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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.

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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 con-

centration, 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 Ser¹⁹–Cys²⁴ and Gly²⁵–Cys³⁷, as well as Pro¹³–Cys²⁴ and Glu²⁹–Asn³⁶, 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 Arg⁵, Lys¹⁰, Glu²⁹, Tyr³⁰, His³⁵ and Gln³⁸. In a parallel study, our group used an alternative strategy that consisted in oxidizing the two solvent-exposed tryptophans (Trp²¹ and Trp²³) 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 (Asn¹⁴Asp) 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 ratio 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 $A_{280}^{1\text{mg/mL}} = 1.40$ or $\epsilon_{279}^{\text{M}} = 140\,000\text{ M}^{-1}\text{ cm}^{-1}$ 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

		CDRH1		CDRH2			
A6	1	EVQLVESGA	EVKKPGASVKV	SCKASGYIFTN	YGISWVRKAPGQGLEWVAWISGYNGLIKS	60	
H8	1	EVQLVESGGGL	VKPGGSLRL	SCAASGFTFGGY	SMNWVRQAPGKLEWVSSISSSSSYIYY	60	
M8	1	QVQLVQSGAEV	KMPGESLKIS	CKGSAYNFSN	YWIWVRQMPGEGLEWMGIVYPGDSHIRY	60	
G7	1	QVQLVQSGAEV	KMPGESLKIS	CKGSAYNFSN	YWIWVRQMPGEGLEWVGIVYPGDSHIRY	60	
				CDRH3			
A6	61	AQRFQGRV	TLTTDISTTTAY	MELRSLRSD	TAVYYCARSGMYKDGSGSYTG--MDVWGQG	118	
H8	61	ADSVKGRFT	ISRDNAKNSLY	LQMNSLRAE	TAVYYCARQAVR---GPARG--MDVWGQG	114	
M8	61	RPSFQGQV	TISADKSI	SFVYLQWSS	LKASGTAIYYCARQSIIPVG-GLMRGDAFDIWGQG	119	
G7	61	RPSFQGQV	TISADKSI	SFVYLQWSS	LKASGTAIYYCARQSIKPVG-GLMRGDAFDIWGQG	119	
		LINKER		CDRL1			
A6	119	TTVTVSSGGGG	SGGGSGGGG	SDIQMTQSP	SSLASVGDRTITCRAS---QNIDKFLNW	175	
H8	115	TTVTVSSGGGG	SGGGSGGGG	SDIQMTQSP	SSLSTSVGDRTITCRAS---HSISSYVNW	171	
M8	120	TMVTVSSGGGG	SGGGSGGGG	SHVILTQ-PP	SVSVSPGQTATITCSGDQLEHMERLSICW	178	
G7	120	TMVTVSSGGGG	SGGGSGGGG	SHVILTQ-PP	SVSVSPGQTATITCSGDQLEHMERLSICW	178	
		CDRL2		CDRL3			
A6	176	FQQKPGKAP	RLLIYGATN	LDSGVPSRIS	SGSGTDFTLTIS	SLQPEDFATYHCQQSY-IT	234
H8	172	FQQKPGKAP	KLISATSTL	QNAVPSRFS	SGSGTDFTLTIS	SLQPEDSATYYCQQSY-SH	230
M8	179	FQQKPGQSP	VVVIYQAT	SRPPGIPER	FSGYSNTATLT	ISGAQSVDDADYYCQAWDFIS	238
G7	179	FHQKPGQSP	VVVIYQAT	YRPPGIPER	FSGYSNTATLT	ISGAQSVDDADYYCQAWDFIS	238
A6	235	PRSGGQGT	KLKLEIKRAAAEQ	KLISEEDL	NGAAHHHHHH	271	
H8	231	SVTFGQGT	RLKLEIKRAAAEQ	KLISEEDL	NGAAHHHHHH	267	
M8	239	ELVFGSGT	QLTVLSAAAEQ	KLISEEDL	NGAAHHHHHH	275	
G7	239	ELVFGSGT	QLTVLSAAAEQ	KLISEEDL	NGAAHHHHHH	275	

Fig. 1. Amino acid sequence alignment of three Hev b 6.02-specific scFvs selected from human repertoire, and the matured clone G7. These sequences include the c-myc C-terminal tag followed by a hexameric His tag. CDRs and FRs are indicated in agreement with Kabat definition (<http://www.bioinf.org.uk/abs/>).

neutralized with 1 M Tris–HCl, pH 7 and recovered as explained above. After each round of panning, supernatants of single phage antibody clones were analyzed for specific binding to Hev b 6.02 and Hev b 6.02-BSA in microtitre ELISA plates (Corning, NY, USA) coated at 3 µg/mL in 50 mM bicarbonate buffer. The phage binding was revealed by incubation with horseradish peroxidase (HRP)-conjugated anti-M13 (Amersham Pharmacia Biotech).

