Hydrogen Exchange Nuclear Magnetic Resonance Spectroscopy Mapping of Antibody Epitopes on the House Dust Mite Allergen Der p 2*

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New strategies for allergen-specific immunotherapy have focused on reducing IgE reactivity of purified recombinant allergens while maintaining T-cell epitopes. Previously, we showed that disrupting the disulfide bonds of the major house dust mite allergen Der p 2 resulted in 10–100-fold less skin test reactivity in mite-allergic subjects but did not change in vitro T-cell proliferative responses. To provide a more complete picture of the antigenic surface of Der p 2, we report here the identification of three epitopes using hydrogen protection nuclear magnetic resonance spectroscopy. The epitopes are defined by monoclonal antibodies that are able to inhibit IgE antibody binding to the allergen. Each monoclonal antibody affected the amide exchange rate of 2–3 continuous residues in different regions of Der p 2. Based on these data, a number of other residues were predicted to belong to each epitope, and this prediction was tested for monoclonal antibody 7A1 by generating alanine point mutants. The results indicate that only a small number of residues within the predicted epitope are functionally important for antibody binding. The molecular definition of these three epitopes will enable us to target limited positions for mutagenesis and to expand our studies of hypoallergenic variants for immunotherapy.

Epidemiologic studies suggest that between 10 and 20% of the world population exhibits some form of IgE-mediated hypersensitivity, which is manifested as asthma, atopic dermatitis, or allergic rhinitis (1). A number of studies have shown that sensitivity to house dust mite allergens is the most important risk factor for asthma (1, 2). More than 10 mite allergens have been defined, and the 14-kDa Group 2 allergens (Der p 21 and Der p 22) are considered major allergens because of the fact that 80–90% of patients have specific IgE Ab to these allergens (3).

Previously we reported that Der p 2 is structurally a member of the immunoglobulin superfamily, although the function of the allergen remains unknown (4). Therapy for allergic disease includes allergen avoidance, pharmacotherapy, and allergen-specific immunotherapy. Recently, new strategies for immunotherapy have been proposed with the aim of improving efficacy, patient compliance, and associated risks (5). Our studies have focused on the generation of hypoallergenic variants; the underlying hypothesis is that reducing IgE reactivity will reduce IgE-mediated side effects (6). The mapping of epitopes on Der p 2 and Der f 2 is an important step toward the development of hypoallergenic variants. Using murine mAb and sera from mite-allergic subjects, we have shown that the epitopes on the Group 2 allergens are conformational and that the three disulfide bonds stabilize this structure (6, 7). Mutational analysis of surface residues found that substitution of threonine for lysine at position 100 had reduced avidity for mAb 7A1, and mutations of residues 44–46 affected the avidity of a second mAb, aDpX (8). A third mAb, 6D6, belongs to a group of mAb that recognize the different naturally occurring isof orm s of Der p 2 at residue 114‡ (9, 10). Although the above studies have been informative, they provide an incomplete map of the epitopes and do not necessarily identify those residues on Der p 2 that contribute significantly to the binding energy.

Epitopes can also be mapped by measuring changes in amide hydrogen exchange rates of the antigen that occur as a result of the formation of an immune complex (11–13). These rates can be conveniently measured using NMR techniques. This method was first used by Patterson et al. (11) to localize the epitope of an anti-lysochrome c mAb. We have subsequently measured similar effects in a number of anti-lysozyme antibodies (13). In general, those residues that are in the structural epitope (those that either contact the Ab or are buried by it) show the largest reduction in exchange rates.

Once the location of the epitope has been obtained using amide exchange measurements the residues, which are important for mAb binding, can be systematically identified using scanning alanine mutagenesis (14–16). The mutation to alanine was generally validated by determining the effect on the rate of amide hydrogen exchange using NMR. These methods have also been applied to the study of the Dengue virus structural proteins (17) and to the study of influenza virus structures (18).

