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BODY DISTRIBUTION OF *BOTHROPS ASPER* (TERCIOPELO) SNAKE VENOM MYOTOXIN AND ITS RELATIONSHIP TO PATHOLOGICAL CHANGES

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E. MORENO and J. M. GUTIÉRREZ. Body distribution of *Bothrops asper* (terciopelo) snake venom myotoxin and its relationship to pathological changes. *Toxicon* **26**, 403–409, 1988. — The distribution of ^{125}I-labelled *Bothrops asper* myotoxin following i.m. and i.v. injections was studied in mice. After i.m. administration the toxin was concentrated in the injected gastrocnemius muscle, with relatively little binding to other tissues. Upon i.v. injection the highest radioactivity was detected in liver, kidneys, lungs, spleen and blood. A conspicuous decrease in myotoxin concentration occurred during the first hour, whereas the rate of decrease was reduced at later time periods. Only the injected skeletal muscle was clearly damaged after i.m. inoculation, as judged by histology and by the decrease in tissue creatine kinase contents. Contralateral, non-injected gastrocnemius was not affected by the toxin. Histological observations carried out after i.v. administration of the toxin revealed moderate alterations only in lungs, with a slight increase in serum levels of the enzymes creatine kinase and alanine aminotransferase.

INTRODUCTION

A TOXIC phospholipase A_2 was isolated from the venom of the snake *Bothrops asper* (GUTIÉRREZ *et al.*, 1984*a*). Initially, it was described as a myotoxin since it induced drastic pathologic effects in skeletal muscle after i.m. injections (GUTIÉRREZ *et al.*, 1984*a, b*). However, more recent data indicated that it has a wider pharmacological profile, inhibiting coagulation and inducing edema and lethality (GUTIÉRREZ *et al.*, 1986). Thus, *B. asper* myotoxin resembles other toxic phospholipases A_2 in that it has several sites of action (ROSENBERG, 1979). The ability of a toxin to affect a given tissue or organ *in vivo* depends not only on the susceptibility of the cells, but also on the capacity of the toxin to reach the tissue. Therefore, in order to gain a better understanding of the pathologic effects induced by *B. asper* myotoxin, we have studied its distribution in different tissues after both i.m. and i.v. injections, as well as the sites of tissue damage.

MATERIALS AND METHODS

Toxin

B. asper myotoxin was isolated after two cycles of ion-exchange chromatography on CM-Sephadex C-25, according to the method of GUTIÉRREZ *et al.* (1986). Homogeneity was demonstrated by polyacrylamide disc gel electrophoresis (REISFELD *et al.*, 1962).

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Distribution of radioactively-labelled myotoxin

Fifty micrograms of myotoxin and 50 μg of purified rabbit IgG were radioactively labelled with 1 mCi of ^{125}I (New England Nuclear) according to the method described by HUNTER (1978), using chloramine T as oxidant. Protein conjugates were separated from the free ^{125}I by gel filtration on Sephadex G-50 using phosphate-buffered saline with 0.1% bovine serum albumin as eluant. The specific activities of ^{125}I-myotoxin and ^{125}I-IgG were 14 μCi/μg and 16 μCi/μg, respectively. White mice (18 – 20 g) were injected either i.m. in the right gastrocnemius or i.v. in the dorsal tail vein with 100 μl of ^{125}I-myotoxin (7.8×10 cpm) or 100 μl of ^{125}I-IgG (9.5×10 cpm). Mice (4 per group) were killed by chloroform inhalation at 30 min, 1 hr, 4 hr, 8 hr, and 20 hr. The different organs were obtained, weighed, and their radioactivity measured in a gamma scintillation counter. Results were expressed as cpm/mg tissue and cpm/organ.

