Eds C. Bon and M. Goyffon
Envenomings and their Treatments

Evaluation of the neutralizing ability of antivenoms for the treatment of snake bite envenoming in Central America

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Résumé

De nombreuses envenimations ophidiennes en Amérique centrale sont dues aux crotales (famille des Viperidae) parmi lesquels *Bothrops asper* est le principal responsable. Quelques accidents sont provoqués par les serpents corail (famille des Elapidés, genre *Micrurus*). Des sérums anti-venimeux (SAV) polyvalents et anti-serpent corail sont produits depuis 1967 à l'Institut Clodomiro Picado, Costa Rica. Les envenimations par crotales sont sévères et complexes, et caractérisées par des lésions locales et des désordres systémiques. L'envenimation par *Micrurus* sp. est caractérisée par un syndrome neurotoxique paralytique. Une série de tests de laboratoire a été adaptée à l'Institut Clodomiro Picado pour évaluer le pouvoir neutralisant des sérums antivenimeux de l'Amérique Centrale. Ces tests portent sur le pouvoir neutralisant des effets létaux, hémorragiques, œdémateux, myotoxiques, coagulants, défibrinants, neurotoxiques, protéolytiques, et des activités phospholipase A_2 et hyaluronidase des venins. Ils permettent donc une évaluation précise des sérums antivenimeux distribués en Amérique Centrale. Les résultats indiquent que les sérums antivenimeux fabriqués au Costa Rica sont efficaces contre les venins des espèces d'Amérique Centrale testées, tandis que d'autres produits disponibles dans la région ont un pouvoir neutralisant moins efficace. Ces observations montrent la nécessité d'un contrôle de qualité rigoureux des sérums antivenimeux.

Summary

Most snake-bite envenomings in Central America are inflicted by pit vipers (family Viperidae, subfamily Crotalinae); *Bothrops asper* is responsible for most accidents. A few envenomings are caused by coral snakes (family Elapidae, genus *Micrurus*). Polyvalent and *Micrurus* antivenoms have been produced in Costa Rica since 1967. Envenomings induced by pit vipers are severe and complex and are characterized by local and systemic pathophysiological alterations. Neurotoxic paralysis is the most severe effect in *Micrurus* envenomings. A series of laboratory assays has been adapted at the Instituto Clodomiro Picado for evaluating the neutralizing ability of antivenoms in Central America. It includes tests for the neutralization of lethal, haemorrhagic, oedema-forming, myotoxic, coagulant, defibrinating, neurotoxic, proteolytic, phospholipase A_2 and hyaluronidase activities. Thus, these assays allow for a complete, detailed evaluation of antivenoms distributed in Central America. Our studies indicate that antivenoms produced in Costa Rica are effective against the Central American venoms tested, whereas other products that are commercially available in the region are less effective in neutralizing the activities of several venoms. Our observations stress the need for rigorous quality control of antivenoms.

Introduction

Snake-bite envenomings are an important medical problem in Central America (Bolaños, 1982; Gutiérrez, 1995). The large majority of accidents are inflicted by

pit vipers (family Viperidae, subfamily Crotalinae), whereas a small number of cases are due to coral snakes (family Elapidae, genus *Micrurus*) (Bolaños, 1982; Gutiérrez, 1995).

Since 1967, polyvalent (Crotalinae) and anti-coral antivenoms have been produced in Costa Rica and are used in the region for the treatment of pit viper and coral snake envenomings, respectively (Bolaños & Cerdas, 1978, 1980). Traditionally, the efficacy of antivenoms has been tested only against the lethal effect of venoms; however, the pathophysiology of pit-viper envenomings includes effects such as haemorrhage, oedema, myotoxicity, coagulopathy, cardiovascular shock and renal failure (Rosenfeld, 1971; Gutiérrez, 1995). Thus, a more rational evaluation of antivenoms should include tests for the neutralization of these specific effects.

During the last decade, we at the Instituto Clodomiro Picado (ICP) have studied the ability of antivenoms produced in Costa Rica, as well as other commercially available products, to neutralize the most relevant toxic and enzymatic activities of Central American snake venoms. A review of these studies is presented here.

General methods for neutralization studies

The following general principles have been adapted for the neutralization studies performed in our laboratory:

(a) *Selection of venom dose*: Before a neutralization test, a venom dose (challenge dose) must be selected for each assay, by performing dose–response studies. A dose that gives a submaximal response is selected; for lethality, a maximal response (100% lethality) is required.

