

Allergen mimotopes for 3-dimensional epitope search and induction of antibodies inhibiting human IgE

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ABSTRACT There is no definite information available on the structural characteristics of IgE binding epitopes on allergenic molecules, although it is widely accepted that most of them are conformational. In the current study we aimed to characterize the IgE epitope of Bet v 1, the major birch pollen allergen, by the application of phage display peptide libraries. We purified IgE specific for Bet v 1 from allergic patients' sera to select mimotopes representing artificial IgE epitopes by biopanning of phage libraries. By linear alignment, it was not possible to attribute mimotope sequences to the primary structure of Bet v 1. We developed a computer-aided, 3-dimensional coarse-grained epitope search. The 3-dimensional search, followed by statistical analysis, revealed an exposed area on the Bet v 1 molecule (located between residues 9–22 and 104–123) as the IgE binding structure. The IgE epitope was located at a 30 Å distance from a previously described IgG epitope and the respective mimotope, designated Bet mim E. Such mimotopes could potentially be used for the induction of IgG capable of interfering with the IgE/allergen interaction. To test this hypothesis, we immunized BALB/c mice with the phage-displayed Bet mim E. Immunizations resulted in the induction of Bet v 1-specific IgG, which was able to block the IgE binding to Bet v 1 *in vitro*. Based on these observations, we propose that immunotherapy with IgE mimotopes generated by biopanning result in formation of blocking IgG. We conclude that mimotope immunotherapy may represent a new and promising concept for treatment of type I allergic disease.—Ganglberger, E., Grünberger, K., Spöner, B., Radauer, C., Breiteneder, H., Boltz-Nitulescu, G., Scheiner, O., Jensen-Jarolim, E. Allergen mimotopes for 3-dimensional epitope search and induction of antibodies inhibiting human IgE. *FASEB J.* 14, 2177–2184 (2000)

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TYPE I ALLERGIC REACTIONS represent a health problem of increasing importance for more than 20% of

the European population (1, 2). The symptoms of immediate-type allergy result from the cross-linking of effector cell-bound immunoglobulin E (IgE) by multivalent allergens. In central Europe, one of nine patients with type I allergy shows specific IgE to birch pollen proteins (3), and the majority (95%) are sensitized to the major allergen Bet v 1 (4). Moreover, 60% of these patients show IgE binding exclusively to Bet v 1 (4). For these reasons, Bet v 1 has become the most extensively characterized allergen on both the molecular (5, 6) and immunological level (7, 8). Several examples illustrate the role of conformational epitopes for Bet v 1-IgE interaction: 1) isoforms of Bet v 1 with only a few amino acid variations displayed a significantly reduced IgE binding capacity (9–12); 2) IgE epitopes of Bet v 1 could not be conclusively mapped using sequential peptide fragments (8); and 3) two recombinant fragments of Bet v 1 could not reconstitute the IgE epitope (13). Until now, the interaction of patients' IgE with Bet v 1 was investigated indirectly by using monoclonal mouse or human antibodies (14, 15). Of these, several monoclonal antibodies enhanced IgE binding whereas others reduced IgE binding to Bet v 1 and inhibited the specific histamine release. These opposing effects depended on the distinct epitope specificity of the antibodies (14, 15).

The phage display technique is relatively new, used to define peptide structures that mimic natural epitopes, including conformational B cell epitopes (16–20). Phage peptide libraries consist of filamentous phages, displaying random peptides of defined length on their surface either fused to the phage minor coat protein pIII (21, 22) or, at a higher copy number, to the major coat protein pVIII (23). Such peptide ligands can be selected by biopanning. We have recently selected phage ligands for the monoclonal anti-Bet v 1 IgG1 antibody BIP1 (24). The identified ligand, termed Bet mim 1, represented a structure mimicking a Bet v 1 epitope, which was not

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recognized by patients' IgE. The identified Bet v 1 epitope did thus represent an IgG epitope (24).

