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# Antivenoms for Snakebite Envenomings

José María Gutiérrez*, Guillermo León, Bruno Lomonte and Yamileth Angulo

*Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*

**Abstract:** Animal-derived antivenoms constitute the mainstay in the therapy of snakebite envenoming. Antivenoms are manufactured by immunizing animals, usually horses, with venoms from a single or several medically-relevant snake species. Antivenoms are constituted by either whole IgG molecules or the immunoglobulin fragments $F(ab')_2$ and Fab, obtained by digestion with pepsin and papain, respectively. Differences in the pharmacokinetics of these active substances have pharmacodynamic implications. Novel technological possibilities may improve the quality of antivenoms in the future, as well as their microbial safety. Antivenom administration might induce early and late adverse reactions, whose possible mechanisms are discussed. Owing to the large variety in the composition of snake venoms and to the need to demonstrate neutralization of relevant snake venoms in different countries, a meticulous preclinical and clinical assessment of antivenom efficacy and safety is required before an antivenom is introduced into clinical application. The accessibility of antivenoms in low-income tropical countries is of concern and efforts should be directed at guaranteeing the access of safe and effective antivenoms at affordable prices and their correct clinical use in these countries.

**Keywords:** Snakebite, neglected tropical disease, antivenom, envenoming, fractionation, quality control, pharmacokinetics, viral safety, adverse reactions.

## INTRODUCTION

The pioneering work of Calmette and Phisalix and Bertrand, in 1894, demonstrated that the serum of animals immunized with snake venoms was effective in the neutralization of the toxic effects of venoms, thus setting the stage for the modern therapy of snakebite envenoming based on the parenteral administration of animal-derived antivenoms [1]. Thereafter, developments occurred in various countries, most notably by Vital Brazil in South America [2] and by other workers in the USA, Japan and Australia [3]. These first antivenoms consisted of crude serum and, consequently, the incidence of adverse reactions resulting from their administration was high.

Further work involved the introduction of fractionation of serum, aimed at obtaining more refined products. Initially, salting-out procedures using ammonium sulfate were introduced [4]. Then, pepsin digestion at acidic pH and heat treatment, i.e. thermocoagulation, were developed by Pope [5, 6] in order to obtain more refined preparations. The majority of currently used antivenom fractionation protocols are based on this methodology which yields $F(ab')_2$ antibody fragments [7]. During the last decades, several innovations have been introduced to antivenom manufacture, such as caprylic acid precipitation of non-immunoglobulin proteins [8, 9], ion-exchange and affinity chromatography [10, 11], and papain digestion for obtaining Fab fragments [11]. The improvement of antivenom manufacturing technologies has allowed the availability of safe and effective products that are widely used in the treatment of snakebite envenoming world wide.

*Address correspondence to this author at the Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; Tel: 506-2229-3135; Fax: 506-2292-0485;
E-mail: jose.gutierrez@ucr.ac.cr

Despite these advances in antivenom production, together with an ever growing knowledge on the biochemical composition and toxic actions of snake venoms, there is a current crisis in antivenom manufacture and access in several regions, particularly in sub-Saharan Africa, some regions of Asia and Latin America, and Papua-New Guinea [12-14]. The roots of this problem are complex and go beyond the scientific and technological issues, since they also depend on economic and political factors related to public health on a wider scale. The present review describes the basic technological aspects of antivenom production and focuses on a number of unsolved issues that need to be addressed in order to ensure the improvement in antivenoms and the accessibility and correct use of these life-saving therapeutics.

## STATE OF THE ART IN ANTIVENOM MANUFACTURE

Antivenom manufacturing laboratories are distributed in every continent [15, 16]. However, the universe of antivenom producers is a highly heterogenous *collage* which includes well developed laboratories with up-to-date technology and compliance with good manufacturing practices and other laboratories with notorious deficiencies in their technological base [14]. Many antivenom-producing laboratories belong to public institutions, although there are also private laboratories which generate a significant quota of the total production of antivenoms world wide [15]. Most manufacturers use horses for immunization with venoms, although few producers also use sheep and donkeys [7, 17] and, more recently, llamas [18]. The use of other animals as a source of antivenom antibodies has been described mostly at the experimental level. Antivenoms can be either 'monospecific' or 'polyspecific', depending on whether animals are immunized with the venom of a single snake species or of various species, respectively. The presentation of antivenoms can be either liquid or freeze-dried. The latter are stable at room temperature and, therefore, do not demand a cold chain for their storage and transportation; in addition,

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their shelf-life is more prolonged than that of liquid preparations. However, the process of freeze-drying increments the production cost and, therefore, the final price of antivenoms. Most antivenoms are dispensed in vials or ampoules in a volume of 10 mL, although presentations of larger volumes are also available. Antivenom manufacture and control involves four basic stages: (a) Immunization of animals with relevant snake venoms, (b) bleeding and fractionation of animal blood to obtain the active substance, (c) formulation and dispensing in the final containers, and (d) quality control of the final product. A detailed description of the various aspects involved in antivenom manufacture and quality control can be found in the recently published WHO Guidelines for the Production and Control and Regulation of Snake Antivenom Immunoglobulins [19].

