Cloning, expression, and characterization of recombinant Hev b 3, a *Hevea brasiliensis* protein associated with latex allergy in patients with spina bifida*

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Background: Two natural rubber latex proteins, Hev b 1 and Hev b 3, have been described in spina bifida (SB)–associated latex allergy.
Objective: The aim of this study was to clone and express Hev b 3 and to obtain the immunologic active and soluble recombinant allergen for diagnosis of SB–associated latex allergy.
Methods: A complementary DNA (cDNA) coding for Hev b 3 was amplified from RNA of fresh latex collected from Malaysian rubber trees *(Hevea brasiliensis)*. PCR primers were designed according to sequences of internal peptide fragments of natural (n) Hev b 3. The 5´-end sequence was obtained by specific amplification of cDNA ends. The recombinant (r) Hev b 3 was produced in *Escherichia coli* as a 6xHis tagged protein. Immunoblotting and inhibition assays were performed to characterize the recombinant allergen.
Results: An Hev b 3 cDNA clone of 922 bp encoding a protein of 204 amino acid residues corresponding to a molecular weight of 22.3 kd was obtained. In immunoblots 29/35, latex-allergic patients with SB revealed IgE binding to rHev b 3, as did 4 of 15 of the latex-sensitized group. The presence of all IgE epitopes on rHev b 3 was shown by its ability to abolish all IgE binding to nHev b 3. Hev b 3 is related to Hev b 1 by a sequence identity of 47%. Cross-reactivity between these 2 latex allergens was illustrated by the large extent of inhibition of IgE binding to nHev b 1 by rHev b 3.
Conclusion: rHev b 3 constitutes a suitable in vitro reagent for the diagnosis of latex allergy in patients with SB. The determination of the full sequence of Hev b 3 and the production of the recombinant allergen will allow the epitope mapping and improve diagnostic reagents for latex allergy.
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Key words: *Latex allergy, spina bifida, recombinant Hev b 3, complementary DNA cloning, immunoblots, inhibition studies, cross-reactivity*

Latex is the milky sap produced by the laticiferous cells of the tropical rubber tree *Hevea brasiliensis.* IgE-mediated type I allergy to natural rubber latex proteins occurs predominantly in risk groups with a high degree of exposure to natural rubber latex products. These groups include health care workers (HCW), rubber industry workers, housekeeping personnel, and children with spina bifida (SB) and urogenital abnormalities.[1,2] Clinical manifestations of latex allergy include systemic urticaria, rhinitis, conjunctivitis, bronchospasm, and anaphylaxis.

The prevalence of latex allergy is especially high among patients with SB. The rate of latex sensitization in this population is between 29% and 72%.[3-6] Because of a very early and high degree of exposure to natural rubber proteins through repeated surgery, children with SB are especially prone to sensitization to latex. Whether repeated exposure or a suspected genetic predisposition is the main factor for sensitization remains to be clarified.[6] Despite applying processing strategies to reduce the protein content of the latex end products, enough allergens remain to elicit type I allergy in sensitized patients.[7]

Two *Hevea* latex allergens, Hev b 1 and Hev b 3, have been found to be associated with SB. These 2 proteins are frequently recognized by IgE of SB patients with latex allergy and less often by IgE of latex-allergic HCWs.[8,9] Hev b 1 was identified as the 14.6 kd rubber elongation factor by sequencing tryptic peptides of the purified allergen.[10] Hev b 1 was cloned and its nucleotide sequence determined.[11]

The second SB-associated latex allergen, Hev b 3, has been reported with different molecular weights by several researchers. IgE antibodies in 83% of US patients with SB and in 67% of Finnish patients with SB recognized a 27 kd protein from *Hevea* latex.[9] Sequences of 6 tryptic peptides showed similarities to sequence stretches within the first 130 amino acid residues of Hev b 1. Lu et al[12] isolated a 23 kd protein from the clear liquid that remains after the autocoagulation of rubber particles without the addition of ammonia. The 90 amino acids sequenced from this 23 kd protein showed 45% similarity with Hev

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*The complete cDNA sequence of Hev b 3 is available from the European Molecular Biology Laboratory database under the accession number AJ223388.

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Abbreviations used

EMBL:	European Molecular Biology Laboratory
HCW:	Health care worker
nHev b 3:	Natural Hev b 3
Ni-NTA:	Nickel nitrilotriacetic acid
PCS:	Pharmacia CAP system
pI:	Isoelectric point
PR:	Pathogenesis related
RAST:	Radioallergosorbent test
rHev b 3:	Recombinant Hev b 3
SB:	Spina bifida
SPT:	Skin prick test

b 1 and shared identical sequence motifs with the 27 kd protein described by Alenius et al.[13] The purified 23 kd polypeptide reacted with IgE from 76% of latex-allergic SB patients and induced proliferation of PBMCs in latex-allergic HCWs and patients with SB.[12]

Yeang et al[8] described a 24 kd protein isolated from small rubber particles that was recognized by IgE from latex-allergic patients with SB and named it Hev b 3. A comparison of amino acid sequences of tryptic peptides between the 27 kd allergen,[13] the 23 kd allergen,[12] and Hev b 3 isolated by Yeang et al[14] from Malaysian and Costa Rican latex showed a good consensus in all fragments studied. Mass spectrometry of Hev b 3 gave several species of molecular masses differing by multitudes of 266 d and starting at 22,258 d.

