

Hypoallergenic Variants of the Major Latex Allergen Hev b 6.01 Retaining Human T Lymphocyte Reactivity¹

Alexander C. Drew,^{2*†‡} Nirupama P. Eusebius,^{*†‡} Linda Kenins,^{*†‡} Harini D. de Silva,^{*†‡} Cenk Suphioglu,^{*†‡} Jennifer M. Rolland,^{*‡} and Robyn E. O’Hehir^{*†‡}

Hev b 6.01 is a major allergen of natural rubber latex with sensitization of 70–86% of latex glove-allergic subjects. Recently, we mapped the immunodominant T cell sites of Hev b 6.01 to the highly IgE-reactive hevein (Hev b 6.02) domain. Hev b 6.01 contains 14 cysteine residues with multiple disulphide bridges stabilizing tertiary conformation. With the goal of a standardized specific immunotherapy we developed hypoallergenic Hev b 6.01 mutants by site-directed mutagenesis of selected cysteine residues (3, 12, 17, and 41) within the Hev b 6.02 domain. Peptides corresponding to the Hev b 6.02 domain of two of the mutants were also synthesized. These mutants and peptide variants showed markedly decreased or ablated latex-allergic patient serum IgE binding by immunoblotting and ELISA. Basophil activation testing confirmed markedly decreased activation with successive cysteine substitutions of the mutants and complete abrogation with the Hev b 6.02 (Cys 3, 12, 17, 41 Ala) peptide. Retention of T cell reactivity is crucial for effective specific immunotherapy and all mutants and peptide variants maintained their latex-specific T cell reactivity. The ablated allergenicity but retained T cell reactivity of the Hev b 6.02 (Cys 3, 12, 17, 41 Ala) peptide suggests this peptide is a suitable candidate for inclusion in a latex immunotherapy preparation. *The Journal of Immunology*, 2004, 173: 5872–5879.

Latex allergy is a major cause of occupational asthma among health care workers (HCW)³ (1), largely associated with the use of latex gloves to minimize infection risk in the workplace. Avoidance of exposure and symptomatic treatment for adverse reactions form the mainstay of management for latex allergy with no currently available licensed extract for specific immunotherapy. The extreme anaphylactic potential of currently available unmodified natural rubber latex extracts has limited trials of conventional latex specific immunotherapy due to the high incidence of adverse events (2–8).

With the increasing number of allergens cloned and expressed as recombinant proteins, substantial interest has been shown in the development of hypoallergenic molecules for immunotherapy (9, 10). Allergens mutated to remove the B cell epitopes can prevent cross-linking of IgE on allergic effector cells, including mast cells and basophils, and offer a safer alternative than crude allergen extracts for immunotherapy. Several mechanisms have been proposed for the change in immune function induced by allergen-specific immunotherapy (11–14). Central to most of these mechanisms are changes in the allergen-specific T cell response. Hence, while the removal of B cell epitopes from allergens is needed to

abrogate IgE-mediated adverse reactions, dominant T cell epitopes must be retained to target T cells for effective immunotherapy (10).

Sixteen proteins of natural rubber latex are designated as allergens by the International Union of Immunological Sciences (15). For individuals sensitized by latex glove use, Hev b 6.01 is a major allergen with 70–86% of latex-allergic HCW having specific IgE to Hev b 6.01 (16–19). Hev b 6.01 is a 20-kDa protein (prohevein) that is cleaved naturally into a short 43 aa protein Hev b 6.02 (hevein) and a larger Hev b 6.03 C-terminal fragment (20, 21). The amount of Hev b 6.02 in natural rubber latex is 30 times higher than the amounts of both Hev b 6.01 and Hev b 6.03 (21). The dominant B cell epitopes of Hev b 6.01 are contained in the Hev b 6.02 domain with between 75 and 84% of latex-allergic subjects having IgE to the Hev b 6.02 domain and only 15–40% having IgE to the C-terminal fragment (18, 19, 22).

Allergies to certain plant foods such as avocado, banana, and chestnut are associated with allergy to latex. Known as the “latex-fruit syndrome”, this phenomenon has been shown to be due mainly to cross-reactive Abs that bind both Hev b 6.02 and class I chitinases from these plant foods (23). Class I chitinases are ubiquitous microbial defense proteins that contain Hev b 6.02-like domains and inhibit the growth of bacteria and fungi.

With the high level of sensitization of HCW to Hev b 6.01 and the role of Hev b 6.01 in the latex-fruit syndrome, the development of a hypoallergenic mutant of Hev b 6.01 for safe immunotherapy is desirable. Hev b 6.01 has been cloned and expressed as a recombinant protein and linear B cell epitopes identified (19, 24, 25). These epitopes offer targets for site-directed mutagenesis to disrupt IgE binding, as has been successful for Hev b 5 (26) and Hev b 6.02 (27). However, recognition that B cell epitopes of the Hev b 6.02 domain of Hev b 6.01 are conformational rather than linear in structure (28) and stabilized by four disulphide bonds (29), suggests that site-directed mutagenesis of cysteine residues is a more direct strategy for the generation of a hypoallergenic protein. This approach has been successful for a number of other dominant allergens (30–32).

*Cooperative Research Centre for Asthma, Sydney, Australia; [†]Department of Allergy, Immunology, and Respiratory Medicine, Alfred Hospital, and [‡]Department of Immunology, Monash University Medical School, Melbourne, Victoria, Australia

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² Address correspondence and reprint requests to Dr. Alexander C. Drew, Cooperative Research Centre for Asthma, Department of Allergy, Immunology and Respiratory Medicine, Alfred Hospital, Commercial Road, Melbourne Victoria 3004, Australia. E-mail address: alec.drew@med.monash.edu.au

³ Abbreviations used in this paper: HCW, health care worker; SPT, skin prick test; WT, wild type; GE, latex glove extract; BAT, basophil activation test; SI, stimulation index.

Given that the Hev b 6.02 domain of Hev b 6.01 is the site of major serum IgE reactivity for latex glove users, in this study we targeted the cysteine residues of Hev b 6.02 for site-directed mutagenesis. We present here the construction and characterization of mutants of recombinant Hev b 6.01 (rHev b 6.01) with one to four of the disulphide bonds of the Hev b 6.02 domain sequentially disrupted. In addition, Hev b 6.02 peptide variants corresponding to two of the mutants are evaluated. The rHev b 6.01 mutants and Hev b 6.02 peptide variants are demonstrated to be hypoallergenic, with abrogated IgE binding and poor activation of allergic effector cells, but all retain the desired ability to stimulate T cells from latex-allergic donors.

