



Review

Pathogenic mechanisms underlying adverse reactions induced by intravenous administration of snake antivenoms



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ABSTRACT

Snake antivenoms are formulations of immunoglobulins, or immunoglobulin fragments, purified from the plasma of animals immunized with snake venoms. Their therapeutic success lies in their ability to mitigate the progress of toxic effects induced by snake venom components, when administered intravenously. However, due to diverse factors, such as deficient manufacturing practices, physicochemical characteristics of formulations, or inherent properties of heterologous immunoglobulins, antivenoms can induce undesirable adverse reactions. Based on the time lapse between antivenom administration and the onset of clinical manifestations, the World Health Organization has classified these adverse reactions as: 1 – Early reactions, if they occur within the first hours after antivenom infusion, or 2 – late reactions, when occurring between 5 and 20 days after treatment. While all late reactions are mediated by IgM or IgG antibodies raised in the patient against antivenom proteins, and the consequent formation of immune complexes, several mechanisms may be responsible for the early reactions, such as pyrogenic reactions, IgE-mediated reactions, or non IgE-mediated reactions. This work reviews the hypotheses that have been proposed to explain the mechanisms involved in these adverse reactions to antivenoms. The understanding of these pathogenic mechanisms is necessary for the development of safer products and for the improvement of snakebite envenomation treatment.

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1. Introduction

Snake antivenoms are formulations of immunoglobulins, or immunoglobulin fragments, purified from the plasma of animals immunized with snake venoms. Their parenteral administration is the mainstay treatment for snakebite envenomation (WHO, 2010a), which is an important, albeit neglected, health problem in many regions of the world (Kasturiratne et al., 2008; Williams et al., 2010; Gutiérrez, 2012). Antivenom immunoglobulins bind and neutralize, through various mechanisms, the toxins

present in the venoms which are responsible for the pathophysiological alterations associated with these envenomations (Gutiérrez et al., 2011).

In addition to their therapeutic effect, snake antivenoms can also induce undesirable effects, whose incidence and severity vary between different products (Gutiérrez et al., 2011). The pathogenesis of adverse reactions to antivenoms is not entirely understood. However, it has been related to: 1 – Factors depending on the manufacturing practices, such as contamination of the formulation with endotoxins (Acconci et al., 2000) or viruses (Burnouf et al., 2004; WHO, 2010a); 2 – factors depending on the physicochemical characteristics of the antivenom, such as purity (Segura et al., 2013) and content of protein aggregates (Frommhagen and Fudenberg, 1962); and 3 – factors

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depending on the immunochemical characteristics of heterologous immunoglobulins of antivenoms, such as anticomplementary activity (Sutherland, 1977) and immunogenicity (Herrera et al., 2005; León et al., 2008; Sevcik et al., 2008; Redwan et al., 2009).

Studies on adverse reactions induced by antivenoms have been previously discussed, but conclusive evidences on their mechanisms have not been reached (Lalloo and Theakston, 2003; Morais and Massaldi, 2009; Gutiérrez et al., 2011). Moreover, new findings on this subject have been published, and discrepancies regarding the nomenclature used to designate the different types of adverse reactions have appeared. In this context, the present work presents an updated view of the pathogenic mechanisms involved in the adverse reactions occurring after the administration of snake antivenoms.

2. Nomenclature of adverse reactions

In 1963, Gell and Coombs proposed a nomenclature to classify allergic reactions based on the mechanisms of the underlying disease (Gell and Coombs, 1963). Afterward, they used this arrangement to classify drug-induced hypersensitivities (Coombs and Gell, 1968). According to the Gell-Coombs classification, adverse reactions induced by antivenoms correspond to type I or type III hypersensitivities, depending on whether the antibody mediating the reaction is IgE or IgG, respectively.

In 1981, the World Health Organization (WHO) classified the adverse reactions to antivenoms on the basis of the time lapse between antivenom administration and clinical manifestations of undesirable effects. Such classification includes: 1 – Early reactions, if they occur within 24 h after antivenom administration, or 2 – late reactions occurring between 5 and 24 days after antivenom administration. In turn, early reactions were classified as either anaphylactic or anaphylactoid, depending on whether the reaction is mediated by IgE (anaphylactic) or by other mechanisms (anaphylactoid) (WHO, 1981).

More recently, the World Allergy Organization (WAO) proposed a new global nomenclature for allergy, which is based on the mechanisms initiating the reaction (Johansson et al., 2004). According to this system, the terms “anaphylactic reactions”, “anaphylactoid reactions” and “late reactions” proposed by WHO (1981), are substituted by the terms “IgE-mediated allergy”, “Immediate hypersensitivity” and “IgG-mediated allergy”, respectively.

In 2010, the WHO published the Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins (WHO, 2010a). In this document, adverse reactions are classified as: 1 – early reactions (i.e. pyrogenic reactions and anaphylactic reactions), or 2 – late reactions (i.e. serum sickness). Moreover, the term “anaphylactic reaction” is used to refer to both “non-IgE-mediated reactions” and “IgE-mediated reactions”, thus eliminating the term “anaphylactoid reaction” (WHO, 2010a).

Nowadays, both the Gell-Coombs classification and the WHO classification published in 1981 are considered outdated (Descotes and Choquet-Kastylevsky, 2001; Rajan, 2003). Therefore, some researchers have implemented the WAO nomenclature to describe the adverse reactions

induced by antivenoms (e.g. Isbister et al., 2008). However, many groups involved in the manufacture and study of antivenoms continue to use the nomenclature derived from the WHO classification published in 1981 (Chippaux et al., 2007; Williams et al., 2007; León et al., 2008; Simpson, 2008; Thiansookon and Rojnuckarin, 2008; Caron et al., 2009; Morais and Massaldi, 2009). For the sake of clarity and uniformity, the nomenclature proposed by the WHO guidelines published in 2010 is used in this work.

3. Early reactions

Early reactions to antivenom administration comprise the group of adverse manifestations which occur within 24 h after starting the intravenous administration of snake antivenoms. Early reactions can be classified as: 1 – Pyrogenic reactions, or 2 – anaphylactic reactions, depending on whether their pathogenesis involves the participation of bacterial endotoxins or the involvement of intrinsic (non-microbial) components, such as immunoglobulins, respectively.

