TOXICOLOGY AND APPLIED PHARMACOLOGY **147,** 442–447 (1997)
ARTICLE NO. TO978285

Inhibition of Toxic Activities of *Bothrops asper* Venom and Other Crotalid Snake Venoms by a Novel Neutralizing Mixture

Gadi Borkow,* Jose Maria Gutierrez,† and Michael Ovadia*

**Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel; and †Instituto Clodomiro Picado, Facultad de Microbiologia, Universidad de Costa Rica, Costa Rica*

Received April 17, 1997; accepted August 22, 1997

Inhibition of Toxic Activities of *Bothrops asper* Venom and Other Crotalid Snake Venoms by a Novel Neutralizing Mixture. Borkow, G., Gutierrez, J. M., and Ovadia, M. (1997). *Toxicol. Appl. Pharmacol.* **147, 442–447.**

The majority of snake bites in Central America are caused by *Bothrops asper,* whose venom induce complex local effects such as myonecrosis, edema and especially hemorrhage. These effects are only partially neutralized by the clinically used antivenom, even when administered rapidly after envenomation. Recently we screened 49 substances for antihemorrhagic activity and found that a mixture composed of $CaNa_2$ EDTA, a *B. asper* serum fraction (natural antidote), and the currently used horse polyvalent antiserum is highly effective in the neutralization of local and systemic hemorrhage developing after *B. asper* envenomation (Borkow *et al., Toxicon* 35, 865–877, 1997). In the present study we screened the best six antihemorrhagic compounds for their capacity to neutralize the lethal activity in mice and the proteolytic, hemolytic, and antiattachment activities *in vitro* of the venom. The compounds tested included the currently used horse antivenom, rabbit antiserum against whole *B. asper* venom or against heated venom, *B. asper* and *Natrix tessellata* serum fractions, and $CaNa_2$ EDTA. The constituents of the antihemorrhagic mixture were also the best inhibitors of the other examined toxic activities. Importantly, the mixture effectively neutralized toxic activities of an additional nine venoms from snakes abundant in Central America. This work suggests that the polyvalent antivenom used in Central America could be enriched with a *B. asper* serum fraction producing a more effective antivenom. In addition, the local application of $CaNa_2$ EDTA to neutralize hemorrhagic toxins, immediately after a snake bite, may provide rapid inhibition of local damage caused by the venoms.

In Latin America bites by snakes of the genus *Bothrops*, which belong to the crotalid family, have the greatest medical importance, as they are responsible for more human morbidity than any other group of venomous snakes (Rosenfeld, 1971). Among them the most dangerous species in Central America is *Bothrops asper* (Bolaños, 1982, 1984; Campbell and Lamar, 1989). After envenomation, striking alterations at the site of injection may be caused, due to a complex series of local effects including hemorrhage, myonecrosis, and edema. In severe cases, the tissue damage may lead to serious sequelae such as permanent tissue loss, disability, and sometimes amputation (Campbell and Lamar, 1989; Gutierrez and Lomonte, 1989; Kerrigan, 1991; Nishioka and Silveira, 1992).

In Central America, the polyvalent antivenom produced in horses at the Instituto Clodomiro Picado in Costa Rica is currently being used as the basic therapeutic device for the treatment of crotalid envenomation. Mortality as a consequence of crotalid snake bites has been reduced with the use of this antivenom. However, both clinical and experimental data indicate that it is only partially effective against the local activities of the venoms, such as edema-forming, myotoxic, and, especially, hemorrhagic activities (Gutierrez *et al.*, 1981). As with other antivenoms, these effects are poorly neutralized, even when the antivenom is administered rapidly after envenomation (Reid *et al.*, 1963; McCollough *et al.*, 1970; Warrel *et al.*, 1976; Lomonte, 1985; Gutierrez *et al.*, 1985). It is therefore important to search for other compounds which can effectively neutralize the hemorrhagic and other harmful activities of crotalid venoms. The relevance of this point was stressed by the World Health Organization (WHO, 1981). Some mammals and many snakes have developed natural antidotes in their plasma that neutralize various snake toxins with much higher neutralization capacity compared to specific immunoglobulins (Ovadia *et al.*, 1976; Ovadia and Kochva, 1977; Domont *et al.*, 1991; Weissenberg *et al.*, 1991). Three types of materials are therefore good candidates to neutralize harmful activities of venoms: antivenoms obtained after immunization, natural products which may be found in animals resistant to snake venoms, and synthetic compounds.

