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Factors associated with adverse reactions induced by caprylic acid-fractionated whole IgG preparations: comparison between horse, sheep and camel IgGs

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Abstract

Caprylic acid purification of IgG, currently used in the manufacture of horse-derived antivenoms, was successfully adapted for the preparation of sheep and camel IgG. Sheep IgG had a molecular mass of ~150 kDa, whereas camel IgG presented two bands of molecular masses of ~160 and 100 kDa, the latter corresponding to heavy-chain IgG, which is devoid of light chains. Horse, sheep and camel IgGs were compared by several parameters aiming at predicting their potential for induction of early and late adverse reactions. Horse and sheep IgGs showed a higher anticomplementary activity than camel IgG, and also elicited a higher anti-IgG response than camel IgG, when injected in mice. Horse IgG agglutinated human type O+ erythrocytes, whereas no such reactivity was observed in sheep and camel IgG preparations. A novel procedure was used for the detection of antibodies in human serum against animal IgGs. It was found that a pool of human sera collected in Costa Rica had a higher titer of antibodies directed against horse and sheep IgGs than against camel IgG. Overall, camel IgG showed the lowest potential for the induction of adverse reactions among the three IgGs tested.

Keywords: IgG; Caprylic acid; Anticomplementary activity; Antivenom; Adverse reactions; Anti-IgG antibodies

1. Introduction

Parenteral administration of heterologous antivenom preparations constitutes the mainstay in the therapy of envenomations by animal bites and stings (Theakston et al., 2003). A great variety of antivenoms are currently produced in many laboratories from the serum of animals, mostly horses and sheep, that are immunized with single or multiple venoms. There are three main types of antivenom preparations, depending on the nature of the neutralizing molecules: (a) whole IgG preparations, (b) $F(ab')_2$ preparations, and (c) Fab preparations (Theakston et al., 2003). $F(ab')_2$ and Fab antivenoms are obtained by pepsin or papain digestion of IgGs, respectively, with the resultant elimination of Fc fragment. These three types of antivenoms differ in their pharmacokinetic characteristics, an issue that has evident implications in their pharmacodynamic performance (Gutiérrez et al., 2003; Theakston et al., 2003).

Caprylic acid fractionation of animal sera or plasma constitutes a highly convenient methodology for immunoglobulin purification (Steinbuch and Audran, 1969). This method is currently being used in antivenom production, and can be applied not only in the manufacture of whole

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IgG antivenoms (Rojas et al., 1994; Gutiérrez et al., 2005), but also in the production of $F(ab')_2$ and Fab antivenoms (Dos Santos et al., 1989; León et al., 2000). Whole IgG antivenoms prepared by caprylic acid fractionation of horse plasma have been shown to be highly safe and effective in the treatment of snakebites in Central and South America (Arroyo et al., 1999; Otero et al., 1999). Owing to the simplicity and low cost of this procedure, it may become a highly useful fractionation methodology for the manufacture of antivenoms in regions of the world where a serious crisis in antivenom accessibility currently exists, such as in sub-Saharan Africa (Theakston and Warrell, 2000; Gutiérrez et al., 2005).

Parenteral antivenom administration is often associated with early and delayed adverse reactions (Warrell, 1995), with a high variation in their incidence depending upon the product being administered (Cardoso et al., 1993; Otero et al., 1999; Lalloo and Theakston, 2003). Several mechanisms have been proposed to cause adverse reactions to antivenoms: (a) complement activation due to the presence of Fc in whole IgG preparations, or to the presence of protein aggregates in antivenoms made of whole IgG or its fragments (Sutherland, 1977; Otero et al., 1999); (b) immunogenicity of antivenom proteins which would elicit an anti-IgG antibody response associated with delayed reactions, i.e. serum sickness (León et al., 2001; Lalloo and Theakston, 2003); (c) the presence of preservatives in antivenom preparations, which may elicit various adverse effects (García et al., 2002). However, the actual mechanisms responsible for such adverse reactions are difficult to demonstrate in patients, and thus remain largely unknown. Despite the fact that caprylic acid-fractionated horse-derived whole IgG antivenoms have shown a good safety profile in clinical trials, mild early adverse reactions have been reported in 10–25% of patients receiving these products (Arroyo et al., 1999; Otero et al., 1999). Therefore, it is of relevance to further investigate the mechanisms behind these adverse reactions, in order to minimize them and to make these antivenoms even safer.

