PN ABX-634 C11-004
97722

FINAL REPORT - 1991-1995

Submitted to the office of:

The Science Advisor
U.S. Agency for International Development
Room 320 SA-18
Washington, D.C.
U.S.A. 20523-1818OC

Title of project: Isolation, Characterization and Neutralization of Hemorrhagic Toxins from the Venom of the Snake *Bothrops asper*

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Grant Number:	DHR-5544-6-00-1064-00
A.I.D. Grant Project Officer:	Dr. Miloslav Rechcigl
Project duration:	October 1991 - September 1995

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3) EXECUTIVE SUMMARY

The general purpose of this project was to study the dangerous venom hemorrhagins of *Bothrops asper* and their antidotes naturally found in the homologous serum and in the serum of the non-poisonous snake *Natrix tessellata* in order to analyze the possibility of improving the quality of the antivenom produced by the Institute Clodomiro Picado in the University of Costa Rica and the treatment of the patients.

Purification and characterization of three hemorrhagins found in the venom were already achieved during the first year of the project; the advance made during the second year was very important for understanding the mechanism of action of venom-induced hemorrhage. This highly relevant knowledge represented the scientific basis upon which new therapeutic strategies can be developed. Our findings indicate that BaH1, the main hemorrhagic toxin of *Bothrops asper* venom, induces hemorrhage by first digesting components that form the basement membrane surrounding and supporting capillary vessels. As a consequence of this enzymatic degradation, endothelial cells are indirectly affected, detaching themselves from the basement membrane and losing cytoplasmic material by blebbing and vesiculation. Thus, gaps appear within endothelial cells leading to hemorrhage. In addition, heat-inactivated hemorrhagins did not lose their antigenicity.

The third year was devoted to developing new strategies for counteracting the venom hemorrhagins of *Bothrops asper*.

Among 16 snake and 9 mammalian sera examined, the serum of *Bothrops asper* and that of the non-poisonous snake *Natrix tessellata* was the most potent of the neutralization of the hemorrhagic activity of *Bothrops asper* venom at molar ratio of 3:1 and 4:1, respectively. In addition, the rabbit antiserum produced after injecting heat-inactivated venom, neutralized the hemorrhagic toxins as efficiently as that produced after injecting the active venom, which establishes a new direction in producing antihemorrhagic antiserum. Of 26 synthetic compounds tested, only O-phenanthroline and EDTA salts inhibited the hemorrhagic activity at 1.5-10 mM, but $CaNa_2EDTA$ was the only non-toxic material. Four injection routes - intradermal, intramuscular, intraperitoneal and intravenous - failed to induce any evident toxicity. The effective antihemorrhagic materials were also screened for neutralization of lethal, proteolytic, cytostatic, and hemolytic activities of *Bothrops asper* venom. A fraction of *B. asper* serum was the most effective inhibitor of all tested toxic activities. The horse polyvalent antiserum effectively neutralized the cytostatic and hemolytic activities, while $CaNa_2EDTA$ was an efficient inhibitor of the proteolytic activity of the venom. Intravenous injections and *in situ* administration of these three materials ($CaNa_2EDTA$, *B. asper* serum, horse polyvalent antivenom) revealed that they were the most potent when administered as a mixture, especially in the *in situ* route.

It is, therefore, recommended to use the *in situ* administration and intravenous injections of this mixture for inhibition of the local and systemic hemorrhage and other toxic

effects developing after *Bothrops asper* envenomation.

The antihemorrhagins found in the sera of the non-poisonous water snake (*Natrix tessellata*) and the venomous Fer-de-lance (*Bothrops asper*) have different characteristics. Whereas that of the non-poisonous snake resembles macroglobulins, the antihemorrhagin of *Bothrops asper* has characteristics of α_2-globulins. However, the mechanism of the neutralization process is similar. Both purified antihemorrhagins form an inactive complex with the toxin.

The collaboration between the two laboratories was very important to achieve the results obtained and the complementarity was excellent. During the first two years the Israeli laboratory isolated and characterized the hemorrhagins from the venom of *B. asper* and studied the activity of the purified toxins on endothelial cells *in vitro* and on isolated extracellular matrix components. In addition, the natural antihemorrhagins from the sera of *N. tessellata* and *B. asper* were also purified and characterized in Tel-Aviv, while the Costa Rican lab worked out the details on the ultrastructural alterations occurring in the capillary blood vessels. Through this collaborative effort, it was possible to achieve a significant advance in the understanding of the mechanism of action of this hemorrhagic toxin both at microscopic and ultrastructural levels and the characteristics of the natural antihemorrhagins. This paved the way for the following studies of the third year in which the Israeli lab studied the neutralization of the toxin both *in vivo* and on cultured endothelial cells *in vitro* in order to find the best strategy for inhibition of the hemorrhagic activity of *B. asper* venom. The Costa Rican lab continued to work out the ultrastructural alterations occurring in the capillary blood vessels, the myonecrosis, and the immunological relationship between toxins of this venom. In addition, with the methodologies developed in Israel, the Costa Rican lab isolated and characterized another hemorrhagic metalloproteinase. Through this collaborative effort, it was possible to achieve a significant advance in the understanding of the mechanism of action of this hemorrhagic toxin both at microscopic and ultrastructural levels and the characteristics of the natural antihemorrhagins, which could pave the way for their future clinical usage.

The fourth year was used to expand the study and elucidate the inhibition of additional toxic activities of *B. asper* venom and of other crotalid snake venoms. Three antisera, two natural antidote fractions and one synthetic compound, which exhibited strong antihemorrhagic activity against the whole venom of *B. asper*, were screened for their capability to neutralize the cytostatic, proteolytic, hemolytic and lethal activities of *B. asper* venom. Among the examined compounds, the *B. asper* serum fraction was found to be the most efficient one. It effectively neutralized all the examined toxic effects of the venom, especially the lethal activity. The horse polyvalent antiserum neutralized effectively the cytostatic and hemolytic activities, while the non-toxic salt $CaNa_2EDTA$ neutralized very effectively the proteolytic activity of the venom. It is, therefore, suggested that a mixture of the three compounds be examined for its clinical potentiality in the neutralization of *B. asper* snake envenomation.

The mixture, which effectively neutralizes all major activities of *B. asper* venom was screened for its capability to neutralize the lethal, hemorrhagic, cytostatic and proteolytic activities of additional ten venoms of snakes that inhabit Latin America. The mixture contained 0.15M $CaNa_2EDTA$, 5 mg/ml of *B. asper* serum fraction and 5 mg/ml of a horse polyvalent antiserum. The hemorrhagic, cytostatic and proteolytic activities of all 10 examined crotalid venoms were effectively neutralized, and the lethal activity of the venoms was completely neutralized by double concentrations.

We feel that this successful project should not be wasted and the results achieved could be clinically applied. Therefore, on the basis of the above results we would like to apply for an additional AID grant in order to continue our fruitful collaboration for studying the feasibility of using these types of inhibitors in the clinical treatment of snake-bite envenomation in human patients in Central and Latin America. (Please see details in the three years proposed Future Work at the end of this report). A successful clinical application in Latin America could lead to a better treatment of snakebite patients in most developmental countries all over the world because they have similar problems to overcome.

4) RESEARCH OBJECTIVES

a. Addressed problem: According to the World Health Organization "injuries and death due to snake and spider bites, as well as scorpion stings, occur in most parts of the world and especially in the Tropics, where they may represent a major health problem" (W.H.O. Offset Publication No. 58, p.5, 1981). Medically important worldwide snakes include many vipers and pit vipers with strong hemorrhagic activities.

