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(54) Title: HYPOALLERGENIC HEVEIN FOR IMMUNOTHERAPY OF LATEX ALLERGY

(57) Abstract: The present invention relates to construction of hevein mutants with reduced allergenicity for immunotherapy of latex allergy by co-mutation of two to 29 amino acid residues, especially three or six amino acid residues, on the conformational IgE epitopes. Prefered positions are R5, K10, E29, Y30, H35 and Q38.

WO 2004/108759 A1

Hypoallergenic hevein for immunotherapy of latex allergy

Field of the invention

This invention relates to hypoallergenic hevein for immunotherapy of latex allergy. More particularly, the present invention relates to construction of hevein mutants with reduced allergenicity for immunotherapy of latex allergy by co-mutation of two to 29 amino acid residues, especially three or six amino acid residues, on the conformational IgE epitopes.

Background of the invention

The knowledge of conformational IgE-binding epitopes of allergens is important in the design of specific therapies for immediate type allergy. Nowadays, corticosteroids, anti-histamines and specific immunotherapy (SIT) are used to alleviate symptoms of the immediate reactions. In conventional immunotherapy, varying doses of allergen-containing extracts are introduced in order 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). Usually 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 (3D) fold and conformation-based allergen uptake by antigen presenting cells was hindered. At present, it is possible to modify IgE-binding residues of an allergen where the allergen still retains its 3D-structure. However, the amino acid residues of protein allergens that interact with IgE must first be identified.

Natural rubber latex (NRL) allergy is an important medical and occupational health problem. Natural rubber latex allergy and its major allergen, hevein (Hev b 6.02, 4,7 kDa), provide an advantageous model system for studies of onset of allergy and the design of immunotherapeutical agents. Hevein is a small and stable protein that is recognized by approximately 70 % of NRL allergic patients (10,11). Its 3D-structure has been resolved (12,13) and several hevein homologues have been identified in various plants (14-16). Linear IgE-binding epitopes of hevein have

extensively been studied using overlapping synthetic peptides (17,18). In one study, regions of hevein were transferred 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. No information is, however, available at present about the amino acid residues from hevein that are critical for IgE binding.

Summary of the invention

Recently it has been established that IgE antibodies bind to N-terminal and C-terminal regions of the major natural rubber latex allergen, hevein (Hev b 6.02). In the present application in order to identify the critical amino acids residues that interact with IgE, the hevein sequence was scanned by using site-specific mutations taking into account the sequences seen in homologous proteins. 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 (Arg5, Lys10, Glu29, Tyr30, His35 and Gln38) were identified and characterized further in detail. Based on these six residues, two triple mutants (HΔ3A, HΔ3B) and one hevein mutant where all six residues were mutated (HΔ6), designed, modeled and produced. Structural and functional properties of these combinatory mutants were compared experimentally and *in silico* with those of recombinant hevein. The modeled proteins had native-like 3D-structures, but the IgE-binding affinity of the mutants decreased by three to five orders of magnitude as compared to that of recombinant hevein. Skin prick test reactivity of the triple mutant H3ΔA was drastically reduced and that of the six-residue mutant HΔ6 was completely abolished in all patients examined.

Consequently, an object of the present invention is to provide tools for identification and modification of amino acid residues on conformational epitopes of allergens that interact with IgE.

Another object of the present invention is to provide a mutant hevein protein with highly reduced ability to bind IgE, which could be used for immunotherapy of NRL allergy, and is anticipated to have a low risk of systemic side effects.

Brief description of the drawings

FIG. 1. Schematic presentation of the mutagenesis of hevein. The boxes indicate N-terminal, core and C-terminal areas of hevein according to previous studies (19). The sequence of native hevein is shown in the first row. The amino acids shaded in gray in the second row indicate independently substituted residues in hevein and the amino acids shaded dark gray in the third row correspond to the most effective residues that were introduced in the combinatory mutants.

FIG. 2. Inhibition-ELISA of the 29 single amino acid residue mutations of hevein. Bars indicate the percentage of patients showing less than 80 % in ability to inhibit IgE-binding to native hevein. If more than 25% of the patients (indicated as horizontal dotted line) showed this reduction, the mutant was considered to be potentially significant. Six of the most potential single substitution mutants are indicated by black bars.

FIG. 3. Inhibition-ELISA of the six selected single amino acid residue mutants. The average inhibition of IgE-binding to solid-phase hevein was detected with six selected mutants (indicated as dotted lines) and rHEV (indicated as a dotted line). Sera from five NRL-allergic patients were analyzed against each mutant and rHEV.

FIG. 4. Inhibition-ELISA of the combinatory mutants. Patient-specific inhibition-ELISAs of the combinatory mutants HΔ3A, HΔ3B and HΔ6 were performed at a concentration of 0.1, 10 and 1000 ng/ml. The inhibition curve with recombinant hevein is shown for comparison (indicated as a dotted line).

