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BIOLOGICAL AND BIOCHEMICAL ACTIVITIES OF *VIPERA BERUS* (EUROPEAN VIPER) VENOM

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L. CALDERÓN, B. LOMONTE, J. M. GUTIÉRREZ, A. TARKOWSKI and L. Å. HANSON. Biological and biochemical activities of *Vipera berus* (European viper) venom. *Toxicon* **31**, 743–753, 1993.—*Vipera berus* is widely distributed throughout the northern part of Europe and Asia. Characterization of several toxic effects of its venom in the mouse, as well as of *in vitro* enzymatic activities was performed. *Vipera berus* venom displayed *in vitro* proteolytic, fibrinolytic, anticoagulant, and phospholipase A_2 activities. The i.p. LD_{50} of the venom for Swiss mice was 0.86 μg/g (95% confidence limits 0.71–1.01 μg/g). Significant local tissue-damaging effects, including edema, hemorrhage and myonecrosis, were observed. The local edema was characterized by rapid onset, reaching a maximum after 0.5–1 hr, and with dose-dependent persistence. The hemorrhagic potency was measured by a skin test, giving a minimum hemorrhagic dose value of 3.2 μg. The venom also induced a moderate local myonecrosis, evidenced by histological evaluation of injected tissue (gastrocnemius), and by biochemical parameters (increase of plasma creatine kinase activity, and decrease of muscle residual MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)-reducing activity). Characterization of the venom by SDS-polyacrylamide gel electrophoresis revealed 10 (reduced) or 11 (unreduced) main protein bands, which were further analyzed in relation to mol. wt and relative concentration by densitometry. A rabbit antiserum to *V. berus* venom recognized all main venom bands by immunoblotting. This antiserum cross-reacted to a variable extent with several crotaline venoms, as assessed by enzyme immunoassay.

INTRODUCTION

Vipera berus (European viper, common adder, common viper) is the venomous snake with the widest geographical distribution, ranging from the British Isles throughout Europe, north to the Artic Circle through the Confederation of Independent States, including southern Siberia, and northern People's Republic of China to the Pacific coast. It is absent from Ireland, southern Spain, Italy and the Balkans (MEHRTENS, 1987). Although rarely fatal, envenomations by *V. berus* deserve medical concern (GONZALEZ, 1991). According

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to HABERMEHL (1981), the World Health Organization indicated about 1300 accidents caused annually by this species in Sweden, of which 12% lead to hospitalization, with a mortality rate of 0.3%.

Clinical findings described in patients envenomed by *V. berus* include pain, stinging sensation, cramps, local edema, drowsiness or confusion, acidosis, leukocytosis, hypotension, shock, vomiting, diarrhea, ecchymosis, and angioneurotic edema of the tongue and lips (HABERMEHL, 1981; STAHEL *et al.*, 1985; CEDERHOLM and LENNMARKEN, 1987). Several components have been isolated from the venom of *V. berus* and biochemically characterized (BOFFA *et al.*, 1976; SIIGUR *et al.*, 1979, 1986, 1988; SAMUEL and SIIGUR, 1990). However, very little information is available on the pathobiological effects induced by this venom in experimental animal models. In the present work, a characterization of several toxic effects of *V. berus* venom in the mouse model is presented. Also, enzymatic activities that might have a relationship to biological effects *in vivo* were determined. Finally, due to the taxonomic position of *V. berus* as a representative of the 'Old World' vipers (Viperinae), an antiserum was produced and utilized to investigate its possible antigenic relationship to venoms of a group of 'New World' pit vipers (Crotalinae).

MATERIALS AND METHODS

Venom

Venom of *V. berus* of Russian origin (batch 122F0841) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Lethal activity

The lethal potency of the venom was determined in Swiss Webster mice (16–18 g). Groups of five animals received an i.p. injection of venom in 500 μl of sterile 0.12 M NaCl, 40 mM sodium phosphate buffer, pH 7.2 (PBS). The logarithmically spaced venom doses utilized in the final estimation were 12.7, 16.8, 22.5, 30, and 40 μg. Deaths were scored after 48 hr, and the lethal dose 50% (LD_{50}) was calculated by probit analysis using the computer program described by TREVORS (1986).