## Immunization

Owing to the great diversity of snake species and the complexity and heterogeneity of venom composition, the selection of the venoms to be used for animal immunization in antivenom production is of paramount importance. Several criteria should be considered for this selection, such as: (a) Knowledge of the snake species that cause the highest toll of envenomings in a country or region. This, in turn, demands epidemiological information on snakebite envenomings, which is rather poor in many countries. (b) Knowledge of the immunological cross-neutralization between antivenoms and venoms of various snake species. This aspect requires research-based information on the ability of antivenoms to react and, more importantly, to neutralize the venoms of heterologous species, i.e. species whose venoms are not used in the immunization (e.g. [20]). Proteomic tools have been adapted for the detailed characterization of venoms and their immunoreactivity with antivenoms, a field called 'antivenomics' [21, 22]. (c) Knowledge of the clinical features of envenomings, in order to identify specific envenoming syndromes that could be clearly associated with particular species of snakes, thus allowing the selection of the appropriate antivenom for treatment. Consequently, the selection of venoms to be used in immunization should be based on a meticulous case by case analysis supported by epidemiological, clinical, immunological, and toxinological information. Unfortunately, in many cases, such selection has been decided on a rather arbitrary basis, a trend that should be reversed with the help of scientific information on snakes and their venoms. In general, polyspecific antivenoms are preferred, since their use does not require the precise identification of the snake species responsible for the accident; this is the case of viperid antivenoms used in Latin America where the majority of accidents are caused by *Bothrops* sp venoms [23, 24]. However, there are cases where monospecific antivenoms are preferred, either because the identification of the offending snake is easy, on the basis of clinical presentation (e.g. envenomings by South American *Crotalus durissus* [23]) or because the number of accidents by a given species is very low or its venom is difficult to obtain (e.g., envenomings by *Atractaspis engaddensis*, *Micrurus* sp, *Lachesis* sp, or *Hypnale hypnale*) [25-27].

The preparation of venom pools for immunization is a critical step to ensure the production of effective antivenoms. These pools should include venoms from many individuals of different geographical locations within the distribution range of the species. This is very important in the case of species with wide distribution ranges owing to the well-known phenomenon of intraspecies venom variability [28-30]. Thus, knowledge on the biochemistry and proteomics of medically-relevant snake venoms is relevant for a more rigorous preparation of venom pools for immunization. Since many snake venoms, especially from viperid species, contain proteolytic enzymes, care should be taken as to ensure that venoms are rapidly frozen and freeze-dried after collection. Immunization with venoms should aim at obtaining a high neutralizing antibody titer without significantly harming the animals being immunized. These goals are usually achieved by the use of repeated injections of low doses of venom. Although some manufacturers inactivate venoms in order to reduce toxicity, this strategy may affect the structure of relevant epitopes, thus compromising antibody response; therefore, it is preferred to use native venoms for immunization. A strategy employing multisite injections of low volumes has been highly effective [31]. Immunization protocols are generally based on the use of Freund's complete and incomplete adjuvants, during the first immunizations, followed by subsequent injections of venom with other adjuvants such as aluminum salts, bentonite, alginate or just saline solution [32-34].

The immune response of animals to venoms is complex and needs to be studied in further detail. For instance, a phenomenon of immune suppression has been described when venoms of some viperid species are injected [35, 36]. It is likely that the difficulty in obtaining high titers against some venoms is related to this largely unexplored phenomenon. The effects of venoms on the physiological status of immunized animals need to be also documented. In the case of a polyspecific viperid antivenom produced in Costa Rica, it was shown that immunization provoked mostly local tissue inflammation and a drop in hemoglobin concentration, without affecting other systemic parameters [37]. In the case of horses, the IgG isotype responsible for most of the neutralizing activity of antivenoms corresponds to IgG(T) [38], now classified as $IgG_3$ and $IgG_5$ [39].

## Fractionation of Hyperimmune Plasma

There are three basic formulations of antivenoms in terms of the active substance. The majority of manufacturers produce antivenoms based on divalent $F(ab')_2$ fragments, whereas other antivenoms contain whole IgG molecules, and few antivenoms are based on monovalent Fab fragments (Fig. **1**).

*Whole IgG antivenoms*: These are produced by fractionating hyperimmune horse plasma by either ammonium sulfate precipitation [40] or by caprylic acid (octanoic acid) precipitation of non-immunoglobulin plasma proteins at acidic pH [8, 9]. Caprylic acid fractionation yields antivenoms of higher yield and purity, as compared to ammonium sulfate-fractionated products [9]. Accordingly, clinical trials have demonstrated a better tolerability of

