

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF *BOTHROPS JARARACA* VENOM

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M. BARRAL-NETTO, A. SCHRIEFER, V. VINHAS and A. R. ALMEIDA. Enzyme-linked immunosorbent assay for the detection of *Bothrops jararaca* venom. *Toxicon* **28**, 1053-1061, 1990.—This study reports an enzyme-linked immunosorbent assay for detecting *Bothrops jararaca* venom in fluids, employing the sandwich method with biotin/avidin amplification. The assay exhibits high accuracy in correlating optical densities with venom concentrations ($r = 0.98$), high reproducibility, low background and limited cross-reactivity with venom from other snake genera. Nevertheless, it was unable to distinguish among venoms from different bothropic species. Using this method we evaluated the serum kinetics of *Bothrops jararaca* venom in C57BL/6 mice. High concentrations were found in serum just 15 min after injection (151 ± 41 ng/ml; mean \pm S.D.), followed by a progressive fall (102 ± 46 , 74 ± 39 and 50 ± 22 ng/ml after 1, 3 and 6 hr respectively), being undetectable by 24 hr. Such serum kinetics indicates a pattern of a rapid absorption of venom from the inoculation site, followed by a slow and progressive drop in its serum levels. This ELISA was a reliable tool in the determination of *Bothrops jararaca* venom levels in mouse serum, and may become useful in other fields of bothropic venom research.

INTRODUCTION

THE EMPLOYMENT of the ELISA in the detection of snake venoms was described by THEAKSTON *et al.* (1977), and since then it has been widely used for a variety of aims (COULTER *et al.*, 1974, 1980; KHIN-OHN-LWIN and AYE-AYE-MYINT, 1982; MINTON *et al.*, 1984). In medicine its main applications are the assessment of venom levels in serum of patients (KHIN-OHN-LWIN *et al.*, 1984), the diagnosis of the snake genus involved in envenomation (THEAKSTON, 1984) and prevalence studies which help health care institutions to properly distribute their antivenoms (SILAMUT *et al.*, 1987). From the research standpoint, applications range from biodistribution, kinetics in different tissues (MG-MG-THIN *et al.*, 1985) and clearance (KHIN-OHN-LWIN *et al.*, 1984), to evaluation of immunization protocols and antibody production in immunized animals. ELISA can also be used in the assessment of neutralization power of antiserum (SIMONSEN *et al.*, 1987), avoiding the currently used bioassays (VILLARROEL *et al.*, 1978/1979) as recommended by the International Society On Toxinology (MEYER *et al.*, 1986).

Ophidism is an important public health problem in Brazil (RODRIGUES *et al.*, 1988). *Bothrops*, *Crotalus* and *Micrurus* are commonly found in this country (MINISTERIO DA SAUDE, 1986), with the bothropic genus being mainly responsible for human accidents. The establishment of the kinetics of a venom in the serum is an essential step in the development of an assay capable of calculating the dose of venom inoculated, in which at least three variables are important: (1) victim's body weight, (2) time elapsed since inoculation, and (3) serum venom levels. This knowledge would allow the development of a personalized modality of treatment, where the victim would receive only the amount of antivenom necessary to neutralize the quantity of venom injected. In this study, we developed an ELISA, applying the sandwich method with biotin/avidin amplification, for the detection of *B. jararaca* venom and report on its use in the assessment of serum venom levels in inoculated mice.

MATERIALS AND METHODS

Reagents

The venoms of *Bothrops* (*jararaca*, *jararacussu*, *moojeni*, *cotiara*, *eritromela* and *atrox*), *Lachesis muta* and *Tityus serrulatus* were donated by Instituto Butantan (São Paulo, SP, Brazil). The purified crotoxin was a generous gift of Dr C. DINIZ (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). Tityustoxin purification was kindly performed by Drs I. HENEINE and W. BERALDO (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). The horse F(ab')₂ fraction anti-*B. jararaca* was a gift of Fundação Ezequiel Dias (Belo Horizonte, MG, Brazil). The biotinylated anti-rabbit IgG, the avidin peroxidase complex and the peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma (St Louis, MO, U.S.A.). The rabbit anti-*B. jararaca* purified IgG was obtained by Protein A gel chromatography of sera collected for a rabbit immunized against *B. jararaca* venom. All reagents were stored frozen.