Detailed description of the invention

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). In order 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 antibody-antigen complex supporting the idea of conformational B-cell epitopes (31,32). Based on structural studies, it is reported that the Fab fragment of an

antibody covers an irregular, but flat area of about 20-30 Å^2. Such an area contains on a native antigenic protein 15-22 residues, of which the most important key residues (5-6 amino acids) 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. In deed to date, most allergen IgE epitopes have been located using overlapping synthetic peptides (8-15 amino acids 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. On the other hand, mutagenesis has also been used to reduce IgE antibody binding by breaking the 3D-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 antibodies. Müller et al. (44) predicted the IgE-binding regions of Der p 2 in NMR spectroscopy by hydrogen protection technique where monoclonal antibodies are used to inhibit IgE antibody binding to the allergen. Only few residues from the predicted epitope area on Der p 2 proved to be functionally important for antibody 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 pollen allergen Lol p 5 (8).

In a previous study, regions of hevein were transferred to a nonallergenic, structurally homologous adaptor protein, the amaranth antimicrobial protein (AMP) (19). The conformational IgE-binding areas of hevein were located to its N-terminal and C-terminal regions. Using that chimera-based approach, the specific amino acid residues critical for IgE binding were however not identified. In the present invention, the hevein sequence was scanned by using site-directed mutagenesis in order to identify the amino acids 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 by neutral or small residues or even by residues with the opposite physicochemical properties were used instead of alanine scanning (22).

The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein.

The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) could be used. See Cunningham et al., Science 244:1081-1085 (1989). The resulting mutant molecules can then be tested for biological activity. Besides conservative amino acid substitutions, hevein variants include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitutions with one or more of the amino acid residues having a substituent group.

Amino acid sequence variants of hevein are prepared by introducing appropriate nucleotide changes into the hevein DNA, or by synthesis of the desired hevein polypeptide. Such variants represent insertions, substitutions, and/or specified deletions of, residues within or at one or both of the ends of the amino acid sequence of a naturally occurring hevein. Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence. Any combination of insertion, substitution, and/or specified deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity as defined herein.

Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

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 application, two important IgE-binding amino acids, Arg5 and Lys10 at the N-terminus and Gln38 at the C-terminus, were not located in the same regions where the linear IgE epitopes were previously detected. On the other hand, two important amino acid residues, Glu29 and Tyr30 in the core region, and His35 in the C-terminal area, are within the linear epitopes. These results indicate that linear epitope mapping strategy may generate insufficient information on the amino acids important for IgE-binding in comparison with the structure-based IgE-epitope mapping approach used in the present application.

In the present invention the identity of the amino acids 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 combinatory mutants (HΔ3A, HΔ3B and HΔ6). Structural changes and possible structural constraints were studied by using homology modeling. Models suggested that the mutations would not disturb the folding of the mutated proteins, however, local changes in shape and charge distribution were predicted in the mutant HΔ3A and especially in the mutant HΔ6. In deed, 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.

In the present application, evolution guided site-specific mutagenesis was used together with molecular modeling to identify IgE-interacting amino acids on conformational IgE epitopes of hevein. Modified heveins with drastically decreased IgE binding ability, constructed according to this invention, have "hypoallergenic" properties *in vivo*. Such modified heveins may be used as candidate molecules for immunotherapy of NRL allergy and they are expected to have a low risk of systemic or anaphylactic side effects.

The invention is now described in more detail in the following examples.

Example 1

Design and production of hevein mutants with single amino acid substitutions

The hevein (Protein Data Bank access code 1HEV) NMR structure (13) was used. Sequence database searches using the Swiss-Prot Protein Sequence Database (20) and multiple sequence alignments were performed (19). On the basis of the multiple sequence alignment made using CLUSTAL X (21), the target residues to be mutated were chosen by using the so-called evolutionary approach based on analogous sites in hevein-like proteins (92 aligned protein sequences) (22).

Position-specific rotamer analysis (23) of the side chains of substituted residues was performed using Insight98.0 software (Molecular Simulations Inc., San Diego, CA), which fit statistically and structurally the most preferred amino acid rotamers by calculating enthalpic energies for the changed mutant structure. Simple energy minimization of the structure was performed with Insight98.0 and rotamers without atomic clashes in their current positions were chosen.

The homology-based models of the 3D structures of the hevein combinatory mutants were produced by mutating the residues in question in the Bodil modeling environment (Lehtonen JV, Still D-J, Rantanen V-V, Gyllenberg M and Johnson MS, unpublished; www.abo.fi/fak/mnf/bkf/research/johnson/bodil.html). The final conformation of each mutated residue was chosen so that they follow the side chain conformation of the wild-type residue as much as possible. The molecular surfaces were produced with Bodil. Structure representations were prepared with Molscript 2.1 (24) and Raster3D (25).

In order 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 (Fig. 1). Target amino acid substitutions were designed using information obtained from the analogous sites in the amino acid sequences of plant proteins containing hevein-like domains. Substitutions were tested by using a rotamer-library of existing structures in order to exclude residues inconsistent with the local structural environment and to estimate the possible orientation in 3D-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 weight was confirmed by MALDI-TOF mass

spectrometry. Altogether, 29 hevein-mutants were successfully produced and purified and included in the immunological tests.

