

Mapping of IgE and IgG₄ Antibody-Binding Epitopes in Cyn d 1, the Major Allergen of Bermuda Grass Pollen

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Key Words

Antibody-binding epitope · Cyn d 1 · Dot immunoblotting · Synthetic peptide

Abstract

Background: Bermuda grass pollen (BGP) is an important seasonal aeroallergen worldwide which induces allergic disorders such as allergic rhinitis, conjunctivitis and asthma. Cyn d 1 is the major allergen of BGP. This study is aimed to map human IgE and IgG₄ antibody-binding sequential epitopes on Cyn d 1 by dot immunoblotting. **Methods:** Synthetic peptides (10-mers; 5 overlapping residues) spanning the full length of Cyn d 1 were used for dot immunoblotting to map human IgE and IgG_{1–4} antibody-binding regions with sera from BGP-allergic patients. Synthetic peptides with more overlapping residues were used for further mapping. Essential amino acids in each epitope were examined by single amino acid substitution with alanine. Peptides with sequence polymorphism of epitopes of Cyn d 1 were also synthesized to extrapolate their differences in binding capability. **Results:** Four major IgE-binding epitopes (peptides 15^{–1}, 21, 33^{–2} and 35^{–1}, corresponding to amino acids 70–79, 101–110, 159–167 and 172–181) and 5 major IgG₄-binding epitopes (peptides 15^{–1}, 30^{–2}, 33^{–2}, 35^{–1} and 39, corresponding to amino acids 70–79, 144–153, 159–167, 172–181 and 192–200) were identified. They are all located on the surface of

the simulated Cyn d 1 molecule, and three of them are major epitopes for both IgE and IgG₄. Their critical amino acids were all characterized. Major epitopes for human IgG₁ to IgG₄ are almost identical. **Conclusions:** This is the first study to map the sequential epitopes for human IgE and IgG₄ subclasses in Cyn d 1. It will be helpful for future development in immunotherapy and diagnosis.

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Introduction

About 20% of the populations in the industrialized countries suffer from allergic diseases caused by aeroallergens derived from grass and tree pollen, house dust mites, and animal dander. Aeroallergens can induce type I hypersensitivity such as allergic rhinitis, conjunctivitis and asthma which are a great threat for quality of life and economic expenses [1]. Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an important source of seasonal aeroallergens in tropical and subtropical areas worldwide [2–6]. In Taiwan, the positive rate of immediate skin tests to the crude BGP extract was about 27% in asthmatic patients [2]. Using immunoblotting, several major human IgE-reactive BGP allergens have been identified. Cyn d 1, the most abundant protein in BGP extracts, has been recognized as the major and group 1 allergen of BGP [3–6].

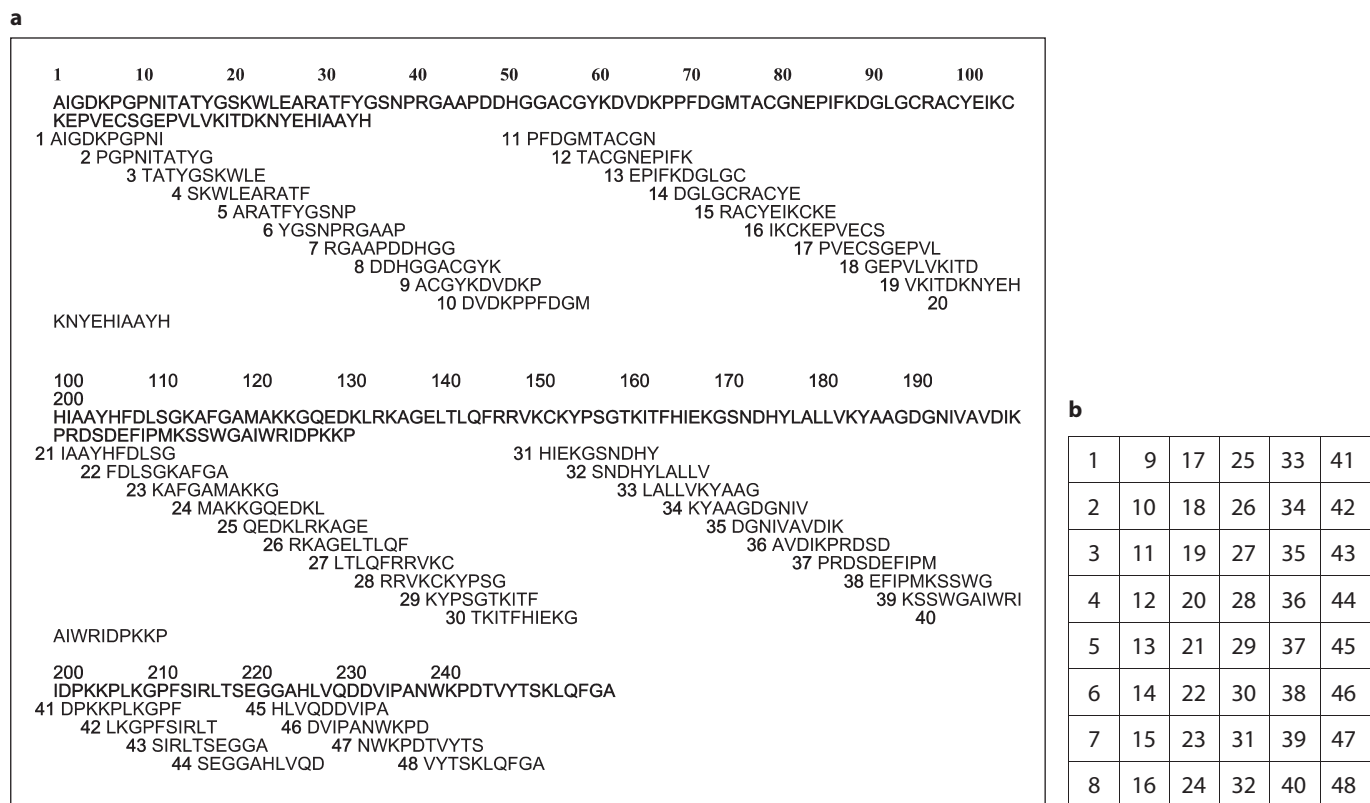


Fig. 1. a Amino acid sequences of Cyn d 1 and synthetic decapeptides. **b** Position of each synthetic decapeptide on the PVDF membrane.

