



Development of an elisa for the diagnosis of reactive IgE antibodies anti-therapeutic horse sera



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ARTICLE INFO

Article history:

Received 5 June 2017

Received in revised form

26 July 2017

Accepted 10 August 2017

Available online 12 August 2017

Keywords:

Anaphylactic

Antivenom

Diagnostic assay

ELISA

Hypersensitivity

Immunological assay

Multiple antigen peptide

ABSTRACT

Hypersensitive diseases that involve IgE reactivity are important concern of public, especially those encompassing the potential pathogenesis from the administration of horse serum-based therapeutics such as antivenoms. A method for the definitive diagnosis of reactive IgE is important for identifying allergic patients to control severe collateral effects during planned and emergency application of immunotherapies when the allergy source cannot be avoided for treatment. To date, no tests have been developed to accompany the wide range of antivenoms produced from horse sera. The aim of this was to develop a cost-effective ELISA of high sensitivity and specificity to detect circulating patient IgE that binds horse IgG₃, the most prevalent antibody class in passive antibody therapies. Horse IgG₃ was purified in a single step on jacalin-Sepharose and absorbed to standard ELISA plates as the capture molecule for reactive human IgE. The direct performance evaluation with allergenic and non-allergenic patient, together with competitive peptides assays, showed high sensitivity and specificity to detect human IgE that recognized horse IgG₃. The analytical sensitivity and ED₅₀ were calculated to be 0.01 $\mu\text{g mL}^{-1}$ and 0.052 $\mu\text{g mL}^{-1}$, respectively. The intra- and inter-assay coefficient of variation ranged from 3.3 to 11.1% and 4.0–8.0%, respectively. The horse IgG₃-based ELISA assay can detect reactive allergenic IgE at picomolar concentrations. The coefficient of variation suggests that it can be easily standardized between laboratories, provide rapid and can be applied to population surveillance. Patient management during treatment for envenomation would be greatly improved by a robust and reliable diagnostic test for preexisting allergies to mitigate life-threatening consequences of hypersensitivity.

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1. Introduction

Hypersensitive diseases are mediated by the activation of reactive IgE. Often, affected individuals are unaware, making it an important concern during the administration of antivenoms and other horse serum-based immunotherapies. A prior or concomitant diagnosis can improve the medical response to pathogenesis involving IgE in allergic patients from planned or emergency treatments when the allergy source cannot be avoided. To date, no such test is available. A common application of antivenom is for snakebites, a frequently devastating environmental and occupational pathology, especially in rural areas of tropical developing countries, where risks from treatment can reach those of the envenomation (Boyer et al., 2013).

Abbreviations used: CBS, 50 mM citrate-buffer saline; CV, coefficient variation; ELISA, enzyme linked immunosorbent assay; holg, horse immunoglobulin; holgG₃, horse immunoglobulin G₃; hulGE, human immunoglobulin E; HPLC, high performance liquid chromatography; ID-holgG₃ ELISA, indirect horse IgG₃ ELISA; IgGC-holgG₃ ELISA, IgG competitive horse IgG₃ ELISA; IACV, intra assay coefficient of variation; IVIg, intravenous Ig; mAb(s), monoclonal antibody(ies); MAP, multiple antigen peptide; Pa, polyacrylate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Antivenoms are efficient immunotherapeutic preparations that can prevent and even reverse the clinical effects of circulating snake toxins (Sutherland and Lovering, 1979; Warrell, 1996; Moran et al., 1998; Morais and Massaldi, 2009). These preparations are normally composed of pepsin-refined immunoglobulin fragments F(ab')₂ that are most often prepared from the plasma or serum of horses hyperimmunized with preparations of the particular toxin(s) in question. The appropriate administration of an antivenom preparation can have a profound and rapid impact on the recovery of a victim (Gutiérrez et al., 2006; Isbister et al., 2012). One of the most severe side effect from treatment are the immediate hypersensitivity reactions to the foreign proteins in snake antivenoms. The prevalence of hypersensitivity disease is not restricted to people living in specific areas. Globally, it has been estimated that 1–40% (Visser and Chapman, 1978; Coetzer and Tilbury, 1981; Wilkinson, 1994; Chen et al., 2000; Isbister et al., 2000) of the population suffer from this problem. However, this prevalence could be higher due the absence of an accurate diagnostic assay (De-Simone et al., 2014), which also prevents determining if the observed allergies are attributable to the presence of entire immunoglobulin in the horse sera-derived product preparations as predicted.

