

# Mutational analysis of the IgE epitopes in the latex allergen Hev b 5

Donald H. Beezhold, PhD,<sup>a</sup> Vicky L. Hickey, BS,<sup>a</sup> and Gordon L. Sussman, MD<sup>b</sup>

Sayre, Pa, and Toronto, Ontario, Canada

**Background:** Hev b 5 is a major latex allergen and potential candidate for an immunotherapy reagent.

**Objective:** The purpose of this study was to produce a hypoallergenic form of Hev b 5.

**Methods:** We used SPOTs analysis with alanine substitution to identify amino acids (AAs) critical for IgE binding and used site-directed mutagenesis to produce recombinant proteins with altered IgE-binding activity.

**Results:** Eleven epitopes were identified (5.1-5.11) in Hev b 5. Individual patients demonstrated variable epitope recognition, with the most intense reactivity to epitopes 5.4 and 5.7. IgE inhibition assays with synthetic peptides indicated that mutating a single epitope would not reduce IgE binding, but rather a combination of epitopes was required. After alanine substitutions to identify the important AAs, site-directed mutagenesis was used to replace the crucial AAs with alanine. Twenty clones with different combinations of altered epitopes were evaluated by means of IgE inhibition assays. Clones with mutations in single epitopes failed to reduce IgE binding, but changes to 8 epitopes (14 AAs) resulted in a 4500-fold reduction in IgE binding. Epitopes 5.7 and 5.9 were found to be cross-reactive, making Hev b 5 a multivalent allergen.

**Conclusions:** We produced a recombinant Hev b 5 protein with significantly reduced IgE-binding activity. Changing a minimum of 3 immunodominant epitopes was required to cause a 100-fold reduction in IgE binding. Changes in 8 epitopes, particularly the cross-reactive epitopes 5.7 and 5.9, were needed to maximize the reduction in IgE binding. Mutants with reduced IgE-binding activity may prove to be valuable reagents for immunotherapy. (*J Allergy Clin Immunol* 2001;107:1069-76.)

**Key words:** Hev b 5, latex allergy, IgE antibodies, recombinant allergens, site-directed mutagenesis

Latex allergy is an important occupational disease in health-care workers.<sup>1-3</sup> The consequences of latex allergy include permanent removal from the workplace for some of these highly trained individuals. Type 1 hypersensitivity reactions develop after repeated exposure to natural rubber latex (NRL) medical devices, such as personal protective gloves.<sup>4</sup> Latex allergens are primarily residual allergenic proteins that originate from the rubber tree *Hevea brasiliensis*.<sup>5-7</sup> At least 11 protein allergens

## Abbreviations used

|      |                             |
|------|-----------------------------|
| AA:  | Amino acid                  |
| AL:  | Ammoniated latex protein    |
| GST: | Glutathione-S-transferase   |
| NAL: | Nonammoniated latex protein |
| NRL: | Natural rubber latex        |
| TBS: | TRIS-buffered saline        |

with variable levels of individual reactivity have been identified. Although no cure for latex allergy exists, progress on allergen identification and characterization makes immunotherapy a real alternative.

Standard immunotherapy protocols involve the repeated injection of allergens over prolonged periods of time, which induces relative immunologic tolerance. Many patients are highly reactive to latex allergens, and therefore this approach includes the risk of severe reactions on injection of the latex allergens.<sup>8</sup> A promising experimental immunotherapy treatment involves DNA vaccines,<sup>9</sup> but a previous study of a DNA vaccine injected into pre-sensitized mice resulted in severe inflammatory reactions.<sup>10</sup> An alternative experimental approach involves modifying specific B-cell epitopes to prevent IgE binding and cross-linking but retaining T-cell epitopes and the ability to induce tolerance.<sup>11,12</sup>

Hev b 5 is a major latex allergen, with a reported prevalence of specific serum IgE of 52% to 92% among health-care workers allergic to latex.<sup>13,14</sup> This protein has been cloned,<sup>13,14</sup> and skin testing with recombinant protein demonstrated a prevalence of 65% reactivity in health-care workers, several of whom were monosensitized individuals.<sup>15</sup> Because this is a major latex allergen and protein to which some patients allergic to NRL are uniquely sensitized, it is a good candidate for modifying B-cell epitopes to produce a potential reagent for immunotherapy.<sup>16</sup>

Hev b 5 is an acidic protein that has been identified in ammoniated latex protein (AL), nonammoniated latex protein (NAL), and NRL gloves.<sup>13-17</sup> This protein appears to be present in low quantities in NAL, but because it is heat resistant,<sup>13</sup> it may represent a greater proportion of the residual protein found on processed latex products. Biochemically, the protein has unique properties because of the high content of glutamic acid (30%) and proline (15%) residues.<sup>13,14</sup> Although this protein has a predicted molecular mass of 16 kd, the protein migrates in SDS-PAGE gels at approximately 36 kd. Inhibition Western blotting demonstrated a broad spectrum of reactive molecules in the 50- to 100-kd range in

From <sup>a</sup>the Laboratory of Immunobiology, Guthrie Research Institute, Sayre; and <sup>b</sup>the Division of Allergy and Clinical Immunology, University of Toronto, Toronto.

Supported by the LEAP Testing Service of the Guthrie Research Institute. Received for publication January 8, 2001; revised February 22, 2001; accepted for publication February 28, 2001.

Reprint requests: Donald Beezhold, PhD, Director, Laboratory of Immunobiology, Guthrie Research Institute, 1 Guthrie Square, Sayre, PA 18840.

Copyright © 2001 by Mosby, Inc

0091-6749/2001 \$35.00 + 0 1/83/115482

doi:10.1067/mai.2001.115482

NAL.<sup>17</sup> Recent studies have identified the B-cell epitopes for this antigen in rabbits, mice, and human subjects<sup>15-18</sup> and the T-cell epitopes in mice and human subjects.<sup>16,18</sup> In this article we have further characterized the human B-cell epitopes and used site-directed mutagenesis to produce recombinant Hev b 5 with reduced IgE-binding activity.

