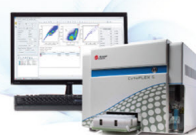


Introducing the **CytoFLEX S** Flow Cytometer
Benchtop Cytometry without Compromises
for Special Applications

[LEARN MORE](#)



**BECKMAN
COULTER**
Life Sciences



Antigenic Determinants of Der p 1: Specificity and Cross-Reactivity Associated with IgE Antibody Recognition

This information is current as
of January 15, 2017.

Jill Glesner, Lisa D. Vailes, Caleb Schlachter, Nicholas
Mank, Wladek Minor, Tomasz Osinski, Maksymilian
Chruszcz, Martin D. Chapman and Anna Pomés

J Immunol published online 30 December 2016
<http://www.jimmunol.org/content/early/2016/12/30/jimmunol.1600072>

-
- Supplementary Material** <http://www.jimmunol.org/content/suppl/2016/12/30/jimmunol.1600072.DCSupplemental.html>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at:
<http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/cgi/alerts/etoc>



Antigenic Determinants of Der p 1: Specificity and Cross-Reactivity Associated with IgE Antibody Recognition

Jill Glesner,* Lisa D. Vailes,* Caleb Schlachter,[†] Nicholas Mank,[†] Wladek Minor,[‡] Tomasz Osinski,[‡] Maksymilian Chruszcz,[†] Martin D. Chapman,* and Anna Pomés*

Der p 1 and Der f 1 are major allergens from *Dermatophagoides pteronyssinus* and *D. farinae*, respectively. An analysis of antigenic determinants on both allergens was performed by site-directed mutagenesis. The analysis was based on the x-ray crystal structures of the allergens in complex with Fab fragments of three murine mAbs that interfere with IgE Ab binding: the two Der p 1-specific mAbs 5H8 and 10B9, and the cross-reactive mAb 4C1. On one hand, selected residues in the epitopes for mAb 5H8 and mAb 4C1 were substituted with amino acids that resulted in impaired Ab binding to Der p 1. On the other hand, an epitope for the Der p 1-specific mAb 10B9, which partially overlaps with mAb 4C1, was created in Der f 1. The mutation of 1–3 aa residues in Der f 1 was sufficient to bind mAb 10B9. These residues form hydrogen bonds with CDRs of the Ab other than H CDR3. This observation unveils an exception to the dominant role of H CDR3 commonly observed in Ag recognition. Overall, this study resulted in the identification of important residues for mAb and IgE Ab recognition in group 1 mite allergens. This information can be used to engineer allergen mutants with reduced IgE Ab binding for immunotherapy. *The Journal of Immunology*, 2017, 198: 000–000.

House dust mites produce proteins that are important inducers of allergic reactions around the world (1). Mite allergies are associated with the development of asthma, allergic rhinitis, and atopic dermatitis. The predominant genus *Dermatophagoides* comprises two mite species, *D. pteronyssinus* and *D. farinae*, which coexist in most geographical areas (2). *D. pteronyssinus* has a preference for temperate and tropical coastal regions, whereas *D. farinae* is more tolerant of low humidity and survives in drier climates (i.e., Midwest region of the United States) (3). The major allergens Der p 1 and Der f 1 play a dominant role in IgE cross-reactivity between both species. Both allergens are cysteine proteases and may be present in large quantities in house dust (up to 100,000 ng/g) (4). The x-ray crystal structure of natural Der f 1 revealed a degree of surface conservation with Der p 1 of ~71% amino acid identity and an overlapping catalytic site (5).

To gain insight into the antigenic relationships between both allergens, x-ray crystal structures of the natural allergen in complex with fragments of murine mAbs were determined. Allergen-Ab

structures provide the most accurate information about the conformational epitopes involved in these interactions. Three mAbs were selected based on their ability to interfere with IgE Ab binding from a set of murine mAbs raised against Der p 1 and Der f 1 (6–8). This set of mAbs comprised just over 3% (2 out of 53) of cross-reactive Abs, and predominantly recognized species-specific epitopes (7, 8). First, the structures of complexes of Der p 1 or Der f 1 with the cross-reactive mAb 4C1 revealed a common epitope between both allergens (9). Recently, the structures of the Der p 1-specific epitopes for mAbs 5H8 and 10B9 were determined (10). Epitopes comprise a limited number of amino acids forming different kinds of interactions with the Ab (hydrogen bonds, cation- π interactions, hydrophobic interactions, etc.). The structural analysis of epitopes, such as the one performed here, informs the engineering of molecules with either reduced ability to bind Abs or with new epitopes. Specific residues can then be replaced by site-directed mutagenesis with amino acids that either decrease or improve Ab binding.

The first goal of the current study was to identify determinants of species specificity and cross-reactivity in Der p 1 and Der f 1 interactions with Abs. For this purpose, the epitopes for species-specific (mAbs 5H8 and 10B9) and cross-reactive (mAb 4C1) Abs were selected for mutagenesis analysis. The Der p 1-specific mAb 10B9 was able to inhibit the binding of the cross-reactive mAb 4C1 to Der p 1, but not to Der f 1 (7). The existence of these two Abs that presumably bound to overlapping or adjacent sites on Der p 1 presented a unique opportunity to assess the molecular basis of recognition. The amino acids that confer specificity to mAb 10B9 were analyzed by engineering a new epitope in Der f 1 that would bind this Ab. The new epitope was created in a step-wise manner by substituting amino acids in Der f 1 to residues in equivalent positions in Der p 1 that were found to participate in mAb 10B9 binding.

The second goal was to identify the main residues involved in specific allergen-Ab interactions and generate mutants with reduced capacity to bind Abs. Most importantly, the mutagenesis analysis led to the identification of residues involved in IgE Ab

*Indoor Biotechnologies Inc., Charlottesville, VA 22903; [†]University of South Carolina, Columbia, SC 29208; and [‡]University of Virginia, Charlottesville, VA 22908

ORCID: 0000-0001-7075-7090 (W.M.); 0000-0001-9933-346X (T.O.); 0000-0001-7521-5485 (M.C.); 0000-0002-0845-3632 (M.D.C.); 0000-0002-8729-1829 (A.P.).

Received for publication January 12, 2016. Accepted for publication November 23, 2016.

This work was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Awards R01AI077653 (to A.P. [contact principal investigator], M.D.C., and M.C.) and R01GM117325 (to W.M.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Address correspondence and reprint requests to Dr. Anna Pomés, Indoor Biotechnologies Inc., 700 Harris Street, Charlottesville, VA 22903. E-mail address: apomes@inbio.com

The online version of this article contains supplemental material.

Abbreviations used in this article: NHS, N-hydroxysuccinimide; PDB, Protein Data Bank; RFU, relative fluorescence unit; S_c, shape complementarity index; SPR, surface plasmon resonance.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/\$30.00

binding given the overlap between mAb and IgE Ab binding sites. Allergens with reduced IgE Ab binding capacity were also produced, and the information obtained about IgE epitopes will contribute to the design of candidate allergens for future immunotherapy against mite allergies.

Materials and Methods

Rational design of mutagenesis

Design of site-directed mutagenesis was based on the structures of Der p 1 or Der f 1 in complex with Fab fragments of either the cross-reactive mAb 4C1 or the Der p 1-specific mAbs 5H8 or 10B9; Protein Data Bank (PDB) accession codes: 3RVV (Der f 1-4C1), 3RVW and 3RVX (Der p 1-4C1), 4PP1 (Der p 1-5H8), and 4PP2 (Der p 1-10B9) (9, 10). Structural images were created with PyMol (11). The shape correlation statistic or shape complementarity index (S_c) was used as a measure of the geometric surface complementarity of protein-protein interfaces based on the relative shape of the opposing surfaces and interactions between the surfaces. The S_c assumes values from 0 to 1, where 1 defines the perfect fit and 0 defines topologically unrelated surfaces (12). The program SC included in the CCP4 program suite was used to calculate the S_c between allergen-Ab complexes (13).

Site-directed mutagenesis of Der p 1 and Der f 1

Der p 1 and Der f 1 mutants were expressed in *Pichia pastoris* and purified by affinity chromatography. Single or multiple mutants were produced: 1) Der p 1 mutants with substitutions in residues involved in mAb 5H8 and/or 4C1 binding, and 2) seven recombinant Der f 1 mutants with 1–4 aa substitutions within the corresponding area of the mAb 10B9 epitope in Der p 1. The templates for mutagenesis were DNA encoding for deglycosylated pro-Der p 1.0105-N52Q and pro-Der f 1.0107-N53Q allergens as previously described (9). The DNA was inserted into *P. pastoris* expression vectors pPICZαC and pPICZαB, respectively, for methanol-inducible expression. The difference in amino acid numbering between Der p 1 and Der f 1 is due to a deletion in Der p 1 of a serine present in Der f 1 at position 8. Site-directed mutagenesis was performed using QuikChange (Stratagene, La Jolla, CA). The sequence of the mutated DNA was confirmed before linearization and transformation into the *P. pastoris* strain KM71.

