

Original article

Characterization of B- and T-cell responses and HLA-DR4 binding motifs of the latex allergen Hev b 6.01 (prohevein) and its post-transcriptionally formed proteins Hev b 6.02 and Hev b 6.03

Background: Multiple immunoglobulin E (IgE)-binding proteins in natural rubber latex extracts have been identified. In the case of Hev b 6 a differentiation was made between the precursor protein prohevein (Hev b 6.01) and its two post-transcriptionally formed proteins, the N-terminal hevein (Hev b 6.02) and the C-terminal domain (Hev b 6.03). All three components act as independent allergens. The aim of this study was a detailed analysis of the T-cell responses and the IgE-binding capacity of Hev b 6.01, Hev b 6.02 and Hev b 6.03 by using these allergens as recombinant maltose-binding fusion (MBP) proteins and the usage of synthetic modified hevein peptides.

Methods: Latex-allergic health care workers (HCWs) suffering from rhinitis, conjunctivitis, contact urticaria and/or asthma with increased specific IgE-antibodies to latex were tested for their IgE-binding capacity and T-cell reactivity (by proliferation response) to the recombinant MBP-rHev b 6.01, MBP-rHev b 6.02, MBP-rHev b 6.03, to native Hev b 6.02, to modified hevein peptides and wheat germ agglutinin (WGA). For testing of the human leucocyte antigen (HLA) class II restriction of MBP-rHev b 6.01 induced peripheral blood mononuclear cell (PBMC) responses, monoclonal antibodies against HLA-DR, HLA-DP or HLA-DQ were added.

Results: Seventeen of 18 (94%) serum samples from latex-allergic HCWs had increased levels of specific IgE to MBP-rHev b 6.01, 16 (89%) to MBP-rHev b 6.02 and 13 (72%) to MBP-rHev b 6.03. A significant difference existed between the specific IgE-values of MBP-rHev b 6.02 and MBP-rHev b 6.03 ($P < 0.01$). Proliferation responses of PBMC of the same 18 latex-allergic patients were positive for MBP-rHev b 6.01 and MBP-rHev b 6.03 in 83 and 67% of the tested PBMC suspension, whereas the proliferation responses induced with MBP-rHev b 6.02 or native Hev b 6.02 were very low (5.6 and 22.2%). Sera from nine additional latex-allergic patients showed specific IgE binding to the native Hev b 6.02, but none of these sera showed specific IgE binding to the modified Hev b 6.02-peptides [whereby all eight cysteine residues were substituted by serine (C → S) or by alanine (C → A)]. Proliferation responses induced by the modified Hev b 6.02 peptides were not significantly different from that induced by Hev b 6.02. Potential HLA-DR4Dw4(DRB1*0401)-restricted T-cell epitopes of Hev b 6.01 predicted by two computer algorithms were only found in the Hev b 6.03-part of Hev b 6.01.

Conclusion: In the Hev b 6.01 precursor the regions responsible for IgE binding and those for inducing the T-cell proliferation responses are settled in different parts of the protein. The Hev b 6.02 domain is responsible for IgE binding and carries discontinuous B-cell epitopes whereas Hev b 6.03 is a better inducer of a proliferation response and contains HLA-DR4-binding motifs.

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Natural rubber latex (NRL) allergy is a 'new' disease whose prevalence reached epidemic proportions in highly exposed populations during the last decade and has developed to be a factor of great importance worldwide

for the diagnosis of intolerance reactions to gloves, occupational asthma and anaphylactic reactions. Exposure to the latex allergens mainly occurs through the respiratory mucosa (e.g. by inhalation of latex-containing

glove powder) and the percutaneous route and may result in sensitization involving specific immunoglobulin E (IgE) antibody binding and subsequently the development of clinically manifested latex allergy (1–4). Much work has been carried out concerning the characterization, purification and cloning of latex allergens in recent years. So far, 16 latex proteins have achieved designation as an allergen (Hev b 1–13) by the International Union of Immunological Sciences, although several more latex proteins with IgE-binding capacity have been identified (5).

Recent studies have shown that Hev b 6.01 (20 kDa; prohevein), a defence-related protein of the rubber tree, is one of the most important latex allergens, especially in health care workers (HCWs) (6). It harbours two allergenic components, the N-terminal hevein (4.7 kDa; Hev b 6.02) and the C-terminal Hev b 6.03 (14 kDa), which are generated after post-transcriptional processing. Hevein, consisting of 43 amino acid residues, is released from the luteoids when the cells are damaged and interacts with a highly glycosylated receptor protein in the envelope around the rubber particles resulting in the coagulation of latex (7). Furthermore, hevein displays strong sequence homologies to several chitin-binding proteins of the N-acetyl glucosamine (GlcNAc) type from wheat [including wheat germ agglutinin (WGA)], barley, and rice (8, 9), whereas Hev b 6.03 displays homologies with potato stress proteins WIN1 and WIN2 (10).

Prohevein-specific IgE antibodies were detected in about 80% of latex-allergic patients. Both hevein and the C-domain were studied for their IgE-binding capacity. While IgE antibodies against purified hevein are found in 70–80% of latex-allergic patients, only 20–30% reacted with the C-domain of prohevein, indicating that hevein is the primary cause of prohevein allergenicity (11). The importance of hevein as a major latex allergen was confirmed by Chen et al. (12). This study revealed that more than 80% of latex-allergic HCWs with positive responses to latex extract by *in vivo* skin prick tests also had positive reactions to hevein. However, less than 30% of the spina bifida patients with latex allergy showed IgE antibodies to hevein in their sera, indicating that for different patient groups the sensitization to latex may be elicited by different allergens retained during the latex allergen exposure. A detailed study of Banerjee et al. (13) demonstrated that the major linear IgE epitopes of prohevein appeared to localize predominantly in the N-domain (hevein molecule) and in the N-terminal region of the C-domain. Using the chimera-based epitope mapping strategy by Karisola et al. (14) the results suggest that the IgE binding ability of hevein is essentially determined by its N-terminal and C-terminal regions and that major IgE-binding epitopes of hevein are conformational. In addition, sensitization to NRL hevein (Hev b 6.02) was described as a main cause of IgE reactivity to class I chitinases in latex avocado and latex banana cross-reactivity (15, 16). Prohevein which is available in

recombinant form (17) as well as hevein are essential reagents in the diagnosis of latex allergy and may be potential candidates for immunotherapy.