Edema-inducing activity

Local edema was quantified in the mouse footpad, by measuring the thickness increase with a low-pressure spring caliper (Oditest, H.C. Krõplin, F.R.G.) as described by VAN LOVEREN *et al.* (1984). Different venom doses (2.5–20 μg) in 50 μl of PBS were injected s.c. in the footpad of groups of five mice (18–21 g), and thickness was measured after 0.5, 1, 3, 6, 9, 24, and 48 hr. Mice were anesthetized by i.p. injection of Mebumal (NordVacc, Sweden; 0.6 mg/10 g body weight).

Hemorrhagic activity

Hemorrhage was determined in groups of four mice (18–21 g), 2 hr after i.d. venom injection (1–16 μg) in 100 μl of PBS, by measuring the diameter of the hemorrhagic spot formed in the internal side of the skin (KONDO *et al.*, 1960). The skin was shaved before injection to visually verify the i.d. application of venom.

Myotoxic activity

Myonecrosis induced by the venom was evaluated by three criteria: histological observation, quantitation of plasma creatine kinase (CK; EC 2.7.3.2) activity, and by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-reduction assay. Groups of five mice received an i.m. venom injection (10–80 μg) in 100 μl of PBS in the right gastrocnemius. Control animals received a similar injection only of PBS. After 3 hr, a blood sample was obtained from the tail for the measurement of plasma CK activity, using a commercial colorimetric assay (Sigma No. 520) (GUTIÉRREZ *et al.*, 1984). After 24 hr, both gastrocnemius muscles were removed, weighed, and homogenized in PBS-1% Triton X-100. The MTT-reducing activity of each homogenate supernatant was determined by incubating 1 ml of homogenate with 0.2 ml of MTT (2.5 mg/ml PBS) for 90 min at 37°C, and then reading the absorbance at 570 nm against a 690 nm reference, as described in detail by LOMONTE *et al.* (1993*b*). The basis of this test is that residual MTT-reducing activity of the injected muscle correlates with the proportion

of unaffected cells in the tissue. For histological evaluation, muscle samples taken 24 after venom injection were fixed with Duboscq-Brasil solution (10% formalin, 50% ethanol, 6.5% acetic acid, 0.45% picric acid), embedded in paraffin and stained with a modified Masson trichrome method (ARCE, 1986).

Enzymatic activities

Proteolytic activity was tested by incubation of venom (30–500 μg/ml) with 1% casein in PBS for 30 min at 37°C. Proteins were then precipitated by addition of trichloroacetic acid, and the increase of absorbance at 280 nm of the supernatants was determined (LOMONTE and GUTIÉRREZ, 1983). Phospholipase A_2 activity was estimated by an indirect hemolytic radial diffusion assay in the presence of egg yolk phospholipids (GUTIÉRREZ *et al.*, 1988), using venom amounts ranging from 0.1–10 μg/well. Fibrinolytic activity was determined on equine fibrin clot using the radial diffusion assay described by GENÉ *et al.* (1989), with venom amounts ranging from 6–100 μg/well. Coagulant activity was tested on human plasma at 37°C, by adding varying amounts of venom (GENÉ *et al.*, 1989). Anticoagulant activity was determined according to the method of ALVARADO and GUTIÉRREZ (1988), by preincubating venom with platelet-poor human plasma at 37°C for 10 min, and then adding $CaCl_2$.

Antiserum production and cross-reactivity studies

A rabbit antiserum against *V. berus* venom was prepared to study the antigenic cross-reactivity between this venom and those from several crotalid species. Venom (1 mg) was emulsified with Freund's complete adjuvant and injected by the i.m. route. On days 21, 36, and 51, respectively, 1 mg doses were given using sodium alginate as a vehicle. The rabbit was bled 14 days after the last injection, and the antibody titer against *V. berus* venom was determined by enzyme-immunoassay (LOMONTE *et al.*, 1991). Serial dilutions of the immune serum were added to plastic wells coated with 1 μg of *V. berus* venom, and bound antibodies were detected using an anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenol phosphate as substrate (Sigma). Normal rabbit serum at the same dilutions was used as a control. In order to evaluate the cross-reactivity between venoms from Old and New World vipers, an appropriate dilution of the anti-*V. berus* serum, corresponding to the linear region of response of the enzyme immunoassay, was tested against a collection of crotalid venoms from Costa Rica. All venoms were used at a concentration of 1 μg/well and binding of the rabbit antibodies was detected as described above. Cross-reactivity was expressed as a percentage, taking as 100% the absorbance readings obtained with *V. berus* venom as antigen.