In order to perform the epitope scanning, single substitution mutants were constructed (table I). Nine hevein mutants were constructed with the megaprimer PCR method (26) and thirteen mutants with the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the instruction manual. The remaining single mutation heveins were constructed with terminal primers.

Both (megaprimer and QuickChange) techniques were used to clone the combinatory mutants (HΔ3A, HΔ3B and HΔ6). These clones were then checked, produced using the Bac-To-Bac™ expression system 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 (Gibco BRL, Life Technologies, Gaihersbur,USA) or in HiQ SFX (HyClone, Logan, USA) medium that was serum-free and depleted of biotin.

Depending on the method to be used, the buffer of the fusion proteins was changed to fresh 25 mM 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, Uppsala, Sweden). Samples were concentrated with an Ultrafree-MC (30,000 NMWL Filter Unit, Millipore Corporation, Bedford, USA) vacuum evaporator or with a MICROSEP (Pall Filtron Corporation, Northborough, USA). All fusion proteins were cleaved with an excess of thrombin (Amersham Biosciences, Piscataway, USA) according to the manufacturer's instructions overnight at room temperature (23-25 °C) or at 37 °C.

Table I. Used primers and their sequences, BamHI and HindIII sites are in underlined, thrombin cleavage sites are in italics and mutation sites are in bold.

Amino acid change	Primer
Hev_5'	5'-TTATTAGGATCC*CTGGTGCCTAGAGGAAGC*GAGCAATGTGGTCG-G-3'
Hev_3'	5'-AACACAAGCTTCTTAGTCTTTGCAATTGCTTTGGC-3'
E1A	5'-GCCTAGAGGAAGC**GCT**CAATGTGGTCGGC-3', 5'-GCCGACCACATTG**AGC**GCTTCCTCTAGGC-3'
Q2V	5'-TAGAGGAAGCGAG**GTG**TGTGGTCGGCAAG-3', 5'-CTTGCCGACCACA**CAC**CTCGCTTCCTCTA-3'
R5A	5'-TATGTGGATCC*GACGACGACGACAAA*GAGCAATGTGGT**GCG**CAA-GCA-3'
Q6V	5'-GCAATGTGGTCGG**GTG**GCAGGTGGCAAGC-3', 5'-GCTTGCCACCTGC**CAC**CCGACCACATTGC-3'
K10A	5'-TTATTAGGATCC*CTGGTGCCTAGAGGAAGC*GAGCAATGTGGTCG-GCAAGCAGGTGGC**GCT**CTCTGCCCCAAT-3'
L11A	5'-AGCAGGTGGCAAG**GCT**TGCCCCAATAACC-3', 5'-GGTTATTGGGGCA**AGC**CTTGCCACCTGCT-3'
P13T	5'-GGCAAGCTCTGC**ACT**AATAACCTATGT-3'
N14A	5'-CAAGCTCTGCCCC**GCT**AACCTATGTTGTA-3', 5'-TACAACATAGGTT**AGC**GGGGCAGAGCTTG-3'
N15L	5'-CTCTGCCCCAAT**CTG**CTATGTTGTAGC-3'
L16A	5'-CTGCCCCAATAAC**GCT**TGTTGTAGCCAGT-3', 5'-ACTGGCTACAACA**AGC**GTTATTGGGGCAG-3'
S19A	5'-TAACCTATGTTGT**GCT**CAGTGGGGGTGGT-3', 3'-ACCACCCCCACTG**AGC**ACAACATAGGTTA-3'

Q20A	5'–ACCACCCCCA**AGC**GCTACAACAT-3'
W21F	5'-TGTTGTAGCCAC**TTC**GGGTGGTGTGGC-3'
W23Y	5'–CCAGTGGGGG**TAC**TGTGGCTCCA-3'
S26A	5'-GGGTGGTGTGGC**GCT**ACTGATGAATAT-3'
T27A	5'-TGGTGTGGCTCC**GCT**GATGAATATTGT-3'
D28A	5'-TGGCTCCACT**GCG**GAATCTTGTT-3'
E29A	5'-TGGCTCCACTGAT**GCT**TATTGTTCACCTG-3', 5'-CAGGTGAACAATAA**AGC**ATCAGTGGAGCCA-3'
Y30F	5'-TCCACTGATGAA**TTC**TGTTCACCTGAT-3'
S32G	5'-AACAC<u>AAGCTT</u>TTTAGTCTTTGCAATTGCTTTGGCAGTTATGATC-AGG**GCC**ACAATATTCATC-3'
P33A	5'-AACAC<u>AAGCTT</u>TTTAGTCTTTGCAATTGCTTTGGCAGTTATGATC-**AGC**TGAACAATAT-3'
D34G	5'-AACAC<u>AAGCTT</u>TTTAGTCTTTGCAATTGCTTTGGCAGTTATG**AC**-**C**AGGTGAACAA-3'
H35A	5'-AACAC<u>AAGCTT</u>TTTAGTCTTTGCAATTGCTTTGGCAGTT**CGC**AT-CAGGTGAA-3'
N36A	5'–AACAC<u>AAGCTT</u>TTTAGTCTTTGCAATTGCTTTGGCA**AGC**ATGAT-CAGG T-3'
Q38V	5'-TGATCATAACTGC**GTG**AGCAATTGCAAAGAC-3', 5'-GTCTTTGCAATTGCT**CAC**GCAGTTATGATCA-3'
S39A	5'-TCATAACTGCCAA**GCT**AATTGCAAAGAC-3', 5'-GTCTTTGCAATT**AGC**TTGGCAGTTATGA-3'
N40A	5'-TAACTGCCAAAGC**GCT**TGCAAAGACT<u>AAG</u>-3', 5'-<u>CTT</u>AGTCTTTGCA**AGC**GCTTTGGCAGTTA-3'

