

Original article

IgE-binding epitopes of the American cockroach Per a 3 allergen

Background: The Per a 3 is a species-specific allergen of the American cockroach (*Periplaneta americana*) related to insect hemolymph proteins and includes four known isoallergens. This study aimed to identify Per a 3 linear IgE-binding epitopes.

Methods: Per a 3 recombinant fragments were generated from the recombinant Per a 3.01 allergen (685 amino acid residues) by using existing restriction sites or by using polymerase chain reaction products, and expressed in *Escherichia coli*. Antigenicities were assessed by immunoblotting, enzyme-linked immunosorbent assay (ELISA), and binding inhibition with human IgE.

Results: Human IgE recognized recombinant fragments 340–425, 466–579, 502–595, and 595–636 as revealed by immunoblotting and ELISA. On the other hand, the N-terminal fragment 1–399, recombinants 410–443, 472–551, 502–579, 606–636, and the C-terminal fragment 636–685 were unable to bind human IgE. Amino acid sequences 400–409, 466–471, 580–595, and 595–605 were shown to be required for IgE binding to the Per a 3.01 allergen, suggesting that the C-terminus contains most of the IgE-binding sites. Four peptides corresponding to these IgE-binding amino acid sequences were synthesized. These peptides reacted with most sera (62.5–87.5%) tested as revealed by ELISA, demonstrating a heterogeneous IgE-binding response. Moreover, preincubation of IgE-positive recombinant proteins and synthetic peptides with atopic IgE resulted in marked inhibition of the IgE binding to Per a 3.01 allergen. Amino acid sequences ⁴⁰⁰TVLRDPVFYQ⁴⁰⁹, ⁴⁶⁶NNVDQI⁴⁷¹, ⁵⁸⁰VDKGHNYCGYPENLLI⁵⁹⁵, and ⁵⁹⁵IPKGKKGGQAY⁶⁰⁵ of the major recombinant American cockroach Per a 3.01 allergen were involved in IgE binding.

Conclusion: These findings will advance our understanding of the antigenic structures responsible for allergenicity to the American cockroach, thereby providing strategies for the development of immunotherapies.

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Key words: Cockroach Per a 3 allergen; IgE-binding epitopes; immunoblotting; synthetic peptides.

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Accepted for publication 29 November 2002

Aerosolized proteins derived from cockroaches induce IgE-mediated hypersensitivity in humans. Among airborne allergens, those from the cockroach play an important role in allergic diseases, especially asthma (1). Previously, we identified two partially purified allergenic fractions, Cr-PI (Per a 3) and Cr-PII (Per a 1), from American cockroach extract (2). Two prominent proteins of 78 and 72 kDa in Per a 3 cause T cell proliferation in cockroach allergic patients (3), and three proteins of 45, 32, and 28 kDa in Per a 1 (4) have been identified as principal *Periplaneta americana* allergens. German cockroach (*Blattella germanica*) allergens, Bla g 1 (5), Bla g Bd90K (function unknown) (6), Bla g 2 (an inactive aspartic protease) (7), Bla g 4 (ligand binding protein) (8) and Bla g 5 (glutathione S-transferase) (9), and American cockroach allergens, Per a 1 (function unknown) (10–13), Per a 3 (insect hemolymph protein) (14–16) and Per a 7 (tropomyosin) (17, 18), have been cloned. Bla g 1 showed 94% nucleotide sequence homology to Bla g Bd90K and

is now designated as Bla g 1.0102. Per a 1 and Bla g 1 of *B. germanica* share significant sequence homology and are cross-reacting allergens between species. Per a 3 is a species-specific allergen of the American cockroach that causes IgE antibody responses in 48–94% of patients allergic to cockroaches. Four isoallergens of Per a 3, Per a 3.01, Per a 3.0201, Per a 3.0202 and Per a 3.0203 were previously identified, and the molecular weight ranging from 79.3, 75.5, 56.2 and 46.7 kDa were reported, respectively (15, 16). While there are no sequence similarity with other known proteins, these aromatic amino acid-rich (16.5–17.3%) Per a 3 allergens were found to have sequence identities (20.1–36.4%) to insect hemolymph proteins. Although several cockroach allergens have been sequenced, information concerning IgE-binding epitopes of cockroach allergens has been unavailable thus far. In the present study, B cell linear epitopes of Per a 3.01, showing 47.4% skin reactivities on 19 cockroach-sensitive asthmatic subjects (GenBank™ accession

number L48018), (15) that are recognized by human IgE were characterized.

