Toxicon 78 (2014) 83–93

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

IgE and IgG epitope mapping by microarray peptide-immunoassay reveals the importance and diversity of the immune response to the IgG3 equine immunoglobulin

Salvatore G. De-Simone [a,b,*], Paloma Napoleão-Pêgo [b], Luiz A.L. Teixeira-Pinto [b], Anibal R. Melgarejo [c], Aniesse S. Aguiar [c], David W. Provance Jr. [a]

[a] Center for Technological Development in Health (CDTS) National Institute of Science and Technology for Innovation in Neglected Diseases (INCT-IDN), FIOCRUZ, Rio de Janeiro, RJ, Brazil
[b] Molecular and Cellular Biology Department, Federal Fluminense University, Niterói, RJ, Brazil
[c] Research and Development Laboratory, Vital Brazil Institute, Niterói, RJ, Brazil

ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 29 November 2013
Accepted 4 December 2013
Available online 13 December 2013

Keywords:
Horse immunoglobulin
B-linear epitopes
IgE epitope
IgG epitope
Anaphylactic reaction
Spot-synthesis

ABSTRACT

The presence of whole horse IgG in therapeutic snake antivenom preparations of high purity is a contamination that can cause IgE-mediated allergic reactions in patients. In this study, the immunodominant IgE and IgG-binding epitopes in horse heavy chain IgG3 were mapped using arrays of overlapping peptides synthesized directly onto activated cellulose membranes. Pooled human sera from patients with and without horse antivenom allergies were used to probe the membrane. We have demonstrated that, for both cases, individuals produce antibodies to epitopes of sequential amino acids of horse heavy chain IgG3, although the signal strength and specificity appear to be distinct between the two groups of patients. A single region was found to contain the dominant allergic IgE epitope. The critical residues involved in the binding of human IgE to the epitope were determined to include four hydrophobic amino acids followed by polar and charged residues that formed a coil structure. This is the first study to describe the specific amino acid sequences involved with the immune recognition of human IgG and IgE to horse antivenom.

1. Introduction

Antivenom is the primary treatment for snake envenoming (Theakston and Warrell, 1991; Lang et al., 2000). These immunobiological compounds contains polyclonal antibodies raised against the one or more venoms and can be obtained in horses, sheep or goats. Intravenous administration of antivenom to patients with snake envenoming prevent or reverse clinical effects by the binding of the antibodies to circulating snake toxins, which neutralizes their activity and promote their elimination (Warrell, 1996). Monovalent antivenoms are raised against a single snake species, while polyvalent antivenoms are raised against more than one specie.

These preparations are normally composed of pepsin-refined immunoglobulin fragments ($F(ab')_2$) that are most

Abbreviations: CBS, 50 mM citrate-buffer saline; hIg, horse immunoglobulin; ELISA, enzyme linked immunosorbent assay; hhcIg, horse heavy chain immunoglobulin; HCS, health control sera; HRP, horseradish peroxidase; NST, not sensitive treatment; ST, sensitive treatment; TBS-CT, tris-buffer saline containing 3% casein and 0.1% Tween 20, pH 7.0; TBS-T, tris-buffer saline, 0.1% Tween 20, pH 7.0; TMB, 3,3′,5,5′-tetramethylbenzidine.

* Corresponding author. Center for Technological Development in Health (CDTS) National Institute of Science and Technology for Innovation in Neglected Diseases (INCT-IDN), FIOCRUZ, Rio de Janeiro, RJ, Brazil. Fax: +55 21 38658183, +55 83 32167436.
E-mail addresses: salvatore.giovanni@pq.cnpq.br, dsimone@ioc.fiocruz.br (S.G. De-Simone).

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http://dx.doi.org/10.1016/j.toxicon.2013.12.001

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). However, immediate hypersensitivity reactions to the foreign proteins in snake antivenoms are the most severe side effects from antivenom treatment. Life threatening reactions can be mediated by patient IgE antibodies (anaphylactic) or non-IgE mechanisms (anaphylactoid) (Chippaux and Goyffon, 1998; Moran et al., 1998; León et al., 2013). Later reactions (5–26 days after administration; serum sickness or type III hypersensitivity) are less severe that involve the immune response of the patient to raise IgG antibodies against the horse proteins (Dart and McNally, 2001; LoVecchio et al., 2003; Vazquez et al., 2013). Published worldwide rates of anaphylactic reactions to antivenom range from 1% (Visser and Chapman, 1978; Christensen, 1981) to 19% (Coetzer and Tilbury, 1982; Isbister et al., 2000) up to a maximum 40% (Wilkinson, 1994). In Brazil, an incidence of about 10–20 cases of anaphylactic reactions for every 20,000 injections has been estimated for the last ten years (Melgarejo, personal communication). Even with these possible side effects, there is good evidence that in cases of severe envenoming the benefits from the treatment with antivenom outweigh the risks of adverse reactions (Warrell, 1996; Chen et al., 2000).

Clinically, anaphylaxis is an exaggerated response of a sensitized patient, someone with circulating IgE antibodies that can react with the proteins administered in the antivenom, which causes the degranulation of mast cells and basophiles. Degranulation leads to the release of histamine, serotonin and other vasoactive substances. An anaphylactic reaction can cause multiple signs and symptoms such as itching, erythema, flushing, urticaria, angioedema, nausea, diarrhea, vomiting and edema of the glottis/larynx, bronchospasm, hypotension, shock and death. Anaphylactoid reactions are clinically similar to anaphylactic reactions, but are not mediated by IgE (Kay, 2001).

Antibodies are proteins produced by B-cells in response to immunogenic substances. Although much is known about the structure-function of this immunological class of proteins, there is limited published evidence about the precise localization of antigenic sites contained within the antibodies themselves (Terness et al., 1995). This is in contrast to their considerable biotechnological and immunotherapeutic importance since the administration of horse immunoglobulin (hIg) preparations in humans carries the inherent risk of inducing an allergic response in a significant number of people (Sutherland and Lovering, 1979; Ellis and Smith, 1988; Demoly et al., 2002; Williams et al., 2007; Morais and Massaldi, 2009). To date, the recognition of hIg by human antibodies and the specific residues involved remain unknown.

Therefore, a detailed molecular characterization of the epitopes in hIg that are bound by human antibodies would greatly contribute to our understanding of the observed adverse reactions. Here, the horse heavy chain of immunoglobulin G3 (hhcIgG3) was examined since it is the major hIg isotype implicated in the snake venom toxins neutralization (Fernandes et al., 2000) and it is the second most prominent antibody in horse serum (Sheoran et al., 2000). In the present study, we have mapped within the entire sequence of hhcIgG3 the epitopes bound by human IgG and IgE along with the critical amino acids within the epitope involved with interactions with human antibodies. The identification of the important amino acids suggests different molecular mechanisms for the binding of IgG and IgE to hhcIgG3.