As one of the most dramatic consequences of *Bothrops asper* envenomation is local and systemic hemorrhage (Moreira *et al.*, 1992), we recently screened five antivenoms, 25 snake and mammalian sera, and 19 synthetic inhibitors for their capacity to neutralize the hemorrhagic activities of the venom of *B. asper* (Borkow *et al.*, 1997). We recommended the *in situ* administration and intravenous injections of a novel neutralizing mixture composed of $CaNa_2$–ethylenediaminetetraacetic acid ($CaNa_2$ EDTA), a *B. asper* serum fraction (natural

0041-008X/97 $25.00

antidote fraction), and the currently used horse polyvalent antiserum. Although this improvement was experimentally verified only in mice, it provided the impetus for this study which aimed to examine the capacity of the most effective antihemorrhagic substances to neutralize other toxic activities of *B. asper* venom as well as venoms of nine additional crotalid snakes distributed in Central America.

METHODS

Materials. All venoms were obtained from snakes kept at Instituto Clodomiro Picado, University of Costa Rica, and were pooled, lyophilized, and stored at −20°C until used. The antivenom against the venoms of *B. asper, Crotalus durissus durissus,* and *Lachesis muta stenophrys,* currently used in Central America, was prepared as previously described (Bolaños and Cerdas, 1980). Antisera against *B. asper* venom or venom heated for 30 min at 60°C were prepared in rabbits by injecting increasing amounts of the mixtures subcutaneously at 1-month intervals. Freund's complete and incomplete adjuvants were used in the immunization. *Bothrops asper* and *Natrix tessellata* 40–60% and 35–55% saturated ammonium sulfate sera fractions (natural antidotes) were prepared as described (Borkow *et al.,* 1994a, 1995a). Transformed murine endothelial cells of capillary origin, established by transformation with the polyoma virus middle oncogene (Bussolino *et al.,* 1992) and primary bovine aortic endothelial cells isolated as described by Schwartz (1978), were grown in Dulbecco's modified Eagle's media supplemented with 10% newborn calf serum, 1% L-glutamine, and 1% penicillin. For experimentation the cells were grown in 96-well uncoated plastic plates (in 0.2 ml medium).

Determination of antihemorrhagic activity. The neutralization of the venom's hemorrhagic activities was determined in two different body sites: in the skin and in the gastrocnemius muscle. The minimal hemorrhagic dose (MHD; MHD is defined as the amount of venom that produces a hemorrhagic spot of about 1 cm in diameter after 2 hr in the skin) for each venom was determined as previously described (Ovadia, 1978). In a separate set of experiments, 40 MHD of *B. asper* venom were mixed with the inhibitors in 1 ml PBS and, immediately, 0.05 ml containing 2 MHD was injected into the dorsolateral aspect of the right gastrocnemius of four mice weighing 25–30 g. Two hours after injection, the mice were killed by cervical dislocation and the injected muscle was removed from the leg. The quantitation of hemorrhage was carried out according to Ownby *et al.* (1984).

Neutralization of venom effect on endothelial cells. The effect of the venom on transformed murine endothelial cells and on primary bovine aortic endothelial cells was examined as described previously (Borkow *et al.,* 1994b, 1995b). Several crotalid and viperid venoms, among them *B. asper* venom, cause cultured cells to round up and detach from the substrate, leaving them viable even after 6 hr of incubation with the venom (Chaim-Matyas and Ovadia, 1987; Borkow *et al.,* 1994b). Twenty microliters PBS containing 50 μg venom and each of the various substances tested were added to the medium in which 10^5 endothelial cells were cultured (0.2 ml final volume). The number of floating cells in supernatant aliquots, withdrawn at various time intervals after the venom addition, was determined by using a cytometer and an inverted microscope. One hundred micrograms of each of the other examined venoms in 200 μl PBS was incubated with or without the neutralizing mixture for 1 hr at 37°C. Then 50 μl of each mixture (25 μg venom) was added to wells containing 10^5 endothelial cells. The number of floating cells was determined as described above.