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nine reduces the side chain to as small as possible without substantially altering the secondary structure. Usually, glycine and proline are not altered, because both of these residues strongly influence the conformational entropy of the peptide chain. This approach has been used to map B-cell epitopes in a number of different model systems. Jin et al. (14) studied 43 different mutants of human growth hormone in combination with 21 different monoclonal antibodies. A study by Dall’Acqua et al. (15) introduced alanine mutations into both the antibody and antigen (lysozyme) and reported the structure of a complex between the antibody and one of the lysozyme mutants. Benjamin and Perdue (16) characterized the interaction of 70 mutants of staphylococcal nuclease with 10 different mAb. All of these studies found that an average of 3 to 4 residues were energetically important to mAb binding. In this study, a residue was classified as being energetically or functionally important if the free energy of binding of the mutant to mAb binding was 1.0 kcal/mol or greater. The studies of growth hormone and staphylococcal nuclease examined virtually the entire surface of the respective proteins, and the study of lysozyme involved residues known to be in contact with Ab from crystallographic studies. In this study, the hydrogen exchange data were used to provide a starting point for a more focal analysis of the epitopes on Der p 2.

EXPERIMENTAL PROCEDURES

Production of rDer p 2—Recombinant Der p 2 (rDer p 2) was expressed and purified from Escherichia coli cultures as previously described (9). Briefly, the protein was recovered from the insoluble fraction of the cell sonicate and resolubilized with 6 M guanidine (one-fifth the original cell culture volume), and after dialysis against buffer (10 mM Tris (Tris base), 1 mM EDTA, pH 8.5), the protein was purified by mAb affinity chromatography. 15N-Labeled protein was obtained by growing the bacteria on a minimal medium with 15N-ammonium sulfate as the sole nitrogen source.

Monoclonal Antibodies—The murine mAb used in this study were produced at the University of Virginia Lymphocyte Culture Center and have been described in detail elsewhere (17). The mAb 7A1, dAbX, and 6D6 were selected (9). The mAb 6A6 and 7A1 were used in this study. The mAb used in this study were produced by Dr. Rob Aalberse and colleagues (18). Radioimmunoassay and ELISA have shown that this panel of 5 mAb defines three antigenic regions on Der p 2 (17), and the mAb 7A1, dAbX, and 6D6 were selected as representative of these regions. For this study, mAb were purified from ascites fluid by precipitation with (NH4)2SO4 followed by affinity chromatography. 15N-Labeled protein was obtained by growing the bacteria on a minimal medium with 15N-ammonium sulfate as the sole nitrogen source.

Monoclonal Ab Inhibition of IgE Binding to rDer p 2—Increasing concentrations of mAb were used to inhibit IgE binding to rDer p 2 in a modified enzyme immunoassay. The antigen was bound directly to the microtiter plate or presented by mAb dAbX. Sera were added along with increasing concentrations of mAb so that the final concentration of serum was 1:4 or 1:8, and the concentration of mAb ranged from 0.01 to 100 μg/ml. IgE binding was detected using biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD) conjugated to horseradish peroxidase (9, 24). The entire coding sequence of each mutant was sequenced to ensure that no unintended changes were introduced. Because a polymerase chain reaction-based method was used to create the mutations, the entire coding sequence of each mutant was sequenced to ensure that no unintended changes were introduced. Competitive Inhibition ELISA—The ability of rDer p 2 and the various alanine mutants to inhibit mAb binding to solid phase rDer p 2 was measured using a goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) conjugated to horseradish peroxidase (9, 24). The results were calculated as the percentage inhibition using the value of maximum inhibition by rDer p 2 as 100% inhibition. The curves were fit to a sigmoidal function method (SPSS Science, Chicago, IL) to determine the concentration of 50% inhibition (IC50). Relative binding constants were calculated, according to Equation 1, as follows.

\[ K_a = IC_{50} / C_{\text{human}} \]  

(Eq. 1)

The change in free energy of the binding reaction as a result of the mutation was calculated from Equation 2 (13),

\[ \Delta G = -RT \ln K_a \]  

(Eq. 2)

where the standard error in the determination of the ΔΔG is ~0.1 kcal/mol. Consequently, a ΔΔG greater than 0.5 kcal/mol was considered significant.