A prerequisite for the understanding of the sensitization process or to design specific immunotherapy strategies in the case of latex allergy is the knowledge of the immunoreactivity of latex allergens at the level of specific T cells. The trimolecular interactions between the class II major histocompatibility complex (MHC) molecule, peptide and the T-cell receptor (TCR) provides the structural basis for the antigen/allergen specificity for T-cell recognition. Accumulation of analytic data on class II-binding peptides as well as the crystallography of human leucocyte antigen (HLA) molecules (18, 19) have led to the development of computer-assisted algorithms, by which the sequences of large proteins can be scanned to predict individual peptides that might bind to distinct MHC molecules (20–23). However, only few studies so far have been conducted to characterize the T-cell responses in latex allergy (24–26).

Investigations of our group (27, 28) concerning the relationship between HLA class II alleles and the IgE-specific immune response to Hev b 6.02 demonstrated increased phenotype frequencies for DR4 and DQ8 among latex-sensitized HCWs with specific IgE-binding to Hev b 6.02. These data suggest DR4 and DQ8 to be operating jointly as susceptibility factor for the allergy to hevein in HCWs.

As a part of our efforts to study the possible pathomechanisms of latex type I allergy, we investigated and compared the IgE-binding capacity and T-cell reactivity to the three recombinant allergens of Hev b 6, to native Hev b 6.02 and to two modified hevein peptides. Moreover, we performed HLA-D typing and analyzed MHC restriction patterns of Hev b 6.01-activated peripheral blood lymphocytes of latex-allergic HCWs. In addition, we predicted potential HLA-DR4Dw4-peptide binding motifs and T-cell epitopes by using specific algorithms.

Material and methods

Patients

Sera and peripheral blood mononuclear cells (PBMC) were collected from 27 latex-allergic hospital employees (Table 1; Pa1–Pa27), especially HCWs (nurses) and doctors, aged between 22 and 61 years (median: 29 years); 26 females and one male; whereby 10 were current smokers, 12 nonsmokers and five ex-smokers. They were diagnosed as having latex-related allergic symptoms like urticaria, rhinitis, conjunctivitis and/or asthma. None of the patients was on immunotherapy and/or corticosteroid therapy. All 27 subjects had positive latex skin prick test results and latex-related specific IgE antibodies (>0.35 kU/l; measured by the CAP-system) (median: 6.71 kU/l; range 0.47–47.3 kU/l). Twenty-three of the 27 latex-sensitized patients were regarded as atopics due to their case history and positive skin prick test reaction to at least two common allergens. Total serum IgE levels of patients ranged between 9.9 and

Table 1. Patients' characteristics

Patient	Total IgE [kU/l]	Latex-specific IgE [kU/l]	Symptoms	HLA-D types	
				DRB1	DQB1
Pa1	160	14.8	C, R, U	0401 1500	0302 0602
Pa2	419	15.0	C, R, U, A	0401 0600	0302 0603-9
Pa3	>2000	28.6	C, R, U	0401 0100	0302 0501
Pa4	48.8	3.07	C, R, U, A	0401 1600	0302 0502
Pa5	65.5	13.5	A, R, C, Ec	0401 0300	0302 0201
Pa6	36.2	3.39	A, U, Ec	0401 0701	0302 0201
Pa7	88.4	5.86	C, R, U	0402 0701	0302 0201
Pa8	35.4	0.47	A, R, U, Ec	0401 1100	0301 0301
Pa9	174.0	11.1	R, K, U	0401 0100	0302 0501
Pa10	856	6.72	C, R, U	1200 0701	0301 0201
Pa11	603	11.9	C, U	0401 0100	0302 0501
Pa12	267	35.8	C, R	n.d.	
Pa13	72.9	1.86	A, C, R	0600 1500	0301 0602
Pa14	157	2.15	A, R, C	0404 0412	0302 0302
Pa15	>2000	19.7	C, R, U	0308 1300	0201 0301
Pa16	1163	6.71	A, R, C, U	1100 1300	0301 0305
Pa17	678	7.19	n.d.	n.d.	
Pa18	668	7.09	n.d.	n.d.	
Pa19	170	8.41	A, C, R	0701 1500	0303 0602
Pa20	46.5	1.01	U, C, R	0400 0600	0302 0603-9
Pa21	883	47.3	U, R	0600 1600	0603-9 1600
Pa22	31.5	0.8	A, U, C, R	0600 1500	0603-9 0602
Pa23	291.0	4.91	A, U, C, R	1100 1100	0403 0300
Pa24	247.0	0.94	A, U, C, R	1100 1100	0301
Pa25	36.2	2.93	A, U, C, R	n.d.	
Pa26	9.89	2.55	U, C, R	1100 1600	0301
Pa27	19.1	1.65	n.d.	0400 1100	0301 0302

A, asthma; C, conjunctivitis; Ec, eczema; R, rhinitis; U, urticaria; n.d., no data available.

above 2000 kU/l (median: 170 kU/l). In addition, PBMC of five healthy control subjects (females) without latex sensitization and atopy were used in selected experiments.

Preparation of the antigens

We used recombinant allergens (rHev b 6.01, rHev b 6.02, rHev b 6.03) as maltose-binding fusion proteins (MBP) with MBP as negative control, the native allergen Hev b 6.02, two synthetic modified hevein peptides (C → S, C → A) and WGA (Sigma, Deisenhofen, Germany) in some of the experiments.

cDNA synthesis and cloning of Hev b 6.01, 6.02, 6.03. Total RNA was extracted from leaves of *Hevea brasiliensis* clone RRIM 600 (kindly provided by the Institut für Nutzpflanzenkunde gemäßiger, subtropischer und tropischer Regionen, Witzenhausen, Germany). Leaves (1 g) were shock frozen in liquid nitrogen, grounded in a mortar and the RNA isolation was performed according to the Total RNA protocol of Qiagen (Hilden, Germany). Five micrograms of the *Hevea* RNA was used for prohevein cDNA synthesis with prohevein specific primers and PCR conditions as described by Rozynek et al. (17). The resulting PCR product allows the ligation into *XmnI/EcoRI* digested fusion vector pMAL-p2 (New England Biolabs, Frankfurt, Germany) after treatment with Klenow enzyme and *EcoRI* digest.

