution (100 µl of a 1 in 2000 dilution in PBS–Tween containing 5% skimmed milk) was added and the samples incubated for 1 h at 37 °C. The plates were washed and 100 µl of substrate medium (10 mM sodium phosphate, pH 7.3 containing 2 mg ml^{-1} of *o*-phenylenediamine dihydrochloride and 0.06% hydrogen peroxide) was added. The plates were then incubated for 7 min at room temperature in the dark. The reaction was stopped by the addition of 2 N H_2SO_4 (50 µl per well). Absorbance was measured at 490 nm with a spectrophotometer (Dynatech MR5000, Labsystem, France).

Iodination of the FtoxG-50 venom. FtoxG-50 venom was iodinated by the enzymatic radiolabeling method described by Rochat et al. (1977) and Tessier et al. (1978). Aah venom toxic fraction (1 mg ml^{-1}) was mixed with 30 µl of lactoperoxidase, 2.24 mCi of ^{125}I Na and 15 µl of 30% H_2O_2. After incubation for 1 min, 15 µl of H_2O_2 was added and the sample incubated for another 1 min. The reaction was stopped by addition of 120 µl of 100 mM tyrosine solution in PBS. The iodinated fraction was separated from free iodine by affinity column chromatography on specific anti-FtoxG-50 $F(ab')_2$ antibodies covalently coupled to EAH-Sepharose 4B.

Determination of Aah FtoxG-50 by radioactivity. The concentration of scorpion toxins in rat sera was determined after trichloroacetic (TCA) precipitation. Sera samples of 50 or 100 µl were mixed with 450 µl or 400 µl of 4% BSA solution and then precipitated with 500 µl of 20% TCA. After 30 min of incubation on ice, the samples were centrifuged at 1500×*g*, supernatants were removed and the radioactivity contained in the pellets was counted in a γ-counter (Pharmacia, Uppsala, Sweden). The TCA precipitation yields of toxic fraction were estimated to be 90% (not shown).

Pharmacokinetic experiments. Adult male Wistar rats (300±20 g) were obtained from Charles River (Saint-Aubin-les-Elbeuf, France). They were housed in temperature-controlled rooms and received water and food *ad libitum* until used. Animals were anesthetized with intraperitoneally (ip) injected sodium pentobarbital (40 mg kg^{-1}). A silastic catheter was implanted into the external jugular vein and used to administer iv solutions (venom and antivenom) and to collect blood samples. All experiments using rodents were performed in accordance with the applicable guidelines and regulations.

Intravenous (iv) or subcutaneous (sc) injections of Aah venom containing or not 10^6 cpm of ^{125}I-FtoxG-50 as radioactive tracer were given to each rat. For the first series of experiments, Aah venom dose (17 µg kg^{-1}) was injected intravenously into the jugular vein of three rats. In a second series of experiments, radiolabeled Aah venom dose (83 µg kg^{-1}) was subcutaneously injected in the back leg. In a third series of experiments, Aah venom dose (250 µg kg^{-1}) was subcutaneously injected in the back leg of three rats. Blood samples (100 µl) were withdrawn and collected in heparinized tubes 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600 min after the venom injection. Toxin concentrations were determined by TCA precipitation (after injection of radiolabeled venom) and ELISA.

Antivenom therapy experiments. One Aah venom dose (83 µg kg^{-1}) containing 10^6 cpm of iodinated FtoxG-50 as radioactive tracer was injected by the sc route. Forty five minutes after envenomation, envenomed animals were divided into four groups of three and received the injection of antivenom antibody fragments (40 mg kg^{-1}) by iv into the jugular vein or im into the back left leg as follows. Group 1 received the injection of $F(ab')_2$ by iv route, group 2 was injected with $F(ab')_2$ by im route, group 3 was injected with Fab by iv route and group 4 received an im injection of Fab. Blood samples were collected in heparinized tubes 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600 min after venom injection and centrifuged at 1500×*g* for 15 min to obtain plasma. The venom concentrations were determined by radioactivity and ELISA tests.

Determination of pharmacokinetic parameters. Toxicokinetic parameters for the venom before and after immunotherapy were determined using the MK-Model software (BioSoft, Cambridge, England). After sc injections, the absorption phase appeared complex so a model-independent approach was used. Area under the curve (AUC) and area under the first moment curve (AUMC) were calculated from zero to the latest experimental time point by log-trapezoidal rule. The elimination rate constant (β) was estimated by log-linear regression for several points in the terminal phase. Extrapolations to infinity

were obtained by: C_t/β for AUC and by: $t \cdot C_t/\beta + C_t/\beta^2$ for AUMC, C_t being the last experimental concentration. The absolute bioavailability (F) was calculated as (AUC sc × D iv)/(AUC iv × D sc). $t_{1/2}$ β represents the elimination half-life. The total body clearance (Cl_T) was calculated as D/AUC, the volume of distribution at the steady state (Vdss) as (D* × AUMC)/AUC^2, and the mean residence time (MRT) as AUMC/AUC.

As previously described in the literature for the pharmacokinetic evaluation of antivenom efficiency (Rivière et al., 1998; Krifi et al., 2005), we chose to inject antivenom antibodies after Tmax was reached to determine the effect of antibodies on the distribution and elimination processes but not on the absorption phase. The effect of F(ab′)$_2$ and Fab fragments on distribution and elimination of Aah venom was calculated over 240 min using the trapezoidal rule.