According to clinical studies reported in the literature, most antivenoms induce early reactions in around 20% of the patients. However, there are products with incidence as high as 88%, or as low as 3% of patients (Table 1). There are no clinical studies reporting the absence of early reactions to antivenoms (Table 1). Such high variability in the incidence of early adverse reactions by antivenoms reflects the high heterogeneity in the safety profile of these products.

3.1. Pyrogenic reactions

Pyrogen is a term used to refer to any substance capable of inducing fever. Pyrogenic reactions (characterized by increase in body temperature, rigors, myalgia, headache, nausea, sweating, chills, increase of the heart rate, and vasodilation with secondary fall in blood pressure), occur during the first hour after initiating the administration of an antivenom contaminated with pyrogenic substances. Adrenaline and anti-pyretics are the standard treatments for this type of reaction. Implementation of Good Manufacturing Practices (GMPs) in antivenom production precludes the appearance of pyrogenic reactions by avoiding the contamination with microbial products (WHO, 2010a).

Many substances induce pyrogenic reactions. However, bacterial lipopolysaccharides (LPS) are the most common pyrogens in biologically-derived pharmaceuticals (Magalhães et al., 2007). LPS are integral components of the outer cell membrane of Gram-negative bacteria. They are amphiphilic molecules formed by 1) the hydrophobic lipid A, which is responsible for the biological activity of LPS; 2) the hydrophilic core oligosaccharide, which provides negative charges that affect the biological activity; and 3) the O-antigen, which contributes to the antigenic properties of the molecule (Hodgson, 2006).

During multiplication and death of bacteria, LPS are released to the environment within macromolecular complexes called endotoxins which, in addition of LPS, contain proteins and phospholipids (Hitchcock et al., 1986). In aqueous solutions, endotoxins form very high molecular

Table 1

Incidence of early (anaphylactic) adverse reactions induced by antivenoms, as reported in several clinical trials.

Manufacturer	Source	Active substance	Incidence of ears	Country of study	Reference
Polonga Tab™	Sheep	Fab	48%	Sri Lanka	Ariaratnam et al., 2001
Protherics UK Ltd	Sheep	Fab	5%	United States	Cannon et al., 2008
	Sheep	Fab	7%	United States	Farrar et al., 2012
Pasteur Merieux	Horse	F(ab') ₂	6%	Martinica	Thomas et al., 1996
Ipser Africa	Horse	F(ab') ₂	6%	Cameroon	Chippaux et al., 1998
Aventis Pasteur	Horse	F(ab') ₂	4%	Cameroon	Chippaux et al., 1999
Bioclon	Horse	F(ab') ₂	11%	Benin	Chippaux et al., 2007
SAIMR	Horse	F(ab') ₂	76%	South Africa	Moran et al., 1998
ViperFab	Horse	F(ab') ₂	13%	France	Haro et al., 1998
Instituto Butantan	Horse	F(ab') ₂	25%	Brasil	Hui et al., 1999
Haffkine	Horse	F(ab') ₂	43%	Sri Lanka	Premawardhena et al., 1999
Haffkine	Horse	F(ab') ₂	81%	Sri Lanka	Ariaratnam et al., 2001
Inst. Butantan	Horse	F(ab') ₂	18%	Brasil	Pardal et al., 2004
Fundação Ezequiel Dias	Horse	F(ab') ₂	19%	Brasil	Pardal et al., 2004
Vins Bioproduct	Horse	F(ab') ₂	81%	Sri Lanka	Gawarammana et al., 2004
Bioclon	Horse	F(ab') ₂	19%	Colombia	Otero-Patiño et al., 2007
Commonwealth Serum Laboratories	Horse	F(ab') ₂	18%	Papua New Guinea	Williams et al., 2007
Saovabha Memorial Institute	Horse	F(ab') ₂	3%	Thailand	Thiansookon and Rojnuckarin, 2008
Haffkine Bio-Pharmaceuticals	Horse	F(ab') ₂	88%	Bangladesh	Amin et al., 2008
Bharat Serums and Vaccines Ltd	Horse	F(ab') ₂	61%	Sri Lanka	Isbister et al., 2012
Instituto Clodomiro Picado	Horse	F(ab') ₂ (caprylic)	29%	Colombia	Otero-Patiño et al., 2012
Instituto Clodomiro Picado	Horse	IgG (sulphate)	52%	Colombia	Otero et al., 1999
Instituto Clodomiro Picado	Horse	IgG (caprylic)	25%	Colombia	Otero et al., 1999
Instituto Clodomiro Picado	Horse	IgG (caprylic)	26%	Nigeria	Abubakar et al., 2010
MicroPharm	Sheep	IgG (caprylic)	19%	Nigeria	Abubakar et al., 2010

weight aggregates. When the critical micellar concentration is reached, endotoxins form supramolecular structures, i.e. micelles (Gutsmann et al., 2007).

The pyrogenic reaction induced by endotoxins starts when the serum LPS binding protein (LBP) binds and transfers LPS aggregates from endotoxin micelles to the cluster of differentiation antigen 14 (CD14). CD14 is a protein that can be found in soluble form or anchored to the plasma membranes of monocytes and macrophages. CD14 assists in the transfer of LPS to a receptor complex formed by the myeloid differentiation 2 protein (MD-2) and the Toll-like receptor 4 (TLR4). Alternatively, MD-2 can directly bind the lipid A of LPS, to form the activated (TLR4/MD-2/LPS) complex (Yong-Cheng et al., 2008; Peri and Piazza, 2012).

As a result, monocytes and macrophages (primarily in liver and lungs) are induced to produce cytokines such as IL-1 β , IL-6, INF- γ and TNF- α (Ogoina, 2011). Through highly complex mechanisms, each one of these cytokines contributes to generate a response in the host. For example, IL-1 β travels through the bloodstream, stimulating cells of the *organum vasculosum* of the *lamina terminalis* (OVLT) to increase the synthesis of prostaglandin E2 (PGE2) (Ott et al., 2010). PGE2 diffuses across the blood–brain barrier and stimulates the heat sensitive neurons in the preoptic region of the anterior hypothalamus, which in response increase the set-point of the body thermal balance (Skarnes et al., 1981), thus contributing to the pyrogenic reaction.

3.2. Anaphylactic reactions

Anaphylactic reactions include a group of early adverse events, which in despite of being produced by different pathogenic mechanisms (e.g. IgE-mediated reactions or non

IgE-mediated reactions), show similar clinical manifestations (i.e. fever, pruritus, urticaria, tachycardia, hypotension, smooth muscle spasms, gastrointestinal symptoms, bronchospasm, respiratory collapse, angioedema, shock, and death).