Localizing antigenic determinants within the polypeptide chains of protein molecules is essential for subsequent investigations of structural and functional characteristics. By identifying and quantifying components in hIg that contribute to immunological discrimination between self and non-self, antivenoms and antitoxins can be improved by new production techniques engineered specifically to minimize activation of a patient's immune response, but maintain efficacy.

2. Material and methods

2.1. Reagents

Amino-PEG_{500}-UC540 cellulose membranes were obtained from Intavis AG Bioanalytical Instruments (Germany). Amino acids for peptide synthesis were purchased from Calbiochem–Novabiochem Corp. (Germany). Acetonitrile, trifluoroacetic acid and other sequence reagents and chemicals were obtained from Merck (Darmstadt, Germany). Rabbit anti-human-IgG and goat anti-human IgE immunoglobulin labeled with alkaline phosphatase were purchased from Abcam plc (USA) and KPL (Kirkegaard & Perri Laboratories, USA), respectively. The immunoperoxidase assay kit for determining the level of IgE in human samples was purchased from Immunology Consultants Lab (Newberg, USA). Super Signal R West Pico and chemiluminescent substrate were acquired from Pierce Biotechnology (Rockford, IL, USA). Bovine serum albumin and Tween 20 were obtained from Sigma–Aldrich.

2.2. Human sera

The pool of human serum that served as the IgG antibodies source was collected from fifteen healthy volunteers who had been treated over a period of 4–12 months with at least one injection of antivenom produced in horses and had no history of hypersensitivity. The source of IgE antibodies was from three selected patients, who had been victims of one or more snake bites and who had presented with a severe anaphylactic reaction when treated with a series of three injections of antivenom (polyvalent antibothropic sera). The concentration of IgE was measured as described below.

The allergic response was confirmed by clinical history and positive response to hIg, and characterized by measuring specific IgE reactivity. A pool consisting of equal volumes of sera from fifteen health individuals who had never received any therapeutically injection products produced in horses were used as a control. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Committee of the Santa Casa de

Misericórdia do Rio de Janeiro (028/10) Hospital and by the Fundação Oswaldo Cruz/IFF (0071/10).

2.3. Determination of IgG and IgE in human sera

The concentrations of human IgG and IgE were determined by enzyme-linked immunosorbent assay (ELISA) in 96-well microtiter plates (Nunc F Immunoplates I; Nunc, Roskilde, Denmark). Briefly, in this assay, the IgG or IgE present in human sera samples reacted with anti-human IgG and IgE antibodies, respectively, previously adsorbed on the surface of a polystyrene microtitre wells. After removal of unbound proteins by washing, a second set of anti-human IgG or anti-human IgE antibodies conjugated with horseradish peroxidase (HRP) were added. After another washing step, the enzymes bound to the immunosorbent were assayed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB). The quantity of bound enzymes varied directly with the concentration of antibodies in the sample; thus, the absorbance, at 450 nm, was a measure of the concentration of IgG and IgE in the test sample. The quantity of IgG and IgE in the test sample were interpolated from a standard curve constructed with known standards and corrected for sample dilution.

2.4. Bioinformatic analysis

DNA sequences derived from the horse (*Equus caballus*) IgG3 heavy chain (GenBank: CAC86339.1) and the human (*Homo sapiens*) IgG3 heavy chain constant regions (GenBank: CAA67886.1) were retrieved from the National Center for Biotechnology Information database.

2.5. Synthesis of the cellulose-membrane-bound peptide array

A library of peptides (Fig. 1C) was designed to represent and cover the entire coding region (354 amino acids) of the heavy chain horse IgG3 protein. Each peptide was 14 amino acids in length and offset from its neighboring peptide by five amino acids. Synthesis was automatically performed onto Amino-PEG_{500} cellulose membranes according to standard SPOT synthesis protocols (Frank, 1992) using an Auto-Spot Robot ASP222 (Intavis, Koeln, Germany). Coupling reactions were followed by acetylation with acetic anhydride (4% v/v) in *N*, *N*-dimethylformamide to block the peptides during subsequent steps. After acetylation, deprotection was performed to remove F-moc protective groups by the addition of piperidine, which rendered the nascent peptides reactive. The remaining amino acids were added by this same process of coupling, blocking and deprotection until the desired peptide was generated. After the addition of the last amino acid in the peptide, amino acid side chains were deprotected using a solution of dichloromethane–trifluoroacetic acid–triisobutylsilane (1:1:0.05, v/v/v) and washed with methanol. Membranes containing the synthetic peptides were either probed immediately or stored at −20 °C until needed. Negative controls [without peptide] and positive controls [IHLVNNESSEVIVHK (*Clostridium tetani*) precursor peptide] were included in each assay.

2.6. Screening of SPOT membranes

SPOT membranes were washed with 50 mM Tris Buffered Saline (TBS; pH 7.0) and then blocked with TBS with 3% casein and 0.1% Tween 20 (TBS-CT) under agitation at room temperature or overnight at 4 °C. After extensive washing with TBS with 0.1% Tween 20 (TBS-T), membranes were incubated for 2 h with diluted human patient sera (1:250 for IgG and 1:100 for IgE) in TBS-CT and then washed again with TBS-T. Subsequently, membranes were incubated with rabbit alkaline phosphatase labeled anti-human IgG (diluted 1:5000) or goat alkaline phosphatase labeled anti-human IgE (diluted 1:1000) prepared in TBS-CT for 1 h, and then washed with TBS-T and CBS (50 mM citrate-buffer saline). The chemiluminescent substrate (CDP Star Ready-to-use Nitro-Block II, Applied Biosystems, U.S.A) was added to detect positive reactions.

2.7. Scanning and measurement of spot signal intensities

Measurements of the spot signal intensities were obtained as described previously (De-Simone et al., 2013b). Briefly, the chemiluminescent signals were measured and a digital image file generated on a MF-ChemiBis 3.2 (DNR Bio-Imaging Systems, Israel) with a resolution of 5 MP. Signal intensities were quantified with TotalLab Software (Nonlinear Dynamics, USA) using algorithms that compared the intensity between background, spot area and negative control to define the empirical probability that the spot signal intensity was distinct from background signals. The spot with the strongest signal on the membrane was reported as having 100% intensity, and all other spots had their intensity values expressed a relative percentage to this intensity. Only spots with intensity values above 30% were considered in the overlapping of reactive peptides.

