

Recombinant Hev b 1: large-scale production and immunological characterization

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Summary

Background Hev b 1 represents one of the most important allergens in *Hevea brasiliensis* latex. It is difficult to get an appropriate amount of native Hev b 1 (nHev b 1) for research purposes.

Objective The aim of this study was to produce sufficient amounts of Hev b 1 by recombinant methods to prove its suitability for latex allergy diagnostics.

Methods We isolated total RNA of *Hevea brasiliensis* leaves and synthesized cDNA by RT-PCR. Recombinant Hev b 1 (rHev b 1) as well as three fragments (amino acid residues 29–137, 48–137, 78–137) were subcloned and expressed as fusion proteins with Maltose-binding protein (MBP) in *Escherichia coli*. The MBP-rHev b 1 fusion protein was examined by RAST with the CAP method, histamine release test and immunoblots with human sera from spina bifida patients as well as from health care workers with latex allergy and monoclonal antibodies.

Results Histamine release test and immunoblots revealed the high allergenicity of the MBP-rHev b 1 construct. By the CAP method, 54 out of 58 serum samples (93%) from latex-sensitized spina bifida patients previously showing immunoglobulin (Ig) E to nHev b 1 exhibited IgE-binding to rHev b 1. Among 71 latex-allergic health care workers tested, 16 (22.5%) had IgE antibodies to rHev b 1. The analysis of the fusion proteins carrying rHev b 1 fragments revealed that the loss of the N-terminal 28 amino acid residues did not affect IgE-binding. In contrast, the lack of the first 47 amino acid residues led to decreased IgE-binding reactivity in two out of four sera tested, whereas the absence of the N-terminal 77 residues abolished IgE-binding in these two sera.

Conclusion The MBP-rHev b 1 fusion protein exhibits a corresponding IgE-binding reactivity to nHev b 1 and may therefore substitute natural Hev b 1 for both *in vitro* diagnostics and research purposes.

Keywords: Latex allergy, *Hevea brasiliensis*, Hev b 1, spina bifida, recombinant allergen

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Introduction

The major latex allergen Hev b 1, also called rubber elongation factor, was initially isolated from latex sap of *Hevea brasiliensis* by Dennis and Light [1]. The enzyme prenyltransferase needs this protein to synthesize multiple cis-1,4-isopren units to rubber particles [2]. With regard to

the whole protein content in latex sap, the portion of Hev b 1 reaches a value between 10 and 60% [1]. Hev b 1 is a very hydrophobic protein with a length of 137 amino acid residues and a molecular mass of 14.6 kDa. Amino acid residues like cysteine, methionine, histidine and tryptophane are completely absent. The N-terminus of Hev b 1 includes a cluster of acidic amino acid residues and is acetylated. The function of this single post-translational modification is unknown, although a protection against proteolysis is supposed [3]. In 1993, Czuppon *et al.* [4]

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isolated the rubber elongation factor out of latex gloves, showed its IgE-binding reactivity and named this new allergen, in accordance with the WHO and IUIS nomenclature [5], Hev b 1. The B-cell epitopes of Hev b 1 were determined by Chen *et al.* [6] using synthetic peptides. They found that the main epitope areas are localized in the carboxy-terminal part of Hev b 1 and in a region between amino acid residues 31–64. The clinical relevance of Hev b 1 has been demonstrated by the high prevalence of IgE antibodies among spina bifida (SB) patients with latex allergy. Studies [7,8] showed that Hev b 1 represented a target for IgE antibodies of up to 80% (67–81%) of SB patients sensitized to latex. Almost 45% of these SB patients were exclusively sensitized to Hev b 1 [7]. The prevalence of the presence of Hev b 1-specific IgE antibodies in latex-sensitized health care workers (HCWs) was reported to be between 18 and 49% by using different serum samples and test methods [7–10].

Since the Hev b 1 protein is tightly associated with rubber particles [1] it was found to be difficult to completely wash it out of the latex gloves during the production procedure. In some protein-rich latex gloves, experiments showed that the Hev b 1 protein and fragments were the dominant components of the whole extractable proteins of the gloves [7]. These results partially explained the high prevalence of seropositive IgE antibodies to Hev b 1 among SB patients who due to repeated surgical interventions had an intensive mucosal contact with latex gloves.

