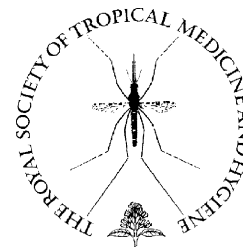




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Human heterophilic antibodies against equine immunoglobulins: assessment of their role in the early adverse reactions to antivenom administration

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Summary The presence of human heterophilic antibodies against horse immunoglobulins (HHA-HI) was determined by ELISA in sera from healthy volunteers and from patients who received equine antivenom for therapy of snake bite envenoming. These patients were selected from two independent clinical studies: one in Colombia in which patients received antivenom constituted by whole IgG ($n=25$); and the other in Brazil where an antivenom constituted by F(ab')₂ fragments was administered ($n=31$). Results show that healthy volunteers have antibodies, mainly of the IgG class, able to react with whole equine IgG. Additionally, patients have IgG antibodies that react both with whole equine IgG and F(ab')₂ fragments. In both clinical studies, no significant differences were observed in the HHA-HI titres between the patients who presented early adverse (anaphylactoid) reactions and those who did not develop them. In addition, no variation in titre was observed in samples collected before and after antivenom administration. These results do not support the hypothesis that the incidence of early adverse reactions to antivenom administration correlates with the titre of HHA-HI in the serum of patients. Nevertheless, participation of these antibodies as part of a multifactorial pathogenic mechanism associated with these reactions cannot be ruled out.

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1. Introduction

Antivenoms constitute the only scientifically validated treatment for snake bite envenoming (Theakston et al., 2003). Antivenoms must have suitable characteristics of identity, effectiveness, purity and security (Good Manufacturing Practices, 1999). The safety of antivenoms is related to the potential transmission of an infectious agent (Burnouf et al., 2004) or to the development of adverse reactions. One type of adverse response to antivenom administration is related to contamination with bacterial endotoxins, thereby inducing a pyrogenic reaction characterised by chills, fever and tremors (Warrell, 1995). However, these reactions are due to the presence of bacterial contaminants and are not caused by the immunoglobulins of antivenoms.

Adverse reactions to antivenom administration can be of two main types. Early adverse reactions (EAR) usually occur during the administration of antivenom and may be either true anaphylactic reactions, which are rarely reported and correspond to type I hypersensitivity (Williams et al., 2007), or de novo anaphylactoid reactions, with an incidence that ranges between 6% and 80% (Chippaux et al., 1998; Gawarammana et al., 2004; Moran et al., 1998). Anaphylactoid reactions occur in patients who have not previously been exposed to antivenom proteins (Sutherland, 1977). These EARs are characterised by urticaria, itching, fever, tachycardia, vomiting, abdominal colic, headache, bronchospasm, hypotension and angio-oedema, although in many cases only urticaria and itching occur (Warrell, 1995). Late adverse reactions occur 5–26 days after the administration of antivenom and mostly correspond to serum sickness, i.e. type III hypersensitivity (LoVecchio et al., 2003).

The mechanisms responsible for EARs have not been conclusively demonstrated. A role has been suggested for the anticomplementary activity of antivenoms in the genesis of EARs (Sutherland, 1977). Such a mechanism has also been suggested as the basis of adverse reactions to intravenous human immunoglobulin administration owing to the formation of protein aggregates during manufacture (Barandun et al., 1962). Despite the fact that complement consumption has not been demonstrated following antivenom administration in clinical cases (Malasit et al., 1986), reduction of anticomplementary activity is achieved in the manufacture of antivenoms through processes such as pepsin digestion (Jones and Landon, 2003) and β -propiolactone treatment (León et al., 2005). However, although pepsin-digested F(ab')₂ antivenoms display a lower in vitro anticomplementary activity than whole IgG antivenoms (Morais et al., 1994), the incidence of EARs following administration of F(ab')₂ antivenoms shows striking variation (Chippaux et al., 1998; Gawarammana et al., 2004; Moran et al., 1998), suggesting that factors other than the nature of the active principle (IgG or F(ab')₂) play a predominant role in the onset of EARs. Furthermore, a recent clinical study comparing two IgG antivenoms with different anticomplementary activity did not show variations in the incidence of EARs (Otero et al., 2006). Therefore, mechanisms other than complement activation must be at play in the onset of these reactions.

