

Peptide mimotopes displayed by phage inhibit antibody binding to Bet v 1, the major birch pollen allergen, and induce specific IgG response in mice

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ABSTRACT The major birch pollen allergen Bet v 1 is one of the most extensively characterized allergens both on the molecular and the immunological level. To define conformational B cell epitopes on Bet v 1, we screened filamentous phage libraries expressing circular or linear nonapeptides to select ligands specific for anti-Bet v 1 murine monoclonal antibodies BIP1 and BIP4. The deduced amino acid sequence of the BIP1 ligand was CFPYCYPSESA, and of the BIP4-ligand, CRQTRTMPGC. Both sequences derived from the circular phage library. Alignments to the sequence of Bet v 1 showed no similarities, indicating that the antibodies most likely recognize discontinuous epitopes. Phages displaying these mimotopes were capable of inhibiting interactions of the anti-Bet v 1 monoclonals with Bet v 1 in a dose-dependent manner in ELISA. In contrast, sequence-identical synthetic peptides were ineffective in blocking the antibody–allergen interactions. This is in agreement with the conformational inhomogeneity of the peptides in solution as observed by nuclear magnetic resonance spectroscopy. Intragastric administration of phages expressing the BIP1 mimotope induced a Bet v 1-specific IgG response in Balb/c mice. We conclude that peptide mimotopes, when displayed on phages, may induce a protective IgG response preventing IgE-mediated allergic reactions, suggesting a possible clinical application.—Jensen-Jarolim, E., Leitner, A., Kalchhauser, H., Zürcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G., Breiteneder, H. Peptide mimotopes displayed by phage inhibit antibody binding to Bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* 12, 1635–1642 (1998)

Key Words: Bet v 1 · allergy · phage · mimotope · oral immunization · monoclonal antibodies

INCIDENCES OF ALLERGIC REACTIONS have increased during recent years, affecting approximately 20% of

the European population (1, 2). Pollen allergies play a dominant role in immunoglobulin E (IgE)²–mediated, immediate-type hypersensitivity reactions, and food allergies have often been revealed to be a consequence of primary sensitization by pollen (3, 4). Birch pollen allergy is to a large extent due to the existence of a single major allergen, Bet v 1, which is recognized via IgE by 96% of birch pollen-allergic patients (5). Bet v 1 is one of the best-characterized allergens. Its cDNA sequence (6), T cell epitopes (7, 8), and crystal structure (9) have been published. Several isoforms of Bet v 1 have been characterized (10) that differ in their ability to bind monoclonal anti-Bet v 1 antibodies as well as human IgE (11–13). In contrast, B cell epitopes of Bet v 1 could not be conclusively mapped by using sequential peptide fragments (14). Synthetic B cell epitopes could be used for neutralizing allergen-specific IgE to inhibit allergic reactions (15–18) or for hyposensitizing patients (19). Immunotherapy with allergen extracts has been applied for many years, but still bears the risk of side effects. New strategies for specific immunotherapy have been suggested, such as hyposensitization with Bet v 1 isoforms, which have a highly reduced capacity to bind IgE (20). However, other immunoglobulin subclasses may also be involved in anaphylactic reactions (21). A hallmark of specific immunotherapy is the induction of allergen-specific IgG antibodies (22). Still, the increase of allergen-specific IgG is not

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² Abbreviations: BSA, bovine serum albumin; COSY, correlated spectroscopy; Ig, immunoglobulin; mAb, monoclonal antibody; Ab, antibody; NMR, nuclear magnetic resonance spectroscopy; rBet v 1, recombinant Bet v 1; nBet v 1, natural Bet v 1; RT, room temperature; ELISA, enzyme-linked immunosorbent assay; IRBM, Istituto di Ricerche di Biologia Molecolare P. Angeletti SPA; PBS, phosphate-buffered saline; pfu, plaque-forming units; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

correlated with the clinical outcome in inhalant allergies (23). Besides beneficial effects, patients may become newly sensitized to some compounds during therapy. To exclude the formation of anaphylactogenic immunoglobulins during immunotherapy, the precise induction of one antibody species to block IgE binding to Bet v 1 is an attractive concept. Murine monoclonal anti-Bet v 1 antibodies have been shown to modulate the IgE binding capacity of Bet v 1 depending on their binding specificity (14).