Because of the tight association with the rubber particles, it is also difficult to get an appropriate amount of purified natural Hev b 1 protein for both diagnostic and research purposes. In particular for the development of methods to quantify the Hev b 1 content in latex products, we need a large amount of highly purified Hev b 1 protein as reference antigen. In this study, we investigated the possibility to produce Hev b 1 in fusion with the Maltose-binding-protein (MBP) by recombinant techniques in *Escherichia coli* (*E. coli*). Further, we compared the immunological properties of MBP-rHev b 1 as well as its fragments with that of natural Hev b 1 by the Pharmacia CAP method using sera from latex-sensitized SB patients as well as HCWs with latex allergy.

Materials and methods

Human sera

Sera were obtained from two groups of clinically well-characterized patients with latex sensitization and/or allergy. In group 1, 58 serum samples from latex-sensitized SB patients (22 females and 36 males) were used. The patients were aged between 2.3 and 21.8 years (mean age: 10.2 years). All the 58 serum samples showed latex-specific

IgE antibodies (range: 0.46 kU/L– ≥ 100 kU/L) as determined by the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) as well as specific IgE to native Hev b 1 as previously tested by the EAST [7]. Out of 58 SB patients, 32 (55%) were diagnosed to have latex allergy by the clinical history. Group 2 included 71 serum samples from HCWs which were consecutively collected in our medical Department. All 71 patients were classified by their clinical history as having latex allergy. In addition to the latex-specific IgE antibodies (range: 1.01 kU/L– ≥ 100 kU/L) and, they all showed positive skin responses to latex extract by skin prick testing (SPT). Two pooled sera were used in the immunoblot experiments. The first one contained sera of 12 HCWs with latex allergy and the second one contained sera of eight SB patients mentioned above. Sera obtained from eight individuals without latex sensitization and showing negative SPT to latex served as negative control.

Skin prick testing

Skin prick tests were performed in all adult patients (HCWs) with latex extract prepared in our Institute as described [7]. Total latex protein concentrations used were 0.001, 0.01 and 0.1 mg/mL in sterile saline solution containing 0.4% phenol. Saline solution (diluent) and a histamine solution (10%) were used as negative and positive controls, respectively. Cutaneous reactions were read after 15 min and were considered as positive when a wheal reaction was at least 3 mm surrounded by an erythema as compared with negative control values (wheal ≤ 2 mm, no erythema).

Monoclonal antibodies

Monoclonal antibodies were produced as described by Chen *et al.* [6]. For binding tests we chose two monoclonal antibodies named II4F9 and II4G9. The first one recognizes Hev b 1 amino acid residues 46–54, the latter binds to Hev b 1 amino acid residues 122–134. A nonrelated monoclonal antibody named II5C9, which has been tested to show no binding reactivity with latex proteins, served as negative control.

Purification of nHev b 1

Isolation and purification of nHev b 1 were performed as recently described in detail [7].

RNA isolation and cDNA synthesis

Total RNA was extracted from one gram chilled leaves of rubber tree *Hevea brasiliensis*, clone RRIM 600, which were kindly provided by the Institute for Agriculture of the Temperate Subtropical and Tropical Zones, Witzenhausen,

Germany. Isolation was performed with the Total RNA kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with 2 µg total RNA using the Superscript II kit in accordance with the protocol of the supplier (Life Technologies, Karlsruhe, Germany).

Amplification of rHev b 1 cDNA

To obtain a Hev b 1-specific amplification we used a 100-µL reaction mixture containing 10 ng latex cDNA, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 2.5 mM MgCl₂, 0.01% (w/v) gelatine (Sigma, Deisenhofen, Germany), 100 µM of each dNTP (Boehringer Mannheim, Germany), 25 pmol of each primer (Hev b 1-51: 5'-CCTGCAGCGCTGAGACGAGACACC-3'/Hev b 1-3': 5'-CCGAATTCAATTCTCTCCATAAAACACC-3') and 2.5 U Taq polymerase (Life Technologies, Karlsruhe, Germany). The reaction product (411 bp) was obtained in a thermal cycler (Biometra, Göttingen, Germany) with an initial denaturation step (95 °C for 3 min) and a total of 35 cycles of denaturation (95 °C for 1 min), annealing (65 °C for 1 min), and extension (72 °C for 2 min), followed by 10 min extension at 72 °C. Eight µL of the PCR-product as well as the negative control sample were run on an 1.5% agarose gel.