There has been little effort to compare horse and sheep IgGs regarding their propensity to induce early and delayed adverse reactions. In addition, other animals may be considered as potential sources of plasma or serum for antivenom production. In Africa, for instance, camelids may become a valuable new source of hyperimmune plasma for antivenom production, as recently shown for an antivenom to treat scorpion stings in Tunisia (Meddeb-Mouelhi et al., 2003). The aim of this work was two fold: (a) to adapt the methodology used for caprylic acid purification of equine IgG to camel plasma, in order to determine if it could be of use in the event of utilizing camels in antivenom production, and (b) to compare IgGs purified by this procedure from the plasma of horses, sheep and camels regarding several characteristics that might be associated with the development of adverse reactions to the administration of antivenoms.

2. Materials and methods

2.1. Plasma fractionation and IgG purification

The procedure described by Rojas et al. (1994) was followed, with several modifications. In the cases of horse and sheep IgG, citrated plasma was the starting material, obtained after sedimentation at 4 °C of blood collected from five adult sheep and ten adult horses, respectively, kept at Instituto Clodomiro Picado. In the case of camel, fractionation was performed on serum, obtained by centrifugation after clotting of blood collected from one healthy adult dromedary (*Camelus dromedarius*) kept in Senegal. For the purification of IgGs, caprylic acid was added to plasma or serum, at a ratio of 5 mL acid added to 100 mL plasma or serum. The mixture was stirred for 2 h at 20–22 °C, filtered through 8 μm filter paper and diafiltered. Then, the preparations were formulated in order to have 0.15 M NaCl, 2.5 g/L phenol and 0.05 g/L thimerosal, at a final pH of 7.0. The three IgG preparations were standardized as to have a final protein concentration of 6 g/L. Finally, the preparations were sterilized by filtration through 0.22 μm membrane and bottled in 10 mL glass vials.

2.2. Electrophoretic analysis

The same amount of IgG protein for each preparation was loaded in SDS-polyacrylamide gels, at an acrylamide concentration of 7.5 g/dL, and electrophoretic separations were performed under non-reducing conditions (Laemmli, 1970). Proteins were stained with Coomassie Brilliant Blue R-250. Molecular mass standards were run in parallel.

2.3. In vitro anticomplementary activity

Dilutions of each IgG preparation, using barbital buffer, pH 7.6 as diluent, were prepared in triplicate. Then, 0.5 mL of fresh human plasma, diluted 1:5 in barbital buffer, were added to 2.0 mL of each IgG dilution. After an incubation of 1 h at 37 °C, 100 μL of a suspension of sheep erythrocytes previously sensitized with rabbit anti-sheep erythrocyte antibodies were added, and the mixture was incubated for 1 h at 37 °C (León et al., 2005). Then, 2 mL of cold barbital buffer were added, the tubes were immediately centrifuged and the absorbance of supernatants at 540 nm was recorded. Human plasma samples were run in parallel by incubating them with barbital buffer, in the absence of heterologous IgG. The rest of the procedure was performed as described, and complement activity in these conditions was considered 100%. Anticomplementary activity of IgG preparations was expressed as percentage, relative to the activity of these controls.

2.4. Immunogenicity of IgGs in mice

Groups of ten CD-1 mice of both sexes (16–18 g) were bled and serum was separated, to obtain a pre-immune serum sample. Then, mice were injected by the intraperitoneal route with 500 μL of either horse, sheep or camel IgG preparation. After 15 days, mice were bled under CO_2 anesthesia into heparinized microcapillary tubes. Then, plasma was separated by centrifugation and the anti-IgG antibody titer was assessed by ELISA. Plates were coated with 100 μL of a 1:1000 dilution of either horse, sheep or camel IgG preparation. After an incubation of 24 h at room temperature, plates were washed five times with 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2 buffer (PBS), and then 100 μL of mouse plasma, diluted 1:2000 in 2% bovine serum albumin (BSA)-PBS, were added to each well. After 1 h of incubation at room temperature, plates were washed five times with PBS, and 100 μL of peroxidase-conjugated anti-mouse IgG, diluted 1:1000 with 2% BSA-PBS were added, followed by an incubation of 1 h at room temperature. Finally, *o*-phenylendiamine (OPD) and H_2O_2 was added for color development, and absorbances recorded at 492 nm.