2.6. Affinity maturation by error-prone PCR

Selected clones from the scFv library were subjected to mutagenesis by error-prone PCR with high, medium and low mutagenesis rate (Cadwell and Joyce, 1992). PCR products were mixed and digested with SfiI and NotI (New England Biolabs, Beverly, MA, USA), gel purified and then ligated into the phagemid pSyn2. Ligated DNA was electroporated into *E. coli* strain TG1 cells and the library variability (mutation rate) was determined. Each new library was subjected to three or four rounds of biopanning using immunotubes. Three cycles of evolution were performed. The sequences of the clones were determined with the primers forward (5'ATA CCT ATT GCC TAC GCC 3') and reverse (5'TTT CAA CAG TCT ATG CGG 3') in the Applied Biosystems 3100 Gene Analyzer (Foster City, CA, USA).

2.7. Soluble single chain antibodies expression and purification

The scFv inserts from the selected clones were ligated into the expression vector Psyn1. This vector allows *E. coli* periplasmic expression of the scFv fused to cmc epitope tag and hexahistidine (His)₆ tag under the control of the *lac* promoter. Soluble scFv was produced as described elsewhere (Nielsen et al., 2006). Finally, the monomers were separated from dimers and higher aggregates by applying the samples onto a Superdex 75 column (Amersham Biosciences, England). Protein concentrations were determined spectrophotometrically using extinction coefficients estimated individually for each clone. Typically, 0.6 mg of purified scFv was obtained from 500 mL of culture of *E. coli* TG1.

2.8. Binding sites analysis using natural variants of Hev b 6.02 by ELISA inhibition assay

ELISA plates were coated with 3 µg/mL Hev b 6.02 diluted in 50 mM carbonate buffer, pH 9.4 (100 µL/well; Costar) and incubated overnight at 4 °C. Wells were blocked with 1% BSA in phosphate-buffered saline, at 37 °C for 2 h. Afterwards, human single chain antibodies were co-incubated with different concentrations (0.005–33 µg/mL) of the inhibitors (Hev b 6.02, Hev b

Table 1

Comparison of the DNA sequences of the V_H and V_L regions (scFv clones A6, H8 and M8) with their closest related germline sequences (IMGT/V-QUEST).

Clone	Germline V, D, J (hc), V, J (hl)	Complete variable region		CDRs		FRs	
		n	Percent	n	Percent	n	Percent
A6 hc	IGHV1-18*01, IGH D3-10*01, IGHJ6*01	42/369 bp	11	25/114 bp	21	17/255 bp	7
H8 hc	IGHV3-21*01, IGH D3-10*01, IGHJ6*01	20/357 bp	6	20/102	20	0/255 bp	0
M8 hc	IGHV5-51*01, IGH D6-19*01, IGHJ3*02	30/372 bp	8	16/114 bp	14	14/258 bp	5
A6 lc	IGKV1-39*01, IGKJ1*01	24/324 bp	7	11/81 bp	14	13/243 bp	5
H8 lc	IGKV1-39*01, IGKJ5*01	25/324 bp	8	15/81 bp	19	10/243 bp	4
M8 lc	IGLV3-1*01, IGLJ1*01	56/333 bp	17	29/93 bp	31	27/240 bp	11

Table 2

Inhibition percentage of IgE binding from natural rubber latex positive sera to native Hev b 6.02 by recombinant antibodies.

Individual	scFv M8	scFv G7	scFv A6	scFv H8	mAb 6E7	scFvs and mAb 6E7
Inhibition percentage						
1	32.6	28.5	22.6	19.0	9.5	24.3
2	14.4	18.5	8.0	6.1	18.8	20.2
3	20.7	18.9	51.3	42.7	9.6	13.2
4	24.9	6.9	–	55.1	20.6	63.4
5	22.0	26.5	8.3	35.7	12.9	15.0
6	8.2	–	–	37.3	30.6	33.6
7	12.1	34.1	14.1	45.3	40.6	71.5
Mean	19.3	22.3	20.9	34.5	20.4	34.5

6.0202, modified Hev b 6.02, and pseudohevein) in PBS for 3 h and were added to the wells for 45 min at 37 °C. The colorimetric reaction was revealed as mentioned above. Results were expressed as percentage of inhibition using the following formula: $[(\text{absorbance in the absence of inhibitor X} - \text{absorbance in the presence of inhibitor X}) / \text{absorbance in the absence of the inhibitor}] \times [100]$.

2.9. IgE inhibition assay using recombinant antibodies

ELISA plates were coated with 3 µg/mL Hev b 6.02 overnight at 4 °C. The plates were blocked with 1% Casein (Sigma, St. Louis, MO) in PBS and incubated 2 h at 37 °C with the human scFvs or monoclonal antibodies (diluted 1:100). After washing, the plates were incubated with 1:10 diluted sera from natural rubber latex allergic patients for 2 h at 37 °C and bound human IgE antibodies were detected as explained before. We use a toxin-specific human scFv like negative control.