For the synthesis of rHev b 6.02 (hevein) and rHev b 6.03 (C-domain) the Hev b 6.01 DNA containing clone pDHPROH1-2 was used as template for subsequent PCR reactions with primer pairs:

PROHEV_5-1: 5'-GAGCAATGTGGTCGGCAAG-3'
HEV_int-3': 5'-ACCGTCGACTTAGTCTTTGCAATTGCTTT
GGC-3' to obtain Hev b 6.02 as well as
PROH-C_int-5': 5'-AGCGGCGAAGGTGTTGGT-3'
PROHEV_3-1: 5'-CAGAAATCTTAATTAATTACTGATGAT
TTCATAACGG-3 for Hev b 6.03.

After treatment with Klenow enzyme the Hev b 6.02 PCR product was cleaved with *SaI* and the Hev b 6.03 PCR product with *EcoRI*. After purification, both amplification products were subcloned into pMAL-p2.

Expression and purification of r-Hev b 6.01, 6.02, 6.03. Cultures in logarithmic stage of the *Escherichia coli* DH5α containing pMAL-p2/Hev b 6.0X were induced by addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 0.3 mM and grown for 60 h at 30–32°C. The bacteria were harvested by centrifugation for 20 min/4000 g at 4°C. The supernatant was discarded and the cells were resuspended in 10 ml column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) per gram of wet cell weight and subsequently frozen overnight at –20°C. After thawing in a water bath, the cells were sonicated in an ice-water bath using a Branson sonifier model 250 (Branson, Danburg, CT, USA) with pulses of 15 s/intensity set to eight for a total time of 3.5 min and centrifuged at 9000 g for 30 min. The supernatant was diluted 1 + 4 with degassed column buffer and loaded onto a Pharmacia XK 16/20 (Amersham Biosciences Europe GmbH, Freiburg, Germany) column packed with amylose beads (bed volume 10 ml). After intensive washing, bound fusion protein was eluted with 10 mM maltose in column buffer. The protein concentration was determined by ESL-assay (Roche, Mannheim, Germany). The OD₂₈₀-difference (optical density) between flow through and column buffer was <0.005.

Preparation of hevein and synthesis of the two modified hevein peptides. Hevein has been purified from fresh *Hevea* latex using ultrafiltration and liquid chromatography on-line coupled electrospray mass spectrometry as described by Chen et al. (12). Two synthetic modified hevein peptides (1–44; C → S; all eight cysteine residues were substituted by serine; C → A: all eight cysteine residues were substituted by alanine) were synthesized stepwise on an Applied Biosystem 432A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using Fmoc strategy as previously described by Chen et al. (29). Peptides were analyzed for their purity by reversed-phase high-performance liquid chromatography (C18 column: 4 × 250 mm) and identified by IonSpray mass spectroscopy on an API III triple-quadrupole mass spectrometer with an IonSpray interface (Sciex, Toronto, Canada).

Antigen-induced PBMC stimulation

Peripheral blood mononuclear cells were separated from the heparinized peripheral blood of the latex-allergic donors by Ficoll–Hypaque gradient centrifugation and adjusted to 1 × 10⁶/ml in RPMI-1640 (Gibco, Eggenstein, Germany) conditioned medium supplemented with 2 mM Glutamax I (Gibco), penicillin/streptomycin and 10% heat-inactivated pooled human AB-sera (Bavarian Red Cross, Munich, Germany). Isolated PBMC were stored at –196°C until use.

For the proliferation assay and for all other experiments, frozen and then thawed PBMC were used. Thawed PBMC were washed extensively for three times and resuspended in complete RPMI-medium. The PBMC proliferation assays

were set up in six replicates in 96-well U-bottom trays in a 200 µl volume generally at a concentration of 5×10^4 cells/well. The native and the recombinant allergens as well as the MBP were added in different concentrations (0.05–10 µg/ml) to the wells at the beginning of the cultures that were performed in a humidified atmosphere at 37°C containing 5% CO₂ for 5 days. Negative controls of cells alone in the presence of RPMI-1640 medium and positive controls of cells incubated with phytohaemagglutinin (7.5 µg/ml) were included in each assay. For the final 16 h, 37 kBq of ³[H]-labelled thymidine-methyl (Amersham Biosciences Europe GmbH) were added to each well, and incorporated radioactivity was measured by liquid scintillation spectrometry.

The stimulation index (SI) was calculated as the ratio of the mean cpm (counts per minute) obtained in the six similar cultures with allergens and that one obtained in the allergen-free culture (RPMI-control). We tentatively set cut-off lines determining positive and negative responses at SI = 2.5 for the latex allergen-driven responses (24, 25). The standard deviation of the six determinations was 10–25%.

HLA restriction study

For testing of the HLA class II restriction of MBP-rHev b 6.01-induced PBMC response, blocking monoclonal antibodies against HLA-DR, HLA-DP or HLA-DQ (Becton Dickinson, Heidelberg, Germany) were added (200 ng/ml) to the PBMC (5×10^5 /ml). PBMC incubated in the absence of the antibodies served as control. After incubation for 2 h at 37°C, the cells were washed two times with medium and stimulated in six parallel determinations (5×10^4 /well) with MBP-rHev b 6.01 (5 µg/ml) or medium for 5 days. The ³[H]-labelled thymidine incorporation of PBMC in the absence and presence of different HLA class II specific monoclonal antibodies was compared and expressed as percentage inhibition. Only values of inhibition higher than 25% were regarded as relevant inhibition of the proliferation response.

HLA class II typing

For HLA class II typing, genomic DNA was extracted from frozen white blood cells using the DNA Easy-Prep kit (Lifecodes, Herentals, Belgium) as recommended by the supplier. DNA typing was performed with Histo Type DNA-DQ and -DR kits (BAG, Lich, Germany) based on the PCR amplification of 24 sequence-specific primer pairs (SSP) for DRB1 and 16 specific primer pairs for DQB1 according to the supplier's protocol. These methods enable the identification of the broad DRB1 and DQB1 alleles equivalent to the serological specification and in some cases a further subdivision. DR4 subtyping was carried out with the Dynal DRB1*04 PCR-SSP subtyping kit allowing the determination of alleles DRB1*0401-DRB1*0423.

