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Dominant Epitopes and Allergic Cross-Reactivity: Complex Formation Between a Fab Fragment of a Monoclonal Murine IgG Antibody and the Major Allergen from Birch Pollen Bet v 1

Osman Mirza,*† Anette Henriksen,‡ Henrik Ipsen,† Jørgen N. Larsen,† Margit Wissenbach,† Michael D. Spangfort,† and Michael Gajhede

The symptoms characteristic of allergic hypersensitivity are caused by the release of mediators, i.e., histamine, from effector cells such as basophils and mast cells. Allergens with more than one B cell epitope cross-link IgE Abs bound to high affinity FcεRI receptors on mast cell surfaces leading to aggregation and subsequent mediator release. Thus, allergen-Ab complexes play a crucial role in the cascade leading to the allergic response. We here report the structure of a 1:1 complex between the major birch pollen allergen Bet v 1 and the Fab fragment from a murine monoclonal IgG1 Ab, BV16, that has been solved to 2.9 Å resolution by x-ray diffraction. The mAb is shown to inhibit the binding of allergic patients’ IgE to Bet v 1, and the allergen-IgG complex may therefore serve as a model for the study of allergen-IgE interactions relevant in allergy. The size of the BV16 epitope is 931 Å² as defined by the Bet v 1 Ab interaction surface. Molecular interactions predicted to occur in the interface are likewise in agreement with earlier observations on Ag-Ab complexes. The epitope is formed by amino acids that are conserved among major allergens from related species within the Fagales order. In combination with a surprisingly high inhibitory capacity of BV16 with respect to allergic patients’ serum IgE binding to Bet v 1, these observations provide experimental support for the proposal of dominant IgE epitopes located in the conserved surface areas. This model will facilitate the development of new and safer vaccines for allergen immunotherapy in the form of mutated allergens.

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Ag. Because tree pollen-allergic patients experience sequential exposure to pollens from hazel, alder, and birch, in Scandinavia over a period of 4 mo, exposure to the conserved surface areas is higher than that of the variable surface areas. High affinity IgE Abs are therefore more likely to be directed to the conserved surface areas than to the variable areas.

We here report a more direct approach to B cell epitope mapping by structural determination of allergen-Ab complexes. This study represents the first analysis of an allergen-Fab complex crystal structure though many studies of Ag-Fab complexes have been analyzed. These include the complexes with HIV1 capsid protein P24 (11), lysozyme (12–15), other Fabs (16), neuraminidase (17–19), staphylococcal nucleases (20), a phosphocholine protein, (HPr) from Escherichia coli (21), and Lyme disease Ag outer surface protein A (22).

X-ray crystallography requires homogeneous reagents for growth of crystals and cannot be performed using polyclonal human serum IgE. In the present study, Fab fragments from monoclonal murine IgG1 (BV16) raised by immunization with purified Bet v 1 have been used as a model system, because monoclonal allergen-specific human IgE is difficult to obtain. The use of IgG instead of IgE in the study of Ag-Ab interaction is justified by the generally accepted mechanism of VDJ gene assembly during early B cell maturation (10), but the system studied remains a model.

The BV16 epitope maps to one of the conserved surface patches described earlier (3). Because BV16 partially inhibits the binding of human serum IgE to Bet v 1, the epitope defined by BV16 is partly or completely overlapping Bet v 1 epitopes recognized by human serum IgE originating from natural exposure. The structure of the Bet v 1-BV16 Fab complex presented here defines the structural basis of a major allergen epitope. The complex provides information about Ab specificity and cross-reactivity having important implications for the design of new and safer vaccines for allergen immunotherapy, e.g., based on reduction of IgE binding by site-directed mutagenesis.

Materials and Methods

Ags and Abs

Pollen extracts from alder (Alnus glutinosa), birch (Betula verrucosa), hazel (Corylus avellana), and hornbeam (Carpinus betulus) were prepared as described (23). Bet v 1 was purified from pollen extract by size exclusion, ion exchange and chelate chromatography as described (25). Recombinant Bet v 1 was expressed in E. coli and purified as previously described (24). Polyclonal anti Bet v 1 rabbit Abs were raised as described (23).