Envenomation due to snake bite constitutes a relevant public health problem in Costa Rica, as well as in the rest of Central America. The majority of snake bites in Central America are caused by pit vipers and among them, by the most dangerous species *B. asper*, locally known as "terciopelo" or "barba amarilla" (Bolanos, 1982). The venom of this species induces a complex series of local and systemic effects characterized by local hemorrhage, myonecrosis, edema and by systemic alterations such as hemorrhage, coagulation disorders, cardiovascular shock and acute renal failure (Gutierrez, 1995). Among these effects, local hemorrhage represents an especially difficult problem for several reasons: a) It develops extremely fast after venom injection; b) It is responsible for blood loss, with the consequent impairment of blood supply to muscle tissue; c) Damage to microvasculature by hemorrhagic toxins also affects the process of skeletal muscle regeneration, since an adequate blood supply is a basic requisite for muscle regeneration. Thus, hemorrhagic toxins are responsible not only for local and systemic hemorrhage and tissue damage, but also for impairment of muscle regeneration, resulting in sequelae such as tissue loss and amputation (Gutierrez and Lomonte, 1989; Moreira *et al.*, 1992)

The horse polyvalent antivenom produced at the Instituto Clodomiro Picado in Costa Rica is currently being used as the basic therapeutic device for the treatment of *Bothrops asper* and other snake bite envenomation in Central America. However, despite its high quality, both clinical and experimental data indicate that the local hemorrhagic activity and other local effects are very poorly neutralized by the antiserum (even when the antivenom is administered rapidly after envenomation, Gutierrez *et al.*, 1981). Moreover, there have been reports of severe allergy to horse serum contradicting the use of horse antivenom in some patients (Kornalik and Vorlova, 1990). For these reasons it is important to search for additional compounds which can effectively neutralize the local hemorrhagic and other harmful activities of crotalid and viperid snake venoms including *Bothrops asper* venom.

b. <u>Adapting to ongoing research</u>: The project fits into the research being carried out in different laboratories concerning the biochemistry of hemorrhagic toxins and their mechanism of action. It has been clearly established that hemorrhagic toxins present in snake venoms are zinc-dependent metalloproteinases (Bjarnason and Fox, 1994), with very specific hydrolytic activity against basal lamina protein components. On the other hand, several ultrastructural studies on the action of hemorrhagic toxins have demonstrated that they induce hemorrhage *per rhexis*, i.e. blood cells leave the vessels through lesions within endothelial cells (Ownby and Geren, 1987; Ownby *et al.*, 1978; 1990). However, it was not clear if these metalloproteinases are cytotoxic to endothelial cells. The way by which hemorrhagic toxins affect endothelial cells and capillary vessels was not resolved at all, and two hypotheses have been proposed: (a) that hemorrhagic toxins degrade basal lamina components as a consequence of which the capillary structure collapses; and (b) that hemorrhagic toxin are cytotoxic to endothelial cells thereby inducing the development of gaps and lesions through which extravasation occurs. One objective of this project was proposed to investigate these hypotheses concerning the hemorrhagic activity of *B. asper* venom.

On this basis, there was an urgent need by the University of Costa Rica to isolate and study hemorrhagic toxins from the venom of this dangerous species (*B. asper*) as there was no information on the amount, nor on the nature and mechanism of action of these toxins. Prior to this project, no significant progress has been made in the understanding of the pathogenesis of hemorrhage induced by the venom of *B. asper*, nor on the biochemical characteristics of the hemorrhagic toxins present in this venom; some data are available on other venoms (Bjarnason and Fox, 1994). Therefore, the Israeli laboratory isolated and characterized the most important hemorrhagic toxins from this venom, while Instituto Clodomiro Picado made every effort necessary to investigate the pathogenesis of this venom in experimental animals. Moreover, it was important to develop new and better therapeutic strategies to counteract these toxins.

In general, at least three types of materials are good candidates to fulfill the mission of venom neutralization:

1) The well known, currently used antivenoms obtained after immunization (immunoglobulins). Their advantage is based on the long experience with their clinical use.

2) Natural antidotes which may be found in the blood of resistant animals (non-immunoglobulins). Many natural antidotes have been purified in this laboratory during the last two decades and have been reviewed by Domont *et al.* (1991). An important advantage of these types of antidotes is their neutralization capacity. They are up to 20 times more efficient than the clinically used horse antivenom (Ovadia *et al.*, 1977; Ovadia, 1978). Plant extracts could be included in this type.

3) Synthetic compounds (various proteolytic inhibitors, especially inhibitors of metalloproteinases, etc.). One of the most important characteristics of these molecules is their small size, which may lead to several advantages: high stability, easy extravasation from the capillaries, quick diffusion in the poisoned tissue to reach the venom, and they do not induce development of antibodies against them.

In this project we decided to examine the strategy of using the advantages of the three types and to develop a mixture containing components of the three types.

We have examined various sera and synthetic compounds and compared their capacity to neutralize the hemorrhagic activity of *B. asper* venom.

The results of this project should have a direct impact on the issue of treatment of snakebite patients, since the improvement in neutralization of hemorrhagic toxins in mice resulted in less tissue damage and better skeletal muscle regeneration.

c. <u>Specific objectives of this project</u>: The overall aim of the project was to study the dangerous venom hemorrhagins of *B. asper* in order to develop new therapeutic strategies which may improve the clinical antivenom produced in Costa Rica.

The specific objectives were:

1. To purify one or more hemorrhagic toxins from the venom of the snake *B. asper* by using chromatographic techniques and to perform a biochemical and toxinological characterization of the toxins.

2. To study the pathogenesis of hemorrhage induced by the most relevant of these toxins in mouse tissue and to investigate the role of pure hemorrhagic toxins in the process of local tissue damage induced by this venom.

3. To analyze the possibility of improving the quality of the antivenom by adding purified hemorrhagic toxins or modified inactive hemorrhagins to the antigenic mixture used to produce antivenom in horses.

4. To study the ability of antidotes naturally found in serums of various animals to inhibit these hemorrhagins.

5. To examine the possibility of using natural and synthetic inhibitors as therapeutic agents mixed with the antivenom clinically used in Latin America.

6. To test the capacity of the improved antivenom mixture to neutralize the venoms of other species of *Bothrops*.

d. Innovative aspects: The project was innovated in the sense that it integrated three different perspectives around the subject of *B. asper* venom-induced hemorrhage: (a) the isolation and characterization of hemorrhagic toxins; (b) the pathogenesis of hemorrhage induced by crude venom and purified toxins; and (c) the isolation and characterization of molecules with potent anti-hemorrhagic activity. All these objectives were finally directed to the design of a potent anti-hemorrhagic mixture that could be of great value in the improvement of the therapy of snakebite envenomation in Latin America.

With regard to the mechanism of hemorrhagic toxins, it has been proposed that a variety of hemorrhagins isolated from several venoms induce microvessel damage by affecting the basal lamina that surrounds endothelial cells in capillaries (Bjarnason and Fox, 1989). However, very few studies have dealt with the correlation between morphological, biochemical and immunocytochemical changes in microvasculature. In this project, biochemical methods determined the effect of purified hemorrhagic toxins on the different components of the basal lamina and ultrastructural studies determined the pathological alterations of endothelial cells. The combination of these experimental strategies provided innovative results that greatly helped in determining the mechanism of microvessel damage.

Development of higher titers of antibodies against the snake venom hemorrhagins was carried out using inactive hemorrhagins obtained after heat treatment, to immunize animals for producing an improved antivenom. Inactive hemorrhagin was administered into the immunized animals in larger doses without affecting the animal. The successful result obtained by this project could be applied worldwide because antisera prepared against venoms containing strong hemorrhagic activity are needed worldwide.