Detection of total and latex-specific IgE antibodies

All sera of the subjects were tested for their concentration of specific IgE to latex allergens and rHev b 6.01 by the CAP system (Pharmacia Diagnostics, Uppsala, Sweden). The results were expressed as kU/l according to the standard curve by Pharmacia Diagnostics. Specific IgE values to rHev b 6.02 and rHev b 6.03 were measured using an enzyme-linked allergosorbent test (EAST) with MBP-rHev b 6.02 and MBP-rHev b 6.03 coupled to CNBr-activated paper discs in our laboratory. After washing and treatment with diethylamine, the allergen discs were incubated with 50 µl of each patient serum for 3 h at room temperature. A Phadezym RAST test kit (Pharmacia Diagnostics) was used to detect specific IgE antibodies. Values greater than 0.35 kU/l were regarded as positive.

Total IgE concentration in sera of the subjects was determined by using the Pharmacia CAP System (Pharmacia Diagnostics), according to the manufacturer's instruction. The measurement range was 2–2000 kU/l.

Predicting HLA-DR binding motifs

Potential HLA-DR4Dw4 restricted T-cell epitopes were predicted according to published DR4Dw4-binding motifs (reviewed in 22, 30–32), using the computer program PROTSHELL (the program PROTSHELL is available from Prof. Dr Claude Muller; Laboratoire National de Santé, Boite Postale 1102, L-1011 Luxembourg).

In addition, the computer algorithm TEPITOPE available from Juergen Hammer (<http://www.vaccinome.com>) for the prediction of human MHC-class-II restricted epitopes was used (33).

Statistics

The Mann–Whitney *U*-test was used to compare the IgE antibody levels with MBP-rHev b 6.01, MBP-rHev b 6.02 and MBP-rHev b 6.03 as well as the proliferation response (SI values) in the patient group. Quantitative data were expressed as the mean values ± standard deviation (SD). Probability (*P*) values below 0.05 were considered significant.

Results

MBP-rHev b 6.01, MBP-rHev b 6.02 and MBP-rHev b 6.03 specific IgE antibodies in the patient sera

All 18 serum samples of latex-allergic HCWs (Pa1–Pa18) had latex-specific IgE antibodies (> 0.35 kU/l) (Table 1). Seventeen of 18 sera (94%) displayed specific IgE antibodies to MBP-rHev b 6.01, 16 (89%) to MBP-rHev b 6.02 and 13 (72%) to MBP-rHev b 6.03 (Fig. 1). A significant difference existed between the specific

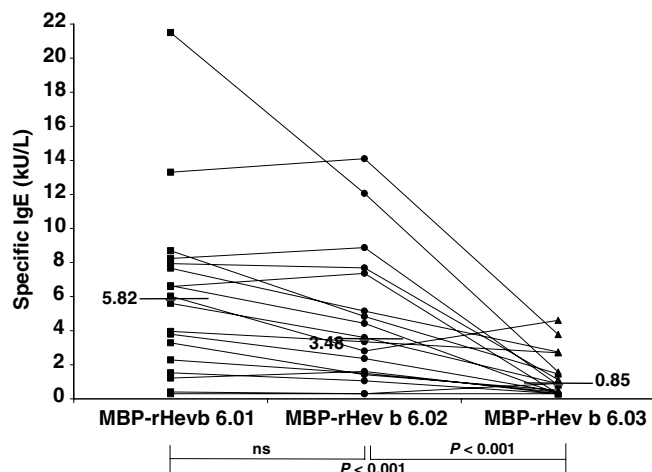


Figure 1. Specific IgE response to MBP-rHev b 6.01, MBP-rHev b 6.02 and MBP-rHev b 6.03. The median values are shown as horizontal lines.

IgE-values of MBP-rHev b 6.02 and MBP-rHev b 6.03 ($P < 0.001$). In addition, most of the latex-allergic patients had only low specific IgE levels to MBP-rHev b 6.03 (median: 0.85 kU/l; Fig. 1). Only four of the 13 (31%) MBP-rHev b 6.03-seropositive patients had an MBP-rHev b 6.03-specific IgE value > 2 kU/l. In contrast, 12 of the 16 (75%) MBP-rHev b 6.02-seropositive patients had an MBP-rHev b 6.02-specific IgE value > 2 kU/l (median: 3.48 kU/l). In all of the 16 serum samples the concentration of anti-MBP-rHev b 6.02 IgE antibodies was > 0.7 kU/l. A significant correlation existed between specific IgE values to MBP-rHev b 6.02 and MBP-rHev b 6.01 ($r = 0.889$; $P < 0.0001$). MBP coupled as control antigen to ImmunoCAP revealed no specific IgE-binding (≤ 0.35 kU/l) in all cases (data not shown).

Proliferation responses induced by MBP-rHev b 6.01, MBP-rHev b 6.02, MBP-rHev b 6.03, Hev b 6.02 and WGA

Peripheral blood mononuclear cells obtained from the same 18 latex-allergic patients (sera tested above for MBP-rHev b 6.01, MBP-rHev b 6.02 and MBP-rHev b 6.03-specific IgE; Pa1-Pa18) were stimulated with the recombinant latex allergens MBP-rHev b 6.01, MBP-rHev b 6.02, MBP-rHev b 6.03 and MBP (negative control) in different concentrations (Fig. 2). Comparison of the SI values indicated significant differences between proliferation responses induced by MBP-rHev b 6.01 and MBP-rHev b 6.02 ($P < 0.001$) as well as by MBP-rHev b 6.03 and MBP-rHev b 6.02 ($P < 0.01$). MBP-rHev b 6.01 and MBP-rHev b 6.03 induced positive proliferation responses [$SI \geq 2.5$; as described in previous publications (24, 25)] in 83% (median SI for stimulation with MBP-rHev b 6.01: 3.9) and 67% (median SI for stimulation with MBP-rHev b 6.03: 2.9), respectively of the tested