Materials and Methods

Subjects

Forty-five latex-allergic subjects (A1–A45) and 18 non-latex-allergic subjects (N1–N18) were recruited from the Alfred Hospital Allergy and Asthma Clinic (Melbourne, Australia). The study was approved by the Alfred Hospital Ethics Committee and written informed consent was obtained from each subject. The latex-allergic subjects had clinical symptoms of IgE-mediated latex hypersensitivity, and either a latex-specific IgE class of 2 to 4 (Kallestad Allercoat EAST, Sanofi-Pasteur Diagnostics, Marnes La Coquette, France) or a positive skin prick test (SPT) to latex extract (Stallergenes, Paris, France). The non-latex-allergic subjects had no clinical history of IgE-mediated latex hypersensitivity and either a latex-specific IgE class 0 or a negative SPT to latex. Fourteen of the control subjects were atopic and four were nonatopic.

Recombinant latex allergens

Hev b 5 and Hev b 6.01 were cloned, expressed, and purified as described previously (33, 34). Site-directed mutagenesis of the pProEx-HTa/Hev b 6.01 construct was conducted using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primer pairs B6C1AF/B6C1AR, B6C2AF/B6C2AR, B6C3AF/B6C3AR, and B6C8AF/B6C8AR (Table I) were used to replace the cysteine residues at amino acid residues 3, 12, 17, and 41, respectively, of the resulting protein with alanine residues. These replacements were conducted sequentially to produce proteins containing 1, 2, 3, or 4 substitutions (rHev b 6.01 mutants 1–4, respectively). After site-directed mutagenesis, the entire coding region of Hev b 6.01 of each construct was sequenced, confirming the nucleotide changes. The constructs were then transformed into *Escherichia coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene) competent cells and the proteins were expressed and purified as for wild-type (WT) rHev b 6.01 (33). To improve solubility for T cell culture studies, WT and rHev b 6.01 mutants were sulfonated as described previously (33).

Latex glove extract

Latex glove extract (GE) was prepared by irrigating latex gloves (Uniglove UG2A, made in Malaysia for UNIMEX trade agents P/L; Melbourne, Australia) with PBS (Sigma-Aldrich, St. Louis, MO; 1 ml/g glove) as described previously (34).

Table I. Oligonucleotides used for site-directed mutagenesis

Primer Pair	Nucleotides ^a	Sequence ^b
B6C1AF/B6C1AR	1–31	5'–GAATTCGAGCAAGCAGGT CGGCAAGCAGGTGGCAAGC–3'
B6C2AF/B6C2AR	19–57	5'–GCAGGTGGCAAGCTCGCA CCCAATAACCTATGTTGTAGC–3'
B6C3AF/B6C3AR	37–74	5'–CCCAATAACCTAGCATGT AGCCAGTGGGGTGGTGTGG–3'
B6C8AF/B6C8AR	108–146	5'–CTGCCAAAGCAATGCAAA AGACAGCGCGAAGGTGTGG–3'

^a From the sequence of Hev b 6.01 (prohevein), GenBank accession number AJ003196.

^b Sequence given for forward primer. Underlined, vector sequence; bold, nucleotides changed for mutagenesis.

Hev b 6.02 peptides

A 43 aa peptide with the sequence of the Hev b 6.02 domain of Hev b 6.01 was synthesized (Mimotopes, Melbourne, Victoria, Australia; peptide quality checked by mass spectrometry or HPLC/mass spectrometry). Additional Hev b 6.02 peptides with alanine substituted for cysteine at amino acid residue 3 (peptide variant 1) or residues 3, 12, 17, and 41 (peptide variant 2) were also synthesized.

Production of Hev b 6.01-specific mAb

mAb production followed established protocols (34). Approval was obtained from the Monash University Animal Ethics Committee. BALB/c mice were immunized with WT rHev b 6.01 and immune mouse spleen cells used to generate hybridomas. Hybridoma supernatants were screened for Abs to WT rHev b 6.01 and GE by performing a duplicate ELISA as described below and positive hybridomas were cloned. Supernatant from one clone, 1A5.4, which showed strong reactivity with GE and WT rHev b 6.01, was used in this study.

Gel electrophoresis and immunoblotting

WT rHev b 6.01 or rHev b 6.01 mutants were subjected to SDS-PAGE on 16% gels and immunoblotting was performed as described previously (34). Human serum was diluted 1/5 and IgE binding was detected using rabbit anti-human IgE (DAKO, Carpinteria, CA) diluted 1/500, followed by goat anti-rabbit IgG-HRP conjugate (Promega, Madison, WI) diluted 1/1000. Hev b 6.01-specific mAb 1A5.4 culture supernatant was diluted 1/100 and mAb binding was detected with goat HRP-conjugated anti-mouse Ig (Silenus, Melbourne, Victoria, Australia) diluted 1/1000. Blots were developed with 4-chloro-1-naphthol.

ELISA for latex-specific human IgE and Hev b 6.01-specific mAb

Serum samples were tested for specific IgE using our standard ELISA protocol (34) with rabbit anti-human IgE diluted 1/1000, followed by swine anti-rabbit IgG-HRP conjugate diluted 1/1000. For the Hev b 6.01-specific mAb 1A5.4, binding was detected using sheep anti-mouse Ig-HRP diluted 1/1000. Background values obtained for sera and mAb on wells uncoated with Ag were subtracted from values obtained on wells coated with Ag.

Inhibition ELISA were conducted by preincubating subjects' sera or the mAb 1A5.4 with Ag before measuring Ab binding as described above. Sera and mAb culture supernatants used in inhibition ELISA were titrated first against the Ags bound to ELISA plates by doubling dilutions. Dilutions that lay in the middle of the linear region of the titration curve were chosen (OD₄₉₀ ~1). Sera and mAb culture supernatant at these dilutions were incubated with Ags at concentrations ranging from 0.0016 to 125 µg/ml for 1 h at room temperature on a rotating wheel before being used in the standard ELISA protocols above.

