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Analysis of B-cell epitopes from the allergen Hev b 6.02 revealed by using blocking antibodies

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ABSTRACT

Hev b 6.02 (hevein), identified as a major allergen from natural rubber latex (NRL), is involved in the latex-fruit syndrome and also acts as a pathogenesis defense-related protein. Its 3D structure has been solved at high resolution, and its linear epitopes have already been reported. However, information about conformational epitopes is still controversial, even though it is relevant for an accurate diagnosis and treatment, as well as for the study of allergen–antibody molecular interactions. We sought to analyze the B-cell epitopes of Hev b 6.02 at a molecular and structural level, using specific recombinant antibodies. We obtained a murine monoclonal antibody (mAb 6E7) and three human single chain fragments (scFvs A6, H8, and G7) anti-Hev b 6.02 that were able to compete for hevein binding with serum IgEs from latex allergic patients. *In vitro* assays showed that the mAb 6E7 and scFv H8 recognized the area of Hev b 6.02 where the aromatic residues are exposed; while the scFv G7 defined the amino and carboxy-terminal regions that lie close to each other, as a different epitope. The structural modeling of the Hev b 6.02–scFv H8 and Hev b 6.02–scFv G7 complexes revealed the putative regions of two conformational epitopes. In one of these, the aromatic residues, as well as polar side chains are important for the interaction, suggesting that they are part of a dominant conformational epitope also presented on the Hev b 6.02–IgE interactions. Antibodies recognizing this important allergen have potential to be used to diagnose and ultimately treat latex allergy.

1. Introduction

It is now widely recognized that IgE-mediated reactions (type I hypersensitivity) and contact dermatitis to NRL proteins are important medical problems, particularly in countries that are striving to attain higher technological standards (Wagner and Breiteneder, 2005). The high-risk population includes individuals exposed to proteins found in products manufactured with this material, such as gloves and surgical devices (Bernstein et al., 2003). Hev b 6.02 (hevein), one of the major NRL allergens existing in high concentration, is a 4.7-kDa lectin that also acts as a pathogenesis defense-related protein (Yeang et al., 2006). Several analyses of different plant-derived food allergens have also shown the presence of this polypeptide chain as a chitin-binding domain in class I endochitinases. This accounts for the cross-reactivity between latex and some fruits (Blanco, 2003).

The first mapping of the linear epitopes of Hev b 6.02, using overlapping peptides in direct or inhibition ELISA was performed by Beezhold et al. (1997) and Banerjee et al. (1997). They independently confirmed that two peptides containing residues Ser^{19}-Cys^{24} and Gly^{25}-Cys^{37}, as well as Pro^{13}-Cys^{24} and Glu^{29}-Asn^{36}, respectively, were responsible for Hev b 6.02-specific IgE binding. Subsequently, Drew et al. (2004) and Raulf-Heimsoth et al. (2004) reported that the tertiary structure of this allergen was relevant for IgE binding. In both works, the Cys residues were mutated to Ser or Ala and, therefore, IgE antibodies did not recognize the mutants. Karisola et al. (2002, 2004), using chimeric-hevein and hevein mutants, reported that IgE conformational epitopes are in the N-terminal and C-terminal regions and suggested that the inter-

Abbreviations: ASA, accessible surface area; BSA, bovine serum albumin; CDR, complementary determining region; ELISA, enzyme-linked immunosorbent assay; FR, framework region; mAb, monoclonal antibody; NRL, natural rubber latex; RMSD, root mean square deviation; scFv, single chain variable fragment; V_H, variable region heavy chain; V_L, variable region light chain.

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acting residues are Arg^{5}, Lys^{10}, Glu^{29}, Tyr^{30}, His^{35} and Gln^{38}. In a parallel study, our group used an alternative strategy that consisted in oxidizing the two solvent-exposed tryptophans (Trp^{21} and Trp^{23}) through chemical modification (Reyes-López et al., 2004), producing a modified Hev b 6.02 that showed only 20% inhibition of IgE binding. It was then suggested that these two residues were important in the IgE–Hev b 6.02 interaction. Recently, we found a natural hevein isoform (Hev b 6.0202) with only one change ($Asn^{14}Asp$) that showed reduced IgE-binding capacity using serum pools of adult and pediatric patients (Reyes-López et al., 2006). These discrepancies suggested the presence of more than one conformational epitope on this small allergen and, therefore, the way in which IgEs recognize Hev b 6.02 remains an open question.

Hybridoma and phage display technology are the methods currently available to produce and isolate allergen-specific antibodies, which are able to inhibit the binding of serum IgE from allergic individuals (Davies et al., 2000; Edwards et al., 2001). Blocking antibodies have been used to learn more about the specific residues that are crucial to allergen–antibody interactions. Nonetheless, to date there are only a few reports on the structure of antibody–allergen complexes (Mirza et al., 2000; Padavattan et al., 2007; Niemi et al., 2007; Li et al., 2008). Furthermore, it has been shown that allergen-specific IgG antibodies, which inhibit the IgE–allergen interaction, have been tested in specific immunotherapy (Flicker and Valenta, 2003).