Identifying patients at risk is an essential step before carrying out surgical interventions that entail latex exposure. Testing requires the availability of reliable, allergen-containing preparations. In particular, patients with SB would profit from a more selective diagnostic screening with well-defined recombinant allergens. The availability of recombinant latex allergens would also make specific immunotherapy of the disease possible. In this study we report the nucleotide and the deduced amino acid sequence of the complementary DNA (cDNA) encoding Hev b 3 and describe the cloning and expression of this recombinant molecule in *Escherichia coli* and its characterization as the 23 kd latex allergen by immunoblots and inhibition experiments.

METHODS

Isolation of total RNA from latex

Fresh latex was collected from regularly tapped Malaysian rubber trees (*H. brasiliensis*, clone RRIM 600). Latex exuding from the tapped trees was collected while it was continuously mixed with an equal volume of RNA extraction buffer (0.1 mol/L tris–hydrochloric acid, 0.3 mol/L lithium chloride, 1 mmol/L EDTA, 10% SDS, pH 9.5) at ambient temperature. This mixture was centrifuged at 100,000*g* for 30 minutes at 15°C. The rubber plug was removed, the aqueous phase was extracted with chloroform/phenol, and total RNA was precipitated with LiCl.

Reverse transcription-PCR amplification and sequencing of Hev b 3

First strand cDNA synthesis was performed by reverse transcribing 0.5 μg of total latex RNA using a modified oligo-dT primer, T_{25}NN (5′-GGAGAAGGA$(T)_{25}$(A/G/C)N-3′). For PCR, 10 μL of the first strand cDNA synthesis was used to amplify part of the coding sequence for Hev b 3. A degenerate primer BW1 was designed according to sequence similarities between Hev b 1 and published peptide fragments of Hev b 3.[12,13] The peptide sequence KYLDFV was chosen (Fig 1, in boldface). The primers used to amplify a fragment of the Hev b 3 cDNA were BW1 (5′-AAGCTTAA(A/G)TA(C/T)TIGA(C/T)TT(C/T)GT-3′, *Hin*dIII restriction site underlined, I stands for inosine) and T_{25}NN. PCR products were cloned into the pCR 2.1 vector (TA Cloning Kit, Invitrogen, NV Leek, Netherlands) in XL-1Blue *E. coli* cells according to the vendor's instructions. The PCR products were sequenced using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Life Science, Little Chalfont, England) with the fluorescent primers fT7 forward and fM13 reverse flanking the vector's multiple cloning site.

To complete the missing 5′-portion of the cDNA, the 5′-AmpliFINDER RACE Kit (Clontech, Palo Alto, Calif) was used according to the vendor's instructions. cDNA synthesis was performed with the T_{25}NN primer, PCR after anchor ligation with the kit's AmpliFINDER anchor primer and the gene-specific internal primer BW4 (5′-AGGTAATGACCGCATATTGCTCAGC-3′, Fig 1, underlined). PCR products were cloned and sequenced as previously described.

The complete cDNA sequence and its deduced amino acid sequence were compared with the European Molecular Biology Laboratory (EMBL) and Swiss-Prot databases.

Construction of the expression plasmid

The complete coding sequence of Hev b 3 was amplified by reverse transcription-PCR from total latex RNA. The primers used in the PCR were BW6 (5′-GATTGGATCCATGGCTGAAGAGGTGGAG-3′, BamHI site underlined) and BW7 (5′-GATCAAGCTTTTATGAATGCCTCATCTCCAAACAC-3′, HindIII restriction site underlined) according to the 5′- and 3′-end of the latex Hev b 3 cDNA (EMBL AJ223388) determined in the previous experiments (Fig 1, *underlined*). The resulting 627 bp long PCR product was ligated into the *Bam*HI and the *Hin*dIII sites of pQE-40 expression vector (Qiagen, Hilden, Germany), which provides a sequence coding for a hexahistidyl (6xHis) affinity tag. The expression construct pQE-40/Hev b 3 was transformed into competent M15[pREP4] *E. coli* cells.

Expression and purification of recombinant Hev b 3

For the expression of Hev b 3, 2 L super broth culture media containing both ampicillin and kanamycin were inoculated with 20 mL of an overnight culture and grown with vigorous shaking to an OD_{600} of 0.7. Expression of Hev b 3 was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 2 mmol/L. After growing for an additional 4 hours at 37°C, the cells were harvested, resuspended in 50 mL denaturing buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L tris, pH 8.0) and stirred at room temperature for 3 hours at 200 rpm. After centrifugation for 30 minutes at 15°C at 20,000 rpm, the highly viscous lysate was treated 3 times with Biocryl BPA-1000 (final dilution 1:100; Toso HAAS, Montgomeryville, Pa).

To purify recombinant Hev b 3, (rHev b 3), 4 mL of 50% w/v nickel nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) were added to the *E. coli* lysate. The batch was stirred for 2 hours at room temperature at 120 rpm, packed into a column, and washed with 25 mL denaturing buffer and 25 mL washing buffer 1 (same composition as denaturing buffer, but pH 6.5) at a flow rate of 0.5 mL/min. By further reducing the pH, the bound protein was