Changes in serum levels of creatine kinase and alanine aminotransferase

Mice (4 per group, 18 – 20 g) were injected with 25 μg (in 100 μl) of *B. asper* myotoxin either i.m. in the right gastrocnemius or i.v. in the dorsal tail vein. Control mice received 100 μl of saline solution. At 1 and 3 hr a blood sample was collected from the tail vein for creatine kinase determination. Mice were sacrificed by chloroform inhalation, their thoracic cavity was opened, and a blood sample was collected from the aorta for the determination of alanine aminotransferase levels. Serum levels of creatine kinase and alanine aminotransferase were quantitated by using the Sigma kits No. 520 C and 59 UV, respectively (Sigma Chemical Co., St. Louis. Mo). In some samples, changes in the isozyme pattern of creatine kinase were also studied electrophoretically using the Sigma kit No. 715 – EP

Quantitation of muscle creatine kinase contents

Mice (4 per group, 18 – 20 g) were injected with 25 μg (in 100 μl) of myotoxin either i.m. in the right gastrocnemius or i.v. in the dorsal tail vein. Control mice received 100 μl of saline solution. At 1 and 3 hr mice were killed by cervical dislocation and both right and left gastrocnemius muscles were obtained, weighed, and immediately homogenized in 5 ml of phosphate-buffered saline solution (pH 7.2) containing 0.1% Triton X-100. Samples were then centrifuged at 10,000 *g* for 20 min and supernatants were collected and assayed for creatine kinase using the Sigma kit No. 520 C. Creatine kinase contents of gastrocnemius were expressed as percentage, taking as 100% the creatine kinase levels of gastrocnemius in mice injected with saline solution.

Histopathological studies

Mice (4 per group, 18 – 20 g) were injected with 25 μg of *B. asper* myotoxin as described. Control mice received 100 μl of saline solution. At several time intervals (1 hr, 3 hr, and 24 hr) mice were killed by cervical dislocation and tissue samples from right and left gastrocnemius, heart, lungs, kidneys, liver and brain were obtained. Tissues were immediately fixed in 10% formaldehyde, processed routinely for histological analysis, and stained with hematoxilin-eosin.

Statistical analysis

The Student's *t*-test was used to determine the significance of the differences between groups injected with toxin and those injected with saline solution in the quantitation of serum enzyme levels. In the analysis of the results of distribution of radiolabelled myotoxin, analysis of variance was used to determine the significance of the differences. When the values were significantly different ($P < 0.05$) by analysis of variance, the significance of the differences between pairs of means at each time period was studied by a Tukey's honestly significant difference test.

RESULTS

Distribution of ^{125}I-labelled myotoxin

Figure 1 shows the time-course of the distribution of ^{125}I-labelled myotoxin in different tissues after i.v. injection. When results are expressed as cpm/organ, liver, kidney, lung, spleen and blood had the highest amount of myotoxin at all time intervals, whereas the organs with the least amount of myotoxin were heart, gastrocnemius and brain. A conspicuous decrease in myotoxin concentration occurred mainly during the first hr after injection, whereas at later time periods the rate of decrease of myotoxin in tissues was slower. When the results are expressed as cpm/mg tissues, statistical analysis indicates that at 30 min the mean values can be arranged into four homogeneous subsets, defined as groups whose highest and lowest means do not differ by more than the shortest significant range for a subset of that size. These subsets are: (1) liver; (2) kidney and lungs; (3) lungs,

