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Mapping human monoclonal IgE epitopes on the major dust mite allergen Der p 2

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Abstract

IgE antibodies drive the symptoms of allergic disease upon crosslinking allergens on mast cells or basophils. If the IgE binding sites on the allergens could be identified, it may be useful for creating new forms of immunotherapy. However, direct knowledge of the human IgE epitopes is limited due to the very low frequency of IgE-producing B cells in blood. A new hybridoma technology using human B cells from house dust mite allergic patients was used to identify four Der p 2-specific human IgE monoclonal antibodies (hIgE mAbs). Their relative binding sites were assessed and compared by immunoassays with three previously studied murine IgG (mIgG) mAbs. Immunoassays showed that the recognition of Der p 2 by the first three hIgE was inhibited by a single mIgG, but the fourth hIgE recognized a different epitope from all the other mAbs. The functional ability of the hIgE that bind different epitopes to crosslink Der p 2 was demonstrated in a mouse model of passive systemic anaphylaxis. NMR analyses of Der p 2 in complex with IgG and IgE antibodies were used to identify specific residues in the epitopes. The combination of immunoassays to distinguish overlapping epitopes and NMR analyses to identify specific residues involved in Ab binding provided the first epitope mapping of human IgE monoclonal antibodies to an allergen. The technologies developed here will be useful in high resolution mapping of human epitopes on other antigens, and the design of improved therapeutics.

Keywords

IgE; Allergen; NMR; Epitope

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Introduction

IgE antibodies are produced by susceptible individuals upon allergen exposure and are key to the symptoms of allergic disease (1, 2). Information about the epitopes recognized by IgE would be useful for designing new molecules for immunotherapy. However, very little is known about IgE antibody binding sites, their biochemical features and their distribution on the allergen molecular surface. Antibodies from different isotypes have the potential to interact with any surface-exposed element of any given antigen (3), but evidence suggests that differences in epitopes exist among isotypes. For example, early epitope mapping studies showed that linear epitopes of synthetic peptides were recognized differently by IgE and IgG (4–9). Recently, different proteins from grass pollen were also reported to be targeted by IgE versus IgG (10). A possible mechanism for the observation that IgE and IgG epitopes are different has been suggested by next generation sequencing studies that found fewer mutations in germ line IgE antibody sequences from allergic individuals than in IgG (11). The differences in isotype may be due to factors related the location where the B cell matures (12). It is also possible that the differences in isotype are driven by the molecular or chemical features of epitopes (13, 14). For example, the analysis of a well characterized epitope recognized by an IgE derived from a combinatorial library suggested that IgE epitopes are potentially more planar than other known epitopes (15). The distribution of IgE epitopes on an allergen may also vary among patients, but some allergens have epitopes clustered in certain areas. For example, a single mAb can block 80% of IgE binding to Phl p 2 (16). All of these studies suggest that a better understanding of the IgE response and localization of epitopes is needed, which would be useful in designing therapies that either seek to block the symptomatic IgE, or modify the allergen for immunotherapy (17–19).

The main reason for the limited knowledge of IgE epitopes is the challenge of cloning IgE antibodies due to the extremely rare B cells harboring IgE genetic information. Instead of being able to study human monoclonal IgE, indirect evidence of human IgE epitopes was originally obtained by site-directed mutagenesis (20, 21) or the analysis of IgE binding to synthetic or recombinant peptides (22). Later on, approaches that take into account the conformational nature of most IgE epitopes on aeroallergens were developed: murine IgG (mIgG) mAbs that inhibit IgE antibody binding have been used as surrogates for IgE in X-ray crystallography studies of allergen-antibody complexes (23). Phage display libraries have also been used to simulate human-like IgE constructs that allowed either an indirect identification of epitopes by immunoassays (24) or directly determined the structure of two allergen-IgE Fab complexes (15, 25). These surrogate technologies have provided valuable information, but further *in vivo* studies would be desirable.

More recently, there has been a return to the challenge of cloning human B cells producing IgE from allergic patients. Anti-peanut IgE antibodies were cloned from patients by selecting for anti-Ara h 2 B cells followed by single-cell RNA sequencing (26). The low frequency of B cells in blood is still an important limiting step for this approach. A novel strategy to clone IgE was followed in the current study, to isolate human IgE monoclonal antibodies by hybridoma technology. This approach was first used to isolate IgE specific for *Aspergillus* allergens (27). The advantage is that the isolated IgE clones contain the natural pairing of the heavy and light chains, as it occurs *in vivo*, which opens a new avenue for the

analysis of IgE epitopes. In this study, four IgE human monoclonal antibodies against Der p 2 were cloned from mite-allergic patients using this new hybridoma technology, the relative positions of their epitopes were analyzed by immunoassays, and residues involved in binding Der p 2 were identified by nuclear magnetic resonance (NMR).

Der p 2 was selected as the model system because of its relevance as a major allergen from the house dust mite *Dermatophagoides pteronyssinus*. A large proportion (≥79%) of mite allergic patients have specific IgE antibodies to this allergen (28, 29). In addition to the medical relevance there are many previous molecular studies of Der p 2, which can provide additional insight. The epitopes of several murine IgG antibodies to Der p 2 were previously studied by hydrogen exchange protection NMR, which identified a few residues protected by the antibodies in complex with the allergen (30, 31). Recently, the X-ray crystal structure of one of them, mAb 7A1, in complex with Der p 2 was determined, and IgE binding sites were identified by site-directed mutagenesis analysis (23). In this study, we capitalized on previous knowledge and utilized NMR studies of methyl labeled Der p 2 in complex with antibodies, both murine IgG and human IgE, to map epitopes. In combination with immunoassays, the information obtained using this approach gives some of the first site-specific information of the human IgE epitopes that will be used for designing molecules with reduced allergenic activity for immunotherapy.

Methods

Human hybridoma generation

IgE-secreting human hybridomas were generated using a methodology that was recently described (27) and is detailed below. Dust mite-specific IgE mAbs were created from dust mite allergic research subjects recruited from within the Vanderbilt University Medical Center. The protocol for recruiting and collecting blood samples from allergic subjects was approved by the Vanderbilt University Medical Center Institutional Review Board (IRB #141330 and 142030). All human study participants provided written informed consent. Diagnosis was based on clinical history and testing serum for the presence and quantity of IgE antibody to dust mite, specifically *Dermatophagoides farinae* and/or *Dermatophagoides pteronyssinus*.

Der p 2-specific IgE antibody levels were measured in human sera by using ImmunoCAPs (d203) in a Thermo Fisher Scientific ImmunoCAP system (Phadia 250 Immunoassay Analyzer; Thermo Fisher Scientific, Portage, Mich.).

IgE-secreting human hybridomas were generated from previously cryopreserved samples that were thawed, washed, and counted before plating. For every 2 million viable cells, the following was added: 30 ml of prefusion medium (ClonaCell-HY 03801; Stemcell Technologies), 20 μl of CpG stock (2.5 mg/ml; ODN 2006), 1 μl each of mouse anti-human kappa (Southern Biotech; 9230–01) and mouse anti-human lambda (Southern Biotech; 9180–01), and 1 million gamma-irradiated NIH3T3 fibroblast line genetically engineered to constitutively express cell-surface human CD154 (CD40 ligand), secreted human B cell activating factor (BAFF) and human IL-21 (provided by Dr. Deepta Bhattacharya; Washington University in St. Louis, St. Louis, MO). The mixture then was plated into 96-

well flat bottom culture plates at 300 μl/well, and incubated at 37°C with 5% CO_2 for 6 days, prior to screening for IgE secretion using an ELISA.