2.5. Antibodies against animal IgG in human serum

In order to detect if normal human serum contains antibodies against animal IgGs, microplates were coated with 100 μL of 1:1000 dilutions of either human, horse, sheep or camel IgGs, purified by caprylic acid fractionation. After washing the plate five times with PBS, 50 μL of biotinylated human IgG (obtained by caprylic acid fractionation from a pool of 10 adult healthy Costa Rican volunteers), diluted 1:500 in 2% BSA-PBS, were added and the plates incubated for 1 h at 20–22 °C. Then, plates were washed five times with PBS, and 100 μL of avidin conjugated with peroxidase, diluted 1:2000 with PBS/2% BSA, were added to each well, followed by an incubation of 1 h at 37 °C. Color was developed by the addition of H_2O_2 and OPD, and the absorbances at 492 nm were recorded. This test avoids the use of an anti-human secondary antibody, in order to rule out the possible interactions of this secondary antibody with animal IgG due to heterophile antibodies known to be present in animal serum (Willman et al., 1999).

2.6. Agglutination of human erythrocytes

The presence of antibodies in IgG preparations able to agglutinate human erythrocytes was tested by adding 100 μL of various dilutions of the three IgG preparations to 50 μL of a 5% suspension of human O+ erythrocytes in PBS. Controls included erythrocytes incubated with PBS alone. After incubation for 1 min at 20–22 °C, tubes were centrifuged at 500*g* for 30 s and macroscopic agglutination observed.

2.7. Statistical analysis

The significance of the differences between the mean values of two experimental groups was determined by the Student's *t*-test. When more than two groups were compared, analysis of variance was performed, followed by a Tukey-Kramer test. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Caprylic acid purification of IgG

The procedure previously used for the purification of horse IgG, based on caprylic acid precipitation of non-IgG plasma proteins (Rojas et al., 1994; Gutiérrez et al., 2005), was successfully applied to sheep plasma and to camel serum. Electrophoretic analysis of the three preparations showed a broad predominant band of 120–150 kDa in horse IgG preparation, a band of ~150 kDa in sheep IgG preparation, and two bands of ~160 and 100 kDa in camel IgG preparation (Fig. 1). These findings agree with previous observations indicating that horse and sheep IgGs have molecular masses of approximately 150 kDa, whereas camel serum contains IgGs of two different molecular masses due to the presence in camelid serum of both

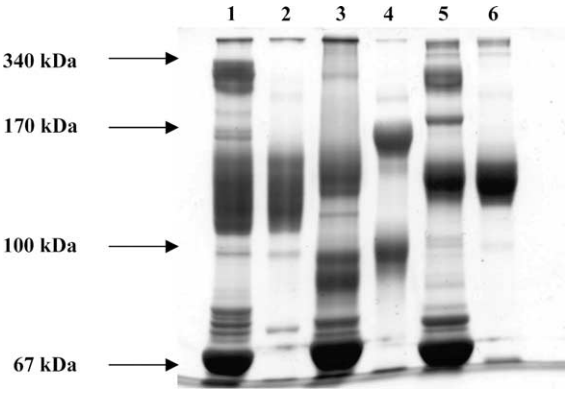

Fig. 1. SDS-PAGE of IgGs isolated from horse and sheep plasma, and from camel serum. Aliquots of each IgG preparation, previously diluted 1:2 in buffer and containing 15 μg protein, were separated by electrophoresis in 7.5% acrylamide gels under non-reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. Lanes 1, 3 and 5 correspond to crude horse plasma, camel serum and sheep plasma, respectively. Lanes 2, 4 and 6 correspond to purified horse, camel and sheep IgGs. Estimated molecular masses are depicted to the left.

conventional, four chain IgGs and heavy chain IgGs devoid of light chains (Hamers-Casterman et al., 1993).

3.2. Anticomplementary activity

A concentration-dependent anticomplementary activity on human complement was observed for horse and sheep IgG preparations. In contrast, no anticomplementary activity was detected for camel IgG, even at the highest concentration tested (Fig. 2).

3.3. Immunogenicity of IgG preparations

Mice injected with either horse, sheep or camel IgGs developed a significant antibody response against these IgGs 15 days after injection. Highest antibody responses were observed for horse and sheep IgGs, whereas mice injected with camel IgG developed a significantly lower antibody response (Fig. 3).