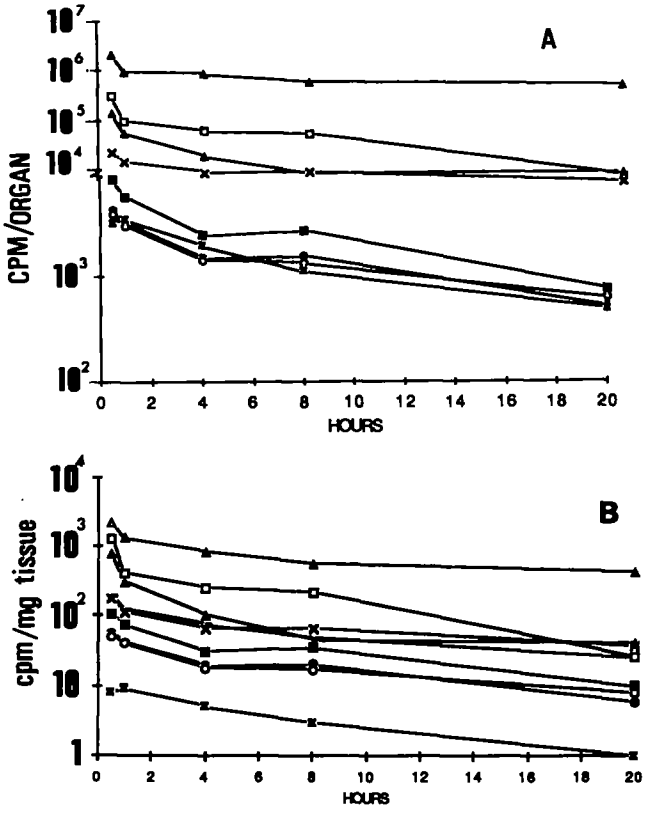

FIG. 1. BODY DISTRIBUTION OF ^{125}I-LABELLED *B. asper* MYOTOXIN AT DIFFERENT TIME INTERVALS AFTER i.v. INJECTION.

Results are expressed as total cpm/organ (A) and cpm/mg wet tissue (B). Each point represents the mean value of four determinations. (●) right gastrocnemius; (○) left gastrocnemius; (□) kidney; (△) liver; (—) blood; (▲) lung; (×) spleen; (*) brain; (■) heart.

blood, spleen and heart; and (4) blood, spleen, heart, right gastrocnemius, left gastrocnemius and brain. When a similar analysis was performed at 1 hr, the means of cpm/mg tissue of the different organs can be placed in three subsets: (1) liver; (2) kidney and lungs; and (3) blood, spleen, heart, right gastrocnemius, left gastrocnemius and brain. The means of cpm/mg tissue when samples were obtained at 4 hr were divided into only two subsets: (1) liver; and (2) the rest of the organs, whereas in samples obtained at 8 hr there were three subsets: (1) liver; (2) kidney; and (3) the rest of the organs. Finally, the means of cpm/mg tissue of samples collected at 20 hr fit into two subsets: (1) liver and (2) the rest of the organs. In conclusion, when data are presented as cpm/mg tissue, liver concentrated the highest amount of myotoxin, followed by kidney and lungs.

In Fig. 2 the time-course of distribution of myotoxin in different tissues after i.m. injection is presented. The highest amount of myotoxin remained at all time periods in the injected gastrocnemius muscle, although there was a considerable reduction 20 hr after injection. In contrast, the left, non-injected gastrocnemius accumulated a relatively low quantity of myotoxin. When results are expressed as cpm/organ, kidney, liver, and blood also had a relatively high amount of the toxin. Between 1 and 4 hr there was small increase in the amount of toxin in liver (from 107,801 to 134,013) and kidney (from 30,475 to 34,770) which occurred simultaneously with a moderate decrease of myotoxin levels in the

right gastrocnemius (Fig. 2). When the results were expressed as cpm/mg tissue, right gastrocnemius accumulated a significantly higher ($P < 0.05$) amount of myotoxin at all time periods. In contrast, the accumulation of myotoxin in the rest of the tissues, expressed as cpm/mg tissue, did not differ significantly between each other ($P > 0.05$) at all time intervals after i.m. injection.