An alternative mechanism of EARs is the formation of immune complexes between human heterophilic antibodies directed against horse immunoglobulins (HHA-HI) and the antibodies present in antivenoms (Herrera et

al., 2005). The presence of HHA-HI in human serum has been described (Henning et al., 2000; Herrera et al., 2005) and a concentration of 0.017 mg/ml of such antibodies has been quantified in immunoglobulin preparations (Sevcik et al., 2008). Increased titres of these antibodies occur in patients undergoing autoimmune processes such as rheumatoid arthritis (Pope and McDuffy, 1981). Thus, equine-derived antivenom administration to patients having HHA-HI could trigger an EAR by means of a type III hypersensitivity mechanism. Similar reactions take place during the administration of plasma to IgA-deficient patients (Sandler et al., 1995) and are involved in allergies to some foods (Ayuso et al., 2000). The objective of this work was to assess whether there is a relationship between the incidence of EARs and the titre of HHA-HI in patients envenomed by snake bites in Colombia and Brazil.

2. Materials and methods

2.1. Clinical study and samples

The titre of HHA-HI was determined in the serum of eight adult healthy volunteers from Costa Rica and in the sera of victims of snake bite envenomings selected from two independent clinical studies: one in Colombia during 2003–2005, in which patients received antivenom constituted by whole IgG molecules ($n=25$); and the other in Brazil during 2002–2004, where an antivenom constituted by F(ab')₂ fragments was administered ($n=31$). For comparative purposes, the samples selected corresponded to a similar number of patients presenting EARs and patients who did not present EARs. These samples correspond to patients included in more extensive clinical trials, and the actual incidence of EARs in these studies will be published elsewhere. All participating patients, or their relatives, as well as the healthy volunteers signed the corresponding informed consent forms.

The number of antivenom vials administered was recorded and, upon antivenom administration, patients were carefully observed for the development of EARs over 24 h following the end of the infusion of the antivenom. The most common manifestations of EARs were nausea, vomiting, colic, abdominal pain, urticaria and chills. Serum samples, obtained from blood collected before antivenom administration, were obtained from all patients. The samples were identified with a code number that was blinded to the analysts who determined the titre of HHA-HI. In addition, titres were compared between samples collected before antivenom infusion and 24 h (IgG antivenom) or 12 h (F(ab')₂ antivenom) after antivenom administration.

2.2. Determination of HHA-HI titres

To determine the HHA-HI titres, microplates were coated with 100 μ L/well of a 5 mg/dL solution of horse IgG or F(ab')₂ fragment, purified by caprylic acid fractionation (León et al., 1997; Rojas et al., 1994). After washing the plates five times with Tris-buffered solution (TBS) (0.05 M Tris, 0.15 M NaCl, 20 μ M ZnCl₂, 1 mM MgCl₂, pH 7.4), 100 μ L of human serum samples, diluted 1:1000 in TBS–2% bovine serum albumin (BSA), was added and the plates were

incubated for 1 h at 20–22 °C. Plates were then washed five times with TBS. For analysis of patient sera, 100 µl of rabbit anti-human IgG conjugated with peroxidase, diluted 1:1000 with TBS–2% BSA, was added to each well. For samples from healthy volunteers, 100 µl of goat anti-human IgG, IgM or IgA, conjugated with alkaline phosphatase, diluted 1:1000 with TBS–2% BSA, was added to each well. Plates were then incubated for 1 h at 20–22 °C. All the conjugates were previously adsorbed by affinity chromatography on Sepharose coupled to equine IgG to assure the absence of heterophilic antibodies in these commercial preparations during the assay. Colour was developed by the addition of *p*-nitrophenyl phosphate for alkaline phosphatase, or H₂O₂ and *o*-phenylenediamine for peroxidase, and the absorbances were recorded at 405 nm or 492 nm, respectively. The titres were expressed as absorbance values (mean ± SD; *n* = 3). Comparisons were made only between samples run in the same plate.

2.3. Determination of rheumatoid factor

The presence of rheumatoid factor was assessed in the sera of patients from Brazil using an agglutination-based kit (Artritest; Wiener Laboratories, Rosario, Argentina). The tests were performed following the manufacturer's instructions.

2.4. Statistical analysis

The significance of the differences between the mean values of the titres in samples from patients who developed EARs and in those who did not, as well as in serum samples before and after antivenom administration, was determined by the Student's *t*-test. The relationship between the number of antivenom vials administered and the incidence of EARs was evaluated by the ϕ^2 test.

