

Allergenic and antigenic determinants of latex allergen Hev b 1: peptide mapping of epitopes recognized by human, murine and rabbit antibodies

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Summary

Background The rubber elongation factor in *Hevea* rubber (Hev b 1) is one of the most important latex allergen and is leading cause of latex type 1 hypersensitivity in children with spina bifida.

Objective The aim of this study was to define the allergenic and antigenic epitopes of Hev b 1.

Methods The immunoglobulin- (Ig)E and IgG antibody binding sites on Hev b 1 allergen were delineated by enzyme linked immunosorbent assay (ELISA) using synthetic overlapping peptides covering the whole Hev b 1 sequence. In order to improve the binding capacity and specificity all peptides were biotinylated at the N-terminal end via a 6-aminohexanoic acid as spacer and then adsorbed to streptavidin pre-coated microtitre plates. Fine mapping to define the essential amino acid residues for the antibody binding was achieved by using overlapping peptides with one amino acid offset.

Results It was demonstrated that the IgE epitopes were located in different regions of Hev b 1 including the C-terminal segment (121–137) and the segments with amino acid residues of 30–49 and 46–64. Two monoclonal antibodies (MoAbs) II2F3 and II4G9 raised against purified Hev b 1 recognized the C-terminal segment only. The results of epitope mapping with three rabbit antisera revealed that five positive peptides, including the epitope peptides 31–49, 46–64 and 121–137, were involved in the antibody-binding sites. Fine mapping on the segments 46–64 and 121–137 showed that the two MoAbs reacted with the peptide 125–134 in the C-terminal region, whereas the peptide with amino acids 124–134 was essential for recognition by human IgE antibodies. Epitopes to rabbit polyclonal IgG and human IgE were also found to be involved in the amino acid residues of 47–59.

Conclusion Our results indicate that the most allergenic/antigenic portions of Hev b 1 allergen are the C-terminal region and the region with amino acid residues of 31–64. In both regions, the minimal IgE-binding epitope is almost identical with the IgG-binding epitope.

Keywords: latex allergy, Hev b 1, B-cell epitope, IgE, monoclonal antibodies, biotinylated peptides, rubber elongation factor

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Introduction

IgE-mediated hypersensitivity reaction to natural rubber latex (latex) has become a serious problem especially among health care workers, who have to use latex-made

gloves regularly for prevention from AIDS and hepatitis etc. [1–5]. In addition, allergic reactions to latex seem to be particularly frequent in patients requiring repeated surgery or long-term hospitalization [6,7]. The responsible allergens are latex proteins which are found in raw latex and in various latex-made products. Immunoblots performed by various investigators with human sera revealed more than 10 protein bands from natural latex and latex glove extract

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which bound IgE antibodies [3,8,9]. The allergenic proteins have molecular weights ranging from 10 to 100 kDa. So far three proteins in natural rubber latex are identified at their primary structure level. They are prohevein with a molecular weight of 20 kDa [10], heveamine of 29.5 kDa [11] and rubber elongation factor (REF) of 14.6 kDa [12]. Recently we have isolated and identified the rubber elongation factor (REF) in latex gloves and in glove powder as one of the most important allergen in latex [13] and designated it as Hev b 1 according to the International Union of Immunological Societies' (IUIS) allergen nomenclature [14].

Many investigators noticed that the 14 kDa protein is the most frequent IgE binding protein in latex [9,16,17]. It was reported that 18–45% of spina bifida patients are latex-sensitized [6,18]. Out of 28 latex-sensitized patients with spina bifida tested by the Pharmacia CAP System (Pharmacia, Uppsala, Sweden), 24 (85%) also showed specific IgE antibodies to Hev b 1 in the enzyme-linked allergosorbent test (EAST) when the protein is coupled with the BrCN-activated cellulose disks (our unpublished results).

The localization of B-cell epitopes is an important step in elucidating the cellular and molecular mechanisms of the immune response as well as a prerequisite for developing new strategies in immunotherapy. Although many efforts have been made to identify latex allergens which bind IgE antibodies in latex-sensitized patients, there is still no report about the analysis of allergenic and antigenic determinants of latex allergens using synthetic peptides. Here we report the identification of IgE binding sites of Hev b 1 by use of synthetic biotinylated peptides and sera of 11 latex-sensitized patients. In addition, autoinhibition assays with corresponding non-biotinylated peptides were performed to check the specificity of the IgE binding reaction. Monoclonal antibodies raised to Hev b 1 protein and sera of three rabbits, which were immunized with Hev b 1 and two protein extracts of latex saps from Thailand and Malaysia, were also investigated for their antibody binding to Hev b 1 peptides. Furthermore, in order to define the essential residues for the binding of antibodies, a fine scanning of allergenic and antigenic determinants was performed using a panel of overlapping peptides with one amino acid offset.

Materials and methods

Purification of Hev b 1

Ammoniated (0.7%) latex sap from Thailand (provided by Kautschuk GmbH, Frankfurt am Main, Germany) was used to isolate the Hev b 1 protein. Eighty millilitres of sap were diluted to 160 mL with 50 mM Tris containing 0.01% Triton X-100, pH 8.5, and then was centrifuged

at 41 000 *g* for 45 min at 10°C. The yellow aqueous layer at the bottom of the centrifuge tube was discarded and the serum free rubbery supernatant was resuspended in 160 mL of 2% (w/v) sodium dodecyl sulfate (SDS) and creamed at room temperature for 30 min. The sample was then centrifuged again at 41 000 *g* for 45 min at 10°C to remove the visible rubber particles, and if necessary, it was centrifuged once more. The supernatant fluid was then filtered through a 0.45 µm filter (Sartorius AG, Göttingen, Germany), and dialysed in distilled water using a cellulose dialysis tubing (Serva, Heidelberg, Germany) with a molecular mass cut-off of 6 kDa for 36 h at 4°C. The dialysate was lyophilized and redissolved in 10 mL 0.1 M ammonium acetate containing 0.01% NaN₃, pH 6.8. The protein solution was then loaded onto a column of Sephadex 75 superfine (2.6 × 90 cm) which was equilibrated and eluted with the same ammonium acetate buffer. Fractions of 2.5 mL were collected and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the 14.6 kDa protein were pooled, desalted by centrifugation through an Amicon membrane, lyophilized and stored at -40°C. The purified Hev b 1 protein showed a homogeneous band in SDS-PAGE by silver staining.