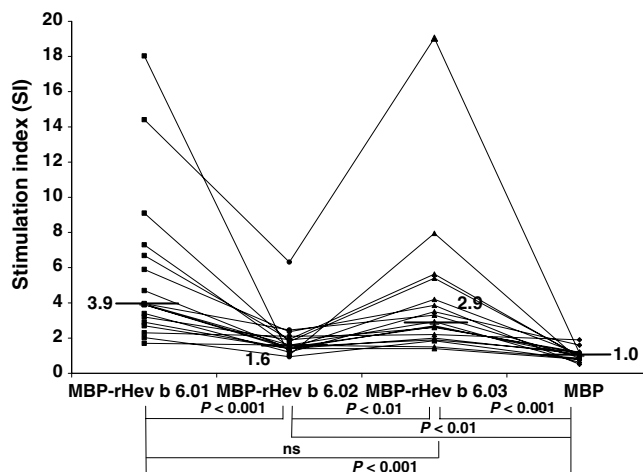


Figure 2. Lymphoproliferation of peripheral blood mononuclear cells (PBMC) in response to incubation with MBP-rHev b 6.01, MBP-rHev b 6.02, MBP-rHev b 6.03 and MBP. PBMC were stimulated with titrated concentrations (10, 5, 1, 0.5, 0.05 μ g/ml) of each antigen and the highest proliferation response indicated as maximal stimulation index values (SI) value for each individual is presented. Each value is expressed as the mean of at least six parallel determinations. The median values are shown as horizontal lines. The cut-off line for positive responses induced was $SI = 2.5$.

patients' PBMC. In contrast, MBP-rHev b 6.02 induced a positive proliferation response only in the PBMC of one of 18 latex-allergic subjects (5.6%) (median SI for stimulation with MBP-rHev b 6.02: 1.6). MBP alone had no effect on the induction of proliferation (median SI for stimulation with MBP: 1.0).

In addition to the PBMC stimulation with recombinant Hev b 6.02 (MBP-rHev b 6.02), the proliferation responses induced by native Hev b 6.02 and WGA (in the same concentration range 0.125–10 μ g/ml) were tested using PBMC of 25 latex-allergic HCWs and of five healthy control subjects without latex sensitization and atopy. Hev b 6.02 induced a positive stimulation response ($SI\text{-value} > 2.5$; median $SI\text{-value}$: 1.9; range 0.4–11.9) only in PBMC of six HCWs (24%), whereas in 21 of 25 PBMC of HCWs (84%) a significant proliferation response to WGA could be measured (median $SI\text{-value}$: 5.7; range 0.5–23.1). In no case the PBMC of the five controls showed an increased proliferation response to Hev b 6.02 (median $SI\text{-value}$: 1.3; range 0.3–1.5), but the PBMC stimulation by WGA was successful in all controls (median $SI\text{-value}$: 6.3; range 2.7–7.3) (Fig. 3).

Specific IgE binding and proliferation response to native Hev b 6.02 and to the synthetic, modified Hev b 6.02-peptides

Sera and PBMC from nine additional latex-allergic patients (Pa19–Pa27) were tested for their specific IgE binding and proliferation response to native Hev b 6.02

and to two modified Hev b 6.02-peptides (1–44; C → S; all eight cysteine residues were substituted by serine; C → A: all eight cysteine residues were substituted by alanine) (Table 2). All nine sera showed IgE binding to the native Hev b 6.02 (median: 0.95 kU/l). In contrast, in none of these sera specific IgE binding to the two modified Hev b 6.02-peptides (C → S and C → A) was detectable. In agreement with the results obtained with the recombinant MBP-rHev b 6.02, the proliferation response induced by native Hev b 6.02 was very low: Only PBMC of two patients showed SI-values ≥ 2.5 (mean $\pm$ SD: 1.97 ± 1.86). Proliferation responses induced by the modified Hev b 6.02 peptides C → S and C → A, were not significantly different from that induced by Hev b 6.02 (SI values mean $\pm$ SD for C → S: 1.83 ± 1.51 ; for C → A: 1.80 ± 1.29).

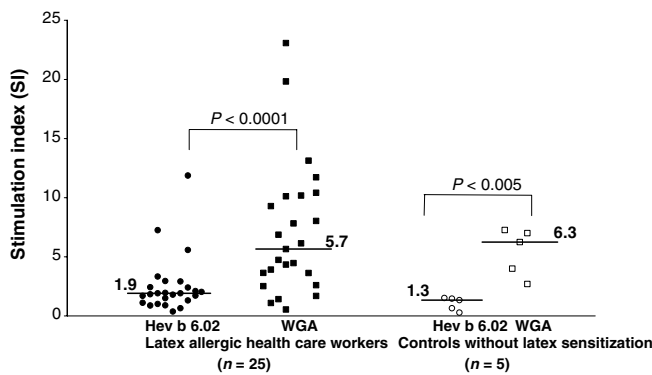


Figure 3. Comparison of the Hev b 6.02- and WGA-induced proliferation responses (the highest individual SI-value is presented) of the peripheral blood mononuclear cells of latex-allergic health care workers ($n = 25$) and controls ($n = 5$) (the median values are shown as horizontal lines). The statistical analysis used was the two-sided Wilcoxon rank sum test for linked samples.

Table 2. Immunoglobulin E binding and peripheral blood mononuclear cell proliferation response to native hevein (Hev b 6.02) and two modified hevein peptides (C → S) and (C → A)

Patient	IgE [kU/l]			Stimulation index [SI]		
	Hev b 6.02	Hev b 6.02 (C → S)	Hev b 6.02 (C → A)	Hev b 6.02	Hev b 6.02 (C → S)	Hev b 6.02 (C → A)
Pa19	3.72	<0.35	<0.35	3.3	1.2	0.8
Pa20	0.76	<0.35	<0.35	1.2	1.2	0.6
Pa21	>17.5	<0.35	<0.35	1.2	1.4	2.4
Pa22	0.63	<0.35	<0.35	0.8	0.9	0.8
Pa23	0.95	<0.35	<0.35	1.3	1.4	1.3
Pa24	1.42	<0.35	<0.35	1.0	2.4	2.3
Pa25	0.66	<0.35	<0.35	1.5	1.0	2.8
Pa26	1.02	<0.35	<0.35	0.9	1.1	1.9
Pa27	0.66	<0.35	<0.35	6.5	4.6	1.1

SI-values in bold indicate positive proliferation response.

