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*J Immunol* 2004; 172:2621-2628; doi: 10.4049/jimmunol.172.4.2621

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Construction of Hevein (Hev b 6.02) with Reduced Allergenicity for Immunotherapy of Latex Allergy by Comutation of Six Amino Acid Residues on the Conformational IgE Epitopes

Piia Karisola,* Jari Mikkola,† Nisse Kalkkinen,‡ Kari J. Airenne,§ Olli H. Laitinen,§ Susanna Repo,¶ Olli T. Pentikäinen,¶ Timo Reunala,¶ Kristiina Turjanmaa,¶ Mark S. Johnson,¶ Timo Palosuo,¶ Markku S. Kulomaa,* and Harri Alenius2†

Recently we have established that IgE Abs bind to conformational epitopes in the N- and C-terminal regions of the major natural rubber latex allergen, hevein (Hev b 6.02). To identify the critical amino acid residues that interact with IgE, the hevein sequence was scanned by using site-specific mutations. Twenty-nine hevein mutants were designed and produced by a baculovirus expression system in insect cells and tested by IgE inhibition-ELISA using sera from 26 latex allergic patients. Six potential IgE-interacting residues of hevein (Arg, Lys, Glu, Tyr, His, and Gln) were identified and characterized further in detail. Based on these six residues, two triple mutants (HΔ3A, HΔ3B) and hevein mutant where all six residues were mutated (HΔ6), were designed, modeled, and produced. Structural and functional properties of these combinatorial mutants were compared experimentally and in silico with those of recombinant hevein. The IgE-binding affinity of the mutants decreased by three to five orders of magnitude as compared with that of recombinant hevein. Skin prick test reactivity of the triple mutant HΔ3A was drastically reduced and that of the six-residue mutant HΔ6 was completely abolished in all patients examined in this study. The approach presented in this paper offers tools for identification and modification of amino acid residues on conformational epitopes of allergens that interact with IgE. Hevein with a highly reduced ability to bind IgE should provide a valuable candidate molecule for immunotherapy of latex allergy and is anticipated to have a low risk of systemic side effects. The Journal of Immunology, 2004, 172: 2621–2628.

The knowledge of conformational IgE-binding epitopes of allergens is important in the design of specific therapies for immediate type allergy. Nowadays, corticosteroids, antihistamines, and specific immunotherapy (SIT)† are used to alleviate symptoms of the immediate reactions. In conventional immunotherapy, varying doses of allergen-containing rubber-tree extracts are introduced to raise the state of unresponsiveness toward the applied allergen. One disadvantage of SIT is that the administration of allergenic material may cause severe and life-threatening anaphylactic side effects (1). This problem can be bypassed by using isoforms of allergens (2), deletion mutants (3), fragments of proteins (4, 5), or linear peptides (6) that lack the original IgE-binding epitopes. The use of engineered “hypoallergenic” variants with reduced IgE reactivity is now being explored in several systems (7, 8). In the first trials, disulphide bridges were eliminated by site-directed mutagenesis (9), but the protein involved failed to achieve a native-like three-dimensional (3-D)-fold and conformation-based allergen uptake by APCs was hindered. At present, it is possible to modify IgE-binding residues of an allergen where the allergen still retains its 3-D structure. However, the amino acid residues of protein allergens that interact with IgE must first be identified.

Natural rubber latex (NRL) allergy and the major latex allergen, hevein (Hev b 6.02, 4.7 kDa), provide an advantageous model pair for immunotherapeutic studies. Hevein is a small and stable protein that is recognized by ~70% of NRL allergic patients (10, 11). Its 3-D structure has been solved (12, 13) and several hevein homologues have been identified (14–16). Linear IgE-binding epitopes of hevein have extensively been studied using overlapping synthetic peptides (17, 18). In our previous study, we transferred regions of hevein into a nonallergenic, structurally homologous adaptor protein, an antimicrobial protein (AMP) from the amaranth (Amaranthus caudatus) (19). Using chimeras thus produced, those portions of the hevein structure that bind IgE were located to the hevein N terminus and C terminus. However, no information is available at present about the amino acid residues on conformational epitopes of hevein that are critical for IgE binding.

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Received for publication September 30, 2003. Accepted for publication December 11, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This study is partly supported by grants from the Academy of Finland, Sigrid Juslinus Foundation (to S.R.), and National Graduate School in Informational and Structural Biology.