Production of mite group 1 natural and recombinant allergens

Natural Der p 1 and Der f 1 (termed nDer p 1 and nDer f 1, respectively) were purified from mite cultures as described previously (5, 9, 10). The recombinant Der p 1 and Der f 1 (termed rDer p 1 and rDer f 1, respectively) wild type and mutant allergens were expressed as proenzymes in *P. pastoris* and purified by affinity chromatography using either mAb 4C1 (for mAb 5H8 and 10B9 epitope mutants) or mAb 5H8 (for mAb 4C1 epitope mutant) coupled to CNBr-activated Sepharose 4B beads (GE Healthcare, Piscataway, NJ). Each sterile filtered supernatant was loaded onto the column and the allergen was eluted with basic elution buffer (0.005 M glycine in 50% ethylene glycol, pH 10). The column fractions were combined, concentrated, and acid dialyzed (0.1 M sodium acetate, pH 4.2) to fully remove the proregion and then dialyzed into PBS.

Measurement of group 1 mite allergens and dose-response curves of epitope mutants by immunoassays

Quantification of expressed allergens was performed by ELISA and by Advanced Protein Assay (Cytoskeleton, Denver, CO). The effect of mutations on Ab binding was assessed by direct binding or ELISA inhibition. The mAb epitope mutants were compared with the allergens with wild type epitopes (recombinant with or without proregion and/or natural Der p 1 or Der f 1) by performing dose-response curves using two-side ELISA. ELISA plates were coated overnight at 4°C with a capture mAb (4C1, 5H8, or the Der f 1-specific 6A8, depending on the assay as explained below).

Standards and samples were added, incubated for 1 h at room temperature, followed by 1 h incubations with a mAb that binds to the epitope that was mutated. Biotinylated mAbs 5H8 or 4C1 (B-5H8 or B-4C1) were used as detection Abs for the mAb 5H8 and 4C1 epitope mutants that had been captured with mAbs 4C1 or 5H8, respectively. The Der f 1 mutants, captured with Der f 1-specific 6A8, were detected with either B-4C1 or B-10B9. The biotinylated Abs were used at dilutions that would provide a sufficient window of Ab binding activity (absorbance up to 3 at 405 nm). This was followed by an incubation with streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO), and subsequent assay development using ABTS/ H_2O_2 as substrate. Absorbance was read at 405 nm using a microplate reader (BioTek Instruments, Winooski, VT).

Affinities of Der f 1 mutants determined by surface plasmon resonance

Affinities of the allergens for mAbs were measured by surface plasmon resonance (SPR) with a Biacore 3000 (GE Healthcare, Marlborough, MA). The nDer p 1, rDer p 1, nDer f 1, and all rDer f 1 mutants were inactivated by overnight incubation with 5 mM E-64 protease inhibitor at 4°C. CM-5 chips were covalently coated by activation with 0.4 M 1-ethyl-3-(3-dimethylamino) propyl carbodiimide hydrochloride and 0.1M *N*-hydroxysuccinimide (NHS), followed by 8 μl/min injection of the mAb 10B9 (90 μg/ml) for 8 min in 50 mM HEPES pH 7.4 and 0.005% Tween 20. Unreacted NHS esters were inactivated with 1.0 M of ethanolamine/NaOH (pH 8.5). The nDer p 1, rDer p 1, nDer f 1 and rDer f 1 mutants were flowed at 30 μl/min for 10 min in a five concentration series ranging from 0 to 1 μM; nDer f 1 was flowed in a concentration series ranging from 0 to 2 μM. The affinity was determined using Langmuir binding with mass transfer and drifting baseline models in the Scrubber2 software suite (BioLogic Software, Campbell, Australia) and Biacore 3000 control software (GE Healthcare).

Inhibition of mAb or IgE Ab binding to Der p 1 or Der f 1 by epitope mutants

Ab binding inhibition assays were performed by ELISA to compare mAb 5H8 and mAb 10B9 epitope mutants for their capacity to inhibit binding of murine IgG mAbs or human IgE Abs to Der p 1. Briefly, for inhibition of mAb binding experiments, mutants, nDer p 1 or nDer f 1 were preincubated for 1 h at room temperature with biotin-labeled mAb 5H8 at 1:2000 dilution to assess 5H8 epitope mutants, or with biotinylated mAb 10B9 at 1:10,000 dilution or more to assess 10B9 epitope mutants. The allergen-Ab mixture was then added to microtiter plates coated with 10 μg/ml Der p 1. The allergens were used as inhibitors at concentrations ranging between 0.001 and 100 μg/ml. After 1 h incubation, plates were washed and incubated for 30 min with streptavidin peroxidase. Plates were developed by addition of ABTS/ H_2O_2 and read as above.

For IgE inhibition experiments, allergens were preincubated for 1 h at room temperature with IgE Abs (plasma diluted to have a ~1–3 window of absorbance or plasma pool diluted 1:2) and then added to microtiter plates coated as above. After a 3 h incubation, plates were washed and incubated for 30 min with peroxidase labeled goat anti-human IgE (or streptavidin peroxidase if anti-human IgE was biotinylated) and developed as above. Plasma from dust mite allergic patients ($n = 17$) used in IgE inhibition and direct Ab binding assays was obtained from PlasmaLab International (Everett, WA) which operates in full compliance of Food and Drug Administration regulations. Informed donor consent was obtained from each individual prior to the first donation. The average of Ab titers were 1883.6 AU/ml total IgE, 171.1 AU/ml Der p 1 and 20.7 AU/ml Der f 1, measured by multiplex array as previously described (14). A pool was made using four plasmas that had higher levels of specific IgE for Der p 1 than for Der f 1. In this plasma pool, the average of Der p 1-specific IgE was 5.8 times larger than the average of Der f 1-specific IgE (147.5 versus 25.4 AU/ml, respectively).

Direct IgE Ab binding assays

Direct IgE Ab binding assays were performed by ImmunoCAP. Biotinylation of the allergens was carried out using the EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format (Pierce; Thermo Fisher Scientific, Rockford, IL), with biotin added at a 10-fold molar excess to the allergens. Zeba Desalt Spin Columns (Pierce; Thermo Fisher Scientific) were used to remove any excess biotin from the biotinylated allergens. A Quant*Tag Biotin Kit (Vector Laboratories, Burlingame, CA) was used to quantify the number of biotins per molecule. IgE detection and quantification in plasma samples were determined using Specific IgE detection streptavidin ImmunoCAP on the Phadia 100 (Thermo Fisher Scientific, Portage, MI). After testing several concentrations of allergen (from 0.25 to 10 μg/ml), streptavidin ImmunoCAPs were loaded with 5 μg per CAP of biotinylated allergen and incubated for 30 min. IgE measurements were performed according to standard ImmunoCAP procedures.

Cysteine protease activity

Cysteine protease activity of the allergen mutants was determined by a fluorometric assay. Purified allergens were diluted to 25 μg/ml in either 50 mM sodium phosphate and 1 mM EDTA buffer (inactive enzyme) or 50 mM sodium phosphate and 1 mM EDTA buffer containing 1 mM reducing agent dithiothreitol (Pierce DTT; Thermo Fisher Scientific) (activated enzyme). The diluted allergens were heated in a 37°C shaking incubator for 10 min. Ten microliters of the heated allergen was added to a

cuvette containing 980 μ l of the corresponding buffer plus 10 μ l of substrate (BOC-Gln-Ala-Arg-MCA; Peptides International, Louisville, KY). The final concentrations were 0.25 μ g/ml for the allergen and 125 μ M for the substrate. Enzymatic activity was measured as the difference in relative fluorescence unit (RFU) at times 0 and 3 min in a Versafluor Fluorometer System (Bio-Rad, Hercules, CA) set at excitation and emission wavelengths of 360 ± 20 and 460 ± 5 nm, respectively.

Results

Site-directed mutagenesis based on crystal structures of allergen-Ab complexes

The rationale for mutagenesis was to identify: 1) determinants of species specificity and cross-reactivity in Der p 1 and Der f 1, and 2) residues involved in IgE Ab binding in mAb epitopes that overlap with IgE Ab binding sites (9, 10). The specificity of the anti-Der p 1 mAb 5H8 was analyzed by substituting important allergen residues involved in Ab interaction with amino acids that would impair Ab binding. In contrast, the specificity of anti-Der p 1 mAb 10B9 was evaluated by creating an epitope in Der f 1 that would bind mAb 10B9. The contact residues involved in these Der p 1 Ab complexes had recently been identified by x-ray crystallography, thus the structures of the 4C1, 5H8 and 10B9 epitopes were known (9, 10). These mAbs have different specificities and the epitopes for mAbs 4C1 and 10B9 overlap (Fig. 1). The epitopes were formed by 14–19 allergen residues from which three to four were selected, taking into account their significant involvement in allergen-Ab binding as explained below. The residues selected for mutagenesis, marked in the Der p 1 and Der f 1 sequences, show the conformational nature of the three epitopes (Figs. 2, 3, Table I).

Mutagenesis of mAb 4C1 epitope

Three amino acids were selected to express a Der p 1 multiple mutant with these substitutions: R156A, Y185V, and D198A (Figs. 2, 3, Table I). Arg¹⁵⁶ was selected due to its dominant role in the interactions between Der p 1 (and Der f 1) and the L chain of 4C1 (Table I). Arg¹⁵⁶ was in a marginal site of the 4C1 epitope (Fig. 2), and participated in four hydrogen bonds with the three CDRs of the L chain of the Ab. Two hydrogen bonds were formed by Arg¹⁵⁶ main chain oxygen atom and side chains of Tyr³² and Arg⁵⁰ from mAb 4C1, and two were formed by Arg¹⁵⁶ side chain with the side chain of Asp⁹² from the Ab (Table I).