Hemolysis assays. The hemolytic assay was carried out according to Quiros *et al.* (1992). Fresh human blood was obtained from a blood bank and stored at 4°C. Prior to use, erythrocytes were washed twice with PBS, pH 7.2, and diluted to 2% (v/v) with 0.5% albumin and 10 mM $CaCl_2$ in the same buffer. Venom (2.5 mg) was incubated with the different potential inhibitors in 0.1 ml PBS for 1 hr at 37°C, followed by addition of 0.9 ml 2% (v/v) washed erythrocytes and the mixtures were incubated for an additional 30 min at 37°C. The tubes were then centrifuged at 1200*g* before hemolysis was determined by measuring the absorbance of the supernatant at 540 nm; 100% hemolysis was determined by measuring the absorbance of supernatants from erythrocytes incubated with distilled water. The inhibitors without the venom were incubated with the cells as controls.

General proteolytic activity. An aliquot of each venom (90 μl containing 12 μg of venom) was incubated with 10 μl of the neutralizing mixture or PBS for 1 hr at 37°C. Azocollase and gelatinase activities were examined as previously described (Ovadia, 1978; Borkow *et al.,* 1993).

Cross reactivity. The cross reactivity between the various venoms and the neutralizing mixture was examined by immunodiffusion tests in 1% agar gels according to the procedure of Ouchterlony, (1967).

RESULTS

The six best antihemorrhagic materials found after screening 49 substances against the venom of *B. asper* (Borkow *et al.,* 1997) were examined during the first part of this study for their capabilities to neutralize other important toxic activities (such as lethal, hemolytic, proteolytic, and cell-detachment) of *B. asper* venom. Of the six tested materials, the most potent antihemorrhagic substance, the *B. asper* serum fraction, was also the most efficient inhibitor of all other toxic venom activities tested. Table 1 shows that the natural antidote (*B. asper* serum fraction) was 3–7 times more effective than the clinically used horse polyvalent antivenom against the hemorrhage, proteolytic, and hemolytic activities of *B. asper* venom. Moreover, the high neutralization capacity of *B. asper* serum fraction was also proved against two additional activities of the venom: the lethal and cell-detachment activities (Figs. 1 and 2). Another natural antidote, the fraction from the serum of the nonpoisonous snake *Natrix tessellata,* also has a higher neutralization capacity than the horse antivenom. In addition, isotonic concentration of the synthetic material $CaNa_2$ EDTA (0.15 M in 0.01 M phosphate buffer, pH 7.2) effectively neutralized the hemorrhagic, proteolytic, and cell-detachment activities of the venom and showed partial activity against the hemolytic activity of the venom (Fig. 2, Table 1). However, $CaNa_2$ EDTA showed only temporal activity in preventing cell detachment in the cell culture system. After more than 1 hr the venom regained cell-detachment activity similar to levels of the control venom (see Discussion).

A mixture of the *B. asper* natural antidote fraction and $CaNa_2$ EDTA with the horse polyvalent antivenom has been shown to be extremely effective in the neutralization of the hemorrhagic activity of the venom (Borkow *et al.,* 1997). Therefore, in subsequent experiments, we examined the capacity of this antihemorrhagic mixture, composed of 5 mg/ml of the horse polyvalent antivenom and 5 mg/ml of a *B. asper* serum fraction in 0.15 M $CaNa_2$ EDTA, to neutralize the proteolytic (azocollase, gelatinase), cell-detachment, and hemolytic activities of *B. asper* venom. Table 2 shows that high dilution of the neutralizing mixture (up to 1:80) neutralized the hemorrhagic activity of *B. asper* venom effectively. The undiluted mixture could also inhibit the cell-detachment, azocollase, and gelatinase activities of the venom. Furthermore, as cross reactivity be-

TABLE 1
Inhibition of Hemorrhagic, Proteolytic, and Hemolytic Activities of *Bothrops asper* Venom by Various Agents