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3 Abbreviations used in this paper: SIT, specific immunotherapy; 3-D, three dimensional; NRL, natural rubber latex; AMP, antimicrobial protein from the amaranth; rHEV, recombinant hevein; SPT, skin prick test; WT, wild type.

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0022-1767/04/$02.00
To characterize the amino acid residues from hevein that interact with IgE, we scanned the hevein sequence by site-directed mutagenesis taking into account the sequences seen in homologous proteins. Six potential IgE-interacting residues were identified and characterized, and based on these residues, three mutant heveins with different combinations of mutated residues were designed and produced. The modeled proteins had native-like 3-D structures, but highly reduced IgE-binding capacity studied both in vitro and in vivo.

Materials and Methods

Sequences, structures, and computational analyses

The hevein (Protein Data Bank access code 1HEV) nuclear magnetic resonance structure (13) was used. Sequence database searches using the Swiss-Prot Protein Sequence Database (20) and multiple sequence alignments were performed as previously described (19). On the basis of the multiple sequence alignment made using CLUSTAL X (21), the target mutants were produced by mutating the residues in question in the structural modeling of pilot molecules (H3A, H3B, and H3D) without atomic clashes in their current positions were chosen. Position-specific rotamer analysis (23) of the side chains of substituted residues was performed using Insight98.0 software (Molecular Simulations, San Diego, CA), which fit statistically and structurally the most preferred amino acid residue rotamers by calculating enthalpic energies for the so-called evolutionary approach based on analogous sites in hevein-like proteins (92 aligned protein sequences) (22).

Production and purification of recombinant proteins

To perform the epitope scanning, single substitution mutants were constructed (Table 1). Nine hevein mutants were constructed with the megaprimer PCR method (26) and 13 mutants with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the instruction manual. The remaining single mutation heveins were constructed with terminal primers. Both (megaprimer and QuickChange; Stratagene) techniques were used to construct and clone the combinatorial mutants (H3A, H3B, and H3D). These clones were then checked and produced using the Bac-To-Bac expression system (Invitrogen, San Diego, CA) in insect cells, and purified as fusion proteins with chicken avidin as previously described (27). Recombinant protein production was performed either in SF-900 II SF (Life Technologies, Gaithersburg, MD) or in HiQ SFX (HyClone, Logan, UT) medium that was serum-free and depleted of antibiotics.

Depending on the method to be used, the buffer of the fusion proteins was changed to fresh 25 or 50 mM Tris·Cl, pH 8, with a desalting column HR10/10 (Amersham Biosciences, Uppsala, Sweden) or with a Fast Desalting column (Amersham Biosciences). Samples were concentrated with an Ultrafree-MC (30,000 NMWL, Filter Unit; Millipore, Bedford, MA) vacuum evaporator or with a MICROSEP ( Pall Filtron, Northborough, MA). All fusion proteins were cleaved with an excess of thrombin (Amersham Biosciences) according to the manufacturer’s instructions overnight at room temperature (23–25°C) or at 37°C.

Characterization of the recombinant proteins

Protease-cleave recombinant hevein (rHEV) and mutants were analyzed by reversed-phase high-performance liquid chromatography on a PepRP

Table 1. Primers used in the study

<table>
<thead>
<tr>
<th>Amino Acid Change</th>
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<td>Hev_5’</td>
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<tr>
<td>Hev_3’</td>
<td>5’-AACAAGCCTTCTTGAATCTTGAATGTTGTCGG-3’</td>
</tr>
<tr>
<td>E1A</td>
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<td>Q3V</td>
<td>5’-TAGAGGAGGGAAGGGTGTGGCTGGCCACAG-3’, 5’-CTTGCGGACACACACTTGGCTCTCTCTA-3’</td>
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<td>R5A</td>
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<tr>
<td>G6V</td>
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<td>K10A</td>
<td>5’-TATAGGATCCCCTGTGCTCTAGAGGAGGGAATGTTGTCGG-3’, 5’-GCTGCGCACTGGCCACACAGTCG-3’</td>
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<td>L11A</td>
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<tr>
<td>P13T</td>
<td>5’-GGCGAAGCTTCCAGAATAAACATACCTAG-3’</td>
</tr>
<tr>
<td>N14A</td>
<td>5’-CAAGCCTGCCCCGCTTAACATAGTTGTA-3’, 5’-TACACACATTAGGTACGGGGCGACATCC-3’</td>
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<td>D34G</td>
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<tr>
<td>S39A</td>
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<tr>
<td>N40A</td>
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<tr>
<td>K42A</td>
<td>5’-CAAGGCTTTAGGTGAAATTGCTGCGGAGGTGGCC-3’, 5’-CTTGCGGACACACACTTGGCTCTCTCTA-3’</td>
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<tr>
<td>E29A + Y30F in H3D</td>
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</tr>
<tr>
<td>Q38V in H3D</td>
<td>5’-GCTAGAAACCTTTTTAGCTGCTGACTTCTG-3’, 5’-GCAAGGCAGTGGGAGGCA-3’</td>
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</tbody>
</table>