## METHODS

### Antibodies

Human sera with anti-Hev b 5 IgE were obtained from 7 health-care workers diagnosed with latex allergy who had positive skin test responses to recombinant (r) Hev b 5 and demonstrated skin test reactivity at 1 ng/mL or less.<sup>15</sup> Rabbit anti-Hev b 5 IgG was obtained from a New Zealand white rabbit immunized with 200 µg of maltose-binding protein/rHev b 5 in a 50:50 mixture of TiterMax adjuvant (CytRx, Norcross, Ga). The rabbit was given a booster injection at 3 months and was bled 4 weeks later, having an anti-rHev b 5 titer of 1:8000, as determined by ELISA.

### Latex proteins

Latex proteins were isolated from AL and NAL by centrifugation to remove the rubber particles and particulates, as previously described.<sup>15,17</sup>

### Synthetic peptides

Synthetic peptides covering 6 previously identified Hev b 5 epitopes<sup>17</sup> were commercially obtained from Genemed Synthesis Inc (San Francisco, Calif) and used in the ELISA assay. The peptides 5.1-LPKNETPEVTKA, 5.2-EETKTEEPAAAP, 5.4-EKEEPTAAPAE, 5.6-EKIEKTEEPAP, 5.7-QTTPEEKPAEP, and 5.8-EEPKHETKETE were resuspended at 10 mg/mL in PBS and stored as aliquots at -80°C.

### SPOTs membranes and alanine substitution

Peptides spanning the entire Hev b 5 sequence were synthesized as a series of decapeptides overlapping by 2 amino acids (AAs) on derivatized cellulose membranes (SPOTs; Sigma-Genosys, The Woodlands, Tex), according to the manufacturer's instructions. On a second membrane, 6 SPOTs representing epitopes 5.1, 5.2, 5.4, 5.6, 5.7, and 5.8 were synthesized for analysis with individual sera. On a third membrane, a series of 12 SPOTs for each of these 6 epitopes were synthesized. For the first SPOT of each row, an 11 AA peptide with the native sequence was synthesized. For each successive spot, a single AA was substituted with an alanine residue in place of the native AA. If the native AA was an alanine, a methionine was substituted.

### Epitope immunoblotting

The SPOTs membranes were wetted in methanol, rinsed with TRIS-buffered saline (TBS), and blocked for 6 hours with 5% BSA protein in 0.05% Tween-20 in TBS. The blocking buffer was removed, and the membrane was incubated overnight with the human sera (diluted 1:10 and preabsorbed with *Escherichia coli* extract). The membrane was washed 3 times with 20 mL of 0.05% Tween-20 in TBS and reacted for 1 hour with mouse monoclonal anti-human IgE (Sigma, St Louis, Mo) diluted 1:1000. After washing 3 times, the membrane was reacted for 1 hour with an alkaline phosphatase-conjugated anti-mouse IgG (Sigma) diluted 1:1000. After washing 5 times, the membrane was placed in development solution (ECF substrate; Amersham Pharmacia Biotech, Inc, Piscataway, NJ). Chemifluorescence was recorded by using a STORM 840 (Molecular Dynamics, Sunnyvale, Calif) and, after densitometry scanning, quantified by using ImageQuant software (Molecular Dynamics).

### rHev b 5 in a glutathione-S-transferase expression vector

The complete coding region for Hev b 5 was amplified from *Hevea brasiliensis* cDNA<sup>15</sup> by means of PCR on a Perkin-Elmer thermal cycler in a 100-µL volume containing 2 mmol/L MgCl<sub>2</sub>, 200 µmol/L of each deoxyribonucleoside triphosphate, 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 µmol/L of each primer: 5'-GAT TAG GAA TTC ATG GCC AGT GTT GAG GTT GAA TC-3' and 5'-TTA ATG AAT TCT TAT TCC TCT GTT TTT TCC ACC GG-3'.

The resulting PCR product was ligated into the plasmid pCR2.1 and transformed into OneShot competent cells (INV F) by using TA cloning (Invitrogen, San Diego, Calif). *E coli*-containing inserts were obtained, plasmids were isolated by *Eco*R1 digestion, and the 456-bp insert was ligated into the *Eco*R1 site of the pGEX-4T-1 (Amersham Pharmacia Biotech) expression vector. Inserts were sequenced and compared with the published sequence of Hev b 5 in the GenBank/EBI data bank (accession number U42640). The pGEX4T-1/Hev b 5 plasmid was inserted into competent BL21 *E coli*, and cultures of *E coli* expressing the glutathione-S-transferase (GST)/Hev b 5 fusion protein were grown. The GST/Hev b 5 fusion protein was isolated from bacterial sonicates with a glutathione column (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The GST construct (pGEX-2T) served as a negative control. The protein concentrations were determined by using the Bradford assay, and the quality of the protein was checked by using SDS-PAGE. The proteins were diluted to 1 mg/mL and stored in aliquots at -80°C.

### Site-directed mutagenesis

Specific point mutations to alanine were introduced into Hev b 5 in pGEX4T-1 by using the QuickChange Site-directed Mutagenesis kit from Stratagene (La Jolla, Calif), according to the manufacturer's instructions. After mutagenesis, each clone was transformed into competent BL21 cells. The sequences for rHev b 5 and each mutated clone were determined by means of cycle sequencing (Applied Biosystems) with BigDye terminators by using overlapping forward and reverse primers.

### IgE inhibition ELISA

The wells of an ELISA assay plate (Nunc-Immuno Plate, Maxiisorp surface) were coated with 1 µg of rHev b 5 in carbonate buffer overnight at 37°C, washed 2 times with 0.05% T-PBS, and blocked with 3% dry milk in T-PBS before use. A dilution plate (Corning) was blocked with 3% nonfat dry milk in T-PBS overnight at 4°C and washed 2 times with T-PBS. One hundred microliters of pooled patient sera diluted 1:10 was mixed with 100 µL of an appropriate inhibitor in the dilution plate. After a 2-hour incubation at 37°C, 100 µL of the mixture was transferred from the dilution plate to the antigen-coated plate and incubated for an additional 3 hours at 37°C. The plate was washed 3 times with T-PBS and reacted with a 1:500 dilution of horseradish peroxidase-labeled anti-human IgE (100 µL, Sigma) for 1 hour at 37°C. A colored reaction product was produced by adding 100 µL of OPD (0.4 mg/mL in phosphate citrate buffer with 0.04% hydrogen peroxide) and incubating for 30 minutes; the reaction was then stopped with H<sub>2</sub>SO<sub>4</sub>. The OD was read at 490 nm.