RESULTS

Effect of mAb on IgE Ab Binding—The mAb used in this study have been characterized by a variety of techniques including inhibition radioimmunoassay and ELISA and have been shown to define three antigenic regions on Der p 2 (17). To demonstrate that these antigenic regions also represent IgE binding regions, we determined the ability of each mAb to inhibit the binding of IgE from allergic sera. Fig. 1, panel A shows the control experiment using the murine-human chimeric IgM mAb 2C8 that contains the 2B12 variable region. The data in panel A clearly show that inhibition of binding of the 2B12-IgE hybrid occurs only when the inhibiting mAb bind to...
the same antigenic region as does the 2B12 mAb itself. For example, only mAbs 1D8, 6D6, 4G7, and 2B12, which were previously shown by classical competitive inhibition studies to bind to the same antigenic regions on Der p 2 (17), are capable of inhibiting the binding of the 2B12-IgE chimeric mAb in a dose-dependent manner. In contrast, mAb 7A1 and aDpX, each of which binds to one of two other nonoverlapping regions, do not inhibit. These results are in complete agreement with a more detailed study on the competitive inhibition of these and other mAb for binding to Der p 2 (17).

Panels B and C show the ability of mAb to competitively inhibit binding of IgE in sera from two house dust mite-allergic subjects, B. R., and M. W. The inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds), aDpX (open squares), and an isotype matched control mAb 10A6 (dashed line). Panels B and C show the inhibition of serum IgE binding from two mite-allergic subjects, B. R., and M. W. The inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds). Panel D shows inhibition of pooled sera IgE Ab by 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), and aDpX (open squares). Values plotted in panels B, C, and D have been corrected for background binding, determined using the mAb 10A6, which was ~5%.

**Amide Exchange Experiments**—Fig. 2 shows sections of HSQC spectra of rDer p 2 for experiments conducted with mAb 7A1. Peaks for residues 73, 94, 97, and 101 are shown. The panels show spectra acquired in H2O (left), after 48 h in D2O (center), and after 48 h in D2O of a complex of Der p 2 and mAb 7A1 (right). These spectra clearly show that residues 94 and 101 were strongly protected from amide proton exchange while complexed with mAb. In contrast residue 97 was weakly protected, and residue 73 was not protected at all. A similar analysis of spectra obtained for rDer p 2 complexed with mAb aDpX and mAb 6D6 (data not shown) showed that residues 72, 73, and 75 were protected by mAb aDpX, and residues 111 and 116 were protected by mAb 6D6. The protected residues are highlighted on the structure of rDer p 2 in Fig. 3. Also displayed in Fig. 3 are the accessible surfaces of those residues that were predicted to be involved in the full structural epitope.

**Fig. 1. mAb inhibition of IgE binding to Der p 2.** Increasing concentrations of mAb were used to inhibit IgE Ab binding to rDer p 2 presented by mAb aDpX in an ELISA. Panel A shows the inhibition of the mouse-human chimeric IgE mAb (containing the mAb 2B12 combining site). Inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds), aDpX (open squares), and an isotype matched control mAb 10A6 (dashed line). Panels B and C show the inhibition of serum IgE binding from two mite-allergic subjects, B. R., and M. W. The inhibitor mAb were 2B12 (open circles), 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), 6D6 (filled diamonds). Panel D shows inhibition of pooled sera IgE Ab by 1D8 (filled circles), 7A1 (filled squares), 4G7 (filled triangles), and aDpX (open squares). Values plotted in panels B, C, and D have been corrected for background binding, determined using the mAb 10A6, which was ~5%.