Omalizumab, which is specific for the unique constant domain of IgE, was used as a capture antibody, coating 384-well black ELISA plates at a concentration of 10 μg/ml. After blocking, 100 μl of supernatant was transferred from each well of the 96-well plates containing B cell lines, using a VIAFLO-384 electronic pipetting device (Integra Biosciences). Secondary antibody (mouse anti-human IgE Fc; Southern biotech, 9160–05) was applied at a 1:1,000 dilution in blocking solution using 25 μl/well. After 10 washes with PBS, fluorogenic peroxidase substrate solution (QuantaBlu; Thermo Scientific 15162) was added at 25 μl/well, as per manufacturer instructions. Relative fluorescence intensity was measured on a Molecular Devices plate reader. Wells were counted as positive if the relative fluorescence intensity was > 5 times background. IgE B cell frequencies then were expressed as the number of IgE positive wells per 10 million peripheral blood mononuclear cells.

HMMA2.5 nonsecreting myeloma cells (provided by Marshall Posner, Mount Sinai, New York, NY) were counted and suspended in cytofusion medium composed of 300 mM sorbitol, 1.0 mg/ml of bovine serum albumin, 0.1 mM calcium acetate, and 0.5 mM magnesium acetate. Cells from IgE positive wells were pipetted gently into microcentrifuge tubes containing 1 ml of cytofusion medium. B cells and HMMA2.5 cells were washed three times in cytofusion medium to ensure buffer equilibration. HMMA2.5 cells were then suspended in cytofusion medium to achieve a concentration of 10 million cells/ml. The HMMA2.5 cell suspension was added to each sample tube and the mixture pipetted into cuvettes (BTX, 450125). Cytofusion was performed using a BTX cuvette holder (BTX Safety stand, model 630B) with a BTX ECM 2001 generator (BTX; 45–0080) programed to run with following settings: a prefusion AC current of 70 V for 40 s, followed by a DC current pulse of 360 V for 0.04 ms and then a post-fusion AC current of 40 V for 9 s. After fusion the content of each cuvette was then added to 20 ml of hypoxanthine-aminopterin-thymidine (HAT) medium containing ouabain, composed of the following: 500 ml of post-fusion medium (Stemcell Technologies, 03805), one vial 50x HAT (Sigma, H0262), and 150 μl of a 1 mg/ml stock of ouabain (Sigma, 013K0750). Fusion products then were plated into 384-well plates and incubated for 14 days before screening hybridomas for IgE antibody production by ELISA.

Wells containing hybridomas producing IgE antibodies were cloned biologically by indexed single cell flow cytometric sorting into 384-well culture plates. Once clonality was achieved, each hybridoma was expanded in post-fusion medium in 75-cm^2 flasks. MAb was expressed by large-scale growth of the hybridoma in serum free medium (Gibco Hybridoma-SFM; Invitrogen, 12045084) in 225-cm^2 flasks. IgE antibody was then purified by immunoaffinity chromatography (Omalizumab covalently coupled to GE Healthcare NHS activated HiTRAP; 17-0717-01) and visualized by SDS-PAGE for purity.

The monoclonal antibodies used are annotated in Table 1.

Immunoassays to assess the relative positions of mAb epitopes on Der p 2

For the two-site immunoassays, a microplate was coated overnight with the murine IgG mAbs (7A1, 1D8 or αDpX, (32), Figure 1) or IgE mAbs (2G1, 1B8 or 2F10; Figure 3) at 10 μg/ml in 50 mM carbonate/bicarbonate buffer, pH 9.6. The plate was washed and blocked with phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 and 1% bovine serum albumin. To the wells coated with murine mAbs (Figure 1), natural Der p 2 (nDer p 2) was added at a concentration of 100 ng/ml, followed by a 1 h incubation and addition of biotinylated human IgE mAbs (1:1,000 and diluted 1:2 across the plate). To the plates coated with IgE mAbs (Figure 3), nDer p 2 was added at the concentration of 100 ng/ml and diluted 1:2 across the plate, followed by a 1 h incubation and addition of biotinylated human IgE mAbs (1:1,000) (same protocol for Figure S2, regardless of the IgG or IgE mAb used for coating). For both immunoassays, addition of Streptavidin Peroxidase (1:1000) (Sigma, St. Louis, MO) for 30 minutes followed, and the plates were then developed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 70 mM citrate phosphate buffer, pH 4.2 and 1:1,000 dilution of H_2O_2. Regarding the allergen, nDer p 2 was affinity purified from mite culture by affinity chromatography using 7A1; the purity was >95% by SDS-PAGE.

For dose-response inhibition experiments of IgE mAb binding to Der p 2, microplates were coated overnight with rDer p 2.0103 at 10 μg/ml, expressed in *Pichia pastoris* as previously described (23). The IgG mAbs were then added at 0.1, 1, 10, 100 μg/ml to the plate wells and incubated 1 h. The plate was washed and IgE mAb (1:500 – 1:50,000) added to wells and incubated 3 h. A 1 h incubation of mouse anti-human IgE Fc-HRP (Southern Biotech, Birmingham, AL) (1:1,000) followed and the plate was developed as above.

For additional inhibition experiments using sera or plasma from mite allergic patients, the maximum concentration of inhibitor (100 μg/ml of IgG) was selected. Microplates were coated overnight with nDer p 2 at 10 μg/ml. The IgG mAbs were then added at 100 μg/ml to the plate wells in duplicate and incubated with plasma from allergic patients (n = 8) at 1:2 dilution for 3 h. A 1 h incubation of mouse anti-human IgE Fc-HRP (Southern Biotech, Birmingham, AL) (1:1,000) followed and the plate was developed and absorbance was read as above.

NMR samples and data acquisition

Der p 2 and Der f 2 have been studied previously by NMR (31, 33–36). Allergen expression and purification followed the optimized protocol of Nakamura, with a few adjustments (37). Benzonase was added prior to sonication, and the insoluble fraction was incubated with 6 M guanidium chloride for 1 hour at room temperature with rapid stirring prior to dialysis and refolding. Assignments were determined in PBS buffer using standard NMR techniques with a [U-^{13}C,^{15}N] Der p 2 sample (29). For maximum sensitivity in a high molecular weight complex with antibodies, a Der p 2 [U-^{2}H,^{15}N, ILV ^{1}H,^{13}C-methyl] sample was made according to Goto et al, with the exception that ^{2}H-glycerol was used instead of ^{2}H-glucose as the primary carbon source (38). This expression protocol labels the Ile δ1 methyl, both methyls of Leu and Val, and otherwise per-deuterates the rest of the protein. For simplicity, we refer to this molecule as the 'methyl labeled Der p 2'.

Complexes were made by mixing 5–8 mg of antibody with an excess of methyl labeled Der p 2 and incubated for at least 30 minutes at room temperature before applying the sample to a 26–600 S200 superdex column flowing at 0.3 ml/min at 4°C with PBS. Three example purifications are overlaid in Figure S1 with an example gel separation of the components. The Der p 2 -mAb fractions (region 2 in Figure S1) were exchanged into ^{2}H-PBS and concentrated to 400 μl. Data was acquired in a 4 mm tube on a Varian 800 MHz spectrometer with a cryogenically cooled probe using the gChmqc pulse sequence, typically for 8–16 hours at 37°C. The structure of Der p 2 in all figures utilizes pdb file 1KTJ (39).