Basophil activation test (BAT)

This assay was modified from Paris-Kohler et al. (35). Aliquots of heparinized blood, 100 µl, were preincubated with stimulation buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, 1 mg/ml BSA, 20 µl/ml heparin, pH 7.4) containing 2 ng/ml IL-3 (R&D Systems, Minneapolis, MN), for 10 min at 37°C, to increase the assay sensitivity. Samples were then incubated for 20 min at 37°C with 100 µl of stimulation buffer containing Ags or, as a control, with stimulation buffer alone. Basophil activation was stopped by incubating at 4°C for 5 min. Normal goat serum (10 µl) was added for 10 min at 4°C to block nonspecific binding of the IgE detection Ab followed by goat anti-human IgE-FITC (Caltag Laboratories, Burlingame, CA) diluted 1/2.5, and mouse anti-human CD63-R-PE (Caltag Laboratories) diluted 1/20 in 20 µl of wash buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 0.27 mM EDTA, pH 7.3). RBC were lysed by incubation with 2 ml of lysing solution (39 mM NH₄Cl, 2.5 mM KHCO₃, 0.2 mM EDTA) for 10 min at room temperature. Cells were washed by centrifugation (250 × g, 5 min at 4°C) and resuspension in wash buffer and analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA). 7-Aminoactinomycin D (Sigma-Aldrich) was added to the samples 5 min before FACS analysis.

Cells were initially gated on high IgE-staining cells to set a forward/side scatter gate encompassing basophils. 7-Aminoactinomycin D staining was used to exclude dead cells and high IgE staining cells were then analyzed for expression of CD63. Results are expressed as the proportion of cells expressing CD63 with background (stimulation buffer alone) values subtracted.

Generation of latex-specific T cell lines

PBMC were separated from heparinized blood by centrifugation on Ficoll Paque (Amersham Biosciences, Uppsala, Sweden) and Hev b 6.01-specific T cell lines generated using our established methods (33, 36). PBMC (2.5×10^6 /well) were stimulated with 20 μ g/ml GE in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA) and 5% screened, heat inactivated human AB serum (Sigma-Aldrich)) in 24-well tissue culture plates (Greiner Labortechnik, Oberösterreich, Austria). After 7 days, lymphoblasts were restimulated (1×10^6 cells/well) for a further week with 20 μ g/ml GE in the presence of an equal number of irradiated (3000 rad) autologous PBMC as APC. On days 9 and 11, 10 U/ml rIL-2 (Cetus, Emeryville, CA) was added. On day 14, lymphoblasts were stimulated as above with 20 μ g/ml sulfonated WT rHev b 6.01 to enrich for Hev b 6.01-specific T cells. Seven days later, resting oligoclonal T cell blasts were washed and tested in proliferation assays as below.

Oligoclonal T cell proliferation assay

Oligoclonal T cell blasts (5×10^4 /well) from the 3-wk cultures were stimulated with GE (50 and 100 μ g/ml), sulfonated WT rHev b 6.01, sulfonated rHev b 6.01 mutants and Hev b 6.02 peptides (10 and 30 μ g/ml), in the presence of equal numbers of APC. Wells containing T cells and APC in the absence of Ag were included to assess background levels of cell proliferation. After 60 h, cells were pulsed with 1 μ Ci of [^{3} H]thymidine (Amersham Biosciences) and 12–16 h later were analyzed by liquid scintillation spectroscopy. Results are expressed as mean cpm for triplicate cultures or as stimulation indices (SI; cpm Ag stimulated cultures divided by cpm unstimulated cultures) with SI >2.5 being defined as positive. Mitogenicity and toxicity of all latex allergens were excluded as described previously (33) (data not shown).

Results

Generation of rHev b 6.01 mutants

To investigate the contribution of disulphide bonds of Hev b 6.01 to its IgE reactivity, alanine residues were substituted for cysteine at positions 3, 12, 17, and 41 of the Hev b 6.02 domain. These cysteine residues pair with other cysteine residues at positions 18, 24, 31, and 37, respectively, to form the first four disulphide bonds of WT Hev b 6.01 (29). Four mutants of rHev b 6.01 were produced: mutant 1, substituted at amino acid residue 3; mutant 2, substituted at amino acid residues 3 and 12; mutant 3 substituted at amino acid residues 3, 12, and 17 and mutant 4, substituted at amino acid residues 3, 12, 17, and 41.

Recombinant WT and mutant Hev b 6.01 proteins were expressed in *E. coli* with a six-histidine tag for purification. These proteins were insoluble and were purified under denaturing conditions. SDS-PAGE under nonreducing conditions showed that the purified recombinant proteins consisted of a major band running at 20 kDa and minor bands at a range of higher molecular masses (Fig. 1a). To demonstrate that these bands were rHev b 6.01 an immunoblot was performed using the Hev b 6.01-specific mAb 1A5.4 as a probe (Fig. 1b). This mAb was raised to WT rHev b 6.01 and recognizes a linear peptide within the Hev b 6.02 sequence (data not shown). The mAb recognized the major band and minor bands of the WT rHev b 6.01 (Fig. 1b, lane 1) and of the four mutants of rHev b 6.01 (Fig. 1b, lanes 2–5), indicating that when expressed in bacteria and purified under denaturing conditions, multimers of rHev b 6.01 were formed. When the rHev b 6.01 preparations were analyzed under reducing conditions by SDS-PAGE a single band at around 24 kDa was observed (data not shown), indicating that the multimers were formed by intermolecular disulphide bonding.

When an immunoblot was probed with serum from a latex-allergic subject (Fig. 1c), WT rHev b 6.01 was shown to be strongly IgE reactive. The major band at around 20 kDa, representing monomeric rHev b 6.01, was IgE reactive, as were the bands representing multimers of WT rHev b 6.01 (Fig. 1c, lane 1). No bands from any of the mutants of rHev b 6.01 reacted with IgE from this

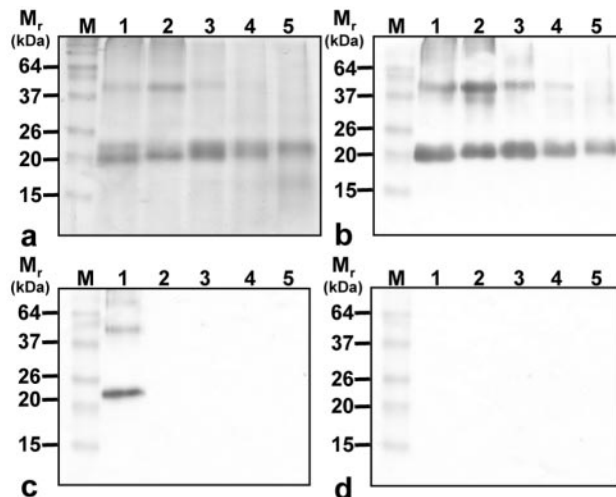


FIGURE 1. Analysis of WT rHev b 6.01 and mutants of rHev b 6.01 by SDS-PAGE and immunoblotting. Recombinant proteins were resolved by 16% SDS-PAGE under nonreducing conditions. Gels were either stained with Coomassie brilliant blue (a) or transferred to nitrocellulose and incubated with the Hev b 6.01-specific mAb 1A5.4 (b), serum from a latex-allergic, Hev b 6.01-sensitized subject (A1) (c), or serum from a non-latex-allergic, atopic subject (N1) (d). Lane 1, WT; lane 2, mutant 1; lane 3, mutant 2; lane 4, mutant 3; lane 5, mutant 4.

subject (Fig. 1c, lanes 2–5). Serum IgE from a non-latex-allergic, atopic subject did not react with either WT rHev b 6.01 or any of the mutants of rHev b 6.01 (Fig. 1d, lanes 1–5).