2.8. Hydropathy

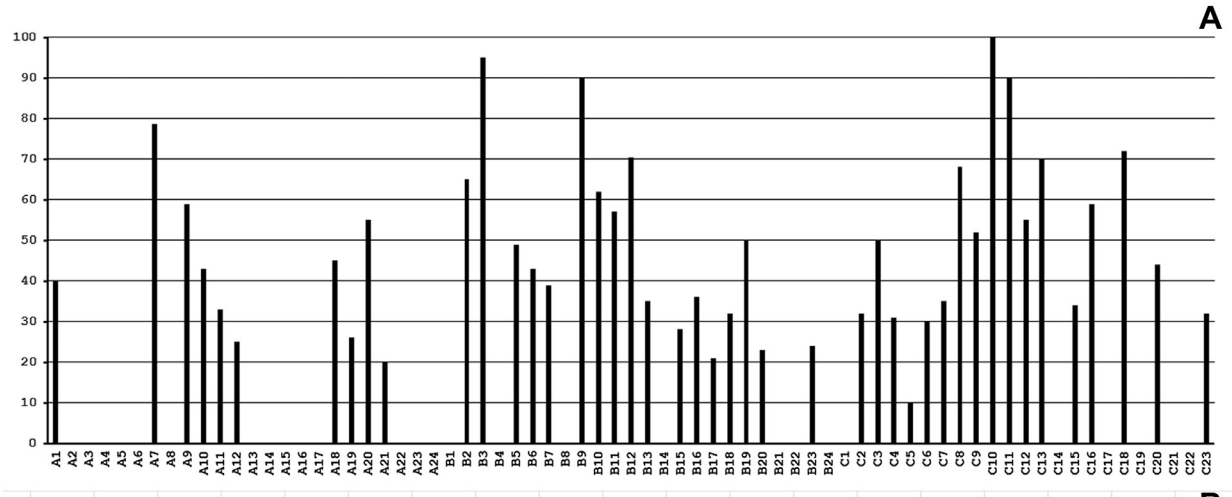
A hydropathy plot with a window size of 9 was used to span the epitopes to define the hydrophobicity of the hhcIgG3 over the length of the peptide sequence (Kyte and Doolittle, 1982).

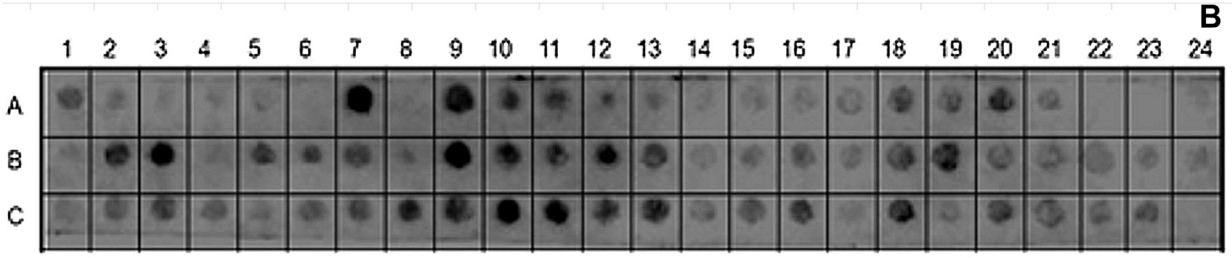
3. Results

3.1. Identification of the immunodominant IgG and IgE epitopes in hIgG3

Two pools of sera were generated to provide either human IgG antibodies or human IgE against horse heavy chain IgG3. The sera with human IgG were stimulated in volunteers that were known not to be sensitive to treatment (NST) by the injection of horse sera-based antivenom. Sera containing human IgE was collected from volunteers who had a known sensitivity (ST) to horse-based immunological treatment. The concentration of IgG from the NST group ranged from 9 to 15 mg/ml as measured by ELISA (data not shown) and IgE in the serum from the ST group was 9–15 mg/ml (group NST) before pooling.

The regions of the primary sequence of hIgG recognized by human IgG and IgE sera were determined using three





C

A1	A-S-T-T-A-P-K-V-F-P-L-A-P-S-C
A2	P-K-V-F-P-L-A-P-S-C-G-T-T-S-D
A3	L-A-P-S-C-G-T-T-S-D-S-T-V-A-L
A4	G-T-T-S-D-S-T-V-A-L-G-C-L-V-S
A5	S-T-V-A-L-G-C-L-V-S-S-Y-F-P-E
A6	G-C-L-V-S-S-Y-F-P-E-P-V-T-V-S
A7	S-Y-F-P-E-P-V-T-V-S-W-N-S-G-T
A8	P-V-T-V-S-W-N-S-G-T-L-T-S-G-V
A9	W-N-S-G-T-L-T-S-G-V-R-T-F-P-S
A10	L-T-S-G-V-R-T-F-P-S-V-L-Q-S-S
A11	R-T-F-P-S-V-L-Q-S-S-G-L-Y-S-L
A12	V-L-Q-S-S-G-L-Y-S-L-S-S-M-V-T
A13	G-L-Y-S-L-S-S-M-V-T-V-P-A-S-S
A14	S-S-M-V-T-V-P-A-S-S-L-E-S-K-T
A15	V-P-A-S-S-L-E-S-K-T-Y-I-C-N-V
A16	L-E-S-K-T-Y-I-C-N-V-A-H-P-A-S
A17	Y-I-C-N-V-A-H-P-A-S-S-T-K-V-D
A18	A-H-P-A-S-S-T-K-V-D-K-R-I-E-P
A19	S-T-K-V-D-K-R-I-E-P-V-L-P-K-P
A20	K-R-I-E-P-V-L-P-K-P-T-T-P-A-P
A21	V-L-P-K-P-T-T-P-A-P-T-V-P-L-T
A22	T-T-P-A-P-T-V-P-L-T-T-T-V-P-V
A23	T-V-P-L-T-T-T-V-P-V-E-T-T-T-P
A24	T-T-V-P-V-E-T-T-T-P-P-C-P-C-E