Currently, the diagnosis of an antivenom allergy involves a patient history, physical examination, and confirmatory tests that identify whether the patient symptoms are of allergic or non-allergic origin. If an allergy is responsible, then the allergens responsible must be identified and the most relevant type of diagnostic testing for identification is provocation. However, this type of testing can be dangerous for the patient, is certainly unpleasant, and cannot be performed for multiple allergens in a single visit to a medical facility. In addition, patients taking certain drugs or patients with skin problems cannot be tested (Galant and Maibach, 1973; Reddy et al., 1978; Brown et al., 1979; King, 1982). In the case of an allergy to antivenom, dermal pretesting is no longer recommended (Cupo et al., 1991; Currie et al., 2008; Thiansookon and Rojnuckarin, 2008). Further, the use of pre-medication before administration of an antivenom also is controversial and not widely practiced. Recent evidence suggests that neither steroids nor antihistamines provide benefits in the treatment of the hypersensitivity (Gawarammana et al., 2004; De Silva et al., 2011).

Nonetheless, *in vitro* techniques for allergy tests can be improved since the specific antigen responsible for immediate allergic hypersensitivity was revealed to be an immunological response characterized as IgE. To gain further insight into the antivenom immune disorder, the allergenic components of horse immune sera have been studied and identified. The major linear IgE-binding epitope was mapped using overlapping synthetic peptides, which showed that the sequence representing the amino acids residues D177 to V189 of the heavy chain of horse IgG3, was recognized by IgE from allergic patient sera (De Simone et al., 2014). Such an epitope is of high diagnostic and clinical value as it can constitute the basis for an epitope-specific immunoassays to identify patients with anaphylactic allergy to antivenom therapy.

However, the validation of candidate epitopes for diagnostic applications is impeded by the lack of rapid and reliable screening procedures as well as issues for generating epitope-specific immunochemical reagents by simple standard procedures. Therefore, our work in the development of an indirect ELISA-based test represents an important improvement in the diagnosis of allergies towards horse Ig preparations (holG3). The holG3-ELISA system requires no standard curve and quantifies allergen-specific IgE at picomolar concentrations. The holG3-ELISA technique detects the presence of circulating specific IgE antibodies permitting *in vitro* diagnosis of IgE-mediated hypersensitivity.

2. Material and methods

2.1. Materials and reagents

Reagents for solid phase peptide synthesis were from Merck-Calbiochem (Darmstadt, Germany) except 3,3'-diaminodipropylamine and *N*-benzyloxycarbonyl-L-tyrosine (*N*-cbz-L-Tyr) that were purchased from Fluka AG (Switzerland) and Acros Organics (USA), respectively. Sepharose CL-4B was purchased from GE Healthcare Life Science (SP, Brazil). Bovine serum albumin (BSA), *p*-nitro phenylphosphate (pNPP) and α -D-galactose were obtained from Sigma-Aldrich (St Louis, MO, USA). Horse serum was supplied by INCQS-FIOCRUZ (Rio de Janeiro, Brazil). Rabbit anti-human-IgG and goat anti-human IgE labeled with alkaline phosphatase were purchased from KPL (Kirkegaard & Perri Laboratories, USA).

2.2. Serum sampling

Human serum that served as the IgE antibodies source was collected from selected patients (group A; *n* = 8), who had been victims of one or more snake bites and who had presented with a severe anaphylactic reaction when treated with at least three injections of antivenom (polyvalent anti-bothropic sera). Blood was drawn by venipuncture and after separation; the serum was frozen at –20 °C until use. The allergic response was confirmed by clinical history, a positive response against holG and characterized “in-house” by measuring specific IgE reactivity (De Simone et al., 2014).

Twenty serum samples from healthy individuals, who had never received any intravenously introduce therapeutic products produced from horses serum (group B), were included as negative controls. A third group, group C (*n* = 6), was composed of people who had received at least one dose of horse serum-derived antivenom and tested positive for IgG reactivity against holG and horse albumin with no indications or symptoms of IgE hypersensitivity.