Two additional amino acids more centrally located in the epitope, Tyr¹⁸⁵ and Asp¹⁹⁸, were also mutated. Tyr¹⁸⁵ participated in hydrophobic interactions with the H chain and residue Tyr¹⁰⁴ from H CDR3. The side chain of Asp¹⁹⁸ interacted through one hydrogen bond with the H CDR3 (main chain of Arg¹⁰³) of the Ab. Tyr¹⁸⁵ and Asp¹⁹⁸ form part of the core of the 4C1 binding epitope. This core is composed of seven residues that have almost the same conformation in the Ab-bound form and in the free form of the allergen. Arg¹⁷ was also mutated to Thr but this mutant did not express. As the side chain of Arg¹⁷ is involved in multiple hydrogen bonds and hydrophobic interactions with several residues, the mutation most likely destabilizes Der p 1. Arg¹⁷ was part of an α -helix and interacted with Tyr¹⁰⁴ from H CDR3 and Tyr⁵⁴ from H CDR2. In addition, Arg¹⁷ participated in a cation- π interaction with Tyr¹⁰² from the H chain (3.3 Å) (9). Table I shows details of the allergen-Ab interactions.

Mutagenesis of mAb 5H8 epitope

Four mutants of the mAb 5H8 epitope were expressed: Q53T, Y93S, Q109R-F111Y, and S114R. Amino acids in Der p 1 were substituted to residues that were either present in the equivalent position in Der f 1 (Q53T, Y93S, F111Y), which does not bind 5H8 and/or to larger residues that could generate a steric hindrance and prevent the Ab binding (Q109R-F111Y, S114R) (Figs. 2, 3). The

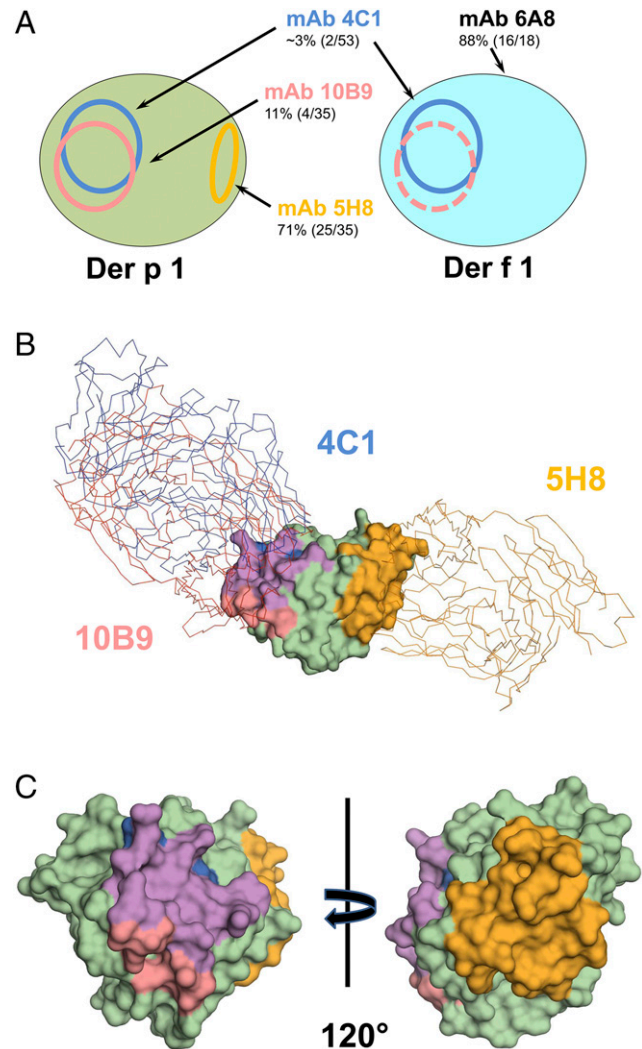


FIGURE 1. Antigenic determinants of group 1 mite allergens. **(A)** Schematic representation of antigenic determinants in Der p 1 and Der f 1 for four mAb previously described (7, 8). Most mAbs raised against Der f 1 were species-specific (6A8; 16 out of 18; 88.9%), whereas only two also bound Der p 1 (4C1; 2 out of 18; 11.1%) (8). Most mAbs raised against Der p 1 were directed to the same epitope (5H8; 25 out of 35; 71.4%) and four were directed to another epitope (10B9; 4 out of 35; 11.4%) (7). In total, only two out of 53 mAbs raised against Der p 1 or Der f 1 were cross-reactive (2 out of 53; 3.8%). **(B)** Epitopes for mAb 4C1 (blue), mAb 10B9 (pink), and mAb 5H8 (orange) are indicated on Der p 1 structure (green). The overlapping region of 4C1 and 10B9 epitopes is marked in lilac. The figure was generated by superposition of crystal structures of Der p 1-Ab complexes (PDB accession codes: 3RVW [Der p 1-4C1], 4PP1 [Der p 1-5H8], and 4PP2 [Der p 1-10B9]). **(C)** Relative position of the three epitopes.

four residues Glu⁵³, Tyr⁹³, Glu¹⁰⁹, and Ser¹¹⁴ were selected from the 12 aa forming hydrogen bonds with 5H8 based on their ability to form strong hydrogen bonds with the Ab. Phe¹¹¹ forms mainly hydrophobic interactions with Trp⁵² and Tyr¹⁰² of the V region of the H chain as well as Tyr⁴⁷ of Der p 1 (Table I) (10). Residues Glu¹⁰⁹ and Phe¹¹¹ are in the core of the epitope, whereas Glu⁵³, Tyr⁹³, and Ser¹¹⁴ are in the rim. An additional criterion was related to the fact that all these residues are significantly different from the corresponding residues in Der f 1 that does not bind this Ab.

Mutagenesis to form a mAb 10B9 epitope in Der f 1

A partial overlap between the epitopes for the cross-reacting mAb 4C1 and the Der p 1-specific mAb 10B9 was taken into consid-

Table I. Mutations for analysis of mAb recognition

Mutations on Allergen ^a	Interacting mAb		Residue	Interaction	Distance (Å) ^c
	Name	Chain CDR ^b			
Der p 1					
R156A	4C1	L CDR1	Tyr ³²	H-bond	2.7, 2.6
		L CDR2	Arg ⁵⁰	H-bond	3.2, 3.2
		L CDR3	Asp ⁹²	Two H-bonds	2.7, 2.8 / 3.0, 3.0
Y185V	5H8	H CDR3	Tyr ¹⁰⁴	Hydrophobic	4.0
D198A		H CDR3	Arg ¹⁰³	H-bond	2.8, 3.0
Q53T		H CDR2	Asp ⁵⁸	H-bond	2.5, 2.7
		H CDR3	Tyr ¹⁰²	H-bond	3.2
Y93S		H CDR1	Thr ³⁰	H-bond	2.5, 2.6
Q109R		H CDR2	Gly ⁵³	H-bond	2.9, 3.1
F111Y		H CDR2	Trp ⁵²	Hydrophobic	
		H CDR3	Tyr ¹⁰²	Hydrophobic	
S114R		L CDR1	Tyr ³²	H-bond	2.9, 3.0
		L CDR2	Tyr ⁵⁰	H-bond	3.1, 3.0
Der f 1 ^d					
S13A	10B9	H CDR2	Tyr ⁵³	H-bond	2.7, 2.6
S19Q		L CDR3	Gly ⁹¹	H-bond	3.3, 3.3
		L CDR3	Asn ⁹²	H-bond	3.3, 3.5
		H CDR3	Tyr ¹⁰⁷	van der Waals	
S180N		H CDR1	Ser ³¹ , Gly ³²	H-bond	2.8, 2.6
		H CDR1	Gly ³²	H-bond	2.8, 2.9
S200N		L CDR2	Arg ⁵³	H-bond	2.5, 2.7

^aResidues in bold indicate the amino acid from the allergen involved in mAb recognition.

^bL refers to Ab L chain, H refers to Ab H chain.

^cThe two values of distance for interactions with mAb 4C1 refer to two forms of the structure of Der p 1 in complex with Fab of the mAb with PDB accession codes 3RVW and 3RVX, respectively.

^dMutations were performed on Der f 1, to create a binding site for mAb 10B9 that is Der p 1-specific, and were based on the structure of the Der p 1-mAb 10B9 complex. H-bond, hydrogen bond.

in agreement with the fact that the epitope for mAb 5H8 resides far from the catalytic area that comprises Cys³⁴ and His¹⁷⁰, as well as from the substrate binding site. The mutant of the mAb 4C1 epitope showed a reduced enzymatic activity (8.3 RFU/min per 0.25 µg/ml) that, interestingly, could be associated with the location of the mutations close to the active site. In particular, the substitution Asp^{198A} is on the same loop as Trp¹⁹², which is very close to the catalytic residue His¹⁷⁰. In addition, it is quite likely that the vicinity of Trp¹⁹² participates in substrate binding. Although disruption of the catalytic site and/or substrate binding by the mutation may have caused the reduction in enzymatic activity, the fact that enzymatic activity was detected indicates overall correct folding of this mutant, together with the fact that IgE binding to this mutant presented by mAb 5H8 could be measured at similar levels as the wild type for a couple of plasmas (data not shown). The triple (S13A S19Q S180N) and quadruple (S13A

S19Q S180N S200N) 10B9 Der f 1 mutants had comparable enzymatic activity to rDer f 1 (data not shown). These results indicate that the mutations did not abolish enzymatic activity. In addition, to show the validation of the expression and purification quality of the mite allergens and the corresponding mutants, group 1 mite allergens were run by SDS-PAGE, displaying the expected m.w. (data for Der p 1 were added as Supplemental Fig. 1).