Material and methods

Human antibodies to cockroach allergens

Human sera were obtained from patients with positive skin test to cockroach extract (15) and assayed for Per 3.01 specific IgE antibodies by enzyme-linked immunosorbent assay (ELISA). Equal volumes of sera from eight patients with Per a 3.01 specific IgE were combined to constitute an atopic serum pool.

Synthetic peptides

Four biotinylated and nonbiotinylated peptides corresponding to IgE-binding amino acid sequences of recombinant fragments were purchased from United Biochemical Research, Inc. (Seattle, WA, USA). They were purified by high-performance liquid chromatography (greater than 85% purity) and characterized using matrix-assisted laser desorption mass spectrometry by the manufacturer.

Recombinant fragments generated by internal restriction enzyme sites and polymerase chain reaction (PCR)

The plasmid with the cDNA encoding for Per a 3.01 (vector pET 30) was isolated and purified by QIAprep[®] Miniprep (QIAGEN, Valencia, CA, USA). The fragment encoding residues 1–399 and 606–636 were generated by digestion of the plasmid with Per a 3.01 DNA using restriction enzymes *EcoRI/AvaI* and *BamHI/HindIII*, respectively (Promega, Madison, WI, USA) by standard methods (19).

PCR amplification of recombinant fragments

The cDNA of Per a 3.01 was combined with oligonucleotide primers specific for the target areas with appropriate restriction enzyme cleavage sites in a solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ and 200 μ M of each dNTP (Pharmacia, Piscataway, NJ, USA). The PCR was performed with an initial denaturation at 94°C for 3 min, and subsequent 35 cycles of amplification was done under the following conditions: annealing at 55°C for 2 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min. The PCR product was separated by agarose gel electrophoresis and purified. The PCR cloning generated nine relevant overlapping cDNAs.

Construction and expression of recombinant fragments of Per a 3.01

The restriction enzyme generated recombinant fragments of Per a 3.01 cDNA were ligated into cloning plasmids pET-30a (residue 1–399) or pET-30c (residue 606–636). The PCR generated recombinant fragments were ligated into pGEM-T vector (Promega, Madison, WI, USA), double digested with *SphI/PstI* and subcloned into pQE-50 (QIAGEN Inc., Valencia, CA, USA). The partial cDNA was double digested either with *EcoRI/HindIII* (residue 378–474) or *BamHI/HindIII* (residues 340–425, 410–443, 466–579, 472–551, 502–579, 502–595, 595–636 and 636–685) and then ligated into pET-30a (residues 340–425, 472–551, 502–579, 502–595, and 636–685), pET-30b (residues 378–474, 410–443 and 466–579) or

pET-30c (residue 595–636). Sequences of recombinant cDNA inserts were determined using a 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The resulting plasmid was transformed into *Escherichia coli* BL21(DE3), and the expression was performed as previously described (15).

SDS-PAGE and immunoblotting

After expression, cells were harvested and sonicated after washing with sonication buffer. The pellets, unpurified recombinants, were resuspended in Sodium dodecyl sulfate sample buffer containing 2-mercaptoethanol for Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and gels were run with discontinuous buffer according to Laemmli's method (20). After electrophoresis, gels were fixed and stained with 0.2 % Coomassie brilliant blue R-250. For immunoblotting, gels were transferred electrophoretically (21) to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Immunodetection was performed using atopic human sera as previously described (11).