Monoclonal hybridoma Abs were derived from BALB/c mice described (i.p. with purified Bet v 1 in IFA. Spleen cells from responding mice were fused with X-63 Ag 8.6.5.3 myeloma cells. Hybridoma cell cultures were grown in DMEM containing 10% (v/v) FCS, and culture supernatants were analyzed by direct ELISA. Positive cultures were cloned and recloned by limiting dilution until all wells derived from single cells were positive. mAbs were produced in Ag-free media. The monoclonality, isotype, and concentration of Ab were determined by isoelectric focusing, immunodiffusion, and single radial immunodiffusion, respectively. Finally, mAb was purified and concentrated by affinity chromatography on protein A-Sepharose CL4B (Pharmacia) according to standard procedures.

Tree pollen-allergic patients’ serum pool was a mix of equal volumes of serum from 15 RAST class 2 or 3 birch pollen allergic individuals.

Cloning and sequencing of Fv genes
cDNA encoding heavy and light chain variable domains of murine mAb BV16 was amplified by PCR (25), cloned in pPCR2.1 (Invitrogen, San Diego, CA) and sequenced according to standard procedures.

Immunoblotting

SDS-PAGE was performed as described (37) using 16% polyacrylamide gels. The gel was subsequently electroblottedted semidry to nitrocellulose, which was processed as described (37).

IgE inhibition assay

The ability of Ab BV16 to inhibit the binding of serum IgE to recombinant Bet v 1 was estimated in an IgE inhibition assay using a serum pool derived from birch-allergic patients. Serum IgE was captured by incubation with anti-IgE coupled to paramagnetic particles. After washing and resuspension in buffer, the Ab preparation tested was added followed by addition of biotinylated Bet v 1. The amount of biotinylated Bet v 1 bound to immobilized Bet v 1 was determined from the relative light units (RLU) measured after incubation with acidimic ester-labeled streptavidin. The degree of inhibition was calculated as (RLUbuffer – RLUbuffer/RLUbuffer) obtained using buffer and respective Abs as inhibitors.

Crystallization of Bet v 1-BV16 Fab complex

Fab fragments were generated by pepsin cleavage, and crystals of the Bet v 1-BV16 Fab complex were produced as described elsewhere (26).

Data collection.

Initially, a room temperature data set was collected. As can be seen from the data in Table I, only diffraction to 3.5 Å was obtained. Consequently, we tried to increase the diffraction limit by using synchrotron radiation and cryogenic techniques. In both cases, integration, reduction, and merging of the data were conducted using DENZO and SCALEPACK (27).

Room temperature experiment. Data were collected in-house using a Rigaku R-axis IIC image plate system with a Rigaku RU200 rotating anode (Rigaku, Tokyo, Japan). The system was equipped with a graphite mono- chromator and a 0.5-mm collimator. The crystal was rotated through 90 frames with an oscillation of 2 degrees, an exposure of 30 min/frame, and a crystal to detector distance of 110 mm. Only one crystal was used for data collection.

Cryogenic temperature experiment. Data were collected at 120K from a single crystal, at the MAX-lab synchrotron facility beamline I11 (University of Lund, Lund, Sweden). Because the precipitant solution was not suitable for cryoexperiments and the crystals were unstable in all of the tested cryoprotectants (glycerol, 2-methyl-2,4-pentanediol (MPD), glu- cose, isopropanol, PEG 600), 10% glycerol was added to the precipitant solution during crystal growth. This did not seem to have any effect on the crystallization of the complex, and significantly improved the crystal stability when subsequently transferring the crystals to a cryobuffer containing 30% glycerol and precipitant. Three data sets were collected from one crystal and subsequently merged together. Collecting data set 1, the crystal was rotated through 60 frames, with an oscillation of 3 degrees, 2 min of exposure per frame, and a crystal to detector distance of 300 mm. A number of overloads were recorded in the resolution region >3.5 Å; these reflections were then collected (data set 2) by rotating the crystal through 90 frames with 5 s exposure and 2 degrees oscillation per frame. To further increase the completeness, data set 3 was collected with 2.5 min of exposure, 2 degree oscillation per frame, and a crystal to detector distance of 200 mm. As can be seen from Table I, the cell dimensions are different at the two temperatures. Either the presence of the glycerol in the crySTALLI- zation solution has changed the packing in the crystal or the crystal has undergone a temperature-dependent phase transition.