Effect of mutations in Der p 1 epitopes for mAb 5H8 and mAb 4C1 on mAb and IgE Ab binding

The capacity of the mAb 5H8 or 4C1 epitope mutants to bind the respective mAb was reduced versus the wild type allergen in different degrees and depended on the mutation according to the displacement of the mAb direct binding or inhibition curves (Fig. 4). The relative effect of the mutations on mAb 5H8 binding was as follows: the double mutant Q109R-F111Y showed the

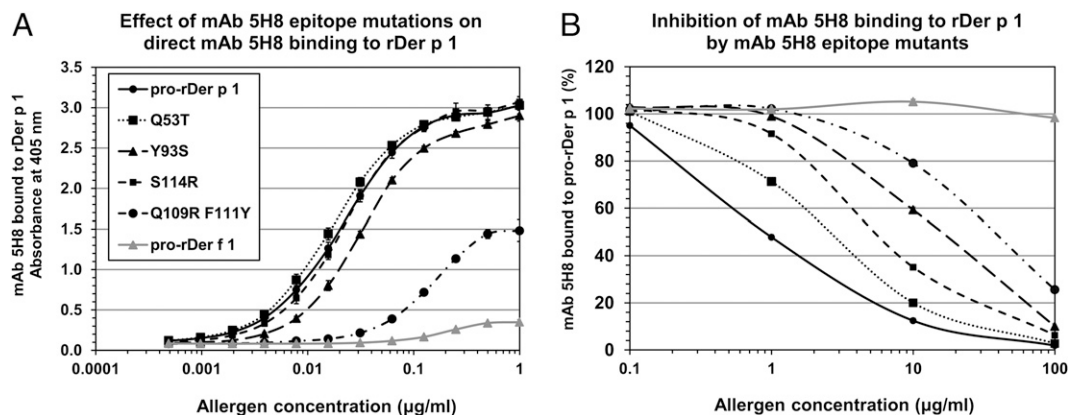


FIGURE 4. Reduction of mAb 5H8 binding due to mutations in the associated epitope. **(A)** Dose-response curves showing the effect of mutations of the 5H8 epitope on mAb 5H8 binding. **(B)** Inhibition assay showing that mutations of the 5H8 epitope reduce the mAb 5H8 binding to pro-rDer p 1. The allergen pro-rDer f 1 was used as a negative control. Data are arithmetic mean $\pm$ SD from an experiment representative of four assays performed.

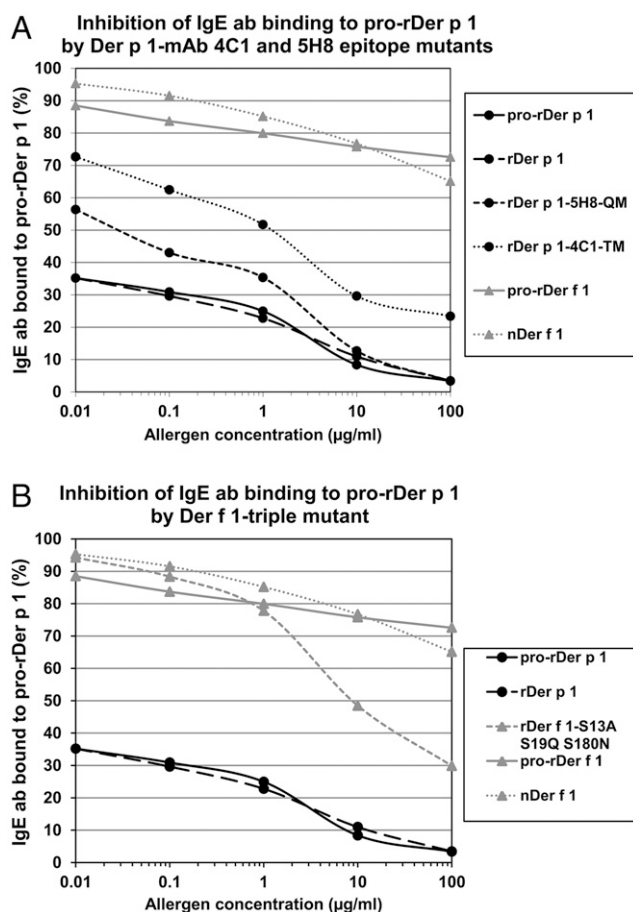


FIGURE 5. Effect of mutations in epitopes for mAbs 4C1, 5H8, and 10B9 on IgE Ab binding to group 1 mite allergens by inhibition assays. **(A)** Reduction of IgE Ab binding to multiple epitope mutants versus the wild type, using pooled plasma from mite-allergic patients ($n = 4$). Inhibitors are the Der p 1 mAb 4C1 epitope triple mutant and the Der p 1 mAb 5H8 epitope quadruple mutant. **(B)** Increase of IgE Ab binding to the Der f 1 triple mutant compared with Der f 1, using the same plasma pool as in (A). Representative experiment out of two performed.

most reduced mAb 5H8 binding (up to 40 times in inhibition assays, Fig. 4B), followed by the other three mutants in this order: Y93S > S114R > Q53T. Gln¹⁰⁹ forms a hydrogen bond with Gly⁵³ of the Ab, and is important for binding the Ab (along with

two water molecules) because it fills a cavity that is located among all three H chain CDRs. Phe¹¹¹ mainly forms the two hydrophobic interactions mentioned above. Therefore, replacement of the polar residue Gln¹⁰⁹ with a more bulky and charged Arg residue, in addition to the F111Y substitution, would have contributed to the reduction of 5H8 binding observed for the mutant Q109R-F111Y.

Interestingly, initial assessment of the effect of individual mutations in the mAb 5H8 epitope on IgE Ab binding showed small or no effect in two mite-allergic patients by IgE inhibition assays and only mutations Q109R-F111Y or S114R showed a reduction of IgE Ab binding (up to 10-fold) (data not shown). Therefore, the mutations Q109R-F111Y, Y93S, and S114R were combined in one mAb 5H8 epitope multiple mutant. Similarly, three mutations in the mAb 4C1 epitope (R156A, Y185V, and D198A), which were previously proven to individually abolish the mAb 4C1 binding and reduce IgE Ab binding up to 10-fold (9), were combined into a mAb 4C1 epitope multiple mutant. The 5H8 and 4C1 multiple mutants were tested in a pool of plasma from four mite-allergic patients and showed a significant reduction in IgE Ab binding by inhibition assays (at least 100-fold for one of the mutants) (Fig. 5A). When tested for individual patients in inhibition assays, a reduction in IgE Ab binding to the mutants versus the wild type was also observed for all plasmas tested ($n = 8$), and the differences between both multiple mutants varied per patient (small for some plasmas and up to >100-fold difference for two of the plasmas tested) (data not shown). Using direct binding assays by ImmunoCAP, a decrease in IgE Ab binding to mutants versus the wild type was also observed for most patients using individual plasma ($n = 12$) (Table II). The maximum reduction in IgE Ab binding versus the wild type was 41.9% for the rDer p 1-5H8-QM, 32.0% for rDer p 1-4C1-TM and 81.7% for the Der f 1 allergen for three different patients (with mean reductions $\pm$ SD for $n = 12$ of $34.0 \pm 5.8\%$, $12.0 \pm 10.5\%$ and $38.7 \pm 24.4\%$, respectively) (Table II). These reductions are significant, considering the polyclonal nature of IgE. The changes observed in IgE Ab binding to the mutants versus the wild type could not be explained by the variability in measurements of protein loaded to the ImmunoCAP (4.0%) or the variability of the ImmunoCAP assays (9.8%). Also, there was no correlation between changes in cysteine protease activity and changes in IgE Ab binding between wild type and mutants. In fact, the largest decrease in IgE binding was found for the mAb 5H8 mutant, which had an equivalent enzymatic activity to the wild type. As expected, IgE Ab binding to Der f 1 did not correlate with binding to Der p 1 (i.e., plasmas 9 and 12) (and

Table II. IgE Ab binding to Der p 1 multiple mutants by direct Ab binding assays (ImmunoCAP)