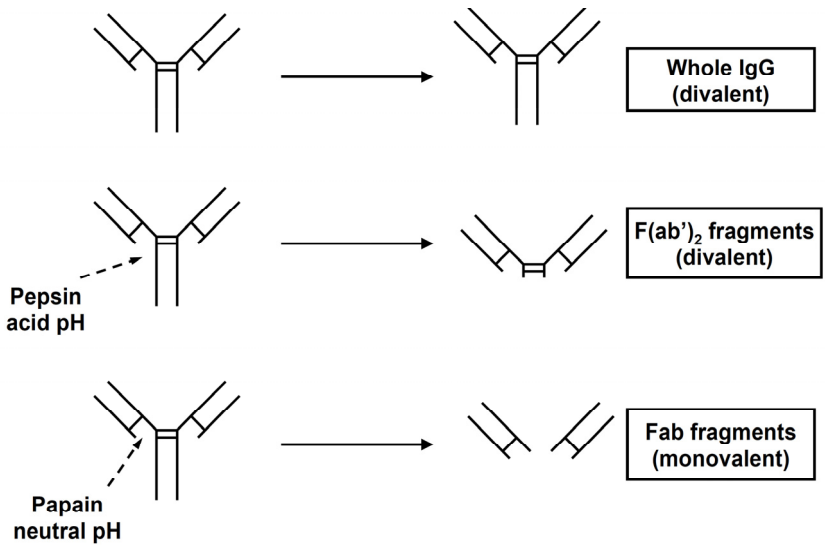


**Fig. (1).** Schematic representation of the three types of active substances constituting antivenoms currently in clinical use. Some manufacturers produce whole IgG antivenoms by purifying antibodies using either ammonium sulfate or caprylic acid fractionation. Many laboratories manufacture antivenoms made of divalent $F(ab')_2$ fragments, obtained by pepsin digestion of plasma at acid pH, thermocoagulation and ammonium sulfate purification of $F(ab')_2$. Few laboratories produce antivenoms made of monovalent Fab fragments by a process based on ammonium sulfate precipitation of IgG followed by papain digestion at neutral pH. Owing to their different molecular masses, these three types of active substance show different pharmacokinetic profiles.

caprylic acid-fractionated antivenom as compared to whole IgG antivenoms produced by ammonium sulfate fractionation [41]. Fig. (**2**) presents the basic fractionation scheme used in Costa Rica for the manufacture of antivenoms by using caprylic acid.

*$F(ab')_2$ antivenoms*: Manufacture of $F(ab')_2$ antivenoms is based on the methodology developed by Pope [5, 6], using pepsin digestion, at acid pH, of plasma proteins, which cleaves the Fc fragment of immunoglobulins and also degrades many other non-immunoglobulin plasma proteins, such as albumin. Then, a heat treatment step, known as 'thermocoagulation', and a series of salting-out steps based on ammonium sulfate precipitation generate a highly purified $F(ab')_2$ preparation (e.g. [42, 43]) (Fig. **3**). The yield of this procedure is affected by the effect that pepsin digestion and heating has on the IgGs. Some manufacturers include additional steps for $F(ab')_2$ purification, such as ion-exchange chromatography [10, 44], and the use of caprylic acid to eliminate lipoproteins that may cause turbidity in antivenom preparations.

*Fab antivenoms*: Antivenoms made of monovalent Fab fragments are produced by the fractionation of sheep-derived plasma. After ammonium sulfate precipitation of IgGs, Fab fragments are generated by papain digestion at neutral pH. Preparations are further purified by ion-exchange chromatography or, in some cases, by affinity chromatography [11].

### Quality Control of Antivenoms

The manufacture of antivenoms involves a meticulous quality control, both in-process and in the final product. This includes a set of physicochemical and biological analyses such as: (a) neutralizing potency, expressed as Median Effective Dose ($ED_{50}$); (b) pyrogenicity (either by the rabbit test or the *Limulus* amebocyte lysate (LAL) test); (c) identity test (by immunochemical methods); (d) protein concentration; (e) determination of pH; (f) purity (by SDS-PAGE or Fast Protein Liquid Chromatography, FPLC); (g) concentration of excipients (sodium chloride, etc.); (h) concentration of preservatives (phenol, cresols); (i) concentration of chemical agents used during fractionation (ammonium sulfate, caprylic acid, etc.); (j) residual moisture (in the case of freeze-dried products); and (k) detection of particulate matter. Manufacturers and regulatory agencies have specifications for these tests. A detailed account of these methods can be found in the WHO guidelines for antivenom production and control [19] and references therein.

## PHARMACOKINETIC CONSIDERATIONS

Antivenom pharmacokinetics is affected by the molecular mass of the active substance, i.e. IgG, $F(ab')_2$ or Fab (see reviews [45, 46]). In general, Fab and $F(ab')_2$ fragments have larger volumes of distribution than IgG. Fab antivenoms distribute more rapidly to tissues than $F(ab')_2$ and IgG. On the other hand, Fab fragments have a shorter elimination half-life than $F(ab')_2$ and IgG, and Fab is cleared from the circulation 35 times faster than IgG due to renal elimination of the former [46, 47]. In contrast, $F(ab')_2$ and IgG are predominantly eliminated by extrarenal mechanisms [46, 47]. When compared in terms of net number of cycles per gram of tissue, IgG cycles 17 and 35 times more often than $F(ab')_2$ and Fab, respectively, in most organs [47]. In summary, Fab fragments have a larger volume of distribution and diffuse more rapidly than IgG and $F(ab')_2$ fragments, thus reaching higher interstitial fluid : plasma concentration ratios; on the other hand, Fab are cleared from the body much faster than $F(ab')_2$ and IgG [45, 46]. These pharmacokinetic differences have evident pharmacodynamic