The phage display technique is an excellent tool for defining peptide structures that mimic natural epitopes, including conformational B cell epitopes (24–28). Phage peptide libraries consist of filamentous phages, displaying random peptides of defined length on their surface; the peptides can be fused to the phage minor coat protein pIII (29, 30) or, at a higher copy number, to the major coat protein pVIII (31). Specific ligands for molecules of interest can be selected from these peptide libraries by biopanning (30, 24–26). The ligands, although differing in sequence, often mimic epitopes on the natural antigen, as shown for human ferritin or *Bordetella pertussis* toxin (27, 28). Furthermore, mimotopes have been shown to effectively induce specific immune responses, as has been shown for a hepatitis B virus surface mimotope (32).

The aim of this study was to use phage-displayed peptide libraries to define ligands mimicking structural epitopes on the allergen Bet v 1 for murine monoclonal antibodies (mAb's) (11). We further aimed to compare the properties of selected phage-displayed peptides and sequence identical synthetic peptides for inhibition of antibody–allergen interactions. The phage displayed peptides were examined for their ability to induce an allergen-specific immune response in mice (12, 13).

MATERIALS AND METHODS

mAb's

mAb's BIP1 and BIP4 were raised by immunizing Balb/c mice with birch pollen extract (Allergon AB, Engelholm, Sweden) as described earlier (11).

Phage libraries

mAb ligands were selected from random phage libraries expressing linear ('pVIII 9aa') (31) or circular ('pVIII 9aa.Cys') (27) nonapeptides fused to pVIII of the filamentous bacteriophage fd. Libraries were kindly provided by IRBM (Istituto di Ricerche di Biologia Molecolare P. Angeletti SPA, Rome, Italy).

Biopanning

Three rounds of biopanning were performed with decreasing amounts of monoclonal antibodies for coating (1.0, 0.1, 0.01 µg per well). Enzyme-linked immunosorbent assay

(ELISA) plates (Nunc, Roskilde, Denmark) were coated with BIP1 or BIP4 mAb's in bicarbonate buffer, pH 8.5, overnight at 4°C in a humid chamber. Wells were blocked with phosphate-buffered saline (PBS) containing 3% (w/v) bovine serum albumin (BSA) for 1 h at 37°C in a humid chamber. 10¹¹ plaque-forming units (pfu) each of freshly prepared wild-type phages of the circular and linear libraries were added together to each well and incubated at 37°C. Unbound phages were removed by washing with Tris-buffered saline (TBS)/0.5% Tween 20. Bound phages were eluted with 0.1 M HCl pH 2.2 containing 1 mg/ml BSA. The eluted phages were immediately neutralized and used for further biopannings.

Amplification and preparation of phages

Escherichia coli XL-1 Blue were grown in SB medium to an OD₆₀₀=1 and infected with eluted phages after biopanning by incubation for 15 min at room temperature (RT). For titer determination of infected *E. coli*, aliquots were plated in serial dilutions on LB plates containing carbenicillin (100 µg/ml). The infected XL-1 Blue culture was grown in SB medium containing carbenicillin (50 µg/ml) and tetracycline (10 µg/ml), 2 h at 37°C. After infection with helper phage VCSM13 (10¹² pfu/ml), the culture was incubated for an additional 2 h at 37°C. Kanamycin (70 µg/ml) was added and the culture was further incubated at 37°C, overnight. Phages were harvested by precipitation with 4% (w/v) polyethylene glycol 8000, 3% (w/v) NaCl on ice for 30 min. After centrifugation, pellets were resuspended in PBS containing 0.15% casein and again centrifuged. Supernatants were immediately used for biopanning experiments.

Colony screening assay

After each biopanning round, infected *E. coli* XL-1 Blue were randomly picked from LB/carbenicillin plates, transferred to new LB/carbenicillin plates, and grown overnight at 37°C. LB plates with colonies were incubated with isopropyl-β-D-thiogalactopyranoside-treated nitrocellulose. Colonies were then transferred with the nitrocellulose to fresh LB/carbenicillin plates and incubated at 30°C overnight. Filters were blocked with 50 mM Tris, 150 mM NaCl, 5 mM MgCl₂, 3% (w/v) BSA, pH 8.0, 30 min at RT and incubated with 50 mM Tris, 150 mM NaCl, 5 mM MgCl₂ containing 400 µg/ml lysozyme (Sigma, St. Louis, Mo.), 20 U/ml DNase (Boehringer-Mannheim, Mannheim, Germany) for 1 h at RT. Filters were saturated with PBS containing 0.5% (wt/vol) bovine serum albumin, and 0.5% (vol/vol) Tween 20 for 1 h at RT. Immunoscreeings with antibodies and isotype control antibodies BIP1 or BIP4, respectively, or with BIP3 (directed against 32 to 68 kDa birch pollen allergens) were performed as described in section 'SDS-PAGE and immunoblotting'. Clones positive in colony screening assay were reamplified in overnight cultures and stored in 20% glycerol at –70°C.