Subcloning and sequencing of the rHev b 1-PCR constructs

The resulting PCR product (411-bp fragment) was subcloned into the pCR2.1 vector using the TA Cloning kit (Invitrogen, Leek, Netherlands). Positive clones were first selected by blue/white screening followed by restriction analysis and finally sequenced on an ABI 310 automatic fluorescent sequencer (PE Biosystems, Weiterstadt, Germany) using the M13 reverse and forward primers. Three clones (pCR-Hev b 1) were found to carry the full length recombinant Hev b 1 (amino acid residues 1–137).

PCR products corresponding to Hev b 1 amino acid residues 1–137, 29–137, 48–137, and 78–137 were amplified in the presence of the Hev b 1 DNA of pCR-Hev b 1 as template by using the primer pairs described below. After treatment of PCR products with Eco 47 III-Eco RI (Hev b 1-51/Hev b 1-3', for full-length rHev b 1), Sca I-Eco RI (Hev b 1-52: 5'-CCGAGTACTACCTTCTCAAACGTC-3'/Hev b 1-3', for rHev b 1 fragment 29–137), SmaI-Eco RI (Hev b 1-53: 5'-CCCGGGTGTGCGATATCATTTGAGGGT-CCGGTGAAGAACGTGGCTGTACCTCC-3'/Hev b 1-3', for rHev b 1 fragment 48–137) and Sna BI-Eco RI (Hev b 1-54: 5'-CCTACGTAGACAGCCGGTTGTTG-3'/Hev b 1-3'; for rHev b 1 fragment 78–137) all four adapted DNA constructs were purified by agarose gel electrophoresis or by Centricon 30 (Millipore, Eschborn, Germany) and were then separately subcloned into XmnI-Eco RI digested pMAL-c2 vector (New England Biolabs, Schwalbach

Germany) yielding four malE-rHev b 1 constructs. After verification by sequencing, all constructs were expressed in *E. coli* strain TB-1.

Overexpression and purification of the Maltose-binding-protein (MBP)-rHev b 1 fusion proteins in E. coli

Ten mL fresh recombinant *E. coli* cells from an overnight culture were grown in one litre Luria broth (LB) medium supplemented with 100 µg/mL (w/v) ampicillin and 2 mg/mL (w/v) glucose at 37 °C for two hours on a laboratory shaker. Expression of the fusion protein was induced by adding 100 mM isopropyl thiogalactoside (IPTG, Roth, Karlsruhe, Germany) up to a final concentration of 0.3 mM and shaking for 4 hours. *E. coli* cells were harvested by centrifugation at 4000 g for 20 min. The pellet was dissolved in 50 mL column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA). *E. coli* cell lysates containing the expressed fusion protein were prepared by sonification in an ice-water-bath with a Branson sonifier-250 (eight intervals of 20 s on level four followed by a time stop of 30 s), followed by centrifugation at 10 000 g for 30 min. The supernatant was loaded onto a 1.5 × 20 cm column filled with a 7-mL bed volume of amylose (New England Biolabs, Schwalbach, Germany). Equilibration and washing were performed with freshly prepared column buffer until the flow-through reached an equal optical density at 280 nm in comparison with the column buffer. The bound fusion protein was eluted in ten 2 mL fractions with column buffer containing 10 mM Maltose.

Factor Xa cleavage of the fusion proteins

For the cleavage of rHev b 1 from the carrier protein MBP, affinity-purified fusion protein was incubated with the protease factor Xa (Mobitech, Goettingen, Germany) in 100 mM phosphate buffered saline, pH 7.5, and at a factor Xa fusion protein ratio of 1:20 (w/w) for 48 h at room temperature under smooth shaking. In case of the MBP-rHev b 1 fusion proteins carrying Hev b 1 fragments 29–137, 48–137 and 78–137, sodium dodecylsulphate (SDS) with a final concentration of 0.04% (w/v) was added in order to increase the cleavage efficiency of factor Xa. In contrast, the full length construct was cleaved in the absence of SDS.

Electrophoresis and immunoblots

Recombinant fusion proteins as well as their factor Xa-cleaved products (digestion mixtures) were separated by one-dimensional SDS polyacrylamide gel electrophoresis (PAGE) and blotted on polyvinylidene difluoride (PVDF) membranes (Pall, Dreieich, Germany) as described before [8]. The blotted proteins were probed with murine mAbs

diluted 1:1000 in dilution buffer (50 mmol/L Na-phosphate, pH 7.5, containing 0.5% w/v. BSA, 0.1% v/v. Tween 20 and 0.02% w/v NaN_3), and bound IgG antibodies were detected with alkaline phosphatase-conjugated goat antimouse IgG (Sigma, Deisenhofen, Germany). The ability of the recombinant proteins to bind with IgE antibodies was analysed by incubating the proteins on a membrane with pooled or single serum samples diluted at 1:10 in the dilution buffer and was detected with alkaline phosphatase-conjugated antihuman IgE antibodies (Sigma), and the colour development of the bands were achieved with a substrate solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and 0.37 mM p-nitro blue tetrazolium chloride (NBT) in 0.1 mmol/L NaHCO_3 containing 10 mmol/L MgCl_2 , pH 9.6. As control, sera from eight individuals without latex sensitization were used.