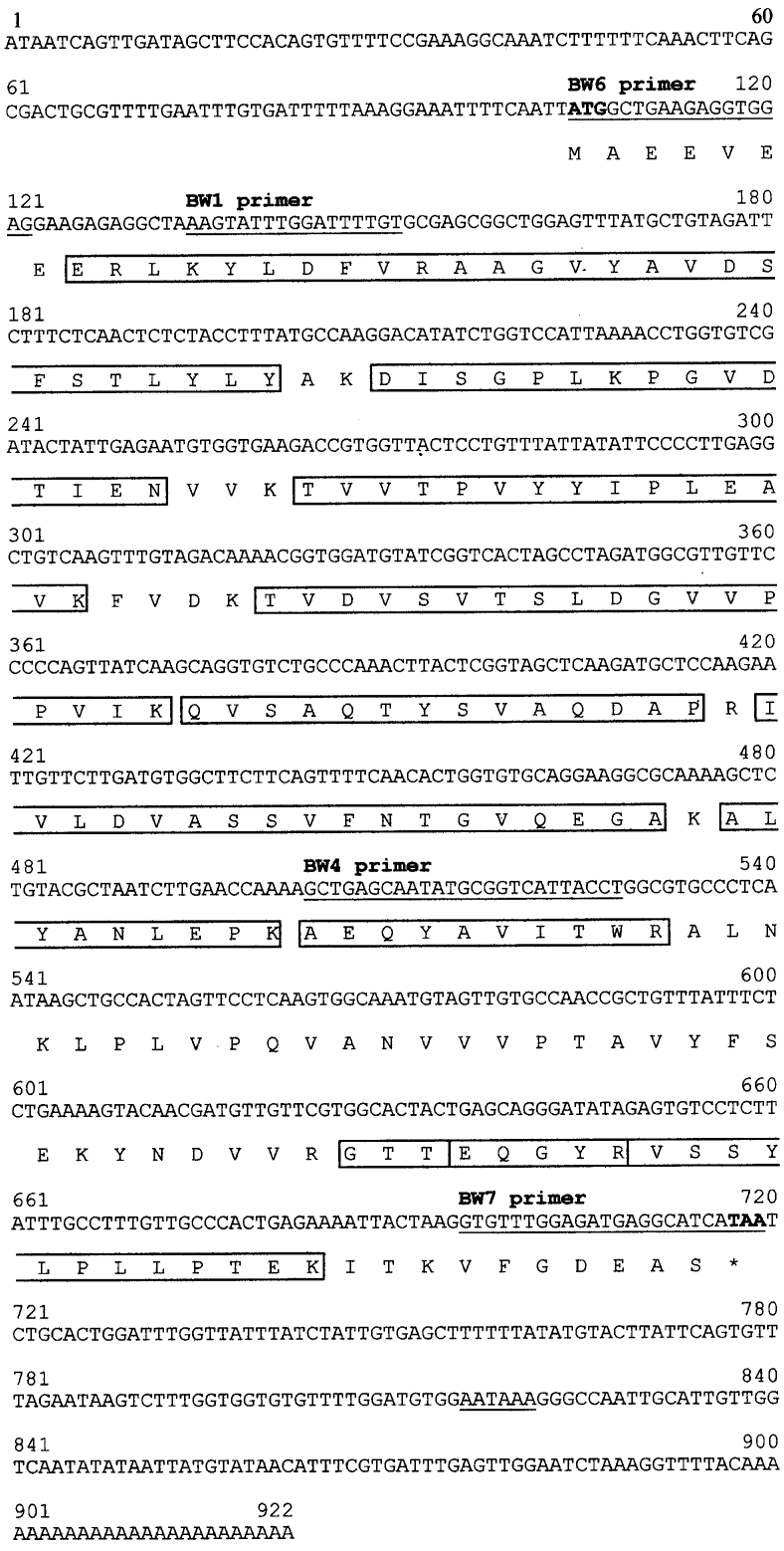

FIG 1. Complete cDNA and deduced amino acid sequences of Hev b 3. The peptide sequence used for the design of the degenerate primer BW1 is in boldface. Positions of all other primers used for the generation of the Hev b 3 sequence and the poly(A)-signal are underlined. Start and the stop codons are in boldface. Sequences of tryptic peptide fragments of the natural 23 kd latex allergen are boxed.[16,17] The stop is indicated by an asterisk. These sequence data are available from the EMBL/GenBank databases under the accession number AJ223388.

eluted with 20 mL washing buffer 2 (same composition as denaturing buffer, but pH 6.0), then with 15 mL elution buffer (same composition as denaturing buffer, but pH 5.5). The course of the purification of rHev b 3 from *E. coli* lysate was monitored by SDS-PAGE on 12% gels.

Refolding of rHev b 3

Pooled fractions of purified rHev b 3 (pH adjusted to 8.0) were mixed with 7 mL 50% Ni-NTA agarose following the batch procedure previously described. The refolding of denatured rHev b 3 immobilized on a Ni-NTA agarose column was carried out using a linear 6 mol/L to 1 mol/L urea gradient in refolding buffer (500 mmol/L sodium chloride, 20% glycerol, 20 mmol/L tris–hydrochloric acid, pH 7.4). The renaturation took place over a period of 2.5 hours at a flow rate of 0.5 mL/min. The refolded Hev b 3 was washed with 1 mol/L urea in refolding buffer containing 100 mmol/L imidazole (Imidazole) and eluted with 1 mol/L urea refolding buffer containing 200 or 300 mmol/L Imidazole. Renatured Hev b 3 was dialyzed against PBS.

Preparation of latex C-serum

Fresh *Hevea* latex was collected in chilled containers from rubber trees (*Hevea brasiliensis* RRIM 600 clone) and then centrifuged at 44,000*g* at 4°C for 1 hour. The aqueous phase, the C serum, was collected and the centrifugation was repeated. The clear aqueous C serum was freeze-dried and stored at –20°C.