Improvement of the quality of the antivenom was achieved by an addition of synthetic inhibitors of the hemorrhagins and by natural antidotes found in serum of the homologous snake *B. asper*.

5) METHODS AND RESULTS

The first part of the work focused on the isolation and characterization of the hemorrhagic toxins in the venom of the snake *B. asper* and studied the mechanism by which these toxins bring about their effects.

a. Isolation and characterization of hemorrhagins: Using the methods of gel filtration, ion-exchange chromatography, hydrophobic interaction and electrophoresis, three hemorrhagic toxins (BaH1, BH2, BH3) and a protease with a weak hemorrhagic activity (BaP1) were isolated from the venom of *B. asper* (Borkow *et al.*, 1993; Gutierrez *et al.*, 1995). These toxins are glycoproteins with similar pIs between 4.5-5.2, but they have different molecular weights. BaH1 is the largest molecule (64 kDa) and the most potent (MHD = 0.18

μg), whereas BH2, BH3 and BaP1 are 26, 55 and 24 kDa, respectively. The main contribution of BH2 and BH3 is not by the direct induction of hemorrhage (MHD = 2, 1.6 μg, respectively), but rather by a synergistic activity with the main hemorrhagic BaH1. No cross-reactivity was found between BaP1 and BaH1, neither was cross-neutralization was observed, indicating that these hemorrhagic toxins have distinct immunological entities (Rucavado *et al.*, 1995)

All hemorrhagins isolated from the venom of *B. asper* show proteolytic activity. The hemorrhagic activity is neutralized by metalloproteinase inhibitors such as EDTA, EGTA and ortho-phenanthroline, but not by other protease inhibitors. The hemorrhagic activity can be restored after the metalloproteinase inhibitors are removed by dialysis and ZnC1 is added, thus indicating the metalloproteinase nature of the toxins (Borkow *et al.*, 1993; Gutierrez *et al.*, 1995).

b. <u>Pathogenesis of the hemorrhage induced by the venom and purified toxins</u>: The venom of *B. asper* induces rapid and drastic pathological effects on capillaries, which are evident macroscopically as early as 5 minutes after venom injection.Two different patterns of endothelial cell alterations occur: in the majority of endothelial cells blebs appear and cytoplasmic projections pinch off to the lumen and the cells become very thin. In other endothelial cells the process of degeneration is characterized by swelling of the endoplasmic reticulum and of the cytosol. It is suggested that basal lamina components are the primary target of the hemorrhagic toxins, as in both degenerative patterns the basal lamina surrounding the capillaries becomes discontinued and disappears as the degenerative stages progress. Both patterns of pathological changes consequently lead to hemorrhage *per rhexis*, i.e., erythrocytes escape through gaps in damaged endothelial cells and not through widened intercellular junctions (Moreira *et al.*, 1992).

Ultrastructural changes induced in the capillaries after injection of BaH1 into the mouse gastrocnemius include the detachment of endothelial cells from the surrounding basal lamina 1 min after the toxin administration. Concomitantly, the number of pinocytotic vesicles decreases, blebs appear, cytoplasmic projections containing cytoplasmic material pinch off to the vascular lumen and the endothelial cells become thin. In capillaries undergoing more advanced degenerative stages gaps or brakes in endothelial cells appear through which erythrocytes escape to the extravascular space (Moreira *et al.*, 1994)

The main conclusion after the general screening of various crotalid and viperid snake venoms on normal bovine and transformed mutine cells cultured *in vitro* is that the examined venoms are not directly cytotoxic to the cells (Borkow *et al.*, 1994a). These venoms, when incubated *in vitro* with the endothelial cells, cause a loss of cell processes which lead to detachment of the cells from the substrate. The cells then become round and most of them aggregate. However, if the venoms are washed from the cells, even after 24 hr of incubation, the cells recuperate, regain their original morphology and proliferate, thus indicating that the effect of the crotalid and viperid venoms on most of the endothelial cells is reversible.

The activity of BaH1 on the endothelial cells is similar to that of the whole venom. Shortly after the addition of BaH1 to the medium, spaces appear between the cultured endothelial cells before any observable damage is observed. These spaces become broader as the incubation is prolonged and the concentration of the toxin is augmented.Prolonged activity of the hemorrhagic toxin causes the cells to become round, to detach from the substrate and float in the medium. However, trypan blue does not stain the cells even after 24 hr of incubation of BaH1 with the cells, indicating that BaH1 is not directly cytotoxic to the cells. Moreover, recultivation of these cells in fresh medium is possible after washing the toxin, even when they were incubated with BaH1 for 24 hr (Borkow *et al.*, 1995). These results are further sustained by a quantitative cytotoxicity assay based on the release of lactic dehydrogenase, which indicates that BaH, despite its potent hemorrhagic activity, does not exert direct cytolytic activity on transformed murine endothelial cells, even at concentrations as high as 65 μg/ml (Lomonte *et al.*, 1994).

The hypothesis that the initial effect of *B. asper* venom and BaH1 is not directed toward the cells but rather on the extracellular components, and that endothelial cell morphological alterations occur as a consequence of the degradation of the basement membrane without any major effect on the viability of the cells, is further validated by the demonstration of the capability of the hemorrhagic toxin BaH1 to degrade a variety of basement membrane components such as Collagen IV, laminin and fibronectin. This activity is inhibited by metalloproteinase inhibitors.

c. <u>Synthetic compounds as inhibitors</u>: Of 19 synthetic compounds tested for their antihemorrhagic capabilities, only metalloproteinase inhibitors, such as EDTA and O-phenanthroline, inhibit the hemorrhagic activity of the crude venom and the purified hemorrhagins at concentrations of 0.5-10 mM. However, only $CaNa_2EDTA$ is non-toxic, even when 0.5 ml of 0.15M are injected into the caudal vein of each white mouse weighing 20-25 gr (Borkow *et al.*, submitted a).

Among various synthetic compounds examined [capable of inhibiting various proteolytic activities] only the metalloproteinase inhibitors including the EDTA salts and 0-phenanthroline inhibited the hemorrhagic activity of the crude venom. The most potent compound was Na_4EDTA which abolished the hemorrhagic activity at the low concentration of 0.5 mM. Fortunately, one of the effective materials, $CaNa_2EDTA$, was not toxic *in vivo*. A later search in the Merck Index revealed that this compound is used as a flavoring and color retention agent in foods and a chelating agent for lead poisoning in animals (Merck Index. 1989). As we were aware of the toxicity of EDTA salts, we injected the white mice with 100-fold concentrations up to 0.15M $CaNa_2EDTA$. Four routes of injection - Intradermal, intramuscular, intraperitoneal or intravenous - did not induce any evident toxicity, whereas the venom hemorrhagic activity was completely inhibited by this compound at 100 fold lower concentration of 1.5 mM.

d. Natural antidotes in snake sera: Among various examined sera, the homologous serum of *B. asper* itself was found to possess the highest antihemorrhagic activity. Thirty μg of this crude serum were sufficient to abolish the hemorrhagic activity of the whole venom. The serum of the non-poisonous snake, *Natrix tessellata*, was almost as efficient. It was, therefore, not surprising that most potent antihemorrhagins were the ammonium sulfate fractions prepared from the sera of *B. asper* and *N. tessellata*. The neutralization capacities of these snake serum fractions were 2-3 times higher than that of the polyvalent horse antivenom. Among 16 snake and 9 mammalian screened for their antihemorrhagic activity against the whole venom of *B. asper*, the homologous serum of *B. asper* demonstrated the highest neutralizing activity, followed by the serum of the non-poisonous snake *Natrix tessellata* at a molar ratio of 3:1 and 4:1, respectively (Borkow *et al.*, submitted a). Moreover, an ammonium sulfate fraction of the *B. asper* serum neutralizes the hemorrhagic, lethal, hemolytic and cytostatic activities of the venom more efficiently than all other examined compounds, including the horse polyvalent antivenom currently used as the basic therapeutic device for the treatment of snakebite envenomation in Central America (Borkow *et al.*, submitted a,b). The ammonium sulfate fraction of *N. tessellata* serum also neutralizes the above toxic activities of the venom, but less effectively. The horse polyvalent antiserum is very effective against the cytostatic and hemolytic activities of the venom, while $CaNa_2EDTA$ is an efficient inhibitor of its proteolytic activity (Borkow *et al.*, submitted b).