Disruption of the disulphide bonds of the Hev b 6.02 domain abolishes IgE reactivity of rHev b 6.01

To investigate IgE binding to rHev b 6.01 mutants in a large population of latex-allergic subjects, a Hev b 6.01-specific IgE ELISA was used (Fig. 2). Sera from 43 latex-allergic and 18 non-latex-allergic subjects were assayed, with the mean plus three SD of the ELISA scores of the non-latex-allergic subjects for each Ag used as the cut-off for a positive reading. In keeping with other studies (16–19), 29 of the latex-allergic subjects had positive levels of IgE binding to WT rHev b 6.01 (29/43; 67%; Fig. 2). In contrast, serum IgE from only 2 of the latex-allergic subjects (5%) was weakly reactive with the mutants of rHev b 6.01. The levels of IgE binding

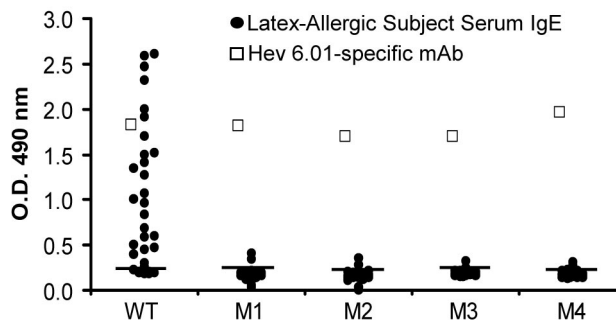


FIGURE 2. IgE binding to WT rHev b 6.01 and mutants of rHev b 6.01 in ELISA. Sera from 43 latex-allergic subjects were assayed by ELISA for IgE binding to WT rHev b 6.01 and mutants of rHev b 6.01 (M1, mutant 1; M2, mutant 2; M3, mutant 3; M4, mutant 4). ●, OD_{490 nm} readings for each subject. The horizontal lines indicate the cut-off for positive IgE binding to WT rHev b 6.01 and mutants of rHev b 6.01 (calculated as the mean of the readings for 18 non-latex-allergic subjects plus three SD for each Ag). The levels of binding of the Hev b 6.01-specific mAb 1A5.4 to WT rHev b 6.01 and mutants of rHev b 6.01 (□) are shown for comparison.

from the serum of these two subjects to mutants of rHev b 6.01 were between 14 and 74 times less than the levels of IgE binding to WT rHev b 6.01 for the same subjects. When the Hev b 6.01-specific mAb 1A5.4 was used in the same assay, it showed comparable reactivity with both WT rHev b 6.01 and the mutants of rHev b 6.01 (Fig. 2). Significant IgE binding to the peptide corresponding to the WT Hev b 6.02 domain was not detected by direct ELISA (data not shown). The results of the ELISA further demonstrate that disruption of the disulphide bonds in the Hev b 6.02 domain completely abrogates or markedly decreases IgE binding to rHev b 6.01.

Mutants of rHev b 6.01 and peptide variants of Hev b 6.02 are poor inhibitors of IgE binding to rHev b 6.01

To further investigate the lack of serum IgE reactivity of rHev b 6.01 mutants and Hev b 6.02 peptide variants, an inhibition ELISA was performed. To establish the optimal inhibitor concentrations for this assay, serum IgE binding to WT rHev b 6.01 for one

latex-allergic, Hev b 6.01-sensitized subject was assessed after preincubation of serum with a range of concentrations of the rHev b 6.01 mutants, the Hev b 6.02 peptide variants or, as controls, WT rHev b 6.01 and WT Hev b 6.02 peptide (Fig. 3, *a* and *b*).

When WT rHev b 6.01 was used to inhibit the binding of IgE to WT rHev b 6.01 (Fig. 3*a*), positive inhibition at low Ag concentrations was evident, increasing to 100% inhibition at higher concentrations. In contrast, all mutants of rHev b 6.01 showed markedly decreased ability to inhibit IgE binding. The rHev b 6.01 mutants 1 and 2 reached a maximum inhibition of 30 and 40%, respectively (Fig. 3*a*). rHev b 6.01 mutants 3 and 4 failed to inhibit IgE binding, even at the highest concentration used (Fig. 3*a*).

As seen with WT rHev b 6.01, WT Hev b 6.02 peptide inhibited IgE binding to rHev b 6.01 at low concentrations, reaching 100% inhibition at higher concentrations. This was in contrast to the lack of significant IgE binding observed in direct ELISA to this Ag. The Hev b 6.02 peptide variants 1 and 2 showed reduced or ablated ability to inhibit IgE to rHev b 6.01, similar to that of the corresponding