Electrophoretic analyses and immunoblotting

Venom was analyzed by polyacrylamide (15%) gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE; LAEMMLI, 1970). Venom was separated at 200 V either unreduced or after reduction with 2-mercaptoethanol for 4 min at 95°C, in a Mini-Protean cell (Bio-Rad, Richmond, CA, U.S.A.). Proteins were stained with Coomassie blue R-250 and the gels were scanned on a GS300 Densitometer (Hoefer Instruments, San Francisco, CA, U.S.A.). Scans were analyzed with the GS365W Electrophoresis Data System (Hoefer) to determine the mol. wt and relative concentrations of the components. Reactivity of the rabbit anti-*V. berus* serum towards the different venom components was tested by immunoblotting. Unreduced venom was separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose at 150 mAmp for 1.5 hr in a Mini-Transblot cell (Bio-Rad), using the buffer described by TOWBIN *et al.* (1979). Proteins on the nitrocellulose were reversibly-stained with amidoblack (SYU and KAHAN, 1987) to check for transfer efficiency. Strips were cut, blocked with bovine serum albumin and casein, and then incubated with either immune or normal rabbit sera. Bound antibodies were detected with an alkaline phosphatase anti-rabbit IgG conjugate, using nitroblue tetrazolium and 5-bromo-4-Cl-3-indolyl phosphate (Sigma) as substrates.

RESULTS

Lethal activity and local tissue-damaging effects

The estimated i.p. LD_{50} of *V. berus* venom was 0.86 ± 0.15 μg/g (14.6 ± 2.6 μg/mouse). The venom induced a marked and rapid edema in the mouse footpad (Fig. 1A), which peaked 0.5–1 hr after injection. This edema was dose-dependent 6–48 hr after injection, but not in the early stages (0.5–1 hr) of the response (Fig. 1A). *Vipera berus* venom also had strong hemorrhagic action, with a minimum hemorrhagic dose (amount of venom inducing a hemorrhage of 10 mm diameter) of 3.2 μg (Fig. 1B). Histological observations confirmed the conspicuous extravasation induced by this venom after i.m. injection (Fig.