The potent antihemorrhagic factors were isolated from the sera of the snakes *B. asper* and *N. tessellata* and were characterized (Borkow *et al.*, 1994b, 1995).

Bothrops asper Serum Antihemorrhagin (BaSAH) is a protein with an isoelectric point of 5.2 and a molecular weight of 66 kDa. It does not form a precipitin line with the main hemorrhagin of *B. asper* snake venom (BaH1), nor with the whole venom, which indicates that the antihemorrhagic factor is not an immunoglobin. It does not digest the hemorrhagic toxin BaH1. ELISA experiments and chromatography of BaSAH with the ^{125}I-labeled hemorrhagic toxin BaH1 demonstrated that the mechanism of the neutralization involves a formation of an inactive soluble complex between the natural antihemorrhagin and BaH1, the main hemorrhagin of *B. asper* venom. The stochiometry of neutralization is 2 molecules of the neutralizing agent BaSAH per each molecule of the hemorrhagic toxin BaH1 (Borkow *et al.*, 1995).

Natrix tessellata Antihemorrhagin (NtAH) is a much larger protein with a molecular weight of about 880 kDa and an isoelectric point of 4.5. Pure NtAH does not form a precipitin line with BaH1, nor with the whole venom and the mechanism of neutralization is similar to that of BaSAH described above. it does not include digestion of the hemorrhagic toxin BaH1, but a formation of an inactive soluble complex between the natural antidote and the main hemorrhagin of *B. asper* venom. Four molecules of NtAH are needed in order to neutralize one molecule of BaH1 (Borkow *et al.*, 1994b).

e. Immunization with inactivated venom: Monovalent rabbit antisera obtained by immunization with heat-inactivated venom of *B. asper* or with heat-inactivated purified BaH1 were compared with the antisera against the native untreated venom or BaH1 (Borkow *et al.*, submitted a). All four rabbit antisera are antihemorrhagic. However, the antiserum against the whole venom is 3-4 times more potent than the antiserum prepared against the isolated hemorrhagic toxin BaH1. This emphasizes the need to use whole venom in the preparation of antisera. Preparation of antisera against heated whole venom is almost as effective as that prepared with untreated venom. This result concurs with the finding that antigenicity of the hemorrhagic components is maintained after being heated 30 min at 60°C, although the hemorrhagic activity is lost (Borkow *et al.*, 1993). This raises the possibility of using heat-inactivated *B. asper* venom together with the native form during the production of antivenom.

f. A neutralizing mixture: Finally, this work focused on the development of a neutralizing mixture which can better counteract the toxic activities of *B. asper* by intravenous injections or *in situ* administration. The ammonium sulfate fraction of *B. asper* serum, the horse polyvalent antivenom and the synthetic $CaNa_2EDTA$ are the most potent antihemorrhagic materials against *B. asper* venom, especially when administered *in situ* as a mixture (Borkow *et al.*, submitted a). This mixture is also effective in neutralizing other important activities of the venom such as the lethal, cytolytic, proteolytic and hemolytic potencies (Borkow *et al.*, submitted b).

g. Intramuscular hemorrhagic activity of the venom alone or after preincubation with the neutralizing materials: The most potent nontoxic antihemorrhagic materials (snake serum fractions, antisera and $CaNa_2EDTA$) were further tested *in vivo* by intramuscular injections. Table 3 shows that all examined factors inhibited the hemorrhage if mixed with the venom immediately before the injection. Among the sera and antisera tested, the snake serum fraction of *B. asper* and the horse polyvalent antivenom were the most efficient antihemorrhagic materials. Complete neutralization was achieved by 5 mg/ml of these materials compared to 30 mg/ml from the other active antisera. The synthetic compound $CaNa_2EDTA$ had the same efficiency when it was injected i.m. at the isotonic concentration of 0.15M.

In the next series of experiments the neutralizing materials were injected after the venom as usually occurs. The best compatible material of each of the three types was examined (the synthetic $CaNa_2EDTA$, *Bothrops* serum fraction, and the horse antivenom) by two routes of injections - intravenous and *in situ* - namely, into the site where the venom had been injected.

h. Comparison between intravenous and *in situ* administration of the non-toxic antihemorrhagic materials: When the i.v. route was used, each inhibitor was injected into the caudal vein of the mice immediately after the intradermal injection of the venom. We can see

that each type of material reduced the hemorrhagic activity, but the mixture of the three types [$CaNa_2EDTA$, *B. asper* serum fraction and the horse polyvalent antivenom] was more potent than each material alone and restrained the hemorrhagic activity to about 5% of the control size. Moreover, much less of each material was needed in the mixture to achieve better activity than each material alone. This may indicate a synergistic effect of these materials in the neutralizing mixture. The antihemorrhagic activity of the mixture was also examined by *in situ* administration; namely, the mixture was administered into the same site where the venom had been previously injected. The inhibition of the hemorrhagic activity in the *in situ* injection was more efficient than the i.v. route, and less of each material was needed to inhibit the hemorrhage when the injection was *in situ* (compared to the intravenous injections). This difference is especially prominent with $CaNa_2EDTA$, which abolished the hemorrhagic activity by *in situ* injection, compared with the i.v. route. Here again, the best neutralization effect was achieved by a mixture of the three neutralizing substances, in which a smaller quantity of each potent material was needed. However, as the interval between the injection of the venom and the administration of the inhibitors was increased, the hemorrhage became stronger; and after half-an-hour it reached the size of the controls (Borkow *et al.*, submitted a), which indicates the importance of an immediate treatment. If we wait about 30 min, the neutralizing mixture is not effective and the hemorrhage is as strong as in the controls. This figure also shows that *in situ* administration of the mixture, which is represented by the dark column in each group, is more effective than the intravenous route represented by the second column at all time intervals. Unlike the intravenous administration, the hemorrhage which appeared after the *in situ* administration of the neutralizing mixture was significantly weaker than the hemorrhage which appeared in the control animals, even when the mixture was injected 20 minutes after the venom. Even a large amount of the venom (12 MHD) could be almost completely neutralized when the neutralizing mixture was administered *in situ* shortly after the venom injection. However, if the venom was injected into the gastrocnemius, the neutralizing mixture was effective only if administered shortly after the venom.

i. <u>Neutralization of the hemorrhage in the lungs</u>: When the venom of *B. asper* reaches the blood, it caused strong hemorrhagic spots in the lungs (i.v. injection of 50 μg). This hemorrhagic activity of the venom could be also neutralized by i.v. injection of the neutralizing mixture, provided that the mixture was injected shortly after the venom. The number and the size of the hemorrhagic spots increased as the interval between the injection of the venom and the neutralizing mixture injections increased. If the interval was longer than 30 minutes the number and size of hemorrhagic spots was similar to those in the animals injected with PBS. It shows that the injection of the antivenom or the neutralizing mixture >30 minutes after the injection of venom was not effective, strengthening again the importance of immediate treatment.

j. Neutralization of additional toxic activities of *Bothrops asper* venom: As the mixture was efficient against the hemorrhagic activity, the next question was: could these materials neutralize additional activities of the *B. asper* venom? The following experiments answer this question positively.