2.3. Purification of horse IgG3

The purification of the holG3 protein was realized by affinity chromatography on a column of jacalin-Sepharose chromatography as described previously (De Simone et al., 2017). Briefly, horse sera (100 mL) was mixed with an equal volume of high salt Tris-buffer (350 mM Tris–HCl, pH 7.4, containing 300 mM NaCl). The solution was filtered (22 μ m) and applied to a column (3 $\times$ 1 cm) containing jacalin lectin conjugated to Sepharose CL4B previously equilibrated with Tris-buffer (175 mM Tris–HCl, pH 7.4, containing 150 mM NaCl). After extensive washing, adsorbed proteins were eluted with 0.8 M α -D-galactose in the same Tris-buffer at a flow rate of 1 mL/min at room temperature. The protein content of the fractions was monitored by the absorbance change at 280 nm on a NanoDrop 2000 spectrophotometer (Thermo Scientific) and the concentration estimated using the extinction coefficient (*E*) of 1.36 for a 0.1% at 280 nm in a 1 cm pathway.

2.4. Electrophoresis

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12% polyacrylamide gels in Laemmli buffers (Laemmli, 1970) under reductive conditions. The gels were Coomassie blue G-250 stained. Phosphorilase B (97.4 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45.0 kDa) and carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa) were used as standards for characterization of molecular mass.

2.5. Peptide synthesis and preparation of the MAP

For preparation of a dendrimeric multi antigen peptide (MAP), a standard solid phase synthesis protocol was followed using the octameric Fmoc₈-Lys₄-Lys₂-Lys-B-Ala Wang resin for the peptide (DVLFTWYVGGGGG) that possessed the IgE epitope. The construct was prepared in an automated peptide synthesizer (PSS8-model, Shimadzu, Kyoto, Japan) and the side chains of octafunctional Fmoc-amino acids were protected with TFA-labile protecting groups as required. Residues corresponding to the monovalent ('tail') part of the construct (see scheme in Fig. 3A), up to the first (bis-Fmoc) Lys residue initiating the dendrimer structure, were incorporated via single couplings. Once sequence assembly was completed, the Fmoc groups were removed and the peptide-resin was cleaved and fully deprotected with TFA/H₂O/EDT/TIS (94/2.5/2.5/1.0 v/v, 90 min). The peptide was precipitated by the addition of chilled diethyl ether followed by centrifugation. The resulting pellet was taken up into aqueous AcOH (10% v/v), dried and stored as a lyophilized powder. When necessary, the MAP was dissolved in water, centrifuged (10,000×g, 60 min, 15 °C) and the supernatant filtered by a centricon 10 filter. Purification of MAPs was performed by RP-HPLC on a Shimadzu C18 column (21.2 × 250 mm, 10 μm) coupled to an HPLC system (Shimadzu, model 6A) at a flow rate of 1.2 mL min⁻¹ using a gradient (7–40%) of solvent B into A [A and B: 0.07% TFA (v/v) in water and acetonitrile, respectively]. UV detection at 220 nm was used to analyze fractions judged and those of adequate purity were pooled and lyophilized. Their identity was confirmed by MS (MALDI-TOF or electrospray).

2.6. Indirect horse IgG3 enzyme-linked immunosorbent assay (ID-holG3 ELISA)

An indirect enzyme linked sorbent assay (ID-ELISA) for holG3 (ID-holG3 ELISA) was prepared in a 96-well plate (C96 Maxisorb Microwell, Nunc, New York, USA) by adding purified holG3 protein in coating buffer (Na₂CO₃–NaHCO₃, pH 9.6) to wells and incubating overnight at 4 °C. Wells were washed three times using PBS-T (PBS with 0.1% Tween 20, pH 7.2), blocked with 2% defatted milk for 2 h at 37 °C and washed three times in PBS-T before the addition of patients sera (50 μl). After a 2 h incubation (37 °C), wells were washed three times with PBS-T and then incubated with goat anti-human IgE conjugated to alkaline phosphatase (50 μl, 1:5000 diln). After another 2 h incubation, wells were washed with PBS-T before detection with p-nitro phenyl-phosphate (pNPP) over a 15 min incubation. Development was stopped with 3 N NaOH and the absorbance measured at 405 nm in a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, Ca, USA).