*BamHI and HindIII sites are underlined, thrombin cleavage sites are in italics, and mutation sites are in bold.
HR 5/5 column (Amersham Biosciences) using a linear gradient of acetonitrile (0—40% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min. Some mutants were run using a 0—100% acetonitrile gradient and a RP HR 5/2 column (Amersham Biosciences) at flow rate of 1.0 ml/min. Chromatography was monitored at 214 and 280 nm. The separated proteins were collected and further analyzed by mass spectrometry.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed using a Bruker Biflex II (Bruker-Daltonics, Bremen, Germany). Approximately 1 pmol (0.5 μl) of the HLPC purified proteins were applied to a thin-layer matrix preparation (0.5 μl of saturated α-cyano-4-hydroxycinnamic acid in acetone) and air-dried. External calibration was performed with insulin (Sigma-Aldrich, St. Louis, MO).

Immunological analysis with sera from NRL-allergic patients and controls

Serum samples were obtained from 26 NRL-allergic patients (23 women and 3 men; mean age, 45 years; age range, 11—72 years), all previously shown to have IgE against hevein. All patients suffered contact urticaria and had positive skin prick test (SPT) responses to the natural hevein. Sera from 19 control subjects without NRL allergy were used as controls in the inhibition-ELISA studies.

Inhibition-ELISA

The inhibition-ELISA for scanning purposes was performed as previously described (19) with the following exceptions. Microtiter plates were coated with native hevein at a concentration of 3 μg/ml, and mutants, mixed with sera, were applied at a concentration of 1 μg/ml. The optimal concentration of mutant protein for the scanning experiments (1 μg/ml) was selected on the basis of preliminary experiments in which several mutant concentrations were tested.

In more detailed studies, inhibition-ELISA was performed with six single-mutation heveins (R5A, K10A, E29A, Y30F, H35A, Q38V) at three different concentrations 0.1, 10, 1000 ng/ml, and some mutants also at 10 μg/ml. With the pilot molecules HΔ3A, HΔ3B, and HΔ6, inhibitions were performed at a concentration of 0.1, 10, and 1000 ng/ml. Sera (diluted 1/10) were obtained from five patients, which were found to recognize AMPN and/or AMPC chimeras in our previous study (19). Thereafter, biotinylated goat anti-human IgE (diluted 1/1000; Vector, Burlingame, CA) was added, followed by streptavidin-conjugated alkaline phosphatase (diluted 1/1000; Zymed, San Francisco, CA), and color substrate (Bio-Rad, Hercules, CA). Developed color was read at 405 nm using an automated ELISA reader (Multiskan MS; Labsystems, Helsinki, Finland).

Skin prick tests

The samples and the negative control to be used in the SPTs were first concentrated by evaporation and then diluted to PBS at concentrations of 0.1 and 1 μg/ml. The tests were performed as previously described (28) with four of the five patients whose sera were used in the detailed ELISA experiments. Histamine at a concentration of 50 μg/ml was used as a positive control.