1) **Proteolytic activity**: The proteolytic activity was examined on azocoll. Whereas saline had no effect on the proteolytic activity of the venom, as expected, $CaNa_2EDTA$ and the serum fractions of *B. asper* and *N. tessellata* completely inhibited the proteolytic activity; horse polyvalent antivenom was less efficient.

2) **Hemolytic activity**: 30% hemolysis of human blood was the maximal hemolytic activity caused by *B. asper* venom. Similarly, the hemolytic activity of the venom which was examined on washed red blood cells was also abolished by the examined inhibitors, apart from $CaNa_2EDTA$, which inhibited about 70% of the hemolytic activity.

3) **Cytostatic activity**: The cytostatic activity of the venom was observed on cultured endothelial cells. The venom caused the cells to detach from the substratum and form floating clusters, but this activity of the venom on endothelial cells was inhibited in the presence of the neutralizing mixture and the cells look normal: the serum fraction of *B. asper* and the horse polyvalent antivenom were the most potent inhibitors. Similar results were obtained with the bovine endothelial cells.

4) **Lethality**: The LD_{50} of the whole venom is 2.5 μg/g. The best neutralizing material was the serum of *B. asper* and all mice injected with 4 LD_{50} of the whole venom preincubated with *B. asper* serum fraction (0.4 mg/ml) survived.

We have recently started to examine the ability of this mixture to neutralize the hemorrhagic, cytostatic and proteolytic activities of an additional ten venoms of crotalid snakes. The following slide shows that the mixture was highly effective in neutralizing the hemorrhagic activity of all examined venoms by low concentrations of the mixture components up to eighty-fold dilution. The neutralizing mixture also inhibited the proteolytic and cytostatic activities of all the examined venoms.

k. Conclusion: In conclusion, this work suggests considering the use of heat-inactivated *B. asper* venom during the production of antivenom; to enrich the polyvalent antivenom, currently used in the treatment of *B. asper* envenomation, with the non-toxic synthetic compound $CaNa_2EDTA$ and a *B. asper* serum fraction. it also suggests examining the possibility of *in situ* administration of the neutralizing mixture in order to counterattack the local hemorrhagic activity of the venom, in addition to the intravenous administration of the neutralizing mixture. The stability of the non-toxic $CaNa_2EDTA$ reinforces the possibility of using this compound as an immediate first-aid in the field, which could be very helpful in retardation of the developing toxicity after a snakebite. When allergy to horse antiserum is found, a mixture of *B. asper* serum fraction with $CaNa_2EDTA$ can be used. Moreover, this study also opens new possibilities for effective neutralization of other crotalid and viperid

snake venoms (Borkow *et al.*, submitted b), as they have toxic activities similar to those of *B. asper* venom and extensive cross-reactivity has been demonstrated (Borkow *et al.*, 1993; Rucavato *et al.*, 1995).

In view of these results, we suggest considering the strategy of using more than one type of neutralizing material in order to counteract effectively all harmful activities of *B. asper* venom and perhaps other crotalid snake venoms. A mixture which includes the three types of materials - the currently used polyvalent antivenom, the natural antidotes (*B. asper* serum fraction) and the synthetic $CaNa_2EDTA$, should be considered as a therapeutic device against the snake bite envenomation of *B. asper*, and probably other cortalid venoms.

Moreover, as most snake bites occur in rural areas, far from medical facilities, we suggest examining the possibility of an immediate *in situ* administration of a neutralizing material into the site of the snake bite as a local first aid in the field, [in order to counterattack the local hemorrhagic activity of the venom,] in addition to the following treatment in the hospital. Until the patient reaches the hospital (and is tested for allergic reactions before the application of antivenom), the immediate local injection may significantly reduce the damage of the envenomation (by neutralizing the hemorrhagic toxins and other local effects developing rapidly *in situ*). $CaNa_2EDTA$ is a stable material and non-toxic and proved to be an effective inhibitor of the hemorrhagic and proteolytic activities of snake venoms, and therefore it is a good candidate for this mission. Furthermore, it should also be emphasized that in many cases only a minority of snake bite victims are hospitalized, and this single injection in the field may be the only available treatment.

Future work should study the feasibility of using these types of inhibitors in the treatment of the [local and systemic effects induced by *B. asper*] snake-bite envenomation in human patients.

On the one hand, it can therefore be concluded that the most efficient neutralizing factor was the *B. asper* serum fraction seen in the following slide. It neutralized all examined activities of the whole venom: the hemolytic, lethal, proteolytic and cytostatic activities, as well as the hemorrhagic activity. Its capability to neutralize the lethal activity of the whole venom was very pronounced compared to the other inhibitors. The general neutralizing capabilities of this fraction against *B. asper* venom were better than the currently used polyvalent antivenom except in the neutralization of the hemorrhage caused when venom was injected into the muscle. On the other hand, $CaNa_2EDTA$ is the most suitable material with almost unlimited shelf life, and very effective antihemorrhagic material, especially when it was administered *in situ*.

6. IMPACT, RELEVANCE AND TECHNOLOGY

This project was of great benefit to the Instituto Clodomiro Picado in particular, and to Costa Rica in general.

a. **Capacity, equipment and expertize**: A significant improvement in the capacity of Instituto Clodomiro Picado (ICP) was obtained. Valuable microscopic equipment was acquired which enables the scientists at ICP to develop research projects in the are of pathology. This equipment, and the training of scientists and technicians along this project, has strengthened the capacity of ICP in the field of experimental pathology. Moreover, great expertize was gained by the Costa Rican investigators on techniques for the isolation and characterization of hemorrhagic toxins. Before this project, no work had been carried out in Costa Rica on the isolation of metalloproteinases. Now, with the technology transferred from Tel Aviv University to Costa Rica on the purification of metalloproteinases, the investigators at ICP have purified a new hemorrhagic toxin and are working on the isolation of other metalloproteinases from snake venoms.

A very important impact of this project for Costa Rica and Latin America is that on the basis of this project, there is a sound hypothesis on the mechanism of action of *B. asper* hemorrhagic toxins. This hypothesis will be useful not only in the understanding of this complex pathologic phenomenon, but also in the design of more effective therapeutic devices to counteract the hemorrhagic activity of venoms. In other words, the basic scientific knowledge gained with this project will be of high value in the more practical task of development of more effective therapies for snakebite envenomation. Since ICP has, over the years, developed an academic leadership in Latin America, this project has strengthened this leadership by enhancing the scientific level of its investigators and by moving forward the understanding of the pathogenesis of venom-induced hemorrhage.

b. **Developing new therapeutical strategies**: At the end of the project, the investigation culminated with the development of a novel and potent neutralizing mixture which is effective in the neutralization of hemorrhagic activity not only of *B. asper* venom, but also of other hemorrhagic venoms around the world. This mixture has proved effective in mice and larger-scale trials are now required to explore the potential of this mixture in the treatment of snakebite victims. The potential benefit of the use of this mixture is very high owing to the impact of venom-induced hemorrhage in the rural population in Latin America.

c. **Establishment of scientific collaboration**: The academic relationship between ICP and TAU has been excellent. Both have benefited mutually and a very productive working relationship has been established. This is evident by the number of scientific publications that have resulted from this project. Most importantly, this excellent academic relationship should be maintained and consolidated in the future.