Study population

Sera from 50 patients with SB aged 7.1 to 22.6 years (median, 10.6 years) were included in this study. Total IgE and latex-specific IgE was determined from sera by the Pharmacia CAP system (PCS) (Pharmacia Upjohn, Uppsala, Sweden).[15] Skin prick tests (SPT) were performed with a commercial latex SPT extract (Alyostal ST-IR prick test standardise: Latex 0903, lot 61893, Stallergènes, Fresnes, France). Sodium hydrochloride served as a negative control and histamine (10 mg/mL) served as a positive control. Provocation tests with latex gloves were performed as described.[16,17] After having washed their hands, patients put one wet hand into a latex glove (Sempermed classic, Lot YE5F7, Semperit, Vienna, Austria) and, as a control, they put their other wet hand into a neoprene glove (Dermaprene, Ansell, Munich, Germany). After wearing these gloves for 30 minutes (or upon showing clinical symptoms), the gloves were taken off and possible clinical reactions were assessed by a physician. Hands were washed again. The single-blind test was assessed as positive if typical immediate-type clinical reactions such as urticaria or Quincke's edema occurred. If the test results were negative, a glove was blown up as an additional test. Other studies have demonstrated significant high allergen levels in the Sempermed classic brand of gloves (more than 500 μg/g).[18,19]

Eleven healthy adults (6 women and 5 men) aged 25 to 39 years with no known allergies served as donors for a nonatopic control serum pool. In addition, 2 atopic patients with high IgE levels for house dust mite (radioallergosorbent test [RAST] = 6) or birch pollen (RAST = 5) but no latex-specific IgE were used as control subjects. Informed consent was obtained from parents of all patients younger than age 18 years.

Immunoblot analysis of latex-allergic SB patients with rHev b 3 and latex C serum extracts

Two μg rHev b 3 or 75 μg latex C serum extract per lane were separated on 12% SDS-PAGE gels, blotted onto nitrocellulose membranes, and treated with blocking buffer (40 mmol/L Na_2HPO_4, 7 mmol/L NaH_2PO_4, 0.5% w/v BSA, 0.05% w/v sodium azide, 0.5% w/v Tween 20, pH 7.5). The membranes were then incubated with patients' sera or plasma (sera diluted in blocking buffer 1:6 or 1:8, plasma diluted 1:2) overnight at 4°C. After washing with blocking buffer, the blots were incubated with iodine radiolabeled 125 anti-human IgE (IBL, Hamburg, Germany) overnight. Binding of patients' IgE to the allergens was visualized on Biomax MS film (Kodak, NY).

Inhibition of IgE binding to latex C serum with patients' sera preincubated with rHev b 3

Patients' sera were preincubated with 20 μg rHev b 3 overnight at 4°C. Seventy-five μg latex C serum proteins per lane were blotted onto a nitrocellulose membrane. The membrane was treated with blocking buffer as previously mentioned and incubated overnight at 4°C with patients' sera previously inhibited with rHev b 3. Afterward, the blots were incubated with ^{125}I-labeled anti-human IgE overnight.

RESULTS

Cloning and sequence analysis of Hev b 3

Degenerate primers designed according to tryptic peptide sequences of Hev b 3 and sequence similarities with Hev b 1 were used to amplify most of the coding region (amino acids 11 to 204) and the complete 3´ end of a Hev b 3 cDNA by reverse transcription-PCR from total latex RNA. The cDNA sequence was completed at the 5´ end using a protocol to amplify cDNA ends by PCR. The complete cDNA of Hev b 3 consists of 922 bp and contains an open reading frame from bases 105 to 719 (Fig 1). The putative polyadenylation signal AATAAA (Fig 1, *underlined*) is present at nucleotide positions 816-821 and 76 bases upstream of the poly(A) sequence. The 5´- and 3´ untranslated regions are 104 bp and 179 bp long, respectively. The complete cDNA sequence of Hev b 3 is available from the EMBL database under the accession number AJ223388.

The Hev b 3 cDNA sequence codes for an acidic protein of 204 amino acid residues with a calculated isoelectric point (pI) of 4.6 and a calculated molecular mass of 22.3 kd. No consensus glycosylation site could be detected in the deduced amino acid sequence, which also did not contain any cysteine residues. All published proteolytic peptide fragments[12,13] (Fig 1, *boxed*) of the proposed Hev b 3 allergen fit into the deduced amino acid sequence of the Hev b 3 cDNA clone, which confirmed that the cloned cDNA encodes the described Hev b 3 latex allergen.

The deduced amino acid sequence of Hev b 3 was compared with sequences in the SWISS-PROT database. Hev b 3 showed an overall identity of 47% to Hev b 1, the rubber elongation factor from *Hevea* latex (SWISS-PROT P15252, Fig 2, *A*). Hev b 3 also shares an identity of 54% with the stress-related protein PvSRP from kidney bean (EMBL U54704, Fig 2, *B*), which, as stated in the database entry, was regulated by heavy metal stress, wounding, and virus infection.