Measurement of cardiopulmonary function. Rats were anesthetized with pentobarbital (40 mg kg^{-1}, ip). Once in deep surgical stage of anesthesia, rats were placed in the supine position on an operating table. The trachea and carotid were cannulated and the carotid was connected to record the arterial blood pressure (Pr3 Db transducer, Gould Inc., Oxnard, CA, USA), the signal being displayed on a computerized pulmonary monitoring system (μMed PMS, London, U.K.). Animals were ventilated with a small animal respiratory pump (Biosevernce, Sheeness, U.K.; 60 strokes/min; 10 ml kg^{-1} body weight). The effect of Aah venom on mean artery pressure and heart rate was evaluated by sc injection of three doses (168, 250 and 500 μg kg^{-1}) of Aah venom into three groups of rats. Then, the effect of antibodies F(ab′)$_2$ or Fab was tested by injecting 40 mg kg^{-1} of antibody fragments by ip or im route, 15 min or 45 min after the sc injection of 250 μg kg^{-1} of Aah venom.

Evaluation of airways resistance. Basal airway reactivity was determined by monitoring enhanced pause (Penh) units with a plesthysmograph that measures respiratory function in unrestrained animals placed in a whole body plesthysmographic chamber (EMKA Technologies, Paris, France). The effect of Aah venom on airway responsiveness was first evaluated by subcutaneously injecting three doses (168, 250 and 500 μg kg^{-1}) of Aah venom into rats. The index of airway obstruction was calculated as: Penh = [Te (expiratory time)/Tr (relaxation time) − 1] × [Pef (peak expiratory flow)/Pif (peak inspiratory flow)] (Eum et al., 1995). For graphic representation, the value for each minute was calculated from the average of three values of Penh recorded every minute. Aah venom and antivenom were administered as described above for experiments on cardiopulmonary function.

Statistics. All results are reported as mean ± standard deviations. Statistical analysis involved non-parametric tests: the Kruskal–Wallis test as a between-group variable followed by the Mann–Whitney U test and SIMSTAT. The level of significance was set at $p < 0.05$.

Results

Toxicokinetic analysis of Aah venom

The toxicokinetics of Aah venom FtoxG-50 were studied in experimentally envenomated anesthetized rats. The plasma concentration of scorpion venom antigen was followed through time by ELISA. After iv injection of Aah venom (17 μg kg^{-1}), the pharmacokinetic curve showed a bi-exponential decline, Aah venom being undetectable 90 min after venom injection (Fig. 1A). The terminal half-life of the venom was about 13 min, indicating that injected venom was eliminated rapidly after administration from the blood compartment. The calculated volume of distribution at the steady state (Vdss) was about two times the rat plasma volume (35 ml kg^{-1}) (Table 1).

In a second set of experiments, two groups of three rats were subcutaneously injected with two doses of Aah venom (83 and 250 μg kg^{-1}) (Fig. 1B). The Cmax Aah venom levels quantified by ELISA for the two doses match plasma venom concentrations

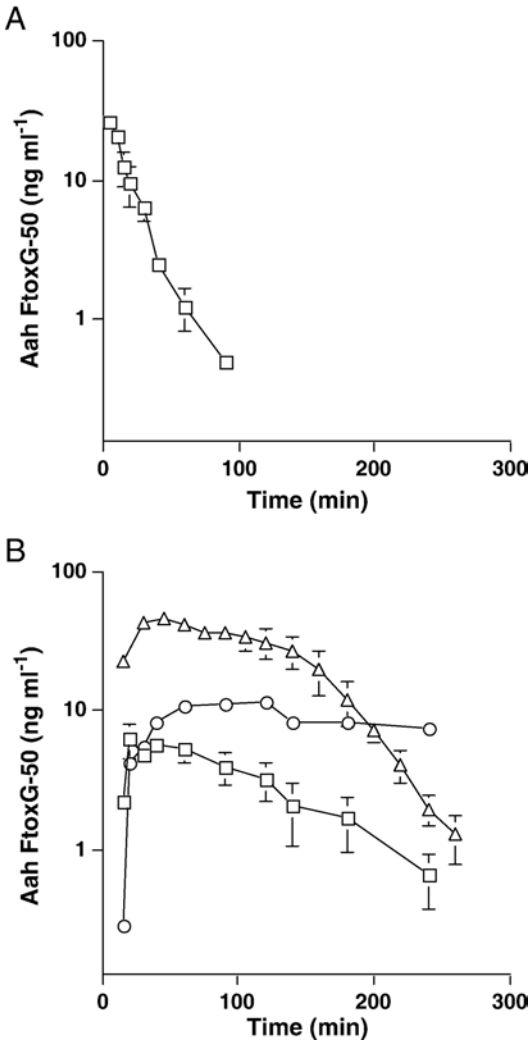

Fig. 1. Pharmacokinetic properties of Aah venom. A: three rats were injected iv with 17 μg kg^{-1} of Aah venom. Blood samples were withdrawn at the indicated times, and Aah venom concentration in plasma samples was determined by ELISA as described in the Methods section. B: groups of three rats were subcutaneously injected with 83 μg kg^{-1} supplemented (circles) or not (squares) with trace amounts of radiolabeled ^{125}I-Aah-FtoxG-50 (10^6 cpm mg^{-1}), and 250 μg kg^{-1} (triangles) of Aah venom, not supplemented with trace amounts of radiolabeled ^{125}I-Aah-FtoxG-50. Blood samples were withdrawn at the times indicated and the plasma was collected. The concentration of Aah FtoxG-50 in plasma was determined by ELISA (squares and triangles) and by counting radioactivity after TCA precipitation (circles).

observed in moderate and severe envenomations in humans (Krifi et al., 1998). The kinetics of scorpion toxin concentrations determined by ELISA method followed parallel curves for the two venom doses. Plasma venom concentrations were maximal 20 to 40 min after venom injection (Tmax) and thereafter showed a bi-exponential decline. The terminal half-lives were between 35 and 53 min (Table 1). The value of the ratio of the AUC determined after administration of Aah venom (17 and 83 μg kg^{-1} respectively) by iv and sc routes indicates complete absorption of Aah venom from its sc injection site (F = 96%). However, for the highest dose, a decrease of the bioavailability is observed (Table 1). The Vdss was significantly higher after sc than after iv injection of venom whatever the dose injected (Table 1).

