Received: July 4, 2008
Accepted: August 20, 2008
Abstract published online: October 13, 2008
Full paper published online: February 28, 2009

J Venom Anim Toxins incl Trop Dis.
V.15, n.1, p. 2-18, 2009.
Review article.
ISSN 1678-9199.

SNAKE ANTIVENOMS: ADVERSE REACTIONS AND PRODUCTION TECHNOLOGY

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ABSTRACT: Antivenoms have been widely used for more than a century for treating snakebites and other accidents with poisonous animals. Despite their efficacy, the use of heterologous antivenoms involves the possibility of adverse reactions due to activation of the immune system. In this paper, alternatives for antivenom production already in use were evaluated in light of their ability to minimize the occurrence of adverse reactions. These effects were classified according to their molecular mechanism as: anaphylactic reactions mediated by IgE, anaphylactoid reactions caused by complement system activation, and pyrogenic reactions produced mainly by the presence of endotoxins in the final product. In the future, antivenoms may be replaced by humanized antibodies, specific neutralizing compounds or vaccination. Meanwhile, improvements in antivenom quality will be focused on the obtainment of a more purified and specific product in compliance with good manufacturing practices and at an affordable cost.

KEY WORDS: snake antivenom, anaphylactic reaction, complement system activation, endotoxins.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Antivenoms have been used successfully for more than a century and up to now constitute the only effective treatment for snakebites and envenomations by other poisonous animals (1-6). Unfortunately, these products are composed of antibodies from immunized animals; hence, the use of heterologous proteins for human treatment involves the possibility of adverse reactions due to activation of the immune system. Unfavorable effects may range from mild symptoms like chills, nausea and fever, to serious problems such as bronchospasms and anaphylactic shock, even when the most refined antivenoms are administered (7-9).

Technologies for snake antivenom production vary according to the production laboratory, even in the same country. Classically, protocols that yield the $F(ab')_2$ fragment, based on pepsin digestion followed by ammonium sulfate fractionation (10) have been used worldwide. More recently, a caprylic acid purification process has been adopted by some countries, including Costa Rica (11, 12) and Thailand (13), while a chromatographic process alone has become the norm in other regions, like Europe and the United States (7, 8, 14). The $F(ab')_2$ product is preferred in Brazil (11, 15), Argentina, Mexico and also in India (16). Whole immunoglobulin antivenoms are used in some countries like Costa Rica, generally associated with the caprylic acid or ammonium sulfate processes. A product based on the Fab fragment is also used in the United States version of the antivenom (7, 8).

In the present study we discuss the possible mechanisms that trigger these side reactions and the possibilities that antivenom-producing laboratories have to minimize or control these difficulties through processing alternatives.

TYPE I HYPERSENSITIVITY (IMMEDIATE HYPERSENSITIVITY, ANAPHYLACTIC REACTION)

This is an early adverse reaction mediated by IgE antibodies, reactive to specific antigens, that are attached to basophil or mast cell Fc receptors (FcεR). The crosslinking of the cell-bound IgE antibodies by the antigen induces degranulation and determines the release of histamine and other pharmacological mediators that lead to several actions including increased vascular permeability, vasodilatation, bronchial and visceral smooth-muscle contraction, mucous secretion and local inflammation (17-19). The systemic presence of antigens, like those existing in heterologous antivenoms, can generate anaphylactic shock. It is an immediate

hypersensitivity reaction characterized by edema in several tissues and drop in blood pressure, secondary to vasodilatation (18). This response usually occurs in atopic subjects previously sensitized by some antivenom component.

Although the probability of anaphylaxis depends on the patient's sensitivity, the production laboratory can implement various processing alternatives to minimize the occurrence of this adverse reaction. The presence of impurities in antivenoms increases the possibility of anaphylactic shock due to IgE antibodies against these subtances (mainly other heterologous animal proteins), especially in atopic individuals. Sera incorrectly purified, or with excessive total protein load, can contribute to the development of this reaction.