Cyn d 1 is a 32- to 34-kDa glycoprotein that gives a 76–100% positive reaction rate with IgE in sera from patients with BGP allergy [6]. The sequences of some cDNA clones of Cyn d 1 were first identified by Smith et al. [7]. However, the cDNAs were truncated in the coding regions [7]. Several full-length cDNA clones encoding Cyn d 1 were thereafter characterized by us [8, 9]. We have confirmed that Cyn d 1 consists of 11 isoforms with sequence polymorphism and differences in isoelectric point or molecular weight [7–11].

Immunotherapy has been used for decades for pollen allergy [12–14]. Because crude pollen extracts used for immunotherapy compose IgE-binding allergens, severe anaphylactic reactions have been repeatedly reported after allergen injection [13, 14]. An ideal vaccine for immunotherapy needs to preserve human IgG antibody-binding epitopes but to avoid human IgE antibody-binding sites [15, 16]. Using mouse monoclonal or rabbit polyclonal antibodies, a number of antibody-binding epitopes have been disclosed for aeroallergens and food allergens [17–23]. We have previously characterized a sequential epitope (peptides 46–64) on Cyn d 1, the major allergen

of BGP [24]. Synthetic peptides have been recently applied for epitope mapping [25–28]. In the present study, we identify human IgE and IgG₄ antibody-binding sequential epitopes on Cyn d 1 and determine the essential amino acids in each epitope by dot immunoblotting. Moreover, the epitopes among different sequences of Cyn d 1 in their IgE and IgG₄ binding capacity were also evaluated.

Material and Methods

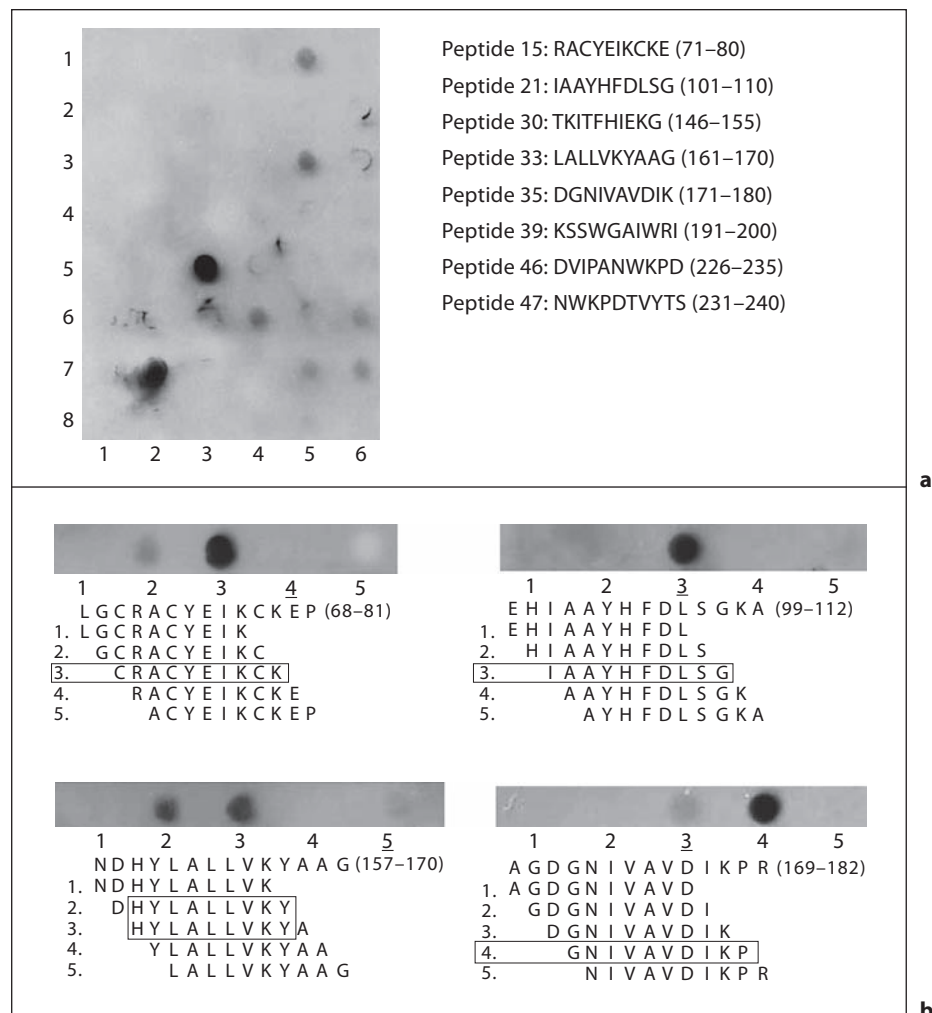
Crude Extract of BGP

Defatted BGP was purchased from International Biologicals (Piedmont, Okla., USA). The crude extract of BGP was prepared as before [6]. Briefly, it was extracted with PBS (pH 8.0) at a ratio of 1:10 (grams per milliliter) with gentle shaking at 4°C for 18 h. After dialysis with distilled water at 4°C overnight, the crude extract was then lyophilized and stored at –20°C.

Peptide Synthesis

The amino acid sequence of Cyn d 1 was based on the report of the NCBI GenBank Database AAB50734.2. Synthetic decapeptides spanning the full length of the Cyn d 1 molecule were pur-

Fig. 2. Mapping of human IgE-binding regions on Cyn d 1. **a** Synthetic overlapping decapeptides of Cyn d 1 were probed with pooled sera from BGP-allergic patients. **b** Final mapping of IgE-binding epitopes on Cyn d 1. Each overlapping peptide was stepwise different from the previous peptide by one residue. Underlined numbers are the original peptide sequences. Amino acid sequences of epitopes are shown in the boxes.



chased from Kelowna International Scientific Inc. (Taipei, Taiwan). After synthesis, they were diluted to 10 µg/µl in PBS and stored at –20°C.