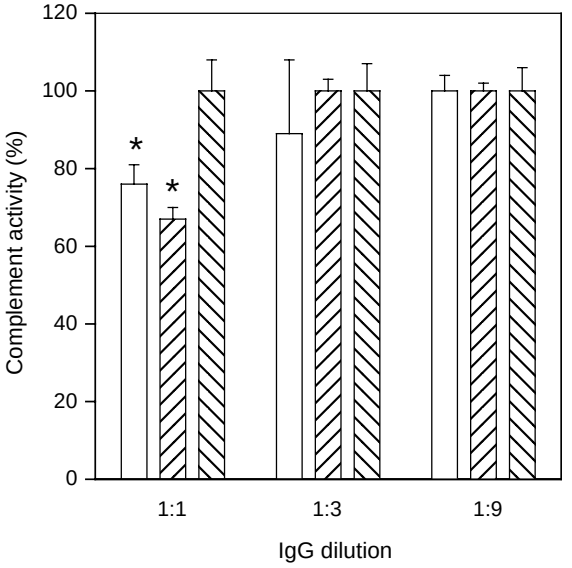

Fig. 2. Anticomplementary activity of IgG preparations. Human plasma was incubated with various dilutions of the three IgG preparations. Then, the mixtures were added to a suspension of sheep erythrocytes previously sensitized with anti-sheep erythrocyte rabbit antibodies. After incubation, tubes were centrifuged and the absorbance of the supernatants was recorded at 540 nm. As control, human plasma was incubated with barbital buffer instead of IgG, and the rest of the procedure performed as described. Anticomplementary activity was evidenced by a reduction in the complement activity of human plasma after incubation with IgGs. Results are presented as mean ± SD. Horse antibodies: empty bars; sheep antibodies, diagonal ascending bars; camel antibodies, diagonal descending bars. Horse and sheep IgGs exerted a significantly higher anticomplementary activity than camel IgG when tested undiluted ($p < 0.05$), whereas no anticomplementary activity was detected at higher dilutions in any of the IgG preparations.

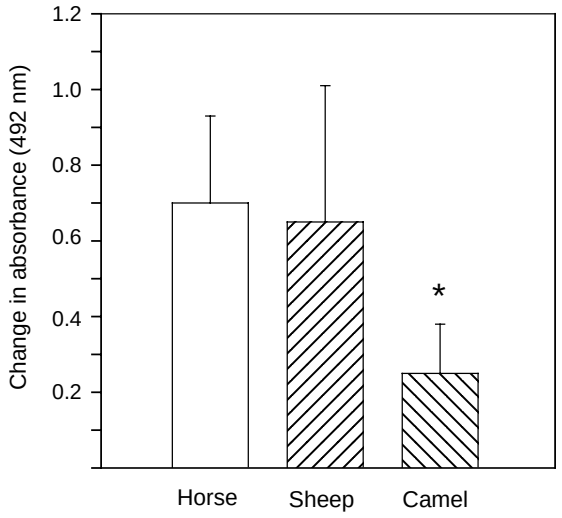

Fig. 3. Antibody response in groups of ten CD-1 mice against IgGs. Mice were bled to obtain a sample of preimmune serum. Then, they received an intraperitoneal injection of 500 μL of either horse, sheep or camel IgG. After two weeks, mice were bled again and serum separated. Anti-IgG response was assessed by ELISA in plates coated with the corresponding IgG used in the immunization. After addition of anti-mouse IgG conjugated with peroxidase followed by the substrate, absorbances were recorded at 492 nm. For each animal, the absorbance of preimmune serum samples was subtracted from the absorbance of samples collected after immunization. Results are presented as mean ± SD. Camel IgG induced a significantly lower antibody response than horse and sheep IgGs ($*p < 0.05$), whereas no significant differences occur in the response to horse and sheep IgGs.

3.4. Antibodies against animal IgGs in human serum

The enzyme immunoassay developed in this work allowed us to compare the titer of anti-IgGs from different species in a pool of human sera, thus allowing the detection of antibodies in human serum against animal IgGs. As shown in Fig. 4, highest absorbances were recorded in plates coated with sheep IgG, followed by horse IgG. In contrast, no significant differences were found when plates were coated with either human or camel IgGs (Fig. 4), evidencing the lack of antibodies against camel IgG in the pool of human sera used.

3.5. Agglutination of human erythrocytes

As shown in Table 1, horse IgGs induced a strong agglutination of human erythrocytes, whereas no macroscopic agglutination was observed when using sheep or camel IgG preparations.