7) PROJECT ACTIVITIES/OUTPUT

a. Published articles

1. Moreira, L., Gutierrez, J.M., Borkow, G. and Ovadia, M. (1992) Ultrastructural alterations in mouse capillary blood vessels after experimental injections of venom from the snake *Bothrops asper* (Terciopelo). Exp. Mol. Pathol., **57**:124-133.
2. 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**:1137-1150.
3. Lomonte, B., Gutierrez, J.M., Borkow, G., Ovadia, M., Tarkowski, A. and Hanson, L.A. (1994) Activity of hemorrhagic toxin BaH-1 from *Bothrops asper* on capillary endothelial cells *in vitro*. Toxicon, **32**:505-510.
4. Moreira, L., Borkow, G., Ovadia, M. and Gutierrez, J.M. (1994) Pathological changes induced by BaH1, a hemorrhagic proteinase isolated from *Bothrops asper* (Terciopelo) snake venom, on mouse capillary blood vessels. Toxicon, **32**:977-987.
5. Borkow, G., Lomonte, B., Gutierrez, J.M. and Ovadia, M. (1994) Effect of various viperidae and crotalidae snake venoms on endothelial cells *in vitro*. Toxicon, **32**:1689-1695.
6. Borkow, G., Gutierrez, J.M. and Ovadia, M. (1994) A potent antihemorrhagin in the serum of the non-poisonous snake *Natrix tessellata*; isolation, characterization and mechanism of neutralization. Biochim. Biophys. Acta, **1201**:482-490.
7. Gutierrez, J.M., Romero, M., Diaz, C., Borkow, G. and Ovadia, M. (1995) Isolation and characterization of a metaloproteinease with a weak hemorrhagic activity from the venom of *Bothrops asper*. Toxicon, **33**:19-29.
8. Rucavado, A., Gutierrez, J.M., Borkow, G. and Ovadia, M. (1995) Immunological studies on BaH1 and BaP1, two hemorrhagic metalloproteinases from the venom of the snake *Bothrops asper* (Terciopelo). Toxicon **33**:1103-1106
9. Borkow, G., Gutierrez, J.M. and Ovadia, M. Activity of BaH1, the main hemorrhagin of *Bothrops asper* venom, on bovine endothelial cells. Toxicon, **33**:1387-1392.
10. Borkow, G., Gutierrez, J.M. and Ovadia, M. (1995) Isolation and characterization of an effective antihemorrhagic factor from the serum of *Bothrops asper* and the mechanism of neutralization. Biochim. Biophys. Acta, **1245**:232-238.

In press

11. Gutierrez, J.M., Romero, M., Nunez, J., Chaves, F., Borkow, G. and Ovadia, M. Skeletal muscle necrosis and regeneration after injection of BaH1, a hemorrhagic metaloproteinase isolated from the venom of the snake *Bothrops asper* (Terciopelo).Exp. Mol. Pathol.

In preparation

12. Borkow, G., Gutierrez, J.M. and Ovadia, M. Inhibition of the hemorrhagic activity of *Bothrops asper* snake venom by a novel neutralizing mixture.
13. Borkow, G., Gutierrez, J.M. and Ovadia, M. Inhibition of toxic activities of *Bothrops asper* and other crotalid snake venoms by a novel mixture.
14. Rucavado, A., Borkow, G., Ovadia, M. and Gutierrez, J.M. Cross reactivity and neutralization of hemorrhagic protein from the venom of *Bothrops asper* and the effect of BP1 on isolated basal lamina components.

b. Presentations in Scientific Meetings

1. Borkow, G., Gutierrez, J.H. and Ovadia, M. (1993). Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*. 4th Pan American Symposium on Animal, Plant and Microbial Toxins, Campinas, Brasil. Toxicon *31*:114-115.
2. Lomonte, B., Gutierrez, J.M., Borkow, G., Ovadia, M., Tarkowski. A. and Hanson, L.A. (1994). Activity of hemorrhagic toxin BaH-1 from *Bothrops asper* snake venom on capillary endothelial cells *in vitro*. XI European Meeting of the International Society on Toxinology, Arezzo, Italy. Toxicon **32**:400-401.
3. Gutierrez, J.M., Moreira, L., Borkow, G. and Ovadia, M. (1994) Pathological changes induced by BaH1, a hemorrhagic proteinase isolated from *Bothrops asper* (Terciopelo) venom on mouse capillary blood vessels. XI European Meeting of the International Society on Toxinology, Arezzo, Italy. Toxicon **32**:398.
4. Borkow, G., Gutierrez, J.M. and Ovadia, M. (1995). Neutralization of hemorrhagic activity of *Bothrops asper* venom by a novel neutralizing mixture. 11th World Congress on Animal, Plant and Microbial Toxins, October, Tel Aviv, Israel. Toxicon **33**:262.
5. Rucavado, A., Borkow, G., Ovadia, M. and Gutierrez, J.M. (1995) X Congreso Centroamericano de Microbiologia. San Jose, Costa Rica, November 1995.

8. PROJECT PRODUCTIVITY

All of the proposed goals were accomplished by the project paving the way for a possible future clinical application by developing new and better therapeutic strategies to counteract the investigated snake toxins. This accomplishment can be divided into two fields:

a. Scientific collaboration: During this project an excellent collaboration was established between the two laboratories. The Costa Rican group focused more on the study of the pathological effects induced by hemorrhagic toxins, not only on blood capillaries but also on muscle cells. In addition, immunological experiments were also carried out in Costa Rica, aiming at the development of more effective antivenoms. On the other hand, the Israeli group put more emphasis on the biochemical characterization of hemorrhagic toxins and antihemorrhagic factors, but particularly on the development of a novel neutralizing mixture,

effective in the neutralization of various toxic activities of *B. asper* venom and particularly the hemorrhagic activity. Thus, the efforts of both groups were complementary.

The Israeli lab who purified the hemorrhagin, sent it to the Costa-Rican lab in which the pathogenesis of the purified toxin, and the ultrastructural alteration in the capillary blood vessels of the mouse were carried out. At the same time, the Israeli lab studied the activity of the toxin on endothelial cells *in vitro* and on isolated extracellular components. This collaboration was important for understanding the mechanism of this hemorrhagic toxin. In addition, the Israeli lab screened various snake sera in order to find a potent natural antidote that could improve the quality of the antivenom produced in the Costa-Rican Institute. Two potent antihemorrhagins with different characteristics were purified from the sera of the non-poisonous water snake and *B. asper* itself. At meetings of Dr. Gutierrez and Dr. Ovadia in Tel Aviv and San Jose, the results achieved were discussed and future work was planned. Dr. Gutierrez received from Dr. Ovadia samples of various sera to continue various aspects of the research in Costa Rica.

The results already obtained in the project have a number of potential uses, some of which will be revealed by continuing the fruitful collaboration after the project ends, as follows (please see Future Work for more details): Firstly, the isolation of a purified hemorrhagic fraction allowed us to study the mechanism of action of this hemorrhagic proteinase. Thus, the purified toxin became an extremely useful tool in discerning the mechanisms by which capillary vessels are disrupted. This, in turn, would allow for the design of new therapeutic strategies to circumvent this pathology.