Expression and purification of rHev b 3

The coding region of Hev b 3 was ligated into the expression vector pQE-40, and rHev b 3 was produced as

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A
Hev b 3    1 MAEEVE.....EERLKYLDFVRAAGVYAVDSFSTLYLYAKDISGPLKPGV  45
Hev b 1    1 ---DEDNQQGQG-G----G--QD-AT---TT--NV--F---K----Q---  50

Hev b 3   46 DTIENVVKTVVTPVY....YIPLEAVKFVDKTVDVSVTSLDGVVPPVIKQ  91
Hev b 1   51 -I--GP--N-AV-L-NRFS---NG-L----S--VA---II-RSL--IV-D 100

Hev b 3   92 VSAQTYSVAQDAPRIVLDVASSVFNTGVQEGAKALYANLEPKAEQYAVIT 141
Hev b 1  101 A-I-VV-AIRA--EAARSL---.LPGQTKIL--VF-GEN*            139

Hev b 3  142 WRALNKLPLVPQVANVVVPTAVYFSEKYNDVVRGTTEQGYRVSSYLPLLP 191

Hev b 3  192 TEKITKVFGDEAS* 204

B
Hev b 3    1 MAEEVEEERLKYLDFVRAAGVYAVDSFSTLYLYAKDISGPLKPGVDTIEN  50
PvSRP      1                          MRCAI--S---ERA-------N-V-D  26

Hev b 3   51 VVKTVVTPVY....YIPLEAVKFVDKTVDVSVTSLDGVVPPVIKQVSAQT  96
PvSRP     27 A-----A---DRFHLV-V-LL-YA-RK-G....E--RH--SNV-K--S-A  72

Hev b 3   97 YSVAQDAPRIVLDVASSVFNTGVQEGAKALYANLEPKAEQYAVITWRALN 146
PvSRP     73 R--VSEVR-...........D--STF--TV-SKY--T---C--SA--K-- 111

Hev b 3  147 KLPLVPQVANVVVPTAVYFSEKYNDVVRGTTEQGYRVSSYLPLLPTEKIT 196
PvSRP    112 Q---F-----A-L-K-A-CT----E-IVSSA-K-----A----V-----A 161

Hev b 3  197 KVFGDEAS* 204
PvSRP    162 ---SGN*   167
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FIG 2. Amino acid sequence alignments of Hev b 3 to homologous proteins. *A,* Hev b 3 shows 47% identity to Hev b 1, another SB-associated latex allergen. *B,* Hev b 3 is 54% identical to the stress-related protein PvSRP from kidney bean *(Phaseolus vulgaris).* Amino acids identical to Hev b 3 are indicated by dashes; gaps in the sequence alignment are indicated by dots.

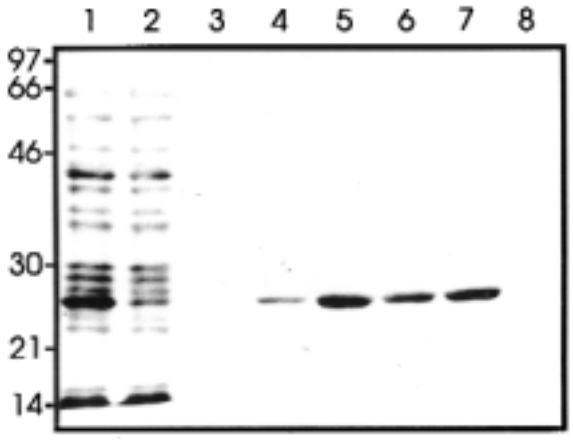

FIG 3. Purification of rHev b 3 from transformed *E. coli* M15 cells. Protein fractions at different stages of purification of rHev b 3 on an Ni-NTA agarose column were loaded onto a 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. Total protein extracted from *E. coli* (lane 1) flow through from the column (lane 2), washing step 1 (lane 3), washing step 2 (lane 4), fractions eluted at pH 5.5 (lanes 5-7), control of completion of elution (lane 8). Molecular weight markers in kd are indicated.

a nonfusion protein with a hexahistidyl tag. After induction of the transformed M15 *E. coli* cells with 2 mmol/L isopropyl β-D-thiogalactopyranoside for 4 hours, rHev b 3 was detected by SDS-PAGE after disrupting the cells with 8 mol/L urea. The recombinant protein was purified under denaturing conditions by Ni-NTA affinity chromatography. Protein samples from consecutive steps of purification are shown in Fig 3.

Pooled fractions of rHev b 3 were refolded while being immobilized on Ni-NTA agarose. The 2 consecutive steps of rHev b 3 on Ni-NTA agarose yielded a product that was free of contaminating *E. coli* proteins when checked on a silver-stained protein gel (data not shown). The recombinant protein was dialyzed against PBS. rHev b 3 remained soluble in PBS after dialysis and was stable in solution when kept at –20°C for at least 4 months. The amount of soluble rHev b 3 that could be purified from *E. coli* cells grown in 1 L super broth medium was 3.7 mg.

Evaluation of the study population and immunoblot analysis of rHev b 3

Fifty latex-sensitized patients with SB were included in this study (Tables I and II). Total IgE ranged from 4.0 kU/L to 1865.0 kU/L (median, 119.0 kU/L). Latex-specific IgE in serum ranged from 0.4 kilounits (kU)/L to 98.6 kU/L (median, 6.0 kU/L). Of the 50 latex-sensitized patients with SB, 35 showed a positive response to the glove provocation test (latex-allergic SB group, Table I) whereas 15 patients with SB were sensitized but had no response to provocation (latex-sensitized SB group, Table II). The clinical symptoms observed were local urticaria, except for one patient who showed mild generalized urticaria (patient 29, Table I). With use of the latex SPT extract of Stallergènes, 18 of 35 latex-allergic patients with SB (51%) and 4 of 15 latex-sensitized patients with SB (27%) had positive results (Tables I and II). The serum pool of 11 nonatopic control subjects showed no specific IgE to latex in SPT.