Protein extracts from latex sap

Ammoniated (0.7%) *Hevea* latex saps from Thailand and Malaysia were diluted (1:1, v/v) with 0.1 M ammonium acetate buffer, pH 6.8, mixed for 30 min and centrifuged at 41 000 *g* and 4°C for 45 min. The aqueous layer containing soluble protein was carefully separated from the creamed rubber particles and filtered through a 0.45 µm membrane filter. The resulting extracts were further dialysed in distilled water across a 6 kDa cut-off cellulose membrane tube for 36 h at 4°C, then lyophilized and stored at -40°C. The protein concentration was determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany).

Antisera

Human sera Sera from 11 latex-allergic subjects were used as a source of IgE antibodies. Three of them were spina bifida patients (SB). All the sera had been tested for their concentrations of specific IgE to latex allergens by the CAP method (Pharmacia, Uppsala, Sweden) and to HEV b 1 in EAST using Hev b 1 coupled to paper disks prepared in our laboratory (Figs 1 and 2). Two other sera from healthy non-allergic adults were used as control (neg 1 and neg 2).

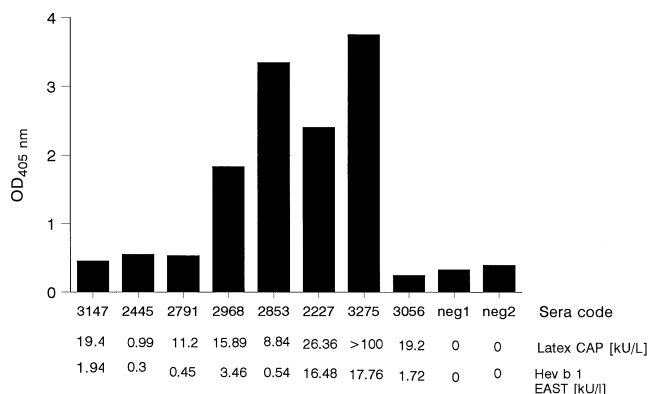


Fig. 1. Binding profiles of IgE antibodies to the Hev b 1 protein. Eight sera from latex-allergic subjects were used to estimate the binding capacity to the microtitre plate-bound Hev b 1 by ELISA. Specific IgE concentrations to latex (CAP method) and to Hev b 1 (EAST) are also shown. Two serum samples from healthy non-allergic adults were used as negative controls (neg 1 and neg 2).

Monoclonal antibodies (MoAbs) BALB/c mice were injected intraperitoneally with 30 µg of Hev b 1 in 200 µL phosphate buffered saline (PBS) and complete Freund's adjuvants (1:1, v/v). Booster injections were administered on days 37 and 88 with the same antigen dose in PBS. On day 91, spleen cells from two immunized mice were fused with NSO myeloma cells (relation 5:1) according to Köhler and Milstein [19]. The hybrid cells producing anti-Hev b 1 antibodies were cloned on agar plates. Two MoAbs named II2F3 and II4G9 were obtained and used for binding tests.

Rabbit antisera Antisera to Hev b 1 (R_H), and to protein extracts of latex saps from Thailand (R_T) and Malaysia (R_M) rubber trees were raised in three New Zealand rabbits by subcutaneous injections of 1 mg protein in 1.5 mL PBS emulsified with complete Freund's adjuvants (1:1; v/v) in small aliquots on the back. Booster injections were prepared in PBS and given at intervals of 4 weeks. After three booster injections, sera were obtained from the marginal ear vein.

Electrophoresis

SDS-PAGE was carried out in a Phast-System (Pharmacia, Uppsala, Sweden) using precast 8–25% gradient gels with molecular mass standards in the range of 14.4–94 kDa as markers. Protein bands were visualized by silver staining.