Purification of immunoglobulins by caprylic acid precipitation of non-IgG plasma proteins, presently offers a cost-effective alternative, which some laboratories have already adopted or are developing (12, 20). The method fabricates a product with a relatively high purity in one step, which makes it suitable as a standalone process, or as the initial step of a purification chain (12, 21). Furthermore, caprylic acid purification reveals a high quality-cost ratio, making it suitable for most countries, even the poorest ones (20, 22). If major expenses are permitted, the use of affinity and ionic exchange columns to purify the final product may be convenient and in fact, it has been proposed to improve its purity (13, 23). Purification by chromatographic techniques alone has already been proposed (14, 24), but with the drawback of a substantially higher cost (25).

The utilization of more specific antibodies reduces the amount of total protein injected into the patient, thereby reducing the probability of anaphylactic shock, so that improvements in immunization schedules and procedures are important to obtain plasma with higher activity per protein unit (specific activity) (24, 26, 27). Several groups have been seeking alternative methods to prepare toxoids through venom detoxification, which would maintain its immunogenicity and minimize the damage to serum-producing animals (28, 29). In the processing field, several authors have proposed the use of affinity chromatography to purify only neutralizing antibodies (30) or IgG isotypes with better neutralizing potency, like horse IgG(T) (14, 31-34). In both cases the total amount of protein injected into the patient would decrease, without losing the required potency.

For more than 100 years, horses have been the animal of choice for production of antivenom and other antisera (4, 5, 10, 35-37). Therefore, many patients are

presently sensitive to horse proteins and at risk for anaphylactic reaction after a second contact. Moreover, horse IgG(T) is highly glycosylated and more immunogenic than other antibody isotypes (2, 38). For patients known to be sensitive to equine proteins, avoidance of antivenoms prepared in this manner is mandatory and should reduce the incidence of anaphylactic shocks. Some production laboratories are now successfully using other animals like sheep as sources of sera (7, 28, 39). Recent studies indicate the use of camels for antivenom production, due to lower *in vitro* complement activation than found in horses and sheep (40, 41). It would be an appropriate option for countries where camels are available (24, 41). At this point, it is important to note that even when human antibodies are employed for treating other diseases, like intravenous immunoglobulins (IGIV), anaphylactic shock and other adverse reactions could still occur (42).

Snake venoms are a complex mixture of peptides, proteins and enzymes, some of which are responsible for their toxic effects. Therefore, several different types of antibodies are needed to neutralize these toxic substances. A promising future alternative for treatment with specific monoclonal or humanized antibodies is likely (24, 43-46). The production of humanized antibodies like single-chain Fv expressed in philamentous phage could replace heterologous antisera, thereby reducing the probability of adverse reaction (47). Studies identifying toxicity factors and the similarity among toxin structures allow the production of antibodies that confer cross neutralization and are a good starting point to develop monoclonal therapy and vaccines (48, 49). Several complex steps are required to develop an effective vaccine against snake venom including: the identification of toxins associated with damaging or even lethal effects from the mixture of peptides and proteins which constitutes snake venoms (50), the detoxification by either chemical or genetic means of vaccine production, the achievement of an effective and safe humoral immune response and, above all, the financial support to carry out all the necessary assays for vaccine development.

Other compounds may be used instead of neutralizing antibodies (51-53). Natural products from plants of different regions, some utilized for centuries in traditional medicine, have been studied (54). Chemical compounds from natural plants – like terpens, steroids and flavonoids – were assayed to determine the inhibition of lethality of *Bothrops jararaca* venom in mice. Although some of the compounds showed a degree of neutralizing activity, none reached 100% protection (55).

Numerous investigations are designed to understand the innate resistance of some animals to snake venoms. Some compounds have been isolated from snakes, opposums and other animals with promising results (56-60). However, the use of substances other than immunoglobulins may not guarantee the absence of anaphylactic reactions. In these cases, the research is at the preclinical level, so results on adverse responses are still unavailable.

COMPLEMENT SYSTEM ACTIVATION (CSA)

Anaphylotoxins, namely C3a, C4a and C5a, are low-molecular-weight active peptides that are generated by complement system activation. They stem from C3, C4, C5 serum complement proteins and are created during complement fixation by Ag-Ab complexes, immunoglobulin aggregates and others. The anaphylotoxins (mainly C5a and to a lesser extent C3a and C4a) stimulate chemotaxis, neutrophil activation and degranulation of basophils and mast cells, with release of pharmacologically active mediators of immediate hypersensitivity (61, 62). The net effects of these activities are histamine- and leucotriene-mediated contraction of vascular smooth muscle, increased vascular permeability and migration of neutrophils and monocytes from blood vessels (19, 61, 62).