The ability of rHev b 3 to bind IgE was evaluated by immunoblots with sera from the 35 latex-allergic patients with SB (Table I; Fig 4, *A*) and 15 latex-sensitized patients with SB (Table II; Fig 4, *B*). Twenty-nine of 35 latex-aller-

TABLE I. Characterization of the study population consisting of 35 latex-allergic patients with SB

Patient No.	Age (y)	Sex	SPT	Total IgE (kU/l)	Latex-specific IgE (kU/l)	Latex CAP class	IgE to rHev b 3
1	17	M	–	194	0.7	2	–
2	12	F	+	117	2.7	2	+
3	18	M	–	107	4.2	3	+
4	10	F	–	44	2.0	2	–
5	11	M	+	182	5.3	3	+
6	8	M	+	970	24.0	4	+
7	15	M	+	43	2.0	2	+
8	13	F	+	33	1.1	2	+
9	8	F	+	68	12.4	3	+
10	11	M	+	691	13.0	3	+
11	10	M	–	85	26.0	4	+
12	6	M	+	458	21.7	4	+
13	10	F	+	53	6.8	3	+
14	11	F	–	112	35.9	4	+
15	18	M	+	1318	18.9	4	+
16	7	M	+	286	45.1	4	+
17	8	M	–	46	1.0	2	+
18	22	M	–	114	6.6	3	+
19	12	M	–	121	1.1	2	+
20	11	M	+	176	46.4	4	+
21	8	M	+	84	7.0	3	+
22	11	M	–	926	14.0	3	–
23	6	M	–	318	6.0	3	–
24	4	M	–	140	4.8	3	+
25	20	M	–	75	0.5	1	–
26	10	F	–	58	22.8	4	+
27	10	M	+	1865	98.6	5	+
28	7	M	–	221	19.5	4	+
29	21	F	+	218	42.3	4	+
30	7	M	–	112	25.8	4	+
31	23	M	+	238	22.1	4	+
32	11	M	+	353	9.4	3	–
33	13	F	–	449	16.7	3	+
34	14	M	–	69	6.0	3	+
35	14	M	+	1170	31.7	4	+

SPT, Skin prick test.

gic patients with SB, that is, 83%, revealed IgE binding to rHev b 3, as did 4 of 15 (27%) of the latex-sensitized SB group. Patients from both groups who had latex PCS classes ≥2 but showed no IgE binding to rHev b 3 were tested with latex C serum as a control for our Hev b 3 immunoblot data. None of these patients revealed reactivity to natural Hev b 3 (nHev b 3) (data not shown). The serum pool of 11 nonatopic control subjects showed no IgE binding to rHev b 3. The sera of the 2 atopic control subjects with high IgE levels to house dust mite (RAST = 6) or birch pollen (RAST = 5) showed no IgE binding to rHev b 3 in immunoblot studies (data not shown).

Inhibition of IgE-binding to nHev b 3 in latex C serum with rHev b 3

Nine rHev b 3 positive sera were selected for immunoblot and inhibition studies on the basis of their positive SPT results (see Table I, patient Nos. 2, 7, 13, 21, 29) or negative SPT results (see Table I, patient Nos. 18, 14, 26; Table II, patient No. 10) and to include a variety of PCS classes (PCS 2-4). All 9 sera displayed IgE antibodies reacting with the 23 kd latex allergen Hev b 3, and 8 of 9 also displayed IgE antibodies reacting with the 14 kd latex allergen Hev b 1 (Fig 5, *A*). We found that the SPT results failed to diagnose latex sensitization in cases in which rHev b 3 was positive. In addition, 7 sera had IgE directed to latex allergens in the 40 to 100 kd molecular weight range (Fig 5, *A*, lanes 2, 3, 5-9).

TABLE II. Characterization of the study population consisting of 15 latex-sensitized patients with SB

Patient No.	Age (y)	Sex	SPT	Total IgE (kU/l)	Latex-specific IgE (kU/l)	Latex CAP class	IgE to rHev b 3
1	14	M	–	244	0.4	2	+
2	6	F	+	24	2.5	2	–
3	21	M	–	4	1.9	3	–
4	15	F	–	1181	14.3	2	–
5	7	F	–	35	0.9	1	–
6	15	F	–	122	0.4	3	–
7	13	F	+	26	4.12	2	–
8	12	F	–	404	1.8	2	–
9	15	F	–	60	2.2	2	–
10	23	F	–	132	1.7	2	+
11	11	M	–	105	0.9	2	–
12	14	F	+	24	1.6	2	–
13	12	M	+	178	26.5	4	+
14	16	F	–	8	0.9	2	+
15	36	M	–	27	0.6	1	—

SPT, Skin prick test.

For inhibition studies, blotted latex C serum proteins were probed with the same set of sera preincubated with 20 μg rHev b 3. IgE-binding to nHev b 3 was totally abolished in 8 of 9 patients (Fig 5, *B*, lanes 1-4 and 6-9). In the case of one patient, the amount of rHev b 3 used was insufficient to block all IgE directed to Hev b 3 (Fig 5, *B*, lane 5). Using 100 μg rHev b 3 in the preincubation, IgE binding to nHev b 3 was completely inhibited (data not shown). Based on the presence of structurally similar epitopes on Hev b 1 and Hev b 3, rHev b 3 was able to block IgE binding to nHev b 1 completely in 4 of 8 patients (Fig 5, *B*, lanes 4, 5, 8, 9). By increasing the dose of rHev b 3 to 100 μg per inhibition, IgE binding to nHev b 1 could be further reduced but not totally abolished (data not shown). IgE binding to higher molecular weight allergens was diminished by rHev b 3 in 5 of 9 patients (Fig 5, *B*, lanes 2, 5, 6, 8).