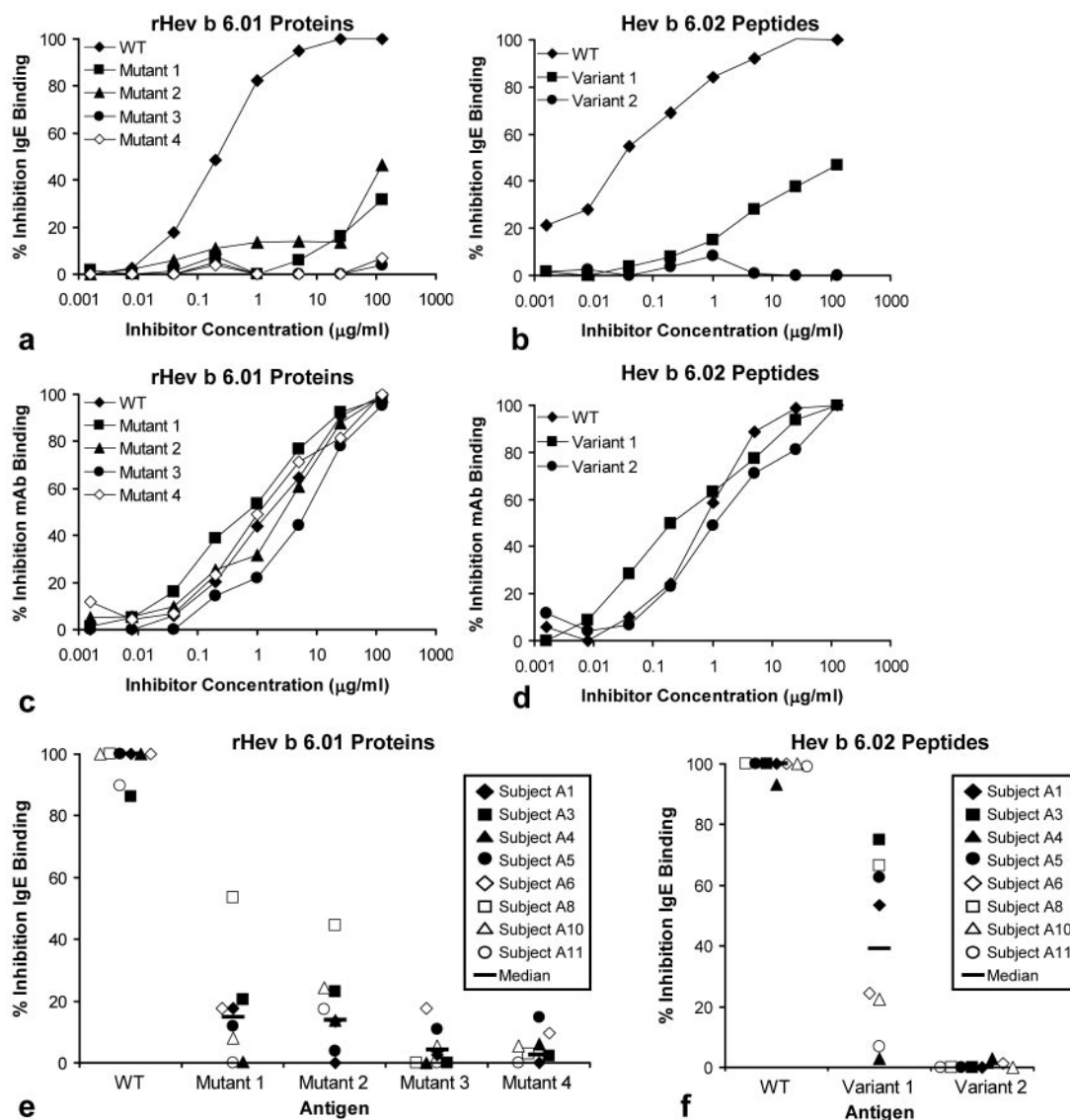


FIGURE 3. Inhibition of IgE binding to WT rHev b 6.01 by rHev b 6.01 mutants and Hev b 6.02 peptide variants. Different concentrations of rHev b 6.01 proteins (*a*) and Hev b 6.02 peptides (*b*) were incubated with the serum of a latex-allergic, Hev b 6.01-sensitized subject or, as a control, the Hev b 6.01-specific mAb 1A5.4 (*c*, recombinant proteins and *d*, Hev b 6.02 peptides). IgE or mAb binding to WT rHev b 6.01 was then assayed by ELISA. Results are presented as percent inhibition. *e* and *f* show the inhibition of IgE binding to WT rHev b 6.01 by rHev b 6.01 proteins (*e*) and Hev b 6.02 peptides (*f*) at 125 μg/ml for serum from eight latex-allergic, Hev b 6.01-sensitized subjects.

recombinant mutant. Hev b 6.02 peptide variant 1 inhibited to a maximum of 40% (Fig. 3*b*) and Hev b 6.02 peptide variant 2 did not inhibit IgE binding (Fig. 3*b*).

To determine the level of nonspecific inhibition inherent in the inhibition assay, an initial nonspecific inhibition assay was performed. The serum from a latex-allergic, Hev b 5-sensitized subject was preincubated with the Ags at 0.001–125 $\mu\text{g/ml}$ and then IgE binding to rHev b 5 was tested by ELISA. The binding of IgE to rHev b 5 was inhibited by 0–12%, depending on the Ag and the Ag concentration (data not shown). Given these results, the level for positive inhibition was set at >12%.

As shown earlier, the binding of the Hev b 6.01-specific mAb 1A5.4 to rHev b 6.01 in a direct ELISA was unaffected by the mutation of cysteine residues (Figs. 1*b* and 2). Therefore, to confirm the ability of all Ags to inhibit Ab binding in the inhibition assay, the binding of mAb 1A5.4 to WT rHev b 6.01 was measured in the presence of a range of concentrations of each Ag (Fig. 3, *c* and *d*). All Ags showed similar inhibition curves, indicating a comparable ability to inhibit mAb binding.

The ability of the rHev b 6.01 proteins and Hev b 6.02 peptides to inhibit IgE binding to WT rHev b 6.01 was investigated for sera from eight latex-allergic, Hev b 6.01-sensitized individuals (Fig. 3, *e* and *f*). Inhibitions were conducted using the various proteins and peptides at 125 $\mu\text{g/ml}$ based on pilot studies with one serum (Fig. 3, *a* and *b*). When WT rHev b 6.01 was used as the inhibitor, inhibition of 86–100% was evident. The mutants of rHev b 6.01 showed markedly decreased ability to inhibit IgE binding for all eight subjects. The rHev b 6.01 mutants 1 and 2 gave maximum inhibitions of 54 and 44%, respectively (Fig. 3*e*). Inhibition with rHev b 6.01 mutants 3 and 4 resulted in levels of maximum inhibition just above the background for the assay, at 18 and 15%, respectively.

WT Hev b 6.02 peptide inhibited IgE binding to rHev b 6.01 giving 93–100% inhibition. For all eight subjects the Hev b 6.02 peptide variants 1 and 2 showed reduced or ablated ability to inhibit IgE binding to rHev b 6.01. Hev b 6.02 peptide variant 1 inhibited to a maximum of 75% (Fig. 3*f*) and Hev b 6.02 peptide variant 2 did not inhibit IgE binding at all (Fig. 3*f*). These results indicate that while a single alanine to cysteine mutation reduced IgE binding to both rHev b 6.01 and Hev b 6.02 peptide, four cysteine substitutions were necessary to abolish IgE binding completely for all subjects.

Markedly decreased basophil activation by rHev b 6.01 mutants and Hev b 6.02 peptide variants

To investigate the biological potency of the rHev b 6.01 mutants and Hev b 6.02 peptide variants, the ability of these Ags to activate basophils was assessed using the BAT (35). Representative BAT flow cytometry plots are shown in Fig. 4, *a–c*, for a latex-allergic, Hev b 6.01-sensitized subject. These plots demonstrate that stimulation with latex allergens caused a marked increase in the proportion of high IgE-staining cells expressing CD63, the phenotype of activated basophils (35).