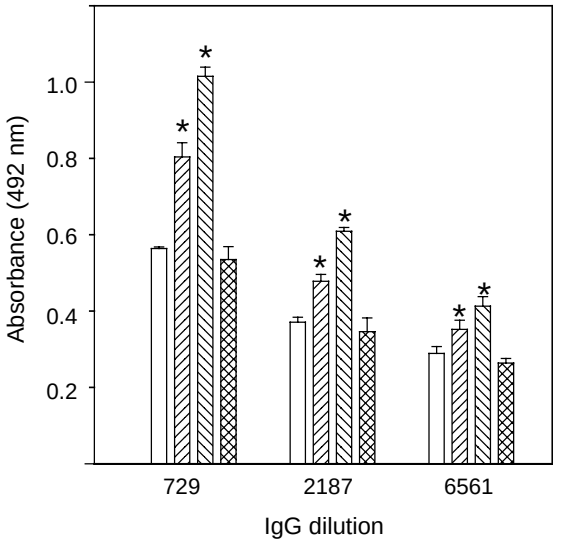

Fig. 4. Detection of antibodies against horse, sheep or camel IgG in a pool of human sera from ten individuals. Plates were coated with 100 μL of a 1:1000 dilution of either human (empty bars), horse (diagonal ascending bars), sheep (diagonal descending bars) or camel (crossed bars) IgGs. Then, various dilutions of biotinylated human IgG were added, followed by an avidin-peroxidase conjugate and peroxidase substrate. Absorbances were recorded at 492 nm. Results are presented as mean ± SD. A significantly higher absorbance ($p < 0.05$) was recorded in wells coated with horse or sheep IgGs than in those coated with human or camel IgGs.

4. Discussion

A very simple and convenient methodology, based on caprylic acid precipitation of non-IgG proteins in plasma or serum, has been adapted in various laboratories for the production of equine-derived antivenoms (Dos Santos et al., 1989; Rojas et al., 1994; Gutiérrez et al., 2005). In this work it has been demonstrated that this procedure can be also applied in the purification of IgGs from sheep and camel sera. In the case of camel serum, this adaptation opens the possibility for the production of this type of antivenom in African countries, where a particularly serious crisis currently exists in the provision of antivenoms (Theakston and Warrell, 2000). Camelids like the dromedary are abundant in sahelian and savanna areas of Africa, where they are highly adapted; this species can yield a large volume of blood. A previous study showed the effectiveness of immunizing dromedaries with scorpion venoms in northern Africa for the production of neutralizing IgGs (Meddeb-Mouelhi et al., 2003). Furthermore, it has been suggested that, owing to the low structural complexity of the antigen-binding sites of heavy-chain camelid antibodies, they may become useful inhibitory molecules (Lauweyers et al., 1998) that may be of value for a variety of purposes, including neutralization of toxins. In our study, two bands of mol. mass of 160 and 100 kDa were observed; they are likely to correspond to classical IgGs having two light and two heavy chains, and IgGs constituted only by two heavy chains, i.e. IgGs devoid of light chains (Hamers-Casterman et al., 1993; Meddeb-Mouelhi et al., 2003). Thus, caprylic acid precipitation allows the purification of both types of IgGs from camelid serum.

Table 1
Agglutination of human group O+ erythrocytes by horse, sheep and camel IgG preparations purified by caprylic acid precipitation of non-IgG plasma proteins

Dilution of IgG	Agglutination		
	Horse IgG	Sheep IgG	Camel IgG
1	4+	–	–
3	3+	–	–
9	–	–	–
27	–	–	–

Dilutions of IgG in PBS were incubated with a 5% suspension of human O+ erythrocytes in PBS. After centrifugation, agglutination was observed macroscopically and graded from –(no agglutination) to 4+(strong agglutination).

Early and late adverse reactions occur in a number of patients receiving antivenoms of different sources and made of different neutralizing molecules (Cardoso et al., 1993; Otero-Patiño et al., 1998). Despite the widely held assumption that the presence of Fc in whole IgG antivenom preparations is the main cause of these adverse reactions (see for example Chippaux, 1998), clinical observations indicate that the mechanisms behind such reactions go beyond the presence of Fc fragments. There are caprylic acid-fractionated whole IgG antivenoms that induce a low incidence of adverse reactions (Otero-Patiño et al., 1998; Otero et al., 1999). Therefore, there is not a clear correlation between the presence of Fc fragment and the incidence of early adverse reactions.