Molecular replacement

Room temperature experiment. Molecular replacement was performed using AMoRe (28). The structure of Bet v 1 (3) and a model of the Fab fragment were used as search probes. Because only the sequence of the complementarity domain of the Fab fragment of BV16 was known at this time, the remaining part of the Fab fragment was described as alanines. The BV16 model was made by assigning coordinates from a known IgG Fab structure (1mlb (29)), to the BV16-Fab/ala sequence, using BIOSYM’S HO- MOLOGY program. There is a 2-residue insert in the CDR H3 loop of the BV16 relative to the model. In this region, a loop that did not introduce any unfavorable intermolecular contacts was built.

Rotation searches was first done with the Bet v 1, the constant, and the variable domains of BV16. This search gave two solutions. The searches were done in the resolution range 10–4 A. Searching for rotation solutions with the whole Fab fragment gave no results. The correlation coefficients were 20.8 and 16.9, significantly higher than the following peak at 10.4. Fixing one solution straightforwardly gave the relative translation of the other. Rigid body refinement of this model gave an R factor of 40% and a correlation coefficient of 64.

Cryogenic temperature experiment. The partially refined complex from the room temperature experiment was used as a search model. Rotation and translation searches were made in the region 8–4 Å. The rotation search

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Overall, the four complexes showed good density throughout the structure. The regions with weak electron density were loop 58–65 and loop 197–204 in the light chain, both showing poor side chain density. In the heavy chain, the loop 131–141 showed poor density, with density gaps in some light chain regions, and one complex (complex 3) showed weak density in one region (complex 1) with a significant contrast to the third peak at 15.9 Å.

The following is based on the cryogenic temperature data only, because none of these regions was at the Bet v 1-BV16 Fab interface.

### Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>Cryoexpt.</th>
<th>Room Temperature Expt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P1</td>
<td>P1</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>$a = 91.7$, $b = 99.1$, $c = 108.9$, $\alpha = 105.7$, $\beta = 98.3$, $\gamma = 97.6$</td>
<td>$a = 63.4$, $b = 84.1$, $c = 93.2$, $\alpha = 88.49$, $\beta = 76.27$, $\gamma = 94.0$</td>
</tr>
<tr>
<td>Maximum resolution (Å)</td>
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<td>3.5</td>
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<tr>
<td>Outer shell</td>
<td>3.00–2.9</td>
<td>3.5–3.45</td>
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<tr>
<td>No. of observations</td>
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<td>65,951</td>
</tr>
<tr>
<td>(Outer shell)</td>
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<td>(2,697)</td>
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<td>No. of unique observations</td>
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<td>(1,442)</td>
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<tr>
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<td>(83.8)</td>
<td>(94.2)</td>
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<td>0.132 (0.297)</td>
</tr>
<tr>
<td>$</td>
<td>|/</td>
<td>|$ in outer shell (%)</td>
</tr>
<tr>
<td>$R_{merge}$ (outer shell)</td>
<td>0.051 (0.268)</td>
<td>0.047 (0.376)</td>
</tr>
<tr>
<td>$</td>
<td>|/</td>
<td>|$ in outer shell (%)</td>
</tr>
</tbody>
</table>