CSA by the Fragment Fc of Heterologous Antibody

In the past, it was presupposed that the presence of Fc fragments in antivenoms was the only, or the most important, cause of anaphylotoxic reactions (63). However, a number of clinical trials have yielded controversial results about the efficacy of snake antivenoms constituted by $F(ab')_2$ and Fab (2, 7, 9, 31). Additionally, clinical studies with whole IgG antibodies produced by caprylic acid purification revealed a relatively low incidence of early adverse reactions (9, 64). These conflicting results can be explained by the presence of variable aggregate levels in the product.

Recently, Otero *et al.* (65) reported a clinical trial that compared two antivenoms composed of IgG processed by caprylic acid purification, with similar aggregate concentration. One of which was treated with β-propiolactone, known to inhibit the complement activation (66). Although the β-propiolactone treated antivenom showed *in vitro* minor anticomplementary activity, no significant difference was found in the number of adverse reactions detected in patients.

CSA by Protein Aggregates

The presence of protein aggregates can also provoke complement system activation. Such aggregates found in antivenoms are originated mainly in the immunoglobulin fraction during production. Aggressive treatments like pepsin digestion and low pH lead to immunoglobulin denaturation that, in turn, diminishes activity and augments aggregate levels (67-69). Given that the presence of Fc fragments has been questioned as an important cause of adverse reactions, pepsin digestion of immunoglobulins should be reconsidered as a necessary process step, due to the important increase of aggregate levels achieved. Despite this, if immunoglobulin digestion is desired, another step to eliminate aggregates should be included (68, 69).

Purification processes have also been implicated in aggregate formation. The second precipitation and resuspension steps that take place in the classic ammonium sulfate fractionation protocol for an IgG or $F(ab')_2$ product (10), seem to increase the aggregates level when compared to caprylic acid purification, in which the immunoglobulins remain in a soluble form continuously (12, 22, 24, 64).

Another important cause of aggregate formation is long storage duration. The final product will lose activity if stored several years due to antibody denaturation, which, in turn, gives rise to an increased aggregate level. Storage temperature of antivenoms in liquid form is probably an important factor in antibody denaturation and increasing aggregate formation. Rojas *et al.* (70) found that antivenom storage at 20°C or more for a year increased the aggregate level as compared with 4°C storage. Furthermore, García *et al.* (63) found differences in the augmentation of aggregate levels among antivenoms stored for three years with different preservatives, meaning that these substances could accelerate the normal denaturation process.

CSA by Immune Complexes (Type III Hypersensitivity)

In 1905, Pirquet and Schick studied the side effects caused by the administration of large quantities of a foreign serum containing antitoxins, a technique used mainly for the treatment of diphtheria and tetanus. In particular, they stressed the fact that symptoms appeared more rapidly after a second exposure to the foreign serum than after the first administration. Their clinical descriptions included fever and rashes, and

some reports of kidney damage with proteinuria, lymphadenopathy and joint symptoms (71).

Type III hypersensitivity is mediated by antigen–antibody complexes. As a consequence of antivenom administration, the patient's immune system reacts by producing antibodies that attach to foreign antibodies (antivenom), resulting in the formation of an immune complex. Such complexes can stimulate an acute inflammatory response that leads to complement activation and leukocyte infiltration, the so-called "serum sickness" syndrome.

This is a systemic late adverse reaction characterized by vasculitis, glomerulonephritis and arthritis due to intravascular formation and deposition of immune complexes that subsequently fix the complement and initiate the hypersensitivity reaction. Patients may develop fever, lymphadenopathy, urticaria and arthritis (19). The classic reaction, that occurs 7 to 15 days after the triggering injection, is known as the primary form of serum sickness. Similar manifestations that appear in a few days following the injection represent the accelerated form of serum sickness, which presumably occurs in subjects already sensitized.