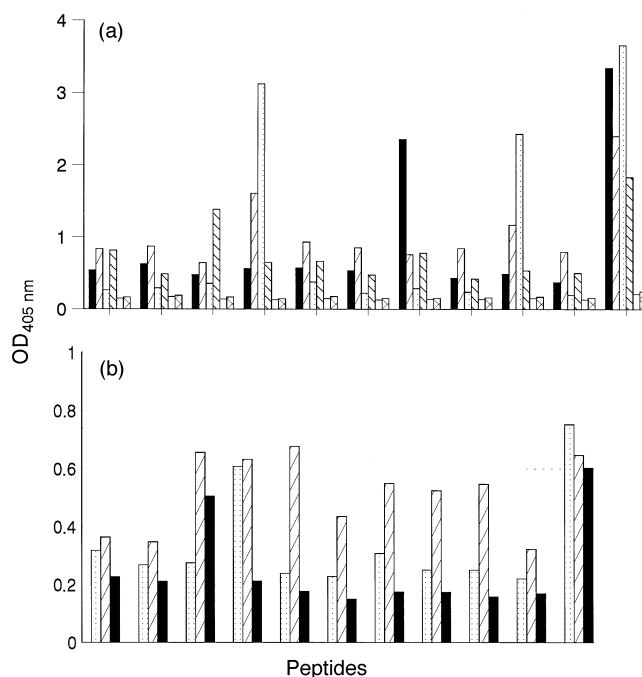


Fig. 2. Allergenicity of nine biotinylated peptides of Hev b 1 (See Table 1 for sequences) to human sera from latex-allergic subjects. (a) Binding profiles of peptides to four sera showing positive reactions to the Hev b 1 protein coated on microtitre plate in ELISA. Two sera from non-allergic persons as well as a non-relevant peptide from the N-terminal region (1–19) of Chi t 1.01 (B-1.01) were used as negative controls. (b) Binding profiles of peptides to sera from three latex-allergic patients with spina bifida (SB). Initial IgE binding data of sera from SB patients: SB-5: latex CAP = 0.35 kU/L, Hev b 1-EAST = 0.36 kU/L; SB-17: latex CAP = > 100, Hev b 1-EAST = 8.30; SB-26: latex CAP = 14.0, Hev b 1-EAST = 2.78. (a) Sera: ■. 2853. ▨. 2227. ▩. 3275. ▪. 2968. □. Neg 1. ▤. Neg 2. (b) Sera: ▩. SB-5. ▨. SB-17. ▪. SB-26.

Peptide synthesis

Peptides were synthesized stepwise on an Applied Biosystems 432A peptide synthesizer using Fmoc strategy [20] as previously described [21]. In order to eliminate the influence of C-terminal ending (-COOH) of the peptide during the antibody binding screening, an amide resin with an excellent acid labile linker of Rink [22] was used which is cleaved by TFA to produce peptide amides. At the N-terminus of the peptide, a Fmoc-protected 6-aminohexanoic acid (Novabiochem, Bad Soden/Ts, Germany) was introduced to act as a spacer and followed by a (D)-biotin-N-hydroxysuccinimide ester (Bachem Biochemica, Heidelberg, Germany) to make an N-terminal biotinylated peptide. All peptides were analysed for their purity by reversed-phase high performance liquid chromatography

(HPLC) (C18 column: 4 × 250 mm) and identified by ion-spray mass spectroscopy on an API III triple-quadrupole mass spectrometer with an IonSpray inter-face (Sciex, Toronto, Canada). When necessary, the peptides were purified by reverse-phase HPLC on a silica bead C18 column (20 × 250 mm) to ensure that their purity was over 75% as determined by analytical RP-HPLC.

Nine biotinylated peptides (eight 19-mer and one 17-mer, designated as B1-19, B16-34, B31-49, B46-64, B61-79, B76-94, B91-109, B106-124 and B121-137, see Table 1) covering the entire sequence of Hev b 1 and overlapped by four amino acid residues each were first synthesized to catch all potential antibody-binding regions of Hev b 1 protein. A 19-mer peptide (B-1.01) from the N-terminus of Chi t 1.01 protein (component III of Chi t I [21]) was also synthesized and biotinylated as control peptide. The peptides for autoinhibition assay were synthesized as free peptides without biotinylation (marked by the letter H). To define the smallest peptide that retains a significant binding ability to antibodies, six biotinylated dodecapeptides with a 6-aminohexanoic acid as spacer covering the C-terminal peptide segment 121–137 and a set of tetradecapeptides corresponding to the whole segment of 46–64 were also synthesized.

All peptides were overlapped with one amino acid offset.

Detection of antibody binding to Hev b 1 and Hev b 1 peptides by ELISA

Presence of antibody-binding epitopes on the peptides of Hev b 1 was examined by ELISA. For preparation of the solid-phase peptide antigen 1 µg of the streptavidin (Sigma, S8276) in 100 µL carbonate/bicarbonate buffer (pH 9.6) was used per well of a microtitre plate (Nunc MaxiSorb) for overnight at 4°C, followed by blocking with 2% (w/v) BSA (Sigma, A7638) in PBS. 0.5 mg of each synthetic peptide were dissolved in 10 mL PBS containing 0.1% BSA and 0.02% sodium azide. Some 100 µL of each peptide solution were then transferred into the corresponding wells. The reaction was allowed to proceed for 2 h at 37°C, and the plates were washed twice with PBS containing 0.1% Tween 20 (PBST). Several sets of immobilized peptides were prepared simultaneously, dried at 37°C and stored at –40°C until use.

The purified Hev b 1 protein was coated on Nunc plates by incubating 100 µL of protein solution (2 µg/well) in

Table 1. Amino acid sequence (one letter code) of Hev b 1 and synthetic biotinylated peptides B1-19 to B121-137. (B1-19 = peptide with sequence of 1-19 functionalized with 6-biotinamido-hexanoic acid)

Hev b 1 sequence:		
1		
AcAEDEDNQGGQGEGLKYLGFVQDAATYAVTTFSNVYLFKDKSGP		
LQPGVDIIIEGPKNVAVPLYNRFSYIPNGALKFVDSTVVASVTIIDRSL		
	137	
PPIVKDASIQQVVSIRAAPAAARSLASSLPGQTKILAKVFGYGEN		
Synthetic peptides		
Code	Region of Hev b 1 protein	Amino acid sequence
B1-19	1-19	B-AEDEDNQGGQGEGLKYLGF-NH ₂
B16-34	16-34	B-YLGFVQDAATYAVTTFSNV-NH ₂
B31-49	31-49	B-FNSVYLFKDKSGPLQPGV-NH ₂
B46-64	46-64	B-QPGVDIIIEGPKNVAVPLY-NH ₂
B61-79	61-79	B-VPLYNRFSYIPNGALKFVD-NH ₂
B76-94	76-94	B-KFVDSTVVASVTIIDRSLP-NH ₂
B91-109	91-109	B-RSLPPIVKDASIQQVVSIR-NH ₂
B106-124	106-124	B-SAIRAAPAAARSLASSLPG-NH ₂
B121-137	121-137	B-SLPGQTKILAKVFGYGEN-NH ₂
B-1.01	1-19 of Chi t 1.01	B-LSADQISTVQASFDKVKGD-NH ₂

carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. After washing with PBST, the wells were blocked with 2% (w/v) BSA in PBST and washed with PBS.