Plasma	IgE Ab Binding (kUA/L)				IgE Ab Binding (%)				Reduction in IgE Ab Binding (%)			
	rDer p 1	rDer p 1-5H8-QM	rDer p 1-4C1-TM	pro-rDer f 1	rDer p 1	rDer p 1-5H8-QM	rDer p 1-4C1-TM	pro-rDer f 1	rDer p 1	rDer p 1-5H8-QM	rDer p 1-4C1-TM	pro-rDer f 1
1	15.1	9.4	12.6	5.1	100.0	62.1	83.4	33.4	0.0	37.9	16.6	66.6
2	4.6	2.7	4.7	4.7	100.0	58.1 ^a	102.0 ^b	101.5 ^b	0.0	41.9 ^b	-2.0 ^a	-1.5 ^a
3	8.9	5.4	7.4	7.4	100.0	60.6	82.9	83.1	0.0	39.4	17.1	16.9
4	3.6	2.4	3.4	1.9	100.0	65.8	93.1	52.2	0.0	34.2	6.9	47.8
5	20.3	14.4	17.7	9.1	100.0	70.9	87.2	44.9	0.0	29.1	12.8	55.1
6	14.5	8.5	9.9	11.3	100.0	58.9	68.0 ^a	77.9	0.0	41.1	32.0 ^b	22.1
7	9.6	7.0	8.3	7.9	100.0	72.7 ^b	86.4	82.1	0.0	27.3 ^a	13.6	17.9
8	11.0	6.5	10.9	5.3	100.0	59.4	99.1	48.3	0.0	40.6	0.9	51.7
9	8.4	6.0	8.3	1.5	100.0	71.2	98.6	18.3 ^a	0.0	28.8	1.4	81.7 ^b
10	38.4	27.4	31.5	17.8	100.0	71.4	82.0	46.4	0.0	28.6	18.0	53.6
11	22.8	16.3	22.4	15.5	100.0	71.5	98.2	68.0	0.0	28.5	1.8	32.0
12	28.6	19.9	21.4	22.8	100.0	69.6	74.8	79.7	0.0	30.4	25.2	20.3
Mean	15.5	10.5	13.2	9.2	100.0	66.0	88.0	61.3	0.0	34.0	12.0	38.7
SD	10.4	7.6	8.4	6.6	0.0	5.8	10.5	24.4	0.0	5.8	10.5	24.4

Lowest % values in a column are indicated by ^a and highest % values by ^b.

served as a different allergen control), because exposure and sensitization to both allergens is expected to differ per patient. These results proved the importance of specific residues in the allergen-Ab interaction and an overlap between IgE and mAb binding sites.

Comparison of Der p 1 recognition by mAb 10B9 versus mAb 4C1

The epitopes for mAb 4C1 and mAb 10B9 significantly overlap and the relative positions of paratopes are rotated (Fig. 6A) (10). Due to this rotation, the H chain of mAb 4C1 overlaps quite closely in space with the L chain of mAb 10B9 (Fig. 6B). Interestingly, a loop containing the H chain CDR3 of mAb 4C1 protrudes to interact with an area on the surface of Der p 1 that also interacts with the H chain CDR3 of mAb 10B9. The latter adopts an α -helical shape upon Ab binding (Fig. 6C, arrow).

Most of the amino acids from Der p 1 that are involved in interactions with the CDR3 are common to both mAbs, but form interactions with different amino acids in the respective Abs (Table III) (15). The kind of interaction also differs in some cases. Mutations Y185V and D198A, designed to reduce mAb 4C1 binding, also affected 10B9 binding by Ab binding assays (data not shown).

As part of the structural analysis of Der p 1 epitopes, the S_c of the interface formed between Der p 1 and each of the mAbs was calculated. These values were 0.624 and 0.649 between Der p 1 and mAb 5H8, and 0.673 and 0.702 between Der p 1 and mAb 10B9; in both cases two copies were found in the asymmetric unit. These values are in a range expected for Ab-antigen complexes (12, 16). However, the S_c values of the interface formed between the mAb 4C1 and either Der p 1 or Der f 1 were 0.763 and 0.782, respectively. Such high S_c values indicate better shape complementarity and are in the range expected for interfaces of oligomeric proteins. The common area shared by the epitopes binding 4C1 and 10B9 Abs has S_c values of 0.761 for 4C1 binding epitope and 0.633 and 0.677 for 10B9 binding epitope (for two copies of complex in the asymmetric unit).

De novo creation of a mAb 10B9 epitope on Der f 1

Seven rDer f 1 mutants were expressed with each containing 1–4 aa substitutions within the corresponding area of the mAb 10B9 epitope in Der p 1. As more substitutions were present in the Der f 1 mutants, the dose-response curves using mAb 10B9 as detection Ab were displaced to the left with increased saturation levels of Ab binding (Fig. 7A). In contrast, parallel dose-response curves using mAb 4C1 as detection Ab revealed that the mutations did not modify mAb 4C1 binding, which indicates that most likely the mutants preserved the same folding as the wild type allergen (in line with the cysteine protease activity mentioned above) (Fig. 7B). These results prove the creation of a binding site for the Der p 1-specific mAb 10B9 in Der f 1, and an increase in mAb 10B9 Ab affinity for the allergen with the addition of up to three to four mutations in Der f 1. The presence of only one mutation was reflected by minimal mAb 10B9 binding. Three double mutants (S13A S19Q, S13A S180N, and S19Q S180N) revealed the relative importance of three residues in mAb binding: $13 > 180 > 19$. The importance of the triple mutant for binding was also observed by inhibition assay (data not shown). These results were confirmed by measuring the affinities of the mutants for the mAb 10B9 binding by SPR. There was an increase of about one or two orders of magnitude by each step of sequential addition of one, two, or three to four mutations to Der f 1, with an ~ 1000 -fold increase in affinity for the mAb 10B9 by the multiple mutants versus Der f 1 (Table IV). The single mutants showed larger affinities (range of K_D 0.25–3 μ M) than the Der f 1 affinity for mAb

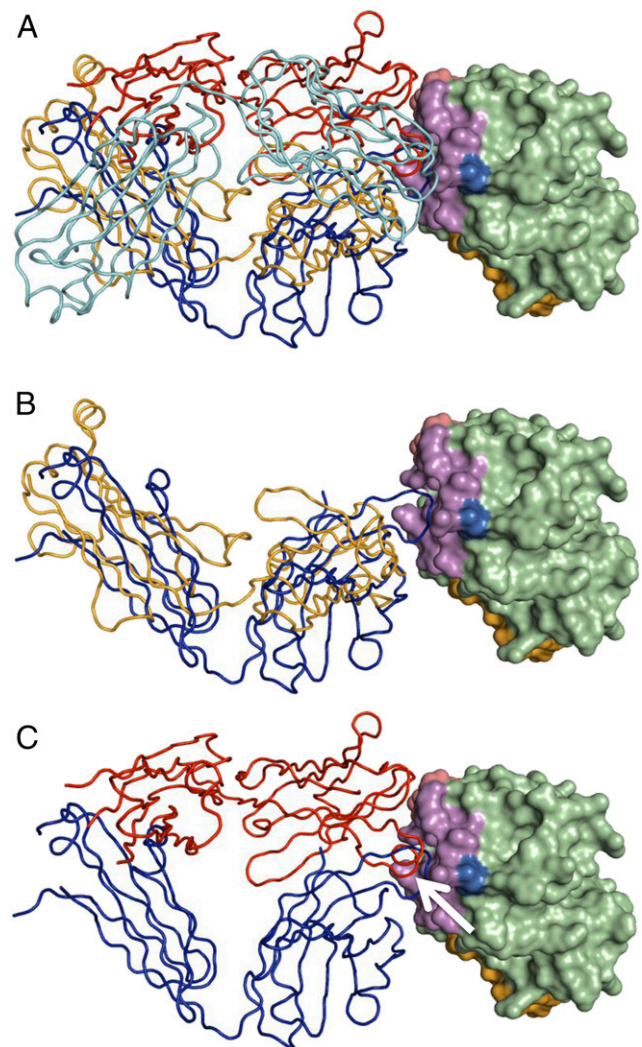


FIGURE 6. Spatial overlap of Fab from mAbs 4C1 and 10B9. **(A)** Superposition of Fab for mAb 4C1 (blue) and 10B9 (red-orange). **(B)** H chain (dark blue) from mAb 4C1 and L chain of mAb 10B9 (orange) coincide in space. **(C)** H chains from mAb 4C1 (dark blue) and mAb 10B9 (red) bind to the same area of Der p 1. White arrow indicates the α -helix formed by the CDR3 from the H chain of mAb 10B9.

10B9 (mean $\pm$ SD, 39.1 ± 3.1 μ M). Overall, the affinities of the single mutants were lower or comparable to the ones for the double mutants (K_D 124–309 nM). Mutations S13A and S180N had a strong combined effect on binding. The triple and quadruple mutants showed affinities for the mAb of 2 ± 19 and 33 ± 19 nM, respectively, that are very close to the affinities measured for rDer p 1 (54 ± 8 nM) and nDer p 1 (1 ± 19 nM). The analysis suggested that the mutation of three or four residues creates epitopes with an affinity within the same order of magnitude as the full epitope in Der p 1 (Table IV). The ability of the rDer f 1 with the three mutations (S13A S19Q S180N) to bind 10B9 was comparable to the ability of rDer f 1 to bind mAb 4C1 ($K_D \sim 18$ nM) (5) (Fig. 8). This indicates that a mAb 10B9 epitope was created in Der f 1.

A comparison of H chain CDRs from four mAbs specific for group 1 mite allergens is shown in Table V, and reviewed in the Discussion.