2.7. Competitive ELISA assays

The same procedures for the ID-ELISA were used with the sera samples premixed. The first assay consisted of a pool of rich IgE patient serum (group A, n = 5, 50 μl) was mixed with an equal volume of a dilution series (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) of a pool of patient sera rich in IgG against holG3 (group B, n = 6) 4 h at 37 °C. The second assay used the same pool of IgE-rich patient serum (50 μl, n = 5) incubated with increasing concentrations of MAP-E. After the incubation time, mixtures were centrifuged (100,000×g, 1 h at 4 °C) before applying to the ID-ELISA.

1 - OD of sample with competitor/OD of sample without competitor
X 100

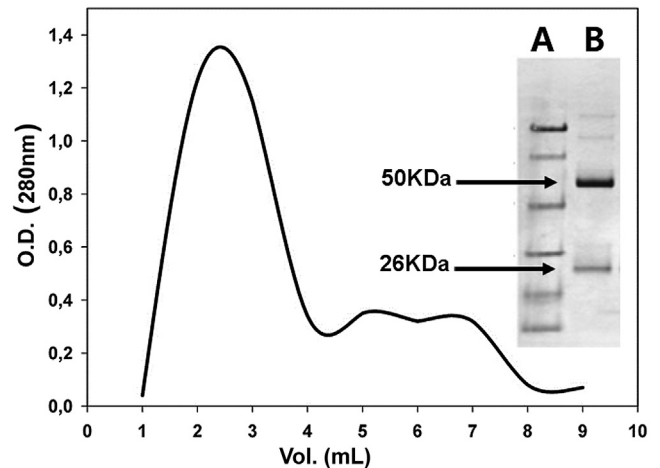


Fig. 1. Affinity purification of horse IgG3 immunoglobulin to jacalin-Sepharose. Jacalin lectin covalently attached to Sepharose CL-4B resin was used to specifically bind holG3 from whole horse blood and eluted with α -D-galactose. The absorbance at 280 nm is plotted against the elution volumes. Analysis of the principle elution peak (1.5–3.5 mL) by SDS-PAGE (12%; inset) shows the heavy and light chains of holG3 under reduced conditions (Lane B). Molecular weight standards (Lane A): phosphorilase B (97.4 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

2.8. Intra assay variation

Intra assay variation describes the variation of results within a data set obtained from one experiment. It is expressed by the intra assay coefficient of variation (IACV) to monitor the deviation within the same assay. Each sample is measured several times, and then % IACV is calculated for each sample by dividing the standard deviation (SD) of a set of measurements by the set mean, and multiplying by 100. Finally, the average of the individual CVs is denoted as intra assay CV.

$$\% \text{ IACV} = \text{Mean of SD} \times 100 / \text{Mean}$$

2.9. Coefficient of variation (CV)

The CV was used to express the precision and repeatability of the ELISA assay. Is a standardized measure of dispersion of a probability distribution or frequency distribution. It was expressed as a percentage, and was defined as the ratio of the standard deviation σ to the mean μ (or its absolute value, $|\mu|$).

3. Results

3.1. Purification of holG3 by jacalin-Sepharose

Horse IgG3 was purified by lectin affinity chromatography on jacalin-Sepharose (Fig. 1) that produced a single major peak. Under native conditions, a gel filtration analysis on HPLC displayed a single major peak that correlated with a molecular mass of 160 kDa and represented the fully assembled holIgG (data not shown). Under reducing conditions on SDS-PAGE, the analysis of the purified holG3 revealed two prominent bands of a molecular mass approximately of 50 kDa (heavy chain) and 26 kDa (light chain) (Fig. 1, insert) confirming that the IgG3 is stabilized by interchain disulphide bridges.

3.2. Optimal conditions for estimation of IgE-specific ELISA

Coating buffer, concentration of individual reagents, temperature, and period of incubation at each step were determined by changing each parameter and keeping the other variables constant. Best reproducibility was obtained when ELISA plates were coated with purified holgG3 in carbonate buffer at a concentration of $30 \mu\text{g mL}^{-1}$, which proved to be the saturating concentration (Fig. 2A; closed circle). Serum from healthy individuals, tested as a pool, did not show any detectable signal (closed triangle).