N-terminal sequence analysis

After blotting the proteins on a PVDF membrane and visualization by Coomassie brilliant Blue R-250 (Roth, Karlsruhe, Germany), protein bands of interest were excised from the blotting-matrix and analysed on a 476-A-Protein-Sequencer (PE Biosystems, Weiterstadt, Germany).

Determination of specific IgE antibodies by the CAP method

To test the IgE-binding reactivity to rHev b 1, the MBP-rHev b 1 fusion protein was employed for the preparation of the ImmunoCAP antigen which was then used to determine IgE antibodies to rHev b 1 by the Pharmacia CAP using reagents and procedures as recommended by the system supplier. A specific IgE-value >0.35 kU/L was regarded as positive.

Histamine release test

Histamine release tests were performed with an enzyme immunoassay kit (Immunotech, Hamburg, Germany) following the protocol provided by the supplier. For the cell challenge or spontaneous histamine release, 200 μL of heparinized blood diluted 1:7 in histamine release buffer were mixed with 100 μL of serially diluted releasing reagents (MBP-rHev b 1, its fragments, MBP) or with 100 μL histamine release buffer, incubated for 30 min at 37 °C. After the incubation, the released histamine-containing supernatant was recovered by centrifugation (900 g for 5 min at 4 °C). The total histamine was determined after cell lysis by addition of 50 μL nondiluted blood to 950 μL of distilled water (1:20 dilution), frozen and thawed suspension. One hundred μL of each supernatant and lysed cell suspension was then transferred in to a clean tube without disturbing the cell pellet and acylated. Afterwards, the

acylated samples were added to a 96-well microtitre plate coated with monoclonal antihistamine antibodies and incubated for two hours at 4 °C with constant shaking in the presence of 200 μL histamine-alkaline phosphate-conjugate. The enzyme immunoassay for histamine is based on the competition between the histamine to be assayed and histamine-alkaline phosphatase (enzyme conjugate) used as tracer for binding to antibodies coated onto microwells. After washing, the chromogenic substrate p-nitrophenylene phosphate was added, followed by incubation for 30 min in the dark at room temperature. The intensity of the colour depended inversely on the histamine concentration in the sample. The histamine concentration was calculated on the basis of the standard curve obtained with standards supplied with the kit. The amount of released specific histamine (percentage) was calculated as follows: challenged release/total histamine $\times$ 100.