In addition, by having a purified hemorrhagic factor it will be possible to add this fraction to the antigenic mixture of venoms that is injected into the horses, at the Institute Clodomiro Picado, for the production of antivenom. Thus, the addition of the hemorrhagic fraction will improve the antibody response of horses against hemorrhagic toxins, thereby improving the therapeutic usefulness of this antivenom, since hemorrhage is one of the most relevant effects in these envenomations. Moreover, biochemical studies have found that various synthetic chemicals and natural antidotes are able to neutralize the hemorrhagic activity of *B. asper* venom. This opens new possibilities of using some of these substances to neutralize the hemorrhagic effect in people bitten by snakes, especially those allergic to horse serum.

This collaborative project has, therefore, had a significant impact on two fronts: (i) It has greatly increased our understanding of the chemical nature of the hemorrhagic toxins present in *B. asper* venom, as well as on the mechanism of action of these toxins. This understanding is extremely useful in the development of new therapeutic devices; (ii) It has resulted in the development of a highly effective neutralizing mixture based on the combination of a chemical product, an antihemorrhagic factor purified from the blood serum of *B. asper* and an antivenom made in horses against the crude venom of *B. asper*.

b. Strengthening of the developing country institution: This project has been particularly useful in strengthening Costa Rican's capacity in the field of toxicology. Thanks to this project, the scientific personnel at the Instituto Clodomiro Picado (ICP, University of Costa Rica) has become involved in the isolation and characterization of metalloproteinases from snake venoms. This has generated an important expertise in the biochemical work related to the purification of these proteases, as well as on the chemical characterization of these toxins. Since proteases are important to snake venoms, not only by inducing hemorrhage but also by causing other effects (coagulopathies, inflammation, etc.), the expertise gained in this project will be extremely helpful in the future development of the research of toxinology at the ICP and, as a consequence, in Costa Rica.

The project has strengthened the ICP at the University of Costa Rica in several ways:

(1) A high quality microscope with photographic and video assembly was obtained. This equipment was badly needed and will allow this group to do more sophisticated and relevant research on the pathological effects induced by snake venoms. In particular, this microscope will allow experiments which enable researchers to visualize pathological alterations in a dynamic fashion.

(2) The methodology adopted in this project for the isolation of hemorrhagic toxins has been successfully transferred to the Institute Clodomiro Picado. Thus, the staff of this Institute is now able to purify hemorrhagic compounds in Costa Rica. This, in turn, will have an impact on the toxinological studies in this country, as well as on the improvement of the antivenom being produced there, since the antigenic mixture used to immunize horses for antivenom production will be enriched with purified hemorrhagic factors, in order to enhance the antibody titers.

(3) The project supported J.M. Gutierrez for a travel to Italy and Israel in order to attend the XI Meeting of the European Division of the International Society of Toxinology, and to visit several laboratories in Rome and Tel Aviv. This trip was very important to discuss many relevant issues in the field of toxin research and also to carry out collaborative research work, in particular with Dr. Ovadia in Tel Aviv University.

9) FUTURE WORK:

This chapter was written as a preproposal for grant application. The excellent collaboration led to the achievement of all proposed goals and yielded 14 refereed articles and presentations in four scientific meetings. We would, therefore, like to continue this fruitful cooperation and perform clinical trials as follows:

a. Clinical studies on the use of the novel neutralizing mixture in the neutralization of hemorrhage: This project was successful in designing a highly effective neutralizing mixture against hemorrhagic activity of a variety of snake venoms. This anti-hemorrhagic mixture is composed of polyvalent antivenom, a fraction from *B. asper*

serum and $CaNa_2EDTA$. Moreover, it was suggested hat the *in situ* injection of $CaNa_2EDTA$ at the site of venom injection would be effective in the neutralization of hemorrhage if the injection is performed soon after envenomation. Now it is necessary to test these neutralizing components in clinical trials to demonstrate their effectiveness in actual snake envenomations. Several studies will be performed on this regard:

(1) Studies of the *in vivo* effects of $CaNa_2EDTA$ and *B. asper* serum fraction in mice: It was shown that $CaNa_2EDTA$ salt is not toxic even at high doses when injected intravenously, intramuscularly and subcutaneously. Now we pretend to carry out a more in depth analysis of the effects of the injection of this salt in mouse skeletal muscle. Groups of mice will be injected either intramuscularly in the gastrocnemius or intravenously with this EDTA salt and the effects in muscle tissue will be examined by following: (i) histopathological changes; (ii) changes in plasma creatine kinase levels, as an indication of muscle damage; (iii) development of edema in muscle tissue. Moreover, other tissues will be examined histologically, such as kidneys, lungs, liver and heart. In this way we will test if this compound induces some toxicity if injected intramuscularly. The same experiments will be performed by injecting *B. asper* serum fraction, in order to study if this protein exerts some toxicity in skeletal muscle and other tissues.

(2) Effect of EDTA injection in snake bitten patients: A clinical trial will be performed in which patients bitten by *B. asper* will be immediately injected at the site of venom inoculation, with $CaNa_2EDTA$ in the doses selected during phase (a). This study will be a randomized trial in which another group of patients will not receive the EDTA salt. Both groups will be treated with polyvalent antivenom in the way it is currently applied in Costa Rica. The development of local and systemic hemorrhage will be studied in these two groups, and a comparison will be made using known clinical parameters, as detailed in the work plan.

(3) Enrichment of polyvalent antivenom with $CaNa_2EDTA$ and the antihemorrhagic fraction from *B. asper* serum. The antihemorrhagic fraction of *B. asper* serum will be purified and added to polyvalent antivenom, together with $CaNa_2EDTA$. This "enriched" antivenom will be compared, in another randomized clinical trial with the currently used polyvalent antivenom. In a group of patients some of them will be treated with the currently used antivenom whereas another group will be treated with the "enriched" antivenom. The clinical and laboratory evolution of these two groups of patients will be evaluated and compared in order to reach conclusions on the improvement of the antivenom by this neutralizing mixture.

(4) Clinical studies in other countries of Latin America. If these clinical trials demonstrate the efficacy of these new neutralizing agents against the venom of *B. asper*, then we will continue with clinical trials in other Latin American countries in which we have collaborations with other research groups. In these cases, treatment will be done in patients bitten by other crotaline snakes such as *Bothrops atrox, Bothrops jararaca* and *Lachesis muta* which are known to induce strong hemorrhage in humans.

b. Studies on the mechanism of action of hemorrhagic toxins: Important advances have been made in this project concerning the mechanism by which hemorrhagic toxins affect capillary vessels. It has been proposed that the initial action has to do with the enzymatic degradation of basal lamina components by the action of hemorrhagic metalloproteinases. It was proposed that hemorrhagic toxins do not affect endothelial cells directly, but instead that after degradation of basal lamina components, the interaction between endothelial cells and extracellular matrix is affected and that some kind of indirect mechanism participates in endothelial cell alterations. In the future we will examine the nature of this indirect mechanism by carrying out experiments in cell culture conditions. Extracellular matrix components will be incubated with hemorrhagic metalloproteinases and the degradation products will be added to endothelial cells after neutralization of metalloproteinase activity with $CaNa_2EDTA$ or by *B. asper* serum inhibitor or by heating at 55°C. Thus, in this way we will test if some product of basal lamina degradation is affecting endothelial cells. This will be studied both functionally and morphologically, by examining the structure of endothelial cells by light and electron microscopy.

c. Proposed workplan for the following three years:

First year:

(1) Studies on the *in vivo* effects of $CaNa_2EDTA$ in mice: This project has clearly shown that the salt $CaNa_2EDTA$ is highly effective in the neutralization of hemorrhagic activity of *B. asper* venom at concentrations of 1.5 mM. It was also shown that, unlike other EDTA salts, this EDTA salt was not toxic even when injected intravenously at a 100-fold concentrations of 150 mM (isotonic solution). During the first year of the continuation of this project we will study in detail the effects caused by intramuscular $CaNa_2EDTA$ injection, in order to demonstrate its inocuity for eventual applications in the treatment of local hemorrhage by snake venoms. The rationale for the intramuscular examination is based on the observation that venom is injected intramuscularly during the snake bites.