K42A	5'-CCAAAGCAATTGC**GCT**GACTAAGAAGCTTG-3', 5'-CAAGCTTCTTAGTC**AGC**GCAATTGCTTTGG-3'
D43A	5'-AAGCAATTGCAAA**GCT**TAAGAAGCTTGTC-3', 5'-GACAAGCTTCTTA**AGC**TTTGCAATTGCTT-3'
E29A+ Y30F in HΔ6	5'-GGCTCCACTGAT**GCATTT**TGTTCACCTGAT-3', 5'-ATCAGGTGAACA**AAATGC**ATCAGTGGAGCC-3'
Q38V in HΔ3B	5'-GACTAAAGCTTTTTAGTCTTTGCAATTGCTT**AC**GCAGTT-3'

Example 2

Characterization of the recombinant proteins

Protease-cleaved recombinant hevein (rHEV) and mutants were analyzed by reversed-phase high-performance liquid chromatography (HPLC) on a PepRP HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) 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, Uppsala, Sweden) 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 (MALDI-TOF) mass spectrometry was carried out using a Bruker Biflex II (Bruker-Daltonic, Bremen, Germany). About 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).

Example 3

Immunological characterization of single residue mutants

The IgE-binding capacity of the mutants was scanned by inhibition-ELISA using sera from 26 NRL allergic patients with anti-hevein IgE antibodies. The mutants had variable capacity to inhibit the binding of IgE antibodies to solid phase hevein (Fig. 2). Based on the scanning, single residue mutants causing less than 80 % inhibition in IgE binding were considered as IgE-interacting residues. If more than one fourth 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 approximately 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 antibodies to hevein. The maximal inhibition of about 60% was obtained with the R5A and K10A mutants at a concentration of 10 and 1 μg/ml, respectively (Fig. 3).

Example 4

Structural studies and molecular modeling of combinatory hevein mutants

In order to identify the combinatorial effects of six selected single residue substitutions, three different combinatory mutants were designed, produced and tested. Two triple mutants, HΔ3A (R5A, E29A, H35A) and HΔ3B (K10A, Y30F, 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, Q38V), contained all six mutations. Also other combinations using these six single residue mutations could be constructed, i.e. mutants having two to six single residue substitutions. In order to avoid structural conflicts due to the introduced mutations at their structural environment, the 3D-structures of the candidate mutants were built using Homodge in Bodil. According to the modeling, the folds of all combinatory mutants (HΔ3A, HΔ3B, HΔ6) were expected to remain fairly unaltered and all four disulphide-bonds should be formed in a way similar to those in wild-type hevein.

The site-specific mutation was performed for the amino acids R5, E29, H35, K10, Y30 and Q38 in the hevein sequence. These six amino acids were substituted with

other amino acids followingly: R5A, K10A, E29A, Y30F, H35A, Q38V. However, other suitable amino acids or variants thereof could be used for substitution.

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, 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 Glu29 with Thr27 and His35 in wild-type 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 Lys10 and the main chain carbonyl oxygen atom of Gln2 due to the K10A mutation. On the other hand, 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 hevein and HΔ6). 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 HΔ6). Evidently, the mutation of R5A results in similar and even stronger structural changes as seen in HΔ3A, 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 HΔ6, E29A and H35A, lead to structural changes similar to those in the HΔ3A structure. However, E29A is located relatively close to the phenyl ring of Y30F enabling hydrophobic interactions between E29A and Y30F.

Example 5

Determining the allergenic capacity of combinatory mutants both in vitro and in vivo

Serum specimens 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

responses to the natural hevein. Sera from 19 control subjects without NRL allergy were used in the inhibition-ELISA studies.

The inhibition-ELISA assays 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-ELISAs were performed with six singly-mutated 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 (Vector, Burlingame, CA, USA, diluted 1:1000) was added, followed by streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, CA, USA, diluted 1:1000) and color substrate (Bio-Rad, Hercules, CA, USA). Developed color was read at 405 nm using an automated ELISA reader (Multiskan MS, Labsystems, Helsinki, Finland).