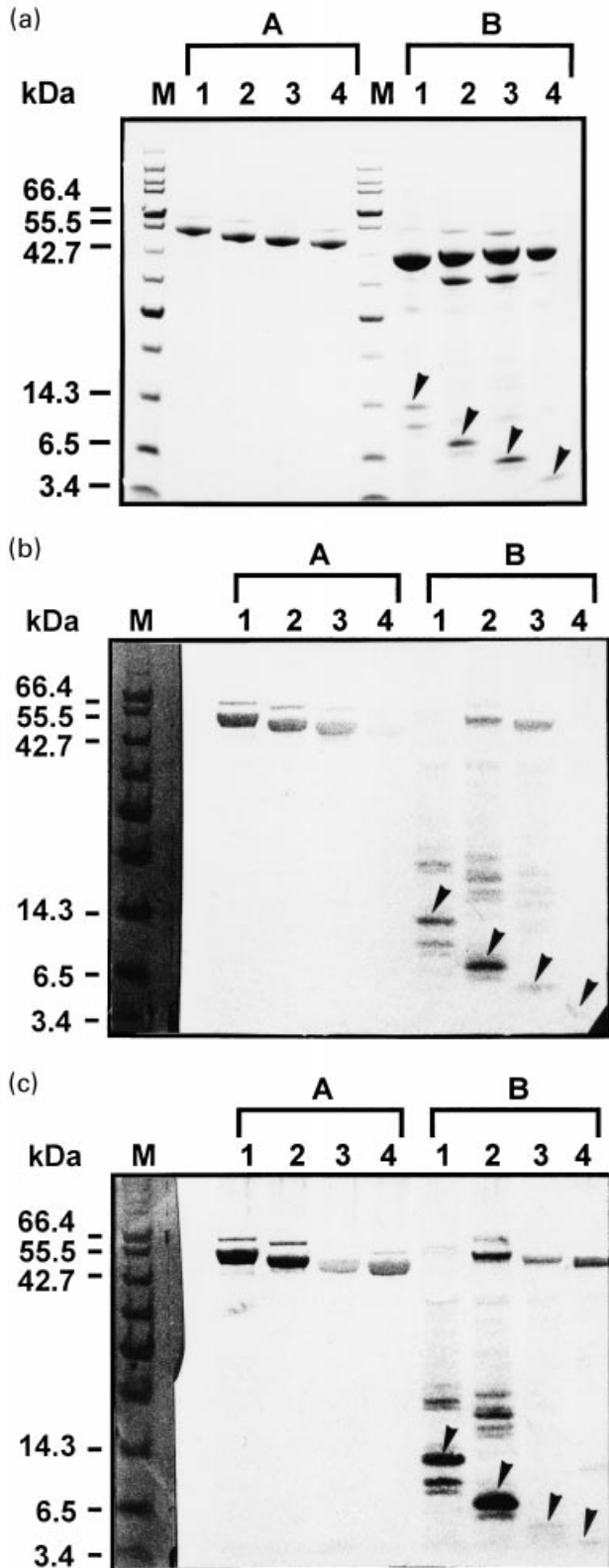
Results

Protein expression and purification of MBP-rHev b 1 and its fragments

cDNA coding for Hev b 1 was obtained by RT PCR from total RNA of *Hevea brasiliensis* leaves using primers designed according to the nucleotide sequence reported previously [11]. After subcloning the Hev b 1 sequence into the pCR2.1 vector, we performed sequencing and found that the deduced amino acid sequence was identical with that reported by Attanyaka *et al.* [11]. The full length Hev b 1 sequence and three fragments were then successfully amplified and subcloned into the pMAL-c2 system.

The yield of the expressed recombinant MBP-rHev b 1 fusion protein (57.3 kDa) after affinity chromatography was on average about 15 mg per litre cell culture. The purity of the MBP-rHev b 1 was >95% as estimated by SDS-PAGE separation. Factor Xa cleavage yielded two main bands with a molecular mass of 14.6 kDa for rHev b 1 and 42.7 kDa for MBP (Fig. 1). N-terminal sequencing of the first seven amino acid residues of rHev b 1 by Edman degradation confirmed the correct sequence.

In the same manner we expressed and purified three rHev b 1 fragments as MBP-fusion proteins carrying a deletion of the first 28, 47 and 77 N-terminal amino acid residues of rHev b 1. The yields of the recombinant rHev b 1 fragments varied from 4 mg/L MBP-rHev b 1 (48–137) to 50 mg/L MBP-rHev b 1 (78–137). In contrast to the full length construct, the rHev b 1 fragments were cleaved in the presence of 0.04% SDS yielding target fragments with a molecular mass of 11.9 kDa for rHev b 1(29–137), 9.8 kDa for rHev b 1(48–137) and 6.5 kDa for the rHev b 1(78–137) fragment. Aliquots of the MBP-rHev b1 fusion



proteins before and after factor Xa cleavage are shown in Fig. 1(a).

Immunoblot analyses of rHev b 1 with human sera and monoclonal antibodies

To investigate the IgE-binding reactivity of all MPB-rHev b 1 constructs we first performed immunoblots by using a pooled serum comprising 12 sera of HCWs who had previously shown IgE antibodies to nHev b 1 (Fig. 1b) and a pooled serum comprising eight sera from SB patients with latex allergy (Fig. 1c). The results in Fig. 1 demonstrate that the full length rHev b 1 as well as its fragments are recognized by both pooled sera independent of the fact if the carrier protein MBP was present or already cleaved off. The 42.7 kDa MPB carrier protein alone shows no IgE-binding reactivity.

In the immunoblots (Fig. 1b,c, lanes B1 and B2) we also found some protein bands which became more clearly after immunostaining and located at the areas between 14 and 30 kDa, indicating that some nonspecific attacks of the protease factor Xa on MBP molecule may happen during the cleavage process. The long incubation time of 48 h at room temperature and the use of SDS detergent, both are essential for a high cleavage yield, may be the reasons for this impairment.