For the detection of IgE antibody binding, 100 µL of serum diluted in PBST containing 0.1% BSA (PBST/BSA) were added in duplicate to Hev b 1 or to peptide-bound microwells and incubated overnight at 4°C. The wells were then washed three times with PBST and the bound IgE antibodies were detected by using alkaline phosphatase labelled goat anti-human IgE antibodies (Sigma, A3525) and the substrate *p*-nitrophenyl phosphate (pNPP; Sigma, N2640) for the color development. The optical density was measured at 405 nm on an ELISA plate reader and the results were expressed as the mean of duplicate wells. The IgE binding assay was optimized by pre-scanning each serum sample with Hev b 1-coated plates at a dilution of 1:2, 1:5, 1:10 and 1:50.

The antigenicity of murine anti-Hev b 1 MoAbs (culture supernatant, 1:20) and rabbit antisera (1:500) to solid-phase peptide antigens was detected utilizing above procedure, except that the second antibody used was alkaline phosphatase-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG (both from Sigma) respectively.

Competitive inhibition assay

In order to verify the binding specificity of human IgE antibodies to the Hev b 1 peptides immobilized on microtitre plates, autoinhibition assays were performed by using the corresponding non-biotinylated peptides. The inhibition assay was identical to the ELISA described above with the exception that 100 µL of diluted sera were pre-incubated overnight at 4°C with 10 µL non-biotinylated peptide solution at concentrations ranging from 0.01 to 1 mg/mL before incubation with solid-phase antigens.

Results

Peptide scanning with human IgE antibodies

Eight serum samples from latex-sensitized and two negative control sera were used to react with solid phase-bound Hev b 1 in ELISA (Fig. 1). The results demonstrated that IgE in four of eight latex-positive serum samples bound strongly to Hev b 1 at a dilution of 1:10. The other four serum samples (patient code numbers: 3147, 3056, 2445 and 2791), however, did not bind to Hev b 1 in ELISA. No binding activity could be observed with these sera even at a higher concentration of IgE (1:2 dilution), despite the fact that they were clearly Hev b 1-positive in EAST.

The four positive sera in ELISA mentioned before (Fig. 1) and three serum samples from patients with spina bifida were used for the peptide scanning. The results in Fig. 2a demonstrate that there was a strong binding of IgE antibodies to peptides B46–64 and B121–137 in sera 3275 and 2227. The binding profiles of sera 2853 and 2968 indicate an elevated IgE reaction to peptides B91–109 and B31–49 respectively. The peptide B46–64 was also identified to be able to bind to IgE in two of three sera from spina bifida patients (Fig. 2b). The third spina bifida serum (SB-26) reacted with peptide B31–49. Interestingly, all but SB-17 sera only reacted with one or two peptides. The IgE in serum SB-17 showed to be able to bind to most of the peptides. No antibody binding was detected to the control peptide B-1.01 or to the negative control sera. In order to confirm the binding specificity of IgE antibodies to Hev b 1 peptides, we performed competitive autoinhibition assays with corresponding non-biotinylated peptides. Figure 3 shows the results of testing human sera (Code: 3275 and 227) on microtitre plate-bound peptides B46–64 and B121–137. The binding of IgE antibodies in serum 3275 to the peptides was completely inhibited by the corresponding peptides H46–64 and H121–137 in solution. The same result was achieved by inhibition of IgE binding in serum 2227 to peptide B46–64. The synthetic peptides were also tested for their ability to inhibit IgE binding to the Hev b 1 protein coated on microtitre plate in ELISA. Result of the inhibition test

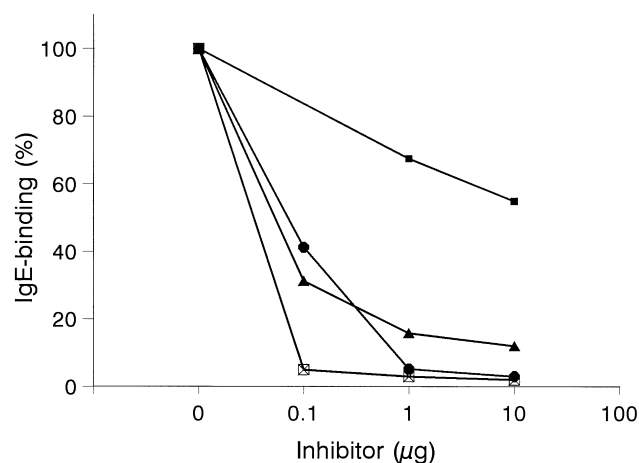


Fig. 3. Autoinhibition of IgE binding to solid phase peptides B46–64 and B121–137. The reaction of peptides B46–64 (A) and B121–137 (B) to IgE antibodies in serum with number 3275 (serum dilution: 1:20), or in serum 2227 (C, serum dilution: 1:10; only to B46–64) was inhibited on an ELISA plate by a series of properly diluted corresponding peptide solution. Binding of IgE antibodies to Hev b 1 could be also inhibited up to 45% by incubating with 10 µg each of peptides H46–64 and H121–137 (D, serum 3275; dilution: 1:50). ●, A. ✕, B. ▲, C. ■, D.