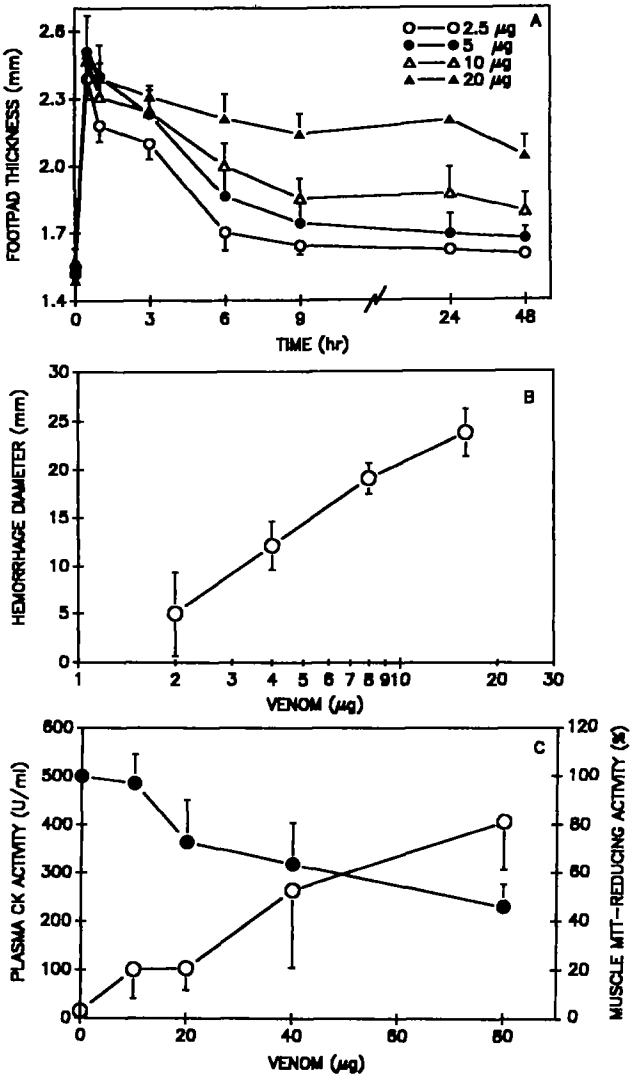

FIG. 1. *In vivo* LOCAL TISSUE-DAMAGING EFFECTS INDUCED IN THE MOUSE BY THE VENOM OF *Vipera berus*.

A. Edema-inducing activity of the venom in the mouse footpad. The indicated doses of venom were injected s.c. and local edema was estimated by measuring the changes in footpad thickness. Each point represents mean ± S.D. of five mice. B. Hemorrhagic activity of the venom in the mouse skin. Different venom doses were injected i.d. and the diameter of the hemorrhagic area was determined 2 hr later. Each point represents mean ± S.D. of four mice. C. Myotoxic activity of the venom in the mouse gastrocnemius muscle. Different venom doses were injected i.m. into the gastrocnemius and 3 hr later plasma creatine kinase levels (○) were determined. After 24 hr, the residual MTT-reducing activity (●) of the tissue was determined and expressed as a percentage of that of the normal contralateral muscle. Each point represents mean ± S.D. of five mice.

2A). In addition, this venom had myotoxic activity, as judged by biochemical indicators of skeletal muscle damage such as plasma CK levels or residual MTT-reducing activity of the injected tissue (Fig. 1C), and by histological evaluation (Fig. 2B).