Activity:	Hemorrhagic[a]		Proteolytic[b]	Hemolytic[c]
Venom dose (μg):	20 (skin)	20 (muscle)	50	5000
Horse polyvalent antivenom	5	60	6	7.4
Bothrops asper natural antidote fraction	5	20	0.8	1.0
Natrix tessellata natural antidote fraction	30	30	4	1.2
Rabbit antiserum against whole venom	30	32	12	2.5
Rabbit antiserum against heated venom	30	40	40	2.5
$CaNa_2$ EDTA (mM)	1.5	150	100	200

Note. The capacity of various substances to neutralize toxic activities of *B. asper* venom was examined.

[a] Hemorrhagic activity: The minimum amount (μg in 50 μl) of each substance which completely neutralized 2 MHD (20 μg) injected into the skin or into the muscle is given.

[b] Proteolytic activity: 50 μg of *B. asper* venom in 0.1 ml PBS was incubated for 1 hr at 37°C with the various compounds. Each mixture was then examined for proteolytic activity on Azocoll. The minimum amount of substance (μg) required to abolish the venom proteolytic activity is shown.

[c] Hemolytic activity: *B. asper* venom (5 mg in 0.1 ml PBS) was incubated for 1 hr at 37°C with the various substances and an hemolysis assay was carried out. The minimum amount of compound (μg) which abolished the venom hemolytic activity is shown.

tween related venoms and toxins is well known (Assakura *et al.*, 1986; Mandelbaum and Assakura, 1988; Mandelbaum *et al.*, 1989), and as immunodiffusion tests showed that this mixture cross reacts clearly with 9 of 11 additional examined venoms of crotalid snakes abundant in Central America (data not shown), this mixture was also examined for its capacity to neutralize toxic activities of those 9 venoms. Indeed, Table 2 shows that the mixture was highly effective in neutralizing the hemorrhagic, cell-detachment, and proteolytic activities not only of *B. asper* venom but of all examined venoms.

DISCUSSION

The basic therapeutic device used in Central America for snake bite envenomation, generally caused by *B. asper* (Bola-

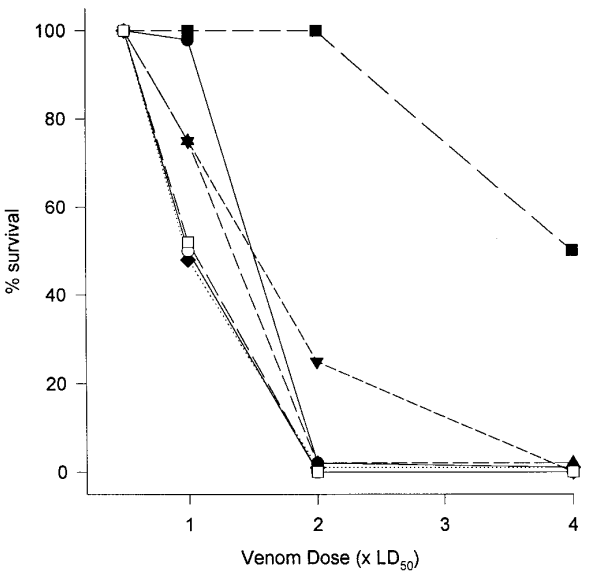

FIG. 1. Inhibition of *Bothrops asper* venom lethal activity. One milligram of horse polyvalent antivenom (●), *Bothrops asper* serum fraction (■), *Natrix tessellata* serum fraction (▲), rabbit antiserum against whole venom (▼), rabbit antiserum against heated venom (◆), 0.15 M $CaNa_2$ EDTA (□) or PBS (○) as control was incubated with 0.25, 0.5, or 1 mg of the whole venom in 2.5 ml PBS for 1 hr at 37°C; 0.5 ml of each mixture (1, 2, and 4 LD50, respectively) was then injected into the caudal vein of four mice weighing 22–25 g. The number of surviving mice was scored after 24 hr.