3.3. Assessment of specific IgE by ELISA

Using the ID-holgG3-ELISA, specific IgE could be measured over a wide range of serum dilutions (Fig. 2B; closed circles and squares). The activity holgG3-specific IgE in the pool of positive sera was confirmed before and after heating for 4 h at 56°C , which would inactivate the IgE reactivity (open circle). In the experiment design, the sera used were obtained from two subjects with a history of antivenom allergy (group A). Two-fold serial dilutions of both sera generated straight lines of reactivity strength with similar slopes when measured by ELISA. The pool of sera from 6 healthy individuals that had no indications of any type of reactivity to holgG3 or antivenom allergies (group C) showed no measurable signal under the same experimental conditions (Fig. 2B; closed triangle).

3.4. Inhibition of the holgE-anti holgG3 by MAPs

To further investigate the specificity of the interaction between patient anti-holgG3 antibodies, present in the sera of allergenic patients, a dendrimeric MAP consisting of peptides spanning the IgE epitope was produced (Fig. 3A; insert) and assessed by a competitive ID-holgG3ELISA (Fig. 3A). An increasing amount of MAP (from 5 to $500 \mu\text{g}$) was combined with pooled patient sera ($50 \mu\text{L}$ diluted 1:10) and after a 2 h incubation at 37°C , the samples were processed in the ID-holgG3-ELISA. Optical density was expressed as percent of the non-MAP treated holgG3-ID-ELISA serum. The results showed that a 50% inhibition of the holgG3

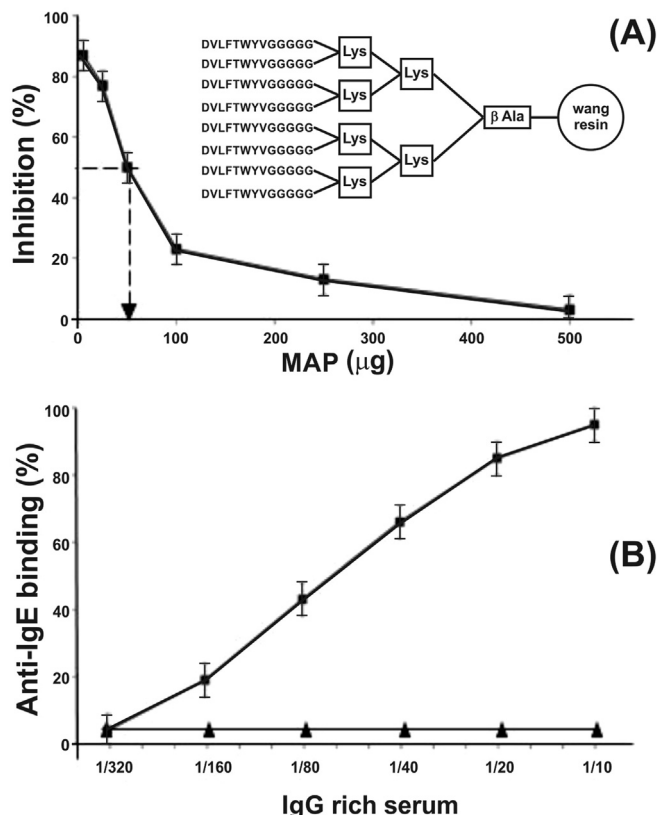


Fig. 3. Interference of a synthetic peptide and anti-holgG3 human IgG on the detection of holgG3 reactive IgE antibodies. Competitive ID-ELISAs were performed by preincubating a pool of sera ($n = 5$) from patients with a clinically confirmed allergy to horse serum-based antivenom with a range of concentrations of either a synthetic peptide containing 8 copies of the IgE linear epitope of holgG3 (Panel A; insert shows half of the MAP structure) or dilutions of holgG anti-holgG3-rich serum from non-allergic patients sera ($n = 5$; Panel B). Measurements represent percent reactivity in the presence of the MAP and percent inhibition by reactive IgG with 100% defined by untreated allergic patient sera. Data points are the media $\pm$ SD of three independent experiments.