All three combinatory mutants inhibited the binding of IgE to solid-phase hevein in a dose-dependent manner (Fig. 5). In two patients (patient 19 and 22), 50 % inhibition in IgE binding was reached with all combinatory mutants (HΔ3A, HΔ3B, HΔ6) at a concentration 10 to 100-fold higher than with rHEV. In another two patients (patient 2 and 8), a 10,000-fold higher amount of mutants was required to achieve 50 % inhibition. In one patient (patient 26), HΔ3B and HΔ6 caused a dramatic reduction in IgE binding, but only a small effect was seen with HΔ3A. 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 HΔ6 mutant containing all six mutations.

Allergenicity *in vivo* of the combinatory mutants was analyzed by skin prick testing (SPT) with four NRL-allergic patients (Table 2). The samples and negative control to be used in the skin prick tests were first concentrated by evaporation and then diluted to PBS at concentrations of 0.1 and 1 μg/ml. The tests were performed (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. Three patients gave strong and one patient gave moderate positive reactions to rHEV. As seen in Table 2, SPT reactivity was abolished in three patients (2, 8, and 22) to the triple mutant HΔ3A and reduced in patient 19 to a borderline positive level. SPT reactivity of one patient (number 19) to the other triple mutant, HΔ3B, was unchanged as compared with rHEV but decreased markedly in the other two patients (2 and 8) and was abolished in patient 22. Interestingly, SPT reactivity was completely abolished with the HΔ6 mutant in all four patients.

Table II. Skin prick test results with combinatorial hevein mutants in four hevein-allergic patients. 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.

	Patient number			
	2	8	19	22
rHEV	+++	++	++	+
HΔ3A	-	-	+	-
HΔ3B	+	+	++	-
HΔ6	-	-	-	-

It should be understood that the detailed description and the specific examples are given for illustration only and various changes and modifications apparent to those skilled in the art will be included in the scope of the invention.

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Claims

1. A hevein mutant with reduced allergenicity, wherein there exists from 2 to 29 site-specific mutations in the hevein amino acid sequence.

2. A hevein mutant of claim 1, wherein the site-specific mutations exist in the N-terminus region (amino acids 1-11) and/or the C-terminus region (amino acids 32-43) of the hevein amino acid sequence.

3. A hevein mutant of claim 1, wherein the site-specific mutations exist in the core region (amino acids 12-31) of the hevein amino acid sequence.

4. A hevein mutant of claim 1, wherein the site-specific mutations exist in two or more of the amino acid residues R5, K10, E29, Y30, H35, Q38.

5. A hevein three-residue mutant HΔ3A (R5X, E29X, H35X), where X is any amino acid residue or variant thereof.

6. A hevein three-residue mutant HΔ3B (K10X, Y30X, Q38X), where X is any amino acid residue or variant thereof.

7. A hevein six-residue mutant HΔ6 (R5X, K10X, E29X, Y30X, H35X, Q38X), where X is any amino acid residue or variant thereof.

8. A hevein three-residue mutant HΔ3A (R5A, E29A, H35A).

9. A hevein three-residue mutant HΔ3B (K10A, Y30F, Q38V).

10. A hevein six-residue mutant HΔ6 (R5A, K10A, E29A, Y30F, H35A, Q38V).

11. Use of hevein mutant with reduced allergenicity for manufacturing of a medicament for immunotherapy of latex allergy.

12. Use of claim 11, wherein the hevein mutant is a hevein three-residue mutant HΔ3A (R5A, E29A, H35A).

13. Use of claim 11, wherein the hevein mutant is a hevein three-residue mutant HΔ3B (K10A, Y30F, Q38V).

14. Use of claim 11, wherein the hevein mutant is a hevein six-residue mutant HΔ6 (R5A, K10A, E29A, Y30F, H35A, Q38V).

15. A method for preventing or treating latex allergy by administering a hevein mutant with reduced allergenicity.

16. A method of claim 15, wherein the hevein mutant is a hevein three-residue mutant HΔ3A (R5A, E29A, H35A).

17. A method of claim 15, wherein the hevein mutant is a hevein three-residue mutant HΔ3B (K10A, Y30F, Q38V).

18. A method of claim 15, wherein the hevein mutant is a hevein six-residue mutant HΔ6 (R5A, K10A, E29A, Y30F, H35A, Q38V).