DNA sequencing

Transfected XL-1 blue bacterial clones positive in the colony screening assay were amplified in overnight cultures. After lysing cells, DNA was isolated by precipitation with polyethylene glycol 8000 (Amresco, Ohio). DNA sequencing was performed by the Sanger dideoxy method using a Thermo Sequenase Cycle Sequencing Kit (Amersham, Little Chalfont, England) with fluorescence-labeled primer 5'-GCT TTA CAC TTT ATG CTT-3' (MWG Biotechnik, Ebersberg, Germany). Sequencing was performed and analyzed by a LI-COR DNA sequencer 4000L (LI-COR Inc., Lincoln, Nebr.).

TABLE 1. Monitoring of phage titers (pfu/ml) during panning experiments			
moAbs	Biopanning		
	Round 1	Round 2	Round 3
BIP1	1.4×10^6	1.5×10^6	4.9×10^8
BIP4	1.6×10^6	1.3×10^7	5.3×10^8

Synthetic peptides

Peptides were synthesized by piChem (Graz, Austria) and purified by high-performance liquid chromatography. For BIP1: CFPYCYPSESA (cyclo 1–5 11-mer); for BIP4: CRQTRTMPGC (cyclo 1–10 10-mer); for controls, a cyclo 1–6 11-mer (CHKLRCDKAIA) and a cyclo 1–10 10-mer (CAISGGYPVC) were synthesized.

ELISA inhibition with purified specific phage clones

ELISA plates (Greiner, Kremsmünster, Austria) were coated with purified BIP1 or BIP4 mAb at a concentration of 1 µg/ml PBS (6 nmol per well) overnight at 4°C, washed with PBS/0.05% (v/v) Tween 20 (PBS/Tween), and saturated with PBS/Tween containing 1% (w/v) BSA for 30 min at RT. Phages were applied in duplicates at 10^7 pfu/ml PBS/Tween/1% BSA for 3 h at 4°C. As controls, preparations containing identical amounts of phages of the original circular and linear library, helper phage VSCM13, and a nonrelated phage clone from a different series of biopannings were used. A dilution series was prepared with rBet v 1 (Biomay Biotechnik Produktions- und Handels GesmbH, Linz, Austria) in PBS (1 mg/ml–0.1 mg/ml) and 1 µl of each dilution was added per 100 µl (corresponding to 60 to 0.06 nmol rBet v 1 per well). As control, recombinant Bet v 2 (profilin) (Biomay Biotechnik Produktions- und Handels GesmbH) was used at identical concentrations. After extensive washing with PBS/Tween, a peroxidase-conjugated sheep anti-M13 phage Ab (Pharmacia, Uppsala, Sweden) was applied for 90 min at RT. The reaction was developed with 2,2'-azino-bis(3-ethylbenzthiazoline) sulfonic acid (Sigma) as substrate. Optical density was measured in an ELISA Reader (Dynatech, Denkendorf, Germany) at 405–490 nm.

NMR spectroscopy

The nuclear magnetic resonance (NMR) spectra were recorded at 600.13 MHz (Bruker DRX 600) from approximately

TABLE 2. Analysis of phage clones positive in colony screening assay with BIP1 by DNA sequencing			
Biopanning Round no.	Colonies		
	Positive/ tested	Deduced aa sequences	Classification
1	1/99	CLYCFHSLYAS	Cyclo (1-4)-11mer
2	2/131	CFPYCYPSESA	Cyclo (1-5)-11mer
3	33/50	CFPYCYPSESA	Cyclo (1-5)-11mer
		CFPYCYPSESP	Cyclo (1-5)-11mer
		CFPYCYPPSLP	Cyclo (1-5)-11mer
		CFPYCYPPSLR	Cyclo (1-5)-11mer

TABLE 3. Analyses of phage clones positive in colony screening assay with BIP4 by DNA sequencing			
Biopanning Round no.	Colonies		
	Positive/ tested	Deduced aa sequences	Classification
1	0/50		
2	0/105		
3	1/112	CRQTRTMPGC	Cyclo (1-10)-10 mer

5 mM solutions in DMSO-d₆ at 300 K in 5 mm tubes unless indicated otherwise. For the COSY (correlated spectroscopy) experiments (phase-sensitive, double quantum-filtered, coherence selection by pulsed field gradients), standard software as supplied by the manufacturer was used (33).