B1	E-T-T-T-P-P-C-P-C-E-C-P-K-C-P
B2	P-C-P-C-E-C-P-K-C-P-A-P-E-L-L
B3	C-P-K-C-P-A-P-E-L-L-G-G-P-S-V
B4	A-P-E-L-L-G-G-P-S-V-F-I-F-P-P
B5	G-G-P-S-V-F-I-F-P-P-K-P-K-D-V
B6	F-I-F-P-P-K-P-K-D-V-L-M-I-T-R
B7	K-P-K-D-V-L-M-I-T-R-T-P-E-V-T
B8	L-M-I-T-R-T-P-E-V-T-C-L-V-V-D
B9	T-P-E-V-T-C-L-V-V-D-V-S-H-D-S
B10	C-L-V-V-D-V-S-H-D-S-S-D-V-L-F
B11	V-S-H-D-S-S-D-V-L-F-T-W-Y-V-D
B12	S-D-V-L-F-T-W-Y-V-D-G-T-E-V-K
B13	T-W-Y-V-D-G-T-E-V-K-T-A-K-T-M
B14	G-T-E-V-K-T-A-K-T-M-P-N-E-E-Q
B15	T-A-K-T-M-P-N-E-E-Q-N-N-S-T-Y
B16	P-N-E-E-Q-N-N-S-T-Y-R-V-V-S-V
B17	N-N-S-T-Y-R-V-V-S-V-L-R-I-Q-H
B18	R-V-V-S-V-L-R-I-Q-H-Q-D-W-L-N
B19	L-R-I-Q-H-Q-D-W-L-N-G-K-K-F-K
B20	Q-D-W-L-N-G-K-K-F-K-C-K-V-N-N
B21	G-K-K-F-K-C-K-V-N-N-Q-A-L-P-A
B22	C-K-V-N-N-Q-A-L-P-A-P-V-E-R-T
B23	Q-A-L-P-A-P-V-E-R-T-I-S-K-A-T
B24	P-V-E-R-T-I-S-K-A-T-G-Q-T-R-V

C1	I-S-K-A-T-G-Q-T-R-V-P-Q-V-Y-V
C2	G-Q-T-R-V-P-Q-V-Y-V-L-A-P-H-P
C3	P-Q-V-Y-V-L-A-P-H-P-D-E-L-S-K
C4	L-A-P-H-P-D-E-L-S-K-N-K-V-S-V
C5	D-E-L-S-K-N-K-V-S-V-T-C-L-V-K
C6	N-K-V-S-V-T-C-L-V-K-D-F-L-P-T
C7	T-C-L-V-K-D-F-L-P-T-D-I-T-V-E
C8	D-F-L-P-T-D-I-T-V-E-W-Q-S-N-E
C9	D-I-T-V-E-W-Q-S-N-E-H-P-E-P-E
C10	W-Q-S-N-E-H-P-E-P-E-G-K-Y-R-T
C11	H-P-E-P-E-G-K-Y-R-T-T-E-A-Q-K
C12	G-K-Y-R-T-T-E-A-Q-K-D-S-D-G-S
C13	T-E-A-Q-K-D-S-D-G-S-Y-F-L-Y-S
C14	D-S-D-G-S-Y-F-L-Y-S-K-L-T-V-E
C15	Y-F-L-Y-S-K-L-T-V-E-T-D-R-W-Q
C16	K-L-T-V-E-T-D-R-W-Q-Q-G-T-T-F
C17	T-D-R-W-Q-Q-G-T-T-F-T-C-V-V-M
C18	Q-G-T-T-F-T-C-V-V-M-H-E-A-L-H
C19	T-C-V-V-M-H-E-A-L-H-N-H-V-M-Q
C20	H-E-A-L-H-N-H-V-M-Q-K-N-V-S-H
C21	H-N-H-V-M-Q-K-N-V-S-H-S-P-G-K

Fig. 1. Binding of human IgG to a cellulose-bound peptide library representing hhcIgG3. An overlapping array of 69 peptides shifted 5 amino acids from each other were probed with a 1:250 dilution of a pool of human sera detected by alkaline phosphatase labeled rabbit anti-human IgG and chemiluminescence. (A) Relative signal intensity of bound human IgG to each position from the membrane. 100% was defined by the positive control and 0% by the negative control. (B) Image of the membrane showing the reactivity at each spot and the positions used to make the measurements presented in panel A. (C) Lists the individual peptides spanning the hhcIgG3 (GenBank ac number: AB100095) and constitute the library with their positions on the membrane. Spots C22 & C23 correspond to the positive peptide controls and the negative control at C24 were a single glycine spotted on the membrane. Only spots with intensities $\geq$30% were considered in the overlapping.

identical libraries consisting of 69 overlapping peptides each. The peptides were directly synthesized simultaneously onto cellulose membranes by standard SPOT protocols. Each synthesized peptide consisted of 14 amino acids and was offset by five amino acids from the previous peptide. Prior to exposing the peptide libraries to test pools of human IgG (NST) or IgE (ST), the membranes were incubated with healthy human control sera (group HCS) to evaluate non-specific antibody binding. No appreciable signal was detected (<30% cutoff; not shown).

The data for the recognition pattern of human IgG binding to the peptide library spanning the hhcIgG3 coding sequence is shown in Fig. 1. In panel A, normalized measured intensities from chemiluminenscence are presented with 100% defined as described in the Materials and Methods. An image from a representative membrane presenting the peptide library is shown in Fig. 1 panel B. A list of the peptides and their coordinates on the membrane are listed in Fig. 1 panel C. The arrangement of the signals is observed in groupings with a single peptide normally presenting a higher signal surrounded or followed by peptides with decreasing intensities. This pattern of varying intensities suggests that certain amino acids within an epitope are of greater importance for antibody binding than others and if they are retained in the peptide there are still interactions between antigen and antibody.

A different pattern was observed with pooled sera of group ST containing human IgE. While human IgG recognized multiple epitopes in hhcIgG3, the sensitive human sera (group ST) that were rich in IgE recognized a single epitope (Fig. 2). This epitope was identified by the strong signal intensity presented in the spot B13 (Fig. 2B). Other spots with small intensities are identifiable from the presentation of the figure (A9, A18, B24), but their intensities were below 30%, which is below the cutoff to be considered an epitope.

From the SPOT membrane analysis, seven prominent IgG epitope regions were identified and are listed in Table 1. The human IgG-binding regions were comprised of the amino acid residues P6-S14, L46-P54, C131-L140, C165-D174, D185-V189, W286-Y298 and T321-T329 of the hhcIgG3 sequence and were designated as epitopes hIgG3-1 through -7, respectively. The epitope recognized by human IgE was represented by the amino acids D177-V189 and encompassed the epitope hIgG3-5 that was recognized by human IgG.