Rabbit immunization

A rabbit was immunized s.c. with 250 ng of *B. jararaca* venom emulsified in 0.5 ml of complete Freund's adjuvant, followed by five other s.c. inoculations of 50 ng of venom emulsified in 0.5 ml of incomplete Freund's adjuvant, in time intervals of 1–2 months. The increase in antibody levels was monitored assaying blood samples, obtained 1 week after each inoculation, with an ELISA using *B. jararaca* venom (10 µg/ml) for coating the plates.

Mice

Male C57BL/6 mice from our own colony, weighing between 18 and 22 g, were maintained on a commercial balanced ration and water *ad libitum*. Sixteen hours prior to inoculation with venom, the animals were arranged in groups of five, receiving no water or ration.

ELISA for venom detection

Polypropylene 96-wells round-bottomed microtiter plates (Hemobag, Campinas, SP, Brazil) were coated with 0.5% F(ab')₂ fraction of horse anti-*B. jararaca* venom in carbonate-bicarbonate buffer (pH 9.6) for 1 hr at 37°C. At this and all other steps the wells were filled with 100 µl volumes. The plates were washed five times with phosphate buffered saline (PBS) containing 0.05% Tween 20 and then incubated overnight at 4°C with 3% low fat milk in carbonate-bicarbonate buffer as a blocking step. After another washing cycle a 1 hr incubation was performed with either (1) different preparations of *B. jararaca* venom (ranging from 1 µg/ml to 300 pg/ml), (2) sera of inoculated mice (diluted 1:5 in PBS), or (3) with different unrelated venom preparations (of several bothropic species and of other genera). The plates were washed again and incubated with rabbit IgG anti-*B. jararaca* venom for 30 min at 37°C. Once again the plates were washed five times and incubated with anti-rabbit biotinylated antibody 1:10,000 in the same conditions mentioned above. Then, after another washing cycle the avidin/peroxidase complex was added to the wells in a dilution of 1:5,000, and the plates incubated as in the biotinylated antibody step. Measurement of the peroxidase bound to the plates was made after a last washing cycle by incubation with peroxidase substrate solution (0.04% *ortho*-phenylenediamine, 0.012% hydrogen peroxide in pH 5.0 citrate-phosphate buffer) for about 15 min at room temperature in the dark, and the reaction was stopped by the addition of 25 µl per well of 8 N sulphuric acid. Spectrophotometric reading using a 492 nm wavelength filter was then performed using a multichannel spectrophotometer (Titertek Multiskan, Flow labs, Scotland).

For some tests we employed the standard peroxidase ELISA method (i.e. without the biotin/avidin amplification system). The difference from the above protocol was that after the incubation step with rabbit IgG anti-*B. jararaca* venom, the plates were washed and then incubated (at 37°C for 30 min) with peroxidase conjugated goat anti-rabbit IgG (Miles Yeda), diluted 1:800. After further washing, incubation with substrate and plate reading was performed as described. Alternatively we employed a co-incubation strategy in the ELISA using the biotin/avidin protocol. *B. jararaca* venom and rabbit IgG anti-*B. jararaca* venom were added simultaneously, and the plates incubated overnight at room temperature. All further steps (from incubation with biotinylated anti-rabbit IgG on) were performed as indicated above.

Serum kinetics studies

C57BL/6 mice were divided in seven groups of five animals each. Six groups were inoculated s.c. in the right hind footpad with 10 µg of *B. jararaca* venom diluted in 20 µl of saline (test groups), while the last group was injected with 20 µl of saline only (control group). Each group was bled at one of the following time points: 15 and 30 min and at 1, 3, 6, 18, and 24 hr after venom inoculation (the same group was bled at 30 min and 24 hr). The control group was bled 1 hr after saline injection. Blood samples (50 to 100 µl) were obtained by puncture of the retroorbital vessels with Pasteur pipettes. Sera were then separated from whole blood and venom levels assessed by ELISA as described above.

Statistical treatment

Using the statistical package Microstat (Ecosoft Co., version 1984), the correlations of log dose of venom and optical densities were made by the least square method and regression analysis.