DISCUSSION

Here we report the cloning and sequencing of Hev b 3, an important *Hevea* latex protein that is a major allergen in SB-associated latex allergy. We have produced Hev b 3 as a recombinant nonfusion protein in *E. coli* and demonstrate that the molecule possesses IgE reactivity, which is comparable to nHev b 3. The presence of IgE epitopes on rHev b 3 could be proved by IgE immunoblots and immunoblot inhibition studies (Figs 4 and 5).

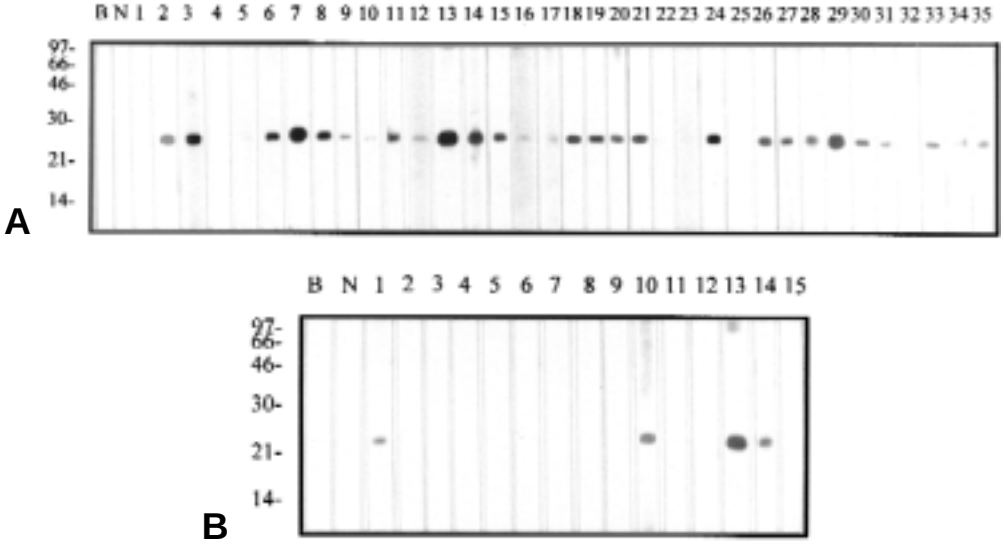

FIG 4. Immunoblot analysis of recombinant Hev b 3 with 50 sera of latex-sensitized patients with SB as listed in Tables I and II. Molecular weight markers are indicated in kd. B: Buffer control without addition of serum. N: Normal human serum pool. **A**, IgE reactivity to recombinant Hev b 3 of 35 sera from latex-allergic patients with SB as listed in Table I. **B,** IgE reactivity to recombinant Hev b 3 of 15 sera from latex-sensitized patients with SB as listed in Table II.

The specificity of IgE binding to rHev b 3 was determined by 2 atopic patients' sera with high IgE levels specific for house dust mite or birch pollen but not to latex. In each case, no IgE binding to rHev b 3 was observed. In addition, a serum of a latex-allergic SB patient with 926 kU/L total IgE (Table I, patient No. 22) and 2 sera of SB patients sensitized to latex (Table II, patient No. 4: 1181 kU/L total IgE; patient No. 8: 404 kU/L total IgE) did not show IgE binding to rHev b 3.

Although Hev b 3 and Hev b 1 are not the only allergens responsible for latex allergy in patients with SB, we are of the opinion that diagnosing 100% of latex-allergic SB patients with these 2 proteins alone is possible. In this study, we have not identified any patient with SB who was either sensitized or allergic to latex who did not have IgE antibodies directed against either Hev b 1 or Hev b 3 (Fig 5, *A*). From this finding, we conclude that these 2 proteins will be sufficient to screen patients with SB for latex sensitization. All other allergens, mainly in the higher molecular weight range, are present in addition to Hev b 1 and Hev b 3. The molecular cloning and sequencing of a Hev b 3 cDNA made the production of the recombinant allergen possible.

Because of the presence of proteases in latex extracts, nHev b 3 has been reported to fragment into several distinct polypeptides of molecular weights from 24 kd to about 5 kd even when stored at –20°C.[8] Exposure to ammonia, which is added to commercial latex, may be an indirect factor for the fragmentation of nHev b 3, because ammonia ruptures the lutoids containing proteases of the B serum, which also causes the fragmentation of nHev b 3.[8] Therefore, the production of the commercially available latex extracts for diagnosis with reproducible amounts of certain latex allergens is problematic. Purified rHev b 3 is free of specific proteases and therefore much more stable than nHev b 3 present in a latex extract (eg, a SPT reagent). In our study, a commercial latex extract failed to produce a positive SPT reaction in 15 of 33 patients (45%) who possessed IgE that bound to rHev b 3 in immunoblot experiments (Tables I and II). Twenty-nine of 35 latex-allergic patients with SB (83%) were identified by immunoblots using rHev b 3, whereas SPT results with the commercial extract were only positive in 18 of 35 (51%) of these latex-allergic patients with SB (Table I).

Purified rHev b 3 is soluble and stable in PBS, a physiologic buffer system, and will therefore constitute an excellent tool for in vivo tests such as SPT or other diagnostic provocation tests. Moreover, it will be possible to use rHev b 3 for in vitro investigations such as antibody (IgE-) determinations or T-cell proliferation studies. rHev b 3 possesses all B cell epitopes that are present on nHev b 3, because it is able to inhibit patients' IgE-binding to nHev b 3 in all cases studied (Fig 5, *B*).