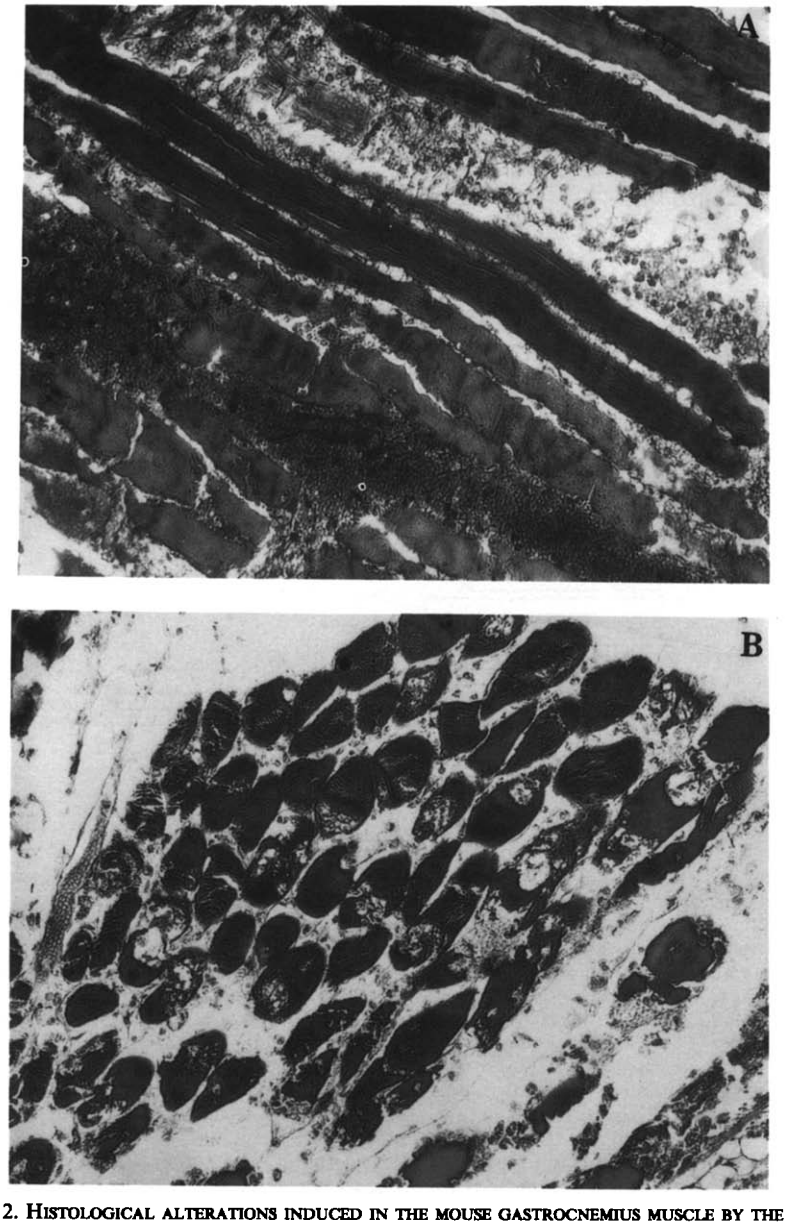

FIG. 2. HISTOLOGICAL ALTERATIONS INDUCED IN THE MOUSE GASTROCNEMIUS MUSCLE BY THE VENOM OF *Vipera berus*.

A. Venom (40 μg) was injected in the gastrocnemius muscle and the tissue was obtained after 24 hr. An area with widespread hemorrhage and a scarce inflammatory infiltrate is seen among the muscle fibers. Modified Masson trichrome stain. Magnification ×40. B. Same as above, showing an area of widespread myonecrosis. Magnification ×100.

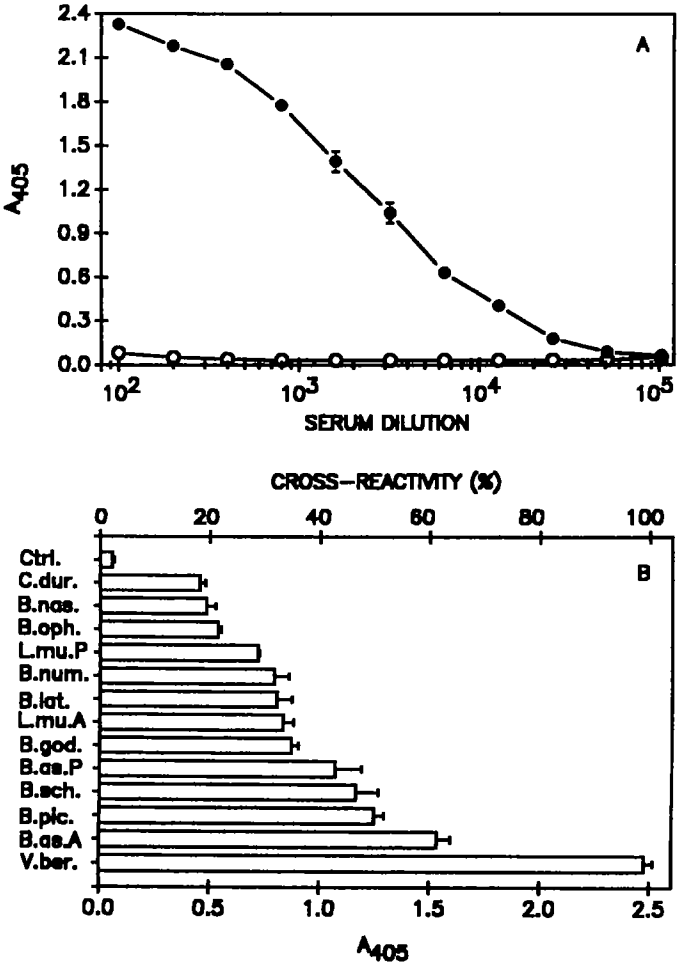

FIG. 3. TITRATION OF AN ANTI-*Vipera berus* RABBIT SERUM AND CROSS-REACTIVITY ANALYSIS USING VENOMS FROM CROTALID SPECIES, BY ENZYME-IMMUNOASSAY.