RESULTS

ELISA for Bothrops jararaca venom detection

The curve "serum venom levels vs optical density" (Fig. 1) obtained from the spectrophotometric evaluation of samples with different concentrations of *B. jararaca* venom revealed high accuracy in correlating optical density (O.D.) with venom concentrations ($r = 0.99$). The comparison of the curves on each and on different plates, revealed a small inter-test and intratest optical reading variation (Table 1).

Cross-reactivity among different snake venoms

In an attempt to investigate the degree of cross-reactivity among different venoms and *B. jararaca* venom using this test, known preparations of several bothropic species as well as of two other genera were assayed and the curves "venom concentration vs optical

TABLE 1. INTRA- AND INTER-ASSAY VARIABILITY AMONG OPTICAL DENSITIES IN THE DETERMINATION OF *B. jararaca* VENOM BY ELISA, PERFORMED IN DUPLICATES IN FOUR TESTS

Venom concentration (ng/ml)	Assays*							
	Test 1		Test 2		Test 3		Test 4	
1000	849	819	952	1033	934	824	1472	1522
300	722	622	842	928	920	805	1432	1500
100	608	618	739	667	759	730	1108	1302
30	492	466	548	536	600	503	920	937
10	262	273	371	364	372	315	474	477
3	127	143	226	235	177	144	214	243
1	50	65	132	137	96	71	75	63
0.3	9	32	80	77	49	32	28	38

*All values represent absorbance readings multiplied by 1000.

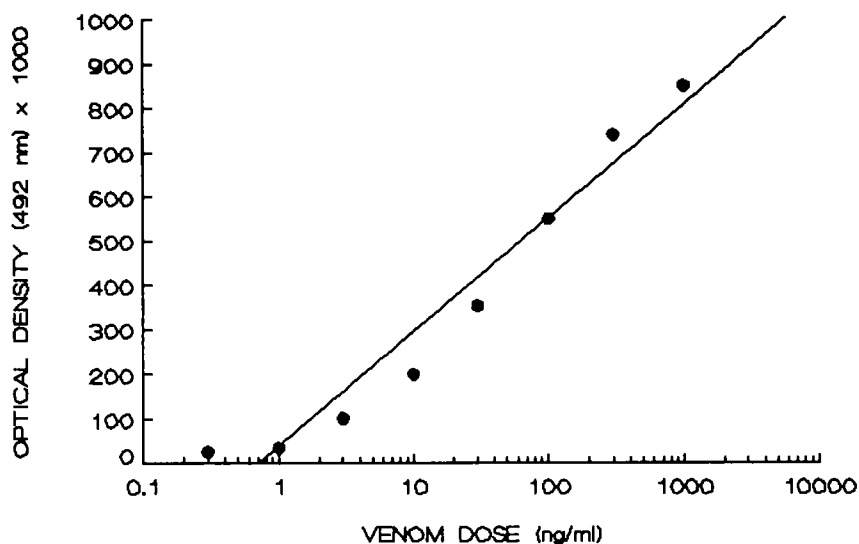


FIG. 1. SOLUTIONS OF *Bothrops jararaca* VENOM, RANGING FROM 0.3 ng/ml to 1 μ g/ml, WERE ASSAYED BY ELISA WITH BIOTIN/AVIDIN AMPLIFICATION.

The resulting optical densities were correlated to the venom concentrations and the linear regression performed by the least squares method ($r = 0.98$). A representative experiment is shown.

density" were plotted (Fig. 2). There was extensive cross-reactivity among venoms of different bothropic species, with the exception of *B. jararacussu*. There was no significant cross-reactivity with venoms of other genera tested, such as *Lachesis* and *Crotalus* (Fig. 2). Limited detection occurred only when they were in extremely high concentrations (in the order of 1 μ g/ml). Purified tityustoxin (from the scorpion *Tityus serrulatus*) was also tested using this assay and showed no cross-reactivity; optical density values were similar to those reflecting background.