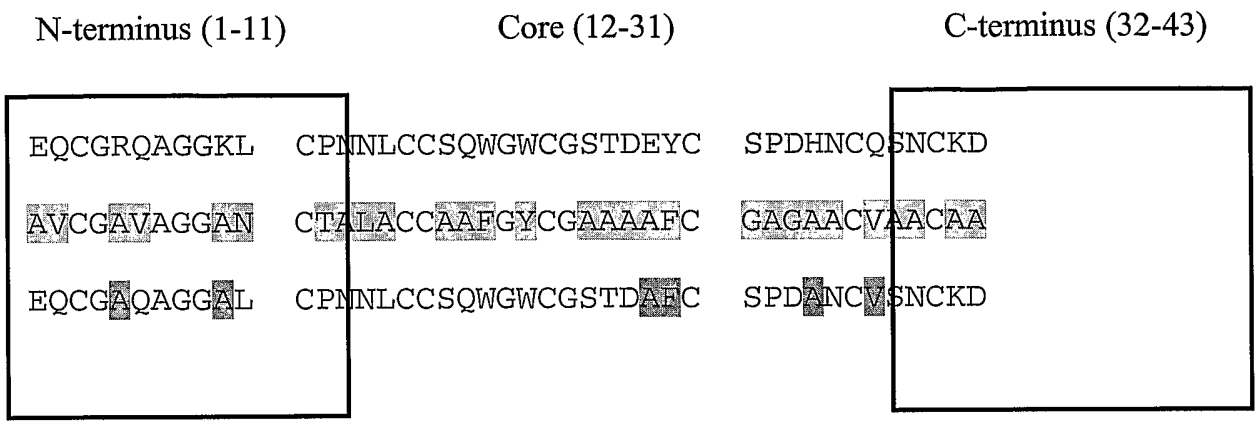
N-terminus (1-11)
Core (12-31)
C-terminus (32-43)
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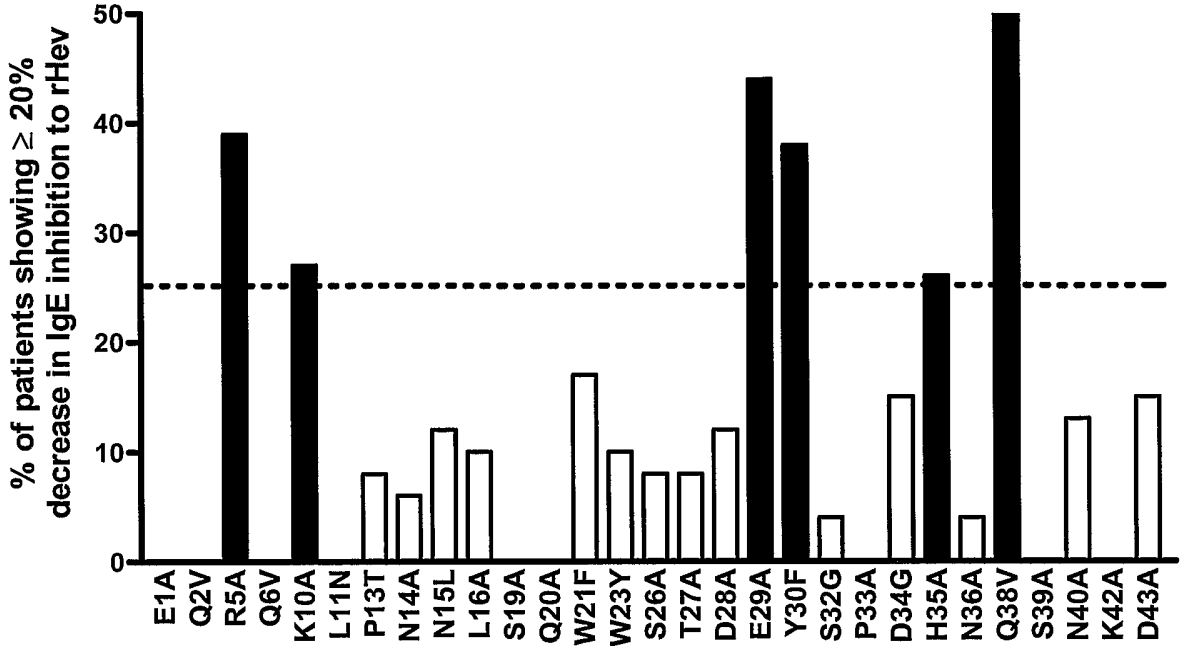
FIG. 1