2.10. Fluorescence measurements

Fluorescence experiments were carried out on a fluorescence spectrometer PerkinElmer BL50 (USA) in a 1 cm quartz cell, with

the FL Winlab software for collecting data. All measurements were done at an excitation of 295 nm and an emission of 320–440 nm, in a cell thermostated at 25 °C. The excitation slit was set to 2.5 and the emission slit to 3.5. The buffering system was PBS pH 7.4, 137 mM NaCl. Hev b 6.02 concentration was 2.11×10^{-6} M for all experiments. Antibodies were prepared in different molar ratios with respect to the allergen. Spectral centers of mass of intrinsic fluorescence (intensity-weighted average emission wavelengths, SCM) were calculated as $\text{SCM} = \sum \lambda I(\lambda) / \sum I(\lambda)$, where λ is the emission wavelength and $I(\lambda)$ represents the fluorescence intensity at wavelength.

In fluorescence quenching experiments aliquots of 3.0 M acrylamide (Aldrich, St. Louis, MO) were added directly to the sample up to a total concentration of 200 mM, with 10 mM increments. The Hev b 6.02 samples were allowed to equilibrate with acrylamide for 5 min before each measurement. For those experiments with Hev b 6.02 in the presence of different molar ratios of recombinant antibodies, Hev b 6.02 was incubated with these antibodies for 12 h before adding the acrylamide solution. Fluorescence values were corrected for dilution by the titrant. Fluorescence was not observed in control titrations with buffer and acrylamide. The decrease in fluorescence intensity is described by the Stern–Volmer

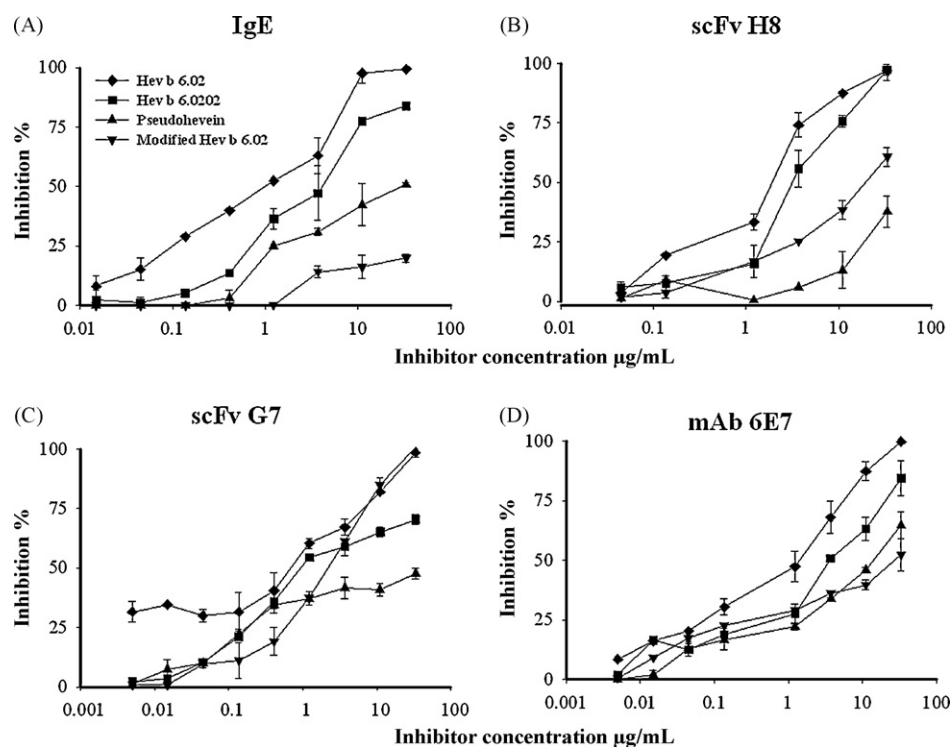


Fig. 2. ELISA inhibition assays. The average inhibition of IgE (A), scFv H8 (B), scFv G7 (C) and mAb 6E7 (D) binding to solid-phase Hev b 6.02 was determined using chemically modified hevein and two natural mutants. The inhibition curve with Hev b 6.02 as the inhibitor is shown for comparison.

equation $F_0/F = 1 + K_{SV} [Q]$, where F_0 and F are the corrected fluorescence intensities in the absence and presence of acrylamide, respectively, K_{SV} is the collisional Stern–Volmer constant, and $[Q]$ is the molar concentration of acrylamide. All measurements were performed in at least triplicate series.

2.11. Sequence alignment and homology modeling of the recombinant antibodies

The three-dimensional structure models of scFvs were built by using the web-based antibody modeling program WAM (Whitelegg and Rees, 2000), ESyPred3D server (Lambert et al., 2002), as well as the program suite Modeller 9v2 (Šali and Blundell, 1993). The framework regions were generated by using homologous antibody templates according to the sequence alignments obtained with BLAST (Protein Data Bank). The best alignments were selected according, not only to the alignment score, but also to the selection of the template structures solved at the highest resolution. CDRs from the light and heavy chains, except CDRH3, were built from homologous loops of the same canonical class. The antibodies were modeled in the form of scFv, with the light and heavy chains connected by a flexible linker segment. The minimization of the structures was performed with UCSF Chimera software (www.cgl.ucsf.edu/chimera) through the MMTK routines.