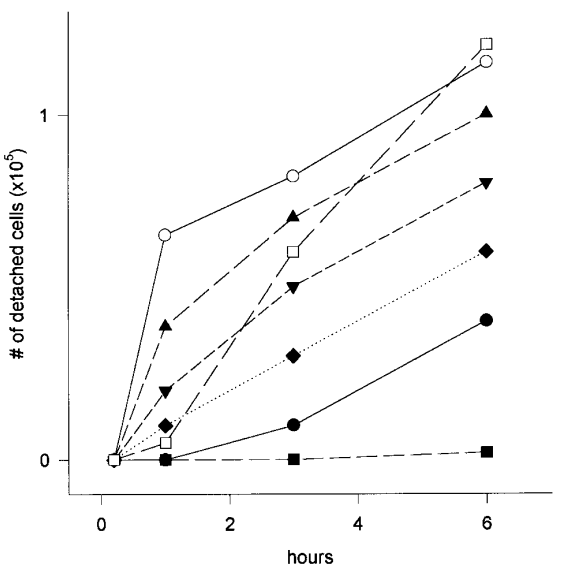

FIG. 2. Inhibition of *Bothrops asper* venom cell-detachment activity. Fifty microliters containing 50 μg *Bothrops asper* venom was mixed with 10 μg horse polyvalent antivenom (●), 10 μg *B. asper* serum fraction (■), 100 μg *Natrix tessellata* serum fraction (▲), 100 μg rabbit antiserum against whole venom (▼), 100 μg rabbit antiserum against heated venom (◆), 0.15 M $CaNa_2$ EDTA (□) in 0.01 M phosphate buffer, pH 7.2, or PBS (○) as control, and immediately added to 150 μl medium in which the 10^5 murine endothelial cells were cultured. The number of floating cells was determined at various time intervals after the venom addition. Similar results were obtained with bovine endothelial cells.

TABLE 2
Neutralization of Toxic Activities of Various Crotalid Snake Venoms by the Neutralizing Mixture

VENOM	Hemorrhagic[a]		Cell-detachment[b]	Azocollase (% inhibition)	Gelatinase
	MHD (μg)	Dilution			
Bothrops asper	10	1:80	100	83 ± 8	100
Bothriechis schlegelii	5	1:12	100	87 ± 4	100
Bothriechis lateralis	10	1:80	100	90 ± 4	100
Bothriechis nigroviridis	>50	—	100	80 ± 7	100
Porthidium godmani	5	1:50	100	92 ± 6	100
Porthidium ophyromegas	10	1:30	100	92 ± 5	100
Porthidium nasutus	15	1:15	100	93 ± 6	100
Atropoides picadoi	2	1:80	100	85 ± 10	100
Crotalus durissus durissus	40	1:15	100	93 ± 6	100
Lachesis muta stenophrys	12	1:2	100	88 ± 6	100

Note. The neutralizing mixture was examined for its capacity to neutralize various toxic venom activities of crotalid snakes abundant in Central America.

[a] Hemorrhagic activity: 400 μl of PBS containing 8 MHD of each venom was incubated with various dilutions of the neutralizing mixture (5 mg/ml of serum fraction and 5 mg/ml horse polyvalent antivenom in 0.15 M $CaNa_2$ EDTA) for 30 min at 37°C, and 100 μl containing 2 MHD of each venom in the mixture was injected into the back skin of three white mice. The highest dilution of the mixture which neutralized the hemorrhagic activity is indicated.

[b] Cell-detachment and proteolytic activities: the effect of the neutralizing mixture on the cell-detachment and proteolytic activities of 25 μg/ml of each venom were examined as described under Methods. Venoms were incubated with the undiluted mixture for 1 hr at 37°C. Each experiment was done in triplicate. The percentage neutralization is indicated. The cell detachment results were obtained with both murine and bovine endothelial cell lines.

ños, 1982, 1984; Campbell and Lamar, 1989), is a horse polyvalent antivenom prepared by using an antigenic mixture composed of the venoms of *B. asper*, *C. durissus durissus*, and *L. muta stenophrys*. Although the mortality resulting from *Bothrops* envenomation was reduced by this antivenom, localized tissue damage was only partially inhibited (Gutierrez *et al.*, 1981).