Human transgenic mouse anaphylaxis experiments

Human FcεRI transgenic mice (B6.Cg-*Fcer1a*^{tm1Knt} Tg(FCER1A) 1Bhk/J were purchased from The Jackson Laboratory (stock #010506), brought out of cryogenic storage, bred and genotyped. Mice were maintained under specific pathogen-free conditions and used in compliance with the revised 2011 “Guide for the care and use of laboratory animals” prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. These double mutant mice express the human Fc fragment of IgE, high affinity I, receptor for alpha polypeptide, (*FCER1A*), under the control of the human *FCER1A* promoter and carry the *FcεR1a*^{tm1Knt} targeted mutation (40). Mice that are hemizygous for the transgene and homozygous for the targeted deletion of the mouse FcεRI respond to experimental induction of anaphylaxis with human IgE. Transgenic mice were sensitized by IP injection with 100μg total purified IgE in PBS and challenged 3 days later by IP injection with 50μg of purified nDer p 2 protein in PBS. Temperature was monitored using implanted temperature probes. Data was combined from two separate experiments containing 12 mice each. The first experiment contained groups: 1B8 alone, 1B8 + 2G1, 2F10 + 1B8, and 2F10 + 2G1. The second experiment contained groups: 2F10 alone, 1B8 + 2G1, 2F10 + 1B8, and 2F10 + 2G1. The protocols for animal care and use for these experiments have been approved by the Institutional Animal Care and Use Committee (IACUC M1700156–00). All personnel are trained on the topic of responsible conduct of research and certified to conduct animal experiments by the institutional IACUC and a verterinarian. Temperatures of sensitized mice and 2F10 alone-sensitized mice were compared independently at each time point following Der p 2 challenge using ANOVA (MATLAB, Mathworks, Inc.). Error bars for the mouse temperature measures represent standard error of the mean.

Results

Isolation of human IgE mAbs

Two research subjects were recruited from Vanderbilt University Medical Center allergy clinic during a routine evaluation (Table 1). The first subject (P1) had eczema and rhinitis, while the second subject (P2) had asthma. Skin prick testing for both subjects demonstrated sensitization to dust mite, having 10–15 mm wheal reactions. The Der p 2-specific IgE antibody levels were 289.2 kU/L for subject P1 and 51.8 kU/L for subject P2. Further patient characteristics are given in Table 2. Peripheral blood mononuclear cells were isolated and cells grown as described previously (27). IgE encoding B cells were identified by screening culture supernatants for the presence of IgE antibody. IgE B cells frequencies and the

number of IgE producing B cells per 10 million PBMCs grown were calculated. Both subjects had nearly identical IgE B cell frequencies, subject P1 had 10 and P2 showed 12 IgE B cells for every 10 million PBMCs cultured. IgE-containing B cell cultures were then immortalized and human hybridomas created without secondary screening for IgE specificity. Once hybridomas were generated they were screened for binding to dust mite allergens. One hybridoma from subject P1 and three from P2 were found to be Der p 2-specific. Additional hybridomas with specificity to allergens of peanut and tree nuts were identified from P2 (data not shown).

Relative epitope mapping on Der p 2 by immunoassays

Three murine IgG mAbs 7A1, 1D8 and α-DpX were used as reference antibodies for epitope mapping of IgE mAbs because they have been reported: 1) to bind to non-overlapping epitopes (31, 32), and 2) to be able to inhibit polyclonal IgE from mite allergic patients (31), which indicates that they overlap with IgE antibodies. We confirmed that these three IgG mAbs 7A1, 1D8 and α-DpX inhibited binding of IgE antibodies from sera/plasma to Der p 2 up to 10.3 %, 23.2 and 36.8%, respectively (with high variability in the percentage of inhibition depending on the subject: average ± std deviation: 2.4 ± 4.6, 5.7 ± 11.2% and 16.7 ± 13.4%, respectively for n =8).

To perform a relative epitope mapping of the four identified human IgE mAbs, we investigated if these IgE mAbs overlapped with any of these three murine mAbs by two-site ELISA and inhibition assays. For the two-site ELISA, each of the murine mAb was used as coating antibody to capture Der p 2, followed by incubation with each of the IgE mAbs. The two-site ELISAs showed that three human IgE mAbs (2G1, 5D10 and 1B8) bound Der p 2 presented by mAb 1D8 and 7A1, but not αDpX (Figures 1 and S2A), which indicates that αDpX and the three IgE mAbs 2G1, 5D10 and 1B8 bind to overlapping epitopes. However, the IgE mAb 2F10 bound Der p 2 presented by each of the three coating murine IgG mAbs (1D8, 7A1 and αDpX) (Figure 1). This result shows that the epitope for mAb 2F10 differs from the mAb IgG epitopes. The differences in binding level of the biotinylated-IgE mAbs are most likely due to differences in the concentration of antibody used (concentrations of purified IgE mAbs before biotinylation ranged from 2.8–5.4 mg/ml), in combination with possible differences in antibody affinity (which was not measured).

To confirm the epitope overlap between the murine IgG mAb αDpX and three human IgEs observed in the two-site ELISAs, inhibition assays were performed (Figure 2). αDpX at 100 μg/ml inhibited 50–71% of the binding of IgE mAbs 2G1, 5D10 and 1B8, but not 2F10, to Der p 2. The murine mAbs 7A1 and 1D8 did not inhibit binding of these IgE mAbs, in a similar way as the negative control, the anti-Der p 1 mAb 4C1. None of the IgG mAbs showed a dose-response inhibition of IgE mAb 2F10 binding to Der p 2 (Figure 2D). Overall, these results showed the interference of murine IgG mAb αDpX with binding of human IgE mAbs 1B8, 2G1 and 5D10 to Der p 2, and confirmed the unique epitope of 2F10.

In addition, to assess relative epitope binding, hIgE mAbs were tested in pairs in two-site ELISAs, using combinations of a non-biotinylated IgE for coating with a biotinylated IgE for detection (Figures 3 and S2B). The three biotinylated IgE mAbs 2G1, 5D10 and 1B8 detected Der p 2 presented by coating IgE mAb 2F10 (Figure 3A). And *vice versa*,

biotinylated 2F10 detected Der p 2 presented by either the IgE mAb 2G1 or 1B8 (Figure 3B). Note that IgE mAb 5D10 and 1B8 are derived from the same patient, and likely represent somatic variants. No reactivity was observed for any mAb pair combination of 2G1, 5D10, and 1B8, which indicates that these three IgE mAbs bind to overlapping epitopes (Figures 3B and S2B).

In summary, recognition of Der p 2 by three hIgE (5D10, 1B8, and 2G1) was inhibited by αDpX, but IgE mAb 2F10 binds to a different site than the epitopes for all the other six mAbs.