**Fig. 2. Representative exchange data for mAb 7A1.** Portions of the HSQC spectra of rDer p 2 containing resonances from residues 73, 94, 97, 101 (panel A), 97 (panel B), and 94 (panel C) are shown. The left column of spectra were obtained for a sample in H2O. The middle column of spectra were obtained from a sample of Der p 2 in D2O. The right column (Protected) of spectra were obtained from a sample that was bound to mAb 7A1 for 48 h in the presence of D2O.

**Fig. 3. Epitopes of mAb 7A1, mAb aDpX, and mAb 6D6.** Residues that were found to have altered exchange rates are mapped on the structure of Der p 2. For each epitope those residues that were found to be protected from amide-proton exchange by mAb are colored red. The molecular surface for the residues predicted to be within each epitope is displayed with cyan-colored dots. The residues that form the aDpX epitope reside primarily on the upper β-sheet whereas those that form the 6D6 epitope reside primarily on the bottom β-sheet.
Functionally important residues in the 7A1 epitope. A, space-filling model showing the residues (white) protected by the 7A1 mAb during the NMR amide-proton protection assay. B, the residues important for mAb 7A1 binding are mapped onto a surface representation of Der p 2. The view is looking down the end of the β-barrel where mAb 7A1 is proposed to interact. The color scheme is as follows: red, residues that when mutated to alanine significant affect the binding of Der p 2 to mAb 7A1. White, Lys-97, was the only residue protected in the amide-protection assay, which, when mutated, significantly affected binding. Green, residues that when mutated to alanine did not affect antibody binding. Blue, residues that were not mutated.

The amino acid residues predicted to be in the epitope for each mAb are listed in Table I.

**Table I**

| Amino acid residues of Der p 2 within predicted epitopes | mAb 7A1 | mAb aDpX | mAb 6D6 |
|--------------------------------------------------------|---------|---------|---------|
| 18–20                                                  | 47–52   | 7–13    |
| 27–37                                                  | 68–81   | 41–55   |
| 56–63                                                  | 84–91   | 70–73   |
| 92–106                                                 | 110–112 | 76, 80  |
| 123–129                                                | 107–120 |         |

**Table II**

| Mutants of Der p 2 and the change in ΔΔG of binding mAb 7A1 | Residue | IC\text{50} | ΔG | Native/mutant | kcal/mol |
|-------------------------------------------------------------|---------|--------------|----|---------------|---------|
|                                                             |         | Native       |    | Mutant        |         |
| His-30                                                      | 2.36    | 5.64         | 0.42| 0.52^         |         |
| Arg-31                                                      | 2.36    | 30.00        | 0.08| 1.51^         |         |
| Lys-33                                                      | 2.36    | 10.82        | 0.22| 0.93^         |         |
| Ser-57                                                      | 2.39    | 10.65        | 0.23| 0.88^         |         |
| Asp-59                                                      | 2.63    | 2.17         | 1.21| −0.11         |         |
| Leu-61                                                      | 2.63    | 2.66         | 0.99| 0.01          |         |
| Val-63                                                      | 2.75    | 3.93         | 0.70| 0.21          |         |
| Asn-93                                                      | 1.80    | 11.04        | 0.16| 1.07          |         |
| Lys-96                                                      | 2.75    | 13.51        | 0.20| 0.94^         |         |
| Ile-97                                                      | 2.61    | 12.79        | 0.20| 0.94^         |         |
| Lys-100                                                     | 2.47    | 2.71         | 0.91| 0.06          |         |
| Ser-101                                                     | 2.47    | 2.83         | 0.87| 0.05          |         |
| Glu-102                                                     | 2.75    | 6.43         | 0.43| 0.50^         |         |
| Asn-103                                                     | 2.47    | 2.96         | 0.84| 0.11          |         |
| Val-105                                                     | 2.61    | 2.42         | 1.08| −0.05         |         |
| Thr-123                                                     | 2.12    | 2.10         | 1.01| −0.07         |         |
| His-124                                                     | 1.80    | 2.39         | 0.75| 0.17          |         |
| Lys-126                                                     | 1.80    | 2.53         | 0.71| 0.20          |         |
| Ile-127                                                     | 2.39    | 2.69         | 0.89| 0.07          |         |
| Arg-128                                                     | 2.39    | 4.29         | 0.56| 0.35          |         |
| Asp-129                                                     | 2.61    | 4.20         | 0.62| 0.28          |         |

* Mutants showing ΔΔG greater than 0.50 kcal/mol.