Patient Sera

Patient sera were collected as described previously [11]. Patients were referred to the Allergy Clinic at the University of Florida due to a history of seasonal allergic rhinitis for at least 1 year. Skin tests were applied to each patient's back and included the standardized allergic extract of BGP (Miles Laboratories, Spokane, Wash., USA). Wheal reactions of greater than 5 mm over albumin-saline controls were considered positive. Twenty-two patients included in this study had registered at least 3+ by the prick test with the BGP extract. BGP-specific IgE antibodies were also measured using the Pharmacia CAP test (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instruction. Three nonatopic serum samples with a very low absorbance value (<0.05) in the CAP test were used as negative controls.

Western Blotting for Human Anti-Cyn d 1 IgE and IgG₄ Antibodies

Briefly, crude BGP extracts (1 µg/lane) fractionated in 12.5% SDS-PAGE were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, Mass., USA). For the detection of specific human IgE, blots were blocked with 3% skimmed milk for 1 h at room temperature (RT) and immersed with serum samples (1:10) overnight at 4°C. They were then immersed with horseradish peroxidase (HRP)-conjugated mouse anti-human IgE monoclonal antibodies (mAb, 1:4,000; SouthernBiotech, Birmingham, Ala., USA) at RT for 5 h. For the detection of specific human IgG₄, blots were blocked overnight with 3% skimmed milk at 4°C and immersed with serum samples (1:500) at RT for 2 h. They were then immersed with biotinylated mouse anti-human IgG₄ mAb (1:20,000; Sigma, St. Louis, Mo., USA) at RT for 2 h and with HRP-streptavidin (1:5,000; Invitrogen, Camarillo, Calif., USA) at RT for 1 h sequentially. The blots were extensively washed with PBS-Tween20 after each treatment. They were then visualized by ECL (Amersham, Little Chalfont, UK).

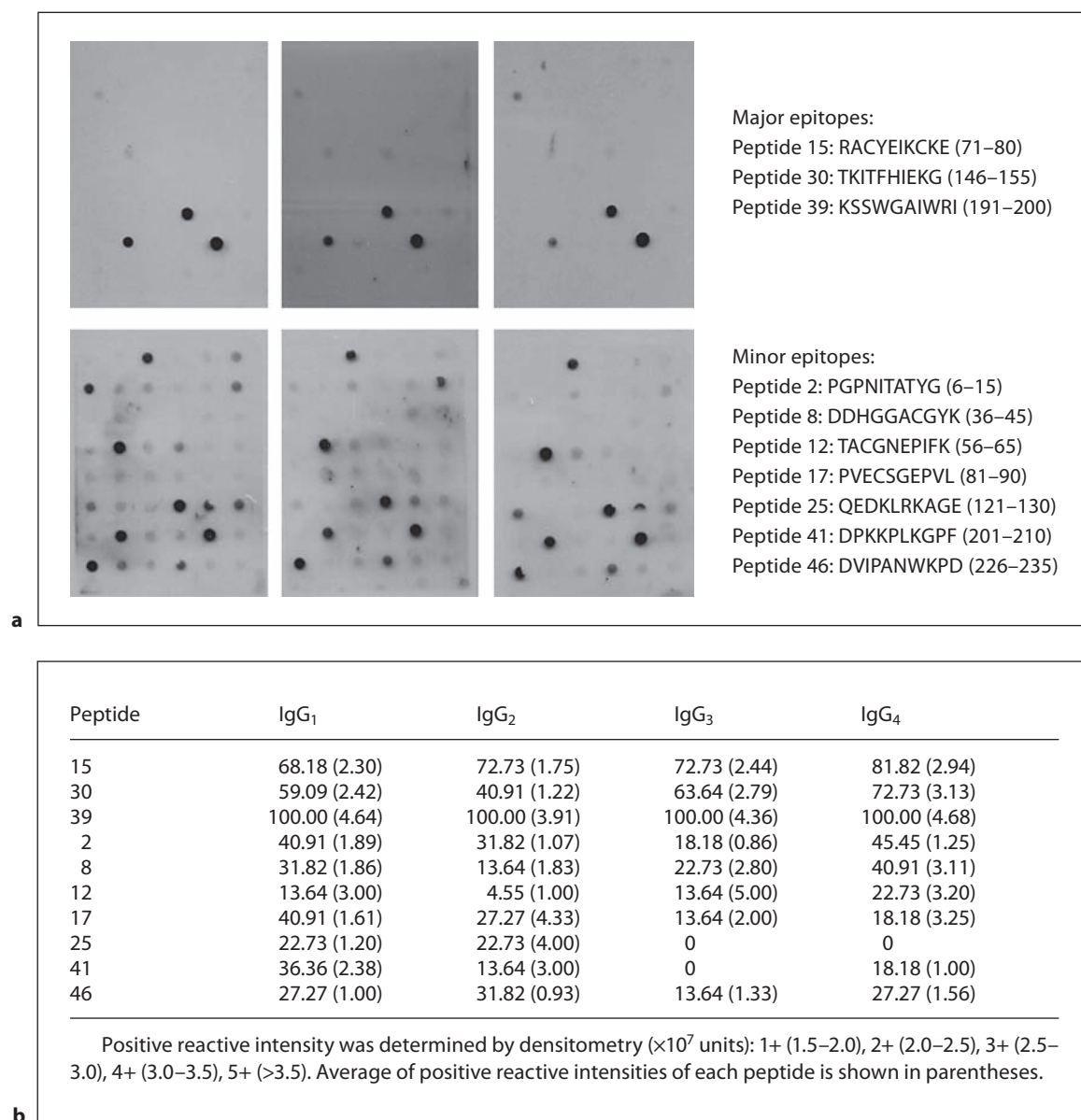


Fig. 3. Mapping of human IgG₄-binding regions on Cyn d 1. **a** Synthetic overlapping decapeptides of Cyn d 1 were probed with each patient's serum. **b** Percentage of positive synthetic peptide-binding reactivity of 22 allergic sera.