The standard treatment for anaphylactic reactions consists of intravenous administration of histamine H1 antagonists (e.g. chlorpheniramine maleate, promethazine or dextrochlorpheniramine), histamine H2 antagonists (e.g. cimetidine or ranitidine), and hydrocortisone (Sutherland and Lovering, 1979; Brian and Vince, 1987; Cupo et al., 1991), although intramuscular administration of 0.1% adrenaline is frequently used as well (WHO, 2010b). On the other hand, clinical studies have shown that pre-treatment with anti-histamines or steroids do not prevent complement activation or the appearance of anaphylactic reactions (Bucarety et al., 1994; Hui et al., 1999). In contrast, administration of low doses of adrenaline is effective in preventing the development of anaphylactic reactions (Sutherland, 1992; Premawardhena et al., 1999; Williams et al., 2007; Habib, 2011; de Silva et al., 2011). Nevertheless, since depending on the dose and administration route adrenaline may induce hypertension, caution is recommended in cases of envenomations characterized by hemorrhage and coagulopathy due to the risk of intracranial hemorrhage (Premawardhena et al., 1999).

3.2.1. IgE-mediated reactions

Early IgE-mediated reactions are adverse events rarely reported as a consequence of antivenom administration. These reactions occur in patients who, due to previous exposition to animal immunoglobulins, produced IgE antibodies towards antivenom proteins. During antivenom administration, these IgE antibodies interacting with the

FcεR present in mast cells and basophils, recognize the antivenom proteins and induce cell degranulation. The release of preformed and *de novo* synthesized vasoactive mediators (e.g. histamine, leukotrienes, PAF, etc.) induce vasodilation and increase in vascular permeability, producing the characteristic clinical manifestations of anaphylaxis (WHO, 1981).

On the other hand, administration of antibiotics is commonly used in the treatment of infections in horses or other animals used to produce antivenoms. However, if this practice is not well controlled and programmed, it involves the risk of introducing antibiotics from animal blood in the final products, i.e. antivenoms. Administration of antivenoms containing traces of antibiotics could induce early reactions in patients presenting IgE towards antibiotics. For prevention, antivenom producers must remove the medicated animals from the bleeding process, until antibiotics have been cleared from blood (EMEA, 2002; WHO, 2010a).

Quarantine periods have been established for the clearance of antibiotics in animals used in the production of meat and milk, in order to prevent antibiotic ingestion and undesirable changes in the color or taste of these products (Stead et al., 2007). Nonetheless, there are not published studies which propose a quarantine period for antibiotic clearance in animals used for antivenom production. Therefore, investigations on the pathogenesis of early reactions observed during antivenom administration must consider the presence of antibiotic traces in the blood of animals used as immunoglobulin source. This is an aspect of the in-process quality control of antivenoms that needs to be strengthened.

3.2.2. Non IgE-mediated reactions

Non IgE-mediated reactions constitute the vast majority of early reactions induced by antivenoms. These reactions occur *de novo* in patients who have not been previously treated with antivenoms (Corrigan et al., 1978; Malasit et al., 1986; Cupo et al., 1991; Otero et al., 1999, 2006; Otero-Patiño et al., 1998, 2007, 2012). Since these reactions are not IgE-mediated, hypersensitivity intradermal tests are useless to predict their occurrence and, consequently, are not recommended (Malasit et al., 1986; Cupo et al., 2001; Thiansookon and Rojnuckarin, 2008).

Non IgE-mediated reactions have been related to antivenom administration using the intravenous route. In contrast, antivenom administration by the intramuscular route is usually not associated with early adverse reactions (Chippaux and Goyffon, 1998). However, antivenoms show the best pharmacokinetic and pharmacodynamic profiles when intravenously administered (Gutiérrez et al., 2003). Therefore, unless conditions do not permit the practice of intravenous infusion (e.g. administration of antivenom in settings different from medical centers), the intravenous route should be used when administering snake antivenoms. Some reports attribute anaphylactic reactions observed during antivenom administration to the velocity of infusion (Holstege et al., 2002). Therefore, a widespread practice in the therapy of snakebitten patients is the slow infusion of antivenom, often achieved by diluting the antivenom in physiological saline solution (Chippaux et al., 1998; Hui et al., 1999; Bentur et al., 2004; de Silva et al.,

2011; Otero-Patiño et al., 2012). However, clinical studies have provided evidence supporting the view that the speed of antivenom infusion does not correlate with the incidence of adverse reactions (Malasit et al., 1986; Isbister et al., 2012). Moreover, for some formulations, the fast intravenous injection (2 mL/min) of undiluted antivenoms induces acceptable incidence and severity of this type of anaphylactic reactions (Abubakar et al., 2010).

The pathogenic mechanisms involved in non IgE-mediated reactions have been investigated by many workers. Fig. 1 presents a time-table of some of the key achievements in the study of these reactions and in the technological efforts introduced to improve the safety of antivenoms. However, despite these research efforts, the pathogenesis of non IgE-mediated reactions is still incompletely understood. Two mechanisms proposed to explain these reactions are: 1 – Antivenom anticomplementary activity (ACA), and 2 – the presence of heterophilic antibodies.

3.2.2.1. Role of the antivenom anticomplementary activity (ACA) in non IgE-mediated reactions. The role of aggregated gamma-globulins in the ACA of human and animal sera was demonstrated in 1962 (Frommhagen and Fudenberg, 1962). In the same year, it was observed that ACA is related to adverse reactions induced by the administration of human intravenous immunoglobulins (IVIGs) (Barandun et al., 1962). Using a similar rationale as that used by Barandun and colleagues, Sutherland (1977) suggested that ACA of antivenoms might have a role in the anaphylactic reactions induced by these immunobiologicals.

Assuming that ACA is causally related to the pathogenesis of non IgE-mediated reactions, four main strategies to reduce antivenom ACA have been proposed: 1 – Reduction of the total amount of protein administered; 2 – enzymatic digestion of immunoglobulins to remove the Fc fragment; 3 – reduction of IgG protein aggregates in antivenoms; and 4 – treatment of immunoglobulins with β-propiolactone, which is known to reduce ACA. However, although these strategies reduce antivenom ACA *in vitro*, none of them have translated into products inducing a lower incidence of anaphylactic reactions in clinical trials (Otero et al., 2006; Otero-Patiño et al., 2012). Despite these conflicting observations, the ACA of antivenoms remains as the most accepted explanation for the pathogenesis of non IgE-mediated reactions (Morais and Massaldi, 2009).