3. Results

Patients included in this study received variable doses of antivenom depending on the severity of the case. In the clinical trial performed in Brazil with F(ab')₂ antivenom, patients received either 4 or 8 vials, whereas in the study performed in Colombia with whole IgG antivenom patients received either 3, 5, 8, 10 or 12 vials. However, despite such variations in antivenom dosage between patients, there was no statistically significant association between the appearance of EARs and the number of antivenom vials administered (*P* > 0.05, ϕ^2 test).

Sera from eight normal volunteers presented HHA-HI that were of the IgG class (Figure 1). No association was found between the titre of HHA-HI and the development of EARs in patients from either population (Brazil or Colombia), since there were no significant differences in the absorbance values between patients who developed EARs and patients who did not (Table 1; Figure 2). Moreover, no significant differences were observed in any patient between the titres of sera collected before and after antivenom administration (results not shown). There was no direct association between the incidence of EARs and the total number of

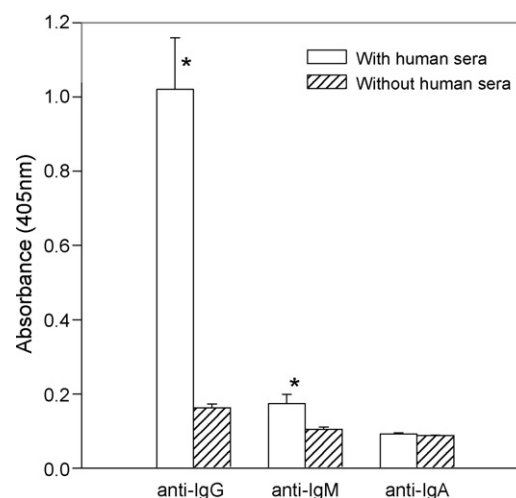


Figure 1 Detection of human heterophilic antibodies against horse immunoglobulins (HHA-HI) in the serum of healthy human volunteers. ELISA plates were coated with a solution of horse whole IgG. The anti-horse IgG titre of serum samples from eight human healthy volunteers was then determined as described in Section 2.2 using either anti-IgG, anti-IgM or anti-IgA conjugated with alkaline phosphatase. Results are presented as the absorbance at 405 nm (mean ± SD) of a 1:1000 serum dilution. *Significant (*P* < 0.05) increment in absorbance was observed for anti-IgG and anti-IgM, but not for anti-IgA.

antivenom vials administered to each patient for either type of antivenom (*P* > 0.05, ϕ^2 test).

Rheumatoid arthritis patients produce rheumatoid factors that are detectable by conventional latex slide agglutination tests using human or horse IgG as antigen (Houssien et al., 1998). Thus, it was relevant to determine whether there is a relationship between adverse reactions to equine antivenom and the occurrence of rheumatoid factor. However, none of the patients who developed EARs in the Brazilian study showed a positive test for rheumatoid factor.

4. Discussion

Anaphylactoid reactions occur in a percentage of patients who receive antivenom for the treatment of snake bite envenoming (Warrell, 1995). Despite the relevance of this clinical phenomenon, the mechanisms behind EARs have not been clearly established. Previous clinical works have examined the role of complement activation in EARs (Malasit et al., 1986; Otero et al., 2006), however no conclusive evidence has been presented in support of this as the main mechanism responsible for EARs. Another possibility is that the incidence of EARs correlates with the total volume of antivenom administered. Although a correlation has been observed between antivenom dose and the incidence of late adverse reactions, i.e. serum sickness (LoVecchio et al., 2003), no such correlation has been demonstrated for EARs (Malasit et al., 1986; Otero et al., 2006). Our present observations further support the concept that the incidence of EARs is not dependent on the antivenom dose used. An alternative mechanism for EARs

Table 1 Detection of human heterophilic antibodies against horse immunoglobulins in the serum of patients treated with equine antivenom in two independent clinical studies

Type of antivenom administered	Antigen used in ELISA	Titre (Abs ₄₉₂) ^a	
		With EARs	Without EARs
IgG	IgG	(n = 11) 0.926 ± 0.143	(n = 14) 1.124 ± 0.240
	F(ab') ₂	0.885 ± 0.141	0.908 ± 0.281
F(ab') ₂	IgG	(n = 8) 0.566 ± 0.048	(n = 23) 0.489 ± 0.122
	F(ab') ₂	0.538 ± 0.064	0.470 ± 0.110

EAR: early adverse reaction.

