Toxicon Vol. 29, No. 4/5, pp. 527–531, 1991.
Printed in Great Britain.

0041–0101/91 $3.00 + .00

SERUM KINETICS OF CROTOXIN FROM *CROTALUS DURISSUS TERRIFICUS* VENOM IN MICE: EVIDENCE FOR A RAPID CLEARANCE

M. BARRAL-NETTO and R. L. VON SOHSTEN

Servico de Imunologia, Hospital Universitario Prof. Edgard Santos, Universidade Federal da Bahia, Salvador-Bahia-Brazil

(*Received* 29 *August* 1990; *accepted* 21 *November* 1990)

M. BARRAL-NETTO and R. L. VON SOHSTEN. Serum kinetics of crotoxin from *Crotalus durissus terrificus* venom in mice: evidence for a rapid clearance. *Toxicon* **29,** 527–531, 1991.—We report on an ELISA for the detection of crotoxin with a detection limit of 1–3 pg/ml of sample. Cross-reactivity with other animal venoms occurred only at concentrations above 1 μg/ml. Serum kinetics of crotoxin were investigated in BALB/c mice after a single 10 μg s.c. dose of venom obtained from *Crotalus durissus terrificus*. Crotoxin levels were 254 ± 141 ng/ml serum ($\bar{X} \pm$ S.E.) 15 min after venom injection, 3.9 ± 0.5 ng/ml serum at 30 min and undetectable thereafter. The rapid clearance of crotoxin from the serum suggests that the test may be unsuitable for the clinical management of envenomation victims.

OPHIDISM is a major public health problem worldwide, particularly in the Third World. In Brazil, snakebites are the leading cause of animal envenomation, which represented approximately 0.04% of all reported deaths during a five-year period (RODRIGUES *et al.*, 1988). The genus *Crotalus* is of special importance because of the high incidence of envenomation and its high mortality rates (Ministerio da Saude do Brasil, 1986). Despite the major advances in the medical sciences, the management of snakebites has not improved much (STAHNKE *et al.*, 1957; Ministerio da Saude do Brasil, 1987), being based on the empirical administration of large amounts of antivenom which is not devoid of severe side effects (NIELSEN *et al.*, 1978). The lack of data regarding the pharmacokinetics of venom toxins makes the task even more difficult. Recently, several groups have successfully employed the enzyme-linked immunosorbent assay (ELISA) for the detection of snake venoms (reviewed by THEAKSTON, 1984), with applications covering detection of venom or anti-venom blood levels in patients and animals (KHIN-OHN-LWIN *et al.*, 1984; SILAMUT *et al.*, 1987), diagnosis of the genus involved (THEAKSTON *et al.*, 1977; COULTER *et al.*, 1980) epidemiological studies (CHIPPAUX and THEAKSTON, 1987; PUGH and THEAKSTON, 1980) and kinetics studies (THEIN-THAN *et al.*, 1985; MG-MG-THWIN *et al.*, 1985). ELISA also has the potential to be used in *in vitro* assays to detect the neutralization potency of specific antisera, instead of the current bioassays. The ELISA technique is rather sensitive, specific and inexpensive. We report on the feasibility of detecting crotoxin (the major toxin of the South American rattlesnake, *Crotalus durissus terrificus* venom) by using a sandwich ELISA together with biotin-avidin amplification. Polypropylene 96-well

microtiter plates (Hemobag, Campinas, Brazil) were coated with 0.05% F(ab)'2 fraction of horse anti-crotalic venom (Instituto Butantan, São Paulo, Brazil) in carbonate–bicarbonate buffer (pH 9.6) for 60 min at 37°C. At all steps we have used 100 μl/well. After five washing cycles (3 min each) with PBS-Tween 0.05%, the plate was blocked using 3% low fat milk overnight at 4°C. Plates were washed and incubated for 60 min at 37°C with either (1) crotoxin (a gift of Dr C. DINIZ; Fundação Ezequiel Dias, Belo Horizonte, Brazil) in phosphate-buffered saline (PBS) at a concentration ranging from 1 μg/ml to 1 pg/ml; (2) sera of mice diluted 1:5 in PBS; or (3) with different unrelated venom preparations. After a washing cycle they were incubated for 30 min at 37°C with rabbit anti-crotoxin IgG (diluted 1:500) obtained in a similar fashion as described elsewhere (BARRAL-NETTO *et al.*, 1990), using 100 μg of crotoxin for initial immunization and 50 ug of the toxin for booster doses. The following incubations with goat-anti-rabbit biotinylated antibody (1:10,000) and avidin-peroxidase conjugate (1:5000) were performed under the same conditions as above. After the final washing cycle, peroxidase substrate solution (0.04% orthophenilenediamine, 0.012% hydrogen peroxide in pH 5.0 citrate-phosphate buffer) was added to the wells and plates were placed in the dark for 10 min. The reaction was stopped by adding 25 μl of 8N H_2SO_4 per well. The reading was performed using a multichannel Spectrophotometer (Titertek Multiskan, Flow Labs, Irvine, Scotland). In order to standardize the ELISA, known concentrations of crotoxin, ranging from 1 μg/ml to 1 pg/ml (diluted in PBS), were assayed by ELISA. The resulting optical densities were correlated to the toxin concentration (logarithms) and the linear regression performed by the least squares method using the Microsoft statistical package (Ecosoft, version 1984). A typical standard curve is depicted in Fig. 1. The correlation coefficients had a mean (±