The distribution of ^{125}I-labelled IgG was also studied. In order to determine if myotoxin binding in skeletal muscle was indeed selective when compared to the binding of IgG, we calculated the changes in the ratio of cpm in muscle/total cpm in all organs at three time periods (1 hr, 4 hr and 20 hr) after i.m. injection of toxin or IgG. It was observed that in the case of myotoxin this ratio decreased only to a small extent in injected gastrocnemius muscle (the ratio was 0.9 at 1 hr, 0.88 at 4 hr, and 0.85 at 20 hr), while this ratio decreased more drastically in mice injected i.m. with IgG (0.9 at 1 hr, 0.67 at 4 hr, and 0.54 at 20 hr).

Histopathology

When *B. asper* myotoxin was injected i.m. there was an extensive myonecrosis in the injected gastrocnemius muscle at 1 hr. The morphology of muscle cells affected by the toxin was identical to that described in previous communications (GUTIÉRREZ *et al.*,

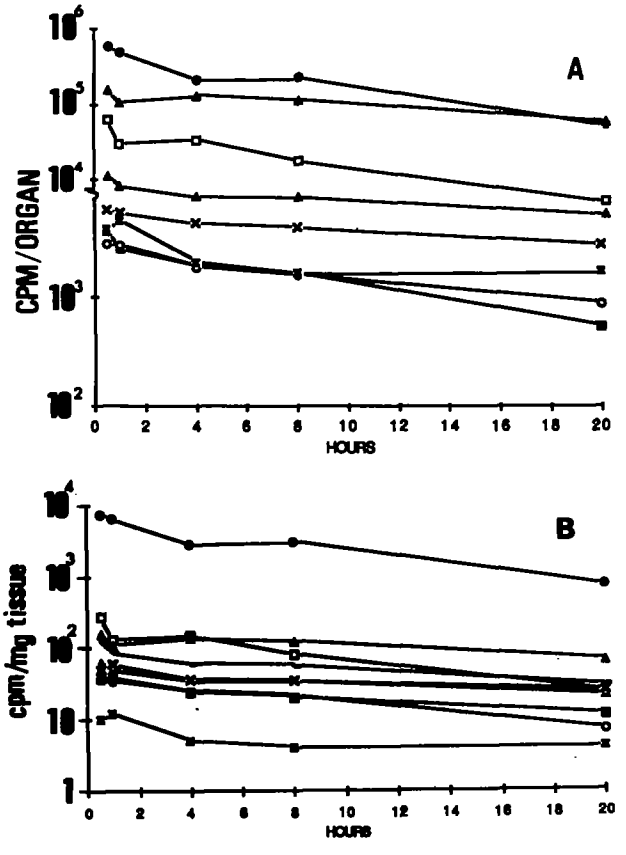

FIG. 2. BODY DISTRIBUTION OF ^{125}I-LABELLED *B. asper* MYOTOXIN AT DIFFERENT TIME INTERVALS AFTER i.m. INJECTION IN THE RIGHT GASTROCNEMIUS.
Results are expressed as total cpm/organ (A) and cpm/mg wet tissue (B). Each point represents the mean value of four determinations. Symbols as in Fig. 1.