Purification of recombinant allergens for ELISA

Recombinant proteins were over-expressed in *E. coli* BL21(DE3) (15). The culture was harvested and the cells were washed with sonication buffer [50 mM Tris-HCl, pH 8, 200 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 5% glycerol]. The pellets were resuspended in sonication buffer containing 2 mM phenylmethylsulfonyl fluoride and the cells were sonicated. The lysate was purified using the rapid affinity column chromatography with pET His-Tag System as described by Novagen (Madison, WI, USA) (15). Purified recombinants were used for ELISA and inhibition study.

Direct ELISA

The reactivities of purified intact Per a 3.01 and IgE-positive mutant proteins, and synthetic peptides with IgE antibodies were evaluated in a direct binding ELISA. ELISA was performed essentially as described previously (11). Optimal concentration of antigen and conjugate were determined by checkerboard titration. Plate was prewashed with washing buffer, phosphate buffer saline (PBS), pH 7.4 containing 0.05% Tween 20 (PBST). Purified recombinants (3.0 μ g/well) or biotin-labeled synthesized peptides (10 ng/well) in coating buffer (0.1 M sodium carbonate, pH 9.6) were used to coat the microtiter plate (Immunolon II; Dynatech, Alexandria, VA, USA) or streptavidin immobilizer plate (Exiqon, Byggestubbe, Denmark) in triplicate and incubated at 37°C for 2 h. In general, five washings were performed between each step. Antigen-coated wells were washed with PBST and then filled with 1% normal goat serum and incubated for 2 h at room temperature. Wells were washed and incubated overnight at 4°C with 1 : 10 dilution of patient serum. After washing, antibody binding was detected using peroxidase-labeled goat anti-human IgE along with 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) containing 0.03% H₂O₂ as a substrate. The absorbance was determined at 415 nm on a SLT SPECTRA Reader (SLT Lab Instruments, Salzburg, Austria).

Inhibition ELISA

Purified Per a 3.01 recombinant allergen (3.0 μ g/well) in coating buffer was used to coat each well in triplicate and incubated at 37°C

for 2 h. After incubation, wells were washed five times with PBST and then filled with 1% normal goat serum and incubated for 2 h at room temperature. Wells were washed and then incubated with patient serum pool which have been preincubated 2 h (1 : 10) at room temperature with the solution of the different concentrations of purified recombinant proteins and nonbiotin-labeled peptides. The wells were incubated at 4°C overnight, and then processed as standard ELISA (described above) using peroxidase-conjugated goat anti-human IgE as the secondary antibody. Per a 3.01 was used as inhibitor for the positive control. Preincubation of atopic sera with IgE-negative recombinant proteins and preincubation of inhibitor with nonallergic sera were used as the negative control. Results were expressed as the percent inhibition of the reaction of antibody without any inhibitor (11).

Results

IgE reactivities of recombinant proteins by immunoblotting

Recombinant fragments of Per a 3.01 were expressed as fusion proteins and assayed for IgE reactivities. The results of immunoblotting of unpurified recombinant proteins using Per a 3.01-skin-test-positive atopic sera are shown in Fig. 1. The intact Per a 3.01 (Fig. 1, lane b), recombinant proteins 378–474, 340–425, 466–579, 502–595 and 595–636 reacted with IgE antibody (Fig. 1, lanes d, f, h, j and l). The fragment lacking the C-terminus, recombinant protein 1–399 (Fig. 1, lane c) was unable to recognize human IgE. In addition, no detectable reactivities were found for recombinant proteins 410–443, 472–551, 502–579, 606–636 and 636–685 (Fig. 1, lanes e, g, i, k and m). Except for nonspecific binding to a band of around 24 kDa found in both nonrecombinant and recombinants (Fig. 1B), no protein from *E. coli* BL21(DE3) was recognized by human IgE antibody (Fig. 1B, lane a).