Hev b 3 shares 47% sequence identity with Hev b 1 on the amino acid level, which results in structural similarities between the two molecules. Interestingly, both allergens also possess a blocked N-terminus.[13,20] Although Hev b 3 is 7.7 kd larger than Hev b 1, areas of sequence identity between the 2 allergens are distributed over the full length of Hev b 1 (Fig 2, *A*), which leads to the formation of similar B-cell epitopes. The immunologic relationship between Hev b 3 and Hev b 1 is demonstrated by the fact that rHev b 3 is able to abolish (Fig 5, *B*, lane 4, 5, 8, 9) or diminish (Fig 5, *B*, lanes 2, 3, 6, 7) IgE binding to nHev b 1. However, in some cases, IgE binding to nHev b 1 was not influenced by preincubating sera with rHev b 3, indicating the presence of epitopes unique to

Hev b 1. In addition, rHev b 3 reduced IgE binding to several latex allergens of higher molecular weight (Fig 5, *B,* lanes 2, 5, 6, 8). Multimeric forms of Hev b 1 have been described.[10] These multimeric forms may be the explanation for the inhibitions observed in this molecular weight range of the immunoblots.

The calculated molecular mass of the deduced amino acid sequence encoded by the Hev b 3 cDNA is 22.3 kd, in accordance with the molecular masses determined for several nHev b 3 species from *Hevea* latex by mass spectrometry, that is, 22 258, 22 524, 22 791, and 23 058 d.[14] The gaps of about 266 d between the different forms of the protein were interpreted by the authors as possible additions of stearolyl groups.[14] The sequence of Hev b 3 does not contain N-glycosylation sites and therefore leaves only limited room for modifications of the molecular weight by post-translational mechanisms, which is in contrast to the earlier reported molecular mass of 27 kd. However, several other researchers have pinpointed the molecular mass to 23 to 24 kd.[8,12] The calculated pI of 4.6 of the deduced amino acid sequence of Hev b 3 is in good agreement with a study of Alenius et al[13] describing a 2-dimensional electrophoresis of the 27 kd Hev b 3 protein revealing one spot with a pI of 4.8.

Hev b 3 shares 54% sequence identity on the amino acid level with the stress response protein PvSRP isolated from kidney bean (EMBL U54704, Fig 2, *B*). This protein was reported in the database entry to be induced by heavy metal ions, wounding, and virus infection. Proteins that are involved in a plant's response to wounding, microbial attack, or environmental stress are classified into 11 families of pathogenesis-related (PR) proteins.[21] It is interesting to note that many plant allergens belong to 1 of the 11 PR families. Examples include allergens such as Bet v 1 from the pollen of white birch and its homologues from apple, celery, and cherry (PR-10 family[22]), as well as the thaumatin-like and osmotin-like allergens of cherries and bell peppers (PR-5 family[23,24]). Hev b 2, a β-1,3-glucanase, and Hev b 6, (pro-) hevein, 2 allergens from *Hevea* latex, are also related to PR proteins; the first belongs to the PR-2 family[25] and the second to the PR-4 family.[26] The sequence identities of Hev b 3 to Hev b 1 and PvSRP of kidney bean imply that Hev b 3 has functions in the synthesis of rubber, as well as in the plant's defense response.

We thank Nyu Ping Chew for technical assistance in *Hevea* RNA isolation.

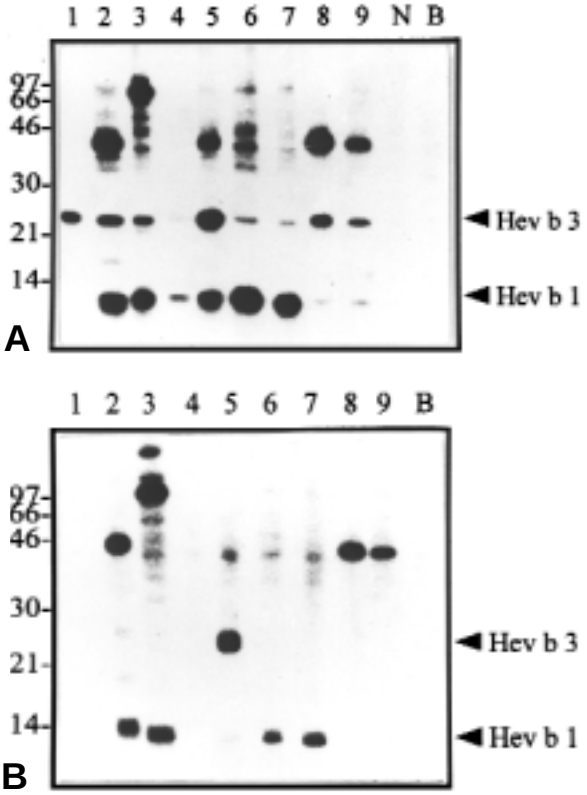

FIG 5. Immunoblot and inhibition experiments of latex C serum allergens with sera of 9 selected latex-sensitized and rHev b 3–positive patients with SB. *Lane 1* shows a patient with negative results of a provocation test (patient 10, Table II), whereas *lanes 2-9* show patients with positive results of provocation tests: patient 2 *(lane 2),* patient 7 *(lane 3)*, patient 13 *(lane 4),* patient 18 *(lane 5),* patient 21 *(lane 6)*, patient 14 *(lane 7)*, patient 26 *(lane 8)*, and patient 29 *(lane 9)*. Patient numbers refer to Table I. **B**: Buffer control without addition of serum. *N*: Normal human serum pool. Molecular weight markers in kd are indicated. IgE binding to Hev b 3 and Hev b 1 are indicated by *arrows.* **A,** IgE binding to allergens present in latex C serum. **B,** The inhibition of IgE binding to allergens present in latex C serum after preincubation of the sera with 20 μg rHev b 3.

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