2.12. Simulated docking of scFv–Hev b 6.02 complexes

The rigid-body docking approach of GRAMM-X (Tovchigrechko and Vakser, 2006) was used to construct models of two scFvs with Hev b 6.02, using the crystal structure obtained at 1.5 Å resolution (PDB code 1Q9B) (Reyes-López et al., 2004). To perform the rotation and translation searches of the allergen around the surface of the antibody-binding region we used RosettaDock (Gray et al., 2003). In the high-resolution refinement step, where the Monte Carlo minimization procedure was applied, the side-chain degrees of freedom were optimized simultaneously, in the context of a detailed all-atom energy function, where short-range hydrogen bonding, van der Waals interactions, and desolvation were the greatest contributors. The search procedure was repeated from different random starting orientations to create 1000 structures. The RMSD values were calculated over the scFv molecules after superposition of the ligand (Hev b 6.02) with the starting model. To rank the results we used Fnat (fraction of native contacts), defined as the residue–residue correct contact number of the predicted complex divided by the number of contacts in the target complex, and the L_{rms} value defined as the main-chain displacement of the predicted ligand against the target structure. Polar contacts were analyzed with the PISA server (Krissinel and Henrick, 2007) and the apolar ones with the PIC server (Tina et al., 2007). The models were visualized with Pymol v1.0r2 (DeLano, 2002).

3. Results

3.1. Selection of Hev b 6.02-specific murine monoclonal antibodies

After immunization with Hev b 6.02 purified from natural rubber latex and covalently bound to BSA, a panel of murine mAbs was obtained. Direct ELISA using Hev b 6.02 in the solid phase screened all the hybridoma candidates. mAb 6E7 (IgG1 κ) presented a high degree of recognition.

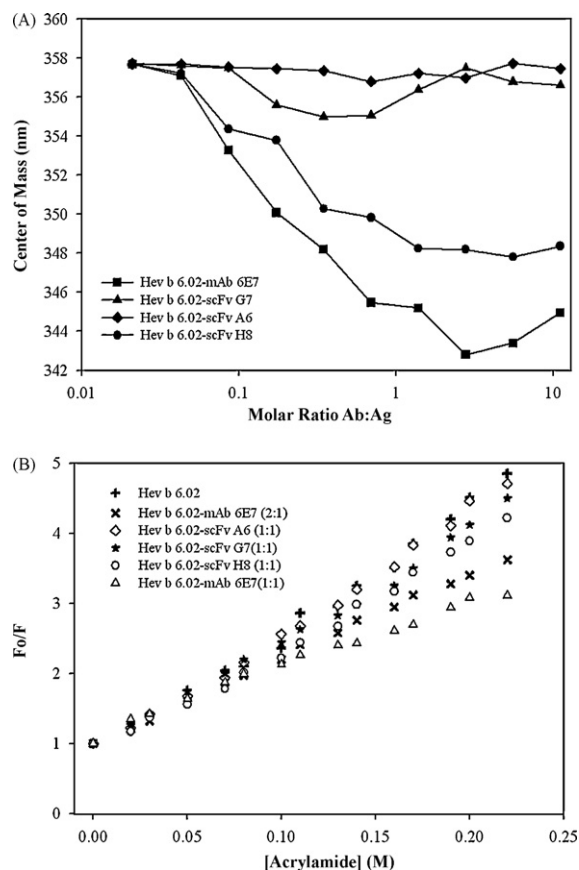


Fig. 3. Tryptophan fluorescence in the identification of Hev b 6.02 epitopes. (A) Tryptophan intrinsic fluorescence of the complexes Hev b 6.02-recombinant antibodies at different molar ratios. (B) Stern–Volmer plots for acrylamide quenching effects on Hev b 6.02 and on its complexes with recombinant antibodies.

3.2. Selection and molecular analysis of the Hev b 6.02-specific human scFvs

A human random combinatorial phage library (Riaño-Umbarila et al., 2005) was screened with immunotubes and microplates coated with Hev b 6.02 and Hev b 6.02-BSA at a low density. Three clones were found to express Hev b 6.02 binding activity, scFv antibodies M8, A6 and H8 (Fig. 1). A sequence alignment of the three light chains (V_L) showed an identity ranging from 39% to 75%, whereas for the three heavy chains (V_H) it ranged from 41% to 49%. Framework regions (FRs) and one complementary determining region of the light chain (V_L -CDR2) were of equal size; CDRL1 and CDRL3 were of different sizes for M8 only. Concerning the V_H s, CDRH3 of different sizes were observed for the three scFvs with 12 (H8), 16 (A6) and 17 (M8) residues, respectively.