A. Titration curve of the anti-*V. berus* serum produced in rabbit. Serial dilutions of antiserum (●), or normal rabbit serum (○), were added to *V. berus*-coated plastic wells. Bound antibodies were detected using an anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenol phosphate as substrate. Absorbances were determined at 405 nm (A_{405}). Each point represents the mean ± S.D. of triplicate wells. B. Cross-reactivity of the anti-*V. berus* antibodies against venoms of crotalid snake species from Costa Rica. *Vipera berus* specific antiserum was utilized at 1 : 1000 dilution and all venoms at 1 μg/well. Cross-reactivity is presented as a percentage of the absorbance readings obtained with the homologous venom (upper axis), or as absolute absorbance units (lower axis). Each bar represents the mean ± S.D. of triplicate wells. All venoms were also tested against normal rabbit serum as a control, giving readings (not shown) below the background level indicated in the figure (Ctrl, control of uncoated wells). C.dur, *Crotalus durissus durissus*; B.nas, *Bothrops nasutus*; B.oph, *B. ophryomegas*; L.mu. P, *Lachesis muta* (Pacific type); B.num, *B. nummifer*; B.lat, *B. lateralis*; L.mu.A, *L. muta* (Atlantic type); B.god, *B. godmani*; B.as.P, *B. asper* (Pacific type); B.sch, *B. schlegelii*; B.pic, *B. picadoi*; B.as.P, *B. asper* (Pacific type); V.ber, *V. berus*.

Enzymatic activities

Vipera berus venom had moderate proteolytic activity on casein, with a specific activity of 59.2 units/mg in the assay system utilized. Phospholipase A_2 activity was detected with

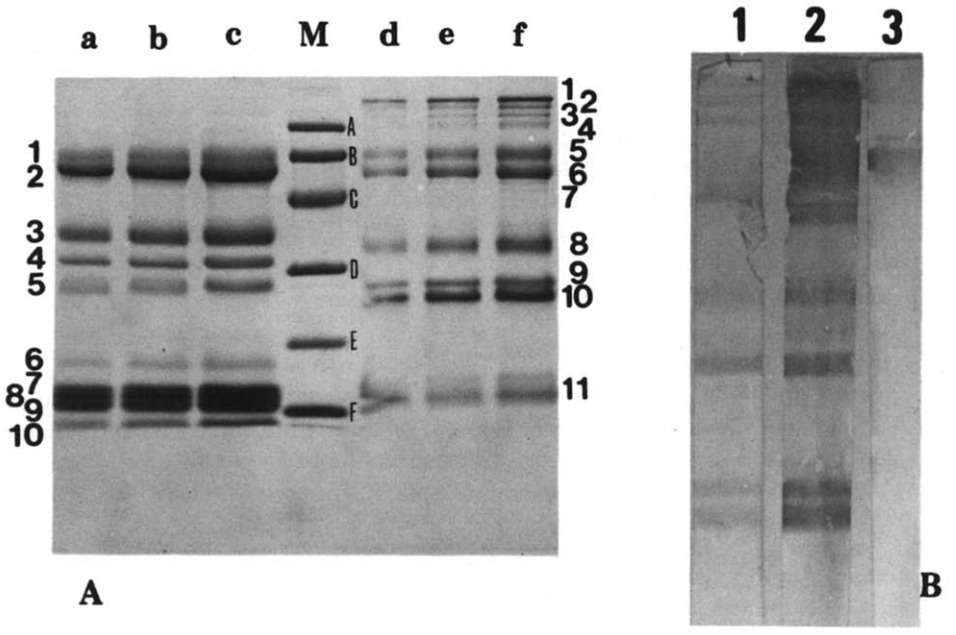

FIG. 4. ELECTROPHORETIC AND IMMUNOBLOTTING ANALYSES OF *Vipera berus* VENOM.
A. SDS-polyacrylamide gel (15%) electrophoresis of the venom after reduction with 2-mercaptoethanol (lanes a,b,c, containing 20, 30, and 50 μg, respectively), or unreduced (lanes d,e,f, containing 20, 30, and 50 μg, respectively). Coomassie blue R-250 stain. M = mol. wt markers (A. 94,000; B. 67,000; C. 43,000; D. 30,000; E. 20,100; F. 14,400). Band numbers, starting from the top, correspond to the desitometric data presented in Table 1. B. Immunoblotting of *V. berus* venom antigens (unreduced) using the rabbit antiserum. 1. Protein pattern revealed by mild (reversible) amidoblack stain; 2. reactivity of the anti-*V. berus* rabbit serum, 1:25 dilution; 3. normal rabbit serum control, 1:25 dilution.