Table 1
Pharmacokinetic parameters of Aah FtoxG-50 in rat

Parameters	Venom (17 μg kg^{-1}) iv route ELISA	Venom (83 μg kg^{-1}) sc route ELISA	Venom (83 μg kg^{-1}) sc route radioactivity	Venom (250 μg kg^{-1}) sc route ELISA
β (min^{-1})	0.053±0.1	0.013±0.001*	0.026±0.04*	0.025±0.01
$t_{1/2}$ β (min)	13±2	53±2*	>300[a]	35±9
Tmax (min)	5	20±8	110±5	40±9
Cmax (ng ml^{-1})	26±3	7±0.2	12±0.3	47±4
AUC_{0-240} (ng min ml^{-1})	280±5	1300±70*	2500±60*	3250±40
AUC_{0-inf} (ng min ml^{-1})	610±100	2900±320	–	7150±1276
$AUMC_{0-\infty}$ (ng min^2 ml^{-1})	11,300±2000	70,000±25,000*	–	577,000±107,000
$MRT_{0-\infty}$ (min)	18±2	110±15	–	94±3
Cl_T/F (ml min^{-1})	4±2	35±3	–	32±1
Vdss/F (ml kg^{-1})	61±22***	2300±530***	–	5300±1080
F (%)	–	96	–	81

Toxicokinetic experiments on Aah FtoxG-50 were performed after sc injection of Aah venom in rats. AUC values were measured by ELISA and by counting the radioactivity after TCA-precipitation, as indicated in the Methods section. *$p<0.05$; ***$p<0.001$. [a]: an estimation of the half-life is shown as an indication since the sampling period was too short for obtaining exact value.

To follow the effect of antivenom on venom toxicokinetics, we used radioiodinated FtoxG-50 as a tracer. The shape of the plasma venom concentration curve obtained and the AUC_{0-240} of radioiodinated venom were significantly different to those quantified by ELISA (Fig. 1B and Table 1). As reported by Krifi et al. (2005), these differences could be due either to a partial degradation of the toxins, such as metabolites, which would be detected by radioactivity counting but not by ELISA or to non-specific adsorption of radioactivity onto precipitable plasma proteins, or both. We considered that pharmacokinetic parameters for Aah venom toxins determined from ELISA data were the most reliable to describe the kinetics of the active toxins. However, these differences have no consequence on the further interpretation of the results because ELISA and radioactivity were used independently to follow the efficiency of antivenom administration. The toxicokinetic properties of radiolabeled toxins were intended to compare the extent of toxin redistribution after injection of various antivenom fragments by different administration routes whereas the ELISA quantification was performed to determine the effect of antivenom on the neutralization of the circulating toxins.

Toxicokinetic analysis of Aah venom after antivenom administration

The effect of antivenom administration on the kinetics of Aah envenomation was examined. Aah venom (83 μg kg^{-1}) containing radiolabeled Aah ^{125}I-FtoxG-50 (10^6 cpm per rat) was injected sc and neutralizing doses of F(ab′)$_2$ or Fab fragments (40 mg kg^{-1}) were injected 45 min later by iv or im routes. Blood samples were collected at various times thereafter, and the plasma concentrations of scorpion toxins were measured by ELISA and/or by radioactivity determination (Fig. 2 and Table 2). The ELISA method allowed the measurement of free Aah toxins since this method cannot measure the toxins complexed with antivenom antibodies, whereas the radioactivity method allowed quantification of all Aah toxins whether free (unbound to antibodies) or complexed with antivenom antibodies. Toxin immunocomplexation and redistribution were followed by comparing the AUC from ELISA and radioactivity data (Rivière et al., 1997, 1998) (Table 2). The iv injection of F(ab′)$_2$ led to the complete and durable immunocomplexation of scorpion toxins in plasma (Fig. 2A): the plasma concentration of free toxins, measured by ELISA, dropped to an undetectable level 20 min after antivenom injection and remained so to the end of the experiment. Total toxin (i.e., free and bound to the antibodies as determined by radioactivity) concentration increased markedly after a short delay and remained at a high level during the experiment (Fig. 2A), the AUC being significantly higher in the presence of antivenom than in its absence (Table 2). This observation indicates that the iv injection of a neutralizing dose of antivenom causes a large redistribution of Aah toxins from the extravascular compartment (tissues) to the blood where they are neutralized by the F(ab′)$_2$ present in excess in the vascular compartment.

The effectiveness of im and iv F(ab′)$_2$ antivenom delivery into rats was compared in the same experimental conditions. Compared to iv injection, the neutralization of plasma venom was slower following im injection (Fig. 2A). The AUC of radioactive toxin after im injection of antivenom was significantly higher than that for control envenomed rats, but lower than the AUC of radioactive toxin measured in iv F(ab′)$_2$ treated envenomed rats. Furthermore, the ELISA AUC after F(ab′)$_2$ iv injection was significantly lower than measured after im injection. Taken altogether, these results demonstrated that the antivenom immunotherapy performed by the im route was less effective than the one carried out by the iv route.