## RESULTS

### Immunodominant Hev b 5 epitopes

We have previously reported 6 epitopes for human IgE (epitopes 1, 2, 4, 6, 7, and 10) that were identified by using SPOTs analysis with sera pooled from 10 health-

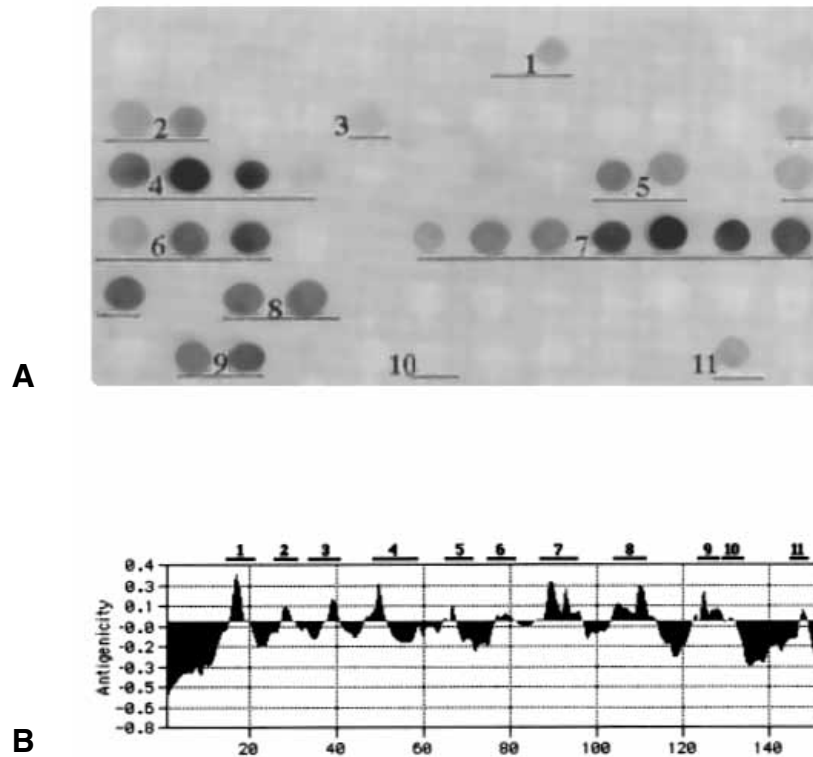


**FIG 1. A**, SPOTs analysis of Hev b 5 epitopes with sera from highly sensitized patients. Each spot represents a sequential decapeptide from the Hev b 5 sequence overlapping by 2 AAs. Membrane was reacted with a 1:10 dilution of pooled sera from 7 individuals allergic to Hev b 5. Epitopes are numbered in the order in which they appear. Epitope 10 was previously identified with sera from patients with latex allergy. Control sera from patients without latex allergy failed to react to the membrane. **B**, Antigenic index for Hev b 5 determined by using the MacVector computer model. Theoretical epitopes are indicated by a positive antigenic index. Actual epitopes determined by SPOTs analysis above are indicated at the top of the figure.

care workers with latex allergy.<sup>17</sup> An additional IgE-binding epitope (epitope 8) was detected by using sera from an individual patient with latex allergy (unpublished data). We now examine the Hev b 5 sequence by using sera from a second group of 7 patients selected because they had a positive skin prick test response to 1 ng/mL or less of rHev b 5. Sera from these highly sensitized patients recognized 10 epitopes, including 6 previously recognized epitopes and 4 additional epitopes (Fig 1). This pooled sera did not recognize one previously identified epitope (epitope 10), and epitope 7 appeared as a broader region than previously observed. The epitopes corresponded to regions of antigenicity, as predicted by using the MacVector computer model (Fig 1). For ease of description, the epitopes were numbered 5.1 to 5.11 in the order that they appear in the molecule (Table I).

For further analysis of the Hev b 5 epitopes, we initially concentrated on the previously identified epitopes (5.1, 5.2, 5.4, 5.6, 5.7, and 5.8) because they were detected with sera from patients with latex allergy, suggesting they are more common epitopes. Recognition of these epitopes was examined individually for patients in this second group by using SPOTs analysis. Fig 2 demonstrates heterogeneity in epitope recognition, with individual patients recognizing 2 to 6 epitopes. All 7 patients

**TABLE I.** Human IgE-binding regions of Hev b 5

| Epitope* | Spot  | AA      | Sequence      |
|----------|-------|---------|---------------|
| 5.1 (1)  | 8     | 15-22   | KNETPEVT      |
| 5.2 (2)  | 13-14 | 27-34   | TKTEEPAA      |
| 5.3      | 17    | 34-42   | APPASEQET     |
| 5.4 (3)  | 24-27 | 50-59   | EEPTAAPAE     |
| 5.5      | 33-34 | 67-74   | EKAEVEK       |
| 5.6 (4)  | 36-39 | 75-82   | IEKTEEPA      |
| 5.7 (5)  | 42-49 | 86-98   | DQTTPEEKPAEPE |
| 5.8 (6)  | 51-52 | 103-111 | EEPKHETK      |
| 5.9      | 62-63 | 124-132 | EKPAEEKPK     |
| 5.10 (7) | NR    | 132-139 | PTTEAAET      |
| 5.11     | 71    | 142-149 | TEVPVEKT      |

NR, Not reactive; this epitope was identified in a previous study.

\*Sequential numbering of Hev b 5 epitopes. Parentheses indicate epitope number designated in a previous study.<sup>17</sup>

reacted weakly to epitope 5.2, whereas there was variable reactivity to the other epitopes. The most intense reactivity for several patients was to epitopes 5.4 and 5.7.