The epitopes are located in various regions of the heavy chain. In comparison to the sequence of human IgG3, the position of epitopes hIgG3-1 and -2 epitopes corresponded to the CH_2 domain in the F(ab')2 of human IgG (Fig. 3). The hIgG3-3 epitope was located in the Fc hinge region, while the epitopes hIgG3-4 through -7 were situated in the CH_3 domain of the Fc region. The epitope bound by human IgE was the same as hIgG3-5 and therefore it was also located in the CH_3 domain of the Fc region. The comparison also showed that each of the epitopes discovered in the horse

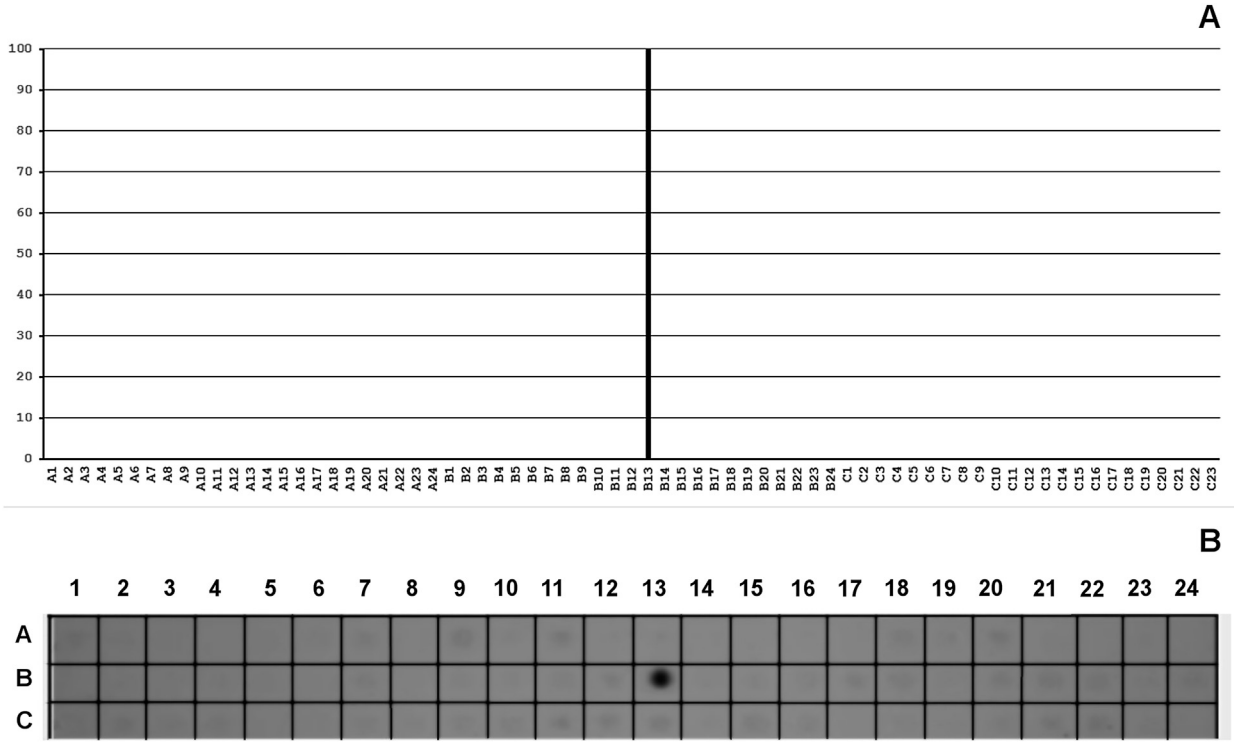


Fig. 2. Analysis of human IgE reactivity to a cellulose-bound synthetic peptide library representing hhcIgG3. A membrane spotted with peptides that span the amino acid sequence of hhcIgG3 was probed with a 1:100 dilution of human sera from volunteers sensitized to horse sera. Bound antibodies were detected by rabbit anti-human IgE labeled with alkaline phosphatase and chemiluminescence. Image of the membrane showing the measured chemiluminescent signal. Relative signal intensity of bound human IgE antibodies with 100% defined by the positive controls and 0% by the negative control. The list of peptides and their positions is the same as in Fig. 1C. Only spots with intensities ≥30% were considered in the overlapping.

Table 1
Defined epitopes in hhcIgG3 recognized by human IgG and IgE antibodies as deduced from Spot synthesis analysis and hydropathy plots. The sequence of each epitope is a result of overlapping of the set of positive spots.

Epitope	IgG-Sequence	IgE-Sequence	Globulin-Domain
hIgG3-1	6PKVFPLAPS14		Variable
hIgG3-2	46LTSGVRTFP54		Variable
hIgG3-3	131CPKCPAPELL140		Hinge
hIgG3-4	165CLVVDVSHD174		CH_2
hIgG3-5	185DGTEV189	177DVLFTWYVDGTEV189	CH_3
hIgG3-6	286WQSNEHPEPEGKY298		CH_3
hIgG3-7	321TDRWQQGTT329		CH_3

heavy chain have numerous differences in the corresponding human sequence (Fig. 3).

3.2. Analysis of individual sera

To investigate possible differences in the pattern of recognition for epitopes between donor sera, the serum from two individual patients from group 2 (anaphylactic donors) were analyzed for IgG antibodies and two sera from patients in group 1 (non anaphylactic donors) were evaluated for IgE bind epitopes. The Spot synthesis results showed that both patients in group 2 had IgG antibodies in their serum that recognized the same pattern of epitopes, but with different intensities, compared to the IgG's in the pool of sera from group 1, while the sera from the patients of group 1 did not recognized the major epitope recognized by the IgE present in the pool of sera from group 2 (data not shown). This result demonstrated that if there was variability in epitope recognition for each individual within the groups of donors. but the variability was not qualitative and did not influence the discovery of the epitopes defined by the pooled sera.