In the present study, we defined an IgE epitope of Bet v 1. This seems of particular interest since IgE mimotopes could be used to precisely induce mono-epitope specific IgG acting potentially as blocking antibodies. We purified IgE from a serum pool of birch pollen allergic patients, which was applied in biopanning experiments. The properties of selected phage-displayed peptides representing mimotopes were compared to the sequence and 3-dimensional structure of Bet v 1 to localize the corresponding IgE epitope. IgE mimotopes were further used in immunization experiments with BALB/c mice for the induction of a blocking type of IgG.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody BIP 1 (25) and 0590B2 (14, kindly provided by Schering-Plough, Dardilly, France) were raised by immunization of BALB/c mice with birch pollen extract and are directed to Bet v 1.

Patients' sera

Sera were collected from 12 patients with positive case history, RAST classes > 4 and skin prick tests to birch pollen. All patients showed intense IgE binding to Bet v 1 in immunoblot analysis.

Affinity purification of patients' antibodies

Five milligrams of recombinant Bet v 1 (rBet v 1) (Biomay, Linz, Austria) were dissolved in coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.8) and mixed with 1.5 g CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). To block any remaining active groups, the gel was incubated with 1 M ethanolamine (pH 9.0), then washed and packed into a glass column.

The serum pool was composed of equal volumes of sera from 12 birch pollen-allergic patients. The serum pool was diluted 1:2 in 0.9% (w/v) NaCl and dialyzed against 0.9% (w/v) NaCl prior to application to the affinity column. For each separation run (two runs were performed), 10 ml of diluted serum was loaded and recirculated overnight at 4°C. After extensive washing with 0.9% (w/v) NaCl, bound antibodies were eluted with 3 M KSCN in 5 mM phosphate buffer (pH 6.5). Eluted fractions were immediately neutralized and dialyzed against 0.9% (w/v) NaCl. Quantity and quality of eluents were checked with dot and blot immunoassays.

Dot assay

Purified immunoglobulins were dotted in triplicate onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Dot blots were air dried and saturated with TBS/0.05% Tween 20/1% powdered milk before incubation with ¹²⁵I-labeled anti-human IgE or IgG (MALT Allergie system, IBL GmbH, Hamburg, Germany). Blots were washed, dried, and exposed to Kodak Biomax MS autoradiography films at -70°C.

Serial dilutions (40 ng, 20 ng, 10 ng, 5 ng/dot) of human

IgE from an immortalized B-cell line (SU-11, 26) and human myeloma IgG were prepared and dotted to nitrocellulose for subsequent quantity estimation by density measurements with a hand-held reflexion densitometer (Vipdens, Brixen, Italy).

SDS-PAGE, IgE, and IgG immunoblotting

Protein extracts were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). Immunoblots were performed as described before, using TBS/0.05% Tween 20/1% powdered milk for blocking, washing, and antibody dilutions. Blotted proteins were incubated with patients' sera (diluted 1:5) overnight. After washing, bound IgE/IgG was detected using ¹²⁵I-labeled rabbit anti-human IgE or IgG (MALT Allergie system) (1:20).

Alternately, blot strips were incubated with BIP 1 (25) or 0590B2 hybridoma supernatant (14). Bound IgG was detected using rabbit anti-mouse IgG (Nordic Immunology, London, U.K.), followed by ¹²⁵I-labeled donkey anti-rabbit IgG (Amersham, Little Chalfont, U.K.), both diluted 1:1000.

Biopanning with phage libraries

Biopannings were performed using phage display libraries expressing random linear (pVIII 9aa, ref 19) or circular (pVIII 9aa.Cys, ref 20) nonapeptides fused to pVIII of the filamentous bacteriophage VSCM13. Libraries were kindly provided by IRBM (Istituto di Ricerche Biologia Molecolare, Rome, Italy).