IgE binding and inhibition studies of recombinants by ELISA

Recombinant proteins were purified for ELISA. The specific human IgE antibodies to intact Per a 3.01 and the recombinant proteins were determined by ELISA with

sera from eight patients with positive skin tests to Per a 3.01, one atopic patient with a negative test to cockroach extract, and one healthy nonallergic subject. The four relevant recombinant proteins, Per a 3.01 : 340–425, Per a 3.01 : 466–579, Per a 3.01 : 502–579 and Per a 3.01 : 595–636 were selected for ELISA and showed positive human IgE-binding that was heterogeneously distributed among the different sera tested (Fig. 2). The IgE from patients 1, 3, 4, 6 and 8 were able to bind all recombinant proteins tested, while IgE from patients 2, 5 and 7 recognized 75, 50 and 25% of proteins tested, respectively. Recombinant proteins 595–636, 502–595 and 340–425, were capable of IgE binding to 87.5% (7 of 8) of the sera tested at a dilution of 1 : 10. On the other hand, recombinant protein 466–579 showed a positive IgE interaction with 62.5% (5 of 8) of the sera analyzed. All of the human IgE recognized the intact Per a 3.01, and no reactivity was detected on recombinant proteins Per a 3.01 : 1–399, Per a 3.01 : 410–433, Per a 3.01:472–551, Per a 3.01 : 502–579, Per a 3.01 : 636–685 and Per a 3.01 : 606–636 (data not shown). In addition, competitive inhibition assays were performed using purified intact Per a 3.01 and recombinant proteins as competitors. Per a 3.01, Per a 3.01: 595–636, Per a 3.01: 502–595, Per a 3.01: 340–425 and Per a 3.01: 466–579 inhibited 99, 93, 91, 87 and 86% of IgE binding to Per a 3.01 at a concentration of 2.0 µg/ml in solution, respectively (Fig. 3). No significant inhibition was observed when IgE-positive recombinants were preincubated with cockroach skin-test-negative atopic sera (data not shown). In addition, no significant inhibition was observed when atopic sera were preincubated with Per a 3.01: 1–339 (Fig. 3) and other IgE-negative recombinant proteins (data not shown). These results and results of the immunoblotting indicate that the amino acid sequences 400–409, 466–471, 580–595, and 595–605 of Per 3.01 directly involved in the interaction to human IgE. Results are summarized, and a schematic map of the location of recombinant fragments on the complete Per a 3.01 molecule is present in Fig. 4.

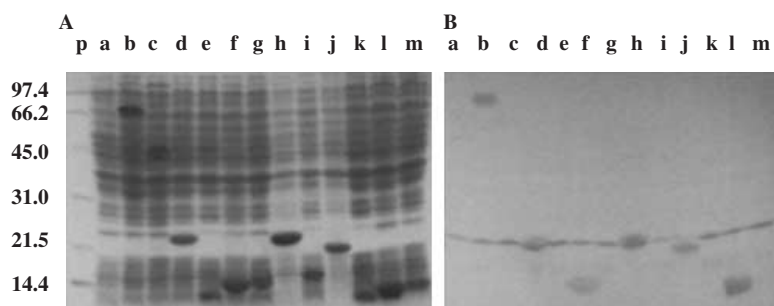


Figure 1. SDS-PAGE (A) and immunoblotting (B) of Per a 3.01 and recombinant proteins. Proteins were stained with Coomassie brilliant blue (A), or probed with atopic human IgE (B). Lanes (a–m): *Escherichia coli* BL21(DE3) (lane a), Per a 3.01: 1–685 (lane b), Per a 3.01: 1–399 (lane c), Per a 3.01: 378–474 (lane d), Per a 3.01: 410–443 (lane e), Per a 3.01: 340–425 (lane f), Per a 3.01: 472–551 (lane g), Per a 3.01: 466–579 (lane h), Per a 3.01: 502–579 (lane i), Per a 3.01: 502–595 (lane j), Per a 3.01: 606–636 (lane k), Per a 3.01: 595–636 (lane l) and Per a 3.01: 636–685 (lane m). Numbers at left indicate sizes of protein markers in kDa (lane p).