We also studied the effects of the injection of Fab fragments on Aah venom toxicokinetics (Fig. 2B). The neutralization of plasma venom following injection of Fab, either by iv or im routes, was slower than that following iv injection of F(ab′)$_2$. This delayed neutralization of venom toxins in plasma is associated with the delayed redistribution of venom toxins from the extravascular compartment (Fig. 2B). Interestingly, the extent of the overall redistribution and that of the immunocomplexation did not differ significantly between the two types of immunoglobulin fragments following iv administration. Indeed, the AUC of radioactive toxins was much larger after injection of Fab than the one determined in control animals (in the absence of antivenom immunotherapy), whatever the route of injection (Table 2). However, Fab injected by im route more efficiently redistributed

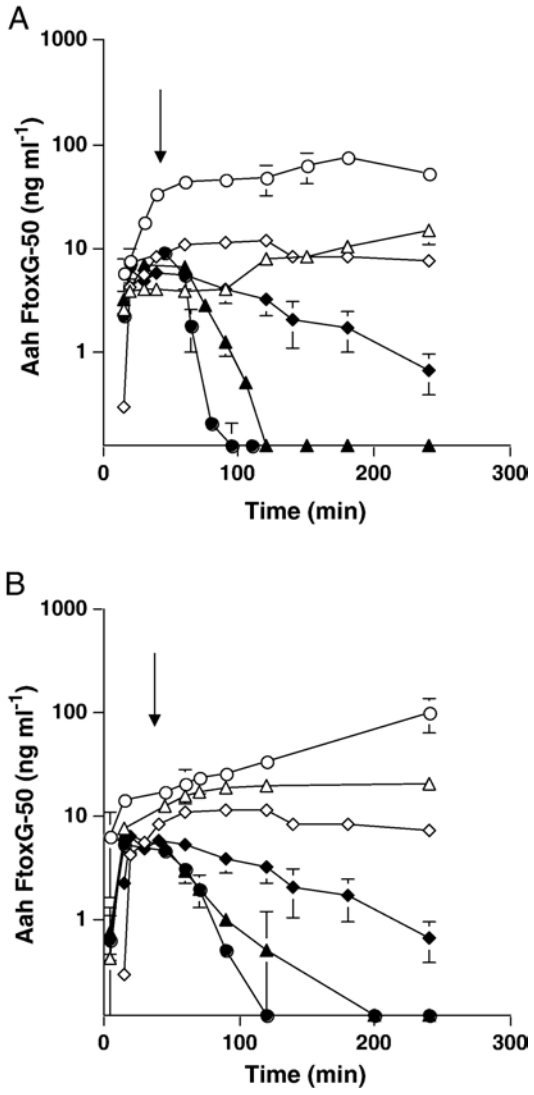

Fig. 2. Effect of antivenom injection on the pharmacokinetic properties of Aah FtoxG-50 venom. Groups of three rats were given sc injections of Aah venom (83 μg kg^{-1}) supplemented with a trace amount of radiolabeled ^{125}I-Aah-FtoxG-50 (10^6 cpm mg^{-1}). Rats were then injected 45 min later with 40 mg kg^{-1} of $F(ab')_2$ antivenom (A) or Fab antivenom (B) by iv (circles) or im route (triangles). Blood samples were withdrawn at the indicated times. Control animals received the sc injection of venom but not antivenom (lozenges). The concentration of Aah FtoxG-50 in plasma samples was determined by ELISA (closed symbols) and by counting radioactivity after TCA precipitation (open symbols). Arrows indicate the time of injection of antivenom.

Aah toxins than did $F(ab')_2$ injected by the same route (Fig. 2B, Table 2). In fact, the extent of venom toxins complexed by Fab was similar for both routes (im or iv) of injection.

Cardiovascular functions in rats envenomed with Aah venom

Effect of Aah venom on hemodynamic variables in anesthetized rats before and after immunotherapy

Four groups of anesthetized rats were subcutaneously injected with three doses of venom (168, 250 and 500 μg kg^{-1}) or with 150 mM NaCl at zero time, and the mean arterial pressure and heart rate were recorded for 100 min (Fig. 3). There is a large increase in arterial pressure after sc injection of 168 and 250 μg kg^{-1} of Aah venom. The hypertensive phase started 20 min after venom injection and lasted 40 min. This effect was maximal 40 to 50 min after envenomation. Afterwards, the mean arterial pressure returned to baseline values. For the largest dose of Aah venom (500 μg kg^{-1}), the increase in arterial pressure was reduced. The hypertension phase was followed at the two larger doses by a decrease in the arterial pressure, which was maximal 90 min after venom injection (Fig. 3). The heart rate was not significantly affected after the sc injection of the lowest dose of Aah venom (168 μg kg^{-1}). A significant decrease of arterial pressure, starting 30 to 40 min after the injection of Aah venom, was observed for the two higher doses (250 and 500 μg kg^{-1}) (Fig. 3). Surprisingly, the bradycardia does not seem to be dose-dependent (it is more pronounced for 250 μg kg^{-1} than for 500 μg kg^{-1}) (Fig. 3).

In a second series of experiments, eight groups of rats received the injection of 40 mg kg^{-1} of antivenom $F(ab')_2$ either by ip or by im route, 15 or 45 min after the sc injection of Aah venom (250 μg kg^{-1}) (Fig. 4). Mean arterial pressure and heart rate were recorded in the function of time and their values, determined at two times after Aah venom injection for blood pressure (45 and 90 min) and at 50 min for heart rate, are represented in Fig. 4. All fragments were efficient in preventing early hypertension and late hypotension when injected 15 min after envenomation. Fab was more efficient than $F(ab')_2$ to prevent the hypertension, whatever their route of administration. $F(ab')_2$ injected by im was the least efficient treatment (Fig. 4A). When injected late (45 min after envenomation), all fragments displayed the same partial efficacy to inhibit the hypertension phase, whereas they all prevented the decrease of blood pressure, irrespective of route of administration. Both antibody fragments prevented bradycardia irrespective of time and delay of administration, except for $F(ab')_2$ fragments when injected by im route 45 min after envenomation (Fig. 4B).

Effect of Aah venom on lungs before and after administration of antivenom

Pulmonary edema is a common cardiovascular manifestation of severe scorpion envenomation. We therefore followed the airway resistance (AR) of unrestrained rats envenomed with 168, 250 or 500 μg kg^{-1} Aah venom by analyzing enhanced pause (Penh) for 300 min. Saline-treated rats served as controls (Fig. 5A). AR (Penh) increased for at least 60 min after venom injection and then remained at a high value up to 200 min after envenomation and finally returned to control values after 250–300 min (Fig. 5A). The dose of 250 μg kg^{-1} caused the earliest appearance (60 min) and the maximal Penh enhancement (1.7±0.18) (Fig. 5A).