3.3. Critical residues of the linear epitope for human IgG binding

The overlap of residues in the peptides located in the positive spots suggested the underlined peptide amino acid sequence (176SDVLFTWYV**DGTEV**189) as the putative epitope for human IgG. A set of eight peptides (P13–P20) were synthesized to contain truncated and/or substituted amino acids for refining the amino acid sequence required for human IgG binding. The amino acids Lys190 and Thr191 did not contribute to binding as shown by the 100% reactivity for the two peptides P13 (181TWYVDGTEVKTA192) and P14 (181TWYVDGTEVAAA192), in which those residues were replaced by alanine (Fig. 4B). The mutation of the N-terminal block sequence 181TWYV184 also did not cause any change in the pattern of reactivity with human IgG antibodies as observed with P15. However, the suppression

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HORSE    ASTTAPKVFPLAPSCGTTSDSTVALGCLVSSYFPEPVTVSWNSGTLTSGVRTFPSVLQSS 60
HUMAN    ASTTGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS 60

HORSE    GLYSLSSMVTVPASSLESKTYICNVAHPASSTKVDKRIEPVLPK-------PTTPAPTVP 113
HUMAN    GLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTPLGDTTHTCPRCPEPKSC 120

HORSE    LT--------------TTVPVETTTP----PCPCECPKCPAPELLGGPSVFIFPPKPKDV 155
HUMAN    DTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDT 180

HORSE    LMITRTPEVTCLVVDVSHDSSDVLFTWYVDGTEVKTAKTMPNEEQNNSTYRVVSVLRIQH 215
HUMAN    LMISRTPEVTCVVVDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTVLH 240

HORSE    QDWLNGKKFKCKVNNQALPAPVERT-SKATGQTRVPQVYVLAPHPDELSKNKVSVTCLVK 275
HUMAN    QDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVK 300

HORSE    DFLPTDITVEWQSNEHPEPEGKYRTTEAQKDSDGSYFLYSKLTVETDRWQQGTTFTCVVM 335
HUMAN    GFYPSDIAVEWESSG--QPENNYNTTPPMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVM 358

HORSE    HEALHNHVMQKNVSHSPGK 355
HUMAN    HEALHNRFTQKSLSLSPGK 377
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Fig. 3. Localization of the antigenic determinants in hhcIgG3 recognized by human antibodies in comparison to the sequence of human IgG3. Epitopes bound by human IgG are displayed in pink and binding by human IgE is shown in green. Dashes represent the absence of comparative amino acids and provide better sequence alignment. The numbers on the left and right sides indicate amino acid positions. Specific differences within the horse and human sequences contained within epitopes are highlighted in yellow. Overall, the identity between hhcIgG3 (GenBank: CAC86339.1) and human IgG3 (GenBank: CAA67886.1) was 66%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

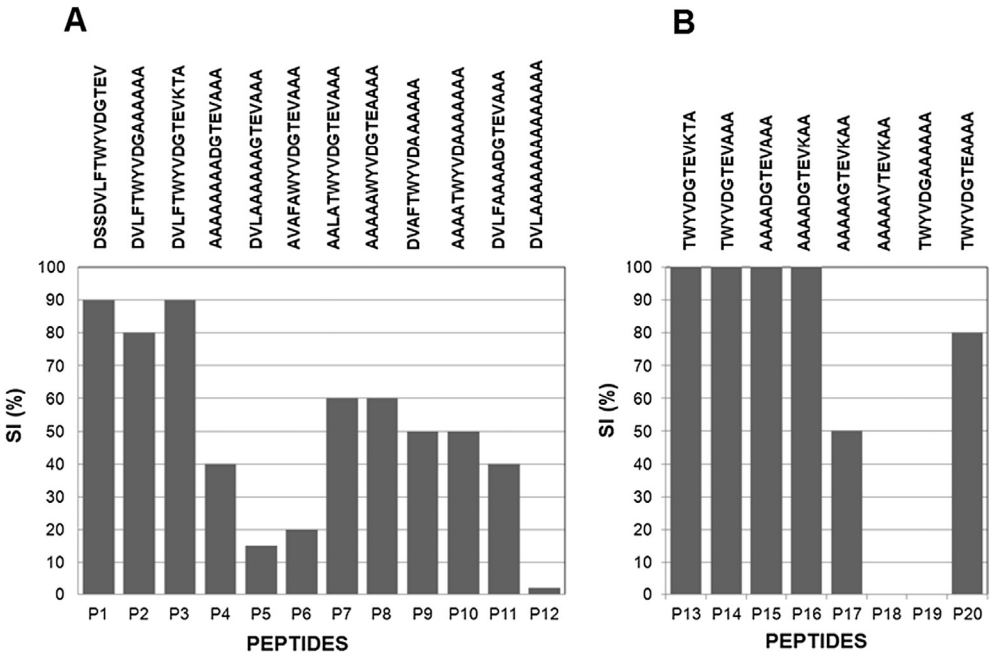

Fig. 4. Analysis of critical amino acids in hIgG3 for epitope recognition by human antibodies. Two mini-peptide arrays were prepared by Spot technology that varied a sequence by substituting adjacent groups of amino acids with alanine (A) or glycine (G). The arrays were probed with pooled human sera followed by anti-human second secondaries and chemiluminescence. The intensities for each peptide are plotted based on calculations relative to 100% for the positive controls and 0% for the negative control. The peptide sequences are listed above the plots. (A) The epitope TWYBDGTEVKTA and variations recognition by human IgG antibodies. (B) The epitope DSSDVLFTWYVDGTEV and variations binding by human IgE antibodies. The data are the average of three (≤4–7%) independent experiments. SI, signal intensity.

of Val189 in P20 decreased the binding by 20%. An even greater decrease, by a factor of 2, was observed for P17 when Asp (D185) was changed to alanine, which supports its important role in recognition by human IgG. The critical amino acids were refined by the peptides P18 and P19. The substitution of the N-terminal Gly186 residue for the hydrophobic residue Val186 in P18 affected the binding of human IgG drastically and led to a complete loss of the IgG binding. The absence of Thr187 and Glu188 in P19 also completely abolished interaction with human IgG. These findings indicated that the five-residue peptide containing the sequence 185DGTEV189 was the minimal effective sequence peptide for 100% human IgG binding and that the three residues 186GTE188 were absolutely critical for IgG-binding.

3.4. Critical residues of the linear epitope for human IgE binding

For further investigation, twelve peptides (P1–12) were designed and synthesized with mutations on the right and left sides of the central critical building block of the putative IgE-binding. This region was chosen because of their exposure to the solvent and because together they constitute one of the most accessible surface areas (Fig. 5) of the molecule to the immune system. To determine the influence of the adjacent amino acids and the crucial residues for conversion to binding by human IgE, a longer sequence (**174DSSDVLFTWYVDGTEV189**) was analyzed than for human IgG. Fig. 4B shows the reactive intensity of the modified peptides after being probed with the IgE hyperimmune patients' sera.