NMR analyses of complexes and mapping epitopes to the structure

Previously, an analysis of the crystal structure and NMR data from Der p 2 in complex with an ScFv of 7A1 demonstrated that surface exposed methyl groups would provide the best signal to noise and most reliable epitope information (23). These probe residues were identified: I97, I28, I121, V40, V63, V81, V105, V116, L17, and L61. The analysis showed that the Der p 2 methyls responded to both remote conformational changes due to antibody complexation, and that resonances from residues immediately in the epitope disappeared. In more technical terms, the close proximity of methyls in the epitope to the large protonated antibody caused the resonances to broaden through a mechanism called cross-relaxation, thus making them hard to observe (41). Figures 4A and 4B show both of these effects when Der p 2 is bound to hIgE 2F10. The methyls of residues L61, V63, and V105 have broadened below detection, while I97 has shifted. Figure 4C shows that these residues are in close proximity on Der p 2. Using the knowledge that I97 is buried in the 7A1 epitope (23), and that 7A1 does not inhibit 2F10 binding to Der p 2 (Figure 2), the shift of I97 is suggested to be a distal conformational change due to the complex formation. Therefore, the 2F10 epitope is proposed to center around V105 as shown in Figure 4D. Figure 4G shows a combination Venn diagram and color legend for Figures 4E & 4F demonstrating that a few residues may overlap between the proposed 2F10 and 7A1 epitopes although this overlap is not functionally important.

Next, we analyzed the data for the complex of Der p 2 and αDPX, shown in Figure S3A&B. V81 showed the largest chemical shift changes, with a small change to V40, and possibly a new shift for I88, although this is speculative. Figure S3C shows how these residues are in close proximity on Der p 2. Combining this information with previous knowledge that disruption of the disulfide bond between C73 and C78 affects αDPX binding (32, 42, 43), and that residues A72, C73, and Y75 were protected by αDPX (as suggested by previous hydrogen exchange experiments; these residues were not detected in the current study since only Ile, Leu and Val were labelled) (31), Figure S3D shows the proposed αDPX epitope with the residues annotated in Figure S3E.

Der p 2 in complex with each of the hIgE mAbs 2G1, 5D10, or 1B8 all showed nearly identical chemical shift changes (Figure S4). There were small changes in the Ile residues, but a few notable changes in the Val, Leu region seen in Figure 5A: Similar to the αDPX complex, V81 and V40 showed shift changes but they were smaller and in different directions. The shifts of V116 were so far novel to the 5D10 family of antibodies. There are possibly new shifts for the adjacent L117 that are subtly different for these three antibodies.

Since αDPX inhibits these hIgE there is likely significant overlap in the epitopes, but since the shift of 116 is different from the αDPX NMR data, the IgE epitope is proposed to be further from V40 and closer to V116; see figure 5C. The epitope overlap is shown on figure 5D&E while figure 5F again provides an explicit Venn diagram of the residues in the epitopes and is a color-coded legend for Figure 5.

The final antibody complex explored was Der p 2 with the mIgG 1D8. There were numerous chemical shift changes scattered throughout Der p 2 (Figure S5) so additional data are necessary to help interpret the NMR shifts. 1D8 belongs to a family of antibodies including 6D6 and 2B12 that have overlapping epitopes. 1D8 can differentiate Der p 2 isoforms via residue differences at position 114 (33, 44, 45). Hydrogen exchange protection also suggested residue 111 and 116 were in the 6D6 epitope, which is a related antibody to 1D8. Similarly, another antibody in this family, 2B12, has a reduced affinity for mutations at residues 45–48 (32, 43). Therefore, we suggest the chemical shift changes and broadening of 116 represent changes explicitly in the epitope region. Next, 1D8 partially inhibited the 5D10 family of IgE, indicating likely some epitope overlap reflected in the shift changes to V81, which are however at different chemical shifts from the previous antibody complexes. These differences in the V81 complex shifts likely indicate that 1D8 is not binding to the same epitope as 5D10/2G1/1B8 or αDPX. Finally, the shift changes to I121 are unique to 1D8 versus all the other antibodies studied. Looking at the available data, it is likely that the 1D8 epitope spreads out from V116 to I121 and partially overlaps with the 5D10 family of epitopes near N44 (Figure S4C&D). This suggests that 1D8 might partially overlap with the 2F10 epitope, although functionally the two antibodies do not inhibit each other.

Figure 6 summarizes all the proposed epitopes in this study, with a Venn diagram indicating the overlap of residues involved in each. The combination of immunoassays and NMR data identified specific residues of Der p 2 that were influenced by antibody binding, and confirmed the overlap of human IgE mAb 5D10, 1B8, and 2G1 and the murine IgG mAb αDPX. NMR data also identified different residues influenced by binding of IgG murine mAbs 1D8 and 7A1 and the human IgE mAb 2F10, consistent with three functionally non-overlapping epitopes. Dashed lines highlight the proposed human IgE epitopes for 5D10/1B8/2G1 and 2F10 in Figure 6 A&B. The methodology described herein can be directly applied to other allergen epitopes, and more generally to other antigen-antibody interactions.

Human transgenic mouse model of anaphylaxis

The *in vitro* immunoassays and NMR data support that the four human IgE mAbs recognize two separate epitopes defined by 5D10/1B8/2G1 and 2F10 mAbs. Theoretically, antibodies recognizing non-overlapping epitopes are sufficient to initiate allergic symptoms when they induce cross-linking of the high affinity IgE receptor (FcεRI) by binding the allergen. To functionally test this hypothesis *in vivo*, a mouse model of passive systemic anaphylaxis (PSA) was created to test antibodies that bind these two epitopes. The model tested whether FcεRI was cross-linked by allergen and thus whether the purified human IgE passively transferred to the mice were capable of functioning alone and/or together. Mice were sensitized using the purified human IgE mAbs specific to Der p 2 three days prior to

challenge with 50 μg of purified natural Der p 2 protein. Anaphylaxis was measured by the decrease in body temperature using implanted temperature probes. Figure 7 shows that the hIgE mAbs which bind the predicted same antigenic site (1B8 & 2G1) do not induce a temperature decrease while IgE mAbs which bind different antigenic sites (2F10+2G1, or 2F10+1B8) exhibited significant anaphylaxis. The temperature data from the different groups were compared by ANOVA using a p value of less than 0.05 to assess significance. From 15–35 minutes after challenge, both the 2F10+1B8 and 2F10+2G1 sensitized mice were significantly lower in temperature from the other three conditions, indicating anaphylaxis. After 35 minutes, the 2F10+2G1 mice continued to be significantly lower until 85 minutes. The decrease in temperature displayed by the IgE groups 2F10+2G1 and 2F10+1B8 reflects systemic anaphylactic reactions which could have only occurred through their ability to simultaneously bind Der p 2 and cross-link FcεRI.

Discussion

Defining allergenic epitopes recognized by human IgE antibodies has long been a goal of allergen research which could ultimately lead to innovations in allergy therapeutics. This study presents the first epitope mapping of four human monoclonal IgE antibodies to Der p 2. This is one of the most important allergens associated with mite allergy, and the development of diseases such as atopic dermatitis and asthma (46). The isolation of human IgE mAbs was possible thanks to the recent development of human IgE monoclonal antibody (mAb) technology (27). Sequencing of the DNA encoding IgE from individual human B cells has the advantage that the expressed antibodies have a natural heavy and light chain pairing. In addition, the isolation of monoclonal antibodies allows for studying clone-specific epitopes, which is not possible using polyclonal IgE from allergic subjects. This approach is a major advance in the antibody field given the low frequency of IgE-producing B cells in blood. One of the limitations of the agnostic approach used in our hybridoma technology is that the specific B cell type encoding the IgE antibody cannot be defined. The Croote et al study that described IgE sequences against Ara h 2 found most of the B cells were plasmablasts (26). However, plasmablasts do not grow in the B cell culture system used in our hybridoma methodology.