^ The mutant N93A failed to interact with the aDpX control mAb.

**DISCUSSION**

To date, no data exist describing IgE Ab binding sites for any allergen at the molecular level. The lack of allergen-specific
monoclonal IgE Ab precludes epitope mapping using crystallographic analysis of allergen-antibody complexes. The NMR methods used here and in other studies (11–13) have clearly shown that it is possible to obtain an approximate mapping of epitopes using this technique. The fidelity of the epitope map depends on several factors, the most significant being the ability to assign resonances and follow the exchange kinetics of these resonances. In the case of Der p 2 89 residues were assigned at pH 3.2. Of these, 31 residues exhibited exchange rates that were either too rapid or too slow to evaluate for protection. Although the NMR experiments were performed under conditions that minimized amide exchange (pH 3.2) the time required to prepare the sample and acquire the NMR spectra was significantly longer than the half-life of the amide proton. This time allowed potentially protected residues with very fast exchange rates in the free protein to exchange with the deuterated buffer. Additional residues were removed from the analysis if their amide hydrogens exchanged too slowly for a significant difference in protection to be seen with the incubation times used here.

Even in the context of these experimental constraints, the residues that were protected are in close proximity to other residues that have been previously implicated to influence the binding of that particular mAb. For mAb 7A1, this study showed that residues 101, 97, and 94 were protected whereas previous studies had shown that mutations of Lys-100 reduce the affinity of Der p 2 for mAb 7A1 (8). For the second mAb, αDpX, the protected residues were 72, 73, and 75. Residue Cys-73 forms a disulfide bond with Cys-78, and mutants that lacked this disulfide, as well as chemically reduced Der p 2, showed reduced affinity for αDpX and IgE Ab (6, 7). The final mAb examined was 6D6, which belongs to a group of mAb that recognizes different naturally occurring Der p 2 isoforms at position 114, either asparagine or aspartic acid (9, 10). The protected residues for mAb 6D6 were 111 and 116. Interestingly, mutation of Lys-100 to alanine had no effect whereas previous studies showed that mutation to threonine did affect 7A1 binding (8), although, in the latter study, mutation to arginine had no effect. Other studies also suggest that different mutations of the same residue can have varying effects on antibody binding (24, 25).

Because the protection data for all three mAbs correlate well with previous mutagenesis data it is likely that the protected residues are within the structural epitope. Finding only 2–3 residues with different exchange rates in Der p 2 is consistent with studies in other systems. For example, in a study of exchange rate differences for three mAb binding to lysozyme, 2–5 residues within the structural epitope for a given mAb were protected (12). Other studies that used this method to localize mAb epitopes generally compared exchange rates for free antigen versus antigen protected by mAb (11, 13). Based on those results, we chose to only measure the differences in amide exchange after 48 h to rapidly assess where the mAb might bind. This time was chosen, because it resulted in a large difference between the strongly protected residues that are contained in the epitope versus residues with different exchange rates due to long range conformational changes (12, 13). A more extensive examination of exchange rates with more time points may reveal other protected residues. Although the results shown in Fig. 1, panel A show no competitive inhibition (i.e. no outright blockage) of 2B12 binding by the 7A1 and αDpX mAb, it is still possible that the affinity of one mAb could be affected by the presence of another mAb, although there is no evidence for such in this study or others previously reported (17) in which reciprocal inhibition studies were conducted.