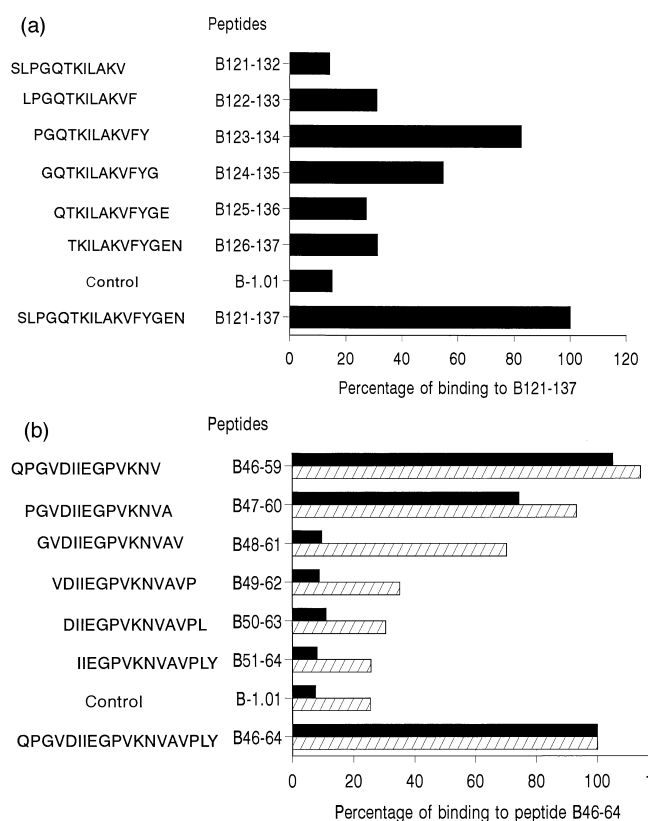


Fig. 4. Fine scanning of B-cell epitopes in the C-terminal region of *Hev b 1* (121-137) and the region of 46-64. (a) Binding profiles of IgE antibodies in serum 3275 (1:20) to peptides derived from the C-terminal sequence (121-137). Six biotinylated dodecapeptides overlapping by 11 amino acids and covering the whole sequence of 121-137 were used to determine the minimal binding site in this region. Results are expressed as the percentage of binding to the full-length peptide B121-137. (b) Binding of IgE antibodies in sera 3275 (1:20) and 2227 (1:10) to peptides representing the B46-64 sequence of *Hev b 1*. Six tetradecapeptides overlapping by 13 amino acids were used in the assay. Results are expressed as percentage of binding to the full length peptide B46-64. The peptide B-1.01 from the N-terminal region (1-19) of *Chi t 1.01* was used as negative control. ■, 3275. ▨, 2227.

using serum 3275 as IgE source is shown in Fig. 3 (D). It was found that the IgE binding to the whole *Hev b 1* protein could be inhibited up to 45% by adding a mixture of 10 μ g non-biotinylated peptides H46-64 and H121-137. By comparison, the IgE binding of sera 3275 and 2227 to peptides B46-64 and B121-137 could be completely inhibited by pre-incubating with 1 μ g of *Hev b 1* protein (data not shown).

To define the sequences of IgE epitopes, two sets of overlapping peptides with one amino acid offset derived from the C-terminal segment of *Hev b 1* (B121-137) and

from the segment of B46-64 were synthesized, biotinylated and subsequently used for determination of the IgE binding activity in ELISA. Figure 4a shows that the peptides B123-134 and B124-135 from the C-terminal region were recognized by serum 3275. The binding activity was strongly dependent on the residues of proline, glycine and tyrosine in position 123, 124 and 134 (P^{123} , G^{124} and Y^{134}). It decreased to about 50% if amino acid P^{123} was omitted in the sequence, and the peptide lost almost all binding activity when Y^{134} or G^{124} were absent in the sequences. These results suggest that the C-terminal minimal epitope of IgE antibodies in serum 3275 contains the amino acids P G Q T K I L A K V F Y (123-134).

Six tetradecapeptides with one amino acid offset from the segment of B46-64 were tested with IgE antibodies of sera 2227 and 3275. The results are demonstrated in Fig. 4b. The residues of A V P L Y in position 60-64 do not seem to substantially contribute to the IgE binding reaction with either of the sera. A proline residue (P) in position 47, however, plays an important role in the binding reaction. The absence of the P^{47} in sequence resulted in a complete loss of binding to serum 3275, while the other serum (2227) did not bind to peptide when G^{48} was also omitted. This pattern of reactivity suggests the existence of an IgE epitope within the peptide sequence of P G V D I I E G P V K N V (47-59) with 13 residues. However, the positive results by the peptides of B46-59 and B47-60 make it impossible to decide whether the C-terminal amino acids of the sequence B46-59 take also a part in the binding activity to IgE antibodies.

Peptide scanning with murine MoAbs II4G9 and II2F3

The reactivity of each MoAb with nine overlapping peptides (B1-19 to B121-137) was assessed. Both MoAbs were found to react strongly to peptide B121-137 (Fig. 5a). A lower reactivity was also observed with peptide B76-94. A detailed mapping using the six overlapping dodecapeptides showed that the minimal epitope of MoAbs contained amino acid residues of Q T K I L A K V F Y. Again, the tyrosine in position 134 (Y^{134}) was necessary for the binding of both MoAbs. Moreover, lacking of Q^{125} resulted in a dramatic decrease of binding to II4G9 and a complete loss of recognition by II2F3 (Fig. 5b).