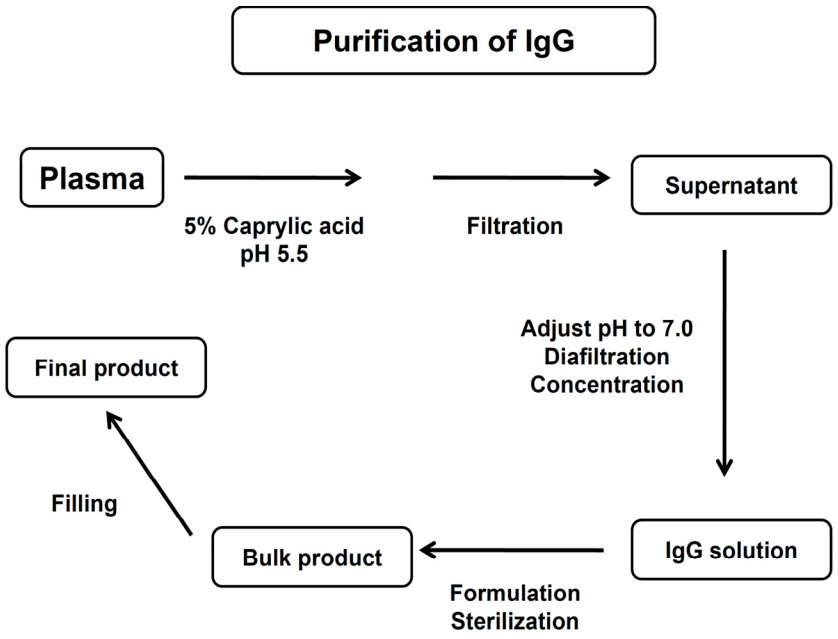


**Fig. (2).** Scheme of the basic methodology used for the manufacture of whole IgG antivenoms by caprylic acid precipitation of non-immunoglobulin plasma proteins.

implications. In the case of viperid snake venoms, composed of high molecular mass toxins that may reach the central blood compartment at later time intervals after subcutaneous or intramuscular injection, as occurs in human envenoming cases, the rapid elimination of Fab fragments is a problem because, by the time toxins are reaching the bloodstream, Fab concentration in blood may be too low. This results in the phenomenon known as 'recurrence of envenoming', in which signs and symptoms of envenoming reappear several hours after antivenom infusion [48, 49]. Thus, a treatment protocol based on repeated administration of antivenom has been developed to compensate for this problem [49]. The selection of the most appropriate type of antivenom active substance has to be determined on the basis of a careful analysis of the toxicokinetics of venoms to be neutralized. In some cases, it is likely that a combination of IgG or $F(ab')_2$ fragment, which would remain in circulation for long time, and a low molecular mass fragment, such as Fab, which would diffuse rapidly to the extravascular compartment, may become a good combination to neutralize toxins that act on the circulation and those that rapidly diffuse to the tissue compartments [50].

## NOVEL TECHNOLOGICAL POSSIBILITIES FOR THE MANUFACTURE AND CONTROL OF ANTIVENOMS

Antivenoms of satisfactory safety and efficacy are produced with the methodologies described above. However, innovations in antivenom technology are being pursued. Some of these developments remain in the experimental realm, although their introduction in antivenom manufacture may be considered in the near future.

*Improvement of immunization schemes*: Venoms are complex mixtures of hundreds of proteins, some of which are toxic whereas others are not. Thus, by immunizing animals with crude venoms, antibodies are raised against toxicologically-relevant and irrelevant venom proteins. If non-toxic proteins predominate, the immune response to relevant toxins may be reduced or affected. Therefore, it is necessary to develop novel immunization strategies aimed at directing the immune response against relevant toxic proteins. This could be achieved by the use of recombinant toxins, such as the sphingomyelinase D from the venoms of the medically-relevant spiders of the genus *Loxosceles* [51]. In the case of snake venoms, this strategy could be used for venoms whose toxicity depends on a single or few toxins, like the South American rattlesnake *Crotalus durissus*, in which case a single toxin, the dimeric phospholipase $A_2$ complex crotoxin, is responsible for the predominant neurotoxic, myotoxic and renal alterations characteristic of these envenomings [52]. A promising novel avenue is DNA immunization, whereby animals are injected with cDNA coding for relevant toxins [53]. Moreover, by using bioinformatic tools, it is possible to design cDNA sequences encoding for immunogenic and structurally-relevant epitopes. The design of 'epitope strings', based on the preparation of single synthetic multiepitope DNA immunogens, has proved successful at the experimental level [54]. DNA immunization has the additional advantage of eliminating the need to collect and maintain snakes in captivity. On the other hand, it is necessary to develop novel adjuvants and immunization schemes on the basis of an improved knowledge on the immune responses of animals to venoms. The identification of possible immune-suppressor components of venoms and the design of more effective immunization schedules are important goals.

*Introduction of novel steps in plasma fractionation*: Although the purity of many current antivenom preparations is satisfactory, it is necessary to explore novel ways to further increase the purity, and hence the safety, of the active substance in antivenoms. Examples of such innovations are the use of membrane-based methods involving precipitation, microfiltration and hydrophobic interaction-based membrane adsorption [55] and dedicated steps aimed at the removal or