Comparison among different ELISA procedures

Searching for optimization of the test, including time spent and sensitivity, we compared the standardized ELISA with two variants using the same peroxidase system. When employing the standard peroxidase test the background was much higher than with the biotin/avidin system, the test being unable to discriminate among high venom concentrations (above 1 μ g/ml). Furthermore the correlation between optical density and venom concentration did not follow the smoothly ascending pattern of that obtained with the biotin/avidin system (Table 2). In the comparison between both biotin/avidin assays ("sequential incubation" and "co-incubation") the plates were incubated with venom preparations ranging from 100 pg/ml to 1 mg/ml and the curves obtained by spectrophotometric reading of the optical densities of those samples were plotted in Fig. 3. The curve obtained from sequential incubation presents a plateau when venom concentrations are higher than 1 μ g/ml, then it starts to drop as the venom concentration decreases. The co-incubation curve, on the other hand, shows a more complex pattern, ascending as concentration of venom decreases, reaching a peak between concentrations of 10 ng/ml and 1 ng/ml, and then dropping in a pattern similar to that seen in the sequential

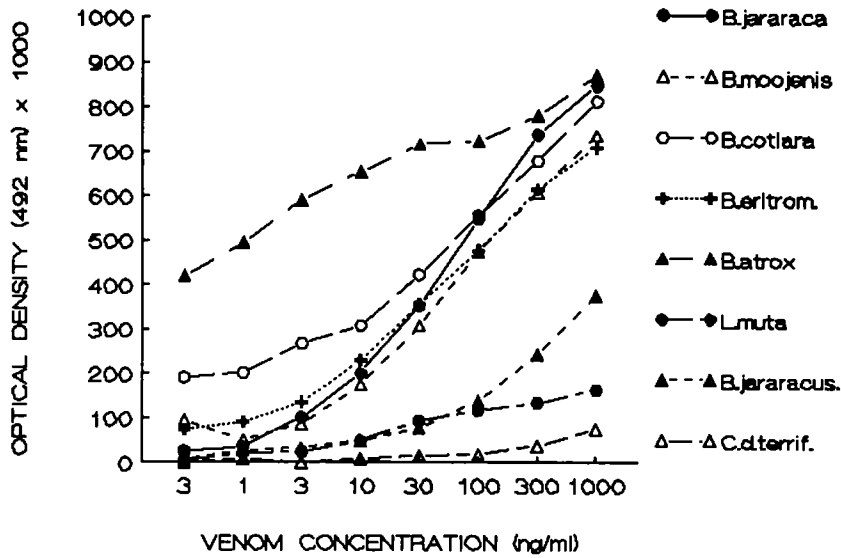


FIG. 2. DIFFERENT VENOM PREPARATIONS OF VARIOUS BOTHROPIC SPECIES, *Lachesis* AND OF *Crotalus durissus terrificus* WERE ASSAYED BY ELISA FOR *Bothrops jararaca* VENOM DETECTION. The resulting optical densities were plotted against their concentrations. (*B. eritrom.* = *B. eritromelas*; *B. jararacus.* = *B. jararacussu*; *C.d. terrif.* = *Crotalus durissus terrificus*).

incubation assay. From the figure we can further state that the co-incubation test shows a sensitivity higher than that of the sequential incubation test, since at low venom concentrations (below 10 ng/ml) it gave absorbance values higher than those obtained by the sequential incubation method.

Serum kinetics of Bothrops jararaca venom

We used the sequential incubation biotin/avidin assay in the evaluation of the serum kinetics of bothropic venom (*B. jararaca*) in mice. The results of the spectrophotometric

TABLE 2. COMPARISON OF ABSORBANCES OF KNOWN VENOM CONCENTRATIONS ASSAYED BY ELISAs EMPLOYING EITHER BIOTIN/AVIDIN OR STANDARD PEROXIDASE SYSTEM

Venom concentration (ng/ml)	Absorbance (492 nm)*	
	Standard peroxidase	Biotin/avidin
1000	† †	849 816
300	1910 †	739 698
100	1677 1840	548 559
30	1211 1360	353 388
10	621 766	200 214
3	242 347	101 93
1	72 119	35 47
0.3	29 59	26 25

*Values represent absorbance readings multiplied by 1000.
 †Absorbances above measurable range.