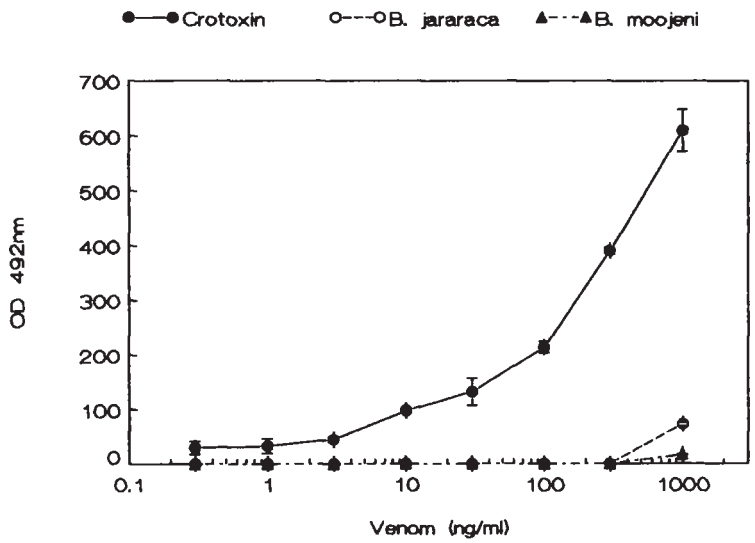

FIG. 1. SENSITIVITY AND SPECIFICITY OF AN ELISA FOR DETECTION OF CROTOXIN FROM *C. durissus terrificus*.

Venom preparations (0.1 to 1000 ng/ml) were assayed by ELISA for crotoxin detection. The resulting optical densities were plotted against their concentrations. Cross-reactivity appeared with *B. moojeni* (filled triangles) and *B. jararaca* (open circles) at concentrations above 300 ng/ml. Venom from *T. serrulatus*, *L. muta*, *B. atrox* and *B. cotiara* gave no reading. Values expressed as means ± S.E. ($n = 3$).

S.E.) of $r = 0.954$ (± 0.019; $n = 7$). The intra-test variation for a given toxin concentration was always less than 10%. When tested against PBS, our ELISA has a detection limit of 3 pg of crotoxin/ml of sample. In order to perform the serum kinetics of crotoxin, groups of five BALB/c mice weighing 15 to 22 g and obtained from our own colony were injected s.c. with 10 μg of crotalic venom in 0.02 ml of PBS into the left hind footpad; controls received PBS alone. The ethical standards proposed by MEIER *et al.* (1986) were followed. Blood samples were obtained from the retro-orbital vessels. Toxin levels were obtained by interpolating the optical densities in the standard crotoxin curve (performed for each experiment). The detection limit in each test was defined as the mean plus two standard deviations of the readings obtained from control mice sera. Figure 2 shows the serum kinetics of crotoxin in mice. The detection limit of the venom was 2.2 ng/ml and peak concentrations occurred at 15 min (254 $\pm$ 141 ng/ml serum; $X \pm$ S.E.; $n = 5$) with a sharp decline at 30 min (3.9 $\pm$ 0.5 ng/ml; $n = 5$). No toxin was detected at 60 min, 180 min or 24 hr (Fig. 2). Our detection limits ranged from 1–3 ng/ml, similar to those reported in the literature (THEAKSTON *et al.*, 1977; KHIN-OHN-LWIN *et al.*, 1984; SILAMUT *et al.*, 1987; BARRAL-NETTO *et al.*, 1990). Since venom concentrations in body fluids are in the nanogram range (KHIN-OHN-LWIN *et al.*, 1984; HO *et al.*, 1986), our test could be useful for investigational purposes *in vivo*, such as the detection of the real amount of inoculated venom based on an equation correlating blood levels, victim's body weight and time elapsed since the accident. However, the rapid clearance of crotoxin from circulation represents a severe limitation to this proposal. Since victims usually take hours to reach specialized medical attention, no toxin would be detected, if crotoxin kinetics in humans are similar to the results reported here. Our results were readily reproducible, with minimal inter-test variations. The ELISA is characterized by low background and high sensitivity. There was no advantage in using rabbit anti-crotoxin IgG instead of whole serum; it is possible that affinity-purified, venom-specific IgG would be better. An