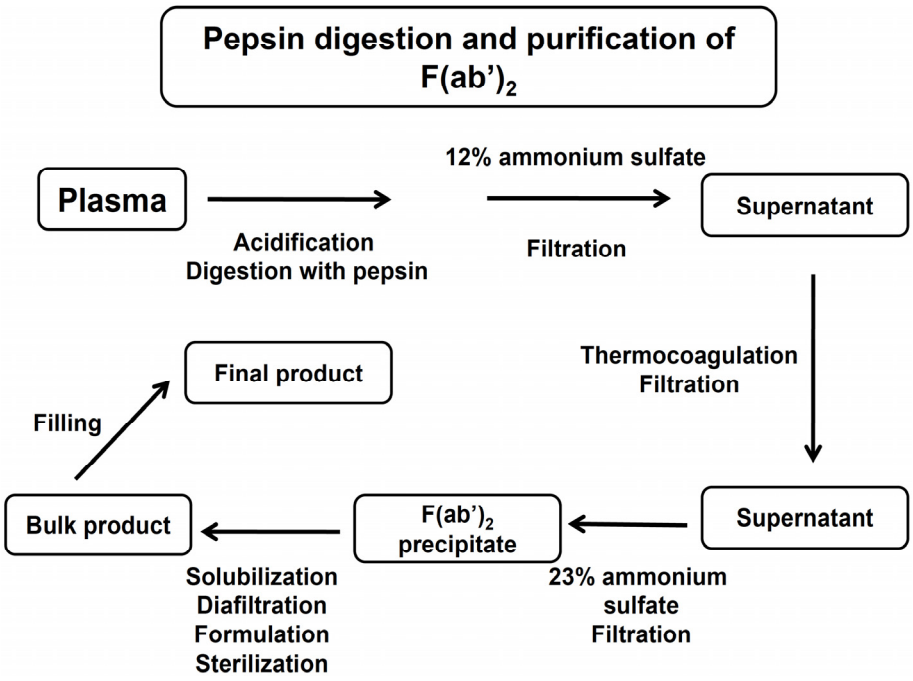


**Fig. (3).** Scheme of the basic methodology used for the manufacture of $F(ab')_2$ fragment antivenoms by pepsin digestion at acid pH, thermocoagulation and ammonium sulfate precipitation.

inactivation of viruses, such as pasteurization [10], nanofiltration [56] or solvent-detergent treatment [57]. Any innovation in the technology of antivenom production should be carefully analyzed from a cost-benefit perspective, in which the involved increments in production costs should be considered *vis-à-vis* the need to make antivenoms accessible at affordable prices to the countries and regions that require these immunobiologicals.

*Monoclonal antibodies*: Monoclonal antibodies of high affinity and neutralizing capacity can be generated against snake venom toxins (see review [58]). However, the application of hybridoma technology for antivenom production has the disadvantage that most snake venoms are comprised of several hundred proteins, many of which are likely to play a role in toxicity of medically-relevant species [59, 60]. This would require a combination of several monoclonal antibodies, which increases the complexity of this approach. However, there are cases of venoms whose toxicity depends on a single or few toxins, thus opening a window of opportunity for monoclonal antibodies. Moreover, a monoclonal antibody preparation could be added to polyclonal antibody-based antivenoms to enhance the neutralizing titer against a particular toxin. The reactogenicity of monoclonal antibodies can be reduced by the generation of 'chimeric' or 'humanized' recombinant antibodies [61, 62].

*Recombinant antibody fragments*: Recombinant scFv fragments have been produced against some toxins [63-67]. Another alternative is the use of recombinant 'single domain antibodies' comprised by the variable region of the heavy chain-only camelid IgG [68, 69]. These and other strategies in the field of antibody engineering offer interesting possibilities for the design of neutralizing antibody fragments of high affinity that could be applied to antivenom production. One problem here is that the pharmacokinetic profile of such small neutralizing molecules is characterized by a rapid elimination, thus precluding their efficacy in the case of envenomings by snakes in which toxins may reach the bloodstream at later time intervals. This problem could be circumvented by the engineering of molecules with varying half-lives.

*Improvement of the stability of antivenoms*: Liquid antivenoms have to be stored at 2-8 ºC, thus precluding their distribution to places where the cold chain cannot be properly maintained, as in many low-income countries. Thus, the development of novel liquid antivenom formulations, stable at room temperature, is a highly relevant task. Possibilities include the use of stabilizers, such as sucrose or sorbitol [70, 71].

*Reducing the use of animals in the assessment of antivenom potency*: Despite the fact that the mouse lethality test remains as the gold standard for antivenom potency assessment [12, 19], there is a need to develop *in vitro* laboratory assays to assess the neutralizing ability of antivenoms, with the consequent reduction in the use of animals. Owing to the complexity and variability of snake venoms, this is a difficult goal. However, promising advances have been made using techniques such as enzyme immunoassays [72, 73], neutralization of enzymatic activities [74] and proteomic analysis of immunodepletion of venom components, a field known as 'antivenomics' [22]. Moreover, the use of fertile hens' eggs to assess toxicity of venoms, at stages that precede the development of pain sensitivity, has been described [75]. As a deeper knowledge on the biochemical composition and mechanisms of action of venoms is gained, novel *in vitro* assays should be developed to evaluate the neutralizing ability of antivenoms.