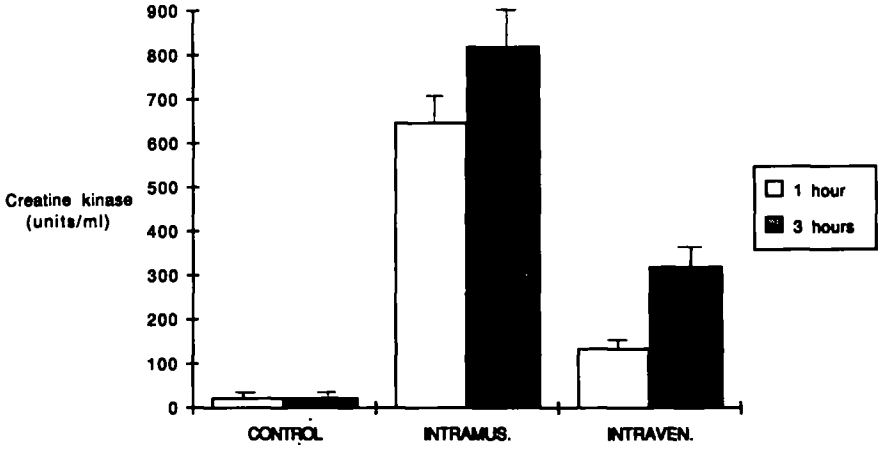

FIG. 3. CHANGES IN SERUM LEVELS OF CREATINE KINASE 1 AND 3 HR AFTER i.m. AND i.v. INJECTION OF 25 μg OF *B. asper* MYOTOXIN.
Results are expressed as mean ± SEM (n = 4). Values increased significantly ($P < 0.05$) at both time intervals after i.m. and i.v. injections.

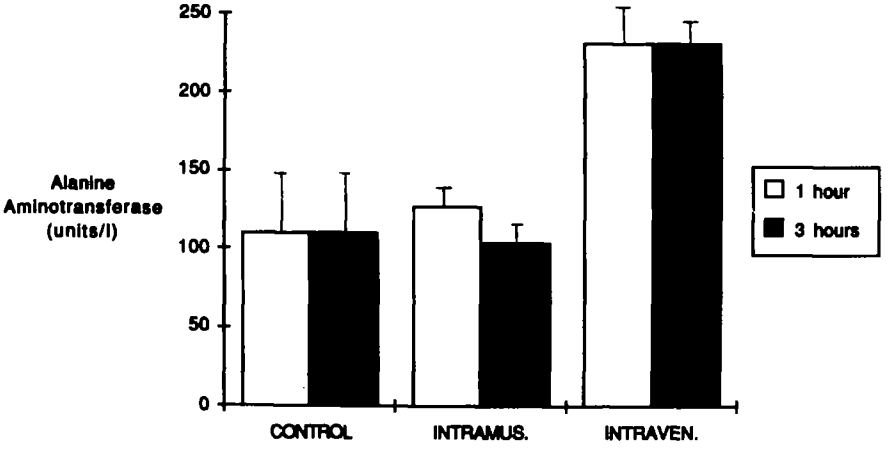

FIG. 4. CHANGES IN SERUM LEVELS OF ALANINE AMINOTRANSFERASE 1 AND 3 HR AFER i.m. AND i.v. INJECTION OF 25 μg OF *B. asper* MYOTOXIN.
Results are expressed as mean ± SEM (n = 4). Values increased significantly ($P < 0.05$) at both time intervals after i.v. injection but not after i.m. injection.

1984*a,b*). Observations made on the left, non-injected gastrocnemius, indicate that muscle cells were not affected, with the exception of few necrotic fibers at 3 and 24 hr. Regarding other tissues, there were no evident morphological changes in kidneys, heart, lungs, brain, spleen and liver after i.m. injection of the toxin.

A different histopathological picture was observed when the myotoxin was administered by the i.v. route. In these cases, there was only a very mild myonecrosis in both right and left gastrocnemius muscles at 1, 3 and 24 hr. The only noticeable morphological alterations were observed in the lungs which presented a moderate

hyperemia and inflammatory reaction at 3 and 24 hr. Other organs did not show evident pathological changes at these time periods.

Changes in serum enzyme levels

Figures 3 and 4 show the changes in serum levels of creatine kinase and alanine aminotransferase respectively 1 and 3 hr after myotoxin injection. Creatine kinase increased significantly ($P < 0.05$) at both time intervals, with the highest increments observed after i.m. injections. Only isozyme CK-MM, typical of skeletal muscle, was elevated in these experiments indicating that creatine kinase release from heart and brain was almost absent. On the other hand, there was not a significant increase ($P > 0.05$) in serum levels of alanine aminotransferase after i.m. injection of the toxin, although this enzyme showed a small but significant elevation when the toxin was injected i.v. (Fig. 4).