3.2.2.1.1. Reduction of antivenom ACA by decreasing the total load of protein administered. Since the first commercial antivenoms prepared by Calmette (1894) to be used in India and Vietnam, it was observed that crude serum or poorly-purified immunoglobulin preparations induce a high incidence of adverse reactions. Therefore, in order to obtain more purified formulations, strategies such as “salting out” (Atkinson, 1900; Gibson, 1906) and pepsin digestion (Pope, 1939) were adapted to protocols for antivenom production. More recently, purification of immunoglobulins by caprylic acid precipitation and ion-exchange chromatography have been introduced with the same purpose (dos Santos et al., 1989; Rojas et al., 1994; Raweerith and Ratanabanangkoon, 2003; Simssiriwong et al., 2012). Various laboratories combine some of these

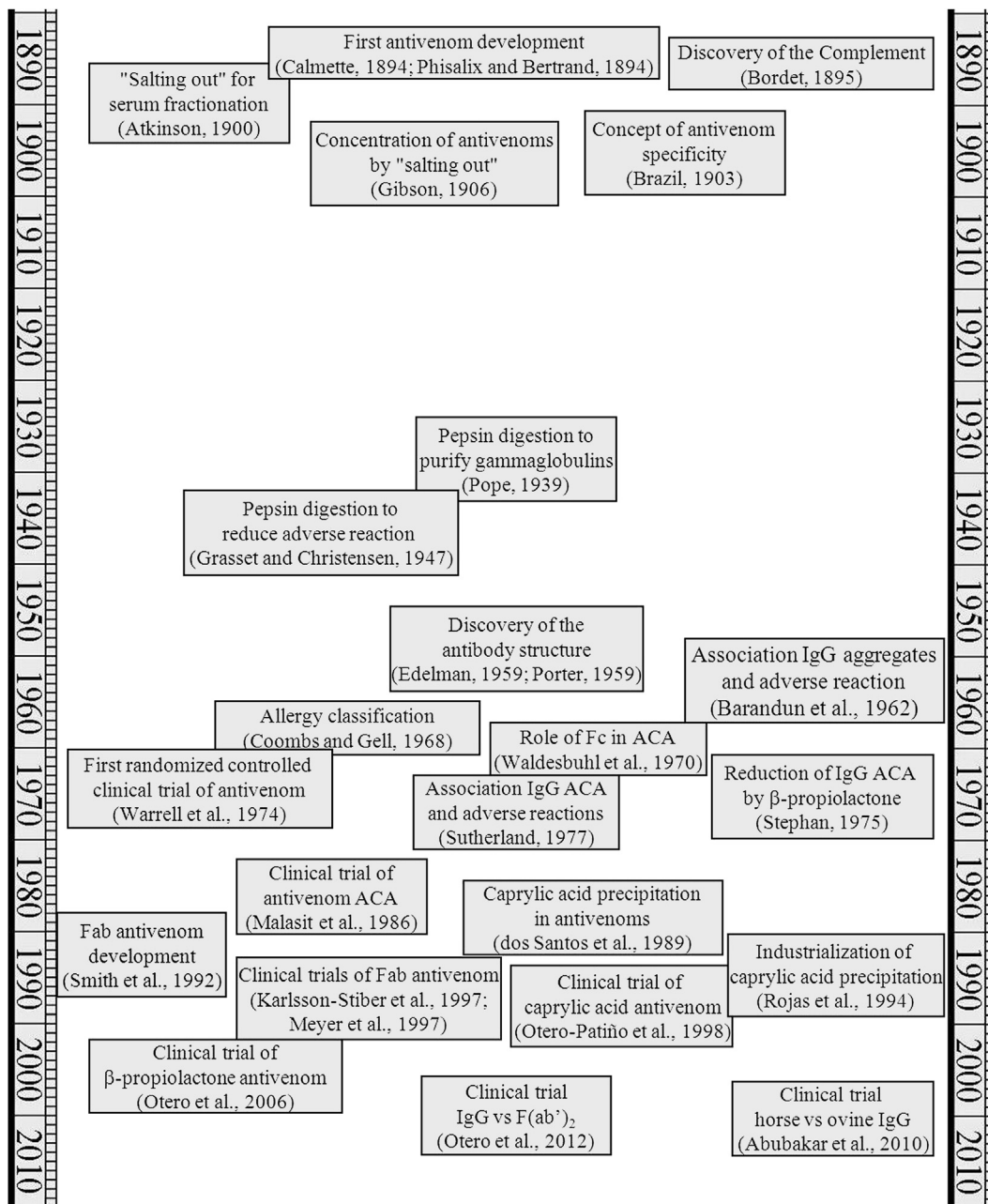


Fig. 1. Time-table of some of the key achievements in the study of adverse reactions induced by antivenoms and some technological efforts introduced to improve the safety of antivenoms.

methodologies, such as pepsin digestion, salting out, and chromatographic separation (Raw et al., 1991; Grandgeorge et al., 1996). Many of these advances have resulted in antivenoms of higher purity and lower total protein content.

The assumption that the incidence of anaphylactic reactions decreases as the total heterologous protein administered is reduced (Malasit et al., 1986; Corrigan et al., 1978; Cardoso et al., 1993; Jorge et al., 1995) is supported by

some evidence indicating that ACA decreases as the total antivenom protein is lowered (León et al., 2001). However, no difference has been observed in the incidence of non IgE-mediated reactions induced by the same antivenom administered at different doses, i.e. at different protein loads (Cardoso et al., 1993; Jorge et al., 1995; Otero et al., 2006). Nevertheless, regardless of these observations, the goal of reducing the total amount of heterologous protein administered in a treatment is considered important. This

goal can be achieved by, at least, three ways: 1 – Using more purified products (Grandgeorge et al., 1996); 2 – increasing the antibody titers in immunized horses, through innovations in immunization schemes, resulting in antivenoms of high neutralizing potency and low protein concentration (Segura et al., 2013); and 3 – preventing antivenom overdosing, a goal that can be achieved by rigorous clinical assessment of antivenom efficacy (Theakston et al., 1992; Riviére et al., 1997; Otero et al., 1999).