Peptide scanning with rabbit polyclonal antibodies (IgG)

The binding ability of nine peptides (B1-19 to B121-137) to three polyclonal rabbit antisera raised against *Hev b 1* (RH) and against protein extracts from latex sap from Thailand (Rt) and Malaysia (Rm) is shown in Fig. 6a. All three antisera were found to have a similar binding pattern to the

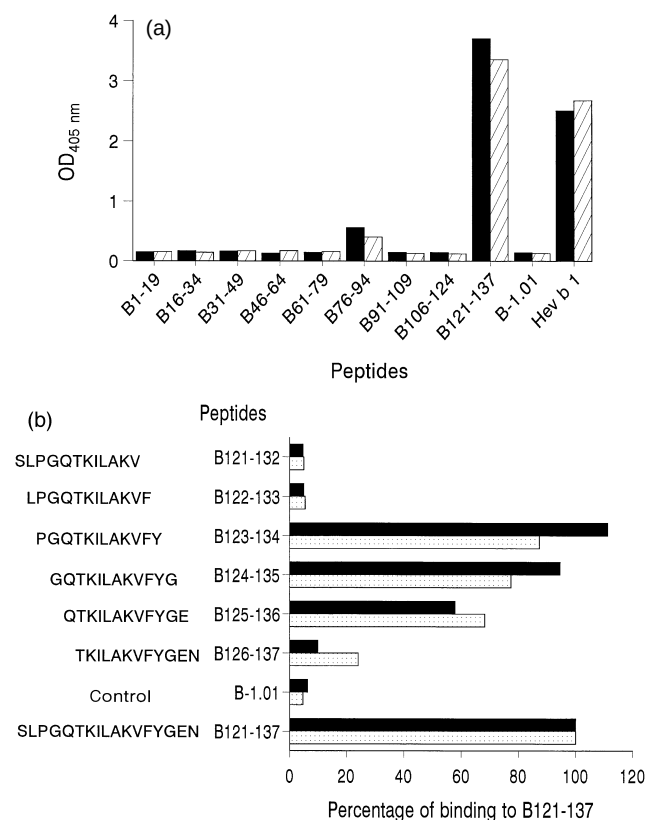


Fig. 5. Reactions of MoAbs to Hev b 1 and Hev b 1-peptides. (a) Binding of MoAbs II4G9 and II2F3 to nine overlapping biotinylated peptides derived from Hev b 1 (see Table 1 for peptide sequences); Solid phase-bound peptide B-1.01 and the Hev b 1 were used as controls. (b) Peptide scan with biotinylated dodecapeptides derived from the C-terminal segment of Hev b 1 (121-137) for binding to II2F3 and II4G9 MoAbs as measured with ELISA. Results are expressed as percentage of binding to the full-length peptide B121-137. (a) ■, MoAb II4G9. ▨, MoAb II2F3. (b) ■, MoAbs II2F3. ▨, MoAb II4G9.

Hev b 1 peptides. They reacted strongly to peptides B31-49, B46-64, B91-109 and B121-137. Sera of R_M and R_T were also reactive to peptide B61-79, whereas the rabbit serum against Hev b 1 (R_H) showed only a slight binding ability to peptide B61-79. A lower reactivity to B76-94 was also observed by serum R_M. A fine mapping with six overlapping tetradecapeptides derived from B46-64 was performed to find out the essential amino acid residues in this region. The results presented in Fig. 6b show that all three rabbit antisera recognized the peptides B46-59 and B47-60 indicating that one minimal epitope to polyclonal rabbit sera is located in the segment with amino acids P G V D I I E G P V K N V (47-59). R_T was also found to be able to react to peptide B50-63. Interestingly, all six peptides were recognized by antiserum R_H raised against Hev b 1. This phenomenon

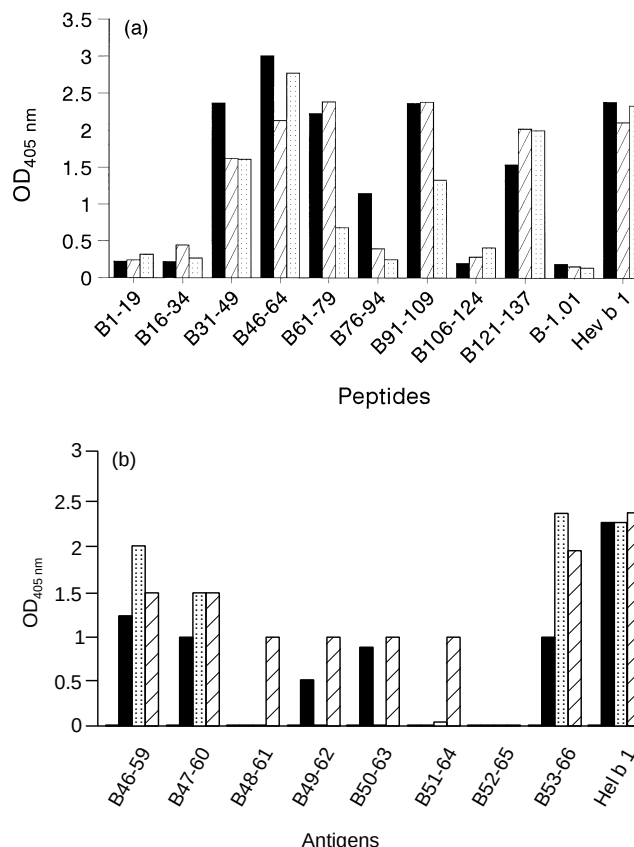


Fig. 6. Antibody-binding profiles of three rabbit antisera to Hev b 1-peptides. (a) Antigenicity of nine biotinylated peptides from Hev b 1. (For peptide sequences, see Table 1). Sera: ■, R_M. ▨, R_T. ▤, R_H. (b) The binding ability of rabbit polyclonal antibodies to peptides representing the B46-64 sequence of Hev b 1. Six tetradecapeptides shown in Fig 4b were used in the assay. Antiserum R_H was raised against Hev b 1; antisera R_T and R_M were raised against protein extracts of latex sap from Thailand and Malaysia. Solid phase-bound Hev b 1 and Chi t 1.01 peptide (B-1.01) were used as controls. R_N is a negative rabbit serum.

indicates that the minimal epitope to R_H in this region is involved in amino acid residues of 51-59 (I I E G P V K N V), or there might be two or more epitopes present in peptide B46-64 reacting to the polyclonal rabbit serum. In addition, an IgG binding determinant of 13 residues can be resolved to amino acids 50-62 (D I I E G P V K N V A V P) based on the fact that peptides B49-62 and B50-63 are recognized by serum R_T.