Four successive panning rounds with affinity-purified human antibodies were performed according to the method of Parmely and Smith (22). For each round, 1 µg IgE in 0.1 M NaHCO₃ (pH 8.5) was coated on ELISA plates (Nunc, Roskilde, Denmark). After blocking with phosphate-buffered saline (PBS)/3% (w/v) bovine serum albumin at 37°C for 1 h, ~10⁸ pfu of freshly prepared phages from each library were pooled and incubated with coated antibodies. After extensive washing, bound phages were eluted and amplified by infecting freshly grown *Escherichia coli* XL 1-blue and superinfection with helper phage VSCM13 (10¹² pfu/ml). Amplified phage particles were precipitated with PEG, resuspended in TBS/0.15% (w/v) casein, and used for the next panning round. Phagemide DNA was prepared from overnight cultures of single phage clones; DNA sequencing was performed by the Sanger dideoxy method using a Thermo Sequenase cycle sequencing kit (Amersham) with fluorescent-labeled primers and analyzed by a LI-COR DNA Sequencer 4000L (LI-COR Inc., Lincoln, Nebr.).

3-Dimensional epitope search

The 3-dimensional coarse-grained epitope search was based on the X-ray structure of Bet v 1 (available in PDB format at the EMBL protein database, entry 1BV1). For determination/fixation, each amino acid was localized using the coordinates of its Cβ atom (for glycine, the Cα atom). Based on this Cβ grid, neighboring amino acids were found in a distance smaller than 6.1 Å (upper limit of Cβ-Cβ distance). Fitting of only two amino acids consecutively induced a broader attempt, which allowed gaps (non fitting amino acids within the nonapeptide sequence). The model was further simplified by classifying all amino acids according to the chemical character of their residues: a group of polar amino acids (N, Q, H, M, S, T), a group of lipophilic amino acids (A, C, G, I, L, F, P, W, Y, V), a group

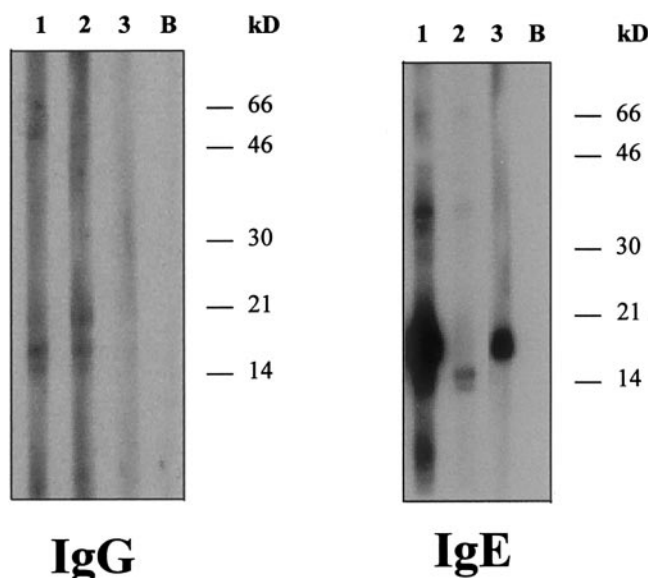


Figure 1. Immunoreactivity of affinity-purified Bet v 1-specific human immunoglobulins was checked by immunoblot analysis on birch pollen extract. Birch pollen extract was blotted to nitrocellulose and incubated with a pool of sera from birch pollen allergic patients (lane 1), the effluent of the affinity column coupled with rBet v 1 (lane 2), the eluent fraction from the affinity column (lane 3). Bound IgE or IgG was detected by ^{125} I-labeled anti-human IgE or IgG antibodies, respectively. The autoradiogram is shown. Note the absence of specific IgG in the eluent fraction.

of acidic amino acids (D, E), and basic amino acids (R, K). All hits were statistically evaluated.

Intraperitoneal immunization of BALB/c mice

Female BALB/c mice, 3–5 wk of age, were used for intraperitoneal (i.p.) immunization with phages (Biovendor Biotechnology, Brno, Czech Republic). For i.p. administration, 2×10^7 pfu phages were diluted to a volume of 100 μ l in PBS. Three groups of three mice each were immunized on days 0, 14, and 28. Group A received phages expressing Bet mim E (CQQFLSVRALC), group B received phages displaying a control peptide (CFFAWRSLPNC) derived from biopannings with a murine monoclonal antibody directed to a different birch pollen protein, and group C received the same amount of PBS only.