TABLE 1. QUANTITATIVE DENSITOMETRIC ANALYSIS OF THE COMPONENTS OF *Vipera berus* VENOM SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS*

Unreduced venom			Reduced venom		
Band	Mol. wt	%	Band	Mol. wt	%
1	125,800	8.2	1	72,600	9.9
2	114,900	3.6	2	62,000	19.1
3	106,100	2.0	3	37,000	13.0
4	98,500	2.8	4	31.400	5.8
5	68,000	13.1	5	26,300	5.6
6	57,200	13.4	6	18,000	4.9
7	54,000	1.9	7	16,000	7.7
8	32,800	19.5	8	15,600	11.7
9	25,700	7.7	9	15,100	15.6
10	23,800	18.9	10	14,200	6.7
11	15,200	9.0			

*Data corresponding to the electrophoretic separations shown in Fig. 4A.

0.3 μg venom/well, or more, by the indirect hemolytic assay, and the minimum hemolytic dose (venom dose causing a lytic halo of 15 mm diameter) was approximately 1.5 μg. The fibrinolytic activity was low, since even with 100 μg venom/well, a lytic halo of 10 mm diameter in the fibrin clot (minimum fibrinolytic dose) was not achieved. This venom did not show coagulant activity on human plasma *in vitro*, even when as much as 200 μg venom was tested. However, a moderate, but significant anticoagulant activity *in vitro* was observed. Using 20 mg of venom, the plasma recalcification time was 19.8 ± 6.8 min, in contrast to 7.0 ± 0.4 min for control. At a dose of 200 μg of venom, plasma was unclottable during a 2 hr observation period.

Cross-reactivity with crotalid venoms

The titer of anti-*V. berus* antibodies detected in the serum from a hyperimmunized rabbit using the enzyme-immunoassay was approximately 1 : 40,000 (Fig. 3A). Using this serum, significant cross-reactivities with several crotalid venoms were obtained, ranging from 62% for the venom of *Bothrops asper* (Atlantic region of Costa Rica) to 18%, in the case of *Crotalus durissus durissus* venom (Fig. 3B).

Electrophoretic and immunoblotting analyses

The SDS-PAGE patterns of *V. berus* venom (unreduced and reduced) are shown in Fig. 4A. The mol.wt and relative proportions of the components resolved are presented in Table 1. Immunoblots indicated that all venoms bands resolved by SDS-PAGE and visible by protein stain were recognized by antibodies present in the rabbit antiserum (Fig. 4B). There was an unexpected reaction of normal rabbit serum antibodies with the venom band of 68,000 mol. wt (Fig. 4B). The experiment was repeated using normal rabbit sera from two different animals, with the same result.

DISCUSSION

The venom of *V. berus* was highly lethal to mice, with an LD_{50} (i.p.) of 0.86 μg/g body weight. This estimation is very similar to that report by RUSSELL (1967) and by MINTON (reviewed by TU, 1977) of 0.80 μg/g. This lethal potency, similar to that of most *Vipera* venoms (reviewed by TU, 1977), was higher than that of all Central American crotalid venoms, with the exception of *Crotalus durissus durissus* (BOLAÑOS, 1972). The lethal potency of *V. berus* venom was comparable to that of neurotoxic Elapidae venoms, such as that of *Micrurus* spp. (BOLAÑOS, 1972). However, these estimations cannot be extrapolated to the human, in which a relatively low fatality rate is observed (HABERMEHL, 1981). Nevertheless, severe cases of envenomation due to *V. berus* may occur (HABERMEHL, 1981; STAHEL *et al.*, 1985; CEDERHOLM and LENNMARKEN, 1987; GONZALEZ, 1991).

Experimentally, *V. berus* venom induced significant local tissue-damaging effects, including edema, hemorrhage, and myonecrosis. The local edema formation was characterized by rapid onset, reaching a maximum of 0.5–1 hr. After this early peak, the persistence of edema was related to venom dose. With the higher doses tested, considerable swelling of the footpad persisted for more than 2 days. This edema pattern was very similar to that recently described for *Bothrops asper* venom (LOMONTE *et al.*, 1993*a*).