The hemorrhagic activity is one of the most significant pathophysiological effects induced by *B. asper* venom (Moreira *et al.*, 1992), as well as by most crotalid and viperid snake venoms (Ovadia, 1978; Ownby *et al.*, 1978; Ohsaka, 1979). For this reason, we recently examined the capacity of this antivenom, as well as other antivenoms, snake sera, natural antidote fractions, and synthetic inhibitors, to neutralize the hemorrhagic activity of *B. asper* venom. We found that a mixture of the currently used polyvalent antivenom, the *B. asper* serum fraction, and $CaNa_2$ EDTA neutralized the hemorrhagic activity of the venom very effectively, especially when administered *in situ* (Borkow *et al.*, 1997).

In the present study we examined the capacity of the six best antihemorrhagic materials, including the horse polyvalent antivenom, to neutralize other toxic activities of *B. asper* venom. On one hand, we conclude that the most efficient neutralizing factor was the *B. asper* serum fraction. It neutralized all examined activities of the venom: the lethal, proteolytic, hemolytic, cell-detachment, and hemorrhagic activities. Its capacity to neutralize the lethal activity of the whole venom was very pronounced compared to the other inhibitors. The general neutralizing capacities of this fraction against *B. asper* venom were higher than the currently used polyvalent antivenom. Similarly, 20- and 6-fold higher neutralization capacities of natural antidotes were found in *Vipera palestinae* and *Crotalus atrox* sera, respectively, as compared to commercial polyvalent antivenoms produced for clinical use in Israel and North America (Ovadia and Kochva, 1977; Weissenberg *et al.*, 1991). On the other hand, $CaNa_2$ EDTA, which is currently used as a color retention and flavoring agent in foods and as a chelating agent for lead poisoning (Merck Index, 1989), is the most stable material tested with almost unlimited shelf-life and is a very effective antihemorrhagic agent (Table 1; Borkow *et al.*, 1997). Because of its capacity to effectively neutralize the hemorrhagic toxins of the venom at isotonic concentrations, $CaNa_2$ EDTA was recommended for use as a component of an antihemorrhagic mixture (Borkow *et al.*, 1997). Moreover, this work shows that this synthetic material is also an effective inhibitor of other venom activities (Table 1 and Fig. 2). The temporal activity of $CaNa_2$ EDTA in preventing cell detachment can be explained by the presence of phosphate in the cell culture medium which can precipitate the calcium from the solution.

We suggest that an immediate *in situ* administration of 0.15 M $CaNa_2$ EDTA, with or without the serum fraction, to counterattack the local hemorrhagic activity of the crotalid venoms, followed by intravenous administration of the polyvalent antivenom in the hospital, may prove to be a beneficial procedure. The stability of the nontoxic $CaNa_2$ EDTA compound reinforces the possibility of using this substance as a local immediate first aid procedure in the field, by injecting the inhibitor into the site of the snake bite. The immediate local injection of $CaNa_2$ EDTA could reduce the damage of the envenomation significantly by neutralizing the hemorrhagic toxins rapidly *in situ* and by preventing the spread of the hemorrhagic effects

and other toxic agents. This hypothesis is currently being evaluated in our laboratories. Current work also indicates the possible application of $CaNa_2$ EDTA in horse immunization for antivenom production (manuscript in preparation).

A mixture of the horse antivenom, the *B. asper* natural antidote fraction, and $CaNa_2$ EDTA was the most effective inhibitor of the hemorrhagic activity of *B. asper* venom (Borkow *et al.*, 1997). The cross reactivity of the polyvalent antivenom with nine other crotalid venoms abundant in Central America led us to examine the capacity of this mixture to counteract nine additional crotalid venoms.

This work shows that enrichment of the clinically used antivenom with a *B. asper* serum fraction not only enhances the neutralization of *B. asper* envenomation, but also can be effective in the neutralization of other crotalid snake envenomations occurring in Central America. It also increases the plausibility of enriching other existing antivenoms with the natural *B. asper* serum fraction, and opens the gate for their enrichment with additional natural antidote fractions from sera of other medically important snakes. Further work should therefore examine the enrichment of the clinically used antivenoms with such natural antidote fractions that can be isolated or produced.