The human IgE mAbs cloned from 2 individuals are inferred to be relevant to the IgE response in other patients from the following information. The epitopes of three of the hIgE coming from 2 different patients (5D10, 1B8 and 2G1, Table 1) overlapped with mIgG αDpX, which inhibited up to 37% of IgE antibody binding to Der p 2 in 8 individually tested sera (33). Similarly, a previous study reported a maximum inhibition of IgE antibody binding of 10–50% for 1D8, 7A1, and αDPX for 2 individuals and pooled sera from 7 mite allergic patients (31). This indicates the relevance of these regions to the patient IgE response in general. The overlap of the three human IgE mAb epitopes could indicate that the area recognized by these antibodies is immunodominant for Der p 2. Two of the IgE mAb (1B8 and 5D10) belonged to the same patient and turned out to have nearly identical heavy and light chain variable gene segment rearrangements. A recent study of IgG antibodies generated during peanut oral immunotherapy demonstrated that three different patients generated antibodies against Ara h 2 with very similar sequences (47). This

suggested convergent selection by B cells and likely indicated that a specific epitope of Ara h 2 was being targeted, as seems to be the case for this Der p 2 area.

The fourth human IgE mAb bound to a site different from epitopes for the other 3 IgE and 2 IgG mAb. The identification of the separate epitope was confirmed with a mouse model of passive systemic anaphylaxis. The decrease in temperature displayed by the mice given IgE groups 2F10+2G1 and 2F10+1B8 reflects significant systemic anaphylactic reactions which could have only occurred through their ability to cross-link FcεRI. This shows that the structural and immunoassay data are able to predict which antibodies are capable of functioning together to activate mediator release and which are not. Because 1B8 and 2G1 bind the same site, they would be predicted to not have the ability to cross-link FcεRI in the presence of allergen.

The addition of the NMR strategy used here allowed the direct observation of conformational changes in specific methyl groups in the allergen when bound to the antibodies. A similar strategy has been used previously in smaller Fab-antigen complexes (23, 48). Previously, detection of ^{15}N labeled allergens (Art v 1 and Bet v 1) in complex with polyclonal IgE was attempted, but the relaxation of ^{15}N is much faster, leading to poorer signal to noise, hence, the changes were difficult to discern (49, 50). Additionally, the ^{15}N data in those studies was acquired with an excess of free allergen, which likely dominated the spectra (51). Methyl labeling and per-deuteration offers superior sensitivity for NMR detection of the allergen-antibody complex, which is in a molecular weight range that is typically difficult to observe by NMR. This is a tradeoff between fewer observables (only Ile, Val, and Leu methyl resonances) with easier detection versus more probes (typically ^{15}N backbone atoms) at significantly lower sensitivity. With fewer observables, it is worth commenting that the proposed epitopes herein likely include more residues than observed in crystal structure epitopes. Therefore, these regions should be considered as the framework for further experiments.

The data is likely to be useful in the design of improved therapeutics, including hypoallergens that specifically target these human epitopes. The recent study of an X-ray crystal structure of Der p 2 in complex with IgG mAb 7A1 has provided a model for how knowledge of residues involved in IgE antibody binding led to the expression of modified allergens with reduced IgE antibody binding capacity (23). The modification of only one IgE epitope is not expected to be sufficient to significantly reduce mediator release activity, when other epitopes are involved in the full IgE response (52). The current study extends epitope mapping to the identification of 2 additional human IgE binding sites that will be further explored to create hypoallergens. Other studies have already proposed mutations of Der p 2 that might be useful for immunotherapy (42–44, 53, 54). Reginald and Chew studied 21 single alanine mutations of Der p 2 and concluded that 5 mutations substantially affected serum IgE binding: N10, E25, K77, K96 and E102 (55). Of these, N10 is in the 1D8 predicted epitope, K77 is in the 5D10/1B8/2G1 predicted epitope, K96 is in the 7A1 epitope, and E102 is adjacent to the 2F10 epitope. This suggests again that the monoclonal antibodies studied here are relevant to the patient IgE repertoire of allergic subjects. Thus, these IgE mAbs are representative of the human polyclonal IgE antibody response. The information

gained from the NMR epitope mapping analysis should be useful for the design of hypoallergens for immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

- 4 human Der p 2-specific monoclonal IgE were found with a new hybridoma technology.
- The human IgE recognize 2 non-overlapping epitopes on Der p 2.
- NMR identified residues in the epitopes of human IgE and murine IgG antibodies.

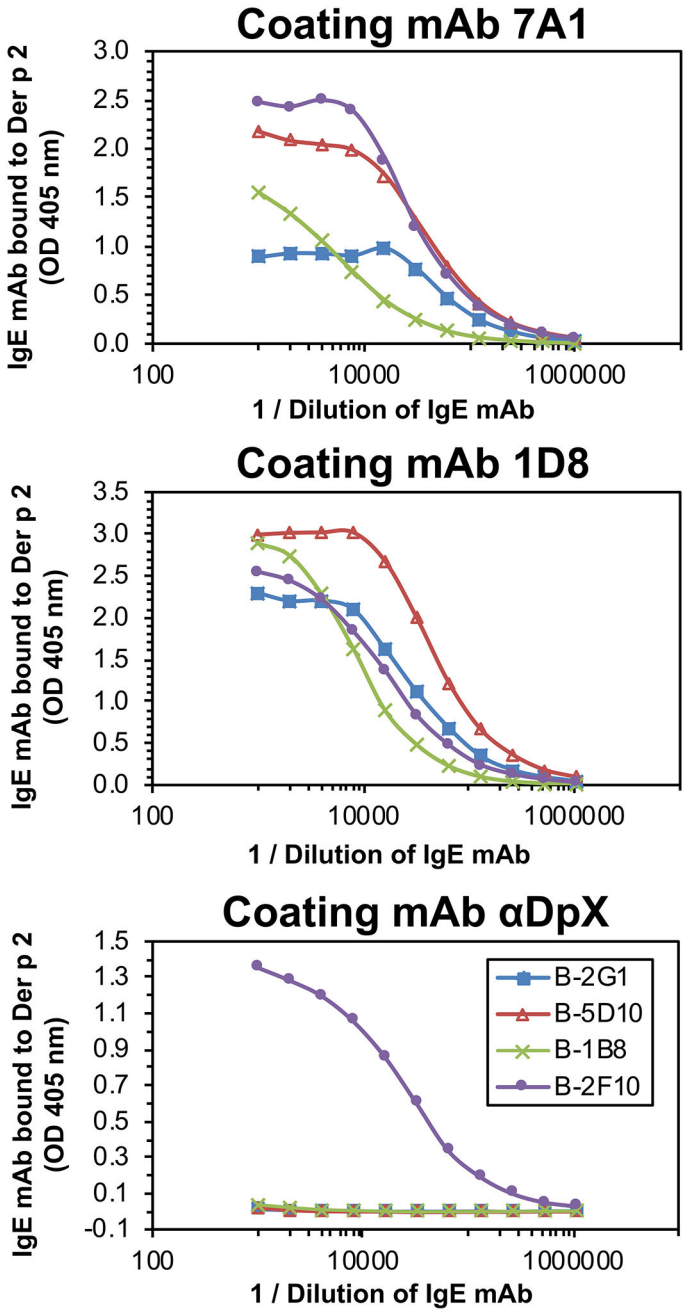

Figure 1.

Dose response curves of human IgE mAb binding to Der p 2 (rDer p 2.0103) presented by murine IgG mAbs coating the plate (7A1, 1D8 and αDpX from top to bottom). The two-site ELISA with all the different antibody combinations were done in duplicate with single data points, and a representative experiment is shown (see also Figure S2).