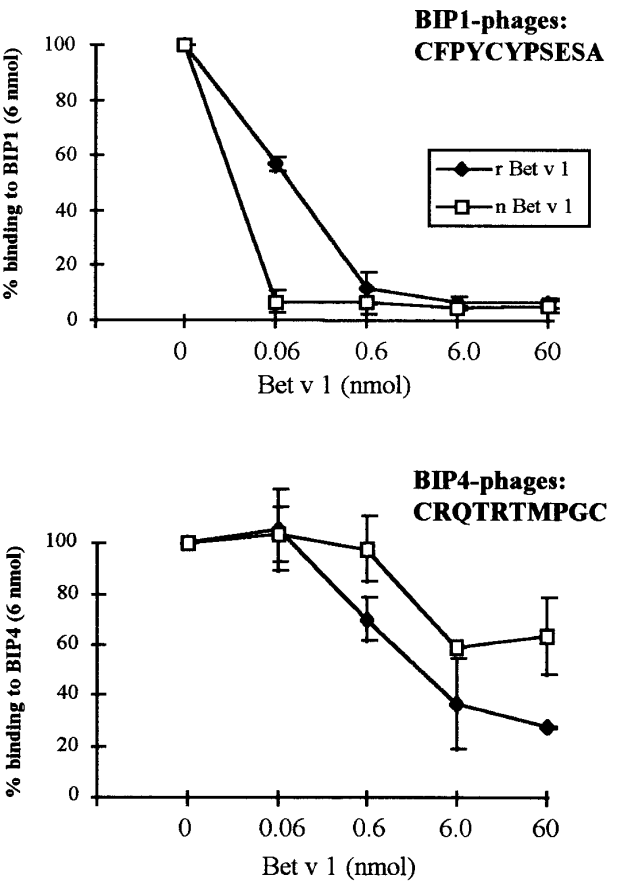


Figure 1. Natural (nBet v 1) and recombinant Bet v 1 (rBet v 1) inhibit competitively the interaction of monoclonals BIP1 and BIP4 with phages expressing peptides CFPYCYPSESA or CRQTRTMPGC. ELISA plates were coated with monoclonals and incubated with 10^6 pfu phages per well. nBet v 1 or rBet v 1 were added simultaneously. Bound phages were detected by peroxidase-conjugated sheep anti-M13 phage antibody. The signal intensity obtained with the uninhibited phage preparation was set equal to 100% and the values compared. No binding was observed with the amplified original libraries and a nonrelated phage clone from a different set of experiments. Using recombinant profilin for control, no inhibition could be achieved.