A potent hemorrhagic action of the venom was observed, both histologically and by the quantitative macroscopic skin test. The estimated potency (3.2 μg minimum hemorrhagic dose) was slightly lower than that of most crotalid venoms of the genus *Bothrops* studied (GUTIÉRREZ *et al.*, 1985). A hemorrhagic protein has been isolated from *V. berus* venom and biochemically characterized as a zinc-containing metalloprotease of 56,300 mol. wt, with proteolytic activity on casein and fibrinogen (SAMUEL and SIIGUR, 1990).

Myonecrosis has not previously been described as an effect of *V. berus* venom. These results showed a moderate, but clear myotoxic action of this venom at the site of injection. Skeletal muscle damage was corroborated histologically, as well as inferred by the biochemical tests. There was a dose-dependent increase of plasma CK activity, and decrease of muscle residual MTT-reducing activity, with good correlation between these two parameters. The increase in plasma CK activity obtained was approximately half that induced by the same dose of a known myotoxic venom such as *B. asper* (data not shown). Myotoxic activity in venoms from several *Vipera* species, although not in that of *V. berus* from Czechoslovakia, was recently reported by MEBS and LANGELUDECCKE (1992), using a qualitative histological technique. Geographical variations may account for the difference with the present study. The lack of reports on the myotoxic action of this venom may be due to the low amounts of venom injected following human snakebites; however, it could also have been missed in clinical evaluations.

The proteolytic activity of the venom, using casein as substrate, was 59.2 U/mg. This value is lower than that of most crotalid venoms, tested under the same conditions (LOMONTE and GUTIÉRREZ, 1983). Despite the fact that hemorrhagic factors found in snake venoms are proteases (BJARNASON and FOX, 1988–89), studies performed with several venoms indicate that hemorrhagic and proteolytic activities do not correlate (GUTIÉRREZ *et al.*, 1985), probably because hemorrhagic toxins are metalloproteases which act only on highly specific substrates (BJARNASON and FOX, 1988–89). Present results provide further support to this hypothesis, since *V. berus* venom was highly hemorrhagic but only slightly proteolytic. However, protease inhibitors have been found in this venom (SIIGUR *et al.*, 1988) in addition to proteases (SIIGUR *et al.*, 1979).

The phospholipase A_2 activity of *V. berus* venom has been described previously (BÜCHERL *et al.*, 1968; SIIGUR *et al.*, 1979; TAN and PONNUDURAI, 1990). BOFFA *et al.*, (1976) purified a phospholipase A_2 occurring as a 14,000 mol. wt monomer or 39,000 mol. wt trimer, with anticoagulant activity. Both phospholipase and anticoagulant activities were confirmed in this study of the whole venom. Very low fibrinolytic activity was observed, which agrees with the low proteolytic activity. The venom lacked the ability to directly activate coagulation of human plasma *in vitro*, even at high concentrations (700 μg/ml).

Immunoblotting using *V. berus* antiserum showed that all components resolved by SDS-gel electrophoresis were recognized by antibodies, and therefore immunogenic. Since viperids and crotalids probably evolved from common ancestors (PHELPS, 1981), some degree of antigenic conservation is to be expected. Indeed, using an enzyme-immunoassay, variable degrees of cross-reactivity were shown between antigens of *V. berus* and of crotalid venoms. Interestingly, the venoms with high cross-reactivity with *V. berus* venom in the enzyme-immunoassay, were those with high hemorrhage activities (*B. asper* Atlantic type, *B. picadoi*). MANDELBAUM *et al.* (1989) have reported cross-neutralization of *V. lebetina* venom hemorrhagic activity by antisera raised against purified hemorrhagic toxins from *B. jararaca* and *B. neuwiedi*. Recent studies by BORKOW *et al.* (1993) indicate antigenic cross-reactivity between purified hemorrhagic factors from *B. asper* and *Vipera*

palestinae. MEBS *et al.* (1988) have also documented considerable cross-neutralizations of hemorrhagic activities of venoms from crotalid and viperid snakes, using a variety of antivenoms. Thus, at least part of the cross-reactivity observed in this work is probably due to common antigenic structures on hemorrhagic toxins.

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