Epitope for mAb 10B9 overlaps with IgE Ab binding site

To investigate if the residues that had been mutated to create a mAb 10B9 epitope in Der f 1 were involved in IgE Ab binding, the

Table III. Amino acids involved in allergen-Ab interactions at the level of H chain CDR3 of mAbs 4C1 and 10B9

Residues Involved in Der p 1-mAb 4C1 Interaction		Residues Involved in Der p 1-mAb 10B9 Interaction	
Der p 1	mAb 4C1	Der p 1	mAb 10B9
<u>Glu</u> ¹³	<u>Arg</u> ¹⁰⁷	<u>Glu</u> ¹³	<u>Arg</u> ⁹⁸
—	—	<u>Asp</u> ¹⁵	<u>Tyr</u> ¹⁰⁷
<u>Arg</u> ¹⁷	<u>Tyr</u> ¹⁰⁴	<u>Arg</u> ¹⁷	<u>Asn</u> ¹⁰⁵
—	Tyr ¹⁰² (cation- π)	—	—
—	<u>Glu</u> ¹⁰⁶	—	—
(Thr ¹⁸¹ in Der f 1)	—	—	—
<u>Tyr</u> ¹⁸⁵	Close contacts with Tyr ¹⁰⁴ and Pro ¹⁰⁵	<u>Tyr</u> ¹⁸⁵	<u>Asn</u> ¹⁰⁵
<u>Asp</u> ¹⁹⁸	Arg ¹⁰³	<u>Asp</u> ¹⁹⁸	<u>Tyr</u> ¹⁰⁶
<u>Tyr</u> ^{203a}	Hydrophobic and H-bond with Tyr ¹⁰⁴	—	—

Most interactions are hydrogen bonds unless indicated. Distances were measured using Coot (15) and a cut-off of 3.3 Å.

Der p 1 residues recognized by both Abs are underlined.

^aTyr²⁰³ forms a 3.32 Å bond with Tyr¹⁰⁴, which is most likely not important for direct allergen-Ab interaction, and a hydrophobic contact with Tyr¹⁰⁴. This residue is probably more important for keeping Arg¹⁷ in proper orientation, to facilitate a strong interaction between Arg¹⁷ and the Ab.

—, not present; H-bond, hydrogen bond.

Der f 1 triple mutant was compared with Der p 1 and Der f 1 by IgE inhibition assay. A plasma pool was used containing four selected plasmas based on their higher (at least 1.5-fold) specific IgE Ab levels to Der p 1 versus Der f 1. Der p 1 had the strongest capacity to inhibit IgE Ab binding to Der p 1, followed by the triple Der f 1 mutant, and finally by Der f 1, which had an inhibition curve more displaced to the right versus the other two curves (Fig. 5B). The relative effect of the three molecules showed that addition of the three mutations conferred to Der f 1 a higher capacity to bind IgE Abs. These results indicate that the mAb 10B9 epitope is involved in IgE Ab binding.

Discussion

Most of the interactions of inhalant allergens with IgE Abs depend on conformational epitopes defined by the three-dimensional structure of the allergen (17). In this study, the antigenic determinants of Der p 1 and Der f 1 were analyzed by site-directed mutagenesis based on four x-ray crystal structures of group 1 dust mite allergens in complex with Fab fragments of mAbs. The mAbs 5H8, 4C1, and 10B9 were selected based on their capacity to interfere with IgE Ab binding and/or their immunodominance (6–8). The mutagenesis analysis of the mAb specificity was designed based on two complementary approaches: 1) to reduce binding of mAbs 5H8 and 4C1 to Der p 1, and 2) to create a new epitope for the Der p 1-specific mAb 10B9 in Der f 1. The ultimate goal was to identify IgE Ab binding sites by analyzing the effect of amino acid substitutions on IgE Ab binding, given the overlap between IgE and mAb binding epitopes.

For the first approach, specific Der p 1 residues in the epitopes for mAb 5H8 and 4C1 were substituted with amino acids that were expected to impair Ab binding due to: 1) their presence in equivalent positions in Der f 1 that does not bind the Ab, 2) their larger size that could lead to steric clashes, or 3) a change in charge or polarity. Individual substitutions affected mAb binding in different degrees, revealing the relative importance of specific residues for Ab recognition. Overall, substitutions in the mAb epitopes had a stronger effect on mAb binding than on IgE Ab binding. A 10–100 fold reduction of mAb binding could be easily achieved with 1–2 aa substitutions, whereas a 2–10 fold reduction of IgE

Ab binding was observed here for mAb 5H8 epitope mutants and in our previous study for single mAb 4C1 epitope mutants (9). This is an expected result given the polyclonal nature of IgE. Only the binding of a fraction of the population of polyclonal anti-Der p 1 IgE Abs produced by B cell clones would be impaired by a single mutation. As expected, single mutations did not abolish mAb recognition because the allergen-Ab interaction involves several residues. Therefore, mutants with multiple amino acid substitutions in the epitopes for either mAb 5H8 or 4C1 were expressed by combining single mutations that were proven effective in the reduction of Ab binding. The effect of these multiple mutants on IgE Ab binding was significant by inhibition and direct Ab binding assays. In inhibition assays, up to at least 100-fold reduction in IgE Ab binding was observed for a mutant in pooled plasma and in two out of eight individual patients tested. The reductions in direct IgE Ab binding to the mutants versus the wild type by ImmunoCAP were significant when considering the polyclonal nature of IgE Abs. The number of epitopes involved in the IgE response to Der p 1 is unknown. A reduction of 50 or 33% would be expected if one out of two or three epitopes involved in IgE response (with equivalent amounts of Ab per epitope), respectively, were abolished. The maximum reductions of 42 and 32% observed for each of the two mutants are in line with the existence of at least two to three immunodominant epitopes on the allergen. The different effect of epitope mutations per patient in-

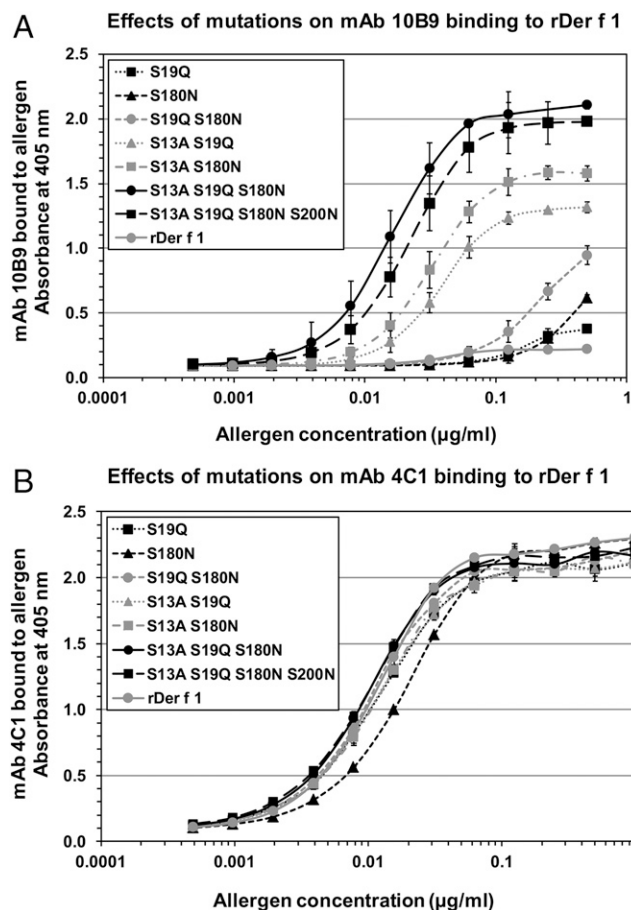


FIGURE 7. Dose-response curves of rDer f 1 wild type and mutants. (A) As the number of mutations in rDer f 1 increases, the ability to bind mAb 10B9 also increases. (B) As positive controls, mutations in rDer f 1 to create a mAb 10B9 epitope site did not interfere with mAb 4C1 binding. (B) Overall, mutating only three to four residues led to the strongest binding of the mAb 10B9. Data are arithmetic mean $\pm$ SD of duplicates from one representative experiment out of two to four experiments performed for each panel.

Table IV. Summary of SPR measurements for allergen-mAb 10B9 interactions

Construct ^a	K_D		Maximum Response Units	
	Mean	SD	Mean	SD
nDer f 1	39.1 μ M	3.1 μ M	3560	217
S19Q	3 μ M	1 μ M	589	172
S180N	250 nM	20 nM	59.0	2.00
S19Q S180N	268 nM	1 nM	25.5	3.00
S13A S180N	124 nM	38 nM	36.5	1.64
S13A S19Q	309 nM	77 nM	216	26.3
S13A S19Q S180N	2 nM	19 nM	97.0	3.00
S13A S19Q S180N S200N	33 nM	19 nM	93.6	7.64
rDer p 1	54 nM	8 nM	99.3	2.16
nDer p 1	1 nM	19 nM	251	41.7

^aPrefix n, natural; prefix r, recombinant.

indicates differences of IgE epitope recognition by patient. Overall, these results proved that important IgE Ab binding sites had been identified on Der p 1.