ACKNOWLEDGMENTS

This study was supported by AID Grant No. DHR-5544-6-00-1064-00. We thank Dr. John McCormick for his helpful comments.

REFERENCES

Assakura, M. T., Reichl, A. P., and Mandelbaum, F. R. (1986). Comparison of immunological, biochemical and biophysical properties of three hemorrhagic factors isolated from the venom of *Bothrops jararaca (jararaca). Toxicon* **24,** 943–946.

Bolanos, R., and Cerdas, L. (1980). Produccion y control de sueros antiofidicos en Costa Rica. *Boletin de la Oficina Sanitaria Panamericana* **88,** 184–196.

Bolanos, R. (1982). Las serpientes venenosas de Centro America y el problema del ofidismo. Primera parte. Aspectos zoologicos, epidemiologicos y biomedicos. *Revta Cont. Cienc. Med.* **3,** 165–184.

Bolanos, R. (1984). *Serpientes, Venenos y Ofidismo en Centroamerica.* Universidad de Costa Rica, San Jose. [Editorial]

Borkow, G., Gutierrez, J. M., and Ovadia, M. (1993). Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*. *Toxicon* **31,** 1137–50.

Borkow, G., Gutierrez, J. M., and Ovadia, M. (1994a). A potent antihemorrhagin from the serum of the non-poisonous water snake *Natrix tessellata:* Isolation, characterization and neutralization mechanism. *Biochim. Biophys. Acta* **1201,** 482–490.

Borkow, G., Lomonte, B., Gutierrez, J. M., and Ovadia, M. (1994b). Effect of various Viperidae and Crotalidae snake venoms on endothelial cells cultured *in vitro. Toxicon* **32,** 1689–1695.

Borkow, G., Gutierrez, J. M., and Ovadia, M. (1995a). Isolation, characterization and neutralization mechanism of an antihemorrhagic factor from the serum of *Bothrops asper*. *Biochim. Biophys. Acta* **1245,** 232–38.

Borkow, G., Gutierrez, J. M., and Ovadia, M. (1995b). *In vitro* activity of BaH1, the main hemorrhagic toxin of *Bothrops asper* snake venom on bovine endothelial cells. *Toxicon* **33,** 1387–1391.

Borkow, G., Gutierrez, J. M., and Ovadia, M. (1997). Inhibition of the hemorrhagic activity of *Bothrops asper* venom by a novel neutralizing mixture. *Toxicon* **35,** 865–877.

Bussolino, F., Guglielmetti, A., Ghigo, D., Percarmona, G., and Bosia, A. (1992). Protein kinase C activation in murine endothelioma cell lines containing middle T antigen stimulated by platelet-activating factor. *J. Lipid. Mediat.* **5,** 109–113.

Campbell, J. A., and Lamar, W. W. (1989). *The Venomous Reptiles of Latin America.* Comstock, London.

Chaim-Matyas, A., and Ovadia, M. (1987). Cytotoxic activity of various snake venoms on melanoma B16F10 and chondrosarcoma. *Life Sci.* **40,** 1601–1607.

Domont. G. B., Perales, J., and Moussatche, H. (1991). Natural anti-snake venom proteins. *Toxicon* **29,** 1183–1194.

Gutierrez, J. M., Chaves, F., Bolanos, R., Cerdas, L., Rojas, E., Arroyo, O., and Portilla, E. (1981). Neutralizacion de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon* **19,** 493–500.

Gutierrez, J. M., Gene, J. A., Rojas, G., and Odell, G. V. (1985). Neutralization of proteolytic and hemorrhagic activities of Costa Rican snake venoms by a polyvalent antivenom. *Toxicon* **23,** 887–893.

Gutierrez, J. M., and Lomonte, B. (1989). Local tissue damage induced by *Bothrops* snake venoms: A review. *Memorias Inst. Butantan* **51,** 211–223.

Kerrigan, K. R. (1991). Venomous snakebite in Eastern Ecuador. *Am. J. Trop. Med. Hyg.* **44,** 93–99.

Lomonte, B. (1985). Edema-forming activity of bushmaster (*Lachesis muta stenophrys*) and Central American rattlesnake (*Crotalus durissus durissus*) venoms and neutralization by a polyvalent antivenom. *Toxicon* **23,** 173–176.