The DNA sequences of these scFvs (GenBank accession nos. EU934735, EU934736 and EU934737) were compared with germline sequences deposited in the IMGT database (Lefranc, 2003). The heavy chains of A6 and H8 are related to VH1 and VH3 families, respectively, while M8 is related to VH5. The light chains of A6 and H8 belong to the Vk1 family, whereas M8 is related to V λ 3. Notably, there were more variations in the V_L -CDRs (14–31%) than in the FRs (5–11%). In the V_H -CDR regions, the variation level accounts for 14% to 21%, while for the FRs, it ranged from 0% to 7% (Table 1). It was very interesting to observe that the rate and distribution of somatic mutations were comparable with those observed for two important human antibodies anti-allergen; IgG anti-Bet v 1 (Denepoux et al., 2000) and IgE anti-Phl p 1 (Flicker et al., 2006).

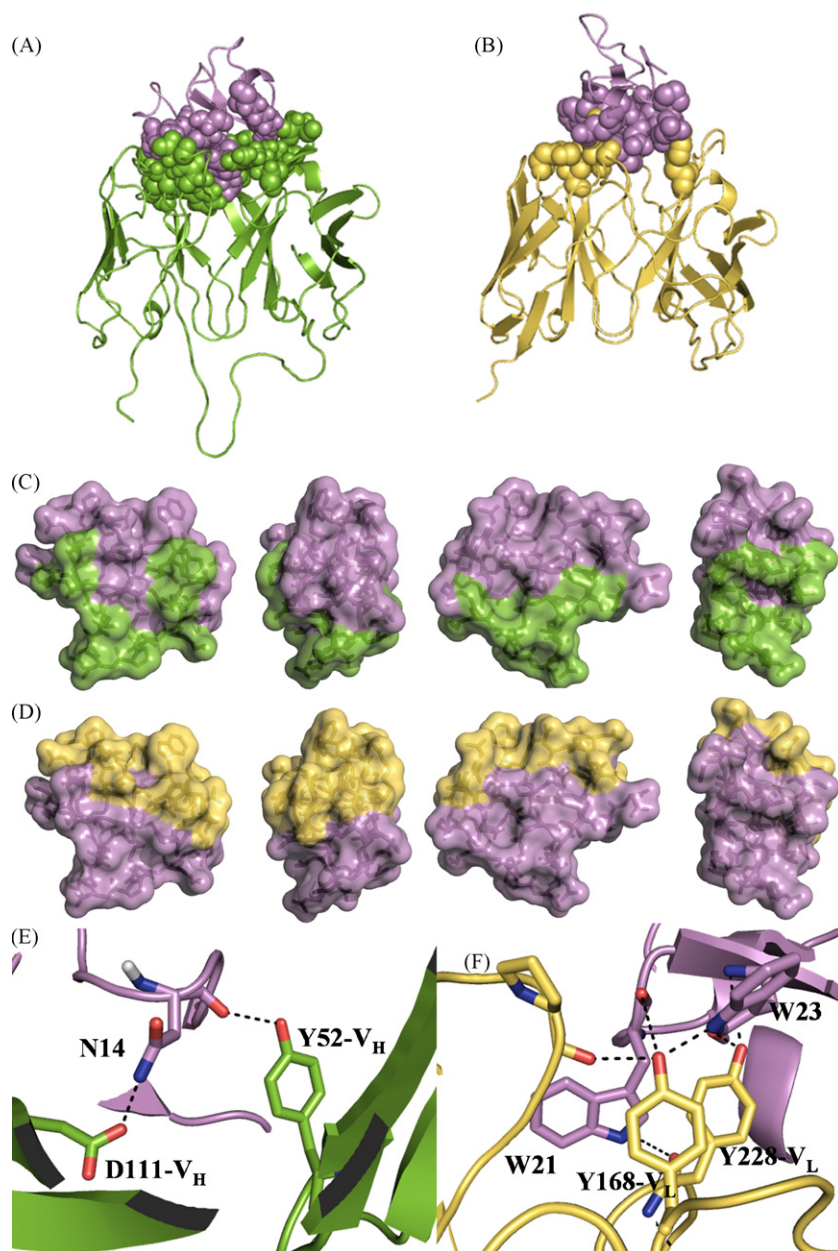


Fig. 4. Models for the interaction of recombinant antibodies with Hev b 6.02. (A and B) Front views of hevein (violet)–scFv G7 (green) and hevein–scFv H8 (yellow), respectively. Hotspot residues are shown as spheres; (C and D) conformational epitope on the hevein surface when interacts with scFv G7, and with H8, respectively. The four views are shown rotated every 90°; (E and F) some interactions at the Hev b 6.02–antibody interfaces. Hydrogen bonds are colored black.