Hev b 1-specific monoclonal antibodies and four individual serum samples from latex-sensitized SB patients were also used in the immunoblot experiments. The results summarized in Fig. 2 confirmed the binding reactivity of the mAbs to rHev b 1 and to the corresponding fragments (Fig. 2d-f, lane 2 and 3). IgE antibodies in the four serum samples from latex-sensitized SB patients (Fig. 2; lanes 4-7) clearly recognized both nHev b 1 (Fig. 2a) and rHev b 1 (Fig. 2b) as well as MBP-rHev b 1 (Fig. 2c). However, the fourth serum (lane 7) showed only a weak intensity of the reaction to the rHev b 1 on the blot. Regarding the rHev b 1 fragments, we observed no influence on IgE-binding when the N-terminal 28 amino acid residues were absent. For two single sera, however, we observed a reduced IgE-binding reactivity when the first 47 amino acid residues at the N-terminus were absent (Fig. 2e; lanes 4 and 7). Both sera showed no detectable IgE-binding when the first 77 amino acid residues were removed (Fig. 2f, lanes 4 and 7).

Fig. 1. Coomassie-stained SDS-PAGE (a) and immunoblot analysis of the Maltose-binding-protein-rHev b 1 constructs and its rHev b 1 fragments using a pooled serum from Hev b 1-sensitized HCWs (b) and Hev b 1-sensitive SB patients (c) before (A) and after (B) factor Xa cleavage. The cleaved target proteins are indicated by arrows. Lane M: molecular mass markers. Lane 1: MBP-rHev b 1, lane 2: MBP-rHev b 1 (29-137), lane 3: MBP-rHev b 1 (48-137), lane 4: MBP-rHev b 1(78-137).

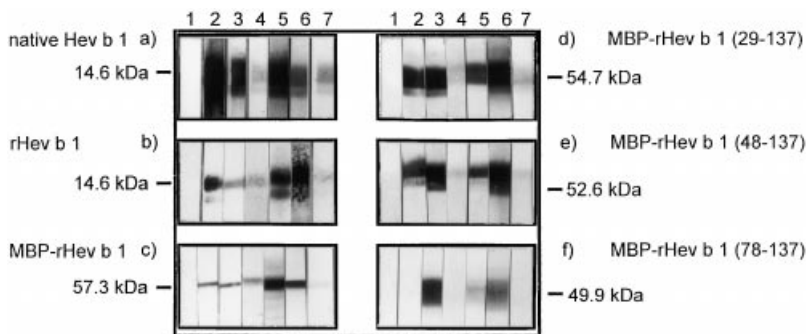


Fig. 2. Immunoblot analysis of the MBP-rHev b 1 fusion proteins with monoclonal antibodies and individual sera of latex-sensitized SB patients. From left to right, lane 1 represents the reaction with mAb II5C9 (negative control), lane 2: mAb II4F9 (recognizing Hev b 1 residues 46–54), lane 3: mAb II4G9 (recognizing Hev b 1 residues 122–134), lanes 4–7: four serum samples from Hev b 1-sensitive SB patients. The following proteins were tested: (a) nHev b 1, 5 µg; (b) rHev b 1, 1 µg; (c) MBP-rHev b 1, 1 µg; (d) MBP-rHev b 1 (29–137), 5 µg; (e) MBP-rHev b 1 (48–137), 5 µg; (f) MBP-rHev b 1 (78–137), 5 µg.

Determination of specific IgE to the MBP-rHev b 1 fusion protein by the CAP system

To examine the IgE-binding reactivity of rHev b 1 MBP-rHev b 1 ImmunoCAPs were prepared as capture antigen for the use in the Pharmacia CAP system. A total of 58 serum samples from latex-sensitized SB patients previously showing IgE to nHev b 1 in the enzyme allerge sorbent test (EAST) were examined (Fig. 3). From these 54 (93%) from 58 sera tested showed positive IgE values to MBP-rHev b 1. The IgE values range from 0.41 to ≥ 100 kU/L (median:

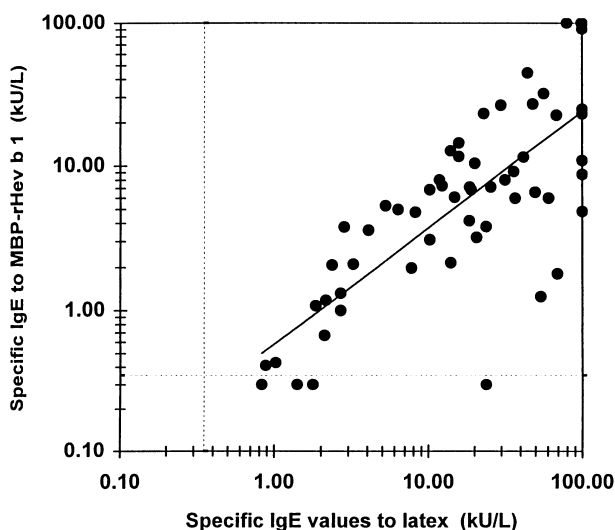


Fig. 3. Scatter diagram of specific IgE values to MBP-rHev b 1 and to whole latex allergens in sera of SB patients by the CAP-method. 58 SB patients previously showing IgE antibodies to nHev b 1 and latex were analysed with MBP-rHev b 1 ImmunoCAP as target allergen. The cut-off value for positivity (0.35 kU/L) is indicated by a dotted line.

5.64 kU/L). In most cases, the IgE values determined by the CAP method were greater than those obtained by EAST (median 3.60 kU/L) which is most likely due to the larger allergen-coated surface of the former ones. Four sera which previously showed low levels of IgE antibodies (0.42, 0.57, 0.74 and 1.23 kU/L, respectively) by EAST were negative by the CAP method with MBP-rHev b 1 as antigen.

A total of 16 (22%) out of 71 sera from latex-allergic HCWs showed IgE antibodies to rHev b 1. The IgE antibody levels ranged from 0.37 to 66.20 kU/L and had a mean level of 7.90 kU/L. Ten sera previously showing positive IgE to nHev b 1 by the EAST (IgE-levels: 0.63–2.71 kU/L) showed negative results by the CAP system with rHev b 1.

Ten sera showing positive IgE values to MBP-rHev b 1 by the CAP system were tested in the EAST for the IgE-binding reactivity to MBP protein alone, where the MBP-coated paper disks were used. No positive result was observed (data not shown).

Histamine release induced by the MBP-rHev b 1 fusion protein

Basophils from one latex-allergic SB patient was challenged with purified MBP-rHev b 1 or MBP-rHev b 1 fragments at protein concentrations between 0.005 µg/mL to 5 µg/mL. As control, challenge tests with MBP at concentrations between 0.05 µg/mL and 5 µg/mL were also performed. The measured amount of released histamine is shown in Fig. 4. The MBP-rHev b 1 and the fragment MBP-rHev b 1 (29–137) induced a dose-related histamine release, and the maximum release of challenged histamine was found at 0.5 µg/mL for MBP-rHev b 1 as well as MBP-rHev b 1 (29–137), whereas the other MBP-rHev b 1 fragments and MBP alone did not trigger the histamine release.

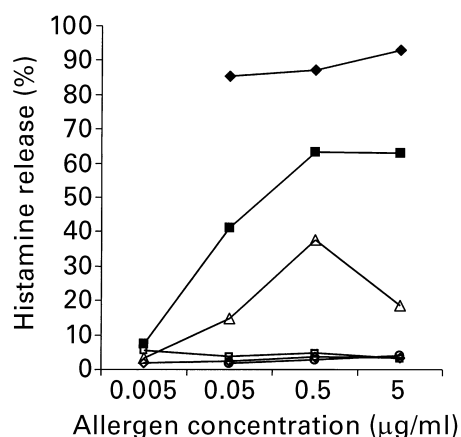


Fig. 4. Histamine release assay. Blood collected from one SB patient was challenged with increasing concentrations of latex protein extract (◆), MBP-rHev b 1 (■), MBP-rHev b 1 (29–137) (△), MBP-rHev b 1 (48–137) (◇), MBP-rHev b 1 (78–137) (□) and MBP (○). The histamine released from basophils was determined by immunoassay and expressed as a percentage of the ratio between the released and the total cellular histamines.