% of patients showing ≥ 20% decrease in IgE inhibition to rHev
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40
30
20
10
0
E1A
Q2V
R5A
Q6V
K10A
L11N
P13T
N14A
N15L
L16A
S19A
Q20A
W21F
W23Y
S26A
T27A
D28A
E29A
Y30F
S32G
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H35A
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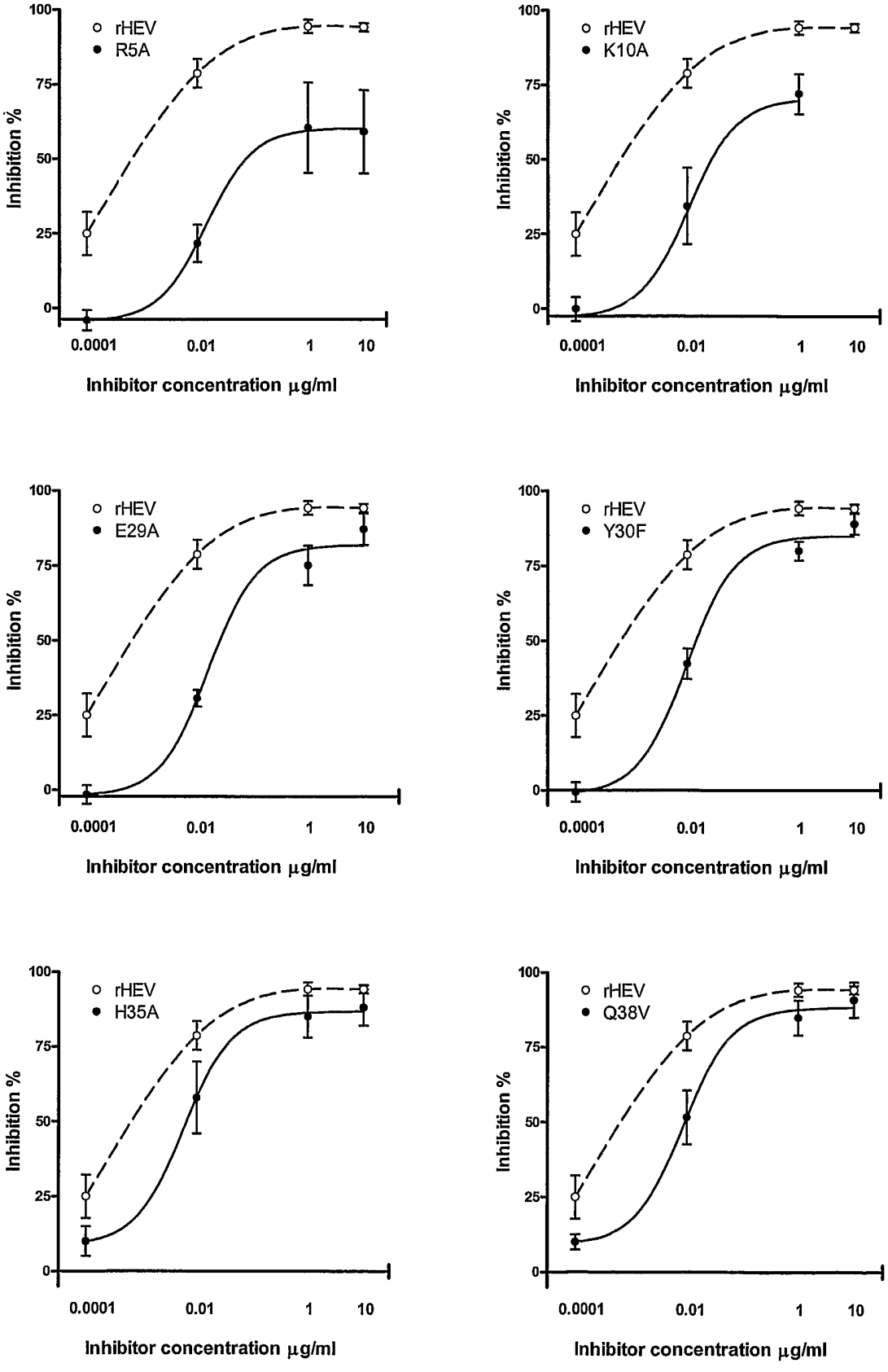
Q38V
S39A
N40A
K42A
D43A

FIG 2.

FIG. 3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R5A
K10A
E29A
Y30F
H35A
Q38V
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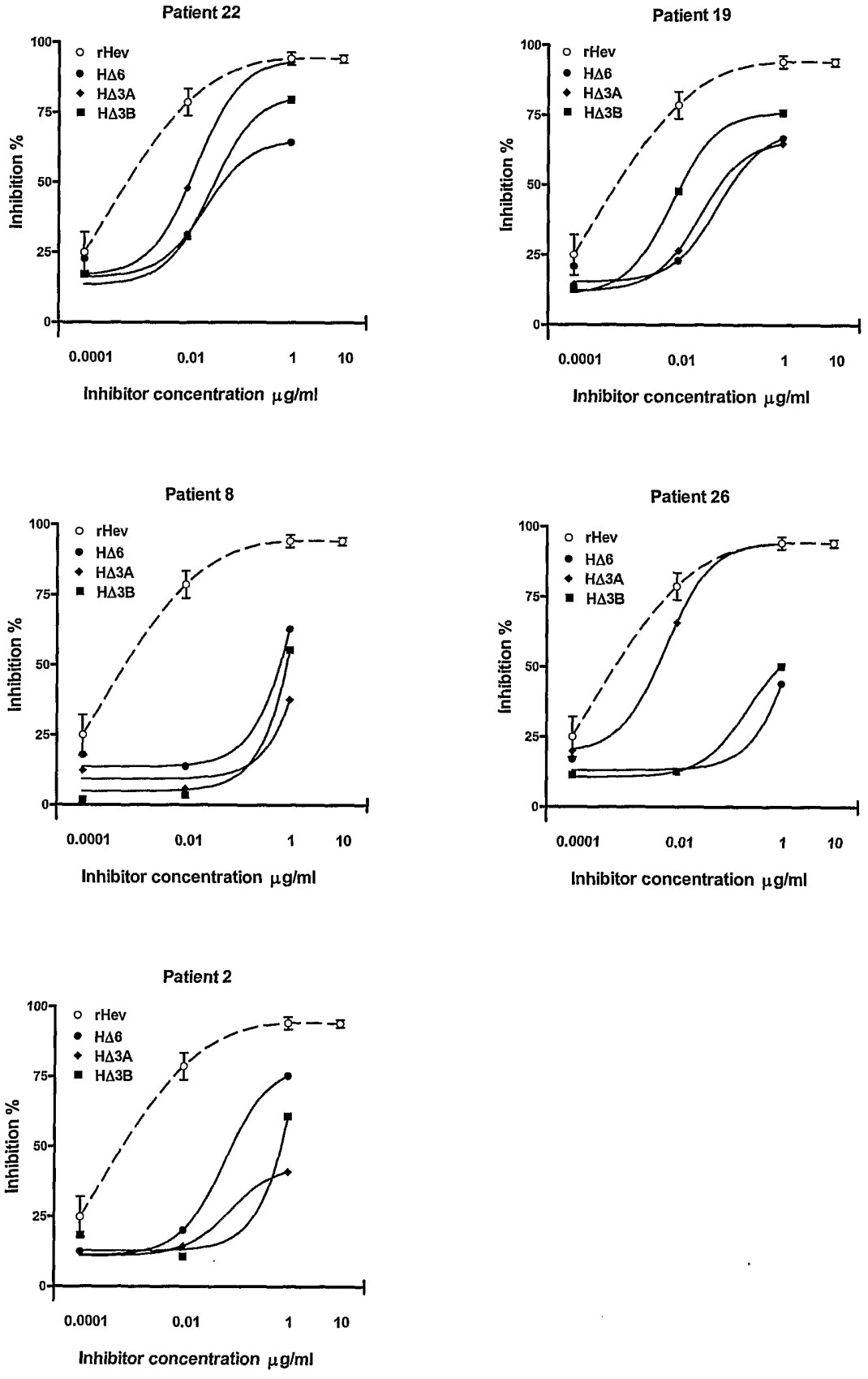
25
0
0.0001
0.01
1
10
Inhibitor concentration µg/ml