3.3. Affinity maturation

To determine whether the increase in the affinity of the scFv could improve the IgE–Hev b 6.02 inhibition, three cycles of directed evolution were carried out with the clone M8. In the first cycle, the library (7.3×10^6 variants; mutation rate 1.1%) was evaluated by phage display against Hev b 6.02 by three rounds of biopanning. The improved recognition of the variant F6 was determined by three mutations in the frameworks of the heavy chain: Met⁴⁸Val, Ile⁷⁶Phe and Gly⁹⁰Ser. This variant was subjected to a second maturation cycle, where the library size was 2.4×10^6 variants and the mutation rate 1%. In this case, the clone F7 was selected. The changes were located at V_H–CDR3: (Ile¹⁰²Lys) FRV_H, (Met¹²¹Val), FRV_L; (Ser²⁰⁸Thr), FRV_L; (Ser²⁵²Gly) and FRV_L (Ala²⁵⁵Gly). For the third cycle (2.3×10^6 variants and mutation rate 1%), the clone G7

was chosen and its DNA sequence showed only two changes with respect to clone F7, FRV_L (Gln¹⁸⁰His) and V_L–CDR2 (Ser¹⁹⁶Tyr).

3.4. Inhibition of IgE binding to Hev b 6.02 by recombinant antibodies

ELISA inhibition assays were performed to verify whether the *in vitro* selected anti-hevein murine mAb (6E7) and human scFvs (M8, G7, A6 and H8) had epitopes in common with the IgEs from latex allergic patients (Table 2). Sera from seven allergic individuals showed that the binding of each recombinant antibody inhibited up to 55% of the IgE–Hev b 6.02 interaction. The combination of the four scFvs and the mAb 6E7 inhibited more than 70% for the sera of two out of the seven patients. These results demonstrate the polyclonal nature of the Hev b 6.02-specific IgE response. Analyzing the results

obtained for the matured G7 compared with M8, we observed only a slight improvement in the percentage of inhibition.

3.5. Mapping of the binding sites of the recombinant antibodies using natural and chemically modified variants of Hev b 6.02

For identification and comparison of the Hev b 6.02 regions recognized by scFvs, mAb and native IgE, two of its natural isoforms and one chemically modified variant were used. Hev b 6.0202 has a single mutation in the position N¹⁴D (Reyes-López et al., 2006), while pseudohevein presents six mutations Trp²¹Tyr, Thr²⁷Ser, Glu²⁹Asp, Asp³⁴Ser, His³⁵Lys and Asp⁴³Gly. For the chemically modified hevein both, Trp²¹ and Trp²³ were oxidized as described previously (Reyes-López et al., 2004). Increasing concentrations of the proteins were added to each recombinant antibody and the residual binding activity to Hev b 6.02 was measured as a percentage of inhibition by ELISA (Fig. 2). The inhibitory effect of Hev b 6.0202 (86%), pseudohevein (50%) and modified hevein (20%) at the highest concentration in the IgE–Hev b 6.02 interaction is shown (Fig. 2A). This establishes the importance of the tryptophans' solvent-accessible surface area (ASA), and some residues located in the carboxy-terminal region, to be recognized by IgEs. In the scFv H8–Hev b 6.02 interaction, only the modified allergen (60%) and pseudohevein (37%) showed changes in the percentage of inhibition (Fig. 2B). For the scFv G7–Hev b 6.02 interaction, the isoform Hev b 6.0202 inhibited 70%, while pseudohevein inhibited 48.5% (Fig. 2C). The highest concentration of pseudohevein and modified hevein showed reduced mAb 6E7-binding capacity, 36% and 58%, respectively, whereas Hev b 6.0202 only reduced it by 15% (Fig. 2D).

3.6. Fluorescence measurements

Two different experiments were performed to analyze the interaction of hevein with the recombinant antibodies. We used the intrinsic fluorescence of the tryptophan residues Trp²¹ and Trp²³ (ASA of 165.9 and 112.1 Å², respectively) and, the acrylamide-dependent quenching. In the first case, the binding of H8 and mAb 6E7 to Hev b 6.02 promotes an emission shift from 358 to 348 and 345 nm, respectively (Fig. 3A). These results are explained by a decrease in the polarity of the environment of the tryptophans' area (Lakowicz, 1986). In the second case, acrylamide was used to quench the fluorescence of hevein and the antibodies either alone or while in a complex. Fig. 3B shows a Stern–Volmer analysis of the acrylamide-dependent quenching of hevein–antibody complexes, where important differences are observed. The Hev b 6.02–mAb 6E7 complex showed a substantial decrease in the K_{SV} value ($12.1 \pm 0.15 \text{ M}^{-1}$) compared to that of Hev b 6.02 ($17.68 \pm 0.4 \text{ M}^{-1}$); the Hev b 6.02–scFv G7 and Hev b 6.02–scFv H8 complexes produced a slightly decrease, and Hev b 6.02–scFv A6 did not affect the K_{SV} value (Fig. 3B). These results indicated that in addition to a change in the polar nature of the environment of the tryptophan residues, they also became less accessible to acrylamide. Therefore, it may be concluded that at least one Trp is in fact involved in interactions with the mAb 6E7 and the scFv H8.