It is possible that binding of mAb to Der p 2 causes a conformational change in a region recognized by another mAb. Indeed, using three mAb specific for the antigen hen egg white lysozyme, we have shown that upon binding, conformational effects are observed at sites distal to the epitope (12, 13). However, these conformational effects are not sufficient to prevent binding of another mAb at the distal site. In that case and in the present study, if there are long range changes in amide protection, i.e. protected residues remote from the bound antibody that are within the epitope of another antibody, the affinity must be affected on the basis of thermodynamic arguments. However, it is impossible to predict the size or direction of the change from the current data, but they cannot be larger than the typical remote protection factors of about 1–2 kcal/mol (12, 13). Although we have not conducted experiments with Der p 2 similar to the lysozyme experiments, one would expect to see similar results, i.e. long range conformational effects. Nevertheless, also similar to the studies in the lysozyme-anti-lysozyme system, it is clear from the results shown in Fig. 1 (panel A) and from studies previously reported on mAb binding to Der p 2 (17) that the failure of the 7A1 and αDpX mAb to inhibit 2B12 binding cannot be accounted for by conformational effects. This strongly supports the conclusion that inhibition by
the other mAbs is due to binding at the same site as the 2B12 mAb. The same arguments apply directly to the data on mAb inhibition of binding of IgE from patient serum, whether it be from one patient or a pooled serum from several patients. Binding of a mAb inhibits the binding of any IgE that binds to the same, or overlapping, epitope(s). It is important to recognize that binding of a single mAb can significantly reduce total patient IgE binding even when the patient IgE can be expected to have maximum heterogeneity as in the case of the pooled sera.

To further characterize the epitopes localized by the exchange data, we utilized the known buried surface area of other protein-antibody complexes to predict which residues may be contained within epitopes on Der p 2. A potential difficulty associated with this method of identifying the structural epitope is illustrated for mAb aDpX. Previous mutagenesis studies implicated residues 44–46 in the binding of aDpX to Der p 2 (8). These residues are 15–19 Å from the protected residues and are thus well outside the predicted epitope. It is possible that the residues protected by mAb aDpX (amino acids 72, 73, and 75) are on the opposite side of the epitope from residues 44–46, and the true center of the epitope lies between these two clusters. A more likely possibility is that alteration of residues 44–46 resulted in significant changes in the conformation of Der p 2. In the original study (8) these residues were changed from NQN to HPP. The 44–46 HIPP mutant reacted poorly with both aDpX and 7A1, as well as with the other two mAbs studied. The epitopes for the aDpX and the 7A1 antibodies are shown here to be quite distant. This implies that the triple mutation likely affects the structure of Der p 2 and that residues 44–46 do not directly contact aDpX. An evaluation of the structure of the HPP mutant by another technique, such as NMR, would help resolve this issue.

Although all three of the mAb studied here will bind simultaneously to Der p 2, the predicted epitope for mAb aDpX overlaps considerably with the predicted epitope for mAb 6D6. Because both mAb can bind Der p 2 at the same time (17), the residues that are protected must not be central to the epitopes. This gives the impression that the mAb aDpX epitope is rotated away from the protected residues of mAb 6D6 or vice versa. In the case of lysozyme, two mAbs were shown to interact with the same residues but with different atoms, and the two mAb could simultaneously bind the antigen (26). These results provide the precedent that epitopes of mAb 6D6 and mAb aDpX could be closely associated and still simultaneously bind Der p 2.