### Synthetic peptides of Hev b 5 epitopes

Using synthetic peptides, we assessed the contribution of epitopes 5.1, 5.2, 5.4, 5.6, 5.7, and 5.8 to the reactivity of pooled anti-Hev b 5 IgE by using IgE inhibition

**TABLE II.** Inhibition of IgE binding to rHev b 5 by natural protein or synthetic peptides

|       | GST<br>(n = 4) | rHev b 5<br>(n = 4) | AL<br>(n = 4) | NAL<br>(n = 4) | Synthetic peptides of Hev b 5*          |                               |                          |                     |
|-------|----------------|---------------------|---------------|----------------|-----------------------------------------|-------------------------------|--------------------------|---------------------|
|       |                |                     |               |                | 5.1, 5.2, 5.4, 5.6, 5.7, 5.8<br>(n = 3) | 5.2, 5.4, 5.7, 5.8<br>(n = 2) | 5.4, 5.7, 5.8<br>(n = 3) | 5.1, 5.6<br>(n = 2) |
| 50 µg | 11.8 ± 1.7     | 88.1 ± 1.4          | 43.5 ± 4.0    | 45.2 ± 2.9     | 35.2 ± 1.1                              | 34.3 ± 3.9                    | 31.3 ± 2.6               | 10.9 ± 3.5          |
| 10 µg | 8.5 ± 3.2      | 81.1 ± 2.0          | 39.5 ± 3.6    | 40.4 ± 1.9     | 28.7 ± 3.3                              | 25.9 ± 5.5                    | 22.2 ± 3.9               | 8.9 ± 6.6           |
| 1 µg  | 5.8 ± 3.8      | 64.2 ± 2.6          | 22.8 ± 3.7    | 22.8 ± 5.2     | 12.0 ± 0.6                              | 13.4 ± 5.0                    | 13.2 ± 1.9               | 5.8 ± 5.7           |

Data represent means ± SD of at least 2 inhibition ELISA assays.

\*Synthetic peptides were mixed in equal quantities before addition to the pooled sera.

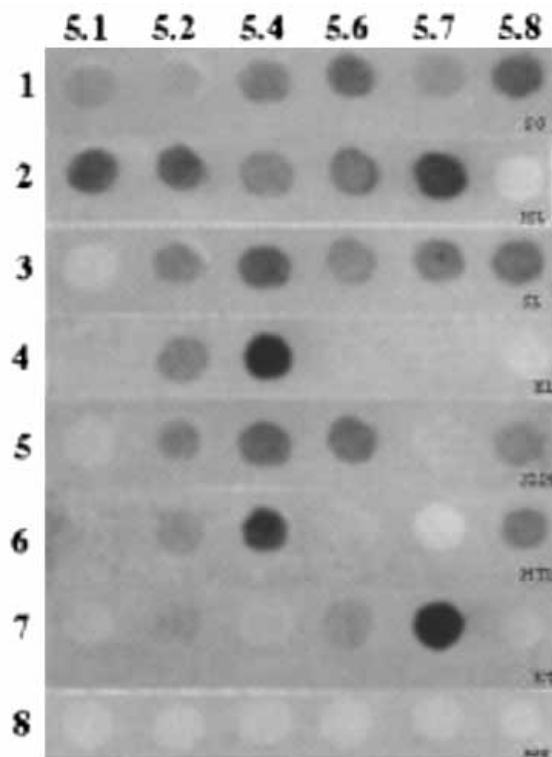


**FIG 2.** Heterogeneity in individual recognition of Hev b 5 epitopes. Six epitopes (5.1, 5.2, 5.4, 5.6, 5.7, and 5.8) were synthesized as 12 AA peptides on a derivatized cellulose membrane strip. The strip was then reacted with individual patient sera (lanes 1-7) and processed for IgE reactivity. A serum pool from 3 individuals (lane 8) with high IgE but without latex allergy served as the negative control.

ELISA. Each epitope was synthesized as an 11 (5.2, 5.4, 5.6, 5.7, and 5.8) or 12 (5.1) AA peptide and used as an inhibitor in the ELISA. As shown in Table II, IgE to Hev b 5 was inhibited by rHev b 5 (positive control) but not by the vehicle control (GST). By using natural latex protein as a positive control, both AL and NAL equally inhibited anti-Hev b 5 IgE. The maximum amount of inhibition that could be achieved with 100 µg of native protein was 62% (data not shown). Because it took approximately 100 times more natural latex protein than rHev b 5 to induce a similar amount of inhibition, the data suggest that less than 1% of natural latex protein is Hev b 5. Individual synthetic peptides failed to produce significant inhibition in the ELISA; however, combinations of synthetic pep-

tides produced up to 35% inhibition (Table II). Inhibition by 3 peptides (5.4, 5.7, and 5.8) nearly equaled the level of inhibition found with 6 (5.1, 5.2, 5.4, 5.6, 5.7, and 5.8). The maximum amount of inhibition that could be achieved with a mixture of 100 µg of each peptide was 42% (data not shown), which is similar to the inhibition achieved with 50 µg of natural protein.

### Alanine substitution analysis

Alanine substitution has been widely used to study epitopes because its substitution removes the side chain contribution to the binding. Alanine substitution analysis was used to identify the AAs important for IgE binding within each epitope. For each epitope, 12 peptides were synthesized on a SPOTs membrane, with the native sequence as the first SPOT and the remaining 11 SPOTs each having successive AAs substituted with an alanine or with a methionine if the native sequence was alanine. IgE binding to the native epitope sequence (Fig 3, column N) was more intense for epitopes 5.4, 5.7, and 5.8 than for epitopes 5.1, 5.2, or 5.6. In each epitope, substitution of several key AAs significantly reduced or eliminated IgE binding (Fig 3). IgE binding to the altered sequence was assessed by using densitometry scanning and compared with binding by the native sequence to identify substitutions that significantly reduced IgE reactivity (Table III). The AAs initially targeted for change by site-directed mutagenesis are indicated in the last column. When possible, we chose AAs that were centrally located in the epitope and induced at least 70% inhibition. Because this pool of human sera did not react to epitope 5.10, the critical AA for this epitope was set at position 134 on the basis of the reactivity of rabbit anti-Hev b 5 (data not shown) to the alanine substitution SPOTs membrane.