3.7. Homology modeling, simulated docking of scFvs–Hev b 6.02 complexes and epitope analysis

For the homology modeling, we compared all the models obtained as described in Section 2, and selected the best results for each scFv. To further elucidate the structural characteristics of hevein bound to the scFvs, the described filter and selection procedure yielded a final model for the docking complex Hev b 6.02–scFv G7 and one for the Hev b 6.02–scFv H8 complex (Fig. 4). In the

Table 3
Antibody interface residues.

Hev b 6.02–scFv G7	Hev b 6.02–scFv H8
Number of residues	
16	15
Buried surface area (Å ²)	
787.7	613.8
P-value	
0.415 (Hev b 6.02), 0.515 (scFv G7)	0.533 (Hev b 6.02), 0.459 (scFv H8)
Residues involved in polar interactions	
Hev b 6.02–scFv G7 (CDRs)	Hev b 6.02–scFv H8 (CDRs)
Gln2–His109 (H3)	Arg5–Gly103 (H3)
Lys10–Tyr196 (L2)	Gln6–Gly103 (H3)
Leu11–Gln193 (L2)	Ser19–Tyr228 (L3)
Cys12–Ser175 (L1)	Gln20–Tyr228 (L3)
Asn14–Tyr52 (H2), Asp111 (H3)	Trp21–Tyr168 (L1)
Asn15–Tyr52 (H2), Arg59 (H2), Trp234 (L3)	Trp23–Tyr168 (L1), Tyr228 (L3)
Ser26–Asp55 (H2)	Gln29–His163 (L1)
Asn40–Asp235 (L3)	Pro33–Tyr59 (H2)
Lys42–Asp55 (H2)	Asp34–Tyr59 (H2)

A contact is made when two side chains have at least one nonhydrogen atom pair within 4 Å.

The P-value is a measure of interface specificity. A value of $P > 0.5$ means that the interface is less hydrophobic. On the other hand, $P < 0.5$ indicates that the interface can be interaction-specific (Krissinel and Henrick, 2007).

first case, the buried accessible surface area was 1485 Å², involving 26 residues of the paratope, and 16 residues of the epitope (Fig. 4A, C and E). Asparagine 14 establishes three hydrogen bonds with residues located in the deep cavity of the antibody, validating the *in vitro* assays (Fig. 2C). This complex reveals a slightly more hydrophilic interface than the average found in antibody–antigen interfaces (Lo Conte et al., 1999). Table 3 lists the interface residues of the resulting models and their interactions. Comparatively, H8 established ten hydrogen bonds with the CDRs, including more hydrophobic residues that made contact with the two aromatic residues (Trp²¹ and Trp²³) in hevein (Fig. 4B, D and F).

For comparison, the coordinates of Hev b 6.02 were submitted to several servers for the prediction of conformational epitopes (Ponomarenko and Bourne, 2007). The server PPIRED (Bradford and Westhead, 2005) predicted three discontinuous epitopes; the first involved Gln², Ser¹⁹, Trp²¹, Trp²³–Ser²⁶ and Glu²⁹ and Tyr³⁰, the second Gly⁹, Leu¹¹–Asn¹⁵, Ser³⁹ and Asn⁴⁰; and the third conformational epitope involved the residues Pro¹³–Cys¹⁷, Cys²⁴–Thr²⁷ and Lys⁴², which are in accordance with our docking results.

4. Discussion

It is well recognized that the latex allergen Hev b 6.02 is an important cause of type I hypersensitivity in latex gloves users, particularly health care workers, children submitted to multiple operations, and more recently laboratory scientists. The fact that several studies have associated Hev b 6.02 sensitization with the latex-fruit syndrome highlights the importance of this small and stable allergen. Such associations establish cross-reactivity as a consequence of the presence of hevein-like domains in some fruits (Blanco, 2003). In this study, we present a structural characterization based on molecular modeling and *in vitro* experiments, of putative B-cell epitopes of Hev b 6.02, using three human scFv antibody fragments and one murine monoclonal antibody. We observed that these recombinant antibodies inhibited the binding of human IgEs to Hev b 6.02, to a varying degree depending on each individual, with a maximum value of 55% for scFv H8, 51% for scFv A6, 37% for scFv G7, and 41% for mAb 6E7. Similar results have been reported for different monoclonal antibodies anti-allergens (Mirza

et al., 2000; Padavattan et al., 2007; Naik et al., 2008; Flicker et al., 2006). Notably, in our case, a synergic effect was observed when a mixture of all the recombinant antibodies was able to inhibit the IgE binding to the allergen, up to 71% in some patients (Table 2). It is evident that this battery of antibodies recognizes different regions of the Hev b 6.02 surface, thus resembling the IgE polyclonal response from allergic patients and allowing for the individual analysis of each B-cell epitope. The overlapping between IgE and IgG epitopes could be useful for the identification of immunodominant regions that are essential for diagnostic and therapeutic studies (van Neerven et al., 2006). For these reasons, the hevein-specific antibodies might be used as potential blocking antibodies, as well as diagnostic and research tools.