Results

Design and production of hevein mutants with single amino acid substitutions

To investigate in detail the interactions between IgE and the allergen, the hevein molecule was scanned with single amino acid substitutions using site-specific mutagenesis (Fig. 1). Only glycines, alanines, and cysteines were excluded from mutagenesis because of their anticipated low potential of being involved in interactions with IgE. Target amino acid substitutions were designed using information obtained from the analogous sites in the amino acid sequences of 92 plant proteins containing hevein-like domains. Substitutions were tested by using a rotamer-library of existing structures to exclude residues inconsistent with the local structural environment and to estimate the possible orientation in 3-D environment. After construction of the gene, production and affinity purification of the fusion protein, the substitution mutants were cleaved apart from the purification tag (avidin) and their molecular mass was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Altogether, 29 hevein-mutants were successfully produced and purified and included in the immunological tests.

Inhibition-ELISA of the 29 single aa residue mutations of hevein. Bars indicate the percentage of patients showing less than 80% inhibition of IgE binding to solid phase hevein (Fig. 2). Based on the scanning, single residue mutants causing <80% inhibition in IgE binding were considered as IgE-interacting residues. If >25% of the patients showed this reduction, the mutant was considered to be potentially significant, and it was selected for further studies (Fig. 2). According to these criteria, six mutants (R5A, K10A, E29A, Y30F, H35A, and Q38V) were selected for more detailed immunological studies using sera from five reference patients with verified NRL allergy. Reference patients were selected so that their IgE-reactivity pattern was heterogenic and covered the spectrum of IgE reactivities to hevein seen in sera used for scanning experiments. An ~10- to 100-fold higher amount of the mutants were required to inhibit IgE binding to hevein by 50% as compared with rHEV (Fig. 3). The mutants R5A and K10A had the strongest reduction in the ability to inhibit binding of IgE Abs to hevein. The maximal inhibition of ~60% was obtained with the R5A and K10A mutants at concentrations of 10 and 1 μg/ml, respectively (Fig. 3).

Structural studies and molecular modeling of combinatorial hevein mutants

To identify the combinatorial effects of six selected single residue substitutions, three different combinatorial mutants were designed,
produced, and tested. Two triple mutants, HΔ3A (R5A, E29A, and H35A) and HΔ3B (K10A, Y30F, and Q38V), contained one amino acid substitution near the N terminus, one in the core region, and one near the C terminus. The six-residue mutant, HΔ6 (R5A, K10A, E29A, Y30F, H35A, and Q38V), contained all six mutations. To avoid structural conflicts due to the introduced mutations at their structural environment, the 3-D structures of the candidate mutants were predicted using Homodock in Bodil. According to the modeling, the folds of all combinatory mutants (HΔ3A, HΔ3B, and HΔ6) were expected to remain fairly unaltered, and all four disulphide-bonds should be formed in a way similar to those in WT hevein.

In HΔ3A the charge distribution of the surface is quite drastically changed, because large areas of positive and negative charges are missing (Fig. 4; compare WT and HΔ3A) that may interfere with the binding of IgE. The mutation R5A would eliminate several strong interactions involving positively charged arginine (Arg5), polar glutamines (Gln6, Gln20, and Gln38), and asparagine (Asn36) in that area. However, the other polar residues can still interact with each other and stabilize the overall structure. The interaction of glutamate (Glu29) with threonine (Thr27) and histidine (His35) in WT hevein also would be eliminated by the mutations E29A and H35A. The surface topology of the HΔ3B is less altered than that of HΔ3A and HΔ6. The major change in HΔ3B results from the loss of the hydrogen bond between lysine (Lys10) and the main chain carbonyl oxygen atom of glutamine (Gln2) due to the K10A mutation. In contrast, regardless of the effects of the K10A mutation, the structure is stabilized by the N-terminal disulphide bridge (Cys3-Cys18).

In HΔ6 the mutations R5A, Q38V, E29A, H35A, and especially K10A, induce a clear change in the shape and the charge distribution of the surface where they are located (Fig. 4; compare WT...
and Glu29, and their substitution with alanine markedly and even stronger structural changes as seen in H\(\Delta3A\) and H\(\Delta6\) changes the surface charge distribution (Fig. 4; compare WT hevein and H\(\Delta6\)). Three of the mutated residues are charged, Arg5, Lys10, and Glu29, and their substitution with alanine markedly changes the surface charge distribution (Fig. 4; compare WT hevein and H\(\Delta6\)). Evidently, the mutation of R5A results in similar and even stronger structural changes as seen in H\(\Delta3A\), because Q38V is mutated at the same time. The mutation K10A destroys an important interaction near the N terminus by substantially changing the shape and the charge distribution of the surface. The other two mutations in H\(\Delta6\), E29A, and H35A, lead to structural changes similar to those in the H\(\Delta3A\) structure. However, E29A is located relatively close to the phenyl ring of Y30F enabling hydrophobic interactions between E29A and Y30F.