Antibody fragments (40 mg kg^{-1}) were injected ip or im 15 min (Fig. 5B) or 45 min (Fig. 5C) after sc injection of 250 μg kg^{-1} of Aah venom. When injected by ip 15 min after venom injection, both fragments completely prevented the appearance

Table 2
Toxicokinetic parameters of Aah FtoxG-50 after immunotherapy

A — radioactivity counting Parameters	Venom (sc route)	Venom+F(ab′)$_2$ (iv route)	Venom+F(ab′)$_2$ (im route)	Venom+Fab (iv route)	Venom+Fab (im route)
AUC_{0-240} (ng min^{-1} ml^{-1})	2500±60	4000±230*#	3000±100*#♦	3900±400*	3800±100*♦
B— ELISA Parameters	**Venom sc route**	**Venom+F(ab′)$_2$ iv route**	**Venom+F(ab′)$_2$ im route**	**Venom+Fab iv route**	**Venom+Fab im route**
AUC_{0-240} (ng min^{-1} ml^{-1})	1300±70	400±90*#	600±80*#	550±100*#	650±60*#

Toxicokinetic experiments on Aah FtoxG-50 were performed after sc injection of Aah venom (83 μg kg^{-1}). AUC values were measured by ELISA and by counting the radioactivity after TCA precipitation in the absence and after iv or im antivenom immunotherapy, as indicated in the Methods section. *$p<0.05$ comparison between venom and antivenom parameters; #$p<0.05$ comparison of route of administration; ♦$p<0.05$ comparison between different antibody fragments injected by the same route.

of AR: Penh values were similar to those for control rats. The im injection of Fab was as effective than the ip one, while the im administration of F(ab′)$_2$ was much less effective (Fig. 5B). The administration of antibody fragments 45 min after venom injection was less effective than when applied 15 min after the venom: almost no effect was noted with F(ab′)$_2$ whereas Fab fragments, either ip and im, significantly reduced the pulmonary effects of Aah venom (Fig. 5C).

Discussion

Antivenom immunotherapy is the only specific treatment for scorpion envenomations, though the efficacy of such treatment is controversial. This is probably due to the absence of rigorous guidelines for its administration (Abroug et al., 1999). We therefore undertook experimental studies to determine the effectiveness of antivenom administration in

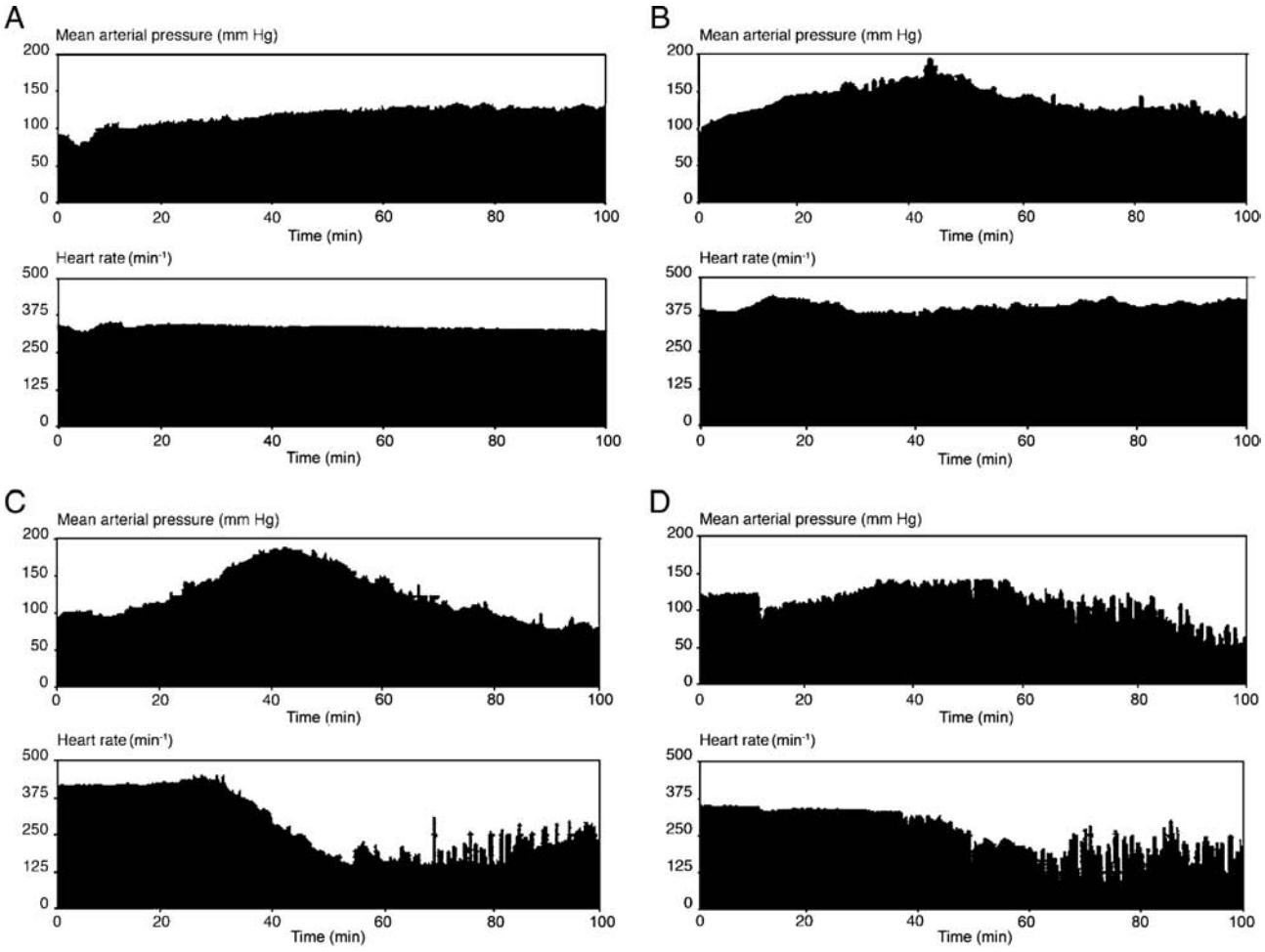

Fig. 3. Cardiovascular effects of Aah venom. anesthetized rats were injected sc with NaCl (150 mM) (A) or 168 μg kg^{-1} (B), 250 μg kg^{-1} (C) or 500 μg kg^{-1} (D) of Aah venom. Mean arterial pressure and heart rate were recorded for 100 min.