The introduction of the tripeptide **DSS** and **KTA** in the N- and C-terminal positions, respectively, of the base epitope peptide (**177DVLFTWYVDGTEV189)** neither increased nor decreased the IgE binding (peptides P1 and P3). Conversely, the hydrophobic sequence **182TWYVD186** proved to be important since its replacement with **AAAAA** had a negative effect on the IgE binding (peptide P5). Likewise, the result for peptide P6 showed that the replacement of **D177**, **L179** and **T181** with Ala significantly diminished its IgE-binding capacity, while the substitution of **TEV** had little effect on the binding (peptide P9). The exclusion of **T181** from the block **181TWYVDGTEV1**89 also did not decrease the IgE bind (peptides P7 and P8), but the subtraction of the block **180 FTWYVDGTEV189** drastically affected recognition of peptide P12 by human IgE. The results suggest that the residues **177-DVLF-180** and the hydrophobic sequence **181TWYV184** (in peptide P11) contained within the linear epitope are critical for human IgE binding to hhcIgG3.

3.5. Accessibility of the antigenic determinants

A hydropathy plot representing the location of the seven immunodominants epitopes determined with a window of 9 amino acids is shown in Fig. 5. This plot shows that the immunogenic peptide sequences that are more exposed to the molecular surface of the hIg are: hIgG3-5 (**185DGTEV189**) and hIgG3-6 (**286WQSNEHPEPEGKY298**).

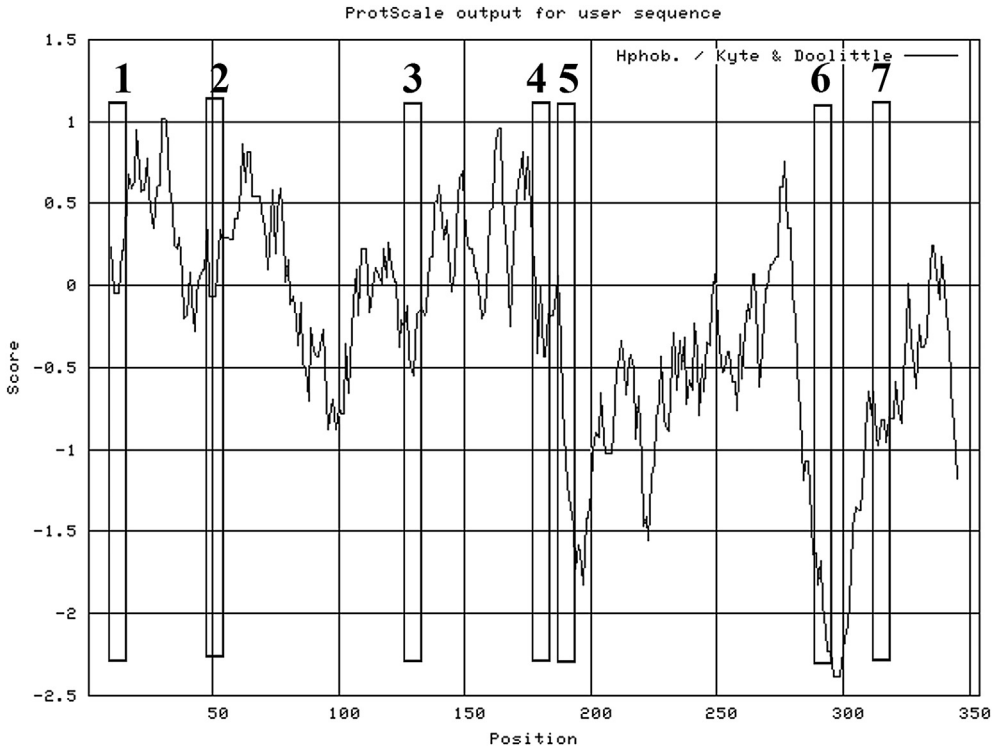

Fig. 5. Hydropathy profile of hhcIgG (window size 9) representing the location of the different immunodominant epitopes. Solid lines indicate the regions of the epitope location corresponding to the positions of the amino acids. Numbers 1–7 refer to epitopes P6-S14, L46-P54, C131-L140, C165-D174, D185-V189, W286-Y298 and T321-T329, respectively, as listed in Table 1. D177-V189 (DVLFTWYVDGTEV) corresponds to the epitope IgE.

These segments were determined to be the most reactive to binding by human antibodies as seen in Figs. 1 and 2.

3.6. Ethics statement

We confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

4. Discussion

Snake bite envenoming is a major health care concern throughout the world (Gutiérrez et al., 2006). An effective medical intervention that provides passive immunity is antivenom prepared in horses. However, hypersensitivity to horse-based sera therapies is a potentially life threatening reaction in patients. Although the worldwide incidence of mortality is not high, it is an important health issue since it is a constant possibility. Various studies have concentrated on the potential of equine immunobiologicals to provoke reactions in patients of either an early type (anaphylactoid) (Moran et al., 1998; Chen et al., 2000) or of late onset (serum sickness) (León et al., 2001). The major allergenic molecule involved has been identified as whole immunoglobulins present in the immunological preparations of horse sera (León et al., 2008). The onset of allergic reactions can be minimized by premedication of patients before administering antivenom (Cupo et al., 1991; Gawarammana et al., 2004; Williams et al., 2007; de Silva et al., 2011). Yet, to advance antivenom therapies through improved production methods, it is important to understand the molecular events related to adverse reactions. Our approach was to identify the epitopes in a horse immunoglobulin, the active constituent of antivenom, recognized by human IgG and IgE because the generation of human IgG relates to serum sickness and IgE plays a critical role in the etiology of allergen-specific responses.

Here, we identified the amino acid sequences of the linear epitopes that constitute the antigenic sites in the horse IgG3 protein heavy chain, the second most prevalent horse sera Ig (Sheoran et al., 2000) and the first involved in snake bites toxin recognition (Fernandes et al., 2000). The epitopes were located and mapped using the Spot-synthesis technique. Since the minimum antigenic site has been demonstrated to consist of five-residues, which is sufficient to bind a detectable quantity of recognizing antibodies (De-Simone et al., 2013a), the panel of peptides representing hhcIgG3 were offset by five amino acids. Furthermore, this avoided scission of antigenic sites to provide full coverage of the coding regions for all possible epitope regions.

This library of peptides was probed with sera pooled from three distinct groups of people. Group 1 ($n = 15$) sera provided primarily human IgG and was collected from patients who had received multiple doses of horse immunological preparations without presenting any indications

of sensitivity. Group 2 consisted of three patients that were clinically recognized as being hypersensitive to horse serum and provided human IgE against hhcIgG3. The third group ($n = 15$) served as a control and was obtained from individuals who had never been exposed to horse sera preparations. The concentration of the two distinct classes of immunoglobulins in the human sera ranged from 9 to 15 mg/ml for IgG and 320–410 ng/ml for IgE, which equates to 10,000 to 1 million fold bias towards IgG than IgE (Ledin et al., 2008). To account for this bias, the optimal dilution of each pool was empirically determined for detection of positive peptides spots. For IgG, the best detection occurred with a dilution of 1:250 and 1:100 for IgE detection. The difference between bias and the detection dilution suggests that human IgE had a greater affinity for hhcIgG3 than human IgG.