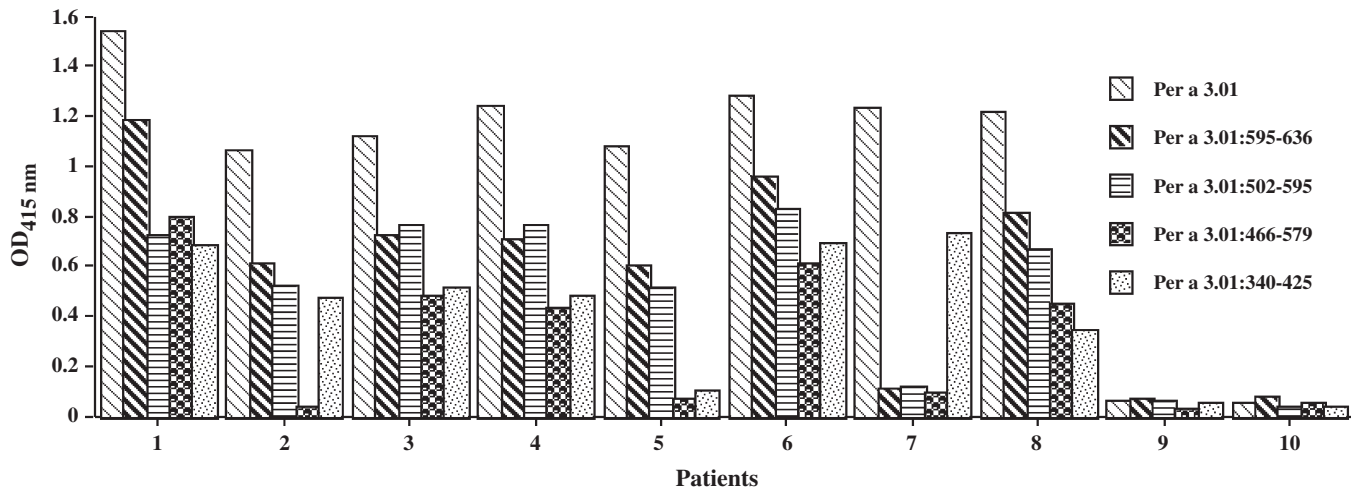


Figure 2. Binding profiles of IgE antibodies to Per a 3.01 and relevant recombinant proteins by ELISA. Eight atopic sera and serum samples from cockroach skin-test-negative atopic (patient 9) and healthy nonallergic subjects (patient 10) were analyzed.

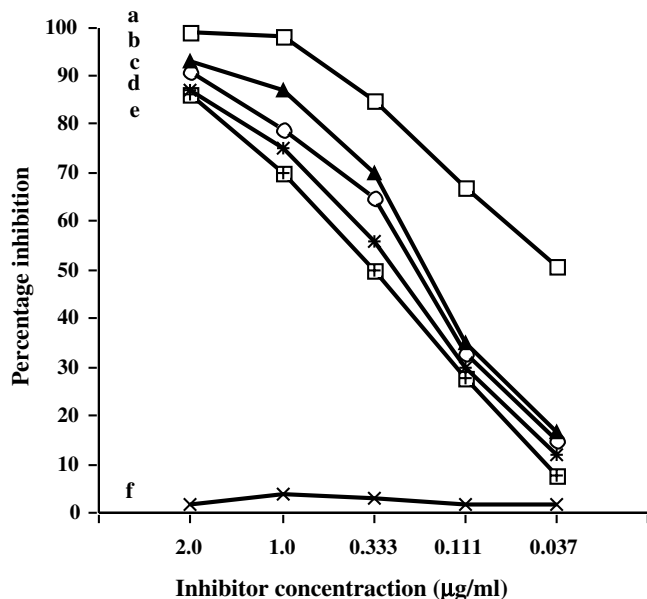


Figure 3. Binding inhibition of IgE to Per a 3.01 by Per a 3.01 (a), Per a 3.01: 595–636 (b), Per a 3.01: 502–595 (c), Per a 3.01: 340–425 (d), Per a 3.01: 466–579 (e), and Per a 3.01: 1–399 (f). Microtiter plates were coated with purified Per a 3.01, and results are average of experiment performed in triplicate as described.