Blood samples from the tail vein were taken on day 0 (preimmune serum), day 21, and day 38.

Inhibition experiments

Approximately 1 μ g/cm natural Bet v 1 (nBet v 1) purified from birch pollen extract using ion exchange chromatography, followed by reversed phase HPLC, was separated by preparative 15% SDS-PAGE and blotted onto nitrocellulose. Immunoblotting was performed as described above, with slight modifications: after blocking, strips were first incubated with mouse immune sera or, for control purposes, with preimmune sera (1:250). After washing, strips were incubated with sera from allergic patients (1:5). After washing, bound IgE were detected with ^{125} I-labeled anti-human IgE (MALT Allergie system) and visualized by autoradiography. The percentage reduction of IgE binding in relation to the

uninhibited control (set to 100%) was determined by densitometric analysis.

RESULTS

Generation of mimotopes

To select peptide ligands of IgE specifically directed to the major birch pollen allergen Bet v 1, we purified specific antibodies from a serum pool of birch pollen-allergic patients using an affinity chromatography column coupled with recombinant Bet v 1 (rBet v 1). Comparing affinity-purified antibodies with serial dilutions of monoclonal IgE and myeloma IgG in immunodot assays indicated an IgG:IgE ratio of 5:1 among purified antibodies (data not shown). Similar IgE/IgG antibody ratios were described earlier in fractions containing allergen-specific IgE, where the IgG was identified as anti-IgE autoantibodies (27). The total yield was estimated at 42 μ g specific IgE from 10 ml patients' serum. To check the immunoreactivity of purified antibodies compared with the serum pool, we performed IgE and IgG immunoblots showing that the purified IgE was intact regarding its binding as well as its constant domain (Fig. 1). Moreover, IgG, which coeluted with Bet v 1-specific IgE, was not directed to Bet v 1 or other birch pollen proteins (Fig. 1). Thus, the eluted antibody preparation contained—in addition to IgG, most likely acting as anti-IgE autoantibodies (27)—polyclonal anti-Bet v 1 IgE.

These purified antibodies were used in biopanning experiments. We screened a repertoire of $\sim 2 \times 10^8$ different peptides by combining the 'pVIII 9aa' and the 'pVIII 9aa.Cys' libraries (19, 20, 23). After the fourth round of selections, we defined five different peptides of possible ligands by random selection. The deduced amino acid sequences are listed in Table 1.

Localization of the major IgE epitope of Bet v 1

Although linear alignments of the mimotope derived amino acid sequences with the known amino acid sequence of Bet v 1 using the GCG package (Genetics Computer Groups, Inc., Madison, Wis.) failed, a

TABLE 1.

Amino acid sequence	Library	Designated
C QQQFLSVRAL C	pVIII 9aa.Cys	Bet mim E
C GGTPPSLPT C	pVIII 9aa.Cys	
C GGTRRPIAS C	pVIII 9aa.Cys	
C SEYREPLL C	pVIII 9aa	
AGTHTPGAT	pVIII 9aa	Bet mim 1
TSKASELAV	pVIII 9aa	
CFPYCYPSESA	pVIII 9aa.Cys	

3-dimensional epitope search resulted in statistically significant amino acid similarities. Our approach was first based on homology between neighboring residues permitting gaps. In another step of abstraction, the amino acids were classified in four different categories depending on the chemical character of their residues. Based on this simplification, alignment with the derived sequences was achieved without admission of gaps. Both approaches showed several possible conformable amino acid positions. Therefore, all hits were statistically evaluated. Significant accumulations in two regions, including amino acids 9–22 and 104–113, were found (Fig. 2A). These two regions were neighboring and located on the surface of the molecule. They covered an area of totally 500 Å² and very likely compose a discontinuous IgE epitope. As exemplified in Fig. 2A, the area found was localized between alpha-helix α 1 and beta strands β 1, β 6, and β 7. The corresponding mimotope was designated Bet mim E.