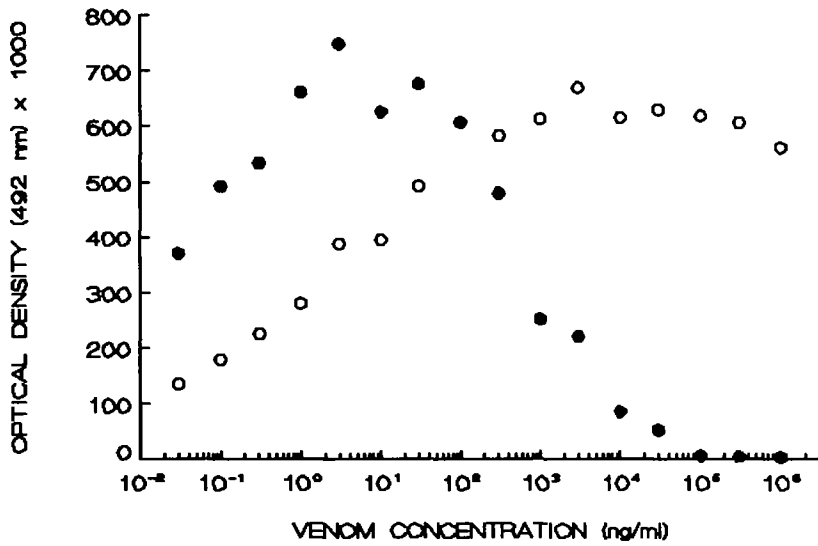


FIG. 3. DIFFERENT KNOWN PREPARATIONS OF *Bothrops jararaca* VENOM WERE ASSAYED BY ELISA APPLYING EITHER SEQUENTIAL INCUBATION OF VENOM AND ITS ANTI-VENOM (○) OR CO-INCUBATION OF BOTH (●).

The resulting optical densities were plotted against venom concentrations.

assays of the sera samples are shown in Fig. 4. There was a rapid absorption of the venom from the site of inoculation to the blood, with venom blood concentration high just 15 min after inoculation. After this high concentration, the serum levels of venom dropped slowly and progressively, and venom was not detectable 24 hr after injection.

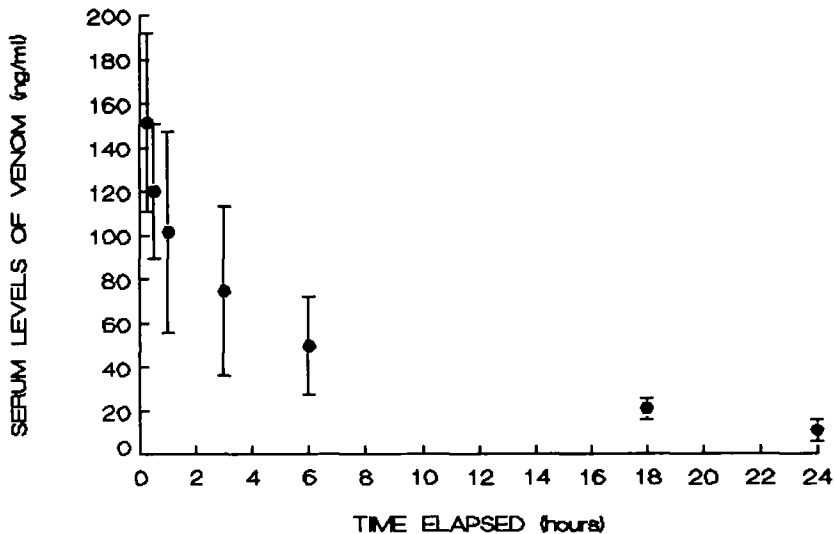


FIG. 4. SEVEN GROUPS OF FIVE C57BL/6 MICE WERE INJECTED WITH 10 µg OF *Bothrops jararaca* VENOM IN THE HIND FOOTPAD AND BLED 15 AND 30 MIN AND 1, 3, 6, 18 AND 24 HR AFTER INOCULATION.

The serum samples were assayed by ELISA for *Bothrops jararaca* venom detection. Points represent means of groups' values, and error bars represent standard deviations.

values obtained from serum samples of the control group of mice (11.8 ± 1.4 ng/ml), the lowest amount of venom reliably detected by the test was 14.6 ng/ml, which corresponds to the mean plus two standard deviations of the hypothetical venom levels encountered in the blood samples of this group (by interpolating absorbance values in the standard curve made with known venom concentrations).