## ANTIVENOM EFFICACY

Antivenoms should be capable of neutralizing, in a timely fashion, the most relevant toxic components of venoms to which the antivenom is designed. Neutralization of venom toxins by antivenom antibodies can be

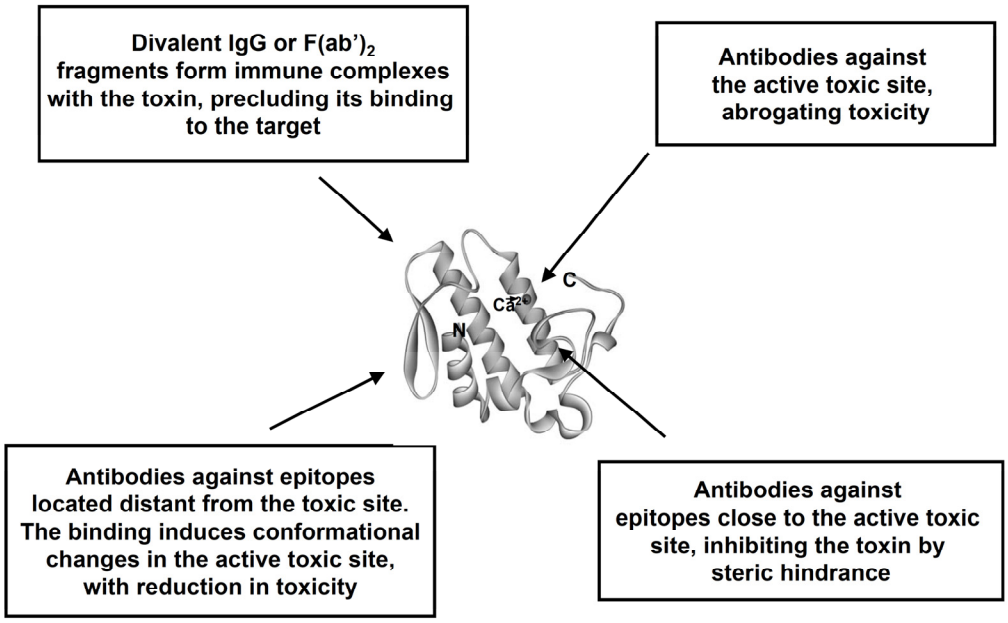


**Fig. (4).** Four mechanisms by which antivenom antibodies, or antibody fragments, may neutralize the toxic activity of snake venom toxins, here illustrated for the case of the phospholipase $A_2$ from *Agkistrodon piscivorus piscivorus* (PDB code: 1VAP [123]), The mechanism based in the formation of multimolecular immune complexes between antibodies and toxins (upper left) occurs only in the case of divalent IgG and $F(ab')_2$ fragments, but not in the case of monovalent Fab fragments. The other three mechanisms of neutralization operate for the three types of active substance (IgG, $F(ab')_2$ and Fab). The sphere in the structure represents the calcium atom of the active site of the phospholipase $A_2$ which is essential for catalytic activity. N corresponds to the N-terminus and C to the C-terminus of the protein. Adapted from [7].

accomplished by, at least, four different mechanisms: (a) Antibody paratopes recognize epitopes located in the toxic site of a particular toxin. (b) The epitope may be located in the vicinity of the toxic site, neutralization being accomplished by steric hindrance. (c) Antibodies bind to an epitope at a molecular region distant from the toxic site, but antibody binding induces conformational changes in the toxin that reduce its capacity to interact with targets and to provoke toxicity. (d) Divalent IgG and $F(ab')_2$ molecules are able to form multivalent immunocomplexes with venom toxins, limiting the ability of toxins to interact with their targets and promoting the elimination of immunocomplexes by the mononuclear phagocyte system (Fig. **4**).

## Preclinical Testing of Antivenom Efficacy

The traditional way to assess the neutralizing potency of antivenoms is by determining their ability to neutralize the lethal activity of a venom in experimental animals, usually mice. For this, a 'challenge dose' of venom is selected (usually 3 to 5 Median Lethal Doses, $LD_{50}$). This dose of venom is incubated with various dilutions of the antivenom and then aliquots of the mixtures, containing the challenge dose of venom, are injected in animals. Deaths are recorded and results are expressed as the Median Effective Dose ($ED_{50}$), defined as the volume of antivenom, or the antivenom/venom ratio, in which 50% of the injected mice survive [12, 76]. Neutralizing activity is usually expressed in terms of mg venom, or number of $LD_{50}$s, neutralized per mL antivenom. Most laboratories use the intravenous route in the assessment of antivenom $ED_{50}$ [76], although other laboratories prefer the intraperitoneal route [77, 78]. Since this protocol, i.e. incubating venom and antivenom prior to injection, does not reproduce the actual circumstances of snakebite envenomings, some authors have used an alternative protocol based on the injection of venom in mice followed by the intravenous administration of antivenom [79]. However, the standardization of this procedure is not simple and, therefore, the protocol based on preincubation of venom and antivenom is used world wide in the assessment of antivenom neutralizing efficacy [19, 76].