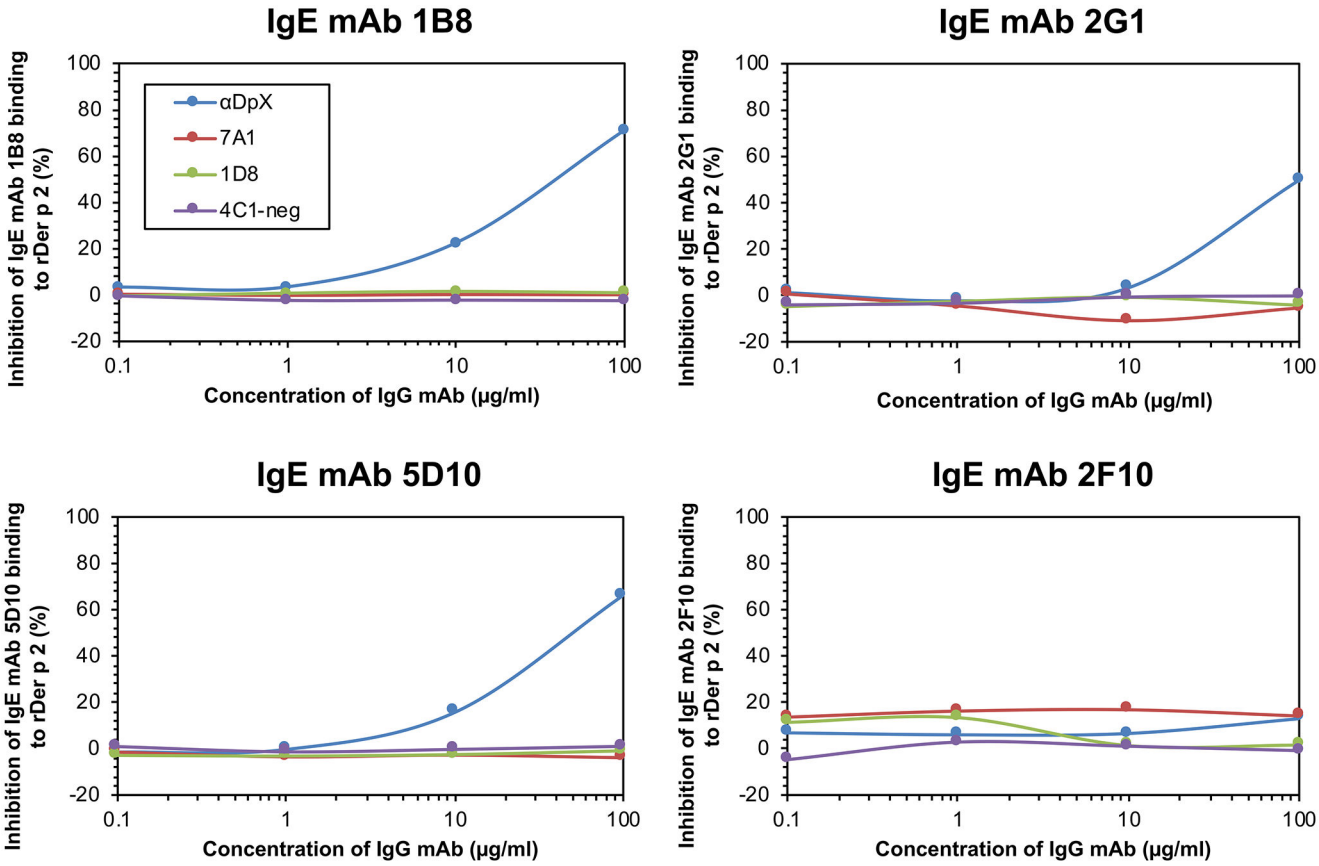

Figure 2.

Immunoassays showing inhibition of human IgE mAb binding to Der p 2 by murine IgG mAbs (αDpX in blue, 7A1 in red and 1D8 in green). The Der p 1 mAb 4C1 (purple) served as a negative control. Each plot is representative of 2–3 experiments, where data was obtained in single (shown) or duplicate. Human IgE mAb are 1B8, 2G1, 5D10 and 2F10.

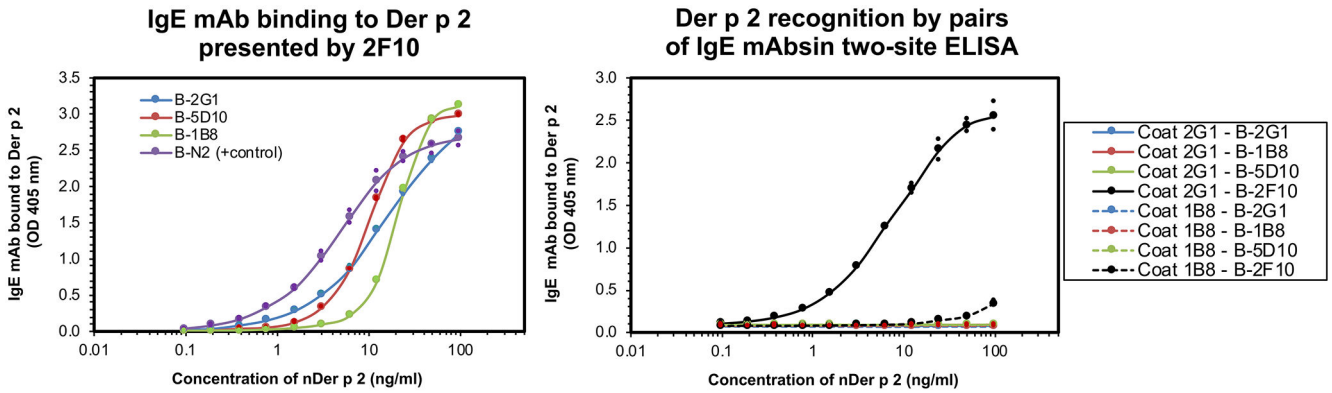

Figure 3.

Dose response curves of IgE mAb pairs: **Left)** Three biotinylated IgE mAbs (2G1, 5D10 and 1B8) detected Der p 2 presented by coating IgE mAb 2F10. Plot is representative of two experiments. Data are average (in the curve) of two duplicates that are shown as smaller symbols around the average. **Right)** Dose-response curves of each of the four biotinylated IgE mAbs binding to Der p 2 presented by either coating 2G1 or 1B8 IgE mAbs. Epitopes of 3 IgE mAbs (2G1, 5D10 and 1B8) overlapped, but mAb 2F10 bound to a different site. Plot is representative of 2–3 experiments and data are average (in the curve) of two duplicates that are shown as smaller symbols around the average.