The BAT has been shown to be sensitive and specific for the diagnosis of latex allergy with a crude latex extract (37). To demonstrate the specificity of the BAT when using recombinant latex allergens, the BAT was performed on 11 latex-allergic subjects and 2 non-latex-allergic, atopic subjects (Table I). The subjects' basophils were stimulated with WT rHev b 6.01 and rHev b 5 at 10 $\mu\text{g/ml}$. In all cases, activation over 1–2% was only seen when the subject had serum-specific IgE to the relevant recombinant allergen (Table II).

To investigate the ability of mutants of rHev b 6.01 to activate basophils, a dose-response profile was first generated. Basophils

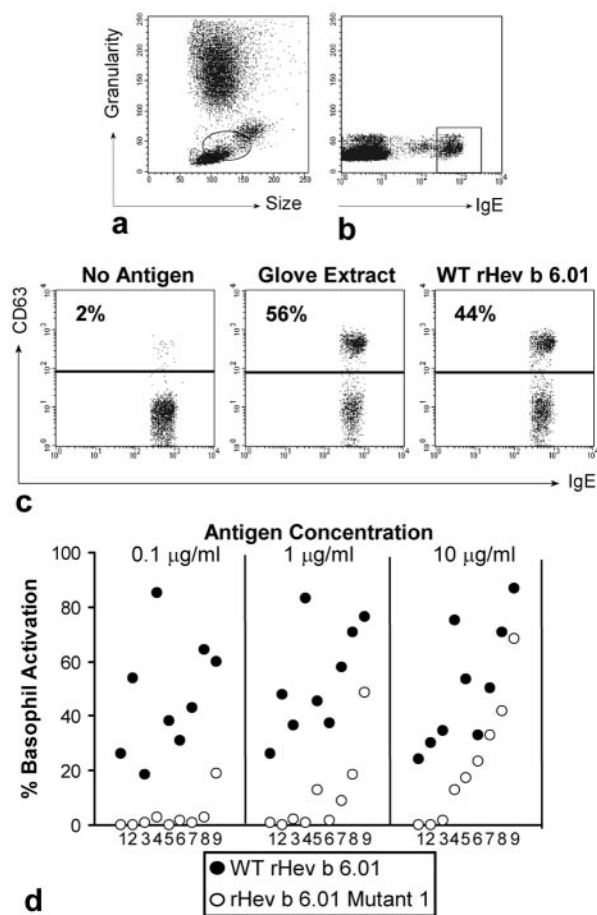


FIGURE 4. BAT. Whole blood from a latex-allergic, Hev b 6.01-sensitized subject was incubated with GE and WT rHev b 6.01 at final concentrations of 0.1, 1, and 10 $\mu\text{g/ml}$. After staining with FITC-labeled anti-human IgE and PE-labeled anti-human CD63 Ab, cells were analyzed by flow cytometry, defining basophils on the basis of scatter (*a*) and high IgE staining (*b*). The percentage of activated basophils expressing CD63 was determined in response to stimulation with GE and rHev b 6.01 (*c*). The activation of basophils from nine latex-allergic, Hev b 6.01-sensitized subjects (A1–9) by WT rHev b 6.01 and rHev b 6.01 mutant 1 (*d*). Percent basophil activation is given after the subtraction of background activation with no Ag present.

from nine latex-allergic, Hev b 6.01-sensitized subjects were stimulated with varying concentrations of both WT rHev b 6.01 and rHev b 6.01 mutant 1. Stimulation with WT rHev b 6.01 at concentrations of 0.1, 1, and 10 $\mu\text{g/ml}$ resulted in activation of basophils from all subjects, with levels of activation varying between 18 and 87% (Fig. 4*d*). Although high levels of activation of basophils were evident after stimulation with WT rHev b 6.01 at 0.1 $\mu\text{g/ml}$, rHev b 6.01 mutant 1 failed, at that concentration, to produce positive levels of basophil activation in eight of the nine subjects. Stimulation with 10 $\mu\text{g/ml}$ rHev b 6.01 mutant 1 resulted in activation of basophils from all but three subjects, although the levels seen were decreased compared with those seen with WT rHev b 6.01 at the same concentration.

For the majority of subjects tested, basophils were activated to some degree by rHev b 6.01 mutant 1 at 10 $\mu\text{g/ml}$ (Fig. 4*d*). Therefore, the ability of rHev b 6.01 mutants with further cysteine substitutions to activate basophils was compared at this concentration (Fig. 5*a*). The Hev b 6.02 peptide variants were also tested for their ability to activate basophils (Fig. 5*b*). The peptides were used to stimulate basophils at 2 $\mu\text{g/ml}$ to give approximately equimolar

Table II. IgE to recombinant latex allergens and basophil activation

Subject ^a	rHev b 6.01-Specific IgE ^b	% Basophil Activation by rHev b 6.01 ^c	rHev b 5-Specific IgE ^b	% Basophil Activation by rHev b 5 ^c
A1	+++	31	—	0
A2	+++	26	—	0
A3	+	45	—	0
A4	+++	94	—	0
A5	+++	71	+++	64
A6	+++	33	—	1
A7	+++	50	—	0
A8	+++	50	+++	60
A9	+	65	+++	91
A10	—	1	+++	42
A11	+++	44	—	2
N1	—	1	—	2
N2	—	2	—	1

^a Subjects A1–A11, latex-allergic; subjects N1 and N2, non-latex-allergic and atopic.

^b Determined by specific-IgE ELISA. +++ = OD_{490 nm} > 1; ++ = OD_{490 nm} > 0.5 and ≤ 1; +, > cut-off and OD_{490 nm} ≤ 0.5; —, ≤ cut-off.

^c Percent basophil activation with 10 μg/ml Ag after the subtraction of background activation with no Ag present.

Ag concentration with the recombinant proteins. As seen previously, WT rHev b 6.01 activated basophils from all six subjects tested with values ranging from 35 to 99% (Fig. 5a). The mutants of rHev b 6.01 elicited decreased basophil activation with an increasing number of cysteine substitutions in four of the six subjects (Fig. 5a, subjects A3, A4, A8, and A10). Maximum basophil activation of 42% was seen with mutant 1 for these four subjects, with a maximum of 15% for mutant 2, 4% for mutant 3, and 21% for mutant 4. For the other two subjects, little decrease in basophil activation was seen when basophils were stimulated with the rHev b 6.01 mutants 1, 2, and 3, compared with stimulation with WT rHev b 6.01 (Fig. 5a, subjects A9, A11). Some decrease was evident for mutant 4 in one of these two subjects (Fig. 5a, subject A11).