(b) *Incubation of venom and antivenom*: On the basis of the venom dose selected, a constant amount of venom is mixed with various dilutions of antivenom in order to obtain several antivenom:venom ratios. Controls containing venom with no antivenom and others containing only antivenom are included. Incubations are carried out at 37 °C for 30 min, and then aliquots containing the challenge dose of venom are tested in the corresponding assay systems.

(c) *Expression of the neutralizing ability of antivenoms*: Neutralizing ability is expressed as the effective dose 50% (ED_{50}), defined as microlitres of antivenom per milligram of venom at which the effect of venom has been reduced by 50% (Gutiérrez *et al.*, 1990). For coagulant activity, neutralization is expressed as the effective dose (ED), defined as microlitres of antivenom per milligram of venom at which the coagulation time of plasma is prolonged three times as compared with that of plasma incubated with venom alone (Gené *et al.*, 1989).

This basic protocol is consistent and reliable, although it does not reproduce the actual dynamics of envenoming since venom and antivenom are not incubated before injection. Thus, several experimental approaches have been used in which venom is injected and then, at several intervals, various volumes of antivenom are administered intravenously (see for example Gutiérrez *et al.*, 1985, 1987). The ED_{50} is estimated when antivenom is injected immediately after envenoming.

Neutralization of effects involved in local tissue damage

Local haemorrhage, oedema and myonecrosis are common consequences of crotaline snake envenomings in Central America and are responsible for significant tissue loss and sequelae (Bolaños, 1982; Gutiérrez, 1995). Antivenoms have been evaluated for the neutralization of these effects.

Haemorrhage

The skin test developed by Kondo *et al.* (1960), as modified by Gutiérrez *et al.* (1985), has been used in experiments in which venom and antivenom are preincubated. The polyvalent ICP antivenom has proved effective against the venoms of 10 Central American crotaline species (Gutiérrez *et al.*, 1985, 1987; Rojas *et al.*, 1987). Other antivenoms commercially available in Central America are equally effective (Bogarín *et al.*, 1995). The cross-reactivity of haemorrhagic toxins has also been demonstrated with other venoms and antivenoms (Mebs *et al.*, 1988; Mandelbaum *et al.*, 1989). Thus, haemorrhagic toxins, which are zinc-dependent metalloproteinases (Bjarnason & Fox, 1994), appear to have common neutralizing epitopes.

Myonecrosis

The experimental assessment of the local myotoxicity of venoms has been a subject of debate. Several procedures have been used, including histology (Ownby *et al.*, 1983; Kouyoumdjian *et al.*, 1986) and measurement of the release of muscle-derived enzymes such as creatine kinase (Gutiérrez *et al.*, 1981, 1987; Melo & Suárez-Kurtz, 1987), cytotoxic effects on myoblasts and myotubes in cell culture (Hayes & Bieber, 1986; Bultrín *et al.*, 1993), changes in the MTT-reducing activity of muscle extracts (Lomonte *et al.*, 1993) and effects on a chick biventer-cervicis preparation (Barfaraz & Harvey, 1994). We have mainly used quantification of plasma creatine kinase levels, together with a histological evaluation of muscle sections.

The polyvalent ICP antivenom is effective in neutralizing local myotoxicity induced by *Bothrops asper* and *Lachesis muta* venoms (Gutiérrez *et al.*, 1981, 1987). Moreover, polyvalent antivenom was effective in neutralizing myotoxin I from *B. asper* venom (Lomonte *et al.*, 1987). In addition, Lomonte *et al.* (1991) observed that antivenoms from the ICP, the Instituto Butantan (Brazil) and the Instituto Nacional de Salud (Colombia) have high antibody titres, as estimated by enzyme-linked immunosorbent assay, against three myotoxic phospholipases A_2 isolated from the venoms of *B. asper*, *B. atrox* and *B. moojeni*. In contrast, MYN antivenom (Mexico) had very low levels of anti-myotoxin antibodies.

Oedema

The mouse foot-pad assay described by Yamakawa *et al.* (1976) has been used to study oedema induced by Central American crotaline snake venoms. Poor neutralization of the oedema-forming activity of Costa Rican crotaline venoms by polyvalent ICP antivenom was observed in preincubation experiments (Lomonte,

1985; Gutiérrez *et al.*, 1986), and the oedema increased when large volumes of antivenom were incubated with venom (Gutiérrez *et al.*, 1986). We have suggested that the release of pharmacologically active peptides from non-immunoglobulin proteins present in antivenoms after incubation with venom proteases affects the results of the assay.