3.2.2.1.2. Reduction of antivenom ACA by enzymatic digestion. Pepsin digestion is a procedure by which the Fc fragment of immunoglobulins is enzymatically removed from the whole IgG molecule to produce the binding-antigen F(ab')₂ fragments. This procedure was developed by Parfentjev in 1936 and improved by Pope in 1939, with the intention of obtaining more purified antitoxins and antivenoms as a consequence of the digestion of contaminant non-IgG plasma proteins. At that time, it was unknown that pepsin digestion resulted in the cleavage of Fc fragment, since the immunoglobulin structure had not been elucidated yet (Edelman, 1959; Porter, 1959), and was only in the subsequent decades when the role of Fc fragment in ACA was demonstrated (Taranta and Franklin, 1961; Waldesbuhl et al., 1970). Therefore, neither Parfentjev nor Pope conceived the role of pepsin digestion as a strategy to remove the Fc fragment, since their main goal was to eliminate proteins without neutralizing activity.

During the years following Pope's work, it was assumed that the highest purity of pepsin-digested antivenoms was associated to the reduction of the propensity of these formulations to induce adverse reactions (Grasset and Christensen, 1947). In fact, an analysis performed in 1964 showed that many snake antivenoms producers implemented pepsin digestion step in their manufacturing procedures (Taub, 1964). Successive studies demonstrated the increasing acceptance of pepsin digestion in the antivenom industry (Chippaux and Goyffon, 1983; Theakston and Warrell, 1991). Currently, most of snake antivenom manufacturing protocols around of the world use pepsin digestion as a key step, to generate F(ab')₂ fragments as active substance, together with additional steps involving salting-out and, sometimes, chromatography (Raw et al., 1991; Grandgeorge et al., 1996; Jones and Landon, 2002, 2003; Morais and Massaldi, 2005).

There has been a widespread belief that F(ab')₂ antivenoms have a better safety profile than whole IgG formulations, and it has been largely assumed that this is mostly due to the lack of Fc fragment in the former (see for example Grandgeorge et al., 1996; Thiansookon and Rojnuckarin, 2008). Thus, since the Fc region is responsible for complement activation by the classical pathway after the formation of antigen–antibody complexes (Waldesbuhl et al., 1970), it has been thought that its removal generates products inducing lower incidence of adverse reactions. However, this presumption has not been supported by experimental and clinical evidence, since: 1 – Non IgE-mediated reactions are not associated with consumption of components of the complement cascade (Malasit et al., 1986; Stone et al., 2013); 2 – equine immunoglobulins are unable to activate the human complement

system by the classical pathway (Helms and Allen, 1970); 3 – F(ab')₂ antivenoms have ACA despite lacking the Fc fragment (Sutherland, 1977; Morais et al., 1994; León et al., 2001); and 4 – several clinical trials have shown that F(ab')₂ antivenoms induce early reactions of variable incidence depending on the product (see Table 1). Thus, although pepsin digested antivenoms have a lower ACA *in vitro* than equivalent whole-IgG antivenoms (León et al., 2001), clinical studies comparing equivalent antivenoms using IgG and F(ab')₂ as active substance show similar incidence of early reactions for both formulations (Otero-Patiño et al., 1998, 2012). A recent study has shown that, *in vitro*, a whole IgG antivenom induces the generation of both C3a and C5a anaphylatoxins, whereas two F(ab')₂ antivenoms only generate C3a (Baptistão and Tambourgi, unpublished results), thus suggesting that removal of Fc reduces the generation of C5a by antivenoms *in vitro*.

Likewise, some researchers and manufacturers have introduced the digestion of immunoglobulins by papain in the fractionation protocols, with the aim of removing Fc fragment and generating monovalent Fab neutralizing fragments (Smith et al., 1992; Laing et al., 1995; Mhatre et al., 1995; Landon and Smith, 1996). Like pepsin-digested antivenoms, the incidence of adverse reactions by Fab antivenoms varies (Meyer et al., 1997; Ariaratnam et al., 2001; Dart et al., 2001), although a recent meta-analysis performed on the Fab antivenom used in the USA evidenced a very low incidence of adverse reactions with this product (Schaeffer et al., 2012).

3.2.2.1.3. Reduction of antivenom ACA by reducing the content of protein aggregates. ACA has been also associated with the presence of protein (immunoglobulin) aggregates in the products (Frommhagen and Fudenberg, 1962). Since the 1960s, various innovations in the technologies to manufacture human immunoglobulin preparations have aimed at reducing the percentage of high molecular mass protein aggregates in these products (Barandun et al., 1962). Thus, assessment of the percentage of protein aggregates in antivenom preparations has been used as a quality control test (Rojas et al., 1994; Grandgeorge et al., 1996; Otero et al., 1999; García et al., 2002; Vargas et al., 2011). On the other hand, a study in which the content of aggregates in an antivenom was increased by heating did not show a parallel increment in ACA (León et al., 2005). IgG dimers are present in antivenoms, being generated during precipitation steps required for immunoglobulin purification or formulation (García et al., 2002). Alternatively, they can be produced by the interaction of idiotype/anti-idiotype networks generated by the mixing of plasma from several individuals, as occurs in the production of antivenoms (Roux and Tankersley, 1990). Additionally, immunoglobulin aggregates can be produced as a consequence of inadequate lyophilization, thus affecting the physicochemical characteristics of the antivenom. However, properly lyophilized antivenoms do not induce higher incidence of adverse reactions than their homologue liquid formulations (da Silva and Tavares, 2012).

Whole IgG antivenoms produced by ammonium sulphate precipitation contain a higher extent of protein aggregates than caprylic acid-fractionated whole IgG antivenoms, and this correlates with a higher incidence of

early adverse reactions with the former antivenom (Otero et al., 1999). Thus, there is evidence suggesting that protein aggregates in antivenoms contribute to the development of early adverse reactions, possibly by inducing complement activation, although more studies are required to further explore this hypothesis. Consequently, fractionation protocols aimed at preventing the formation of protein aggregates, or at eliminating such aggregates, result in antivenoms of lower ACA.