IgE binding and inhibition studies of synthetic peptides by ELISA

Biotinylated and nonbiotinylated peptides corresponding to IgE-binding amino acid sequences 400–409 (peptide 400–409), 466–471 (peptide 466–471), 580–595 (peptide 580–595) and 595–605 (peptide 595–605) were synthesized. Binding of specific human IgE antibodies against four solid phase-bound biotinylated peptides was demonstrated by ELISA with same sera used for the analysis

of recombinants. Synthetic peptides showed positive IgE-binding that was heterogeneously distributed among the different sera tested, and no antibody binding was detected in the negative control sera (Fig. 5). The IgE from patients 1, 3, 4, 6 and 8 were able to bind all peptides tested, while IgE from patients 2, 5 and 7 recognized 75, 50 and 25% of peptides tested, respectively. Peptides 595–605, 580–595 and 400–409 were capable of IgE binding to 87.5% (7 of 8) of sera tested, and peptide 466–471 showed a positive IgE interaction with 62.5% (5 of 8) of the sera analyzed. In order to confirm the binding specificity of IgE antibodies to Per a 3.01 peptides, competitive inhibition assays were performed. The eight sera from Per a 3.01-skin-test-positive patients were combined, and corresponding nonbiotinylated peptides were used as competitors for the inhibition of IgE binding to Per a 3.01. Peptides 595–605, 580–595, 400–409 and 466–471 inhibited 40, 37, 30, and 25% of IgE binding to Per a 3.01 at concentration of 2.0 µg/ml in solution, respectively (Fig. 6). No significant inhibition was observed when peptides were preincubated with negative control sera.

Discussion

Reported evidence indicates that allergen degradation products are natural targets of B cell recognition, underlying the importance of continuous epitopes (22). There is an increasing amount of data showing that immunotherapy by repeated subcutaneous injection is an effective method in the treatment of allergy (23). Immunotherapy with specific recombinant allergens or epitopes, rather than crude allergen vaccine mixtures, may prove to be a more effective regimen. Recently, small peptides carrying only one IgE-binding epitope have been