Dot Immunoblotting for Human IgE-Binding Epitopes

Dot immunoblotting was modified from our previous report [9]. Briefly, synthetic peptides (5 μ g/dot) were adsorbed onto PVDF membranes with a Bio-Dot Apparatus (Bio-Rad, Richmond, Calif., USA). After blocking, the membranes were immersed with pooled serum samples (1:10) overnight at 4°C. They were then reacted with HRP-conjugated mouse anti-human IgE mAb (1:4,000) at RT for 5 h and visualized by ECL.

Dot Immunoblotting for Human IgG₁₋₄-Binding Epitopes

As above, synthetic peptides (5 μ g/dot) were adsorbed onto PVDF membranes. After blocking, the membranes were immersed with individual serum samples (1:500) for 2 h at RT. After washes, they were incubated with biotin-labeled mouse anti-human IgG₁, IgG₂, IgG₃ and IgG₄ mAb (1:5,000 for IgG₁ and IgG₃; 1:20,000 for IgG₂ and IgG₄) at RT for 2 h. They were then reacted with HRP-streptavidin (1:5,000) for 1 h at RT. The intensity of ECL signals was analyzed using a densitometer (ImageQuant TL, Array Version 7.0; Amersham Biosciences).

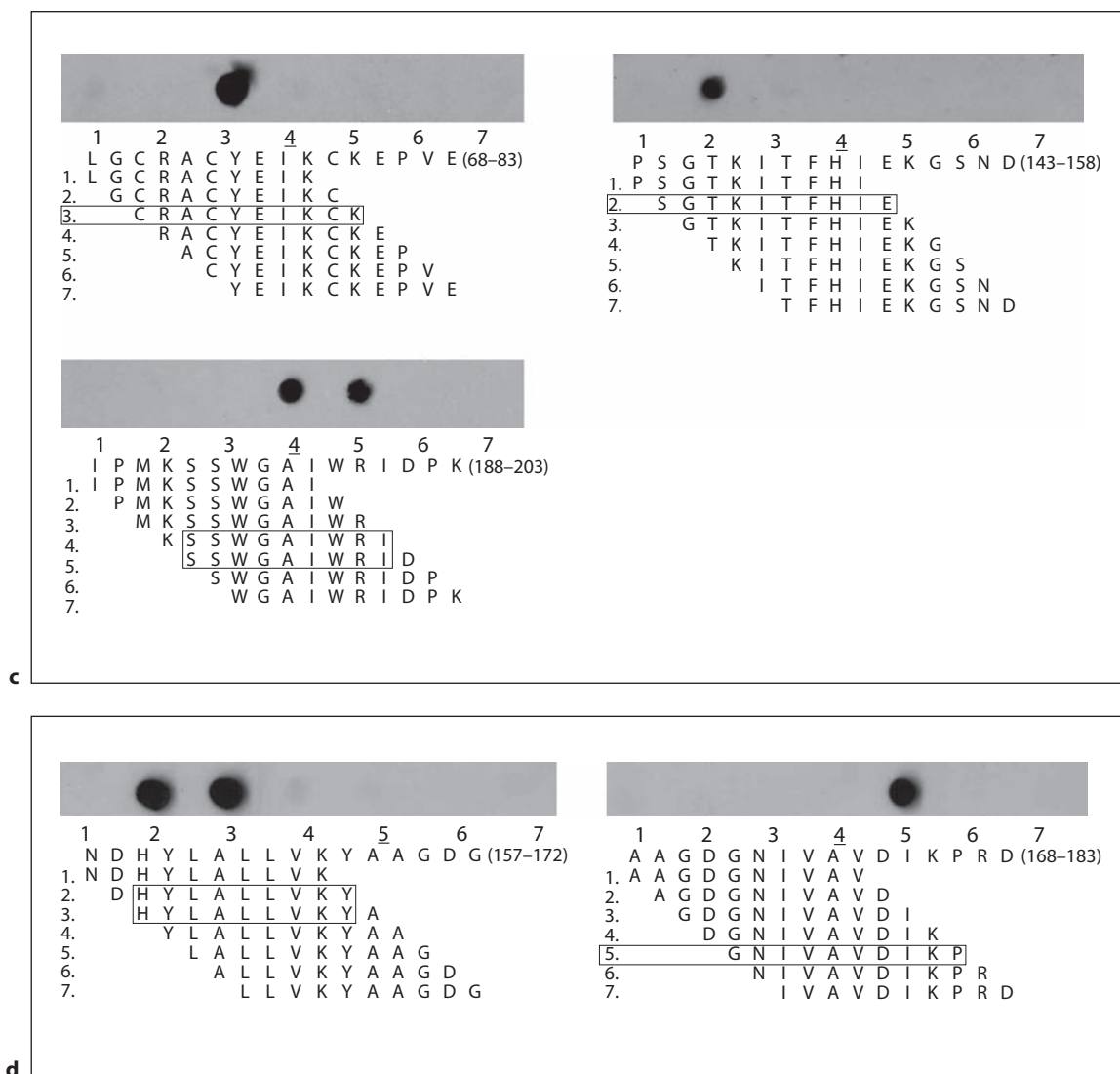


Fig. 3. Mapping of human IgG₄-binding regions on Cyn d 1. **c** Final mapping of IgG₄-binding epitopes on Cyn d 1. **d** Cross-reactivity between human IgE- and IgG₄-binding epitopes on Cyn d 1. IgG₄-binding reactivity was found in two of human IgE epitopes (peptides 33⁻² and 35⁺¹, amino acids 159–167 and 172–181, respectively). Underlined numbers are the original peptide sequences. Amino acid sequences of epitopes are shown in the boxes.

Analysis of Essential Amino Acid in Human IgE- and IgG₄-Binding Epitopes

The amino acids critical for human IgE and IgG₄ recognition on Cyn d 1 were identified by synthetic peptides with single amino acid replacement with alanine at each position. All the procedures are the same with the immunodot assays described as above.