Anticomplementary activity has been proposed as a key mechanism in the induction of early adverse reactions after administration of human and heterologous IgG preparations (Barandun et al., 1962; Sutherland, 1977). Previous studies have demonstrated anticomplementary activity in both whole IgG and $F(ab')_2$ equine-derived antivenoms (Montero et al., 1989; Otero et al., 1999; León et al., 2001). Our observations indicate that horse and sheep IgGs exert this activity on human serum, whereas camel IgGs did not activate complement in these experimental conditions. The reason behind this interesting observation is not known, but may have to do with the peculiar biochemical features of camel IgGs (Hamers-Casterman et al., 1993), a hypothesis that remains open for future studies.

The development of serum sickness is the most common manifestation of late adverse reaction after administration of heterologous antivenoms (Warrell, 1995), although its actual incidence has not been properly investigated. Serum sickness, which is a typical type III hypersensivity reaction, is directly proportional to the immunogenicity of heterologous antivenoms. In order to predict the propensity of

development of this type of late adverse reaction in the three types of IgGs analyzed in this study, we immunized groups of mice with the same amount of protein from each IgG preparation. Observations indicate that horse and sheep IgG induce a similar antibody response in mice, thus raising doubts on the concept that horse IgG is more immunogenic than sheep IgG (Landon and Smith, 1996), at least in this experimental model. In contrast, a significantly lower anti-IgG antibody response developed in mice injected with camel IgG, suggesting that it is less immunogenic than horse and sheep IgGs. Furthermore, single-domain antibody fragments of camelid IgG, comprising only the variable domain of heavy chain antibodies, are effective antigen-binding fragments devoid of immunogenicity (Cortez-Retamozo et al., 2002).

When incubated with human erythrocytes, horse IgG induced agglutination, thus evidencing the presence of antibodies against erythrocyte membrane components. These antibodies might be specific for such antigens or may correspond to 'heterophile' antibodies, which are polyspecific and agglutinate erythrocytes of various species (Levinson and Miller, 2002). In contrast, no such agglutination was observed when sheep or camel IgGs were incubated with human erythrocytes. The clinical significance of antibodies agglutinating erythrocytes in equine-derived antivenoms has not been established, although our observations indicate that no deleterious effects on erythrocytes occur after intravenous injection of horse IgG antivenom in rabbits (León et al., 2005). Nevertheless, the role played by these antibodies in the early adverse reactions to antivenoms requires further investigation.

A number of workers have shown that human serum contains antibodies against IgGs from various animals, even when they have not been previously injected with these IgGs. It has been speculated that this antibody response may be secondary to sensitization to animal IgG either by ingestion through the food or by exposure to animal hair and dander (Hunter and Budd, 1980; Bernhisel-Broadbent et al., 1991; Ayuso et al., 2000). Since there is a conspicuous cross-reactivity between IgGs of different animal sources (Esteves and Binaghi, 1972), exposure or ingestion of IgG from an animal species may sensitize people to IgG of a different animal source. Regardless of the mechanism of sensitization, the exposure of humans, particularly in rural areas, to horses, sheep or camel may induce a sensitization to animal IgG. Thus, when an intravenous infusion of relatively large amounts of these heterologous proteins is performed during antivenom therapy, such antibodies may react with animal IgGs in the circulation, forming immune complexes and activating the complement system, with the consequent release of anaphylatoxins and the onset of early adverse effects. Such mechanism would be involved only in early adverse reactions and not in late adverse effects associated with serum sickness.

The immunoassay developed in this study evidenced the presence of anti-sheep and anti-horse IgGs in the pool of human sera used, whereas the absorbances of wells coated with camel IgGs were similar to those of wells coated with human IgG. The fact that the pool of sera tested corresponds to blood samples collected in Costa Rica agrees with this result, as sheep and especially horses are abundant in this country, whereas there has been no previous exposure to camel serum proteins in these people. Hence, it is proposed that a potential mechanism for the induction of early adverse reactions to heterologous antivenom administration might be the existence of anti-animal IgG antibodies in the serum of patients receiving antivenom, as a consequence of previous sensitization to these proteins. To the best of our knowledge, this represents a novel mechanism to explain the early adverse reactions in heterologous antivenom administration.

In conclusion, the methodology for IgG purification based on the precipitation of non-IgG proteins by caprylic acid was successfully adapted for the purification of sheep and camel IgGs. When the different IgG preparations were compared for their potential for induction of adverse reactions, camel IgG showed the lowest propensity to induce adverse reactions. This supports the use of camelid antibodies in the preparation of antivenoms.

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