DISCUSSION

The ELISA reported herein for detection of bothropic venom is characterized by low background, high reproducibility and high efficiency in calculating serum levels of venom from optical densities. Thus, this test is a very reliable means of evaluating bothropic venom concentrations, allowing its use in the study of the serum kinetics of this venom. Its use for clinical purposes, however, is not established. Bothrops venom is a mosaic of different proteins, many of which may not be important toxic components. A reliable ELISA test for clinical use must specifically evaluate the serum levels of relevant toxic fractions.

The presence of high background readings is a major problem in enzyme immunoassays, and some solutions have been discussed elsewhere (HO *et al.*, 1986). In our tests, the use of low fat milk resulted in very low background when compared to the same assay without overnight incubation with milk (data not shown). Those background levels were even lower when the reagents were diluted with 3% low fat milk in phosphate buffered saline (PBS) instead of PBS alone in the other steps of the test (data not shown).

The test showed high specificity for detecting bothropic venom as assessed by the low cross-reactivity with venoms of other snake genera and of the scorpion *T. serrulatus*, but failed to distinguish the *B. jararaca* venom from several other bothropic venoms. The only bothropic venom tested that displayed low cross-reactivity with *B. jararaca* venom was that of the *B. jararacussu* species, which is in accordance with the lower antigenic similarity of this venom with that of *B. jararaca* already discussed by VILLARROEL *et al.* (1974a,b, 1976/1977). The venom of *B. atrox* gave higher O.D. values than those obtained from the venom of *B. jararaca* used for immunization. It is possible that *B. atrox* venom contains a high proportion of the immunogenic proteins and this point deserves further examination. The sensitivity was also high, detecting venom in concentrations of 1–3 ng/ml, which are lower than the average circulating levels of venom, reported for other snake venoms (KHIN-OHN-LWIN *et al.*, 1984; SILAMUT *et al.*, 1987), in bitten patients.

The strange pattern observed in the co-incubation test was probably due to removal of the rabbit anti-*Bothrops* IgG by an excess of antigen. At high concentrations (greater than 10 ng/ml) there was an inverse relation between venom levels and O.D. In venom concentrations lower than 1 ng/ml, the pattern was similar to that disclosed by the sequential incubation assays. On the other hand, the co-incubation test displayed a sensitivity greater than that of the sequential incubation assay at low venom concentrations, as shown by the higher absorbances at similar venom concentrations (Fig. 3). The possibility of having similar O.D.s with two largely different venom concentrations, precludes the use of the co-incubation test in the evaluation of serum venom levels. Such procedure may have a place within research laboratories for the detection of very low venom concentrations, outside the range of reliability of the sequential incubation assay.

The described ELISA has many potential uses besides assessing the kinetics of bothropic venom. For instance it might be employed in epidemiological studies. From the purely

clinical standpoint, however, its use is precluded by the long time it takes and the lack of specificity in determining the actual levels of toxic components of the venom. With precoated plates, it takes about 5 hr which is unusable since a 3 hr delay in treatment significantly increases morbidity. Nevertheless, it may be possible to develop an optimized version that would match the time requirements for clinical use (DHALI WAL *et al.*, 1983) and the need for specificity in the detection of the toxic components of the venom.

The kinetics of *B. jararaca* venom in mice sera disclosed early elevated venom concentrations followed by a progressive drop in serum levels. The inoculations were done s.c. in the hind footpad in an attempt to resemble the most frequently found conditions in human accidents (i.e. s.c. or i.m. bites in the lower limbs). There was a fast absorption of venom from the site of inoculation, with high serum venom levels just 15 min after injection. If the same happens in humans, it correlates well with the increased morbidity associated with the delay of treatment. However, the slow clearance of the venom from the serum after the early peak is indicative of a slow rate of diffusion to other tissues. This might explain the overall low mortality involved in accidents with this snake genus (RODRIGUES *et al.*, 1988). The patients normally receive anti-ophidic therapy several hours after accidents, causing an increased frequency of local sequelae, but because of the slow penetration into vital organs, the treatment may still be capable of preventing the patient's death.

Lastly, the determination of the kinetics is also important if one is attempting to develop a personalized modality of treatment, in which the amount of antivenom to be given to the patient is determined on the basis of the quantity of venom inoculated. In such an approach we envision an equation correlating the variables (including victim's body weight, serum venom level and time elapsed since inoculation) to the amount of inoculated venom. A better knowledge of venom serum kinetics in different animal species, especially those more similar to man, may help define an approach.

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