Epitopes of Cyn d 1 with Sequence Polymorphism

Sequence polymorphism of Cyn d 1 had been identified [7–9]. Epitopes specific for human IgE and IgG₄-antibodies on different

sequences of Cyn d 1 were synthesized to compare with the binding capacity of wild type using the immunodot assays described as above.

Protein Structure Homology Modeling

As the crystal structure of Cyn d 1 has not been identified, the structure homology modeling method was used to extrapolate the simulated structure of the Cyn d 1 molecule from the known three-dimensional structure templates of group 1 pollen allergens using an automated protein homology-modeling server (SWISS-MODEL) [29].

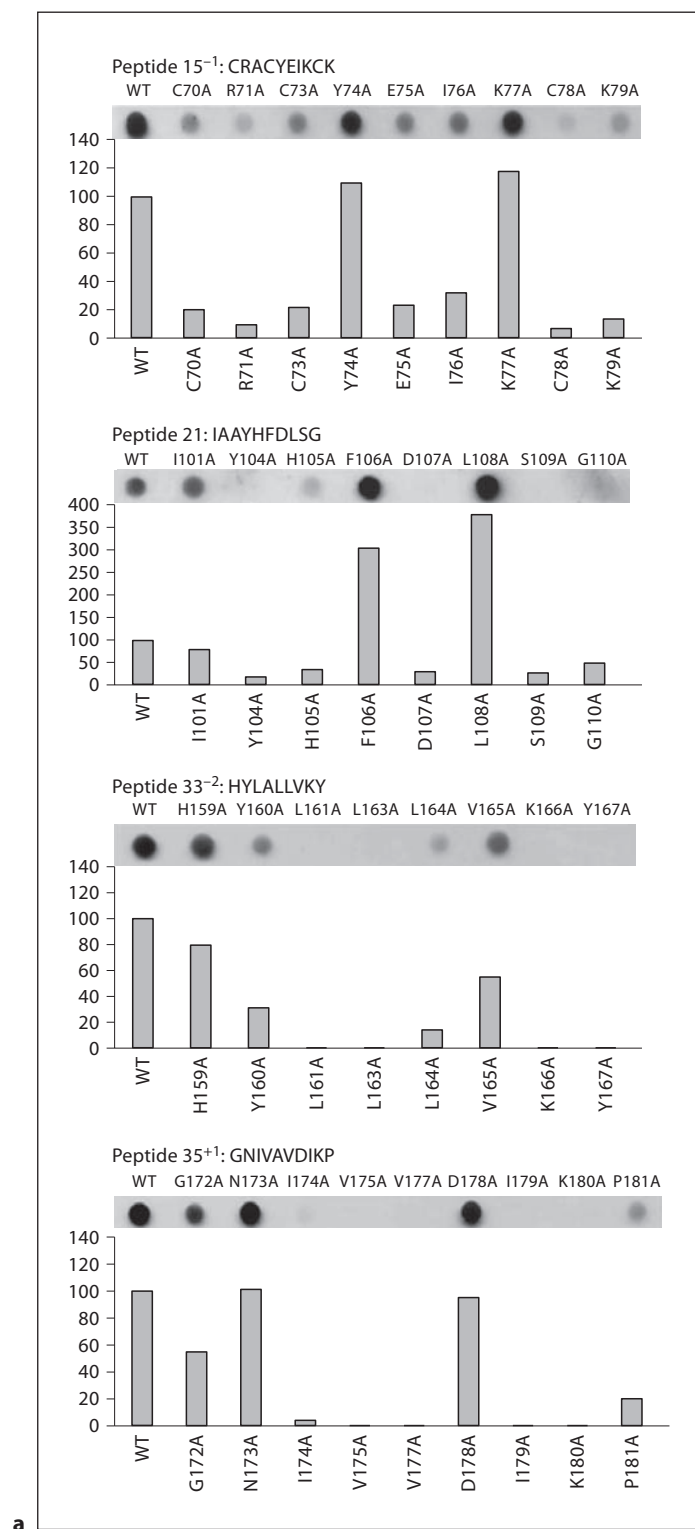


Fig. 4. Identification of critical amino acids in IgE (a) and IgG₄ (b) epitopes of Cyn d 1 using synthetic peptides with single amino acid substitution with alanine respectively. Immunodot blotting was performed as before using pooled patients' sera.

Results

Western Blot Analysis

All 22 patients had positive BGP-specific IgE reactions in the Pharmacia CAP test. Western blot assays were further used to confirm that they all contained specific IgE and IgG₄ antibodies to Cyn d 1, but not the controls (data not shown).