FIG 4.


Patient 22
Patient 19
Patient 8
Patient 26
Patient 2
rHev
HΔ6
HΔ3A
HΔ3B
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25
0
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0.01
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10
Inhibitor concentration μg/ml

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SEQUENCE LISTING

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<150> FI 20030850

<151> 2003-06-06

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<221> PEPTIDE

<222> (1)..(43)

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20 25 30

Pro Asp His Asn Cys Gln Ser Asn Cys Lys Asp
35 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2004/000344

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/415, A61K 39/35, A61P 37/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM. ABS DATA, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Karisola Piia et al., "1030 Identification of IgE-Binding Amino Acids on the Conformational Epitopes of Hevein (Hev b 602)" 58th Annual Meeting of the American Academy of Allergy, Asthma and Immunology; New York, NY, USA; March 01-06, 2002, Journal of Allergy and clinical Immunology, January, 2002, vol. 109, Supplement 1, page 333, ISSN 0091-6749, abstract	1-2,11,15
Y	--	2-10,12-14, 16-18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2004	09 -11- 2004
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Therese Persson/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2004/000344

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03037921 A1 (COOPERATIVE RESEARCH CENTRE FOR ASTHMA), 8 May 2003 (08.05.2003), page 23, lines 27-29; page 29, lines 25-26; page 30, lines 16-28, page 36, lines 20-27; page 39, line 26 - page 40, line 3; page 40, lines 15-25; page 42, lines 13-20; examples 6-12	1,11,15
Y		2-10,12-14, 16-18
	--	
X	Alenius H. et al., "Natural rubber latex allergy", Occupational and Environmental Medicine, 2002, Vol. 59, pages 419-424	1-18
Y		2-10,12-14, 16-18
	--	
Y	Banerjee B. et al., "IgE from Latex-Allergic Patients Binds to Cloned and Expressed B Cell Epitopes of Prohevein", The Journal of Immunology, 1997, Vol. 159, pages 5724-5732, table II	3-10,12-14, 16-18
	--	
Y	Karisola P. et al., "The Major Conformational IgE-binding Epitopes of Hevein (Hev b6.02) Are Identified by a Novel Chimera-based Allergen Epitope Mapping Strategy", The Journal of Biological Chemistry, 2002, Vol. 277, No. 25, pages 22656-22661	2,4-10, 12-14,16-18
	--	
P,X	Karisola, P. et al., "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 1", The Journal of Immunology, 2004, vol. 172, no. 4, page 2621 - page 2628, abstract; figure 1; page 2624, column 1, paragraph 1	1-18
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 2004/000344

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Reyes-López, Céasar A. et al, "Insights into a conformational epitope of Hev b 6.02 (hevein)", Biochemical and Biophysical Research Communications, 2004, vol. 314, page 123 - page 130, abstract; figure 1; page 127, column 2, page 128, column 2, paragraph 2	1-4,11,15
P,Y		5-10,12-14, 16-18

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2004/000344

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 15-18
because they relate to subject matter not required to be searched by this Authority, namely:

see next sheet

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Claims 15-18 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/10/2004

International application No.
PCT/FI 2004/000344

WO	03037921	A1	08/05/2003	AU	PR849001 D	00/00/0000
				EP	1451216 A	01/09/2004