The control group 3 was an important component of the experimental design because it was reported that healthy people may well present antibodies that cross-react against the hinge region of hIg (Terness et al., 1995). In our assays using the sera of Group 3, no cross reactivity was detected (data not shown). Conversely, the Group 1 sera readily identified seven major IgG epitopes while for group 2 there was only a single major IgE epitope (Table 1). Two of the human IgG antigenic determinants were located in the variable region of the F(ab')2, one epitope in the hinge region, four to the Fc region, one in the CH_2 domain and three in the CH_3 domain (Table 1). These finding confirmed previous reports suggesting that exaggerated human exposure to horse immunological preparations generates a strong IgG-based response (Rojnuckarin et al., 1998; Sevcik et al., 2008). Furthermore, the results strongly suggest that the presence of antibodies against the Fc regions was due to the presence of intact hhcIgG3 in these preparations since only two correlated with the F(ab')2 that are isolated from the pepsin digest of horse immunoglobulin during preparation.

An important aspect of site recognition is regulated by the MHC of the host with the response to each site being under separate genetic control (Okuda et al., 1978, 1979). This separation allows the immunodominance of antibodies directed against a given site to vary between host species and even between individuals within a given species such that frame shifts in the amino acid sequence of epitopes recognized by antibodies can be observed (Atassi, 1982). A comparison between the sites recognized in hhcIgG3 by human and rabbit antibodies are consistent with MHC of the host being a major cause for frame shifts in the epitope sequences and the level of antibody response. Previous results using the same strategy with rabbit hyperimmune sera raised against hhcIgG3 identified 11 immunodominant regions distributed throughout the antibody heavy chain (De-Simone et al., 2013b). The regions defined as −3 and −5 in the human response displayed a 1–2 residue shift in amino acids compared to rabbit, while regions −1 and −2 exhibited left and right shifts of 1 and 5 residues, respectively. Six other antigenic regions recognized by the immunized rabbit sera were not reactive with human IgG (De-Simone et al., 2013b). This result stresses the necessity to confirm antibody responses observed in other systems directly with those in humans.

Sera was only available from three patients hypersensitive to horse sera for use in identifying the epitopes recognized by human IgE since such patients are difficult to recruit. Notwithstanding, a single immunodominant antigenic determinant was identified that contained thirteen amino acids (D177-V189; Fig. 2 and Table 1). This antigenic determinant was located in the CH_3 region as expected for a reaction against contaminating whole hhcIgG3 molecules. Interestingly, it contained an overlapping sequence of 5 residues 185–189 (DGTEV) that was recognized by human IgG. This epitope forms a loop in the residues 177DVLFTWYVDGTEV189 and its high signal intensity during detection suggested that the loop was maintained in the peptide (spot B13, Fig. 2).

Since amino acid characteristics in an epitope contribute to antigenicity, the hydrophobicity, polarity and charge of each were determined (Fig. 5). The relative hydrophobicity of the epitope, as determined by the Kyte and Doolittle method, identified the IgE epitope as a relatively hydrophobic region of the protein (Fig. 5). Of the 14 amino acids, hydrophobic residues were the most frequent (6/14), followed by polar residues (4/14) and charged residues (4/14). To determine the critical amino acids, a panel of peptides with various mutations were synthesized. A major change in the IgE reactivity was observed with the peptides containing the hydrophobic sequence (181TWYV184) replaced with truncated peptides or substituted amino acids. The sequence 177-DVLF-180 was also shown to be necessary for the binding to IgE. This was suggested by the results whereby the reactivity to P5–P8 and P11 were markedly reduced (15–50% reactivity) by substitution with Ala. With regard to the remaining residues between positions 185–189, all showed rather weak but important reactivity (~40% reactivity) in Ala-scanning experiments (P4 and P11), indicating that 185-DGTEV-189 are associated with the reactivity of P4 and P11 with IgE. Taken together, thirteen residues (**177DVLFTWYVDGTEV189)** were concluded to be critical for the IgE-binding.

It is interesting to note that as many as four of the thirteen critical residues have an electrically charged side chain, being likely to form salt bridges with IgE. Indeed, salt bridges are known to significantly contribute to specificity. Differing from the situation for human IgG binding to the same epitope region that was restricted to five amino acids (**185DGTEV189**) with three critical residues (**186GTE188**), the determined critical residues in the IgE epitope were mostly hydrophobic. This result led us to conclude that hydrophobic interactions, which are generally known to have essential roles in stabilizing protein–protein interactions (Kobayashi et al., 2010), are directly involved in the binding of hIg by human IgE considering that the side chains of Try (W), Tyr (Y), Phe (F) and Val (V) participate in a hydrophobic cluster made from hydrophobic side chains of adjacent residues (Val-178, Phe-180, W182, Tyr-183 and V-184), which would strengthen the hydrophobic interaction with IgE.

Considering the high level of similarity between the sequence of human and horse IgG3, an alignment was performed to understand the absence of self-recognition. The alignment shows that the main difference between the human and the horse IgG3 proteins structure are in the

residues V189-H190 and for IgE either D177, L179 or T181 (Fig. 3). These amino acids correspond to the critical residues for human IgG and IgE binding to hhcIgG3.

5. Conclusions

In conclusion, we have demonstrated that individuals with and without hypersensitivity to antivenom produce antibodies to sequential epitopes of hhcIgG3, although the signal strength and specificity appear to be distinct in the both groups of patients. The major IgE- epitope of hhcIgG3 is included in the CH_3 domain of the Fc region (Fig. 2; spot B13, region 116–130) and consisted of nine amino acid residues. The principal IgG-binding epitope was restricted to five critical amino acids, which are part of the IgE epitope. Each class of human immunoglobulin appeared to bind through different mechanisms. The determined critical residues of 185DGTEV189 for IgG and 181TWYVDGTEV189 for IgE suggests that binding occurs through electrostatic and hydrophobic interactions, respectively. This is the first study to describe the specific amino acid sequences involved in the immune recognition of horse antivenom. Overall, our data should advance the development of processes to produce hypoallergenic recombinant human immunoglobulin mutants that are practical antigens for allergen-specific immunotherapy and for the diagnosis of allergies to hIg.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work received financial assistance from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and FIOCRUZ (PROEP) to SGS.

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