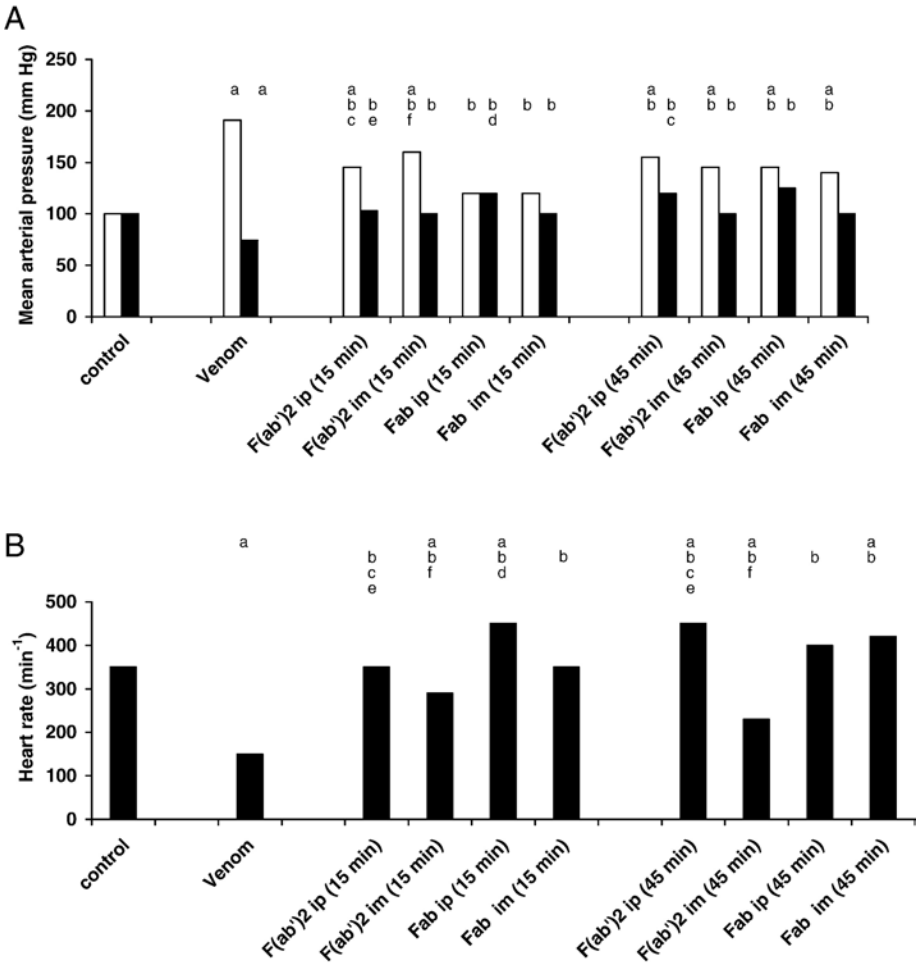

Fig. 4. Effect of antivenom administration on mean arterial pressure and heart rate. Anesthetized rats were sc injected with 250 μg kg^{-1} Aah venom and 15 min or 45 min later $F(ab')_2$ or Fab (40 mg kg^{-1}) was injected by the ip or the im route. A: mean arterial pressure was measured 45 (open histograms) and 90 (closed histograms) min after envenomation. B: heart rate was recorded after 50 min. Control animals received sc injections of 150 mM NaCl or 250 μg kg^{-1} Aah venom. a: $p<0.05$ comparison between control and venom/antivenom parameters; b: $p<0.05$ comparison between venom and antivenom parameters; c: $p<0.05$ comparison between different $F(ab')_2$ injected by iv and im; d: $p<0.05$ comparison between Fab injected by iv and im; e: $p<0.05$ comparison between different antibody fragments injected by ip; f: $p<0.05$ comparison between different antibody fragments injected by im.

envenomed animals, based on observations of its effect on the kinetics and dynamics of the toxic venom components.

We drew three main conclusions from our toxicokinetic studies. Firstly, we found that Aah venom had very fast kinetics in the rat with an elimination half-life of about 13 min after iv injection and between 35 and 53 min after sc injection. These half-life values are similar to those for *Tityus serrulatus* venom in mice, determined by Revelo et al. (1996), but significantly shorter than those reported by others for *Androctonus amoreuxi*, *Buthus occitanus mardochei*, *Centruroides limpidus limpidus* and *Androctonus mauritanicus mauritanicus* venoms, respectively (Ismail et al., 1983; Pépin-Covatta et al., 1996; Calderon-Aranda et al., 1999; Krifi et al., 2001; El-Hafny et al., 2002). This difference may be due to the different animal models studied (rat and rabbit) or to the different methods used to quantitate plasma venom (radioactive methods and ELISA). Secondly, the absorption of Aah venom was rapid, in agreement with other pharmacokinetic studies of scorpion venoms (Ismail et al., 1994; Ismail and Abd-Elsalam, 1998; Revelo et al., 1996; Calderon-Aranda et al., 1999; Krifi et al., 2001, 2005). The fast absorption of Aah venom is consistent with the rapid onset of clinical symptoms observed after scorpion stings. Thirdly, the biodistribution of Aah venom in the rat differed considerably depending on the route of administration (iv or sc). This was already reported by Pépin-Covatta et al. (1996) and Calderon-Aranda et al. (1999) for sc injection of *B. occitanus mardochei* and *C. limpidus* venoms. However, no such difference was reported for the venom of *Buthus occitanus tunetanus* (Krifi et al., 2001). The extent of distribution depends on two main physicochemical properties of the toxin: protein binding and the partition coefficient. We selected rats with similar body weights for the experiments, so only the protein binding parameter appears to be involved. *A. australis* venom proteins may bind to both circulating and stationary proteins with different consequences. When injected iv, venom components might bind plasma proteins tightly and consequently have a small volume of distribution. When injected sc, venom components may also be distributed through the lymphatic system to reach their cell