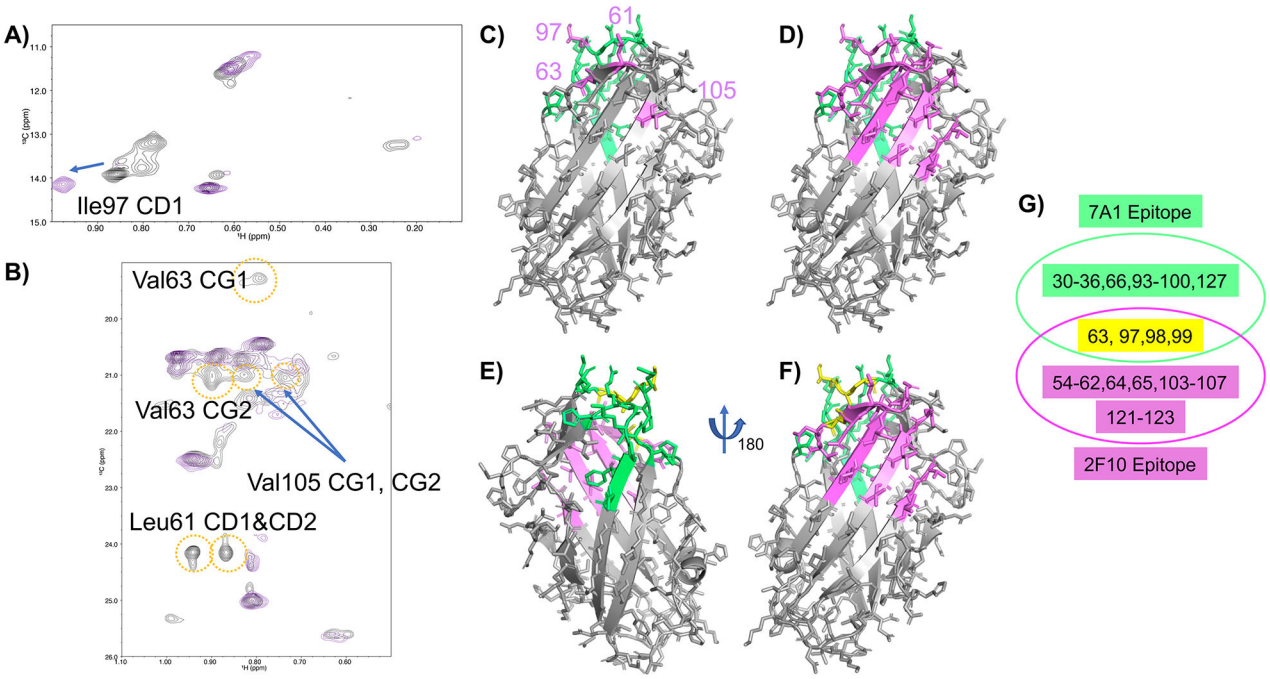

Figure 4.

Interactions of Der p 2 with hIgE 2F10 and mIgG 7A1. Panels A) and B) show NMR ^{1}H-^{13}C HMQC data of methyl labeled Der p 2 alone (black contours) and Der p 2 in complex with 2F10 (purple contours). A) is the Ile region and B) is Val and Leu region. Arrows indicate shifts and dotted circles indicate resonances absent in the complex. C) Der p 2. The 7A1 epitope is colored pale green, as previously identified (23). Residues identified in the 2F10 interactions are indicated in violet. D) Adjacent residues proposed to be in the 2F10 epitope are colored violet. Panels E) and F) show the structure of Der p 2 rotated 180 degrees on the y axis and colored as shown in G) to indicate the epitopes of 7A1 and 2F10.

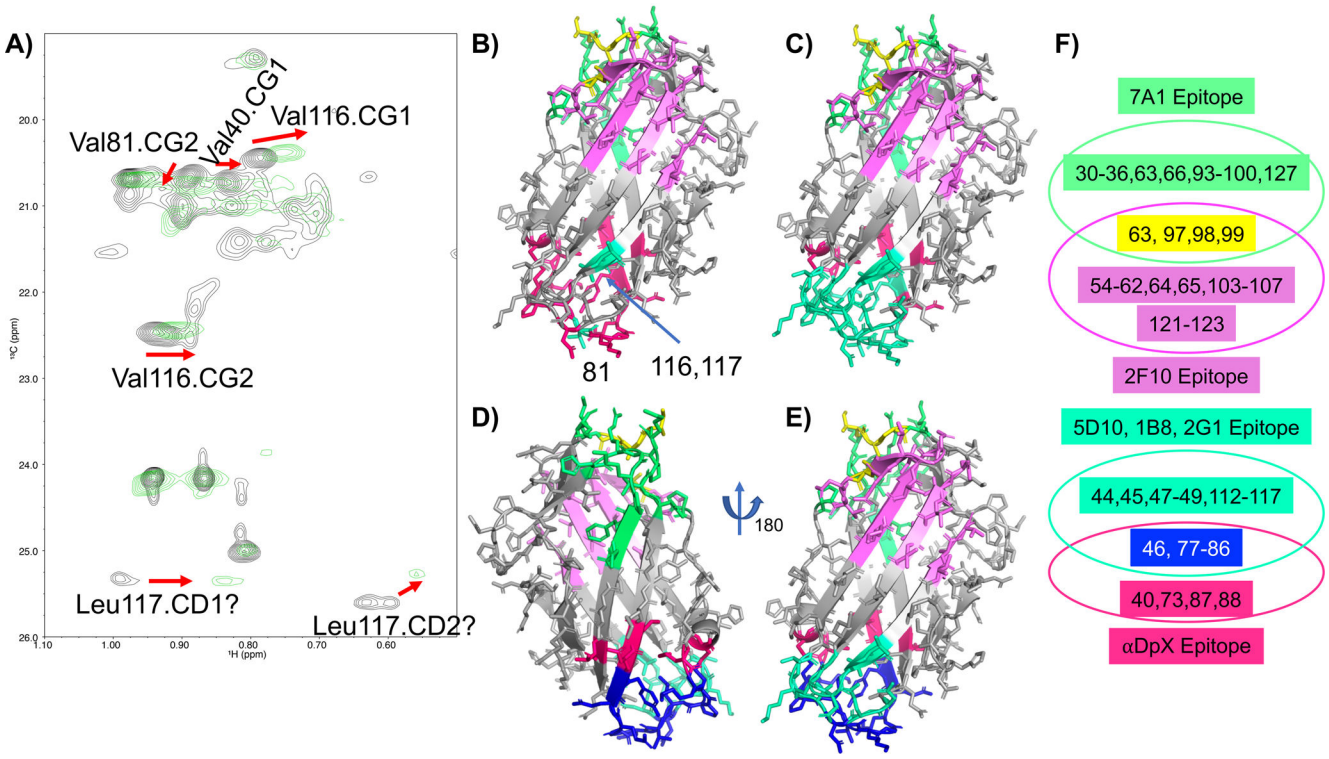

Figure 5.

Interactions of Der p 2 with hIgE 5D10 and related 2G1 and 1B8. Panel A) shows NMR ^{1}H-^{13}C HMQC data of methyl labeled Der p 2 alone (black contours) and Der p 2 in complex with 5D10 (green contours) in the Val and Leu region. Annotations indicate shifts in the complex. Question marks indicate uncertain assignments. B) Der p 2. Residues identified in the 5D10 interactions are indicated in greencyan. C) Adjacent residues proposed to be in the 5D10 epitope are colored greencyan. Panels D) and E) show the structure of Der p 2 rotated 180 degrees on the y axis and colored as shown in F) to indicate the epitopes analyzed herein.