Inhibition experiments with anti-HLA-DR, anti-HLA-DQ and anti-HLA-DP

For testing the HLA class II restriction of a latex allergen-induced PBMC proliferation response, inhibition experiments with MBP-rHev b 6.01 as a stimulus were performed in PBMC of eight HCWs (Table 3; Fig. 4). After preincubation with anti-HLA-DR the proliferation responses induced by MBP-rHev b 6.01 were inhibited in all eight cases in the range between 45 and 86% (median: 51.9% inhibition). The inhibition frequency with anti-HLA-DQ and anti-HLA-DP was lower (with both antibodies only an inhibition in five and seven of eight

Table 3. HLA status and T-cell reactivity induced by MBP-rHev b 6.01 of eight latex-allergic health care workers

Patient	HLA status	T-cell reactivity (SI-value) induced by MBP-rHev b 6.01*
Pa1	DR4 (0401), DR15, DQ8, DQ6	7.3 ± 1.6 (100 $\pm$ 21.6%)
Pa2	DR4 (0401), DR6 (13), DQ8	2.7 ± 0.7 (100 $\pm$ 26.5%)
Pa3	DR4 (0401), DR1, DQ8, DQ5	3.4 ± 0.5 (100 $\pm$ 13.9%)
Pa6	DR4, DR3, DQ8, DQ2	9.5 ± 1.0 (100 $\pm$ 10.1%)
Pa8	DR4 (0401), DR11, DQ7, DQ7	3.9 ± 1.1 (100 $\pm$ 28.2%)
Pa9	DR4 (0401), DR1, DQ8, DQ5	9.1 ± 0.5 (100 $\pm$ 5.5%)
Pa10	DR12, DR7, DQ7, DQ2	4.7 ± 1.4 (100 $\pm$ 29.8%)
Pa14	DR4 (0404), DR4 (0412), DQ8, DQ8	3.2 ± 1.0 (100 $\pm$ 31.2%)

* Median of the SI-values of six results and the standard deviation (SD) are presented. The numbers in brackets indicate the respective SI-value as 100% and the SD-value as percentage variation.

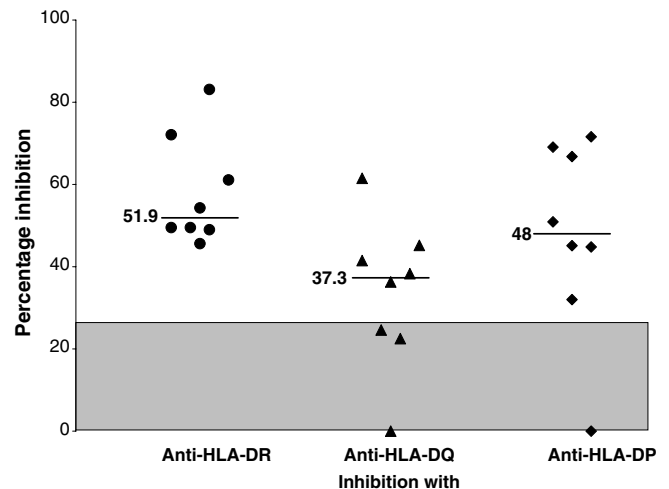


Figure 4. Inhibition of MBP-rHev b 6.01-induced proliferation response by preincubation of peripheral blood mononuclear cells (PBMC) with anti-HLA-DR, -DQ or -DP. PBMC incubated with MBP-rHev b 6.01 (5 μ g/ml) in the absence of antibodies served as control (0% inhibition). The proliferation responses (SI values) in the presence of the different HLA class II-specific monoclonal antibodies were calculated as percentage of inhibition of the SI value in the absence of the respective antibodies (Table 3). Percentage inhibition values $\geq 25\%$ were decided as relevant inhibition.

Table 4. Predicted potential HLA-DR4Dw4(DRB1*0401)-binding motifs in the Hev b 6.01 molecule using the PROTSHELL algorithm

	Relative position (P1 xx P4 x P6 xx P9)			
	P1	P4	P6	P9
Stringent conditions†	FYWILV	MAVLQ (no R, K)	TSVAG	AGSTQN (polar, aliphatic)
Corresponding region in Hev b 6.01‡	130–138: IVDQCSNGG			
Less stringent conditions†	FYWILV	Variable	TSVAG	AGSTQN (MVLHPKPY)
Corresponding regions in Hev b 6.01‡	146–154: FRQLD T DG K 174–182: FNPLF S VM K 90–98: WR SKY GW TA 96–104: WT AFG GP V G 99–107: FC GPV GA H G 72–80: IN AAS AY C S 115–123: IS VTW T GT G 163–171: IN Y Q F VD C G			

† Anchor positions (P1, P4, P6 and P9) according to motifs of Sinigaglia and Hammer (32). Main residues (marked in bold and underlined type) in HLA-DR4Dw4(DRB1*0401) ligands are shown. The first anchor is set as relative position 1.
‡ Epitopes are aligned to the respective motifs. Aligned anchor residues are marked in bold and underlined type. Residues with less relevance as anchor position are marked cursive and with broken lines.

cases, respectively, and with median inhibition values of 37.3 and 48%, respectively). As described previously (28), inhibition experiments with Hev b 6.02 as a stimulus were performed successfully only in PBMCs of two HCWs with latex allergy. In these two cases anti-HLA-DQ and anti-HLA-DP were able to inhibit the proliferation, whereas anti-HLA-DR had no significant influence on the proliferation response.