For control purposes, the 3-dimensional coarse-grained epitope search was also applied for the localization of Bet mim 1, an IgG epitope of a monoclonal murine anti-Bet v 1 antibody (BIP1; 24, 25) (Fig. 2B). The program identified the IgG epitope at positions 58–67, located in substantial distance of Bet mim E.

Induction of antibodies inhibiting human IgE binding

Phages displaying the peptide CQQFLSVRALC, derived from the pVIII 9aa.Cys (20) library, were over-represented in round 4 of the biopannings. Therefore, we selected this dominant clone for immunization experiments in BALB/c mice. For control, phages displaying a peptide (CFFAWRSLPNC) derived from biopannings with a monoclonal antibody (BIP 3), directed to a different birch pollen protein (25), or PBS alone, were delivered to BALB/c mice i.p. The specific IgG response in sera of mice was analyzed by immunoblot (Fig. 3). All BALB/c mice immunized with phages displaying the Bet v 1 IgE epitope developed IgG, which reacted with the 17 kDa allergen Bet v 1 *in vitro* (Fig. 3A). In contrast, sera of mice immunized with phages displaying the control peptide CFFAWRSLPNC or PBS did not show any Bet v 1 specific reactivity (data not shown). The specificity of IgG induced by immunization with Bet mim E phages was further tested by inhibition experiments. IgG binding of mice immunized with phages displaying the peptide CQQFLSVRALC was effectively inhibited by preincubation of mouse immune sera with recombinant (r) or natural (n) Bet v 1, but not after preincubation with an unrelated protein (rBet v 2, birch profilin) (Fig. 3B). These experiments indicated that the peptide CQQ-

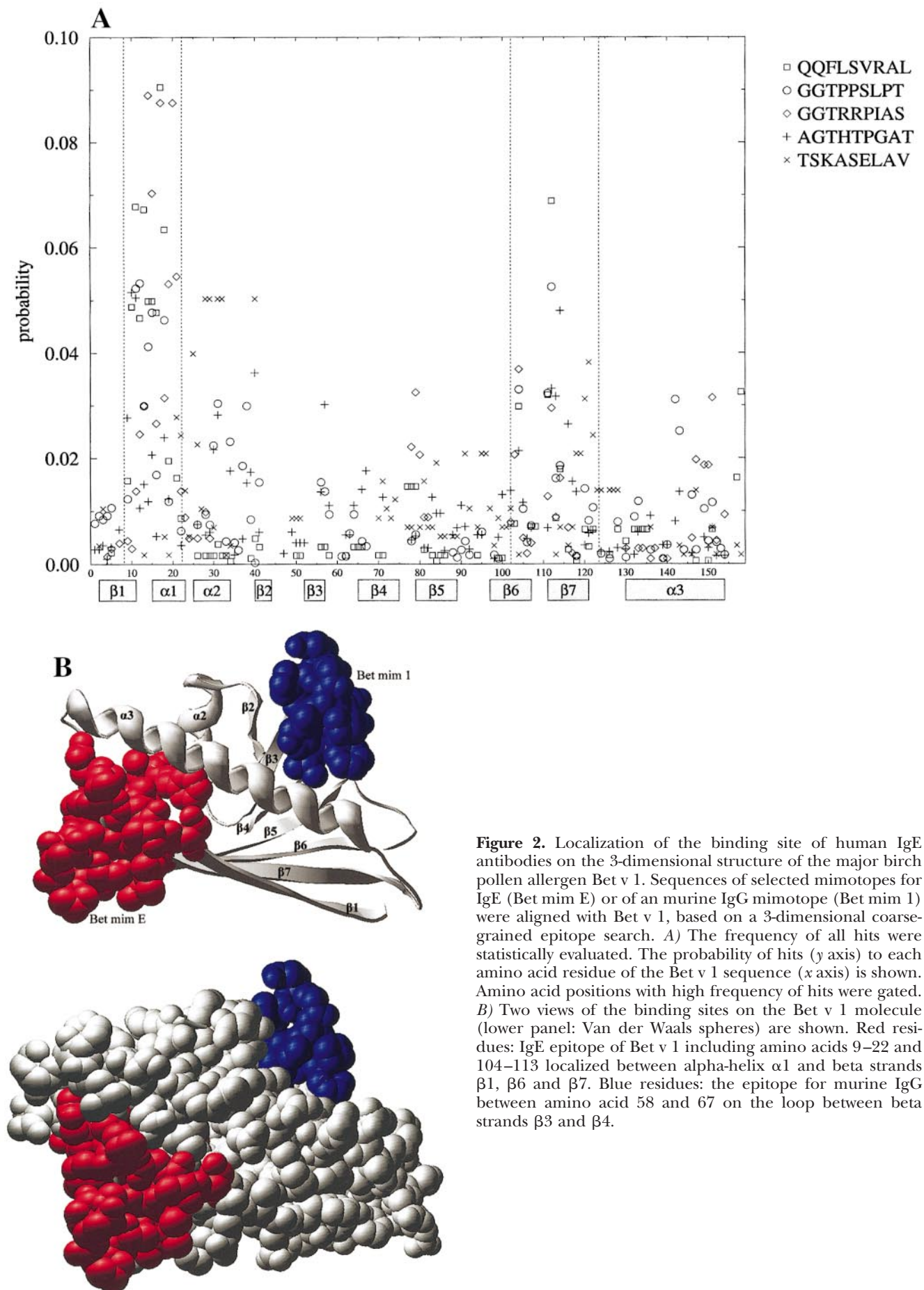
FLSVRALC selected by polyclonal patients' IgE directed to Bet v 1 mimicked a Bet v 1 epitope.