targets, i.e., sodium channels of excitable nerve cells, for which they have a great affinity. Previous major toxicodynamic analyses were done on animals injected iv with scorpion venoms (Tarasiuk et al., 1998; Zeghal et al., 2000, Abroug et al., 2003; Ouanes-Besbes et al., 2005), and thus may not reflect what occurs following an actual envenomation. Targets reached by venom components injected iv in experimental studies may not be the same as those affected following a scorpion sting and/or may be targets reached with different kinetics.

We then proceeded to a toxicodynamic analysis of Aah venom effects. Cardiovascular and respiratory distress occur following severe scorpion envenomation (Freire-Maia et al., 1974; Sofer and Gueron, 1988; Abroug et al., 1991; Bawaskar and Bawaskar, 1991; 1994; D'Suze et al., 1999), including hypertension, bradycardia, tachycardia, hypotension, bronchorrhea and pulmonary edema (Bouaziz et al., 1996; Krifi et al., 1998). In this study, we used a rat model to attempt to mimic an accidental envenomation in human. For this purpose, we administered Aah venom sc to rats and determined the cardiovascular and pulmonary effects. We observed complex hemodynamic alterations: blood pressure rose shortly after sc venom injection followed by a hypotension phase with high venom doses of 250 and 500 μg kg^{-1}. Similar effects on blood pressure have been described in humans following scorpion envenomations. Zeghal et al. (2000) and Andrade et al. (2004) observed these effects on blood pressure in rats given *B. occitanus* and *T. serrulatus* venoms iv, respectively. *Leiurus quinquestriatus* envenomed dogs (Tarasiuk et al., 1998) had only a direct hypotension phase. A slight increase in cardiac rhythm was observed shortly after injection with the lowest doses (168 and 250 μg kg^{-1}) of venom. Bradycardia was recorded 50 min after envenomation with the two highest doses (250 and 500 μg kg^{-1}) only. There was a dose-dependent upper airway obstruction within 50 min of venom administration. This obstruction was reversible whatever the dose injected. These symptoms and their time courses are consistent with what is observed in human victims. They also correlated strongly with the time required for toxic venom components to reach their maximal concentrations in plasma.

Gutierrez et al. (2003) emphasized the importance of detailed analyses of pharmacokinetic–pharmacodynamic relationships for each venom–antivenom system to determine the most appropriate type of neutralizing molecule for each particular venom. We used the described rat model to test the immunoneutralizing effect of antivenom antibodies on Aah venom toxicokinetics. We first tested the effect of two antivenom immunoglobulin fragments (Fab and $F(ab')_2$) injected by different routes (iv and im) on plasma sequestration and redistribution of venom toxins. These two parameters are key elements for the effectiveness of antivenom immunotherapy for viper and scorpion envenomations (Audebert et al., 1994;