### Model building and refinement

The following is based on the cryogenic temperature data only, because this data set had the highest resolution. The refinement of the structure was conducted using initially the X-PLOR 3.851 program (30), and in the later stages of refinement CNS (0.5 β Version) (31). The molecular replacement solutions from AMoRe were subjected to rigid body refinement, refining all 12 polypeptide chains as individual groups. The program o was used for manual fitting (32). A combination of torsion angle and cartesian dynamics including minimization and simulated annealing procedures were applied using the Engh-Huber parameters (33); 5% of the data was used for cross-validation. For the following rounds of refinement, strict noncrystallographic symmetry (ncs) was used. Torsion angle slowcool protocol runs (starting from 3000 K) and grouped B factor refinement (one B factor per residue) followed. After convergence of the $R_{merge}$, manual electron density fitting was performed followed by positional refinement and restrained individual B factor refinement. The $R_{merge}$ now converged at 35%. Inspection of the electron density revealed that two of the complexes returned good density overall, whereas one complex (complex 3) showed weak density in the outer shell.
the main chain. Attempts to find alternative conformation with omit maps did not give unambiguous results. Previously, this region has been reported to be disordered (34). All N-terminals parts of the Fab molecules are well ordered, whereas the BV16 C-terminals could not be fitted properly. Additionally, the loops 60–65, 94–97, and 122–129 and residues 1–7, 36–40, and 155–157 showed weak density in one of the four bet v 1s. In one light chain, the loops 125–133, 167–174, and 180–187 and residues 11–16, 22–27, and 138–145 showed weak side chain density. Because none of these regions is problematic in the room temperature structure, these problems are attributed to static disorder as a consequence of the phase transition.

In the Bet v 1 molecules, D93 lies outside the allowed Ramachadran regions, which was also the case in the structure of native Bet v 1. Two residues from the light chain are in the disallowed regions, these are residues L30 and L68, both located in loop regions, with relatively well-defined density. In the heavy chain residue, H54 falls in the disallowed regions; again good density supports the conformation. Refinement statistics were calculated using CNS and PROCHECK (35).

The mean $B$ values for complexes 1 and 2 are 54.1 and 57.8 Å$^2$, and for complexes 3 and 4, they are 76.1 and 71.7 Å$^2$. One of the complex molecules with the higher mean $B$ value includes the bet v 1 with poor density, and the other includes the light chain with poor density. The clear difference in mean $B$ values of the two pairs is probably due to the mentioned static disorder in the crystal and is an effect of the cryocooling.

**Results and Discussion**

**Characterization of murine monoclonal BV16 Ab**

A panel of murine mAbs was obtained after immunization with Bet v 1 purified from a birch pollen extract. Screening of the hybridoma candidates aimed at obtaining both Bet v 1 specific as well as Abs cross-reactive between the homologous major allergens from alder and hazel. For this purpose, all hybridomas were screened in direct ELISA using alder, birch, and hazel pollen extract, respectively. BV16 was selected as a cross-reactive Ab, subsequently confirmed by immunoblotting using pollen extracts from the Fagales species birch, hazel, alder, hornbeam, and recombinant Bet v 1 (Fig. 1). Weak reactivity with the hazel major allergen is often encountered in this experimental setup. However, the reactivity with the major hazel allergen is comparable with that of the homologous major allergens. The result is most likely due to incomplete re-folding of the hazel allergen after denaturing electrophoresis as indicated by Ipsen and Larsen (36).

The ability of Bet v 1-specific rabbit polyclonal and murine mAb BV16 to inhibit the binding of Bet v 1 to specific serum IgE...
derived from birch-allergic patients was addressed in an IgE inhibition assay (Fig. 2). The Bet v 1-specific rabbit Abs were raised by repeated immunization using Bet v 1 purified from a pollen extract in IFA. By this procedure, the Ag will be partly denatured, and the resulting Ab will contain specificities directed at any conformation of the Ag. As a consequence, these Abs showed 100% inhibition in agreement with the notion that epitopes recognized by human-specific serum IgE are predominantly conformational and thus constitute only a fraction of those defined by the rabbit antiserum. Surprisingly, the mAb BV16 was able to inhibit ~40% (relative to the polyclonal rabbit Ab) of the binding of specific serum IgE to Bet v 1. This high proportion of inhibition not only shows that the surface area defined by the BV16 epitope is relevant also in terms of IgE specificities but also supports the concept of dominating IgE epitopes. Because the BV16 epitope is located in one of the conserved surface patches described earlier (3), it furthermore supports the notion that conserved surface areas constitute dominant epitopes for high affinity IgE.

Structural analysis of complex

The allergen Bet v 1 forms a 1:1 complex with BV16 Fab. The main structural feature of the allergen Bet v 1 is a seven-stranded anti-parallel $\beta$ sheet that wraps around a 25-residue-long C-terminal amphipathic $\alpha$ helix. The $\beta$ sheet and the C-terminal part of the long helix are separated by two consecutive helices. Between the two helices and the $\beta$ sheet, there is a larger cavity and a forked tunnel through the structure.