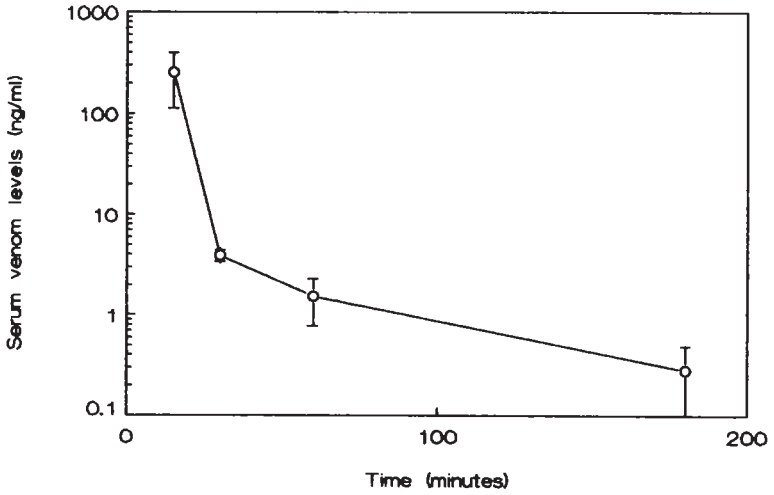

FIG. 2. SERUM KINETICS OF CROTOXIN IN MICE.
Groups of five BALB/c mice were injected s.c. with 10 μg of venom from *C. d. terrificus* in the footpad, and bled at different time intervals. Sera were assayed by crotoxin-specific ELISA. Values expressed as mean $\pm$ S.E. ($n = 5$) crotoxin concentrations in ng/ml of serum. At 6 and 24 hr post-injection no venom was detected in serum.

interesting point in analyzing the usefulness of venom detection by ELISA is the species specificity. In our experiments significant cross-reactivity appeared only at 1000-fold higher concentrations of the heterologous venoms (Fig. 1). Since the concentration of venoms in the circulation is in the range of nanograms, this cross-reactivity is of no practical importance. THEAKSTON *et al.* (1977) report no significant cross-reactivity of clinical importance in 14 different venoms using a double sandwich ELISA. Recent immunological data suggest that crotoxin homologs are present in a large number of rattlesnakes (HENDERSON and BIEBER, 1986). Mojave toxin from *C.s. scutulatus* and concolor toxin from *C.v. concolor* are related to crotoxin (cited in KAISER and AIRD, 1987). KAISER and AIRD (1987) found the Uracoan rattlesnake (*C. vegrandis*) toxin to be homologous with crotoxin from *C. durissus terrificus* venom. There is no geographical overlap among these species, and that should pose no problems to the practical use of anti-crotoxin ELISA. However, the rapid disappearance of crotoxin from serum, and the time necessary to perform the test are the major limitations for evaluation of crotoxin levels by ELISA in a real accident. A proper clinical diagnosis would provide better and faster results in an acute situation. Although not useful for diagnostic purposes, our test contributed to unraveling the pharmacokinetics of crotoxin, and similar studies are necessary in man.

Acknowledgements—The authors thank Mr VANILSON SOUZA and Mr MARCONES SILVA for their excellent animal care and technical support, and Mr JACKSON LEMOS for secretarial assistance. R. L. VON SOHSTEN is a recipient of a Conselho Nacional de Pesquisas (CNPq) fellowship. This project was supported by Financiadora de Estudos e Projetos (FINEP).

REFERENCES

BARRAL-NETTO, M., SCHRIEFER, A., VINHAS, V. and ALMEIDA, A. R. (1990) Enzyme-linked immunosorbent assay for the detection of bothropic venom. *Toxicon* **28**, 1053–1061.