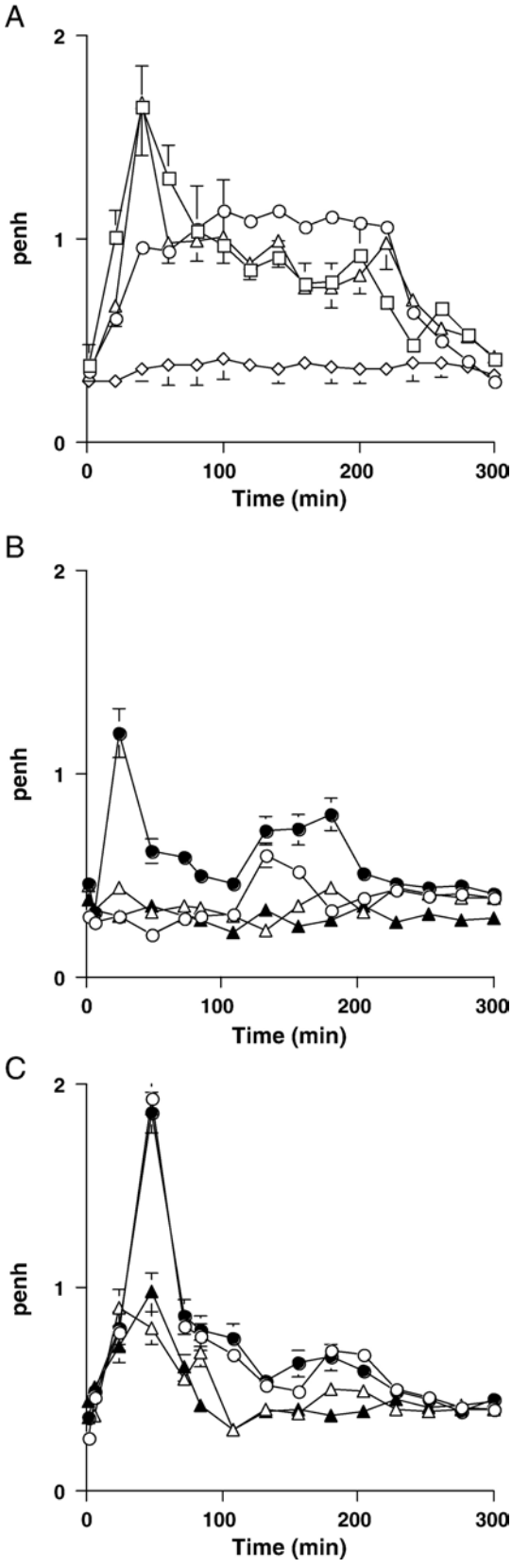

Fig. 5. Pulmonary effects of Aah venom before and after immunotherapy. The airway resistance of unrestrained Aah envenomated rats was followed. A: the dose response effect was tested after subcutaneous injection of 168 μg kg^{-1} (circles), 250 μg kg^{-1} (squares) or 500 μg kg^{-1} (triangles) of Aah venom. Control animals (lozenges) received 150 mM NaCl. Airway reactivity was determined by monitoring enhanced pause (Penh) units as described in the Methods section. B and C: rats were given 250 μg kg^{-1} Aah venom and 15 min (B) or 45 min (C) later an ip (open symbols) or im (closed symbols) injection of 40 mg kg^{-1} antivenom, consisting of either $F(ab')_2$ (circles) or Fab (triangles) fragments.

Rivière et al., 1997; Calderon-Aranda et al., 1999; Krifi et al., 2001; 2005). Fab and $F(ab')_2$ similarly redistributed venom components from the extravascular compartment to the central one. Whereas Fab mediated redistribution by both the iv and im routes equally well, $F(ab')_2$ was more effective when injected iv than im. This result is explained by the different pharmacokinetic profiles for Fab and $F(ab')_2$ due to their molecular mass. Bazin-Redureau et al. (1997) showed that the volume of distribution of Fab in rats after iv injection was three times greater than that of $F(ab')_2$, demonstrating the ability of Fab to diffuse into the extracellular water volume. Fab also has a much shorter half-life after iv injection (Rivière et al., 1997) and a faster absorption after im injection than $F(ab')_2$ (Rivière et al., 1998). Both fragments less potently affected the process of plasma sequestration by the im route than the iv route, which is explained by their respective Tmax values of 48 h and 12 h (Pépin-Covatta et al., 1995; Rivière et al., 1998), compared with that of Aah venom (20 to 40 min). According to kinetic markers – the neutralization of circulating toxins and the redistribution of toxins from peripheral compartments – iv injection of specific antivenom was the most effective immunotherapy against Aah envenomation. $F(ab')_2$ and Fab were equally potent.

Although the nature, chronology and mechanisms of the cardiac and hemodynamic effects following scorpion envenomation have been extensively studied, only a few studies have investigated the blocking effects of antivenoms (Tarasiuk et al., 1998; Andrade et al., 2004; Abroug et al., 2003; Ouanes-Besbes et al., 2005). We showed that $F(ab')_2$ given ip or im shortly after venom injection (15 min) only partially prevented the increase of peripheral resistance, whereas Fab was efficient whatever the route of injection used. Fab is more effective than $F(ab')_2$ because of its larger distribution volume and faster absorption. Even after delayed injection (45 min), both antibody fragments prevented hypotension, a symptom observed in 18% of patients stung by *A. australis* (Bouaziz et al., 1996) and that may lead to collapse and circulatory arrest. $F(ab')_2$ and Fab also prevented bradycardia: Fab was effective through both ip and im routes, whereas $F(ab')_2$ was only effective if administered ip. Tarasiuk et al. (1998) reported that $F(ab')_2$ from antivenom given at the same time or within 10 min of iv administration of *L. quinquestriatus* venom neutralizes bradycardia in dogs. Delayed injection of antivenom was totally ineffective. The authors suggested that the ineffectiveness of delayed antivenom treatment was a consequence of the rapid release of various mediators from sympathetic and parasympathetic nerve terminals, an indirect effect of scorpion venom (Freire-Maia et al., 1995; Tarasiuk et al., 1998; Zeghal et al., 2000; Ouanes-Besbes et al., 2005), although other studies have indicated that scorpion toxins also exert a direct effect on the cardiopulmonary system (Bouaziz et al., 1996; Teixeira, 2001; Rogers, 1996; De-Matos et al., 2001). We think that the lack of efficiency of antivenom antibodies observed in the previous studies is mainly explained by the significant differences between toxicokinetic properties of venom administered iv or sc, probably leading to the different toxicodynamics that we observed. Indeed, Ismail and Abd-Elsalam (1998) found that $F(ab')_2$ had neutralization effects in rats administered venom sc even if the fragments were injected 30 min after envenomation. We also observed potent therapeutic effects of antivenom on the respiratory system in envenomed rats. When injected ip, shortly after Aah venom administration, Fab and $F(ab')_2$ similarly prevented the increase of airway resistance. Fab was also effective when given im, whereas $F(ab')_2$ was not. Fab, but not $F(ab')_2$, decreased airway resistance after delayed injection, irrespective of the route of injection. Due to the volume of distribution of Fab, its ability to neutralize scorpion toxins outside the vascular compartment, "en route" to or at the site of action might explain this result. Scorpion venoms contain small toxins which have a rapid initial absorption, a large distribution in the extravascular compartment and a short half-life. These venoms may be efficiently neutralized by small molecular weight antibodies including Fab which possess adapted pharmacokinetic parameters. Moreover, venom toxins/Fab complexes have a molecular weight smaller than the filtration threshold of the renal glomeruli and may therefore be eliminated rapidly in urine, as described for Fab/colchicine complexes (Sabouraud et al., 1992).

Finally, we propose that iv injection of a mixture of Fab and $F(ab')_2$ from antivenom might counteract the pathophysiological effects of scorpion venoms. If these antibody fragments cannot be administered iv, the im injection of Fab could be beneficial; Fab was more effective than $F(ab')_2$ by the im route. Currently, we are working to determine the appropriate dose of antivenom and the best ratio of Fab to $F(ab')_2$ to be used according to the degree of scorpion envenomation.

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