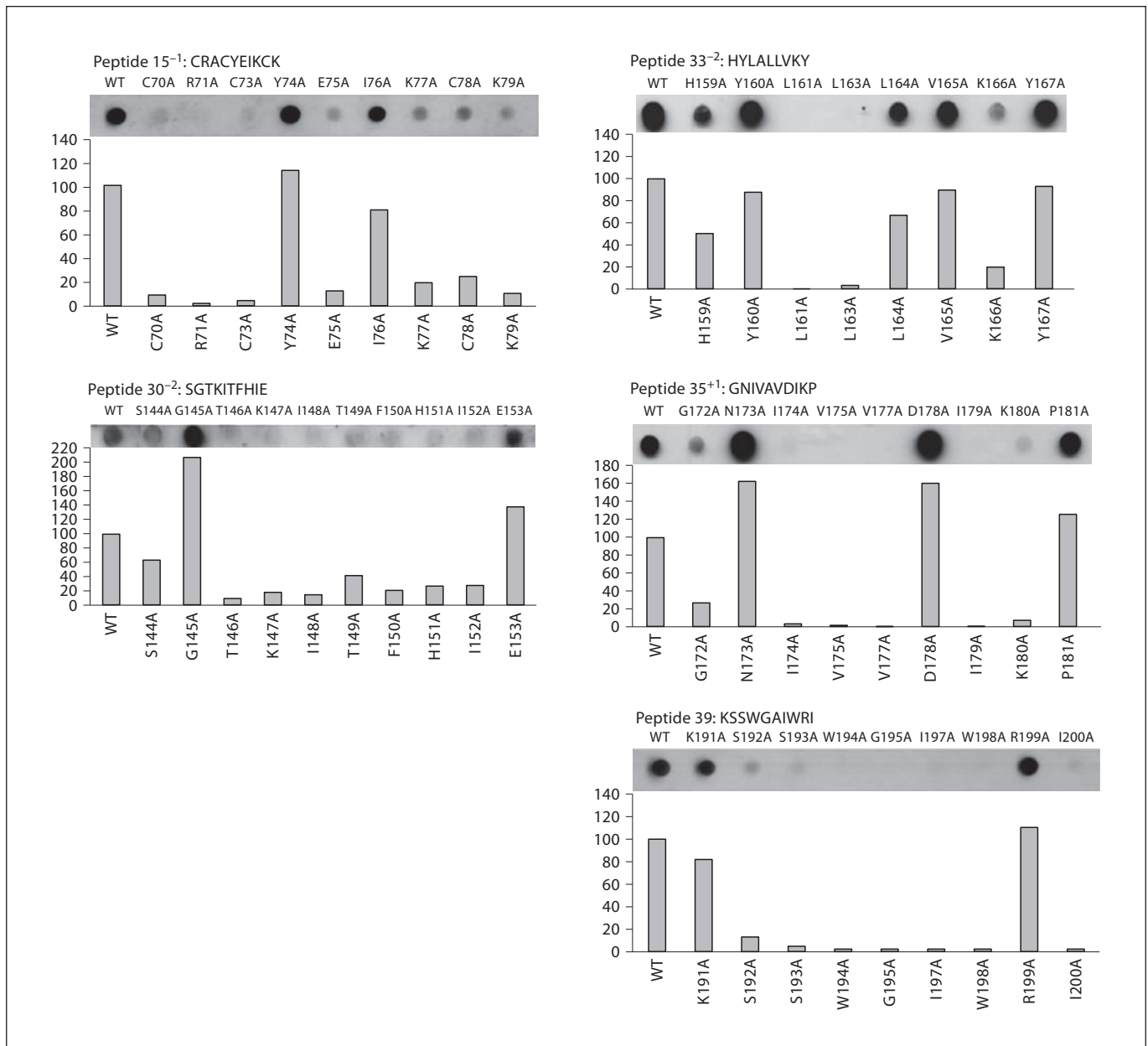
Mapping of Human IgE-Binding Epitopes on Cyn d 1

Forty-eight synthetic decapeptides with 5 overlapping residues were synthesized first to cover the full length of Cyn d 1 (fig. 1a) and applied onto PVDF membrane (fig. 1b). Using pooled sera from allergic patients with strong IgE-binding reactivity to Cyn d 1 in Western blotting, the immunodot assay showed 2 strong positive human IgE-binding spots on peptides 15 and 21 (amino acids 71–80 and 101–110, respectively), as well as 6 weak positive spots on peptides 30, 33, 35, 39, 46 and 47 (amino acids 146–155, 161–170, 171–180, 191–200, 226–235 and 231–240, respectively; fig. 2a). Synthetic decapeptides of positive spots with 9 overlapping residues were further synthesized. Strong positive reactions were observed for 4 major IgE-binding epitopes on peptides 15⁻¹, 21, 33⁻² and 35⁺¹ (amino acids 70–79, 101–110, 159–167 and 172–181, respectively; fig. 2b). Three control sera also showed negative reactivity (data not shown).

Mapping of Human IgG₁₋₄-Binding Epitopes on Cyn d 1

Using synthetic decapeptides with 5 overlapping residues, Cyn d 1-specific binding regions of each patient's serum IgG antibodies were also examined by immunoblotting. Among 22 allergic sera, several positive human IgG₄-binding regions were repeatedly identified. Results of 6 samples are shown in figure 3a. Peptides 15, 30 and 39 (amino acids 71–80, 146–155 and 191–200, respectively) had high positive reaction rates (81.8, 72.7 and 100%, respectively) and were recognized as major IgG₄-binding regions (fig. 3b). Whereas further mapping of them using decapeptides with 9 overlapping residues disclosed strong positive reactions on IgG₄-binding epitopes in peptides 15⁻¹, 30⁻² and 39 (amino acids 70–79, 144–153 and 192–200, respectively; fig. 3c), mapping of 7 minor regions (positive rates between 20–60%) failed to show strong positive reactivity (data not shown).

In addition, decapeptides with 5 overlapping residues were also applied for mapping of potential human IgG₁, IgG₂ and IgG₃ antibody-binding regions. Their findings are summarized in figure 3b. Compared with IgG₁, IgG₂ and IgG₃, specific IgG₄ consistently showed the highest



4b

positive reaction rates and intensities to peptides 15, 30 and 39 (amino acids 71–80, 146–155 and 191–200, respectively).

Cross-Reactivity between Human IgE- and IgG₄-Binding Epitopes on Cyn d 1

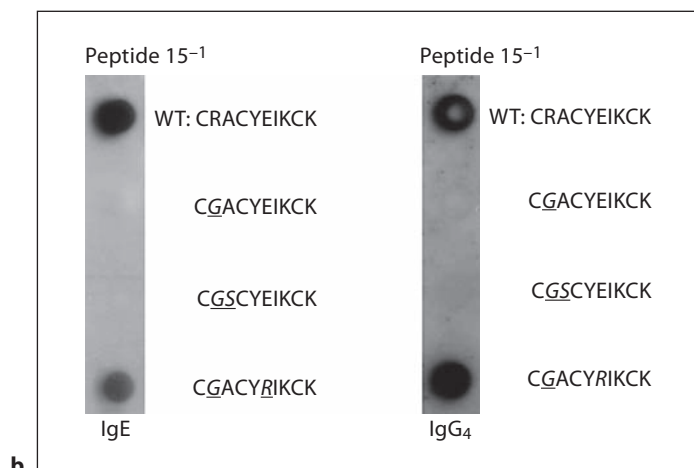
The above epitope mapping has disclosed an epitope (peptides 15⁻¹) to be recognized by specific IgE and IgG₄ simultaneously. Whether other epitopes on Cyn d 1 could

be also recognized by both of them was further examined. Indeed, strong IgG₄-binding reactivity was also found in another two human IgE epitopes (peptides 33⁻² and 35⁺¹, corresponding to amino acids 159–167 and 172–181), but not in the other IgE epitope (peptides 21, amino acids 101–110; fig. 3d). On the other hand, major IgG₄-binding peptides 30⁻² and 39 (amino acids 144–153 and 192–200, respectively) failed to show strong IgE-binding reactivity (data not shown).