### Site-directed mutagenesis

Using site-directed mutagenesis to change the cDNA, we generated 20 recombinant Hev b 5 clones with a single mutated epitope or a combination of mutated epitopes (Table IV). Each change was confirmed by sequence analysis. The ability of the recombinant proteins to inhibit the binding of IgE to rHev b 5 was assessed by using inhibition ELISA. Fig 4 shows the inhibition curves for clones with various combinations of mutations. GST used as a negative control failed to cause significant inhibition. Reduced IgE-binding activity was evident by the curve shifting to the right.

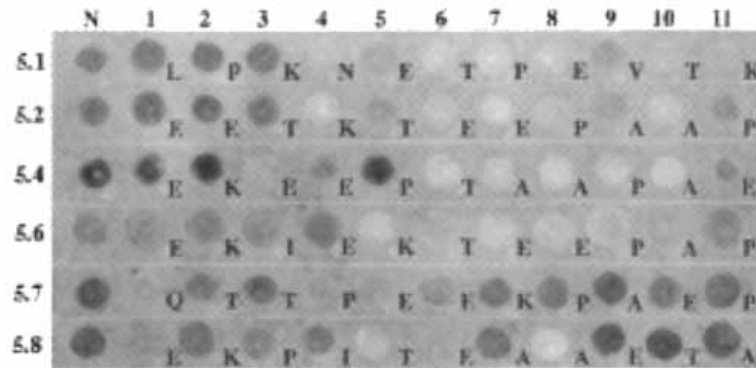


**FIG 3.** Alanine substitution analysis of Hev b 5 immunodominant epitopes. Alanine substitution SPOTs membrane reacted with pooled sera from 7 highly sensitized patients. Epitope designation is indicated at the left. Column N represents the native sequence, and in subsequent columns the AA that was changed to alanine is indicated to the right of each spot.

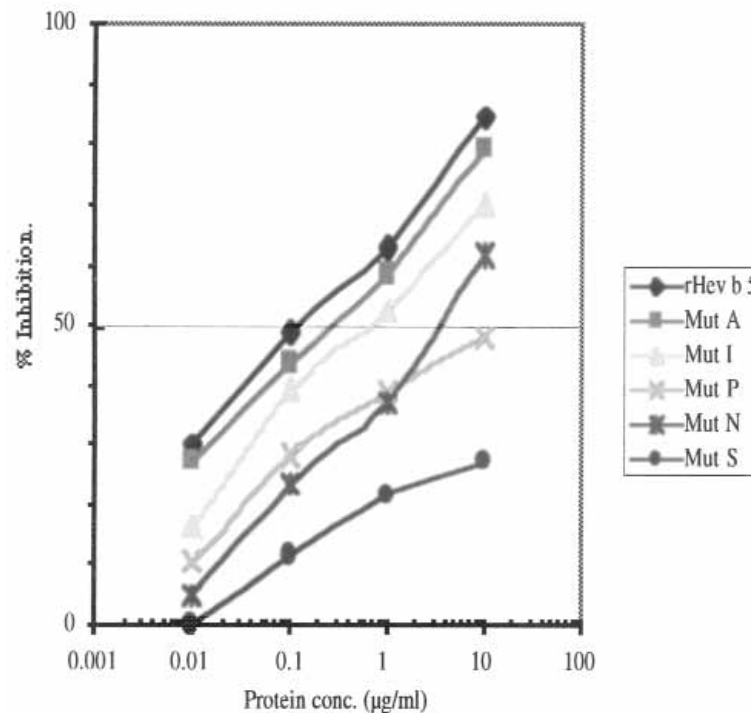


**FIG 4.** Plot of the inhibition ELISA curves for selected mutant (*Mut*) proteins. The 50% inhibition level used for comparison of the clones is indicated.

To compare mutant clones, we used regression analysis to calculate the amount of protein necessary to produce a 50% inhibition in IgE binding (Table IV). Single mutations only produced a 2- to 3-fold reduction in IgE binding. Changes in multiple epitopes (clones H-M) resulted in a 6- to 20-fold reduction in the IgE binding. A single mutation in each of 7 epitopes (M) only resulted in a 20-fold reduction in IgE binding. Because we expected greater reductions, we examined the possibility that the mutations we made in the 3 immunodominant epitopes (5.4, 5.7, and 5.8) were not effective in eliminating binding to the epitope. Mutation N incorporated one addi-

tional AA change in epitopes 5.4 (E<sup>50</sup>→A), 5.7 (Q<sup>87</sup>→A), and 5.8 (K<sup>110</sup>→A) but did not further reduce (27-fold vs 20-fold) IgE binding. Because epitope 5.7 appeared to be a large epitope (Fig 1) or perhaps several epitopes, an additional change of 2 AAs (K<sup>93</sup>→A and P<sup>94</sup>→A) was also tested. This resulted in a significant decrease in IgE binding (mutation O vs I and mutation R vs N), indicating that the C-terminal region of the epitope still had significant IgE binding in mutation N.

Next we examined the contribution of the newly identified epitopes 5.5 and 5.9 by mutating 2 AAs in epitope 5.9 (K<sup>125</sup>→A and P<sup>126</sup>→A) and 1 in epitope 5.5 (E<sup>70</sup>→A).