Discussion

cDNA encoding the rubber elongation factor (Hev b 1) of rubber tree *Hevea brasiliensis* was cloned by several laboratories [2,11,12] and it was also tried to overexpress the recombinant Hev b 1 to study its immunological properties [11]. However, no study has been yet reported on the success to produce an appropriate amount of recombinant Hev b 1 for latex allergy research, except for a few immunoblot analyses. In our present work, starting with total RNA from *Hevea brasiliensis* leaves, we have successfully subcloned, expressed and purified the full-length rHev b 1 and three rHev b 1 fragments in fusion with MBP. The yield of recombinant fusion protein ranges from 10 to 20 mg per litre cell culture. The high yield of recombinant Hev b 1 provides an additional antigen source for the latex allergy research. In the present study, the obtained MBP-rHev b 1 fusion protein was not only used in immunoblots and *in vivo* histamine release, it was also examined by the CAP method, a standardized diagnostic system for *in vitro* IgE determination. The immunoblot analysis with pooled (Fig. 1b,c) and single sera (Fig. 2) as well as the monoclonal antibodies (Fig. 2) revealed a distinct binding reactivity of the recombinant proteins with IgE and IgG antibodies, independent whether the carrier protein MBP was present or not.

The test results by the Pharmacia CAP-method using sera from 58 latex-sensitized SB patients (Fig. 3) confirmed that Hev b 1 is a most important latex allergen for children with SB. 54 (93%) of 58 sera previously showing IgE antibodies to native Hev b 1 exhibited positive IgE to the MBP-rHev b

1 fusion protein. Why the remaining four sera with low IgE-values to nHev b 1 (0.42–1.23 kU/L) did not show IgE-binding to rHev b 1 is unknown but it may have two reasons: First, the high background by the EAST for nHev b 1-test may give rise to a false positive result and second, the possible conformational difference between native and recombinant Hev b 1 may cause a different binding reactivity to IgE antibodies. In comparison with the results obtained by the EAST [7] in our previous work, a greater discrepancy was seen in the sera from HCWs. Only 16 (22.5%) out of 71 sera from latex-allergic HCWs showed IgE to rHev b 1. This result is more similar to the findings reported by other research groups [9,10] who tested nHev b 1 using sera from adult patients by immunoblotting. But the seropositive prevalence is much lower than 49% which was observed previously by EAST [7]. Even though the sera used in this study were different from those studied in previous work, the reason causing significant differences regarding to the IgE-binding reactivity to native and recombinant Hev b 1 by the different test methods should be further investigated. Nevertheless, our results by the SB patients and in the immunoblots indicate that rHev b 1 has the corresponding IgE-binding reactivity to nHev b 1. In addition, the use of the CAP method with rHev b 1 as solid phase antigen may provide a test with higher specificity and thus offers a reliable diagnostic tool for the determination of specific IgE to Hev b 1.

The fact that the IgE epitopes of Hev b 1 are located at different parts of the molecule was reported previously [6]. The IgE-binding determinants were found to be involved mainly in the C-terminal and in amino acid residues 30–64 of the Hev b 1 molecule. Results of the EAST experiment revealed that a synthetic peptide corresponding to 30–60 amino acid residues of Hev b 1 was recognized by IgE antibodies in 43 out of 58 sera from patients (11 HCWs and 47 SB patients) with confirmed IgE to Hev b 1 (our unpublished results). In order to show that the recombinant Hev b 1 may have the same property, we expressed Hev b 1 fragments lacking different parts of its N-terminal amino acid residues. Results of immunoblots using pooled and single sera showed that the deletion of the first 28 amino acid residues had obviously no general influence on its IgE-binding ability. In contrast, when the first 77 amino acid residues of Hev b 1 were absent, the fragment lost its IgE-binding capacity in two out of four tested SB patient sera. This indicates that in these two cases the IgE-binding epitopes are located in the first 77 amino acid residues of Hev b 1. Alternatively, the first 77 amino acid residues are important to the structure of an epitope located within the following sequence.

Since the Hev b 1 molecule is tightly associated with rubber particles in latex products, it is more difficult to wash it out during the leaching process in the production. It can be

assumed therefore that Hev b 1 may be the last one among the latex proteins which are leached out of latex products during the washing procedure. The Hev b 1 content in the products is consequently a useful marker for the evaluation of the allergenicity of rubber products. A two-site ELISA based on the use of two monoclonal IgG antibodies to Hev b 1 was recently developed for the quantification of Hev b 1 content in rubber products [13]. The production and isolation of highly purified recombinant MBP-rHev b 1 and the confirmation of its IgE and IgG binding reactivity corresponding to the native one provide thus a defined reference antigen which has a considerable advantage for the establishment of a standardized assay used by different research centres.

In conclusion, we could demonstrate by various applications that MBP-rHev b 1 has comparable allergenic properties like its natural counterpart and is therefore a useful tool for the evaluation of specific IgE in allergic patients.

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