WT Hev b 6.02 peptide was able to activate basophils from the six subjects with values of 54–99% (Fig. 5b). Five of the six subjects showed a decrease in basophil activation after stimulation with Hev b 6.02 peptide variant 1, with 0–96% basophil activation. Basophil activation was negligible for all of the six subjects after stimulation with the Hev b 6.02 peptide variant 2, with a maximum basophil activation of 5%. The subjects that had retained basophil activation when stimulated with the rHev b 6.01 mutants showed little or no activation when stimulated with the peptide variant 2 at an equimolar concentration.

rHev b 6.01 mutants and Hev b 6.02 peptide variants retain the ability to stimulate proliferation of allergen-specific T cells

Oligoclonal Hev b 6.01-specific CD4⁺ T cell lines were generated from the PMBC of five latex-allergic, Hev b 6.01-sensitized subjects. The proliferation of these T cell lines was measured after stimulation with GE, WT rHev b 6.01, rHev b 6.01 mutants 1 and 4, WT Hev b 6.02 peptide, and Hev b 6.02 peptide variants 1 and 2. A representative proliferation assay is shown in Fig. 6a. Hev b 6.01-specific oligoclonal T cell blasts from all five subjects (Fig. 6b) showed significant proliferation to GE, WT rHev b 6.01, and WT Hev b 6.02 peptide (SI > 2.5). In all five cases, proliferation was retained to the rHev b 6.01 mutants and Hev b 6.02 peptide variants.

Discussion

The latex allergen Hev b 6.01 is an important cause of latex sensitization in latex-glove users, particularly HCWs and laboratory

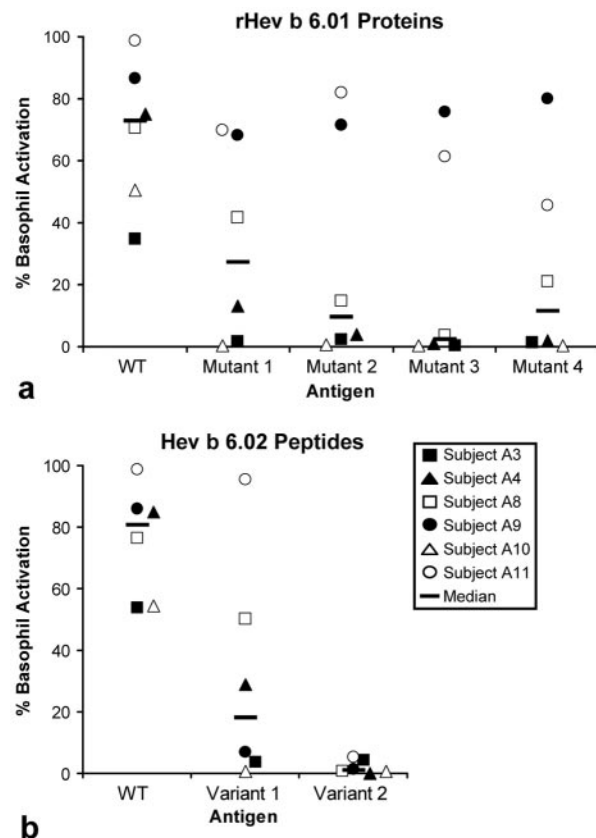


FIGURE 5. Activation of basophils by rHev b 6.01 mutants and Hev b 6.02 peptide variants. The activation of basophils from six latex-allergic, Hev b 6.01-sensitized subjects (A3, A4, A8, A9, A10, and A11) after stimulation with WT rHev b 6.01 and the four rHev b 6.01 mutants at 10 μg/ml (a). Basophil activation following stimulation with WT Hev b 6.02 peptide and Hev b 6.02 peptide variants 1 and 2 at 2 μg/ml (b). Each symbol indicates a different subject. Percent basophil activation is given after the subtraction of background activation with no Ag present.

scientists. Hev b 6.01 and the other major latex glove allergen Hev b 5, are important triggers of occupational asthma and allergy (36, 38). The association of Hev b 6.01 sensitization with the development of latex-fruit syndrome further emphasizes the importance of this allergen and the desirability of a safe and effective desensitizing vaccine. To date, the high anaphylactic potential of current unmodified natural latex extracts has prevented the development of specific immunotherapy for the treatment and prevention of latex allergy. However, technology for molecular cloning and sequencing of major allergens has facilitated the identification of critical T cell epitopes to permit development of safe, T cell-targeted therapies. T cell epitope-based peptide vaccines have been successful in trials for bee venom allergy (39) and cat allergy (40); the use of dominant T cell epitope peptides resulted in down-regulation of response to the whole molecule consistent with intramolecular suppression. Alternatively, the ability to genetically alter and express recombinant modified proteins has stimulated interest in the generation of hypoallergenic mutant proteins for potential use as immunotherapeutic vaccines for allergy (10).

Highly conformationally dependent molecules such as Hev b 6.01, stabilized by the presence of multiple disulphide bonds, suggest a strategy of site-directed mutagenesis of cysteine residues preventing disulphide bridging, for disruption of conformational B cell epitopes and abrogation of IgE binding. A decrease in IgE-binding potential minimizes the risk for anaphylaxis and increases the likelihood of development of proteins or peptides suitable for