To test the hypothesis of blocking IgG antibodies, we investigated whether IgG specific for an IgE epitope was able to affect the binding of human IgE to allergens. Nitrocellulose-blotted purified allergens were first preincubated with sera from mice immunized with phages displaying Bet mim E, and for control purposes, with preimmune sera or a monoclonal antibody previously shown to block IgE binding (14). As demonstrated in Fig. 4, IgE binding of a serum pool from birch pollen allergic patients to purified nBet v 1 was strongly inhibited after preincubation of the membrane with IgG to Bet mim E or with monoclonal antibody 0590B2 14; Fig. 4, lanes 3 and 4). In contrast, IgE binding was not affected by a monoclonal IgG antibody (BIP 1) directed to a distinct Bet v 1 epitope (Bet mim 1) that was not recognized by IgE (24) (Fig. 4, lane 5).

DISCUSSION

Attempts to identify IgE epitopes of Bet v 1 on a molecular basis have proved unsuccessful so far, probably because these epitopes are conformational and discontinuous (8–13). In previous studies, we applied the phage display technique for defining peptide structures mimicking natural epitopes of Bet v 1 (24, 28). Moreover, we have demonstrated that phage mimotopes are powerful immunogenic vectors for the precise induction of epitope specific IgG (24, 28–30). In these experiments, the induced IgG enhanced the IgE binding to Bet v 1 and, thereby, the allergic reactivity. Consequently, the aim of the present study was to identify the major IgE epitope of Bet v 1 and to direct an IgG response toward this IgE epitope for blocking the allergic reactions. We defined IgE mimotopes of Bet v 1 by biopanning of phage display libraries.