In the current study, differences in recognition of two Abs that bind overlapping epitopes were evaluated in detail by comparing the epitopes for mAbs 4C1 and 10B9. Structural evidence for recognition of a single epitope by two distinct Abs has been reported previously with different kinds of overlaps for different sets of molecules. Two Abs bound hemagglutinin of influenza virus with similar affinities and orientation, and one-third of the interactions were conserved in the two complexes (18). The interaction of two Abs with influenza virus neuraminidase involved a rotation (of 72°) of the two Abs binding a common epitope. The same set of residues was used in interactions differing sterically and chemically in both complexes (19). In the current study, more than 70% of the 10B9 binding epitope overlaps with the epitope for the mAb 4C1. However, the 4C1 paratope is rotated counterclockwise by ~90° with respect to the 10B9 paratope, and the H chain CDR3s adopt different conformations (an unusual α -helix induced upon mAb 10B9 binding versus a loop for mAb 4C1) (10). Interestingly, although the H chain CDR3 of mAbs 10B9 and 4C1 recognize a similar area on Der p 1, a superposition of the

structures of both complexes showed that overall the H chain of mAb 4C1 overlaps with the L chain of mAb 10B9 in the space. The H chain CDR2s from both mAbs bind to completely different areas of Der p 1, despite their high amino acid sequence identity (75% for CDR2s versus 60% for CDR1s and 27% for CDR3s). Most of the allergen residues involved in recognition by the H chain CDR3 are the same for both mAbs but interact in different ways (Table III). Most interestingly, four residues in common, Glu¹³, Arg¹⁷, Tyr¹⁸⁵, and Asp¹⁹⁸, are not sufficient for binding mAb 10B9 to Der f 1. To create a mAb 10B9 binding site in Der f 1, selected residues were substituted with amino acids present in equivalent positions in Der p 1 and not involved in mAb 4C1 binding. The sequential substitutions S13A, S19Q, and S180N in Der f 1 to the equivalent residues in Der p 1 led to the creation of a mAb 10B9 binding site. Ab binding immunoassays showed an increase in allergen-Ab affinity as additional mutations were added to form the epitope, and SPR confirmed an exponential increase in binding affinities (up to ~1000 fold). This de novo creation of an epitope led to three interesting conclusions. First, only few residues (1–3) were sufficient to form an Ab binding site (although additional residues are expected to be involved in the full mAb 10B9 epitope). This observation is in line with a study which proved that a limited number of mutations in the Ag binding site can alter specificity or add a distinct specificity to create a bispecific Ab (20).

Second, residues mutated to bulkier amino acids than serine (glutamine and asparagine) resulted in mAb 10B9 binding. This is in contrast with the mutations to bulkier residues performed for mAb 5H8, which were designed to lead to steric clashes that would prevent binding. To interpret these results, a hypothesis was postulated that the molecular surfaces involved in binding the mAb 4C1 to Der p 1 would be more complementary and close to each other than those involved in mAb 10B9 binding. Consequently, few bulkier residues in Der p 1 (absent in Der f 1) would act as an anchor for 10B9 to bind above the level of 4C1 binding. This possibility was further investigated by analyzing the surface complementarity of allergen-Ab interfaces (12). In agreement with the hypothesis, the calculated S_c was significantly higher for the interface formed between the mAb 4C1 and both Der p 1 or Der f 1 than the same statistic calculated for the interface formed between mAb 10B9 or 5H8 and Der p 1 (16). The S_c was also significantly higher for the common area on the Der p 1 surface shared between mAbs 4C1 and 10B9. This suggests a better ability of 4C1 to bind these two allergens despite small conformational differences in its epitopes on the surface of Der p 1 and Der f 1, and may also contribute to the fact that 10B9 and 5H8 are binding only to Der p 1. The shape complementarity analysis led to formulate a possible reason of cross-reactivity for mAb 4C1 and lack thereof for mAbs 10B9 and 5H8.

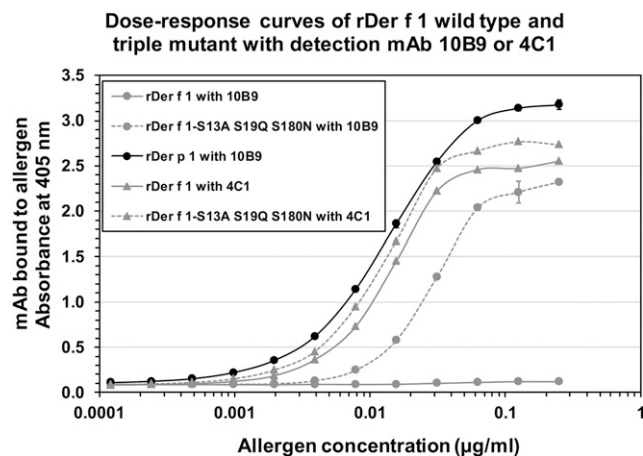


FIGURE 8. Evaluation of new epitope for mAb 10B9 on rDer f 1 triple mutant. The rDer f 1 with three mutations (S13A S19Q S180N) was able to bind 10B9, but not the wild type rDer f 1. This ability was comparable to the ability of rDer f 1 to bind mAb 4C1. As positive controls, mAb 4C1 recognizes both wild type and mutated rDer f 1 (both presented by mAb 6A8), and mAb 10B9 recognizes rDer p 1 presented by mAb 5H8. Data are indicated in circles for mAb 10B9 and in triangles for mAb 4C1. Data for mAb 10B9 are arithmetic mean $\pm$ SD of duplicates from a representative experiment out of four.

Table V. Comparison of H chain CDR length and sequence identities among four mAbs against group 1 mite allergens

	Length (Amino Acids)					SD
	4C1	10B9	5H8	6A8	Mean	
CDR1	6	5	5	5	5.3	0.5
CDR2	16	16	17	17	16.5	0.6
CDR3	12	14	10	12	12.0	2.0 ^a
Sequence Identity (%)						
4C1-CDR1	—	60.0	40.0	50.0	50.0	26.3
4C1-CDR2	—	75.0	33.3	33.3	47.2	32.9
4C1-CDR3	—	27.3	30.0	22.2	26.5 ^b	36.9
10B9-CDR1	60.0	—	50.0	25.0	45.0	31.2
10B9-CDR2	75.0	—	26.7	26.7	42.8	36.6
10B9-CDR3	27.3	—	50.0	44.4	40.6 ^b	31.3
5H8-CDR1	40.0	50.0	—	25.0	38.3	32.5
5H8-CDR2	33.3	26.7	—	43.8	34.6	33.5
5H8-CDR3	30.0	50.0	—	12.5	30.8 ^b	37.8
6A8-CDR1	50.0	25.0	25.0	—	33.3	35.4
6A8-CDR2	33.3	26.7	43.8	—	34.6	33.5
6A8-CDR3	22.2	44.4	12.5	—	26.4 ^b	39.2

CDR as defined in (5).

^aCDR3 is the most variable CDR in length.

^bCDR3 is the most variable CDR in sequence (lowest means of CDR sequence homologies with three other mAb).

—, 100% identity.

Finally, our results illustrate an exception to the general idea that the H chain CDR3 is sufficient for most Ab specificities and has a dominant role in Ag recognition (21). A comparison of H chain CDRs from four mAbs specific for group 1 mite allergens (4C1, 10B9, 5H8, and anti-Der f 1 6A8) showed the highest variability in length and sequence for CDR3 (Table V). In agreement with our observations, H chain CDR3s have been reported to be the most variable in length, sequence, and structure (21, 22). However, most of the residues interacting with the H chain CDR3 from the mAb 4C1 are also present in Der f 1, but are not sufficient for binding the mAb 10B9. The creation of a new mAb 10B9 binding site in Der f 1 proved that additional hydrogen bonds involving other CDRs (CDR1 and CDR2 from H chain and CDR2 and CDR3 from L chain) were required for mAb 10B9 binding. These results are in line with recent observations that H chain CDR3 diversity is not required for Ag recognition by synthetic Abs (23).

Mapping of binding sites for human IgE and IgG Abs on Der p 1 has been performed previously either by immunoassays using recombinant allergen fragments or by phage display technology (24–27). In the early 1990s, the use of small peptides limited the first approach to the identification of mostly linear epitopes, such as residues 155–187 (25). Nevertheless, three regions (60–80, 81–94, and 101–111) strongly bound IgE and appeared to be close in space in the folded molecule, most likely comprising a conformational IgE Ab binding site. In another study, peptides with more than 30 aa were required for consistent Ab binding, which is in agreement with the importance of conformational epitopes for IgE Ab binding to Der p 1 (24). Later on, phage display technology had been used to identify mimotopes from phage peptide libraries screened with Abs specific for Der p 1 (26, 27). Szalai et al. (26) identified five mimotopes in Der p 1 corresponding to a single patch in conserved areas between Der p 1 and Der f 1. This patch includes a few of the residues that form the cross-reactive mAb 4C1 epitope. Furmonaviciene et al. (27) found a Der p 1 sequence (Leu¹⁴⁷–Glu¹⁶⁰) that also comprises part of this epitope. Some of the sequences reported by these studies contain residues that were identified here as IgE Ab binding sites (i.e., Arg¹⁵⁶) by combining high resolution x-ray crystallography with detailed site-directed mutagenesis analysis. This strategy allowed the precise identification of only a few (3 to 4) amino acids that are important for allergen-Ab recognition while considering the three-dimensional

structure of the allergen that forms conformational epitopes. The analysis of antigenic determinants performed here for Der p 1 and Der f 1, and previously for the cockroach allergen Bla g 2, can also be applied to other major mite allergens such as Der p 2 and the recently reported Der p 23 for which the three-dimensional structures are known (28–31). The information generated provides a basis for the design of allergen mutants showing reduced IgE Ab binding with potential for future use in immunotherapy.