Discussion

For the first time, the allergenic and antigenic determinants of a well defined latex allergen have been elucidated by

mapping the whole molecule and localizing the IgE and IgG binding sequences using synthetic overlapping peptides.

The first effort to identify continuous allergenic determinants using synthetic overlapping peptides was made by Atassi and Atassi [23]. Since that time, several reports on allergenic epitopes using peptides, either as free peptide or attached to polyethylene pins, have been published [24–26]. However, because of the small amount of IgE relative to the other immunoglobulin isotypes (IgG, IgA and IgM) present in serum and of the lower binding affinity of small peptides in comparison to the whole protein, it is often difficult to obtain reproducible and quantitative binding using a direct binding ELISA in IgE immunoassays [25,27]. For these reasons, the synthetic peptides of *Hev b 1* were prepared by biotinylation at the N-terminus via a 6-aminohexanoic acid molecule as spacer and followed by a reaction with immobilized streptavidin on microtitre plates prior to reaction with the patient sera. In this way, an optimal immobilization of the peptides can be achieved regardless of the differences of peptide sequences. The strong biotin/streptavidin binding ensures that no leaching of the bound peptides occurs while reaction with the antibody takes place. Furthermore, the peptides thus immobilized on solid phase are able to keep their native configuration, and the binding reaction of peptide to antibody is conducted without steric hindrance due to the spacer molecule between the peptide chain and biotin molecule.

In addition, the applied test system may also influence the results. The results in Fig. 1 show that the positive IgE binding reaction in EAST could turn out to be negative in ELISA (see sera numbers 3056 and 3147). This is possible because of the different immobilization of allergens on the two solid phases and/or the different spatial configuration of the allergen and relevant epitopes in ELISA and EAST. In the EAST system the *Hev b 1* protein is covalently bound, mainly through the amino groups, to BrCN- activated cellulose disks, while binding of *Hev b 1* to microtitre plate in the ELISA system is based on a non-covalent hydrophobic interaction between the protein and the solid phase. Seven human serum samples were used to test the binding ability of nine biotinylated peptides covering the whole *Hev b 1* sequence. The obtained results, both in competitive and direct ELISA, indicated that the IgE binding sites could be well defined by using biotinylated peptides. As expected, the IgE epitopes differed from patient to patient, probably due to the allergen presentation to the immune system, e.g. the way sensitization to *Hev b 1* occurred. The most allergenic/antigenic portions of the *Hev b 1* allergen are the C-terminal region and the region with amino acid residues of 31–64.

The fact that the binding activity of serum 3275 to peptides B46–64 and B121–137 could be completely

blocked by 1 µg *Hev b 1* protein and that only 45% of the IgE binding to *Hev b 1* were inhibited by using a mixture containing 10 µg of H46–64 and H121–137 each suggests that there may exist other epitope(s) in the protein. As shown in Fig. 2a, serum 3275 did not react with any other peptide than B46–64 and B121–137 in direct ELISA indicating that the additional epitopes are either conformational or they are linear, but missed by being cut at a critical point in peptide synthesis.

To delineate the IgE/IgG antigenic epitopes more precisely, we also employed a panel of overlapping peptides with one amino acid offset from the C-terminal segment and the segment of B46–64. A noteworthy result of our study was the demonstration that the minimal sequence of IgE and IgG MoAbs binding in the C-terminal region of *Hev b 1* is almost identical except for one amino acid residue. The antibody binding site was narrowed to the essential sequence of G Q T K I L A K V F Y (124–134) for IgE (serum 3275) and Q T K I L A K V F Y (125–134) for murine monoclonal IgG.

The presence of tyrosine residue in position 134 (Y¹³⁴) played a key role in antibody binding. Exclusion of Y¹³⁴ in the sequence resulted in a complete loss of binding activity to both IgE and IgG MoAbs. Unlike Y¹³⁴, the residues of P¹²³ G¹²⁴ Q¹²⁵ appeared to share equally in antibody binding. Successive omission of the three residues from the N-terminus of the peptide resulted in a gradual reduction of binding capacity to antibodies (Figs 4a and 5b). More information about the contribution to antibody binding by individual residues within this epitope will be obtained by comparing the antibody binding ability of analogs containing single amino acid substitution.

Sequence similarity of the antibody binding sites to IgE and polyclonal rabbit sera was also observed by fine mapping of the segment B46–64 (Figs 4b and 6b). IgE antibodies in sera 3275 and 2227 as well as all three rabbit antisera strongly reacted with the peptides B46–59 and B47–60 indicating that one minimal epitope to IgE and polyclonal rabbit IgG is located in the segment with amino acids P G V D I I E G P V K N V (47–59). These results, however, do not permit to determine whether those amino acids in the C-terminus, i.e. V⁵⁹, N⁵⁸ and K⁵⁷, are also relevant with the binding activity to antibodies.

Since the ammoniated latex sap is the most important raw material for glove manufacturing, two rabbits were immunized with two protein extracts from these saps of different origin. In comparison, one rabbit was injected with purified *Hev b 1* protein. The results of binding studies indicate that the reaction pattern of nine synthetic *Hev b 1* peptides B1–19 to B121–137 is very similar within three rabbit sera, regardless whether antibodies were raised

to whole latex proteins or only to purified Hev b 1. This suggests that Hev b 1 is a potent immunogen in latex protein extracts. Since Hev b 1 was found in fresh latex sap to be tightly bound to rubber particles and to be absent in the latex solution phase [15], our results indicate that Hev b 1 can be partially released in ammonia solution. The comparison of these binding regions of Hev b 1 with that bound to IgE and monoclonal IgG antibodies shows that only the C-terminal peptide B121-137 is recognized by all antibodies used in this study, and the investigated IgE-binding peptides (B31-49, B46-64, B91-109 and B121-137) are involved in peptides recognized by polyclonal rabbit antisera. In addition, the sea R_T and R_M also react strongly with peptide B61-79.