Genetic analysis of HLA-DRB1, DRB3, DRB4, DRB5, and DQB1 alleles

Human leucocyte antigen class II alleles of DRB were determined by using the PCR-SSOP method with 28 SSOPs, which allowed a differentiation between 13 HLA DRB1 types, 1 DRB4 type and two different DRB5 types and 20 SSOPs from the 11th Histocompatibility Workshop or commercial SSP kits were used to determine 15 different DQB1 alleles (28) in 23 of the 27 HCWs (Pa1–Pa27) tested on their specific IgE and T-cell response (Table 1). The analysis of the typing data revealed remarkably high frequencies of DQB1*0302 (DQ8) (11 of 23; 48%) and DRB1*0401-04 (DR4) (13 of 23; 56%). The most prominent DR4 suballele was *0401 (69%).

Predictions of MHC II peptide binding motifs of Hev b 6.01

According to the approach of Hammer, Sinigaglia and colleagues (32, 34) (summarized in 22, 30, 31), potential HLA-DR4Dw4 restricted T-cell epitopes were predicted to published DR4Dw4-binding motifs using the computer program PROTSHELL. Using a stringent level of amino acid composition for the anchor positions P1, P4, P6 and P9 (Table 4), only one HLA-DR4Dw4 (HLA-DRB1*0401) binding motif was found in the region 130–138. Calculating with less stringent criteria (e.g. P4 is variable), HLA-DR4Dw4 binding motifs were also found

in the regions comprising residues 146–154, 174–182, 90–98, 96–104, 99–107, 72–80, 115–123 and 163–171. Potential HLA-DR4Dw4 (DRB1*0401)-restricted T-cell epitopes were only found in the Hev b 6.03-part of Hev b 6.01; no predicted binding motif was part of the N-terminal hevein amino acid (aa) sequence (1–43) (Fig. 5).

A similar, but not identical result could be obtained by using the program TEPITOPE. Again, no HLA-DR4Dw4 binding motif was predicted in the hevein region. In contrast to the results obtained by PROTSHELL, using the most stringent criteria (1% threshold values) TEPITOPE calculates the region 146–154 as HLA-DR4Dw4 binding motif. Using a threshold value of 6%, additional HLA-DR4Dw4 binding motifs were predicted in the regions comprising residues 60–68, 117–125, 130–138, 139–147, 163–171, 165–173, 174–182 and 178–186. Only four identical regions were predicted by both algorithms.

Discussion

In this study the impact of the specific IgE and PBMC responses to the latex precursor allergen Hev b 6.01 and its post-transcriptionally formed proteins Hev b 6.02 and Hev b 6.03 were examined in a group of latex-allergic HCWs. HCWs belonged to the risk group for latex sensitization and in most of them specific IgE antibodies against Hev b 5 and Hev b 6.01/Hev b 6.02 could be demonstrated. In a randomly selective group of latex-allergic HCWs our study confirmed the importance of Hev b 6.01 and Hev b 6.02 as relevant allergens by using recombinant proteins fused to MBP. In contrast to Hev b 6.02 representing the N-terminal 4.7 kDa region with a pronounced IgE-binding capacity, the IgE-binding of the 14 kDa C-domain Hev b 6.03 was of minor importance. Approximately 72% of our tested HCWs had a positive

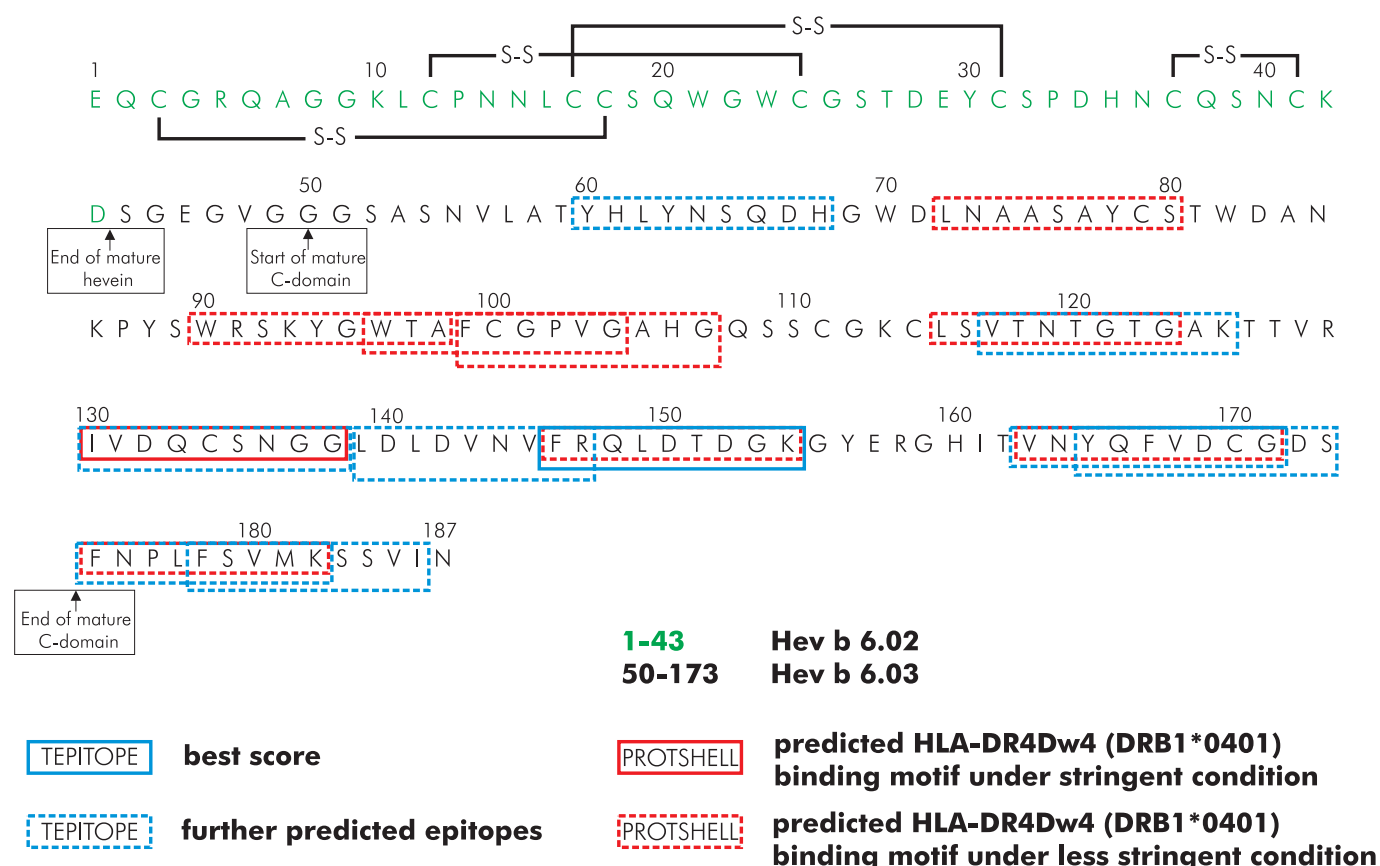


Figure 5. Amino acid sequence of Hev b 6.01 (prohevein) and the predicted HLA-DR4Dw4 binding motifs using PROTSHELL- and TEPITOPE-algorithms (stringent and less stringent conditions).