3.2.2.1.4. Reduction of antivenom ACA by β -propiolactone treatment. Treatment of immunoglobulins with β -propiolactone is a procedure developed to reduce ACA in IVIg preparations (Stephan, 1975). This procedure was first used at the experimental setting in antivenoms by Guidolin et al. (1997). This treatment significantly reduces the antivenom ACA (León et al., 2001). However, a clinical comparison of two antivenoms constituted by whole IgG purified by caprylic acid precipitation, one treated with β -propiolactone and the other produced without this treatment, showed that both formulations induced a similar incidence of anaphylactic reactions (Otero et al., 2006). Taken together, and despite some evidences implicating ACA as the culprit for early adverse reactions to antivenoms, several studies described above raise important doubts on the role of ACA in non IgE-mediated adverse reactions to antivenoms. The recent study of Stone et al. (2013) provides compelling evidence that intravenous administration of an antivenom that induces a high incidence of anaphylactic reactions does not reduce the serum complement levels. Clearly, more research is needed to clarify this issue.

3.2.2.2. The role of natural antibodies in the pathogenesis of the non IgE-mediated reactions. Natural antibodies are immunoglobulins whose presence in plasma is not stimulated by infection or by the intentional immunization with their corresponding antigens but, instead, occur by the natural post-thymic and/or bone marrow exposure of immune cells to an antigen (Kaveri, 2012). There are two types of natural antibodies: 1 – Autoantibodies (i.e. antibodies recognizing self-antigens), and 2 – heterophilic antibodies (i.e. antibodies towards molecules originating from a different species). Both types of natural antibodies have been associated with the pathogenesis of non IgE-mediated reactions to antivenoms.

3.2.2.2.1. Autoantibodies in the antivenom. Rheumatoid factor and anti-idiotypic antibodies are examples of autoantibodies recognizing antigens in the antibody itself (Nelson et al., 1985; Levinson and Miller, 2002). The probability of recognition between homologous antibodies increases as the number of individuals included in preparing the pool of plasma increases (Morgan et al., 1979; Roux and Tankersley, 1990). Since production of antivenoms starts with a pool of plasma from several, sometimes many, individuals, idiotype/anti-idiotype dimers can be formed in a concentration proportional to the number of animals providing plasma (Roux and Tankersley, 1990). Spontaneous dimerization has been observed in pooled human, bovine and mouse IgG (Roux and Tankersley, 1990). However, the demonstration of this phenomenon in antivenoms is pending. In IVIg production, dimers formed by this mechanism have been associated to the hypotension

described after the intravenous administration of this product (Kroez et al., 2003).

3.2.2.2.2. Heterophilic antibodies in the antivenom. Heterophilic antibodies are present in all species. The first report of antibodies agglutinating and lysing heterologous erythrocytes was made in 1875 by Leonard Landois (Hughes and Gardner, 2002). Afterward, it was demonstrated that antibodies towards human erythrocytes are present in the plasma of animals used as immunoglobulin source for antivenom production, such as equines, ovines, and camelids (Herrera et al., 2005; Sehrawat and Singh, 2006).

In the case of equine-derived antivenoms, anti-human erythrocyte antibodies are conserved after immunization with snake venoms and are dragged along the antivenom production process from plasma up to the final product (León et al., 2007). These antibodies belong to the IgG3 and IgG5 classes (formerly IgG(T) class) (Wagner et al., 2004), which do not participate in complement-mediated erythrophagocytosis or red blood cell lysis (Nakamura and Katsura, 1964; Helms and Allen, 1970; Banks and McGuire, 1975). Consequently, the participation of anti-erythrocyte antibodies present in antivenoms in the pathogenesis of adverse reactions has not yet been demonstrated (León et al., 2007). Antivenoms also contain antibodies towards other human cells such as mast cells, neutrophils and endothelium (our unpublished observations). Interestingly, it was recently found that non IgE-mediated reactions are characterized by high levels of mast cell degranulation in patients, a phenomenon that might be triggered by non allergen-specific activation of mast cells, which may be related to the quality of antivenom preparations, as well as a priming effect on the immune response by the venom itself (Stone et al., 2013). It is necessary to further investigate the role of antibodies in antivenoms against human cells in the pathogenesis of adverse reactions are pending.

3.2.2.2.3. Heterophilic antibodies in human plasma. Production of heterophilic antibodies is stimulated by contact with animals (Prince et al., 1973; Spitzauer et al., 1994, 1995), administration of vaccines (Després and Grant, 1998), or ingestion of food (Hunter and Budd, 1980). Therefore, heterophilic antibodies are present in the plasma of all people (Lacroix-Desmazes et al., 1995). Human plasma contains antibodies (IgG and IgE) towards animal antigens such as albumin (Werfel et al., 1997; Restani et al., 1997; Cahen et al., 1998; Eysink et al., 2002), myoglobin (Fuentes et al., 2004), and immunoglobulins (Bernhisel-Broadbent et al., 1991; Werfel et al., 1997; Kricka, 1999; Willman et al., 1999; Ayuso et al., 2000).

It has been proposed that heterophilic antibodies could be related to the pathogenesis of allergy towards some foods (Sampson, 1989; Werfel et al., 1997), through a mechanism that involves the formation of immune complexes (Paganelli et al., 1979, 1981) not associated to a reduction in the complement lytic activity (Hubsy et al., 1990).

Horses are the most commonly used animals to produce antivenoms (Lalloo and Theakston, 2003; Gutiérrez et al., 2003), and human heterophilic antibodies towards equine immunoglobulins have been described (Henning et al.,

2000). On this basis, it has been proposed that immune complexes formed by antivenom proteins (regardless of whether antivenom is formulated with whole IgG or F(ab')₂ fragments) and heterophilic antibodies, may be involved in the pathogenesis of non IgE-mediated reactions induced by equine-derived antivenoms (Herrera et al., 2005; León et al., 2008). Similarly, IgA-deficient patients produce natural anti-IgA antibodies, which have been associated with the pathogenesis of adverse reactions that develop when these patients are exposed to the IgA present in normal plasma or in some IVIg formulations (Vyas et al., 1968, 1969, 1975; Sandler et al., 1994; Koskinen et al., 1995).

Equine-derived antivenoms, formulated with whole immunoglobulins purified by precipitation with caprylic acid, induce an incidence of anaphylactic-type reactions ranging between 15 and 25% in humans (Otero et al., 1999, 2006; Abubakar et al., 2010; Otero-Patiño et al., 2012), whereas these reactions are largely absent in horses and cows (Estrada et al., 2010). This strongly suggests that, in addition to the physicochemical properties of the formulation, specific and individual characteristics (e.g. presence of heterophilic responses in the recipient species) determine the incidence and severity of non IgE-mediated reactions.