To diminish the incidence of this reaction, it is important to reduce antivenom reactivity to the immune system. In this way, the solutions proposed above to attenuate type I anaphylactic reaction may be useful in this case as well. León *et al.* (72) showed that equine IgG induces a higher anti-immunoglobulin response in mice, in comparison to $F(ab')_2$. Nevertheless, the digestion process gives rise to an important activity loss through antibody denaturation so the amount of foreign protein present in a dose of $F(ab')_2$ antivenom should be larger than that in a whole IgG-based antivenom (67). Therefore, by considering that the total amount of heterologous protein administered plays a more determinant role in the occurrence of serum sickness than the type of antibody preparation utilized, it is doubtful to establish *a priori* which molecule induces less response (72). Alternatives including vaccination and the use of humanized antibodies or other neutralizing substances may be the best answer in a near future.

PYROGENIC REACTIONS

Antivenom contamination by endotoxins is the main cause of pyrogenic reactions in patients. Bacterial endotoxins consist of lipopolysaccharide, a major component of the outer cell membrane of gram-negative bacteria. Endotoxins present strong

biological effects on humans and other mammals when reach their bloodstream during bacterial infection or via intravenous application of a contaminated medicine.

Endotoxins are known to cause fever at very low doses and septic shock at higher doses (73). The threshold level of endotoxin for intravenous applications is set to 5 endotoxin units (EU) per kilogram of body weight per hour. The term EU describes the biological activity of an endotoxin (74). The molecular mechanism of toxicity is not completely understood but it has been related to the interaction with TLR4 (Toll-like receptor 4) and/or LPB (LPS binding protein) receptors and to the activation of monocytes and other cell components of the immune system that carry out TNF and other cytokine production (75-79).

Higher levels of endotoxins are related to bacterial infection or digestive tract injuries, but contamination at low concentrations can be found in pharmaceutical products. Contamination of antivenom products with endotoxins take place if preventive measures are not followed during processing (80). The presence of low doses of endotoxins in antivenoms generate an important increase of mild, early adverse reactions (generally fever) in patients (9). To avoid endotoxins, the production laboratories must implement strict quality requirements in facilities, raw materials, process systems and equipment.

Endotoxins are very stable molecules of varying size; their biologically active part can survive extremes of temperature and pH in comparison to proteins. Temperatures from 180 to 250°C and acids or alkalis of at least 0.1 M must be chosen to destroy endotoxins in laboratory equipment (74). Therefore, it represents a challenge to remove endotoxins from biological fluids including proteins. In addition to this, endotoxin shows a strong association with proteins, so steps that involve protein concentration also involve endotoxin concentration and steps that involve protein purification of other protein involve endotoxin elimination (81). Thus, ammonium sulfate fractionation process tends to increase endotoxin level more than the caprylic acid purification of immunoglobulins in a production system, not only because of a higher endotoxin level in the raw materials and a longer process time, but also due to a specific concentration of endotoxins in the final precipitate, which corresponds to the IgG fraction (81).

Finally, if a product is accidentally contaminated and fails to pass the quality control, it should be discarded or reprocessed. Decontamination is a costly alternative, so avoiding endotoxin contamination must be the preferred choice (74). However, in

unexpected cases, it is absolutely necessary to count with a decontamination procedure in order to save a given production batch that otherwise would be discarded. With this goal, several systems including ultrafiltration membranes and chromatography resins coupled to different ligands have shown good capacity to capture and remove endotoxins (73, 74, 82-84). Unfortunately, the use of these systems involve variable yield losses, so this is another reason why they should be applied only to save occasional production batches but not as routine (85).

CONCLUDING REMARKS

In the past century, antitoxic sera were widely used for diphtheria, tetanus and treatment of accidents with poisonous animals (10, 35-37, 71). Nowadays, for tetanus and diphtheria treatments, the antitoxic sera have been replaced by vaccination, antibiotic therapy and human neutralizing antibodies, but for treating envenomation by snakes and others animals, heterologous antivenoms still remain the only effective solution.

Possibly in a near future, antivenoms will be replaced by vaccination and humanized antibodies or/and specific neutralizing compounds. Meanwhile the improvement in antivenom quality must focus on the increase of product purity and the reduction of aggregates, as well as on the implementation of good manufacturing practices (GMP). Unfortunately the incorporation of refined purification techniques to antivenom production process and others commercials factors have carried on an important cost increase, thereby causing an strong antivenom shortage specially in the poorest countries (25, 86-89). The best solution includes best quality antivenoms at an affordable cost (20, 22).

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