**FIGURE 4.** Modeled structures of the combinatorial mutants H\(\Delta3A\), H\(\Delta3B\), and H\(\Delta6\) show the changes of topology and charge distribution in the surface of the protein in comparison with the structure of WT hevein. Negatively (red) and positively (blue)-charged residues and other mutated residues (green) are indicated on the surface of native hevein (WT).

Combinatory mutant H\(\Delta6\) with six single point mutations has dramatically reduced allergicic capacity both in vitro and in vivo.

All three combinatorial mutants inhibited the binding of IgE to solid-phase hevein in a dose-dependent manner (Fig. 5). In two patients (patients 19 and 22), 50% inhibition in IgE binding was reached with all combinatorial mutants (H\(\Delta3A\), H\(\Delta3B\), and H\(\Delta6\)) at a concentration 10- to 100-fold higher than with rHEV. In other two patients (patients 2 and 8), a 10,000-fold higher amount of mutants was required to achieve 50% inhibition. In one patient (patient 26), H\(\Delta3B\) and H\(\Delta6\) caused a dramatic reduction in IgE binding, but only a small effect was seen with H\(\Delta3A\). Although the reactivity of the mutants in vitro varied considerably between patients and between mutants (Fig. 5), the most consistent and strongest reduction in IgE binding was obtained with the H\(\Delta6\) mutant containing all six mutations.

Allergenicity in vivo of the combinatorial mutants was analyzed by SPT with four NRL-allergic patients (Table II). Three patients gave strong and one patient gave moderate positive reactions to rHEV. As seen in Table II, SPT reactivity was abolished in three patients (patients 2, 8, and 22) to the triple mutant H\(\Delta3A\) and reduced in patient 19 to a borderline positive level. SPT reactivity of one patient (patient 19) to the other triple mutant, H\(\Delta3B\), was unchanged as compared with rHEV but decreased markedly in the other two patients (2 and 8) and was abolished in patient 22. SPT reactivity was however completely abolished with the H\(\Delta6\) mutant in all patients tested.

**Discussion**

Conventional allergen-specific immunotherapy with allergen extracts represents one of the few treatment approaches that may eventually cure type I allergy. This approach has, however, suffered from severe problems due to dangerous side-effects including systemic and even anaphylactic reactions (29). The use of allergen isoforms or engineered proteins with reduced IgE-binding ability is now being explored as a means to provide safe and effective allergen specific immunotherapy (30). To design such tools with a low risk of systemic side effects, identification of the IgE-binding epitopes is essential.

X-ray crystallography and NMR studies have provided detailed knowledge of the Ab-Ag complex supporting the idea of conformational B cell epitopes (31, 32). Based on structural studies, it is reported that the Fab of an Ab covers an irregular, but flat area of \(-20–30 \text{ Å}^2\). Such an area contains, on a native antigenic protein, 15–22 residues, of which the most important key residues (5–6 aa) are responsible for stringent binding (33). However, structural studies cannot always be performed, because of limited amount of protein or unsuitable protein sizes for the method. Indeed to date, most allergen IgE epitopes have been located using overlapping synthetic peptides (8–15 aa long) that cover the protein sequence, but such methods only identify linear stretches (34, 35). N-terminal and C-terminal truncations (36–38), fragmentation (39, 40), and in-frame deletion (41) studies have also been successfully used to locate continuous B cell epitopes.