The presence of human IgG towards equine immunoglobulins (both whole IgG and F(ab')₂ fragments) in the serum of snakebitten patients treated with equine antivenoms has been investigated. No correlation was found between the concentration of these heterophilic antibodies and the incidence of anaphylactic reactions (León et al., 2008). However, this issue deserves further exploration, and similar studies analyzing the presence of human IgM towards equine immunoglobulins are pending (Herrera et al., 2005).

Assuming that sheep antibodies are less antigenic than horse antibodies, some researchers suggested that ovine-derived antivenoms should have lower ACA and induce lower incidence of anaphylactic reactions than equine-derived antivenoms (Sjostrom et al., 1994; Landon and Smith, 2003; Redwan et al., 2009). This could be based on the presence of higher levels of heterophilic antibodies in human plasma against immunoglobulins of different species. Evaluation of this hypothesis in mouse models has produced contradictory results (Herrera et al., 2005; Redwan et al., 2009). However, a clinical trial in which patients envenomed by *Echis ocellatus* in Nigeria, who received 0.4 g of an ovine-derived antivenom, showed an incidence of anaphylactic reactions of 19%, in comparison to 26% shown by patients treated with 1.2 g of an equine-derived antivenom, a difference that, although significant (Abubakar et al., 2010), is not too pronounced. These results suggest that ovine and equine immunoglobulins are similarly tolerated by patients. Interestingly, like horse immunoglobulins, the presence of heterophilic antibodies towards sheep immunoglobulins has been demonstrated (Hunter and Budd, 1980).

Other animal species used as immunoglobulin source for the production of experimental antivenoms are goats (Russell et al., 1970), hens (Maya et al., 2002; Meenatchisundaram et al., 2008; de Andrade et al., 2013), llamas (Fernández et al., 2010), and camels (Harrison et al., 2006). Among these, camelid immunoglobulins have

shown lower ACA and immunogenicity, when compared with ovine or equine immunoglobulins (Herrera et al., 2005; Redwan et al., 2009). Nevertheless, clinical trials demonstrating the incidence of adverse reactions induced by camelid-derived antivenoms have not been performed. On the other hand, heterophilic antibody response has been used to discuss the inconvenience of hen-derived antivenoms, owing to the widespread exposition of humans to egg yolk antigens (Sevcik et al., 2008). Owing to the antigenic similarities between immunoglobulins of different species (Vaerman et al., 1971; Esteves and Binaghi, 1972; Kurimoto et al., 1982), exposition to food manufactured from some species (e.g. cows) could induce the production of heterophilic antibodies towards immunoglobulins from other species used to produce antivenoms (e.g. sheep).

4. Late reactions

Late reactions induced by antivenoms correspond to a syndrome traditionally known as “serum sickness”, which corresponds to type III hypersensitivity in the Gell-Coombs classification (Gell and Coombs, 1963). In late reactions, the immune system of the patient receiving the antivenom recognizes the heterologous proteins as foreign and mounts an IgG-based antibody response towards them. In a recent study, it was found that, after antivenom administration, concentration of antibodies in serum towards heterologous immunoglobulins increases from 2 times to more than 100 times, as compared to the basal values (Morais et al., 2012).

The formation of soluble antigen–antibody complexes is responsible for the reaction, which usually manifests clinically between 5 and 20 days after antivenom administration. While there is clinical evidence suggesting that no sensitization is produced in patients after repeated administration of Fab ovine antivenoms (Lavonas et al., 2013), it has been demonstrated that, in the case of whole IgG antivenoms, and probably for F(ab')₂ antivenoms as well, the incidence of late reactions increases with the total amount of heterologous protein administered (LoVecchio et al., 2003). Thus, antivenom protein concentration and dosage are key determinants for the generation of late adverse reactions.

The actual incidence of late adverse reactions to antivenom administration is poorly known, mostly because patients do not usually return to health centers after discharge. Thus, there are few reports on the incidence of late adverse reactions to antivenoms (LoVecchio et al., 2003; Abubakar et al., 2010; Otero-Patiño et al., 2012).

Immune complexes, formed by human IgG and the antivenom proteins that they recognize, activate the complement system through the classical pathway, and also activate neutrophils, thus contributing to the development of manifestations such as fever, myalgia, arthralgia, arthritis, urticaria, lymphadenopathy and gastrointestinal disorders, which become evident from 7 to 15 days after antivenom administration (Nielsen et al., 1978; LoVecchio et al., 2003). Other alterations, such as laryngeal edema, glomerulonephritis, and vasculitis are complications rarely observed in these patients (Baumann et al., 2001; Erdei et al., 2004; Szebeni, 2005). In general, during serum

sickness, laboratory analyses show elevated erythrocyte sedimentation rate, leukocytosis occasionally accompanied by eosinophilia, hematuria, proteinuria and decrease of complement components in serum (e.g. C3, C4 and CH₅₀ activity).

How do complement system and neutrophils become activated by immune complexes is not completely understood. However, some cellular receptors of complement and immunoglobulins, such as C3bR, C5aR and FcγIII, have been implicated as important participants in this activation mechanism.

4.1. Role of C3bR

In the initial phase of late reactions, there is an excess of antivenom proteins in the patient blood, in comparison with the low concentration of human IgG towards antivenom immunoglobulins. Under this circumstance, small soluble immune complexes are formed, and become distributed throughout the body and deposited in anatomical sites such as the synovial membrane of joints and glomerular or other endothelial basement membranes. Then, these immune complexes activate the complement system by the classical pathway, generating anaphylatoxins that stimulate mast cells degranulation (Erdei et al., 1999, 2004). As a result, proteases, leukotrienes, serotonin and, particularly, histamine, are released.

The synthesis and release of vasoactive substances by mast cells promote smooth muscle contraction and, after interacting with their endothelial receptors, stimulate vasodilation and increase in capillary permeability (Erdei et al., 1999, 2004). As a consequence, cutaneous edema and erythema occur (Baumann et al., 2001; Erdei et al., 2004; Szebeni, 2005). Neutrophils are attracted by the chemotactic activity of anaphylatoxins and attempt to phagocytize the immune complexes coated with C3b. However, because immune complexes are often attached to basement membranes, phagocytosis is largely unsuccessful. Instead, the lytic enzymes are released to the interstice, with the consequent damage to neighboring tissue (Köhl, 2001).