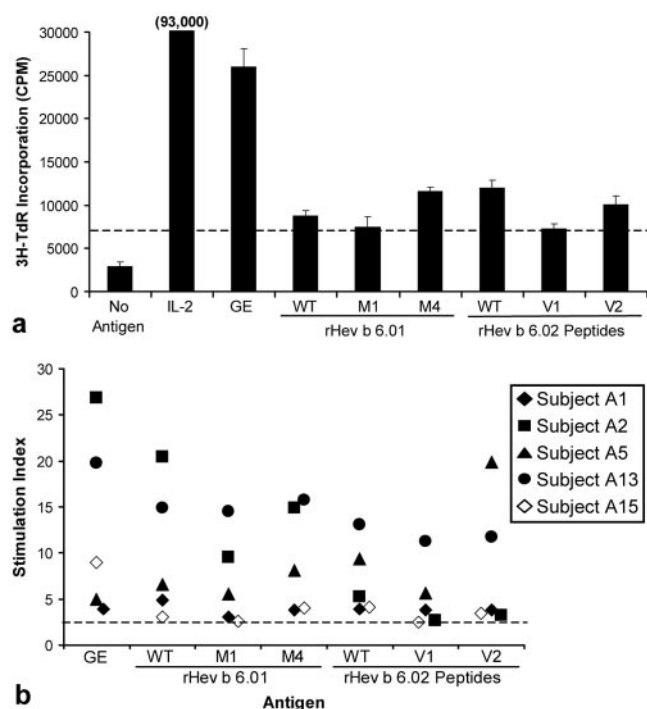


FIGURE 6. Proliferation of Hev b 6.01-specific T cell lines in response to rHev b 6.01 mutants and Hev b 6.02 peptide variants. Oligoclonal T cells from latex-allergic, Hev b 6.01-sensitized subjects were stimulated with GE, WT rHev b 6.01, mutants of rHev b 6.01 (M1, mutant 1; M4, mutant 4), WT Hev b 6.02 peptide, and variants of Hev b 6.02 peptide (V1, variant 1 and V2, variant 2), in the presence of equal numbers of irradiated APCs. Proliferation as correlated with incorporation of tritiated thymidine was determined. The horizontal lines indicate the cut-off level of positive proliferation (>2.5 times no Ag cpm). *a*, Typical proliferative response of an oligoclonal T cell line (subject A15). Results are shown as mean cpm $\pm$ SD for triplicate cultures. Control cultures contained no Ag, 10% IL-2, or GE. *b*, Proliferative response of oligoclonal T cell lines from five subjects (A1, A2, A5, A13, and A15). Results are shown as SIs (mean cpm after culture with Ag divided by mean cpm after culture without Ag). The values given are for the maximal proliferative response from cultures with Ags at 10 and 30 μ g/ml. The background responses of T cells cultured with irradiated APCs in the absence of Ag for the five subjects ranged from 473 to 9046 cpm (median 2888 cpm).

use in high dose-specific immunotherapy (32, 33). This strategy was used here to produce hypoallergenic mutants of the major latex allergen Hev b 6.01.

Four mutants of rHev b 6.01 were produced by sequential substitution of four cysteine residues by alanines with disruption of the four disulphide bonds of the Hev b 6.02 domain of rHev b 6.01. Approximately 80% of latex-allergic subjects possess serum IgE specific for the Hev b 6.02 domain of Hev b 6.01. The larger C-terminal fragment, Hev b 6.03, has a lower frequency of serum IgE reactivity, between 15 and 40% (18, 19, 22). Given the higher frequency of serum IgE reactivity to the Hev b 6.02 domain, this was initially targeted for mutagenesis. However, with the potential for allergic effector cell activation in a minority of individuals by the C-terminal fragment of Hev b 6.01, we decided to examine corresponding synthetic peptides derived from the sequence of the Hev b 6.02 domain. These peptides had either the first disulphide bond or all four disulphide bonds disrupted by cysteine to alanine substitutions.

In this study, WT rHev b 6.01 and WT Hev b 6.02 peptide were shown to be IgE-reactive and capable of activating basophils from sensitized subjects. The disruption of the first disulphide bond of

the Hev b 6.02 domain of rHev b 6.01 and the Hev b 6.02 peptide resulted in marked decreases in serum IgE binding and basophil activation. With the disruption of additional disulphide bonds there was a further decrease in both IgE reactivity and the ability to activate basophils, with complete abrogation of basophil activation when three or more cysteines were substituted. Studies of hypoallergenic recombinant forms of other allergens have also demonstrated that mutation of multiple cysteine residues was required to completely abrogate IgE reactivity (30, 31). It has been shown that the conformational IgE epitopes of Hev b 6.02 contain amino acids from both the N- and C-terminal regions of that domain (28). It is likely that disruption of the disulphide bonds of Hev b 6.02 resulted in a destabilization of these IgE-binding epitopes.

Previous studies aimed at generation of hypoallergenic allergen mutants have tested biological activity by SPT (31, 41). Due to the anaphylactic potential of natural latex extracts (42), SPT for latex allergy is not routinely performed in our clinic. As an alternative to SPT, with its inherent safety risks, the BAT was used in this study to measure the relative ability of the WT Ags, rHev b 6.01 mutants, and Hev b 6.02 peptide variants to activate basophils. Although BAT is recognized as a very sensitive and specific assay for the diagnosis of latex allergy (43), there have been no direct comparisons of BAT and skin prick testing with recombinant latex proteins. Although it has been reported that 1 to 10 ng/ml recombinant latex allergen will elicit a positive SPT response (38), we found a lack of activation of basophils with 10 μ g/ml rHev b 6.01 mutants. However, given the different methodologies of the two tests it is not possible to entirely exclude the risk of IgE-mediated adverse events from the mutants in vivo. Further testing of candidate vaccines before use in immunotherapy would include SPT and end-point titration to assist in determination of optimal dose for successful immunotherapy.

It is well established that T cells play a pivotal role in the mechanisms of effective allergen desensitization (12). Without the ability to stimulate T cells, a hypoallergenic mutant would not be useful in immunotherapy. The Hev b 6.01 mutants and peptide variants prepared in this study were shown to have retained T cell reactivity despite ablation of B cell epitopes. Importantly, three of the subjects used to assess T cell reactivity of the mutants and variants had previously been shown to recognize one or both of two highly dominant CD4⁺ T cell reactive sites of Hev b 6.01, i.e., Hev b 6.01 (10–29) and Hev b 6.01 (19–38) (33). Our current results are consistent with retention of these critical immunodominant sites and therefore the ability of our candidate peptide to induce T cell anergy and linked suppression in a successful T cell-targeted immunotherapy strategy.

Latex allergy in most individuals results from sensitization to a number of latex allergens (44). As monosensitization to Hev b 6.01 is uncommon (38), any potential immunotherapy preparation would require the use of more than one hypoallergenic recombinant latex allergen or derived synthetic peptide. Hev b 5 and Hev b 6.01, particularly the Hev b 6.02 domain, are the two major allergens for individuals sensitized by latex glove use and these Ags should be targeted in a latex immunotherapy “vaccine” for this patient group (44). A T cell stimulatory, hypoallergenic desensitizing “vaccine” targeting both these allergens would be a welcome addition to pharmacotherapy for latex allergy, either as a treatment regimen for patients with confirmed allergy or as a possible preventive option. The combination of the dominant Hev b 5 T cell epitope peptides reported by us previously (36) and the Hev b 6.02 peptide variant with four cysteine to alanine substitutions described here would be a strong candidate for inclusion in a therapeutic vaccine.

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