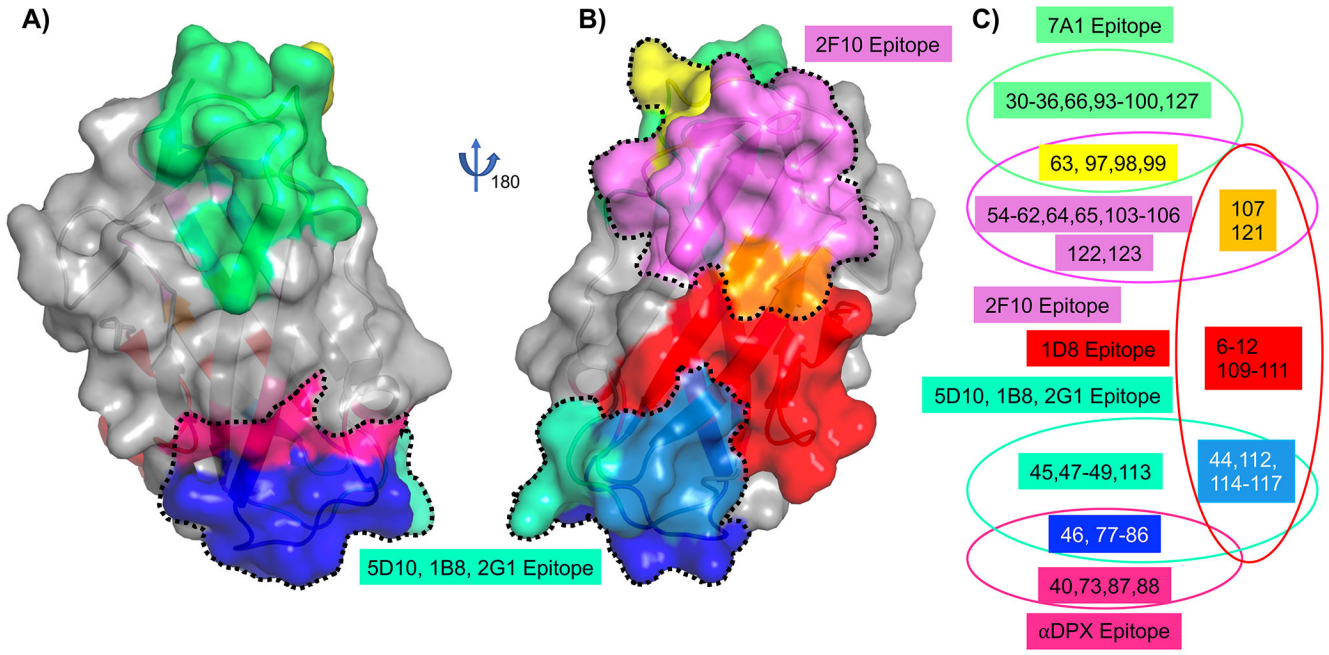

Figure 6.

Epitope mapping summary. Panels A) and B) show Der p 2 rendered as a semi-transparent surface and ribbon diagram. Panel C is a Venn diagram of the residues in the epitopes studied herein. The coloring of the diagram is consistent with the structure rendering. Dashed lines highlight the proposed human IgE epitopes of 5D10/1B8/2G1 and 2F10.

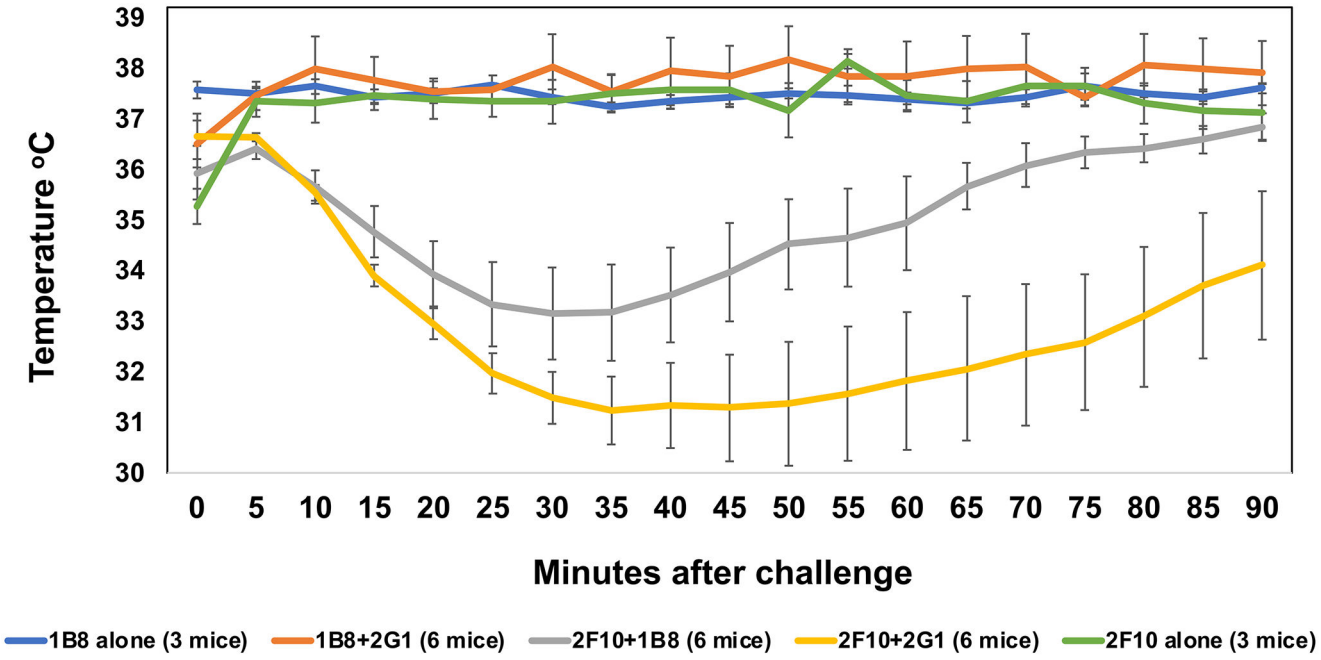

Figure 7.

Human FcεRI transgenic mouse anaphylaxis. Mice were sensitized using purified human IgE mAbs (see legend) specific to Der p 2 three days prior to challenge with 50 μg of purified natural Der p 2 protein. Anaphylaxis is measured as drop in body temperature using implanted temperature probes. Error bars represent the standard error in the mean.

Table 1:

Origin and Isotype of Antibodies

Name	Origin	Isotype	Patient #
2G1	Human	IgE	P1
5D10	Human	IgE	P2
1B8	Human	IgE	P2
2F10	Human	IgE	P2
7A1	Murine	IgG	-
1D8	Murine	IgG	-
αDpX	Murine	IgG	-

Table 2.

Subject allergy history, B cell frequency, and hybridoma yield.

Subject code	Age	Sex	Allergic disease	Total serum IgE (kU IgE/L)	Dust mite, *D. pteronyssinus* SPT (mm wheal)	Dust mite, *D. farinae* SPT (mm wheal)	IgE B cell frequency (per 10^7 PBMCs)	Dust mite-specific IgE hybridomas generated
P1	8	M	FA, Eczema, Rhinitis	ND	10×10	10×10	10	**1**
P2	18	M	FA, Asthma	677	10×8	15×10	12	**3**

Subject age, total serum IgE, and dust mite skin prick testing results are shown. IgE B cell frequencies are expressed as the number of IgE positive cells per 10 million peripheral blood mononuclear cells. Subjects' allergy histories are listed in descending order from most to the least reactive, P1: dust mite, fish, peanut, cashew, egg; P2: peanut, walnut, dust mite, cockroach. The total dust mite-specific IgE expressing human hybridomas generated for each subject is listed. FA, food allergy; SPT = skin prick test; ND = not determined.