As the concentration of anti-antivenom IgG increases, the plasma levels of antivenom immunoglobulins decrease and their proportions approximates to the equivalence. In this circumstance, large and insoluble immune complexes are formed. These immune complexes are easily eliminated by cells of the mononuclear phagocyte system. Finally, when the concentration of antivenom immunoglobulins in plasma reaches very low levels, small soluble immune complexes are formed again. However, these are characterized by an excess of human IgG towards antivenom proteins, having low capacity to activate the complement system. Therefore, at this stage, the signs and symptoms of late reactions tend to disappear (Nielsen et al., 1978).

4.2. Role of the C5aR and FcγIIIR

Events occurring during late reactions can be reproduced experimentally by the Arthus reaction, the most accepted *in vivo* model for the study of type III hypersensitivity (Szalai et al., 2000). In this reaction, the antigen is injected either intradermally or subcutaneously into

animals previously immunized (actively or passively) with this antigen. After 4–8 h, macroscopic evaluation of the injection site evidences a dermal induration and erythema, and microscopic examination reveals the presence of a neutrophil-based inflammatory infiltrate.

The Arthus reaction occurs in C3-deficient mice, but not in C5aR-deficient mice (Baumann et al., 2001), being completely inhibited by the intravenous injection of soluble FcγIIIR (Lerino et al., 1993). Among the consequences of the FcγR activation are the release of platelet activating factor (PAF), cytokines, and other lipid mediators, and the induction of cyclooxygenase and nitric oxide synthase (Jancar and Sánchez, 2005). These results suggest that the concomitant mediation of C5aR and FcγIIIR is necessary to reproduce the Arthus reaction. Nevertheless, how C5a is generated in C3-deficient mice is unknown (Baumann et al., 2001).

5. Conclusions

The safety profile of antivenoms is determined by factors such as manufacturing practices, physicochemical characteristics of products, and intrinsic characteristics of heterologous immunoglobulins. In order to improve clarity and uniformity, we suggest that scientific reports addressing the issue of adverse reactions to snake antivenoms follow the nomenclature proposed by the WHO guidelines published in 2010. According to this arrangement, adverse reactions induced by antivenoms can be classified, depending on the time between antivenom administration and the onset of clinical manifestations, as early reactions (i.e. reactions occurring within the first hours of antivenom infusion) or late reactions (i.e. reactions occurring between 5 and 20 days after treatment). In turn, depending on the mechanism underlying early reactions, these can be categorized as: 1 – Pyrogenic reactions (e.g. reactions induced by endotoxins); 2 – IgE-mediated reactions (e.g. reactions mediated by IgE antibodies towards antivenom proteins); or 3 – non IgE-mediated reactions, whose pathogenesis is still not completely understood. On the other hand, late reactions are induced by immune complexes formed by antivenom proteins and IgM or IgG antibodies raised in the patient against such proteins. A summary of the mechanisms proposed to explain the pathogenesis of adverse reactions induced by snake antivenoms is presented in Fig. 2.

The following tentative conclusions on the pathogenesis of early adverse reactions can be reached on the basis of the experimental and clinical evidence accumulated: a) Some reactions involving fever, rigor, chills, and hypotension are due to the presence of bacterial endotoxins in antivenoms. b) There is evidence of mast cell degranulation in patients receiving antivenom and developing anaphylactic reactions, thus implying that release of mast cell mediators plays a role in these reactions; however, the mechanisms behind such degranulation remain unknown. c) Although protein aggregates in antivenoms contribute to the development of early adverse reactions, and antivenoms are able to activate complement *in vitro*, it is unlikely that antivenom ACA plays a key role in the pathogenesis of anaphylactic reactions.

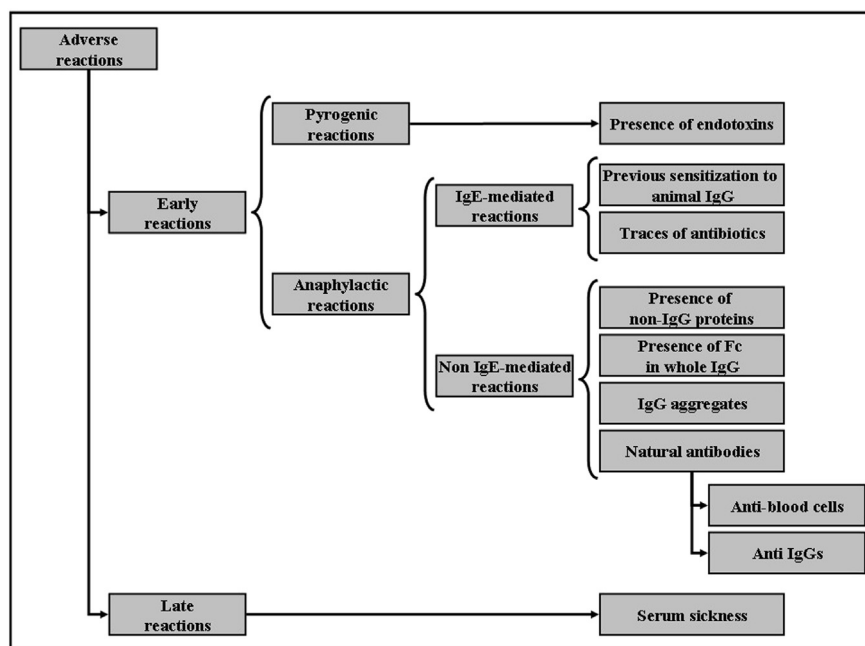


Fig. 2. Mechanisms proposed to explain the pathogenesis of adverse reactions induced by snake antivenoms.

On the other hand, alternative possible mechanisms involved in adverse reactions to antivenoms might include: a) The antibiotic traces in the blood of animals used as immunoglobulin source which may be present in the final product. b) The spontaneous dimerization of pooled equine IgG in antivenoms. c) The presence of anti-endothelium, anti-mast cells, or anti-leukocytes antibodies in antivenoms. d) The presence of human IgM antibodies, in the plasma of patients, towards equine immunoglobulins. Future research is necessary to clarify each of these possible mechanisms. Understanding the role of these mechanisms in the genesis of adverse reactions to antivenoms will undoubtedly contribute to formulate technological solutions that allow the production of safer antivenoms.

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Conflict of interest statement

The authors declare that there are no conflicts of interest regarding this manuscript.

Ethical statement

This review was prepared following international guidelines for the preparation of scientific publications.

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