**TABLE III.** Effect of alanine substitution on IgE binding to Hev b 5 epitopes

| Epitope | Native AA sequence (% inhibition) |                       |                       |                       |                       |                       |                       |                       |                       |                        |                      |  | AA to change |
|---------|-----------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------|----------------------|--|--------------|
| 5.1     | L <sup>13</sup> (4)               | P <sup>14</sup> (-6)  | K <sup>15</sup> (6)   | N <sup>16</sup> (88)  | E <sup>17</sup> (73)  | T <sup>18</sup> (97)  | P <sup>19</sup> (100) | E <sup>20</sup> (96)  | V <sup>21</sup> (55)  | T <sup>22</sup> (84)   | K <sup>23</sup> (73) |  | 18           |
| 5.2     | E <sup>25</sup> (1)               | E <sup>26</sup> (0)   | T <sup>27</sup> (11)  | K <sup>28</sup> (96)  | T <sup>29</sup> (55)  | E <sup>30</sup> (91)  | E <sup>31</sup> (100) | P <sup>32</sup> (87)  | A <sup>33</sup> (62)  | A <sup>34</sup> (91)   | P <sup>35</sup> (40) |  | 31           |
| 5.4     | E <sup>48</sup> (15)              | K <sup>49</sup> (-12) | E <sup>50</sup> (82)  | E <sup>51</sup> (57)  | P <sup>52</sup> (-3)  | T <sup>53</sup> (97)  | A <sup>54</sup> (95)  | A <sup>55</sup> (99)  | P <sup>56</sup> (94)  | A <sup>57</sup> (96)   | E <sup>58</sup> (56) |  | 53           |
| 5.6     | E <sup>73</sup> (19)              | K <sup>74</sup> (1)   | I <sup>75</sup> (16)  | E <sup>76</sup> (-19) | K <sup>77</sup> (91)  | T <sup>78</sup> (67)  | E <sup>79</sup> (92)  | E <sup>80</sup> (84)  | P <sup>81</sup> (70)  | A <sup>82</sup> (55)   | P <sup>83</sup> (2)  |  | 78           |
| 5.7     | Q <sup>87</sup> (73)              | T <sup>88</sup> (35)  | T <sup>89</sup> (18)  | P <sup>90</sup> (67)  | E <sup>91</sup> (72)  | E <sup>92</sup> (65)  | K <sup>93</sup> (32)  | P <sup>94</sup> (37)  | A <sup>95</sup> (6)   | E <sup>96</sup> (25)   | P <sup>97</sup> (21) |  | 91           |
| 5.8     | E <sup>103</sup> (56)             | E <sup>104</sup> (27) | P <sup>105</sup> (48) | K <sup>106</sup> (37) | H <sup>107</sup> (87) | E <sup>108</sup> (73) | T <sup>109</sup> (22) | K <sup>110</sup> (97) | E <sup>111</sup> (-9) | T <sup>112</sup> (-13) | E <sup>113</sup> (5) |  | 108          |

Data in parentheses indicate the percentage inhibition compared with that of the native peptide, as determined by using densitometry. Epitopes 5.3, 5.5, 5.9, 5.10, and 5.11 were not analyzed by means of alanine substitution.

**TABLE IV.** Reduction of IgE-binding activity by mutant Hev b 5 clones

| Clone    | Description<br>(epitopes changed) | Position of AAs<br>changed to alanine                           | Protein required for<br>50% inhibition<br>(mean $\pm$ SE) | Fold change<br>in<br>IgE binding |
|----------|-----------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------|----------------------------------|
| rHev b 5 | Native                            | —                                                               | 0.12 $\pm$ 0.01                                           | —                                |
| Mut A    | 1                                 | 18                                                              | 0.28 $\pm$ 0.03                                           | 2                                |
| Mut B    | 2                                 | 31                                                              | 0.46 $\pm$ 0.09                                           | 4                                |
| Mut C    | 4                                 | 53                                                              | 0.36 $\pm$ 0.06                                           | 3                                |
| Mut D    | 6                                 | 78                                                              | 0.30 $\pm$ 0.04                                           | 2                                |
| Mut E    | 7                                 | 91                                                              | 0.39 $\pm$ 0.03                                           | 3                                |
| Mut F    | 8                                 | 108                                                             | 0.44 $\pm$ 0.07                                           | 4                                |
| Mut G    | 10                                | 134                                                             | 0.28 $\pm$ 0.07                                           | 2                                |
| Mut H    | 4, 7                              | 53, 91                                                          | 0.66 $\pm$ 0.04                                           | 5                                |
| Mut I    | 4, 7, 8                           | 53, 91, 108                                                     | 0.92 $\pm$ 0.19                                           | 8                                |
| Mut J    | 2, 4, 7, 8                        | 31, 53, 91, 108                                                 | 1.18 $\pm$ 0.40                                           | 10                               |
| Mut K    | 1, 2, 4, 7, 8                     | 18, 31, 53, 91, 108                                             | 1.86 $\pm$ 0.13                                           | 16                               |
| Mut L    | 1, 2, 4, 6, 7, 8                  | 18, 31, 53, 78, 91, 108                                         | 1.44 $\pm$ 0.33                                           | 12                               |
| Mut M    | 1, 2, 4, 6, 7, 8, 10              | 18, 31, 53, 78, 91, 108, 134                                    | 2.43 $\pm$ 0.43                                           | 20                               |
| Mut N    | 1, 2, 4, 6, 7, 8, 9, 10           | 18, 31, 50, 53, 78, 87, 91, 108, 110, 134                       | 3.35 $\pm$ 0.62                                           | 27                               |
| Mut O    | 4, 7, 8                           | 53, 91, 93, 94, 108                                             | 3.39 $\pm$ 0.53                                           | 27                               |
| Mut P    | 4, 7, 8, 9*                       | 53, 91, 93, 94, 108, 125, 126                                   | 10.38 $\pm$ 1.19                                          | 84                               |
| Mut Q    | 4, 5, 7, 8, 9*                    | 53, 70, 91, 93, 94, 108, 125, 126                               | 13.59 $\pm$ 1.06                                          | 110                              |
| Mut R    | 1, 2, 4, 6, 7, 8, 9, 10           | 18, 31, 50, 53, 78, 87, 91, 93, 94, 108, 110, 134               | 34.09 $\pm$ 2.93                                          | 277                              |
| Mut S    | 1, 2, 4, 6, 7, 8, 9, 10           | 18, 31, 50, 53, 78, 87, 91, 93, 94, 108, 110, 125, 126, 134     | 556.6 $\pm$ 99.5                                          | 4519                             |
| Mut T    | 1, 2, 4, 5, 6, 7, 8, 9, 10        | 18, 31, 50, 53, 70, 78, 87, 91, 93, 94, 108, 110, 125, 126, 134 | 442.5 $\pm$ 121.1                                         | 3593                             |

Data represent means  $\pm$  SE of at least 3 ELISA assays.