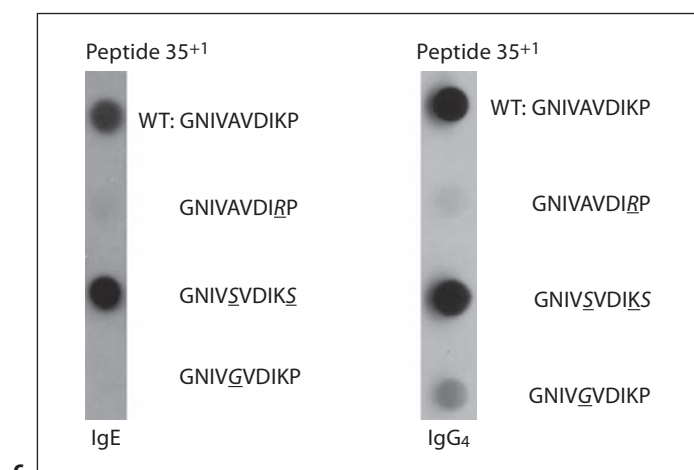
Peptide	Wild type	Isoallergens	Reference
15 ⁻¹ (70–79)	CRACYEIKCK	CG ACYEIKCK [7] CG <u>S</u> CYEIKCK [7, 9] CG ACY R IKCK [8, 9]	
30 ⁻² (144–153)	SGTKITFHIE	S <u>D</u> TKIA F H V E [7, 9] S <u>D</u> TKIT F H V E [9]	
33 ⁻² (159–167)	HYLALLVKY	N YLALLVKY [7, 9]	
35 ⁺¹ (172–181)	GNIVAVDIKP	GNIV G VDIKP [7, 9] GNIV S VDIK S [7, 9] GNIVAVDI R P [8]	
39 (192–200)	SSWGAIWRI	Q SWGAIWRI [7, 9] L SWGAIW R M [9] Q SWGAIW R M [9]	

Wild type according to NCBI GenBank database AAB50734.2

a



b



c

Fig. 5. a Sequence polymorphism of Cyn d 1. One of three polymorphic peptides corresponding to peptide 15⁻¹ (amino acids 70–79) (b) or peptide 35⁺¹ (amino acids 172–181) (c) showed strong positive activities to Cyn d 1-specific IgE and IgG₄ antibodies, respectively.

Essential Amino Acids in IgE- and IgG₄-Binding Epitopes

The amino acids critical for IgE and IgG₄ binding to Cyn d 1 were determined by dot immunoblotting using synthetic peptides with single amino acid substitution by alanine. The results are summarized in figure 4. Some replacements reduced human IgE or IgG₄ antibody-binding capability dramatically, indicating that these amino acids are critical for antibody recognition. Although some of the IgE- and IgG₄-binding epitopes were overlapped, we found that their essential amino acids were not totally identical.

Comparison of Antibody-Binding Activities among Different Sequences of Cyn d 1

After comparing the sequences between wild type and different sequences of Cyn d 1, all the polymorphic peptides located on the above 6 epitopes were synthesized and further probed for human IgE- and IgG₄-binding activities as above (fig. 5a). After comparison with major human IgE- and IgG₄-binding epitopes, two polymorphic peptides corresponding to peptide 15⁻¹ and 35⁺¹ also showed strong binding activities (fig. 5b, c).

Protein Structure Homology Modeling

Using a gapped BLAST query against the SWISS-MODEL template library extracted from PDB, one template with 65.27% identities to Cyn d 1 sequence named 2hcz (Zea m1, a β-expansin and group-1 pollen allergen from maize) was found. The binding region of human IgG₄ and IgE antibodies of Cyn d 1 were shown on the three-dimensional model of Cyn d 1. All of them have part of structure identified on the surface of the protein (fig. 6).

Discussion

Peptide array-based dot immunoblots have been applied to map human IgE and IgG₄ antibody-binding sequential epitopes on Cyn d 1 in the present study. By means of 48 synthetic decapeptides overlapping with 5 amino acids, a variety of human IgE-binding spots on Cyn d 1 (2 strong positive and 6 weak positive) were initially identified. Further mapping of them using decapeptides with 9 overlapping residues disclosed 4 strong positive spots of major sequential IgE epitopes (fig. 2b), that is, 2 weak positive IgE-binding spots evolved into strong positive ones after minor adjustment of sequences of peptides. Although somewhat heterogeneous results