Mandelbaum, F. R., and Assakura, M. T. (1988). Antigenic relationship of hemorrhagic factors and proteases isolated from the venoms of three species of *Bothrops* snakes. *Toxicon* **26,** 379–385.

Mandelbaum, F. R., Serrano, S. M. T., Sakurada, J. K., Rangel, H. A., and Assakura, M. T. (1989). Immunological comparison of hemorrhagic principles present in venoms of the Crotalinae and Viperinae subfamilies. *Toxicon* **27,** 169–177.

McCollough, N. C., and Gennaro, J. F. (1970). Treatment of venomous snake bite in the United States. *Clin. Toxicol.* **3,** 483–489.

Moreira, L., Gutierrez, J. M., Borkow, G., and Ovadia, M. (1992). Ultra structural alterations in mouse capillary blood vessels after experimental injection of venom from the snake *Bothrops asper* (terciopelo). *Exp. Mol. Pathol.* **57,** 124–133.

Nishioka, S. A., and Silveira, P. V. P. (1992). A clinical and epidemiologic study of 292 cases of lance-headed viper bite in a Brazilian teaching hospital. *Am. J. Trop. Med. Hyg.* **47,** 805–810.

Ohsaka, A. (1979). Hemorrhagic, necrotizing and edema-forming effects of snake venoms. In *Snake Venoms* (C. Y. Lee, Ed.), pp. 480–546. Springer-Verlag, Berlin/Heidelberg/New York.

Ouchterlony, O. (1967). Immunodiffusion. In *Handbook of Experimental Immunology* (D. M. Weir, Ed.), pp. 665–706. Davis, Philadelphia.

Ovadia, M., Moav, B., and Kochva, E. (1976). Factors in the blood serum of *Vipera palestinae* neutralizing toxic fractions of its venom. *Anim. Plant Microb. Toxins* **1,** 137–142.

Ovadia, M. (1978). Isolation and characterization of three hemorrhagic factors from the venom of *Vipera palestinae. Toxicon* **16,** 479–487.

Ovadia, M., and Kochva, E. (1977). Neutralization of Viperidae and Elapidae snake venoms by sera of different animals. *Toxicon* **15,** 541–547.

Ownby, C. L., Bjarnason, J., and Tu, A. T. (1978). Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom. *Am. J. Pathol.* **93,** 201–212.

Ownby, C. L., Colberg, T. R., and Odell, G. V. (1984). A new method for

quantitating hemorrhage induced by rattlesnake venoms: Ability of polyvalent antivenom to neutralize hemorrhagic activity. *Toxicon* **22,** 227–233.

Quiros, S., Gene, J. A., Gutierrez, J. M., and Thelestam, M. (1992). Effect of *Bothrops asper* (Fer-de-lance) snake venom on erythrocyte membrane. A comparative study. *Comp. Biochem. Physiol.* **101,** 433–436.

Reid, H. A., Thean, P. C., and Martin, W. J. (1963). Specific antivenene and prednisone in viper-bite poisoning: controlled trial. *Br. Med. J.* **11,** 1378–1180.

Rosenfeld, G. (1971). Symptomatology, pathology, and treatment of snake bites in South America. In *Venomous Animals and Their Venoms* (W. Buchler and E. Buckley, Eds), pp. 345–384. Academic Press, New York.

Schwartz, S. M. (1978). Selection and characterization of bovine aortic endothelial cells. *In Vitro* **14,** 966–970.

Warrel, D.A., Greenwood, B. M., Davidson, N. Mc. D., Ormerod, L. D., and Prentice, C. R. M. (1976). Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (*Naja nigricollis*). *Q. J. Med.* **45,** 1–22.

Weissenberg, S., Ovadia, M., Fleminger, G., and Kochva, E. (1991). Antihemorrhagic factors from the blood serum of the western diamondback rattlesnake *Crotalus atrox. Toxicon* **29,** 807–818.

World Health Organization (WHO) (1981). *Progress in the Characterization of Venoms and Standardization of Antivenoms.* WHO, Geneva. [Offset Publication No. 58]