Numerous structural studies have been performed on Abs including structure determinations of intact IgG Abs (37). There has been special focus on determining the regions involved in the interactions with Ag, the CDRs (38). All BV16 Fab CDRs except L2 are in proximity to Bet v 1. L2 can be seen on Fig. 3 in front of the 3 BV16 Fab strands most to the right, far from the complex interface. Interestingly, superposition of the template CDRs (9) and the corresponding BV16 Fab CDRs shows good agreement in conformation for the loops L1, H1, and H2 but not for L2. This probably reflects that the template CDR conformations are derived from CDRs that actually are in contact with the Ag, and not from loops that do not interact. In previous investigations of Fab-Ag complexes, it is frequently seen that there are CDRs not involved in the complex formation.

Fig. 3 shows an overall view of the complex between Bet v 1 and BV16 Fab. No major conformational changes in the structure of Bet v 1 are seen upon binding to the monoclonal BV16 Fab. The overall rms deviation between free and bound Bet v 1 is 0.605 Å, as calculated by the program o (39).

Fig. 4 shows the contact surfaces found on BV16 and Bet v 1, respectively. Contacts are defined as the molecular surfaces of the two molecules that are <3.2 Å apart. The calculation of the contact surfaces was performed with the program GRASP (40). The buried area on Bet v 1 is calculated to be 931 Å$^2$ (of a total molecular

![FIGURE 3.](http://www.jimmunol.org/)

![FIGURE 4.](http://www.jimmunol.org/)
Table II. Contacts between Bet v 1 and BV16

<table>
<thead>
<tr>
<th>Bet v 1 Residue</th>
<th>BV16 Residue</th>
<th>Hydrogen Bond</th>
<th>CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>E42</td>
<td>R101</td>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>N43</td>
<td>R101</td>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>N43 O</td>
<td>D102 N</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>I44</td>
<td>Y32</td>
<td></td>
<td>H1</td>
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<td>E45</td>
<td>Y32</td>
<td></td>
<td>H1</td>
</tr>
<tr>
<td>E45 OE1</td>
<td>W33 N</td>
<td>+</td>
<td>H3</td>
</tr>
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<td>E45 OE2</td>
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<td>H3</td>
</tr>
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<td>A100</td>
<td></td>
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<tr>
<td>G46 N</td>
<td>T103O</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>N47</td>
<td>Y32</td>
<td></td>
<td>L1</td>
</tr>
<tr>
<td>N47 ND2, OD1</td>
<td>S91 OG</td>
<td>+</td>
<td>L3</td>
</tr>
<tr>
<td>G48</td>
<td>Y32</td>
<td></td>
<td>L1</td>
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<tr>
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<td>S91</td>
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<tr>
<td>P50 O</td>
<td>Y96 OH</td>
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<td>H76</td>
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<td>H2</td>
</tr>
<tr>
<td>K97</td>
<td>D35</td>
<td></td>
<td>H2</td>
</tr>
</tbody>
</table>

a Interactions are defined as distances <4.0 Å (acceptor-donor distance <3.5 Å for hydrogen bonds).

The buried area is re-assembled to be 916 Å² in the influenza virus N9 neuraminidase-protein-Fab complexes (41). For example, the buried area is re-assembled to be 916 Å². This is comparable to what is found for other protein-Fab complexes (41). For example, the buried area is reported to be 916 Å² in the influenza virus N9 neuraminidase-NC41-Fab complex (19).

Given the small sizes of the epitope (only around 10% of the total allergen surface), it is obvious that it is theoretically possible to bind more than one IgE molecule to the same allergen molecule. Because the BV16 Ab cross-reacts with the major allergens from other trees of the Fagales order, BV16 is expected to bind to one of the patches of conserved residues on the molecular surface of 9,119 Å² and the buried area on BV16 Fab is 807 Å² (of 21,648 Å²). This is comparable to what is found for other protein-Fab complexes (41). For example, the buried area is reported to be 916 Å² in the influenza virus N9 neuraminidase-NC41-Fab complex (19).