An alternative procedure which avoids preincubation was developed, in which antivenom is administered intravenously 5 min before venom is injected subcutaneously into the footpad of mice (Gutiérrez *et al.*, 1986). With this method, much better neutralization of the oedema induced by Costa Rican crotaline venoms was observed (Gutiérrez *et al.*, 1986, 1987; Rojas *et al.*, 1987); however, the oedema-forming activity of *Crotalus durissus durissus* venom was not neutralized (Rojas *et al.*, 1987), even though this venom is part of the antigenic mixture used in the production of the antivenom (Bolaños & Cerdas, 1980). It has been speculated that this venom may contain low-relative-molecular-mass, non-immunogenic proteins which cause oedema (Rojas *et al.*, 1987).

Experiments have also been performed involving independent injection of venom and antivenom. When polyvalent ICP antivenom is administered intravenously at various times after envenoming, neutralization of haemorrhage, myonecrosis and oedema is poor, since significant neutralization is observed only when the antivenom is given within the first few minutes after venom injection (Gutiérrez *et al.*, 1981, 1985, 1987). Similar observations have been made with other antivenoms (Homma & Tu, 1970; Russell *et al.*, 1973; Ownby *et al.*, 1986). Thus, despite the fact that polyvalent ICP antivenom contains antibodies effective against haemorrhagic, myotoxic and oedema-forming toxins of Central American crotaline venoms, neutralization is difficult in this type of experiment, probably due to the extremely rapid onset of local effects (Gutiérrez *et al.*, 1980, 1984; Moreira *et al.*, 1992). Since the antivenom is made of undigested immunoglobulin G molecules, it will be important to test whether $F(ab)_2$ or Fab fragments are more effective in neutralizing local effects, owing to their faster distribution in the tissues.

Neutralization of coagulant and defibrinating effects

Central American crotaline venoms are coagulant and induce defibrination after intravenous injection, with the exceptions of the venoms of *Bothriechis lateralis, Porthidium ophryomegas, P. nasutus, Atropoides picadoi* (Gené *et al.*, 1989) and *Agkistrodon bilineatus* (unpublished observations). Since defibrination is a typical finding in pit viper envenomings in Central America (Bolaños, 1982; Gutiérrez, 1995), antivenoms should be tested for their ability to neutralize this effect.

The procedures described by Theakston and Reid (1983), as modified by Gené *et al.* (1989), were used to test the neutralization of coagulant and defibrinating activities. The polyvalent ICP antivenom is effective in neutralizing these activities of Central American crotaline venoms (Gené *et al.*, 1989). Moreover, in the case of *B. asper* venom, neutralization of this effect was achieved even when the antivenom

was administered after envenoming (Chaves *et al.*, 1989). The other three antivenoms were less effective than polyvalent ICP antivenom in neutralizing the coagulant activity of *B. asper* venom (Bogarín *et al.*, 1995).

Neutralization of lethality

Neutralization of lethality is probably the single most important test in evaluating the efficacy of an antivenom. In the case of Central American crotaline venoms, the assay is performed by preincubating venom and antivenom at various ratios and then injecting aliquots of the mixtures, containing four LD_{50}, intraperitoneally into mice. Deaths are recorded over the following 48 h, and the ED_{50} is estimated by either the Spearman-Karber method (World Health Organization, 1981) or by probits (Trevors, 1986).

Polyvalent ICP antivenom was effective in preincubation-type experiments against the venoms of all of the Central American crotaline species tested (Bolaños & Cerdas, 1978; Gutiérrez *et al.*, 1987; Rojas *et al.*, 1987). In contrast, three other antivenoms were ineffective in neutralizing the lethal effect of *B. asper* venom (Bogarín *et al.*, 1995). In experiments with independent injection of venom and antivenom, Gutiérrez *et al.* (1987) demonstrated that polyvalent ICP antivenom neutralizes the lethality of *L. muta* venom when administered after envenoming and is effective even when administered late in the course of envenoming. Similar results have been obtained with the venoms of *B. asper* and *C. d. durissus* (unpublished observations).