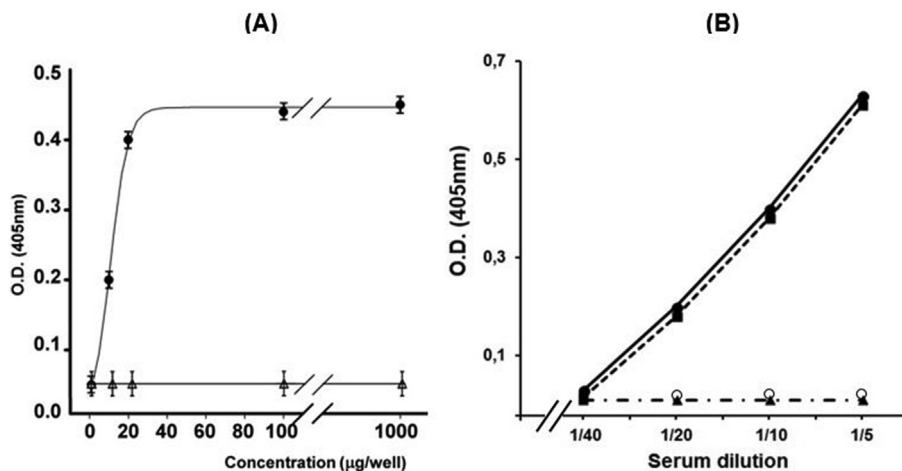


Fig. 2. Detection of human IgE by an ID-ELISA using purified horse IgG3. A, Optical density (405 nm) as a function of antigen concentration added to the wells using a 1:10 dilution of serum from a patient with a clinically defined allergy to antivenom (closed circle) and healthy individuals (closed triangle, $n = 6$). The results are the media $\pm$ SD of three experiments. B, Optical density (405 nm) as a function of serum dilution of two patients with clinical allergy (closed circle and square), healthy individuals (closed triangle) and a pool of the two patients treated at 56°C for 4 h (open circle). In this case the plate was coated with $30 \mu\text{g mL}^{-1}$ of holgG3.

binding human IgE was obtained with 5.2 μg of hulgE-MAP (0.52 $\mu\text{g mL}^{-1}$ of peptide).

3.5. Interference by human IgG anti-holgG3 in the detection of holgG3 specific IgE

The ELISA-positive sera were assayed by ID-holgG3-ELISA before and after mix with a pool of human sera rich in IgG anti-holgG3 (group C, individuals without allergy). Briefly, 50 μL of human holgE-ELISA-positive sera were mixed with the same volume of an increasing serial dilution of a pool sera rich in IgG anti-holgG3, incubated for 4 h at room temperature on a rotator and centrifuged. The supernatant was tested for specific anti-IgE by ID-holgG3-ELISA. The data are presented in Fig. 3B as percent inhibition by the human IgG anti-holgG3. The data show that an increase in the concentration of the human-IgG rich sera in the mixture can interfere with the detection. However, even at the highest concentration (1/10 dilution), IgE can be detected by the ID-holgG3-ELISA.

3.6. Sensitivity, ED_{50} , intra assay variation and coefficient of variation of the ID-holgG3ELISA

The analytical sensitivity and ED_{50} (the dose required to achieve 50% of a positive signal) using the developed ID-holgG3-ELISA method was calculated to be 0.01 $\mu\text{g mL}^{-1}$ and 0.052 $\mu\text{g mL}^{-1}$, respectively. The intra- and inter-assay coefficient of variation (CVs) ranged from 3.31 to 11.12% and 3.99–8.01%, respectively.

3.7. Ethical statement

We confirm that all aspects of the work covered in this manuscript that involved either experimental animals or human patients has been conducted with the approval of all relevant institutional ethics committees, FIOCRUZ (CAAE:23784214.8.000.5248) and Evandro Chagas National Institute of Infectious Diseases (CAAE: 007110). All patients invited to participate in this study provided written informed consent.

4. Discussion

In the present study, we describe a sensitive and specific ID-holgG3-ELISA for the measurement of IgE bind-holgG3 antigen, which has potential applications in identifying persons allergic to antivenoms produced from horse sera and in the evaluation of a progression of hypersensitivity. The antigen used in an immunoassay is the key to acquire high sensitivity and specificity. Therefore, it is important to know the structure and sequence of the antigenic regions. For an allergenic region of a protein, recognized by the binding sites of IgE molecules, they are similar to B-cell epitopes and can be classified into two categories: i) conformational epitopes, in which the involved residues are distantly separated in the sequence and brought into physical proximity by protein folding, and ii) linear sequential epitopes, comprised of a single continuous stretch of amino acids within a protein sequence that can react with anti-protein antibodies. It has been thought that both conformational and sequential epitopes are in general responsible for allergic reactions (Miyaji et al., 2013; Husslik et al., 2016). However, in the case of allergy to horse serum-based antivenom immunoglobulins, we have previously shown that the predominant immunological reactive response of patients with allergies to therapeutic antivenom is confined to a single major epitope, 177DVLFTWYVDG-TEV189, located strictly to the horse IgG3 heavy chain (De-Simone et al., 2014).