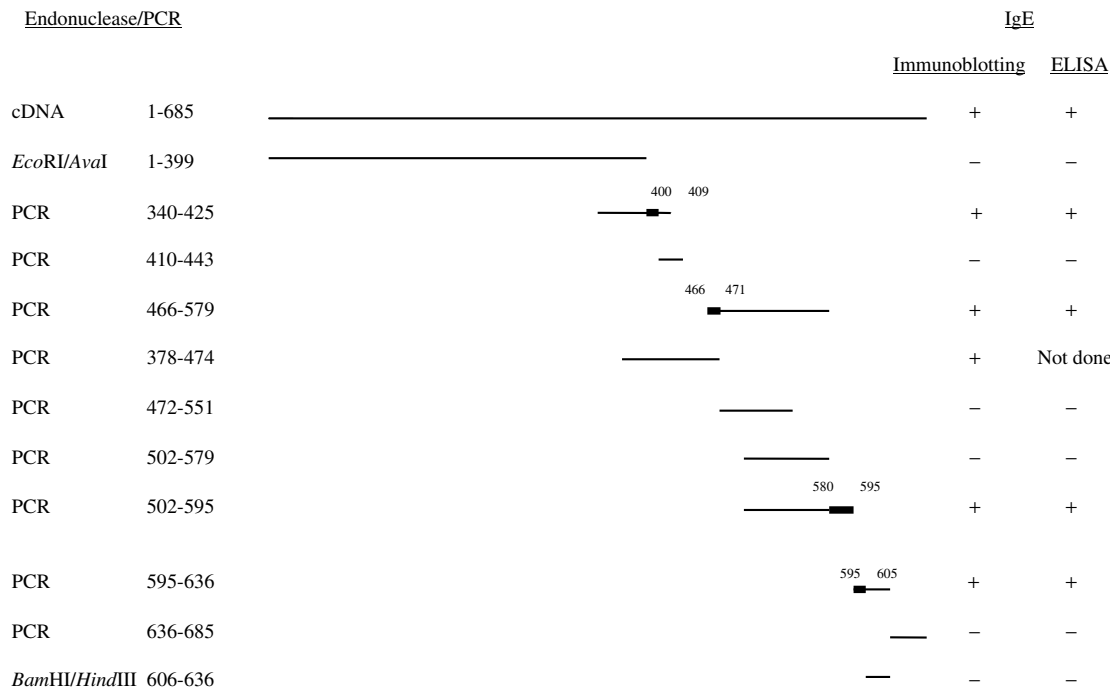


Figure 4. B-cell epitope mapping on Per a 3.01. Schematic map location of deletion mutants on the complete Per a 3.01. — represent the region recognized by human IgE.

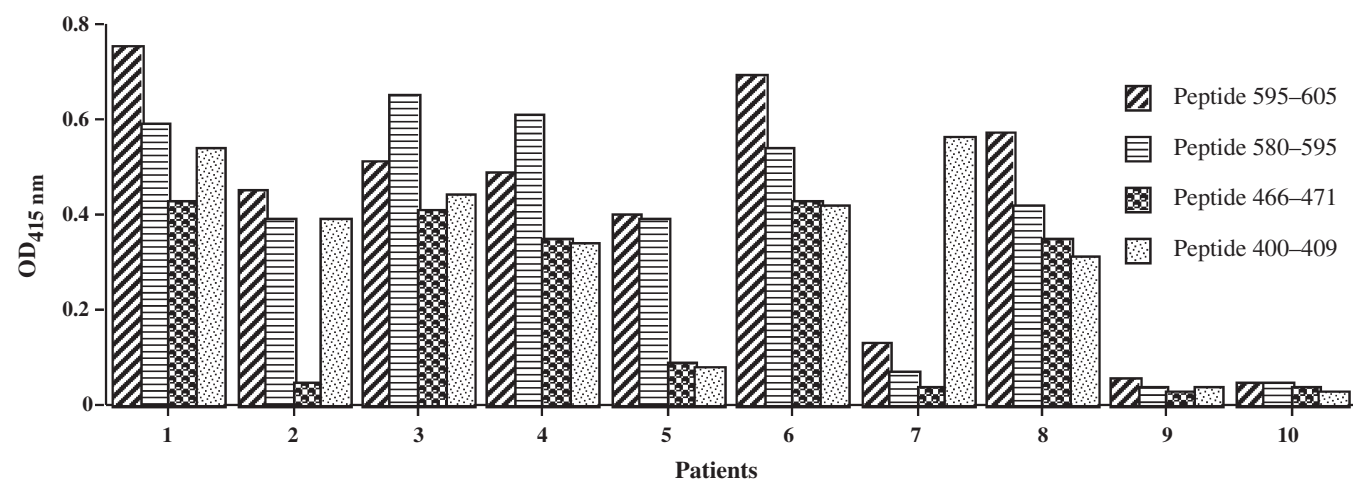


Figure 5. Binding profiles of IgE antibodies to synthetic peptides by ELISA. Eight atopic sera and serum samples from cockroach skin-test-negative atopic (patient 9) and healthy nonallergic subjects (patient 10) were analyzed.

proposed for immunotherapy in order to increase specificity of the immune response (24). These peptides cannot cross-link receptor-bound IgE on basophils and mast cells, thereby preventing undesirable reactions such as histamine release, while inducing blocking IgG antibodies that interfere with the allergen-IgE interaction. Information on IgE-binding epitopes is required for application of allergen immunotherapy. Recently, it has been demonstrated that IgE-binding fragments of bovine dander

allergen (Bos d 2) that are potent stimulators of specific T cell clones is significantly reduced when a segment from either the amino or the carboxyl terminus is deleted (25). Modification of cysteine residues in the house dust mite allergen resulted in reduced binding capacity of Der p 2 to human IgE (26). The CD4⁺ allergen-specific T cell is crucial for IgE induction. Nevertheless, allergens with no or very low IgE-binding capacities are also important in immunotherapy.