Groups of four Swiss-Webster white mice (18-20 grams body weight) will be injected intramuscularly with 100 μl of 150 mM solution of $CaNa_2EDTA$. Inoculations will be performed in the right gastrocnemius muscle and, as controls, mice will be injected with phosphate-buffered saline solution in the left gastrocnemius muscle under otherwise identical conditions. Other groups of animals will be injected intravenously At several time intervals (15 min, 1 hr, 3 hr, 6 hr, 24 hr and 7 days) animals will be sacrificed by ether inhalation and samples from the following tissues will be obtained: gastrocnemius muscle, liver, kidney, lung and heart. Tissues will be fixed in Karnovsky's fixative, dehydrated in serial ethanol solutions and embedded in Spurr resin for histological analysis. In addition, the serum levels of several enzymes (creatine kinase, lactic dehydrogenase) will be measured in order to detect tissue damage. Finally, changes in the general condition of the animals will be evaluated by determining the changes in weight as well as in the overall behavior of the animals. An

identical study will be carried out by injecting the serum fraction from *B. asper* which effectively neutralizes hemorrhagic activity. This part of the project will be performed also during the first year.

The influence of $CaNa_2EDTA$ and the neutralizing fraction from *B. asper* serum will be also examined in the mouse cremaster muscle using intravital microscopy as follows: The cremaster muscle of Swiss Webster white mice will be exposed to various concentrations of $CaNa_2EDTA$ and serum anti-hemorrhagic factor, and the microscopic changes will be followed and recorded in a video system. This will allow a dynamic analysis of the muscle and microvessel damage, as well as on the inflammatory changes developing in muscle tissue. This part of the study will be carried out during the first year.

(2) Further studies on the mechanism of action of BaH1 and BaP1: The metalloproteinases BaP1 and BaH1 will be isolated from the venom of *B. asper* by a combination of ion-exchange chromatography on CM-Sephadex and gel filtration on Sephacryl S-200, according to Gutierrez *et al.* (1995) and Borkow *et al.* (1993). Homogeneity will be tested by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Endothelial cell cultures will be carry out with a human capillary endothelial cell line, as described by Lomonte *et al.* (1994). Initially, cells will be incubated with various concentrations of metalloproteinase BaP1 and cell viability will be examined by measuring the release of lactic dehydrogenase in the medium, using a commercial kit. Cytotoxicity will be also evaluated by the reduction of MTT method. A control of 100% activity will be done by incubating the cells with Triton X-100 and a basal release will be determined by incubating the cells with medium without toxin. In addition to cell viability determinations, cells will be harvested and DNA extracted in order to run agarose gel electrophoresis of DNA (Gavrieli *et al.*, 1992) The pattern of DNA bands will be examined in order to determine if there is apoptotic cell death. In other type of experiment, isolated extracellular matrix proteins (fibronectin, laminin, entactin, collagen type IV and heparan sulfate proteoglycan) will be incubated with metalloproteinase BaP1. After incubation, the reaction will be stopped by the addition of $CaNa_2EDTA$ or the serum inhibitor from *B. asper* or by heat inactivation. The reaction products will be added to endothelial cell cultures in order to study if the products of basal lamina degradation exert an effect on endothelial cells. Finally, the activity of BaP1 and BaH1 will be examined *in vivo* using intravital microscopic system according to Bruno *et al.* (1994) from the Costa-Rican laboratory.

Second year:

(3) Effects of $CaNa_2EDTA$ injection in snake bitten patients: During the second year, a first clinical trial will be performed in Costa Rican hospitals. A group of 40 patients bitten by *B. asper* will be studied in a randomized clinical trial. As soon as they arrive to the hospital, 20 of them will receive an intramuscular injection of $CaNa_2EDTA$ at the site of venom injection, this site is clearly identified by the fang marks, as well as by the patient's description. Immediately, these patients will receive the traditional antivenom treatment (10

vials of polyvalent antivenom administered intravenously in 500 ml of physiologic saline solution). A second group of 20 patients will not receive the EDTA injection and will be treated by the regular intravenous infusion of 10 vials of polyvalent antivenom. The patients included in this work should have evidence of envenomation upon arrival to the hospital. Patients without signs of envenomation will be excluded from this study. The protocols for the clinical trials will be prepared according to the guidelines established by the Ministry of Health of Costa Rica with mild envenomation or

The clinical evolution of these two groups of patients will be followed by studying blood pressure, development of edema, local hemorrhage, local necrosis, coagulopathies, systemic bleeding and renal function. Laboratory tests such as hemoglobin concentration, hematocrit, total leucocyte counts, platelet counts, prothrombin time, serum urea and creatinine and urinary sediment analyses will be performed. Clinical and laboratory analyses will be done every 6 hr since the initiation of the treatment.

(4) Enrichment of polyvalent antivenom with $CaNa_2EDTA$ and the antihemorrhagic fraction from *B. asper* serum: A batch of polyvalent antivenom produced at Instituto Clodomiro Picado will be enriched by the addition of $CaNa_2EDTA$ and the serum fraction of *B. asper* that neutralizes hemorrhage. The enriched antivenom will be analyzed by the quality control protocol currently used for the evaluation of polyvalent antivenom. This protocol includes the following procedures: Safety test, pyrogen test, sterility test, potency test, concentration of protein, concentration of phenol, concentration of chloride, concentration of sulfate and immunological identity. Then, this enriched antivenom will be used in a randomized clinical trial with a similar design to the one described above. A group of 20 patients known to have been bitten by *B. asper* will be treated in the hospital by the intravenous infusion of 10 vials of this enriched antivenom. Another group of 20 patients will be treated by the intravenous infusion of 10 vials of polyvalent antivenom. Again, only patients showing signs and symptoms of envenomations will be included in this study. The clinical evolution of these two groups of patients will be evaluated as described above for the $CaNa_2EDTA$ study.

(5) The use of $CaNa_2EDTA$ during immunization: A mixture of the venom with $CaNa_2EDTA$ will be injected into several horses in order to test the possibility of using this non-poisonous mixture during the immunization of the horses for the production of antibodies without poisoning the animals.

Third year:

(6) Clinical trials in additional countries: If the results of the clinical trial studies performed during the second year are satisfactory, then similar clinical trials will be carried out in other Latin American countries, where the same type of antivenom is used and research groups are working in the snakebite treatment. Tentatively, these studies could be performed in Colombia and Brazil, in addition to Costa Rica.

Successful clinical application may also be significant to most developmental countries in the world as they face similar problems of snakebite treatment.

(7) General agreement: It was agreed that the Israeli laboratory will supply purified BaH1 and the isolated neutralizing fraction of *B. asper* serum. In addition, the Israeli lab will carry out a number of experiments related to preclinical trials.

The clinical trials, their evolution and experiments related to pre- and post-clinical trials will be performed in Costa Rica and/or other Latin American countries.

d. Budget:

For these three years' research of clinical application we shall require the following general budget (detailed budget will be submitted upon request)

	1st year	2nd year	3rd year	Overall
Tel Aviv University	39,000	32,000	22,000	94,000
Costa Rican Institute	34,000	39,000	32,000	105,000
Total	73,000	71,000	54,000	199,000

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