specific IgE response to MBP-rHev b 6.03 and most of them had only low specific IgE levels to this allergen. Alenius et al. (6) reported that 69% of NRL allergic patients ($n = 56$) had IgE antibodies to purified prohevein, whereas 21% of these patients had IgE against the purified prohevein C-domain. Using modified Hev b 6.02 peptides, in which all eight cysteine residues were substituted by serine or alanine, the IgE-binding capacity disappeared, as the 3-D structure of the molecule constituted by the disulphide bridges was destroyed. These data strongly suggest that the correct conformational structure of the small, tightly packed molecule Hev b 6.02 is essential for IgE binding and that the major IgE epitope of Hev b 6.02 is a discontinuous B-cell epitope. Different approaches of B-cell epitope mapping using overlapping peptides in direct or inhibition ELISA by Banerjee et al. (13) and Beezhold et al. (35) respectively led to different results: they demonstrated independently that peptides containing residues 19–14 and 25–37 and 13–24 and 29–36, respectively are responsible for Hev b 6.02-specific IgE binding. In contrast, the novel chimera-based allergen epitope mapping strategy used by Karisola et al. (14) demonstrated that the major IgE-binding epitopes of Hev b 6.02 are conformational and that the N-terminal and the

C-terminal regions of this molecule are essential for IgE binding.

In addition to the IgE binding capacity, the PBMC proliferation responses induced by rHev b 6.01, rHev b 6.02 and rHev b 6.03 were studied. Both MBP-rHev b 6.01 and MBP-rHev b 6.03 were good inducers of proliferation responses because in 83 and 67%, respectively of the tested patients' PBMC a positive proliferation response was measured. In contrast, the ability of MBP-rHev b 6.02 as well as of the native Hev b 6.02 to induce a proliferation response was very low and detectable only in 5.5 and 24% of the patients' PBMC tested. Moreover, using increased allergen concentrations did not increase the proliferation responses (data not shown). In contrast to native as well as recombinant hevein, WGA, which includes four hevein-like sequences in its primary structure, was a good inducer of a proliferation response due to the lectine attributes of this molecule but independent of allergen specificity. The ability of Hev b 6.02 to induce a proliferation response is closely related to the allergic status and not predominantly due to an unspecific mitogenic lectine effect seen in the case of WGA stimulation.

In our previous paper concerning HLA-D association with hevein-specific IgE immune response (28) the data

showed that hevein-specific IgE was strongly associated with the HLA class II alleles DQB1*0302 (DQ8) and DRB1*04 (DR4) (both types are in linkage disequilibrium). To prove this association on the T-cell epitope level, we used available approaches to predict MHC II peptide binding motifs in the Hev b 6.01 molecule. Computer-aided prediction programs, such as PROTSHELL and TEPITOPE for the determination of HLA-DR4Dw4 binding motifs revealed that the Hev b 6.02 region of Hev b 6.01 does not have an HLA-DR4Dw4 binding motif. Although both programs gave similar but not identical results, they predicted potential HLA-DR4Dw4 (DRB1*0401)-restricted T-cell epitopes in the whole Hev b 6.03-part of Hev b 6.01: using less stringent criteria, HLA-DR4Dw4 binding motifs were predicted in the regions comprising residues 130–138, aa 146–154, aa 163–171 and aa 174–182 with both algorithms.

According to our experimental data that Hev b 6.03 was a good inducer of lymphocyte proliferation there seems to be a conformity between the predicted HLA-DR4Dw4(DR1*0401) binding motif and the detected T-cell response. In addition, the absence of predicted HLA-DR4Dw4(DR1*0401) binding motifs as an indicator of the relatively low number of T-cell epitopes recognized in the Hev b 6.02 region could be one explanation for the weak proliferation response of PBMCs against native and recombinant Hev b 6.02.

Inhibition experiments using anti-HLA-DR, -DQ and -DP suggested that the DR and DP binding sites were more important for the proliferation response induced by recombinant Hev b 6.01 than HLA-DQ, whereas HLA-DR binding sites seemed to be not sufficient for the proliferation response induced by Hev b 6.02.

In addition to MHC class II-binding, other factors are important for the induction of a T cell proliferation, e.g. TCR recognition and/or various co-stimulatory molecules. Studies on epitope recognition suggest that it is the

low affinity of a peptide for an MHC class II molecule and/or a TCR that favours a Th2-type response (36). This may be a possible reason for the dominant IgE-response to Hev b 6.02 (induced by Th2-cytokines) and its only minute or suboptimal PBMC activation measured by proliferation. No data concerning cytokine pattern induced by prohevein and its fragments are available now, and our own preliminary results about secretion of cytokines in PBMC supernatants indicated no significant different pattern, with the only exception being IL-6.

In summary, our data indicate that the regions in the important latex allergen Hev b 6.01 (prohevein) responsible for PBMC responses and IgE-binding sites are not identical: the Hev b 6.02 domain was responsible for IgE binding and contains discontinuous B-cell epitopes and Hev b 6.03 was a better inducer of proliferation responses and contains HLA-DR4-binding motifs.

The ability to define 'immunodominant' and 'disease-related' T-cell-reactive regions is of potential practical value in the design of immunotherapeutics. Functional changes that reduce the IgE binding potential need to be determined to produce modified allergens for immunotherapy. A successful approach has been to replace the cysteine residues to prevent the formation of disulphide bonds and dramatically alter the conformation of the native protein.

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