In an effort to reduce the use of mice in the assessment of polyvalent antivenom efficacy, an indirect haemolytic assay in agarose gels has been adapted. A highly significant correlation was observed between neutralization of indirect haemolysis and neutralization of the lethality of *B. asper* venom (Gutiérrez *et al.*, 1988). Thus, this procedure is now routinely used in an in-process estimate of the ED_{50} of polyvalent antivenom, whereas the mouse assay has been kept as the reference method for testing the final product.

Neutralization of enzymatic activities

Crotaline snake venoms exert a variety of enzymatic activities, some of which are related to their pharmacological effects. Neutralization of caseinolytic, fibrinolytic, fibrinogenolytic, hyaluronidase and phospholipase A_2 activities of Central American crotaline venoms has been studied in preincubation-type experiments. Polyvalent ICP antivenom is effective in neutralizating these activities and is particularly effective against the hyaluronidase effect (Gené *et al.*, 1985; Gutiérrez *et al.*, 1985, 1987; Rojas *et al.*, 1987; Gené *et al.*, 1989; Valiente *et al.*, 1992).

Neutralizing ability of *Micrurus* antivenoms

Micrurus spp. venoms induce neurotoxicity, which results in the paralysis of various muscles and, eventually, in respiratory paralysis (Bolaños, 1982; Vital-

Brazil, 1987; Gutiérrez, 1995). A postsynaptic effect predominates in the action of these venoms, although the venom of *M. nigrocinctus* from Costa Rica also induces presynaptic alterations (Goularte *et al.*, 1995). In addition, several *Micrurus* venoms are myotoxic to mice (Gutiérrez *et al.*, 1983, 1992), although this effect has not been observed clinically.

A monovalent *M. nigrocinctus* antivenom is produced at ICP and used in Central America. It is effective against the lethal effect of this venom (Bolaños *et al.*, 1978; Gutiérrez *et al.*, 1991) and the venoms of *M. fulvius* and *M. carinicauda* (Bolaños & Cerdas, 1978). In addition, it neutralizes the myotoxic and phospholipase A_2 activities of *M. nigrocinctus* venom (Gutiérrez *et al.*, 1991; Lomonte *et al.*, 1993). A multivalent *Micrurus* antivenom was prepared at ICP which is effective in neutralizing a large number of *Micrurus* venoms (Bolaños *et al.*, 1978). A monovalent *M. mipartitus* antivenom produced at ICP is used in Costa Rica and Colombia (Bolaños *et al.*, 1975).

Two in-vitro assays have been used to study the neutralizing ability of monovalent *M. nigrocinctus* antivenom. Antivenom was effective in neutralizing the neuromuscular blocking action of homologous venom on an isolated mouse phrenic nerve–diaphragm preparation, both when venom was preincubated with antivenom and when venom and antivenom were added separately (Goularte *et al.*, 1995). This antivenom neutralizes the binding of α-neurotoxins from *M. nigrocinctus* venom to acetylcholine receptors, as seen in an enzyme-linked immunosorbent, non-radioactive assay (Alape *et al.*, 1995). Interestingly, the antivenom reversed the binding of toxins already complexed with the receptor, an observation that might have therapeutic implications. These in-vitro assays are useful in studying the neutralizing ability of *Micrurus* antivenoms.

Final remarks

Despite observations of cross-reactivity between venoms and heterologous antivenoms (e.g. Rosenfeld & Kelen, 1966; Mebs *et al.*, 1988), there is extensive intra- and interspecific variation in the composition of snake venoms (Chippaux *et al.*, 1991). This variability may confer different immunological profiles on venoms from different species or from different populations within the same species. This variation has evident implications for the efficacy of antivenoms, as has been pointed out by many authors (see for example Grasset, 1955). Therefore, antivenoms must be tested against the most relevant snake venoms from the countries where the antivenoms are distributed. In Central America, neutralization assays must be perfomed to test the antivenoms distributed in the region, since some of them are produced using venoms from species distributed in other countries.

It is important to keep in mind that, although the assays described in this review are valuable for evaluating antivenoms, caution should be exercised in extrapolating experimental results to the clinical situation. Clinical trials are required in order to fully assess the neutralizing ability of antivenoms. New

methods are also needed to evaluate the neutralization of other relevant toxic activities, such as cardiovascular shock and renal failure, and in-vitro systems are required that would reduce the use of mice in antivenom testing.

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