Although the neutralization of lethal activity is the most important parameter for testing the capacity of antivenoms to neutralize snake venoms, the complex pathophysiology of snakebite envenoming involves various toxic effects whose neutralization should be tested for an adequate assessment of antivenom neutralizing efficacy. In the case of many viperid snake venoms, for instance, relevant effects include hemorrhagic, coagulant, defibrinogenating, myotoxic and necrotizing activities. A series of simple *in vivo* and *in vitro* laboratory tests have been developed to quantify these toxic activities and to analyze their neutralization by antivenoms [19, 20, 80, 81]. The basic protocol for these assays involves the selection of a challenge dose of venom and the incubation of this dose of venom with various dilutions of antivenom, followed by their testing in the corresponding assays systems. In this way, an overall picture of the antivenom neutralizing profile is achieved. It has been observed that some antivenoms are capable of neutralizing the lethal effect of a venom, but they are ineffective in the neutralization of a specific toxic effect. This principle is illustrated when anti-*Bothrops* sp antivenoms in Latin America are confronted with venoms of *Lachesis* sp. In this case, antivenoms are effective in the neutralization of lethality, but are ineffective in the neutralization of the clinically-relevant coagulant and defibrinogenating effects

induced by *Lachesis* sp venoms [82, 83]. In the case of venoms, such as those of many species of the family Elapidae, whose main toxic effect is neurotoxicity, the neutralization of lethality is a good parameter to assess the efficacy of antivenoms. Many studies have been published on the preclinical assessment of antivenom efficacy, evidencing a large extent of cross-reactivity in some cases, and the lack of neutralization of heterologous venoms in others [77, 84-88]. Such type of preclinical testing is required when a new antivenom is developed or when an existing antivenom is introduced in a new geographical setting.

### Clinical Assessment of Antivenom Efficacy

The definitive proof of the efficacy and safety of antivenoms for the treatment of snakebite envenoming has to be shown in the clinical setting. Consequently, the design and development of clinical trials, including the appropriate training of medical and nursing staff in the performance of these trials, should be actively promoted. Initially, phase II small-scale clinical trials are performed, involving a small number of envenomed patients [48, 89]. Placebo-controlled trials are generally unacceptable on ethical grounds for the clinical testing of antivenoms. The demonstration of antivenom efficacy should be carried out in phase III clinical trials which, ideally, should be controlled, randomized, blinded trials involving a large number of patients. Such types of trials have been performed with antivenoms since the 1970s [90], and a growing number of such studies have been published in various parts of the world [41, 91-93]. In some cases, however, open-label studies are performed, some of which compare results with historical controls [94]. Clinical trials should be based on robust clinical end-points to determine efficacy, such as halting of hemorrhagic manifestations and recovery of coagulation parameters in viperid envenomings.

Clinical trials have demonstrated that, in general terms, antivenoms are highly effective in correcting local and systemic hemorrhage and clotting disturbances induced by viperid venoms and in preventing severe neurotoxic effects induced by elapid and some viperid venoms, provided antivenoms are administered in a timely fashion [95]. In contrast, the efficacy of antivenoms in the neutralization of toxins inducing local tissue damage and in reversing neurotoxic manifestations induced by presynaptically-acting neurotoxic phospholipases $A_2$ is more limited [95]. In the case of local tissue damage, the rapid onset of these effects, and the often prolonged delay in antivenom administration, determine that, by the time antivenom is infused, significant local tissue pathology has already developed [95, 96].

## ANTIVENOM SAFETY

### Adverse Reactions to Antivenom Administration

The intravenous administration of animal-derived antivenoms may result in adverse reactions of variable severity [97, 98]. The incidence of such reactions varies depending on the product being administered, and may range from less than 6% to over 70% of the cases [99-101]. Adverse reactions to antivenoms may be pyrogenic reactions, resultant from the contamination of antivenoms with bacterial lipopolysaccharides [100]. However, since pyrogen testing is a routine procedure in quality control laboratories, these reactions are seldom reported. True anaphylactic reactions, i.e. reactions mediated by IgE, are also infrequent because most people receiving antivenom have not been previously sensitized to horse immunoglobulins. The most frequent adverse reaction corresponds to what has been called 'early adverse reaction' (EAR), which occurs within the first hours of antivenom infusion and is characterized by manifestations such as urticaria, itching, bronchospasm, angioedema, colic, nausea and hypotension [97]. Such EARs occur in people not previously sensitized with horse IgG and their incidence greatly varies among different products. These reactions are treated with antihistamines, steroids and adrenaline [41, 98, 99, 102].

The mechanisms involved in EARs have not been clearly identified. It has been proposed that antivenom-induced complement activation plays a key role in the onset of this reaction; the ability of antivenoms to activate human complement *in vitro* has been repeatedly demonstrated [41, 103-105]. However, the evidence of *in vivo* complement activation is not so clear [106]. It has been assumed that the cleavage of Fc fragment from horse IgG, by either pepsin or papain digestions, would reduce the anticomplementary effect of antivenoms and, consequently, would decrease the EARs to antivenom administration. Although *in vitro* evidence supports the concept that $F(ab')_2$ antivenoms have less anticomplementary activity than whole IgG antivenoms [104], $F(ab')_2$ antivenoms are still able to activate complement [104, 107]. More importantly, clinical trials have evidenced that some $F(ab')_2$ antivenoms and caprylic acid-fractionated IgG antivenoms induce a similar incidence of EARs [41, 100, 108]. It has been proposed that the critical issue in the generation of EARs by antivenoms lies in the physicochemical characteristics of these products, such as total protein concentration, presence of antibody aggregates, and presence of excipients, such as preservatives, that may contribute to these reactions [41, 104, 105, 109]. Thus, strategies aimed at further reducing the incidence of EARs to antivenom administration should be based on the development of products of good physicochemical profile and low protein concentration.