CHIPPAUX, J. B. and THEAKSTON, R. D. G. (1987) Epidemiological studies of snake bite in French Guiana. *Ann. Trop. Med. Parasitol.* **81**, 301–304.

COULTER, A. R., HARRIS, R. D. and SUTHERLAND, S. K. (1980) Enzyme immunoassay for the rapid clinical identification of snake venom. *Med. J. Austr.* **1**, 433–438.

HENDERSON, J. T. and BIEBER, A. L. (1986) Antigenic relationships between Mojave toxin subunits, Mojave toxin and some crotalid venoms. *Toxicon* **24**, 473–482.

HO, M., WARRELL, M. J., WARRELL, D. A., BIDWELL, D. and VOLLER, A. (1986) A critical reappraisal of the use of enzyme-linked immunosorbent assays in the study of snake bite. *Toxicon* **24**, 211–221.

KAISER, I. I. and AIRD, S. D. (1987) A crotoxin homolog from the venom of the Uracoan rattlesnake (*Crotalus vegrandis*). *Toxicon* **25**, 1113–1120.

KHIN-OHN-LWIN, AYE-AYE-MYINT, PE, P., NWE, T. and NAING, M. (1984) Russell's viper venom levels in serum of snake bite victims in Burma. *Trans. Roy. Soc. Trop. Med. Hyg.* **78**, 165–168.

MEIER, J., BANKS, B., GREPPY, E. E., HABERMEHL, G., KORNALIK, F., LEE, C.-Y., MEBS, D., ROSENBERG, P. and THEAKSTON, R. D. G. (1986) Ethical standards and guidelines for animal experiments in toxinological research. *Toxicon* **24**, 327–330.

MG-MG-THWIN, THEIN-THAN and HLA-PE, U. (1985) Relationship of administered dose to blood venom levels in mice following experimental envenomation by Russell's viper (*Vipera russelli*) venom. *Toxicon* **23**, 43–49.

MINISTÉRIO DA SAÚDE DO BRASIL (1986) Report of the "II Reunião técnica sobre ofidismo: Distribuição geográfica das serpentes do Brasil". Brasilia: Centro de Documentação do Ministerio da Saúde.

MINISTÉRIO DA SAÚDE DO BRASIL (1987) Manual de diagnóstico e tratamento de acidentes ofidicos. Brasilia: Centro de Documentação do Ministerio da Saúde.

NIELSEN, H., SORENSEN, H., FABER, V. and SVEHAG, S. E. (1978) Circulating immune complexes, complement activation kinetics and serum sickness following treatment with heterologous anti-snake venom globulin. *Scand. J. Immunol.* **7**, 25–33.

PUGH, R. N. H. and THEAKSTON, R. D. G. (1980) Incidence and mortality of snake bite in Savanna Nigeria. *Lancet* **29**, 1181–1182.

RODRIGUES, D. S., TELES, A. M. S., MACHADO, M. A. M. L., VARGENS, M. M. L., NASCIMENTO, I. M. and PLANZO, T. M. P. (1988) Ofidismo na Bahia: Um problema de Saúde Pública. *Rev. Soc. Bras. Toxicol* **1**, 23–26.

SILAMUT, K., HO, M., LOOAREESUWAN, S., VIRAVAN, C., WUTHIEKANUN, V. and WARRELL, D. (1987) Detection of venom by enzyme linked immunosorbent assay (ELISA) in patients bitten by snakes in Thailand. *Brit. Med. J.* **294**, 402–404.

STAHNKE, H. L., ALLEN, F. M., HORAN, R. V. and TENERY, J. H. (1957) The treatment of snake bite. *Am. J. Trop. Med. Hyg.* **6**, 323–335.

THEAKSTON, R. D. G. (1984) The use of enzyme immunoassay in snake venom research. Proceedings of the 6th European Symposium on Animal, Plant and Microbial toxins (Basle). 9–20.

THEAKSTON, R. D. G., LLOYD-JONES, M. J. and REID, H. A. (1977) Micro-ELISA for detecting and assaying snake venoms and venom antibody. *Lancet* **2**, 639–641.

THEIN-THAN, KYI-THEIN and MG-MG-THWIN (1985) Plasma clearance time of Russell's viper (*Vipera russelli*) antivenom in human snake bite victims. *Trans. Roy. Soc. Trop. Med. Hyg.* **79**, 262–263.