Mut, Mutation.

\*Indicates 1 additional AA change.

†Indicates 2 additional AA changes.

‡Indicates 3 additional AA changes.

Because we had not performed alanine substitution analysis for these epitopes, antigenicity prediction by the MacVector program was used to predict which AAs to change. The addition of a change in epitope 5.5 did not further decrease IgE binding (mutation P vs Q and mutation S vs T). The changes to epitope 5.9 produced a dramatic change in IgE binding, resulting in a clone with a 4500-fold reduction in IgE binding. Interestingly, epitope 5.9 has sequence homology to epitope 5.7, with the sequence EKPAE 124-128 being found in epitope 5.7 at position 92-96. Mutations at K<sup>93</sup> and P<sup>94</sup> in epitope 5.7 and K<sup>125</sup> and P<sup>126</sup> in epitope 5.9 induced the greatest reductions in IgE binding, suggesting that epitope 5.9 is cross-reactive with epitope 5.7.

## DISCUSSION

Patients with latex allergy are often hyperreactive to latex protein, making immunotherapy risky. We have

shown that recombinant latex proteins are an important source of purified latex allergens that may serve to improve in vitro and in vivo diagnostic tests.<sup>15</sup> The production of recombinant proteins and the potential to modify the cDNA for these proteins allow novel approaches for immunotherapy. It has been proposed that site-directed mutagenesis be used to produce hypoallergenic variants of potent allergens for immunotherapy.<sup>11</sup> In this study, sera from patients who were highly reactive to Hev b 5 were used to study the IgE epitopes. With site-directed mutagenesis, we successfully altered the epitopes and reduced IgE binding to the recombinant protein. Although many patients with NRL allergy react to multiple latex allergens, we identified 5 patients who were monosensitized to Hev b 5,<sup>15</sup> suggesting that a hypoallergenic Hev b 5 may serve as an immunotherapy agent for these patients.

Previously, we identified 7 human IgE-binding epitopes in Hev b 5 by using sera from patients with latex

allergy.<sup>17</sup> In this study we used highly reactive patient sera to confirm those findings and to identify 4 additional epitopes. SPOTs analysis with individual sera demonstrated considerable heterogeneity among patients for epitope recognition, with epitopes 5.4, 5.7, and 5.8 being more reactive. Using an inhibition ELISA, we found that Hev b 5 epitopes were equally present in both NAL and AL preparations. With the native protein, we were only able to inhibit 62% of the IgE reactivity to rHev b 5, perhaps reflecting a low abundance of Hev b 5 in the natural latex. Inhibition with synthetic peptides confirmed that the epitopes 5.4, 5.7, and 5.8 were immunodominant. A combination of 6 peptides was needed to produce maximum inhibition (35%). This lower level of inhibition by synthetic peptides possibly reflects a lower affinity for the short peptides compared with the native protein.

An important consideration in evaluating recombinant allergens is the conformation of the protein<sup>19</sup> because conformational changes can drastically alter IgE binding.<sup>20</sup> rHev b 5 is unlikely to be in its native conformation because it is coupled to the bacterial GST protein. Like the maltose-binding protein/Hev b 5 construct,<sup>16</sup> attempts to cleave the recombinant Hev b 5 protein from GST resulted in an unstable molecule that degraded almost immediately. Yet the GST/rHev b 5 construct was able to compete for anti-Hev b 5 IgE at least as strongly as the natural protein, indicating that the relevant epitopes are expressed in the recombinant fusion protein.

Functional changes that reduce IgE binding potential need to be determined to produce modified allergens for immunotherapy. Ferreira et al<sup>11</sup> used information from nonallergenic homologues of Bet v 1 to identify the sites to target. Only one homologue to Hev b 5 has been described,<sup>21</sup> and there is no information on its potential allergenicity. A second successful approach has been to replace cysteine residues to prevent the formation of disulfide bonds and dramatically alter the conformation of the native protein.<sup>20</sup> Hev b 5 has no cysteine residues, and no 3-dimensional structural information is available. Another approach involves alanine substitution analysis, and this approach has been used successfully to alter the soybean allergen Gly m Bd 30k.<sup>22</sup>

Initially, we used alanine substitution to identify specific AAs to target for mutation. Because MacVector proved to be an accurate predictor of antigenicity, we also used this computer model to identify potential changes. Twenty different clones were generated by means of site-directed mutagenesis, with changes to single or multiple epitopes. Combinations of epitopes were evaluated until a sequence was found that dramatically reduced IgE reactivity. Mutation of single epitopes had little influence on IgE binding. Mutating 3 immunodominant epitopes produced an approximately 100-fold reduction in IgE binding. Changes to 5 additional epitopes were required to achieve significant reductions in IgE. The clones with the greatest reduction in IgE reactivity needed modification of 8 epitopes (14 individual AA changes) to produce a dramatic reduction (4500-fold) in IgE binding. Site-directed mutagenesis identified

5.9 as a key epitope; however, because of the number of clones that would be required, we did not analyze all combinations of altered epitopes. Experiments are in progress to develop additional clones with various combinations of 5.9 and other immunodominant epitopes to identify the minimal number of changes required to achieve this level of reduction in IgE binding. The human IgE response is polyclonal and heterogeneous, and thus multiple changes appear necessary to reduce the potential for recognition of the hypoallergenic mutant.