The linear response obtained for holgG3-specific IgE over a wide

range of serum dilutions indicates that the ID-holgG3-ELISA is a suitable assay to unambiguously detect the presence of circulating IgE. The specificity of the reaction detected by the ID-holgG3-ELISA for IgE binding to holgG3 was demonstrated by the significant decrease in the optical density observed after competition with a MAP consisting of a peptide representing the single major epitope (Fig. 3A). Considering that the saturating concentration of the whole holgG3 in the assay was 30 $\mu\text{g/mL}$, the 50% inhibition value of 5.2 μg represents an approximately 1:1 molar ration of MAP to target.

To analyze the suitability of the assay to detect hypersensitivity in the background of a previous IgG-based immunological response to horse serum-based antivenoms, an IgG-rich serum competition assay was performed. The values from the ID-holgG3-ELISA to detect anti-holgG3 IgE were affected (Fig. 3B) by the presence of anti-holgG3 IgG. This observation is not surprising since the major antigenic IgE determinant shares five amino acids (DGTEV) with the major antigenic IgG epitope (De Simone et al., 2014). Yet, the ID-holgG3-ELISA was still able to detect reactive IgE, which suggests that the choice of purified horse IgG3 in the ELISA format is a highly effect method for detecting hypersensitive individuals even when reactive IgG and IgE coexist in patient sera. It also indicates that the quantitative values assay may be able to identify hypersensitive patients with a greater chance of exhibiting symptoms or not due to repression of IgE binding by IgG competition.

Considering that the circulating concentration of IgG is several magnitudes fold higher than IgE (Ledin et al., 2008), our observations suggest that the affinity of reactive IgE is much greater than reactive IgG. This most likely reflects the greater number of amino acids in the IgE epitope compared to the IgG epitope that increase the points of interactions between the immunoglobulin binding site and epitope. Incidental evidence suggests that epitope recognition by IgE, for antivenoms, is a progressive process that begins with IgG recognition. The incidence of hypersensitivity to the first application of an antivenom or envenomation is rare (Isbister et al., 2000; Lamb et al., 2017). However, multiple exposures appears to increase the likelihood of a hypersensitive response. Here, the control group that provided serum rich in IgG reactive for holgG3 had only received a single application of antivenom. In contrast, the hypersensitive individuals had received multiple treatments that presented a high concentration of holgG3-specific IgE antibodies. These observations are consistent with an initial IgG response that progressed to the IgE response. It will be of interest to test this hypothesis on the progression of an initial, IgG-based immune response towards hypersensitivity to other allergies.

Overall, the data suggests that our ID-holgG3-ELISA is an important advancement for the identification of patients that could display anaphylactic reactions to horse serum-based therapies. Further, our evaluations suggest that the greatest benefits will be realized by patients that have multiple exposures to antivenoms or other passive antibody therapies. Additional applications include research on the progression to hypersensitivity and allergies from venoms. The development process highlights the importance of improved choices of experimental conditions and the accurate selection of reagents.

5. Conclusion

The ELISA developed here displayed high sensitivity and would be suitable in routine laboratory tests that may contribute to epidemiological statistics on the presentation of hypersensitive patients to equine therapeutic preparations. The new method, based on singular allergenic molecule, will provide an increased consistency in the testing for allergies challenging results through the use of the purified antigen holgG3, which permits an improved

standardization of the diagnostic test. The performance of the hIgG3-ELISA immunoassay suggests that positive results can be further refined to determine the likelihood of symptomatic and asymptomatic reactions hypersensitive with a small sample volume. The approach utilized can be applied to the detection of other unsuspected sensitivities by focusing on allergenic molecules and mapping the IgE epitope.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank VG Mendes, K Felisbino and PN Pêgo for assistance with testing anti-horse Ig antibodies. SGS received financial assistance from the Brazilian Council for Scientific Research (CNPq-no 467.488.2014–2), Carlos Chagas/Filho Foundation of Research Support of the State of Rio de Janeiro (FAPERJ no 170.009–2008), Higher Education Coordination of Improvement (CAPES- no 291910) and PROEP/FIOCRUZ.

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