To test the predicted epitope of mAb 7A1, single alanine mutants were generated, and the contribution of their side chain to the free energy of mAb binding was determined. Alanine scanning mutagenesis has been used to examine the energetic contribution of side chains to antibody interactions for human growth hormone, Staphylococcal nuclease, and lysozyme (14–16). These studies found that the ΔΔG values ranged between 1 and 4.5 kcal/mol, but the majority of residues showed ΔΔG between 1.0 and 2.0 kcal/mol. Residues with ΔΔG values in the neighborhood of 1.0 kcal/mol or greater were considered to be functionally important. In each case, only 2 to 5 residues met this criterion for any given antibody. The energetically significant residues were found to be discontinuous with respect to the primary sequence. In our study a total of 7 residues showed changes in ΔΔG greater than 0.5 kcal/mol. Of these, 5 would be considered to be functionally important for mAb binding based on the criteria stated above. In studies of this type it is essential to show that no large changes have occurred in the structure because of the alanine mutation. In this study one mutant, K33A, was chosen for a more detailed structural analysis, because it showed a large difference in ΔΔG, and the chemical shift of its amide is resolved in NMR spectra. Although there are a few minor perturbations in the spectra, the NOE analysis indicated that no significant changes occurred because of the substitution of the alanine at position 33 in the uncomplexed Der p 2. In addition, the mutagenesis data reported here are confirmed by a recent report on the effect of mutagenesis of the Der f 2 allergen on binding of two mAb that see the same antigenic regions as do the 7A1 and aDpX (27).

There are two possible explanations for the observation that changing Asn-93 to an alanine significantly affects binding of both the 7A1 and aDpX mAb. First, Asn-93 may indeed have been recognized by both mAb although competitive inhibition showed that cross-inhibition did not occur. The crystal structures of two lysozyme-anti-lysozyme mAb complexes showed that the two mAb could bind to the same residue, albeit at different atoms, and yet not competitively inhibit (26). Second, we have shown that binding of mAb at one region can cause conformational effects elsewhere in the antigen molecule (12, 13). The side chain of Asn-93 forms two hydrogen bonds with the main chain nitrogen and oxygen of Val-94. These same two Val-94 atoms also form hydrogen bonds with main chain atoms of Lys-33 for which independent mutational evidence suggests it is a binding residue. Thus mutation of Asn-93 to alanine could indirectly affect 7A1 binding through Lys-33.

Considering the number of residues found to be protected from exchange, the number of residues found to be energetically important, and the distribution of these residues, Der p 2 and the mAbs that interact with it fit the general paradigm of protein-antibody interactions. The energetics of the interaction of Der p 2 with mAb 7A1 is similar to that found with other antigen-antibody complexes; only a small number of discontinuous residues contribute significantly, as individual amino acids, to the binding affinity. These results will provide insights into the molecular nature of the epitopes of this clinically important antigen and will serve as a basis for the generation of modified forms of Der p 2 for immunotherapy.

Of particular importance to the use of sequence variants in immunotherapy are our results comparing the reactivity of patient IgE with natural and rDer p 2 (6, 9). These studies showed that the IgE of some patients reacted with natural and with rDer p 2. Natural Der p 2 is a mixture of isoforms that differ at positions 40, 47, 127, and 114 (28). The rDer p 2 used in these studies corresponds to the rDer p 2.0101 isoform that carries aspartic acid at position 114. Subsequent experiments have clearly shown that native reactivity could be restored by changing position 114 of the rDer p 2 to asparagine, the amino acid found in the other Der p 2 isoforms. Taken together, these results strongly suggest that the patient sera that did not react with rDer p 2.0101 were specific for a single site that included position 114. In addition, the results presented here suggest that the patient IgE Ab recognized a limited number of epitopes on Der p 2, in that 50% of IgE reactivity could be inhibited by a single mAb (Fig. 1). Overall, these results suggest that Der p 2, as seen by at least a subset of patients, could be considered monovalent (a single epitope) or paucivalent (a few epitopes), rather than polyvalent. Thus, selected amino acid changes within each known epitope may produce a null allergen permitting its use in immunotherapy without fear of hyperreactivity because of preexisting IgE Ab. All changes required to produce this null allergen must be made on the same molecule.

If a separate variant allergen was produced for each epitope and a mixture of these variants used for immunotherapy, each variant in the mixture would possess all the other native epitopes, and although the overall concentration of each epitope would be reduced, the mixture would react with preex-
isting IgE with the risk of inducing hypersensitivity reactions during immunotherapy.

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