Site-directed mutagenesis often leads to less radical changes to the overall protein structure although its effect on the biological function can be substantial (42). Site-directed substitutions are therefore used to locate active sites of protein as well as to identify functional epitopes. In contrast, mutagenesis has also been used to reduce IgE Ab binding by breaking the 3-D structure of an allergen molecule, for example, by eliminating disulphide bridges. Takai et al. (43) applied this approach to the major house dust mite allergen where they substituted six cysteine, six proline, and three lysine residues in Der f 2 and indicated the presence of five epitopes by immunoblot analysis. Mutagenesis has also been used as a supplementary method to identify amino acids interacting with IgE Abs. Müller et al. (44) predicted the IgE-binding regions of Der p 2 in NMR spectroscopy by hydrogen protection technique where mAbs are used to inhibit IgE Ab binding to the allergen. Only a few residues from the predicted epitope area on Der p 2 proved to be functionally important for Ab binding when the residues were tested with alanine point mutants. Similar results were reported for the peanut allergens Ara h 1, Ara h 2, and Ara h 3 (45, 46) and on the ryegrass pollens allergen Lol p 5 (8).
In our previous study, regions of hevein were transferred to a nonallergenic, structurally homologous adaptor protein, AMP(19). The conformational IgE-binding areas of hevein were located to its N- and C-terminal regions. Using that chimera-based approach, we were, however, unable to identify the specific amino acid residues critical for IgE binding. In the present study, we scanned the hevein sequence by using site-directed mutagenesis to identify the amino acid residues of hevein forming the conformational epitope recognized by IgE. This technique was based on the so called “evolutionary approach” where hevein homologues were compared and substitutions made by neutral or small residues or even by residues, with the opposite physicochemical properties used instead of alanine scanning (22).

Previously, Banerjee et al. (17) synthesized overlapping decapeptides of prohevein (Hev b 6.01) and identified two major linear IgE-binding epitopes (residues 19–24 and 25–37) at the N terminus of prohevein. Essentially similar results were also reported by Beezhold et al. (47), who identified two linear IgE epitopes in hevein (residues 13–24 and 29–36). In the present study, two important IgE-binding amino acid residues, Arg⁵ and Lys¹⁰ at the N terminus and Gln38 at the C terminus, were not located in the same Table II. SPT results with combinatorial hevein mutants in four hevein-allergic patients

<table>
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<td>+</td>
<td>++</td>
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<td>HΔ6</td>
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*a Concentration of rHEV and all of the four mutants was 1 µg/ml. One plus (+) denotes a marginal positive reaction (wheal size at least 3 mm in diameter and at least half of the size produced by histamine), ++, refers to a reaction which is equal to that produced by histamine, and +++ to a reaction that is greater than that produced by histamine. SPT results presented here are means of two separate tests.*
regions where the linear IgE epitopes were previously detected. In contrast, two important amino acid residues, Glu^{29} and Tyr^{30} in the core region, and His^{35} in the C-terminal area, are within the linear epitope regions. These results indicate that linear epitope mapping strategy may generate insufficient information on the amino acid residues important for IgE-binding in comparison with the structure-based IgE-epitope mapping approach used in the present study.

The identity of the amino acid residues from hevein that interact with IgE was then successfully used to design “hypoallergenic” mutant hevein by combining three to six of the most significant single residue substitutions within three combinatorial mutants (H\(\Delta 3A\), H\(\Delta 3B\), and H\(\Delta 6\)). Structural changes and possible structural constraints were studied by using homology-based modeling. Predicted structures suggested that the mutations would not disturb the folding of the mutated proteins. Local changes in shape and charge distribution were, however, predicted in the mutant H\(\Delta 3A\), and especially, in the mutant H\(\Delta 6\). Indeed, these local changes may explain why the IgE-binding ability of these mutants was dramatically reduced in inhibition-ELISA and, more importantly, why the SPT reactivity of mutants was significantly reduced or even completely abolished. With regard to SIT, it is crucial that the T cell epitopes of the mutated molecule remain functional. Since the modified hevein shows only minimal changes in the tertiary structure of the protein it is likely that there is minimal interference in T cell epitopes which is essential for successful immunotherapy. In the present approach, we successfully used evolution guided site-specific mutagenesis together with molecular modeling to identify IgE-interacting amino acid residues on conformational IgE epitopes of hevein. Modified heveins with drastically decreased IgE-binding activity, constructed in this study, seem to have “hypoallergenic” properties in vivo. Such modified heveins can be used as candidate molecules for immunotherapy of NRL allergy, and they should have a low risk of systemic or anaphylactic side effects during the treatment.

Acknowledgments

We thank Prof. Gert Vriend for his supervising advice and comments during the early phase of the study. We also thank Irene Helkala, Helena Honkasalo, and Sari Tillander for their excellent technical assistance. And we are grateful to Dr. Hoong Y. Yeang for his gift of the hevein cDNA.

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