In addition to complement activation, other mechanisms have been suggested as playing a role in the onset of EARs, such as: (a) The potential deleterious effects of preservatives included in the formulation of antivenoms [109]. (b) The presence of antibodies in antivenoms that react with blood cells and endothelium; this has been demonstrated in the case of anti-human erythrocyte antibodies in a number of antivenoms [110]. (c) The presence, in human plasma, of antibodies against horse IgGs, which develop probably as a consequence of sensitization to horse proteins through food ingestion or exposure to dander [111-113]. The presence of human antibodies against antivenom IgGs has been demonstrated [114]. In turn, antivenoms may also have antibodies against human plasma proteins. These immunological interactions may result, upon antivenom administration, in the formation of immune complexes, followed by complement activation and the generation of pharmacologically-active mediators that may participate in

the pathogenesis of EARs. Despite the likeliness of this mechanism, a study with clinical samples did not find a correlation between the titer of anti-horse IgG antibodies and the development of EAR in patients bitten by snakes and receiving antivenom [114]. It is likely that several mechanisms are involved in EARs, and further research is necessary to identity them and to ascertain their role in this phenomenon.

In addition to EARs, antivenom administration is also associated with late adverse reactions (LARs), which develop between 5 and 14 days after antivenom infusion and correspond to the typical type III hypersensitive phenomenon known as 'serum sickness' [115, 116]. It is based on the formation of immune complexes between antivenom IgG and human IgGs synthesized against horse IgG. Such complexes, after deposition in blood vessels and joints, induce complement activation, generation of anaphylatoxins and attraction of leukocytes, all of which contribute to the clinical manifestation of serum sickness, i.e. urticaria, itching, fever, arthralgia, myalgia and lymphadenopathy [102]. The incidence of LARs correlates with the dose of antivenom administered, i.e. the total load of foreign protein injected [116].

### Microbial Safety of Antivenoms

Antivenoms should be free of bacterial and fungal contamination, and some antivenoms contain preservatives in their formulation, such as phenol or cresols, to prevent microbial contamination [19]. Sterility is achieved through good manufacturing practices and by a membrane filtration step before dispensing in the final container. Animal-derived antivenoms have never been shown to transmit viruses to humans; however, recent reports of viral zoonotic diseases have raised concerns on the viral safety of antivenoms [19]. Horses and sheep harbor many types of viruses, some of which are pathogenic to humans [19, 117]. Consequently, the need to include steps in the manufacture of antivenoms aimed at removing or inactivating viruses in the final product is being considered. This includes a careful selection and monitoring of the animals used in antivenom production, including clinical examination, hematological and clinical chemistry laboratory testing, and screening for viruses using immunological and molecular tests [117]. In addition, the introduction of viral inactivation or removal steps in the fractionation protocol should be considered. Fortunately, some of the currently used steps in the purification of antivenoms, such as pepsin digestion at low pH and caprylic acid addition, are highly effective for viral inactivation, especially against enveloped viruses [117, 118]. In some cases, additional viral inactivation or removal procedures could be introduced in antivenom manufacture, such as pasteurization [10], storage and formulation at acid pH [117] and nanofiltration [56].

## HOW TO ENSURE THAT ANTIVENOMS REACH THE PEOPLE THAT NEED THEM

Despite significant advances in our understanding of venom composition and variability and in the technological platforms for antivenom manufacture, there is a growing concern for the inaccessibility of antivenoms in many regions of the world, particularly in sub-Saharan Africa, some regions of Asia and Latin America, and Papua-New Guinea [13, 14, 119, 120]. Therefore, the issue of antivenom manufacture and distribution clearly goes beyond the scientific and technological realms, as it involves political, economic and public health considerations as well.

It is necessary to ensure that antivenoms are accessible, at affordable prices, to low-income countries where they are needed. This includes international cooperative efforts to enhance the technological capacity of local antivenom manufacturing laboratories, together with innovative purchasing schemes, involving public and private sectors [120]. Moreover, the issue of antivenom distribution within countries is a critical one, since it should be based on a rigorous epidemiological account of snakebite envenomings and the identification of the most vulnerable regions requiring antivenoms. The use of geographical information system (GIS) technologies [121] and the introduction of compulsory notification of snakebite cases represent necessary tools to have an accurate picture of the areas where antivenoms have to be deployed. Issues associated with antivenom distribution are closely related to antivenom stability and with a meticulous analysis of the cold chain system in different regions, especially in remote rural areas where these accidents are frequent, in order to decide which type of antivenom preparation is most suitable for each region. Furthermore, the strengthening of public health systems, particularly in rural areas where snakebites are frequent, is also mandatory, since many regions of the world are characterized by the absence of health posts in areas of high incidence of snakebites. Finally, the adequate training of health staff, including physicians, nurses and other personnel, on the correct diagnosis of snakebite envenoming and on the adequate use of antivenom is especially important, because even if antivenom is accessible, its incorrect use may result in clinical consequences or in the misuse of this precious drug [120, 122]. Thus, the issue of antivenom manufacture and control has to be closely linked and integrated with public health areas dealing with antivenom accessibility and its correct distribution and use in regions of the world characterized by a high incidence of snakebite envenoming. On a broader perspective, the issue of public education on the prevention and management of snakebite envenomings, especially in rural areas of high snakebite incidence, should complement the tasks discussed above, since a timely transportation of patients to health centers is a crucial aspect in the management of these envenomings. A concerted global effort, being currently promoted by the World Health Organization [14] and by the Global Snake Bite Initiative of the International Society on Toxinology [119], is necessary to fulfill these goals.

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