^a The sera of patients were separated into two groups: patients who presented EARs and those who did not. ELISA was performed as described in Section 2.2 using anti-human IgG conjugated with peroxidase. Results are presented as the absorbance at 492 nm (mean ± SD) of a 1:1000 serum dilution. No significant differences were observed ($P > 0.05$) in the titres of patients having EARs and those who did not develop EARs treated with either whole IgG or F(ab')₂ antivenoms.

has been investigated in this work, i.e. the presence of HHA-HI.

Our results demonstrate the presence of HHA-HI, predominantly of the IgG class, in healthy volunteers from Costa Rica and in patients suffering snake bite envenoming from Colombia and Brazil. However, a correlation between the HHA-HI titre and the development of EARs was not found, thus showing that the incidence of EARs is not directly related with the titre of these antibodies. Nevertheless, a possible involvement of these antibodies as a contributing factor in the mechanism of EARs cannot be ruled out and should be explored further. It might be that for EARs to develop, a definite proportion of HHA-HI and antivenom IgG is required, as occurs in serum sickness following antivenom administration (Nielsen et al., 1978). Therefore, HHA-HI may play a role in EARs when their concentration and the concentration of administered equine IgG or F(ab')₂ fragments reach an optimal proportion in the bloodstream of patients upon antivenom administration, forming immune complexes that trigger the release of inflammatory mediators responsible for the reactions (Jancar and Sánchez, 2005).

Other factors such as the physicochemical characteristics of antivenoms, i.e. total protein concentration, extent of protein aggregate formation and the presence of preservatives, among others, as well as the protocol used for antivenom administration (speed of infusion, dilution of antivenom in saline) are likely to play a role in the development of EARs. A multifactorial mechanism, in which the presence of HHA-HI hereby demonstrated may play a role, is the most likely scenario in the generation of EARs following antivenom administration.

Authors' contributions: RO, FOSF, JLCC, KCB, FHW, CRM, JCLP and CMSM designed the protocols for the clinical trials; GL, AS, MH, BL and JMG designed the protocol for the immunological studies; RO, FOSF, KCB, JLCC and FHW carried out the clinical assessments; GL, AS and MH carried out the immunoassays; GL, AS, MH, BL and JMG performed the analysis and interpretation of the data; GL, RO, FOSF, BL and JMG drafted the manuscript. All authors critically read the manuscript, made suggestions to its final content and

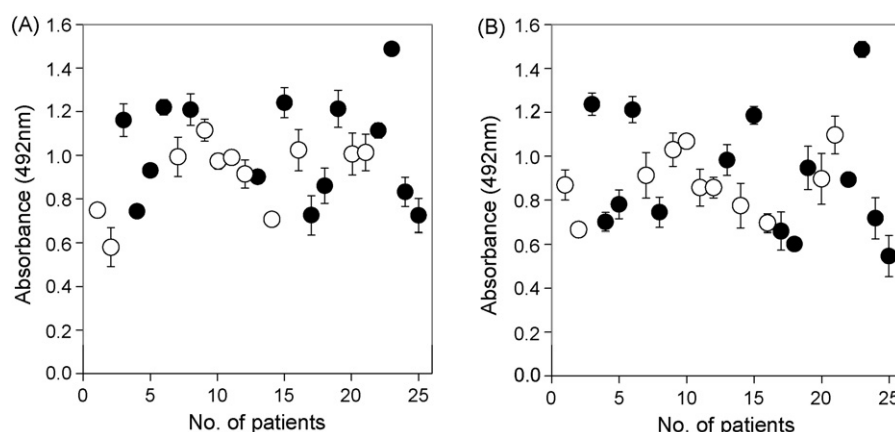


Figure 2 Titres of human heterophilic antibodies against horse immunoglobulins (HHA-HI) in the sera of envenomed patients treated with whole IgG equine antivenom. ELISA plates were coated with either (A) equine IgG or (B) equine F(ab')₂. Assays were performed as described in Section 2.2 using anti-human IgG conjugated with peroxidase. Each symbol represents one patient; empty circles correspond to patients who developed early adverse reactions (EARs), whereas filled circles correspond to those who did not develop EARs. Each sample was run in triplicate. Results are presented as absorbance at 492 nm (mean ± SD) of a 1:1000 serum dilution.

read and approved the final version. GL is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: This study was part of larger clinical investigations with protocols approved by the Human Ethics Committee of the University of Antioquia, the Instituto Nacional de Vigilancia de Medicamentos y Alimentos (INVIMA) of the Colombian Ministry of Health (Record of Proceedings No. 28), the Ethical Committee of Butantan Institute, and the Ethical Research National Committee of Brazil.

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