To localize the major IgE epitope, we developed a computer-aided algorithm based on the 3-dimensional structure and the chemical character of the amino acids. With this new method, it was possible to localize the IgE epitope on the surface of Bet v 1 using mimotopes and the known antigen structure. To our knowledge, a conformational IgE epitope on the 3-dimensional structure of Bet v 1 is shown here for the first time. Our data demonstrate that regions separated on the linear amino acid sequence compose one patch on the surface of the allergen and form a discontinuous IgE epitope. The position hits one of three surface-exposed conserved patches common to all Bet v 1 homologues, which were predicted by X-ray crystallography (6). It is remarkable that the work of Ferreira et al. (12) has shown that point mutations in six critical amino acid posi-



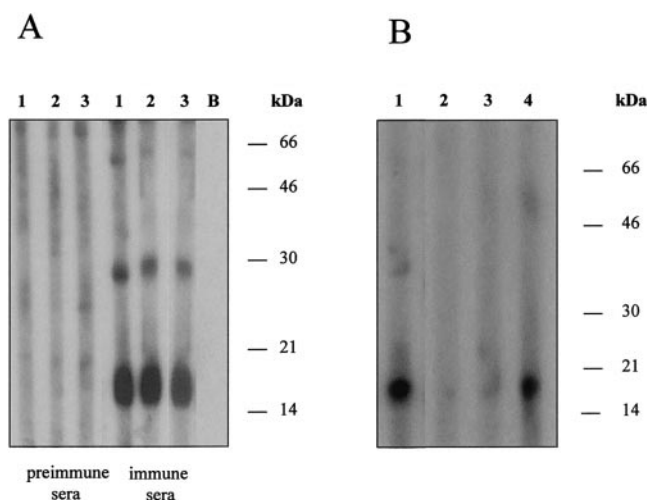


Figure 3. Mice immunized with phages displaying Bet mim E, a conformational IgE epitope of Bet v 1, form IgG to the 17 kDa allergen, which can be specifically inhibited by Bet v 1. *A*) Birch pollen extract was blotted and strips were tested with preimmune and immune sera of three different mice (1:250), lanes 1–3; or buffer, lane B. *B*) IgG from mice immunized with Bet mim E can be specifically inhibited by Bet v 1. Lane 1: uninhibited binding of murine IgG; lane 2: inhibition with nBet v 1; lane 3: inhibition with rBet v 1; lane 4: no inhibition effect with r Bet v 2. Bound IgG was detected by ^{125} I-labeled donkey anti-rabbit antibody. The autoradiogram is shown.

tions lead to a loss of allergenicity and that three of these amino acids (aa 10, 112, 113) are located within the computer determined epitope. Thus, our data based on a rational approach confirm these previous findings deduced from immunological properties of *in vitro* mutagenized Bet v 1.

According to our calculation, Bet mim E is located at a substantial distance from Bet mim 1, an IgG epitope of the monoclonal murine anti-Bet v 1 antibody BIP 1 (24, 25). From serological experiments we concluded that Bet mim 1 does not represent the binding site for IgE (24). Based on sequence alignments, we had previously speculated on a putative binding site of the monoclonal antibody to Bet v 1 (31). With the 3-dimensional, coarse-grained epitope search, this assumption was confirmed in the present study, again matching one of the three conserved patches of Bet v 1 homologues published by Gajhede et al. (6).

Whereas the role of IgE in the pathogenesis of type I allergy is well established, much less is known concerning the effects of allergen-specific IgG. A phenomenon accompanying specific immunotherapy is the induction of allergen-specific IgG antibodies (32). The determined IgG levels were without correlation to the clinical outcome of immunotherapy (33). Recent studies indicated that, depending on the epitope-specificity, IgG may enhance or inhibit IgE binding to Bet v 1 by affecting the 3-dimensional conformation of this molecule (14, 15). In accordance, we could previously demonstrate that

IgG antibodies directed to a Bet v 1 epitope distinct from the IgE epitope enhanced allergic skin reactivity in a murine model (28). Using allergen molecules as a whole for immunotherapy, it is not possible to predict which epitopes will be targeted by IgG resulting in modulation of biological response. Therefore, it is an attractive concept to use ‘artificial’ epitopes for immunotherapy, preferably a type inducing a ‘blocking’ IgG response. Such IgG directed to an IgE epitope may block the IgE–allergen interaction without sensitizing effector cells such as mast cells or basophils via FcεRI.