Laukkanen et al. (2003) obtained two hevein-specific recombinant IgE antibodies from a human scFv library, and proposed them as potential reagents for the analysis of latex allergens. Nevertheless, these antibodies have not been used for the epitope analysis. Consequently, to gaining insights into the location and characterization of the conformational epitopes of this important latex allergen, in the present study three scFvs and one mAb Hev b 6.02-specific were selected. The purified antibodies were able to compete with the binding of sera IgEs from latex allergic patients. Additionally, isoforms and chemically modified Hev b 6.02 were tested with the recombinant antibodies. The structural modeling of different Hev b 6.02–scFv complexes provided complementary information on the Hev b 6.02–IgE interaction.

2. Materials and methods

2.1. Allergens

Hev b 6.02, Hev b 6.0202, pseudohevein (*Hevea brasiliensis* clone GV42) and chemically modified hevein were obtained following the procedures reported by Reyes-López et al. (2004, 2006).

2.2. Characterization of allergenic patient and sera

Twenty adult and pediatric NRL allergic patients were characterized by positive clinical history and skin prick testing. Additionally, sera were tested for reactivity with Hev b 6.02 by direct ELISA.

2.3. IgE ELISA

Hev b 6.02 diluted in 50 mM carbonate buffer, pH 9.6 was coated on microtiter plate (Corning, NY, USA) at 3 μg/mL (100 μL/well) and incubated overnight at 4 °C. The wells were emptied, and the remaining protein binding sites were blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS), at 37 °C for 2 h. After rinsing, 100 μL of latex allergic patients serum (diluted 1:10 in PBS) were added and incubated at 37 °C for 3 h. Biotinylated mouse anti-human IgE (Zymed; diluted 1:1000) was added, followed sequentially by streptavidin conjugated peroxidase (Zymed; diluted 1:2000) after washing. Produced color was read at 490 nm by using an automated Dynatech MR 5000 ELISA reader (Dynatech Laboratories, Inc., Chantilly, Va.). The mean + 3 S.D. of the control individuals was chosen as the threshold value for a positive result.

2.4. Hev b 6.02-specific monoclonal antibodies: production, isotyping and purification

Four to eight-week-old female BALB/c mice were immunized intraperitoneally (i.p.) with Hev b 6.02-BSA conjugated, and emulsified in complete Freund's adjuvant. Thirty and 45 days later, mice were boosted with an identical amount of antigen in incomplete Freund's adjuvant. The mAbs were derived by somatic cell hybridization of Sp2 mouse myeloma cell line to 1.35×10^{7} spleen cells at a radio of 1:10 in the presence of polyethylene glycol (PEG) 1500. Hybridoma cells were selected by Hypoxanthine-Aminopterin-Thymidine (HAT) medium as previously described (Galfré and Milstein, 1981) and further selection of the secreted antibody to Hev b 6.02 were chosen by direct ELISA. Positive cell cultures were cloned at least five times by limiting dilution. Isotype detection was done using a commercial kit (Bio-Rad Laboratories, CA, USA). For the production of ascites, hybridoma cells were i.p. injected into pristine-primed female BALB/c mice and ascites were collected 30 days after injection. Antibodies were purified from hybridoma culture and ascitis fluid by affinity chromatography using a Protein G Column (Amersham Biosciences; Uppsala, Sweden). Protein concentration was measured assuming that $A_{280}^{1\,mg/mL} = 1.40$ or $\varepsilon_{279}^{M} = 140\,000\,M^{-1}\,cm^{-1}$ corresponds to a purified antibody solution with a concentration of 1 mg/mL.

2.5. Hev b 6.02-specific human scFvs phage display selection

A human combinatorial library of scFvs, prepared by Riaño-Umbarila et al. (2005) with diversity close to 1.2×10^{8}, was displayed in filamentous phages and used for the selection of antibodies that recognized Hev b 6.02. Rescue of phagemid particles was done as described by Marks et al. (1991). Before the biopanning, 1 mL of the library (1×10^{13} phage antibodies) in PBS was incubated for 90 min in the presence of different blocking agents (BSA and gelatin). Biopanning was performed by adding the pre-blocked phages to immunotubes (Maxisorp; Nunc, Roskilde, Denmark) and microplates (Covalink; Nunc, Roskilde, Denmark) coated with purified Hev b 6.02 and Hev b 6.02-BSA at the concentrations of 10 μg/mL (cycle 1) and 5 μg/mL (cycles 2–4), then washing 20 times with PBST (PBS with 0.1% Tween 20) and 20 times with PBS. The bound phage antibodies were recovered by the addition of one mL of *E. coli* strain TG1 ($A_{600} = 0.7$) for 30 min without agitation and then 30 min with agitation (37 °C). Finally the cells were plated on agar ampiciline for overnight growth. The panning procedure was repeated twice. In the third round, the selection was performed using a Surface Plasmon Resonance instrument (BIAcore X, Uppsala, Sweden). Twenty-five micrograms of Hev b 6.02 were covalently bound onto a CM5 sensor chip using an equimolar mix of *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide in 10 mM acetate buffer pH 3.0. Approximately 370 resonance units (RU) were coupled. The phage antibodies were injected and eluted with different concentrations of NaOH (2.5–25 mM) and were recovered as explained above. In the fourth round, the selection was performed in immunotubes and microplates, with some variations. After the washing steps, one mL of 100 mM triethylamine (Pierce, Rockford, IL, USA) was added followed by a 10 min incubation, to remove the less stable phage antibodies. The remaining phage antibodies were