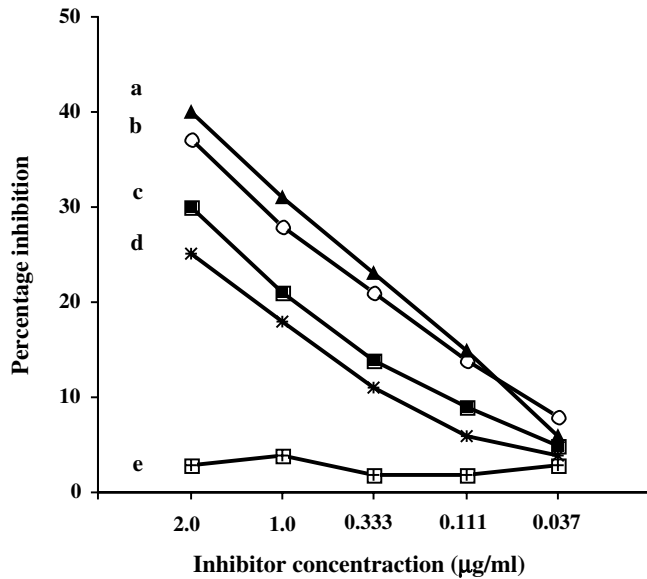


Figure 6. Binding inhibition of IgE to Per a 3.01 by peptide 595–605 (a), peptide 580–595 (b), peptide 400–409 (c), peptide 466–471 (d) and cockroach skin-test-negative atopic sera (e). Microtiter plates were coated with purified Per a 3.01, and results are average of experiment performed in triplicate as described.

In this study, we have designed and performed B cell linear epitopes, excluding conformational epitopes, analysis of the major American cockroach Per a 3.01 allergen using a number of recombinant fragments. Preliminary results of immunoblotting, ELISA, and inhibition studies revealed that the amino acid sequences 400–409, 466–471, 580–595, and 595–605 are involved in human IgE-binding. Moreover, peptides corresponding to these IgE-binding amino acid sequences were synthesized and

characterized further by ELISA and inhibition studies, with results confirming our preliminary findings. In our previous study, 13 potential central antigenic determinants of Per a 3.01 were predicted using the Jameson and Wolf method (27), and two are in the regions of amino acids 582–587 and 597–600 (15). This is in concordance with our experimentally identified IgE-binding sites containing residues 580–595 and 595–605. Other investigations also found close correlation between predicted and determined dominant amino acids (28, 29). In other cases, however, nonpredictive antigenic determinants have shown immunodominance (30). Several algorithms exist for predicting potential antigenic determinants. The correct prediction frequency of these algorithms is 50–60% (31). Thus, antigenic determinant prediction cannot replace experimental epitope determination.

In conclusion, we have shown that the amino acid sequences ⁴⁰⁰TVLRDPVFYQ⁴⁰⁹, ⁴⁶⁶NNVDQI⁴⁷¹, ⁵⁸⁰VDKGHNYCGYPENLLI⁵⁹⁵, and ⁵⁹⁵IPKGKKKGQAY⁶⁰⁵ of the American cockroach Per a 3.01 allergen are involved in human IgE binding. Our current findings advance the understanding of the specific reactivity of the epitopes of cockroach allergens, thereby potentially improving the method of immunotherapy and providing a mean towards developing more specific vaccines with enhanced clinical efficacy.

Acknowledgements

We would like to thank Dr Shue-Fen Luo of the Section of Rheumatology, Allergy, and Immunology, Department of Medicine, Chang-Gung Memorial Hospital, Taipei, Taiwan for providing human atopic sera. This work was supported by grants NSC88–2314-B075A-007 and NSC89–2320-B075A-011 from the National Science Council and grant TCVGH 897302C from Taichung Veterans General Hospital, Taiwan, Republic of China.

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