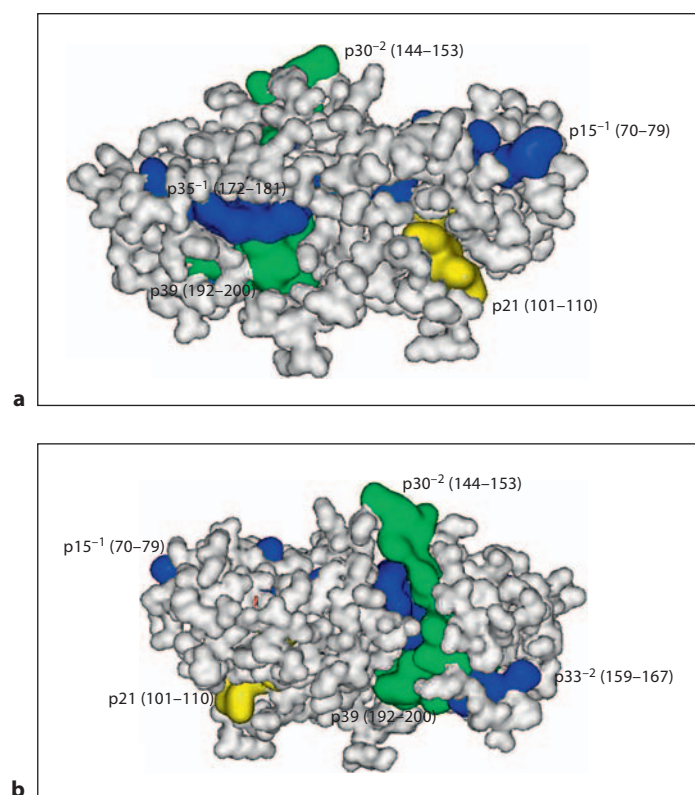


Fig. 6. The binding region of human IgG₄ and IgE antibodies of Cyn d 1 were shown on the three-dimensional model of Cyn d 1. Surface epitopes are colored yellow, green or blue when they are recognized by IgE, IgG₄ or both, respectively. Front face (a) and back face (b).

for IgG₄ binding were found among the 22 patients, 3 strong positive IgG₄-binding spots were almost consistently shown for most of them (fig. 3b).

In agreement with recent reports [25–28], the present study confirms that dot immunoblotting using synthetic peptides is a good tool to determine human sequential IgE and IgG₄ epitopes on allergens. Using computerized modeling, we also simulated the tertiary structure of Cyn d 1 protein and clearly located sequential IgE and IgG₄ antibody-binding epitopes on the surface of simulated Cyn d 1 molecule (fig. 6). As IgE and IgG₄ epitopes may not be sequential, the minor spots in the present study could be the continuous part of discontinuous epitopes and need further study. Among different sequences of Cyn d 1, there are 6 polymorphic peptides belonging to 2 epitopes. We found that two of them have common positive IgE and IgG₄ antibody-binding reactivity. These two sequences should be included in the testing panel to improve the sensitivity of the test for BGP allergy.

The spectra of IgG₁, IgG₂ and IgG₃ antibodies against major epitopes on allergens are rarely explored. Ramos et al. [30] observed that significant serum levels of Blo t 11-specific IgG₁, IgG₂ and IgG₄, but not IgG₃, were detected in Blo t 11-allergic patients. For specific IgG_{1–3} antibodies of Cyn d 1, their binding capability to synthetic decapeptides was not identical. Heterogeneous results for IgG_{1–3} binding were also found among different individuals. However, most of them also had strong binding activities to the three strong positive IgG₄-binding spots in the dot immunoblot using decapeptides with 5 overlapping residues. Therefore, there is no discrepancy among IgG subclasses against major epitopes of allergens. Compared with IgG_{1–3}, IgG₄ also had the highest positive rate of binding activities to those three IgG-binding epitopes. Thus, IgG₄ is the best choice for the detection of antigenicity of Cyn d 1.

Cross-reactivity between IgE and IgG antibodies has been reported. Ramos et al. [30] observed that the pattern of IgE and IgG reactivity to some fragments of Blo t 11 were remarkably similar in dot immunoblots. Using dot immunoblots for epitope mapping of Cyn d 1, we report here that 3 of 4 (75%) IgE-binding epitopes are IgG₄-binding sites, 3 of 5 (60%) IgG₄-binding epitopes are IgE-binding sites. Although both IL-4 and IL-13 can induce B cell class switch from IgM to IgE or IgG₄ [31, 32], partial differences in epitope specificities of IgE and IgG₄ have been substantiated here again.

Elevation of specific IgG₄ has been demonstrated in patients undergoing allergen-specific immunotherapy [12–14]. We and other groups [25, 28] have shown that there are two types of IgE- and IgG₄-binding epitopes: one can be recognized by both IgE and IgG₄ and the other can be only recognized by either IgE or IgG₄. Future studies may compare possible variation of common versus distinct IgE and IgG₄ epitopes after immunotherapy. In our preliminary study, strong IgG₄-binding reactivity was found in two other major IgE-binding epitopes (peptides 33⁻² and 35⁺¹, corresponding to amino acids 159–167 and 172–181), but not vice versa. Interestingly, their sequences are all shifted from the initial sequences (peptide 33 and 35, amino acids 161–170 and 171–180, respectively). It is possible that a minor shift over the sequences of critical amino acids might have a great influence on the binding affinity. It is more likely that epitope mapping using larger peptides may avoid this defect.

Two allergenic regions corresponding to amino acids 120–170 and 224–244 of Cyn d 1 were recently disclosed by mapping of IgE-binding recombinant fragments and

deletion mutants [33]. We have confirmed here that there is indeed a major IgE-binding epitope (peptide 33⁻², amino acids 159–167) among amino acids 120–170 of Cyn d 1. Moreover, we have further revealed a strong major IgG-binding epitope (peptide 30⁻², amino acids 144–153) in this large fragment of Cyn d 1. However, we failed to demonstrate strong IgE-binding reactivity by using synthetic decapeptides with 9 overlapping residues among amino acids 224–244 of Cyn d 1 (data not shown). As our preliminary data of IgE-binding epitopes mapped with 48 decapeptides overlapping with 5 amino acids showed weak reactions to peptides 46 and 47 (amino acids 226–235 and 231–240, respectively; fig. 2a), it is still likely that there is an IgE-binding epitope larger than decapeptides or a conformational epi-

tope. Therefore, larger peptides may be needed to further evaluate the possible IgE-binding epitope at the C-terminal end of Cyn d 1.

To sum up, mapping of human major IgE- and IgG₄-binding epitopes on Cyn d 1 and determination of the critical amino acids among them provide very useful information for future molecular engineering such as site-directed mutagenesis and synthetic peptide vaccines. Above all, creation of a new generation of hypoallergenic variants as immunotherapeutic vaccines is really needed to reduce the lethal allergenicity of Cyn d 1 and to develop safer immunotherapy, that is, to retain its IgG₄ epitopes but to ablate its IgE epitopes or replace some of its amino acids critical for IgE binding.

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