A detailed analysis of Ab-Ag interactions has recently been undertaken (38). They have grouped the interactions based on the size of the Ag: small molecule (hapten) interactions; peptide interactions; and large (protein) interactions. When comparing the interactions seen with the normalized frequency of interaction for each amino acid in the CDRs, it is found that all interacting residues from BV16 Fab have frequencies above 0.2. None of the CDR residues described as “only structural” by MacCallum et al. interacts with the Bet v 1. They also note that large Ags seem to prefer contacts with residues at the extremities of the combining site, e.g., most of L2 and residues H30–H32 in H1. Although BV16 Fab has contacts to both H31 and H32, there are no contacts to L2. This is not uncommon. In the complex between hen egg lysozyme and the Fv fragment of its mAb D11.15 (14), neither L1 nor L2 interacts with the Ag.

Because the BV16 Ab cross-reacts with the major allergens from other trees of the Fagales order, BV16 is expected to bind to one of the patches of conserved residues on the molecular surface of 9,119 Å² and the buried area on BV16 Fab is 807 Å² (of 21,648 Å²). This is comparable to what is found for other protein-Fab complexes (41). For example, the buried area is re-assembled to be 916 Å². This is comparable to what is found for other protein-Fab complexes (41). For example, the buried area is reported to be 916 Å² in the influenza virus N9 neuraminidase-NC41-Fab complex (19).

FIGURE 5. Close-ups of interaction region. Stereo view of the interactions involving all hydrogen bonds formed on complexation. Bet v 1 residues have yellow carbon atoms whereas BV16 Fab residues have green carbon atoms. Only Bet v 1 residues involved in hydrogen bonds are labeled. E45 makes two short hydrogen bonds with the BV16 backbone WH33 NH and GH99, both 2.9 Å. N47 is in position to make hydrogen bonds with SL91 (2.7 Å). In addition, the following hydrogen bonds are formed: N43 to DH102, G46 to TH103, P50 to YL96, and G51 to WH33. This figure has been prepared with MOLSCRIPT and RASTER3D.
of Bet v 1 defined previously (3). Fig. 7 shows the epitope defined by the BV16 Ab and a corresponding molecular surface defined by the residues completely conserved among all Fagales major allergens (3). The surface area defined by the conserved residues is contained in the surface area defined by the interactions with BV16 Fab.

In conclusion, the interactions observed in the Bet v 1-BV16 Fab complex can be described in terms of interactions previously described for Ag-Ab complexes. The size of the Bet v 1 epitope is within the range defined by other complexes. Even though the existence of dominant IgE binding allergen epitopes is a matter of controversy, the concept is supported by the notion that natural exposure to surface patches conserved among structural homologues and isoallergens is higher and therefore more likely to induce high affinity IgE. The results of the IgE inhibition assays clearly suggest that dominant human IgE epitopes are located in conserved surface patches with the size of average Ag-Ab contact areas. These patches are therefore targets for modifications aiming

**FIGURE 6.** Omit maps of the major interaction region. This figures show stereo views of an omit map of the major Bet v 1-BV16 Fab interaction region contoured at 1.3 σ. A, Bet v 1 residues G44, E45, I46, and BV16 Fab residues YH32, WH33, WH104. B, Bet v 1 residues N47, G48, G49, and P50 along with Fab residues WH104 and YL96. These figures have been prepared with DINO (http://www.bioz.unibas.ch/~x-ray/dino) and RASTER3D

**FIGURE 7.** Bet v 1 epitope and patch of conserved residues in the Fagales order. The solvent-accessible surface of Bet v 1 is shown. The surface was calculated with a probe with radius of 1.4 Å. A, Patch of conserved residues among the allergens from the Fagales tree order (gray), comprising residues 41-52; B, gray areas, the residues comprising the BV16 epitope with the same orientation of the molecule. All except one Bet v 1 residue (residue 41) from the conserved patch are involved in contacts with the BV16 Fab. This explains why the BV16 is known to bind to allergens from pollen from other trees. The figure was prepared with SPOCK and RASTER3D.
at modulating the human allergen-specific immune response, with important implications for vaccine development. They also form the molecular basis of clinically observed cross-reactivity of tree pollen-allergic patients toward tree pollen allergens from related species.

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References