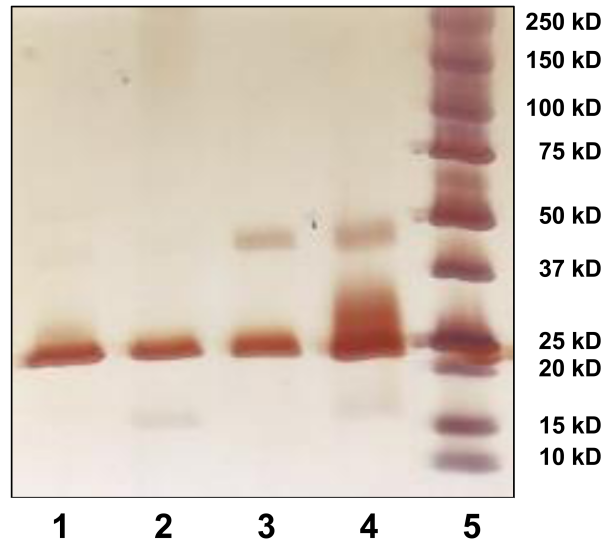
Disclosures

M.D.C. is co-owner of Indoor Biotechnologies Inc., and J.G., L.D.V., and A.P. are employees of Indoor Biotechnologies Inc. The other authors have no financial conflicts of interest.

References

- Platts-Mills, T. A., D. Vervloet, W. R. Thomas, R. C. Aalberse, and M. D. Chapman. 1997. Indoor allergens and asthma: report of the third international workshop. *J. Allergy Clin. Immunol.* 100: S2–S24.
- Arlian, L. G., M. S. Morgan, and J. S. Neal. 2002. Dust mite allergens: ecology and distribution. *Curr. Allergy Asthma Rep.* 2: 401–411.
- Arlian, L. G., J. S. Neal, M. S. Morgan, D. L. Vyszenski-Moher, C. M. Rapp, and A. K. Alexander. 2001. Reducing relative humidity is a practical way to control dust mites and their allergens in homes in temperate climates. *J. Allergy Clin. Immunol.* 107: 99–104.
- Tovey, E. R., M. D. Chapman, and T. A. Platts-Mills. 1981. Mite faeces are a major source of house dust allergens. *Nature* 289: 592–593.
- Chruszcz, M., M. D. Chapman, L. D. Vailes, E. A. Stura, J. M. Saint-Remy, W. Minor, and A. Pomés. 2009. Crystal structures of mite allergens Der f 1 and Der p 1 reveal differences in surface-exposed residues that may influence antibody binding. *J. Mol. Biol.* 386: 520–530.
- Chapman, M. D., W. M. Sutherland, and T. A. Platts-Mills. 1984. Recognition of two *Dermatophagoides pteronyssinus*-specific epitopes on antigen P1 by using monoclonal antibodies: binding to each epitope can be inhibited by serum from dust mite-allergic patients. *J. Immunol.* 133: 2488–2495.
- Chapman, M. D., P. W. Heymann, and T. A. Platts-Mills. 1987. Epitope mapping of two major inhalant allergens, Der p 1 and Der f 1, from mites of the genus *Dermatophagoides*. *J. Immunol.* 139: 1479–1484.
- Heymann, P. W., M. D. Chapman, and T. A. Platts-Mills. 1986. Antigen Der f 1 from the dust mite *Dermatophagoides farinae*: structural comparison with Der p 1 from *Dermatophagoides pteronyssinus* and epitope specificity of murine IgG and human IgE antibodies. *J. Immunol.* 137: 2841–2847.
- Chruszcz, M., A. Pomés, J. Glesner, L. D. Vailes, T. Osinski, P. J. Porebski, K. A. Majorek, P. W. Heymann, T. A. Platts-Mills, W. Minor, and M. D. Chapman. 2012. Molecular determinants for antibody binding on group 1 house dust mite allergens. *J. Biol. Chem.* 287: 7388–7398.
- Osinski, T., A. Pomés, K. A. Majorek, J. Glesner, L. R. Offermann, L. D. Vailes, M. D. Chapman, W. Minor, and M. Chruszcz. 2015. Structural analysis of Der p 1-antibody complexes and comparison with complexes of proteins or peptides with monoclonal antibodies. *J. Immunol.* 195: 307–316.

11. DeLano, W. L. 2002. *The PyMol Molecular Graphics System*. DeLano Scientific, San Carlos, CA.
12. Lawrence, M. C., and P. M. Colman. 1993. Shape complementarity at protein/protein interfaces. *J. Mol. Biol.* 234: 946–950.
13. Collaborative Computational Project, Number 4. 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 50: 760–763.
14. King, E. M., L. D. Vailes, A. Tsay, S. M. Satinover, and M. D. Chapman. 2007. Simultaneous detection of total and allergen-specific IgE by using purified allergens in a fluorescent multiplex array. *J. Allergy Clin. Immunol.* 120: 1126–1131.
15. Emsley, P., and K. Cowtan. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60: 2126–2132.
16. Garcia, K. C., M. Degano, L. R. Pease, M. Huang, P. A. Peterson, L. Teyton, and I. A. Wilson. 1998. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science* 279: 1166–1172.
17. Lombardero, M., P. W. Heymann, T. A. Platts-Mills, J. W. Fox, and M. D. Chapman. 1990. Conformational stability of B cell epitopes on group I and group II *Dermaphagoides* spp. allergens. Effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J. Immunol.* 144: 1353–1360.
18. Fleury, D., R. S. Daniels, J. J. Skehel, M. Knossow, and T. Bizebard. 2000. Structural evidence for recognition of a single epitope by two distinct antibodies. *Proteins* 40: 572–578.
19. Malby, R. L., W. R. Tulip, V. R. Harley, J. L. McKimm-Breschkin, W. G. Laver, R. G. Webster, and P. M. Colman. 1994. The structure of a complex between the NC10 antibody and influenza virus neuraminidase and comparison with the overlapping binding site of the NC41 antibody. *Structure* 2: 733–746.
20. Bostrom, J., S. F. Yu, D. Kan, B. A. Appleton, C. V. Lee, K. Billeci, W. Man, F. Peale, S. Ross, C. Wiesmann, and G. Fuh. 2009. Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site. *Science* 323: 1610–1614.
21. Xu, J. L., and M. M. Davis. 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13: 37–45.
22. Morea, V., A. Tramontano, M. Rustici, C. Chothia, and A. M. Lesk. 1998. Conformations of the third hypervariable region in the VH domain of immunoglobulins. *J. Mol. Biol.* 275: 269–294.
23. Persson, H., W. Ye, A. Wernimont, J. J. Adams, A. Koide, S. Koide, R. Lam, and S. S. Sidhu. 2013. CDR-H3 diversity is not required for antigen recognition by synthetic antibodies. *J. Mol. Biol.* 425: 803–811.
24. Greene, W. K., J. G. Cyster, K. Y. Chua, R. M. O'Brien, and W. R. Thomas. 1991. IgE and IgG binding of peptides expressed from fragments of cDNA encoding the major house dust mite allergen Der p 1. *J. Immunol.* 147: 3768–3773.
25. Greene, W. K., and W. R. Thomas. 1992. IgE binding structures of the major house dust mite allergen Der p 1. *Mol. Immunol.* 29: 257–262.
26. Szalai, K., J. Fuhrmann, T. Pavkov, M. Scheidl, J. Wallmann, K. H. Brämwig, S. Vrtala, O. Scheiner, W. Keller, J. M. Saint-Remy, et al. 2008. Mimotopes identify conformational B-cell epitopes on the two major house dust mite allergens Der p 1 and Der p 2. *Mol. Immunol.* 45: 1308–1317.
27. Furmonaviciene, R., P. J. Tighe, M. R. Clark, H. F. Sewell, and F. Shakib. 1999. The use of phage-peptide libraries to define the epitope specificity of a mouse monoclonal anti-Der p 1 antibody representative of a major component of the human immunoglobulin E anti-Der p 1 response. *Clin. Exp. Allergy* 29: 1563–1571.
28. Woodfolk, J. A., J. Glesner, P. W. Wright, C. L. Kepley, M. Li, M. Himly, L. M. Muehling, A. Gustchina, A. Wlodawer, M. D. Chapman, and A. Pomés. 2016. Antigenic determinants of the bilobal cockroach allergen Bla g 2. *J. Biol. Chem.* 291: 2288–2301.
29. Mueller, G. A., D. C. Benjamin, and G. S. Rule. 1998. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry* 37: 12707–12714.
30. Derewenda, U., J. Li, Z. Derewenda, Z. Dauter, G. A. Mueller, G. S. Rule, and D. C. Benjamin. 2002. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. *J. Mol. Biol.* 318: 189–197.
31. Mueller, G. A., T. A. Randall, J. Glesner, L. C. Pedersen, L. Perera, L. L. Edwards, E. F. DeRose, M. D. Chapman, R. E. London, and A. Pomés. 2016. Serological, genomic and structural analyses of the major mite allergen Der p 23. *Clin. Exp. Allergy* 46: 365–376.



Supplemental Figure S1. Silver stained SDS-PAGE gel showing recombinant Der p 1 wild type (1), multiple epitope mutants rDer p 1-4C1-MM (2) and rDer p 1-5H8-MM (3), and natural Der p 1 (4) (purified in the same way as the allergen that was used to determine the structure of native Der p 1 by X-ray crystallography, which demonstrated correct folding of the allergen). All allergens run at the expected MW (24 kDa) with the occasional formation of 48 kDa dimers at certain concentrations. A larger smear in the natural allergen is most likely due to glycosylation. Molecular weight markers are in lane 5.