Changes in muscle creatine kinase contents

Creatine kinase contents of gastrocnemius muscle injected i.m. with the toxin decreased to 44.1 ± 8.1% and 40.2 ± 5.0% at 1 and 3 hr, respectively, when compared to gastrocnemius injected with saline solution. In contrast, creatine kinase contents of contralateral, non-injected gastrocnemius were not reduced significantly (94.0 ± 9.8% and 95.2 ± 8.2% at 1 and 3 hr, respectively). On the other hand, when the myotoxin was administered i.v., creatine kinase contents of both right and left gastrocnemius remained within normal ranges (96.0 ± 8.1% and 97.3 ± 7.0% of control values at 1 and 3 hr, respectively).

DISCUSSION

The distribution of *B. asper* myotoxin in the body of mice differed considerably depending on the route of injection. After i.m. inoculation myotoxin damages primarily the injected gastrocnemius, as judged by histology and the decrease in creatine kinase levels. These data correlate with the distribution of ^{125}I-myotoxin, since high quantities of it are present in the injected muscle. The almost negligible accumulation of toxin in the contralateral, non-injected gastrocnemius agrees with the histological observations in that there is very little tissue damage. Furthermore, contralateral gastrocnemius creatine kinase contents are reduced only slightly under these conditions.

Intravenous injection of the toxin resulted in a strikingly different distribution pattern and induced a smaller increase in serum creatine kinase levels. Quantitation of muscle creatine kinase contents and histological examination revealed that neither right nor left gastrocnemius were significantly affected. However, the fact that there was a small increase in serum creatine kinase levels indicates that mild systemic myotoxicity occurred. After i.v. injection the myotoxin seems to accumulate in liver, lung, kidney, and spleen, whereas after i.m. injection most of the myotoxin was captured in the right gastrocnemius. However, the accumulation of myotoxin observed in the left, non-injected gastrocnemius after i.v. or i.m. injections was practically the same, probably indicating that similar quantities of toxin were available for the non-injected muscle.

Since significant quantities of myotoxin were present in blood, the radioactivity detected in the contralateral gastrocnemius may reflect in part not a direct attachment of the toxin to muscle cells, as happens in the injected gastrocnemius, but rather the presence of the toxin in the vasculature of skeletal muscle. Nevertheless, as stated before, the slight increase in creatine kinase levels after i.v. administration of the toxin indicates that at

least some myotoxin crosses the capillary wall, reaching and binding to skeletal muscle cells.

The increase of radioactivity observed in liver and kidney between 1 and 4 hr after myotoxin injection in the right gastrocnemius was probably due to accumulation of toxin that was released from skeletal muscle cells at those time periods. Although the cpm detected suggest a modest absolute increase in liver and kidney during that period of time, it must be kept in mind that the relative increment of radioactivity in these organs is probably much higher since there is a constant elimination of the myotoxin from the body, as demonstrated after i.v. injection of this protein. On the other hand, the considerable decrease of myotoxin accumulation in all tissues at 20 hr suggest that it was probably eliminated from the body, perhaps through bile and urine.

It has been described that *B. asper* myotoxin is highly lethal when injected intraventricularly in the brain of mice (GUTIÉRREZ *et al.*, 1986), although its toxicity decreases considerably when injections are performed by the i.v. route. Also, despite the observation that myotoxin is cytotoxic to cardiac myocytes in cell culture (unpublished results), there is negligible myocardial damage when the toxin is administered *in vivo*, as revealed by histology and the normal levels of the isozyme CK-MB in serum. This can be explained by the fact that, although the toxin affects cardiac myocytes and brain neurones when directly exposed to these cells, it does not affect them *in vivo* because there is very little accumulation of toxin in heart and brain. Since microvasculature in these tissues is characterized by the presence of continuous capillaries with low permeability (SIMIONESCU and SIMIONESCU, 1983) it is likely that this constitutes a barrier which prevents the passage of myotoxin from the lumen of blood vessels to the extravascular space.

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