Concerning the primary structure, the three scFv anti-hevein obtained in this work, display CDR loops of the V_H and V_L chains that are, surprisingly, highly identical to those regions of a few available sequences of IgE antibodies raised against different allergens. The BLAST analysis showed that the V_L s of A6 and H8 presented a sequence identity of 81–83%, respectively, with V_L s of scFv antibodies against Phl p 5 (Persson et al., 2007). CDRL3 showed the highest sequence variation, as expected; however, the aromatic residues remained. For the V_H different results were obtained: The H8 V_H had an 85% identity with one ϵV_H from antibodies of grass pollen allergic patients (Andréasson et al., 2006), while the A6 V_H presented a 74% sequence identity with another ϵV_H obtained from allergic patients antibodies (Coker et al., 2005). CDRH1 and CDRH2 loops were quite conserved and the largest differences were found in the CDRH3 regions. In accordance with our results, Niemi et al. (2007) reported a high identity among V_L s of anti-grass pollen, anti-latex and anti-bovine milk antibodies, suggesting the presence of similar epitopes on these allergens. It is worth mentioning that the IgEs anti-hevein reported by Laukkanen et al. (2003) present an identity of 63% when compared to H8, with the highest similarities between CDRL1 and CDRL3.

To further analyze the epitope regions, we performed ELISA inhibition experiments testing two natural isoforms and the allergen chemically modified at the tryptophan residues. These isoforms are polymorphic variants of the same allergen, which present small variations in the discontinuous epitopes that could translate in important changes in the binding strength. We found that each recombinant antibody recognized in a different way the isoallergens tested. The local change in charge distribution present in Hev b 6.0202 might explain why the scFv G7 binding ability was reduced, meaning that Asn¹⁴ is part of the epitope recognized by this antibody. On the contrary, when the milieu of the aromatic residues (Trp²¹ and Trp²³) was changed, the scFv H8 and mAb 6E7-binding abilities were decreased, suggesting their participation in another immunodominant region. Lastly, pseudohevein that includes mutations at the C-terminal region and at the aromatic area (Trp²¹Tyr) showed a reduction in the binding capacity for all the antibodies, thus suggesting the participation of those residues in the antibodies recognition (Fig. 2). Comparatively pseudohevein and modified hevein presented the lowest IgE recognition, indicating that the residues Trp²¹, Trp²³, Thr²⁷, Glu²⁹, Asp³⁴, His³⁵, and Asp⁴³ are relevant for this interaction.

In agreement with the results presented above, when intrinsic fluorescence spectra were recorded in the presence of increasing concentrations of scFv H8 and mAb 6E7, it was observed that the relative emission of the two tryptophans in hevein shifted. The dose-dependent shift in fluorescence emission could be mediated by a conformational change in the protein once the antibodies were bound, or by a change in the local environment of the two tryptophans, reflected as polarity. With regard to the fluorescence quenching with acrylamide, the presence of the two antibodies provoked a decrease in the accessibility to at least one of the

tryptophans. This is consistent with our previous report, where we established the importance of the aromatic residues exposed to the solvent for Hev b 6.02–IgE interaction (Reyes-López et al., 2004).

The crucial event for triggering an allergic response is the allergen–antibody interaction; therefore, determining the structure of these complexes is a way to identify relevant epitopes. These molecular determinants can be predicted by molecular modeling, using suitable software. For this purpose, we obtained two docked models using the scFvs H8 and G7 with hevein. They suggested two putative discontinuous epitopes, the first comprising a patch of exposed aromatic residues surrounded by polar ones, and the latter including the amino and carboxy-terminal ends. These results are in agreement with the experimental assays presented above, and are consistent with some characteristics of antigen–antibody complexes from crystallographic data (Table 3) (Lo Conte et al., 1999; Collis et al., 2003).

It is increasingly evident that aromatic patches on the surface of allergens are important. For example, a common putative IgE epitope present in cysteine protease allergens shows a conserved Tyr localized in the center of the epitopes (Furmonaviciene et al., 2000). In another case, one Trp is a critical residue, recognized by monoclonal antibodies, in the serine protease allergens (Lee et al., 2007). Furthermore, the epitope of albumin 2S, from sunflower seeds, consists of a hydrophobic patch including one Trp and five methionines (Pantoja-Uceda et al., 2004). Recently, the crystal structure of the complex Api m 2–Fab reported by Padavattan et al. (2007) presented an epitope formed by a central hydrophobic patch, which is surrounded by charged and polar residues. Two aromatic residues, a Trp and one Phe, establish six apolar contacts with the Fab. All these results support the idea that aromatic residues participate in networks of co-operative interactions with not only hydrophobic, but also charged and hydrogen bond donor and acceptor groups in the IgG and IgE–allergen interactions.

In summary, the use of oligoclonal antibodies (i.e. mixtures of monoclonal antibodies or human scFvs) proved useful in this kind of studies. The identification of Hev b 6.02 B-cell epitopes will allow for a structure-based rational modification of these regions aimed at producing allergen variants with low IgE-binding activity.

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