Finally, the localization of the epitopes in Hev b 1 should be mentioned. The two main domains that contain epitopes are in segments covered by peptides 46-64 and 121-137. Both epitopes detected by fine mapping in these segments are located just outside the alpha-helices III and IV according to the prediction of the secondary structure of Hev b 1 by Dennis *et al.* [12] based on the Chou-Fasman method [28].

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References

- 1 Turjanmaa K. Incidence of immediate allergy to latex gloves in hospital personnel. *Contact Dermatitis* 1987; 17:270-5.
- 2 Lagier F, Vervloet D, Lhermet I, Poyen D, Charpin D. Prevalence of latex allergy in operating room nurses. *J Allergy Clin Immunol* 1992; 90:319-22.
- 3 Jaeger D, Kleinhans D, Czuppon AB, Baur X. Latex specific proteins causing immediate-type cutaneous, nasal, bronchial, and systemic reactions. *J Allergy Clin Immunol* 1992; 89:673-8.
- 4 Tomazic VJ, Withrow TJ, Fisher BR, Dillard SF. Latex-associated allergies and anaphylactic reactions. *Clinical Immunology and Immunopathology* 1992; 64:89-97.
- 5 Slater JE. Latex allergy. *J Allergy Clin Immunol* 1994; 94:139-49.
- 6 Slater JE, Mostello LA, Shaer C. Rubber-specific IgE in children with spina bifida. *J Urol* 1991; 146:578-9.
- 7 Tosi LL, Slater JE, Shaer C, Mostello LA. Latex allergy in spina bifida patients: prevalence and surgical implications. *J Pediatr Orthop* 1993; 13:709-12.
- 8 Alenius H, Palosuo T, Kelly K *et al.* IgE reactivity to 14-kD and 27-kD natural rubber proteins in latex-allergic children with spina bifida and other congenital anomalies. *Int Arch Allergy Immunol* 1993; 102:61-6.
- 9 Beezhold DH, Sussman GL, Kostyal DA, Chang N-S. Identification of a 46-kD latex protein allergen in health care workers. *Clin Exp Immunol* 1994; 98:408-13.
- 10 Broekaert I, Lee HI, Kush A, Chua NH, Raikhel N. Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc Natl Acad Sci USA* 1990; 87:7633-7.
- 11 Jekel PA, Bernard J, Hartmann H, Beintema JJ. The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. *Eur J Biochem* 1991; 200:123-30.
- 12 Dennis MS, Henzel WJ, Kohr W, Light DR. Amino acid sequence of rubber elongation factor protein associated with rubber particles in *Hevea* latex. *J Biol Chem* 1989; 264:18618-26.
- 13 Czuppon AB, Chen Z, Rennert S *et al.* The rubber elongation factor of rubber trees (*Hevea brasiliensis*) is the major allergen in latex. *J Allergy Clin Immunol* 1993; 92:690-7.
- 14 WHO/IUS Allergen Nomenclature Subcommittee World Health Organization, Geneva, Switzerland, Allergen nomenclature. *Clin Exp Allergy* 1995; 25:27-37.
- 15 Dennis MS, Light DR. Rubber elongation factor from *Hevea brasiliensis*: identification, characterization and role in rubber biosynthesis. *J Biol Chem* 1989; 264:18608-17.
- 16 Alenius H, Turjanmaa K, Palosuo T, Mäkinen-Kiljunen S, Reunala T. Surgical latex glove allergy: Characterization of rubber protein allergens by immunoblotting. *Int Arch Allergy Appl Immunol* 1991; 96:376-80.
- 17 Slater JE, Chhabra SK. Latex antigens. *J Allergy Clin Immunol* 1992; 89:673-8.
- 18 Meeropol E, Kelleher R, Bell S, Leger A. Allergic reactions to rubber in patients with myelodysplasia [Letter]. *N Engl J Med* 1990; 323:1072.
- 19 Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256:495-7.
- 20 Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Peptide Protein Res* 1990; 35:161-214.
- 21 van Kampen V, Becker W-M, Chen Z *et al.* Analysis of B-cell epitopes in the N-terminal region of Chi t I component III using monoclonal antibodies. *Mol Immunol* 1994; 31:1133-40.
- 22 Rink H. Solid phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett* 1987; 28:3787-90.
- 23 Atassi H, Atassi MZ. Localization of the continuous allergenic sites of ragweed allergen Ra3 by a comprehensive synthetic strategy. *FEBS Lett* 1985; 188:96-100.

- 24 Walsh BJ, Howden MEH. A method for detection of IgE binding sequences of allergens based on a modification of epitope mapping. *J Immunol Meth* 1989; 121:275–9.
- 25 Stewart GA, Armstrong L, Krska K et al. Epitope mapping analysis of the major mite allergens using synthetic peptides. In: 'Epitopes of Atopic Allergens'. Edited by Sehon AH, Kraft D and Kunkel G. The UCB Institute of Allergy, Brussels, Belgium, 1990: 41–7.
- 26 Ball G, Shelton MJ, Walsh BJ et al. A major continuous allergenic epitope of bovine B-lactoglobulin recognized by human IgE binding. *Clin Exp Allergy* 1994; 24: 758–64.
- 27 Zhang L, Olsen E, Kisil FT et al. Mapping of antibody binding epitopes of a recombinant Poa p IX allergen. *Mol Immunol* 1992; 29:1383–9.
- 28 Chou PY, Fasman GD. Empirical predictions of protein conformation (Review). *Ann Rev Biochem* 1978; 47:251–76.