Modified allergens for immunotherapy need to have altered B-cell epitopes but not T-cell epitopes<sup>23</sup> to reduce IgE-mediated adverse effects but preserve T-cell recognition and immunogenicity. Other investigators have determined the location of 2 human T-cell epitopes at AAs 46-65 and 109-128 in Hev b 5.<sup>18</sup> Peptide 46-65 was the more common T-cell epitope, stimulating T cells from 5 of 5 donors versus 3 of 5 donors for peptide 109-128. To reduce IgE binding, we made several changes (E<sup>50</sup>→A and T<sup>53</sup>→A) within the dominant T-cell epitope, but it is not known whether this will affect T-cell recognition. Changes to 3 AAs in a dominant T-cell epitope of the Bet v 1 allergen did not alter recognition by a T-cell clone specific for that epitope.<sup>11</sup> The ability for our mutant Hev b 5 clones to stimulate T cells must be determined before use in clinical trials to desensitize patients.

An important discovery in this study was the cross-reactivity between epitopes within the Hev b 5 molecule. We were unable to significantly reduce IgE binding to the clones until both epitopes 5.7 and 5.9 were altered. The sequence similarity (EKPAE) indicates the presence of cross-reacting IgE that was capable of recognizing both epitopes on the same protein. Experiments with rabbit anti-rHev b 5 (unpublished results) indicate that epitopes 5.2 and 5.6, both having the sequence KTEEP, are also cross-reactive. The presence of several cross-reactive epitopes within the same protein makes Hev b 5 a multivalent allergen. Having 2 sites on the same molecule recognized by a single IgE antibody could significantly increase the likelihood of cross-linking the IgE bound to receptors on mast cells and the release of allergic mediators. This multivalent property of Hev b 5 may help to explain the potent nature of latex allergens.

We thank Maren Nalepa, Colleen Landy, Ben McClintic, and Deidre Blake for excellent technical assistance.

## REFERENCES

- Spaner D, Dolovich J, Tarlo S, Sussman G, Butto K. Hypersensitivity to natural latex. *J Allergy Clin Immunol* 1989;83:1135-7.
- Slater JE. Rubber anaphylaxis. *N Engl J Med* 1989;83:1126-30.
- Sussman G, Beezhold D. Allergy to latex rubber. *Ann Intern Med* 1995;122:43-6.
- Tomazic V, Withrow T, Fisher B, Dillard S. Latex-associated allergies and anaphylactic reactions. *Clin Immunol Immunopathol* 1992;64:89-97.
- Carrillo T, Culvas M, Munoz T, Hinojosa M, Moneo J. Contact urticaria and rhinitis from latex surgical gloves. *Contact Dermatitis* 1986;15:69-72.
- Dalrymple SJ, Audley BG. Allergenic proteins in dipped products: factors influencing extractable protein levels. *Rubber Dev* 1992;45:51-60.
- Tomazic VJ, Shampaine EL, Lamanna A, Withrow TJ, Adkinson NF, Hamilton RG. Cornstarch powder on latex products is an allergen carrier.

- er. *J Allergy Clin Immunol* 1994;93:751-8.
8. Leynadier F, Herman D, Vervloet D, Andre C. Specific immunotherapy with a standardized latex extract versus placebo in allergic healthcare workers. *J Allergy Clin Immunol* 2000;106:585-90.
  9. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 1996;2:1122-8.
  10. Slater JE, Colberg-Poley AM. A DNA vaccine for allergen immunotherapy using the latex allergen *Hev b 5*. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankfurt A M* 1997;91:230-5.
  11. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998;12:231-42.
  12. Kraft D, Ferreira G, Vrtala S, Breiteneder H, Ebner C, Valenta R, et al. The importance of recombinant allergens for diagnosis and therapy of IgE mediated allergies. *Int Arch Allergy Immunol* 1999;118:171-6.
  13. Akasawa A, Hsieh L-S, Martin BM, Liu T, Lin Y. A novel acidic allergen, *Hev b 5*, in latex. *J Biol Chem* 1996;271:25389-93.
  14. Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RGO. Identification, cloning, and sequence of a major allergen (*Hev b 5*) from natural rubber latex (*Hevea brasiliensis*). *J Biol Chem* 1996;271:25394-9.
  15. Yip L, Hickey V, Wagner B, Liss G, Slater J, Breiteneder H, et al. Skin prick test reactivity to recombinant latex allergens. *Int Arch Allergy Immunol* 2000;121:292-9.
  16. Slater JE, Paupore EJ, O'Hehir RE. Murine B-cell and T-cell epitopes of the allergen *Hev b 5* from natural rubber latex. *Mol Immunol* 1999;36:135-43.
  17. Beezhold DH, Hickey VL, Slater JE, Sussman GL. Human IgE-binding epitopes of the latex allergen *Hev b 5*. *J Allergy Clin Immunol* 1999;103:1166-72.
  18. de Silva HD, Sutherland MF, Suphioglu C, McLellan SC, Slater JE, Roland JM, et al. Human T-cell epitopes of the latex allergen *Hev b 5* in health care workers. *J Allergy Clin Immunol* 2000;105:1017-24.
  19. Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000;106:409-18.
  20. Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen, Der p 2. *Mol Immunol* 1996;33:399-405.
  21. Ledger SE, Gardner RC. Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var. *deliciosa*)-Plant Mol Biol 1994;25:877-86.
  22. Helm RM, Cockrell G, Connaughton C, West CM, Herman E, Sampson HA, et al. Mutational analysis of the IgE-binding epitopes of P24/gGly m Bd 30K. *J Allergy Clin Immunol* 2000;105:378-84.
  23. Schramm G, Kahlert H, Suck R, Weber B, Stuwe H-T, Muller WD, et al. "Allergen engineering": Variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J Immunol* 1999;162:2406-14.

### Availability of Journal Back Issues

As a service to our subscribers, copies of back issues of *The Journal of Allergy and Clinical Immunology* for the preceding 5 years are maintained and are available for purchase until inventory is depleted. The following quantity discounts are available: 25% off on quantities of 12 to 23, and one third off quantities of 24 or more. Please write to Mosby Subscription Customer Service, 6277 Sea Harbor Dr, Orlando, FL 32887, or call (800) 654-2452 or (407) 345-4000 for information on availability of particular issues and prices. If unavailable from the publisher, photocopies of complete issues may be purchased from Bell & Howell Information and Learning, 300 N Zeeb Rd, Ann Arbor, MI 48106, (313) 761-4700.