Therefore, the IgE mimotopes of Bet v 1 gener-

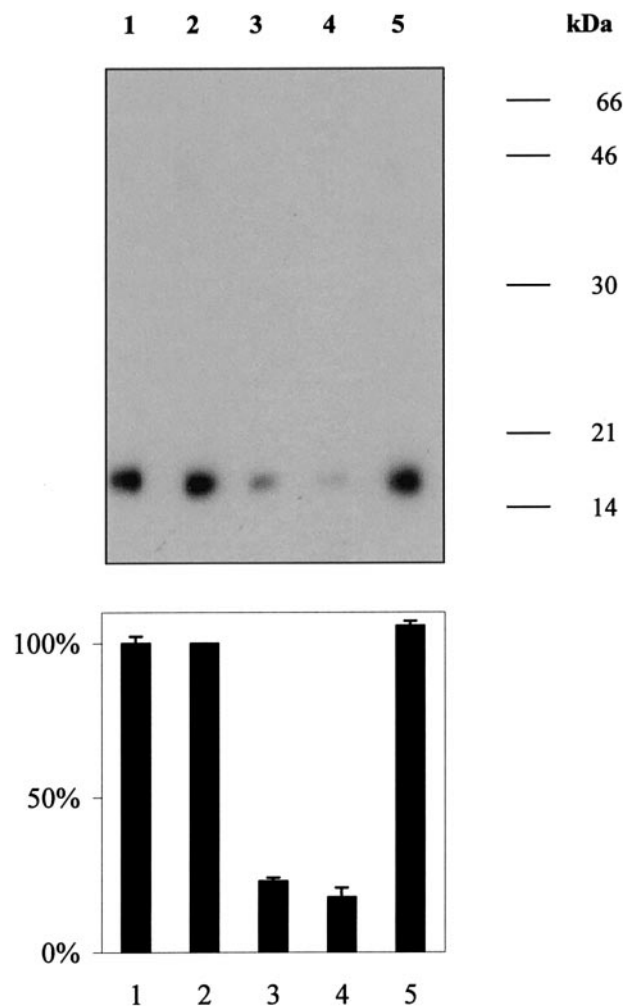


Figure 4. Preincubation of blotted nBet v 1 with mouse IgG to Bet mim E inhibit human IgE binding. Nitrocellulose-blotted purified nBet v 1 was preincubated with mouse sera. Lane 1: no preincubation; lane 2: preincubation with preimmune serum; lane 3: preincubation with immune serum; lane 4: preincubation with 0590B2; lane 5: preincubation with BIP 1. Strips were then exposed to sera from a pool of 12 birch pollen-allergic patients. Bound IgE was detected with ^{125} I-labeled anti-human IgE and visualized by autoradiography. Signal intensities of the autoradiogram were analyzed by densitometrical measurements. Maximum intensity was set equal to 100%. Black columns indicate the relative percent of IgE binding in lanes 1–5.

ated by biopanning of phage display peptide libraries were applied in immunization experiments. Mice immunized with IgE mimotopes showed an IgG response specific for Bet v 1 that was capable of blocking human IgE binding *in vitro*. Based on these observations, we conclude that the precise induction of blocking antibodies through mimotope vaccination inhibit consecutive IgE binding.

In this study, we present a strategy to define discontinuous IgE epitopes with the phage display technology. More important, the selected mimotopes were capable of inducing B-cell responses to the natural conformational IgE epitope. In our opinion, this induction of 'blocking' IgG through IgE mimotope vaccination represents a completely new principle for interfering with IgE-allergen interactions. Moreover, for more than 90% of birch pollen allergic patients who recognize Bet v 1 via IgE and 60% who recognize it exclusively (4), the interference of IgE/Bet v 1 interaction by mimotope immunotherapy could also represent a promising therapeutic concept. **[FJ]**

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