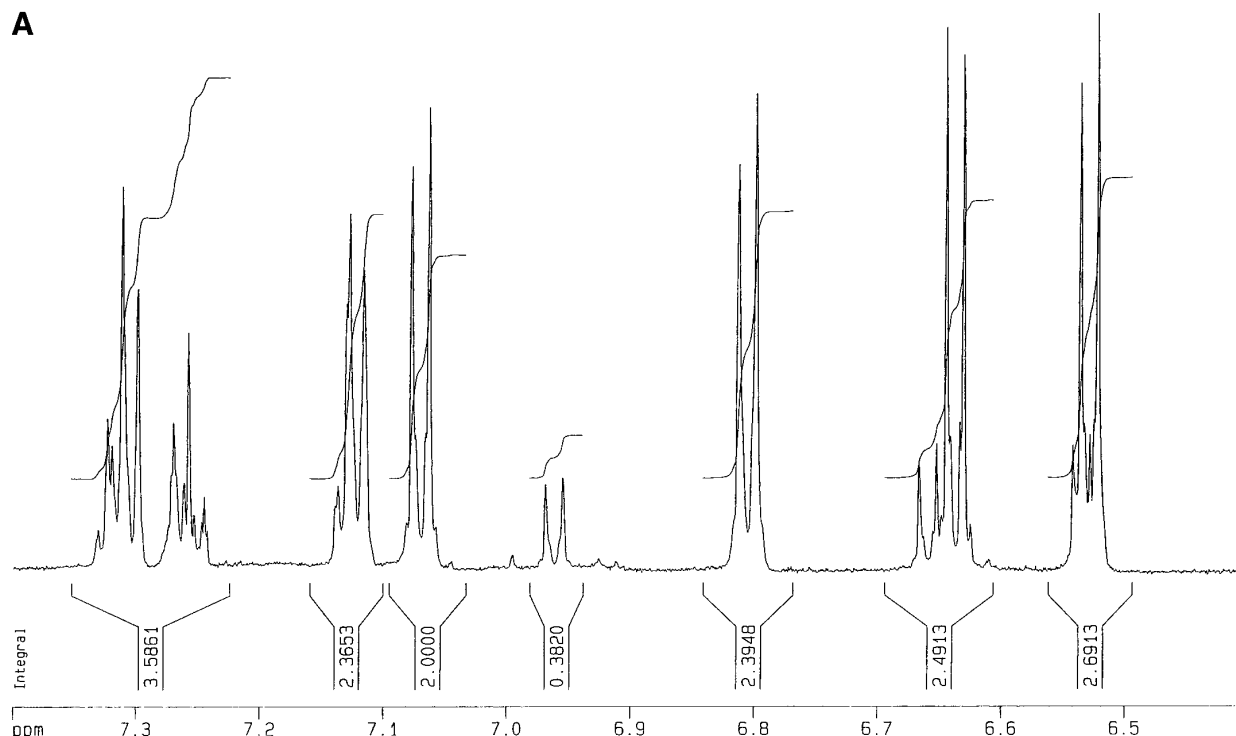
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Figure 2. (See next page for legend.)

Oral immunization of Balb/c mice with BIP1-phages

Balb/c mice, 5–6 wk of age, were used for oral immunization with phages (Biovendor Biotechnology, Brno, Czech Republic). For intragastral administration, 2×10^8 pfu phage were diluted to a volume of 200 μ l in PBS and delivered directly to the stomach by a blunt steel feeding tube. Three groups (of four mice each) were given gavages on day 0, 7, 14, and 35 with BIP1-phages, with wild-type phages or PBS. Blood samples from the tail vein were taken on day 0 (preimmune serum) and on day 49.

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 15% gels under reducing conditions. Blot strips were saturated with PBS containing 0.5% (w/v) BSA, and 0.5% (v/v) Tween 20. Mouse sera (1:250 in blocking buffer) were applied overnight at 4°C. Bound mouse IgG was detected by ^{125} I-labeled sheep anti-mouse IgG (Amersham International Ltd., Amersham, U.K.). Blots were then washed, dried, and exposed to Kodak X-omat film at -70°C .

RESULTS

Specific increase of phage titers during biopanning with BIP1 and BIP4 mAb's

Monoclonal antibodies BIP1 and BIP4 to Bet v 1 (the major birch pollen allergen) were used for biopanning experiments in decreasing amounts from the first to the third round of biopanning. With both mAb's, an increase of phage titers from the first to

the third round were observed (**Table 1**), indicating amplification of phages expressing specific ligands during pannings. This increase of phage titers was associated with an increase of phages binding to BIP1 or BIP4 in immunodot experiments. No binding was observed on dotted isotype control antibodies with equal amounts of wild-type phage or helper phage VSCM13 (data not shown).

Colony screening and sequence analysis

The specificity of selected BIP1 or BIP4 phages was further demonstrated by a colony screening assay. Only XL-1 Blue clones expressing the BIP1-specific phage were recognized by BIP1, but not by BIP4 or BIP3 (11). BIP4 clones were exclusively recognized by BIP4, but not by isotype controls. DNA sequencing of positive clones revealed that biopannings with BIP1 mAb favored selection of cyclo (1–4)- or cyclo (1–5)-11 mer peptides (**Table 2**). After the third panning, the sequence CFPYCYPSESA was found in 7 of 10 analyzed clones.

With BIP4, only one positive colony from 112 tested was detected after the third biopanning round (**Table 3**). DNA sequencing of the positive clone showed the insert CRQTRTMPGC, a cyclo (1–10)-10 mer peptide.

We compared the sequences of the inserts with the sequence (6) and 3-dimensional structure (9) of the Bet v 1 molecule using the Swiss Pdb Viewer software program (Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development SA, Ge-

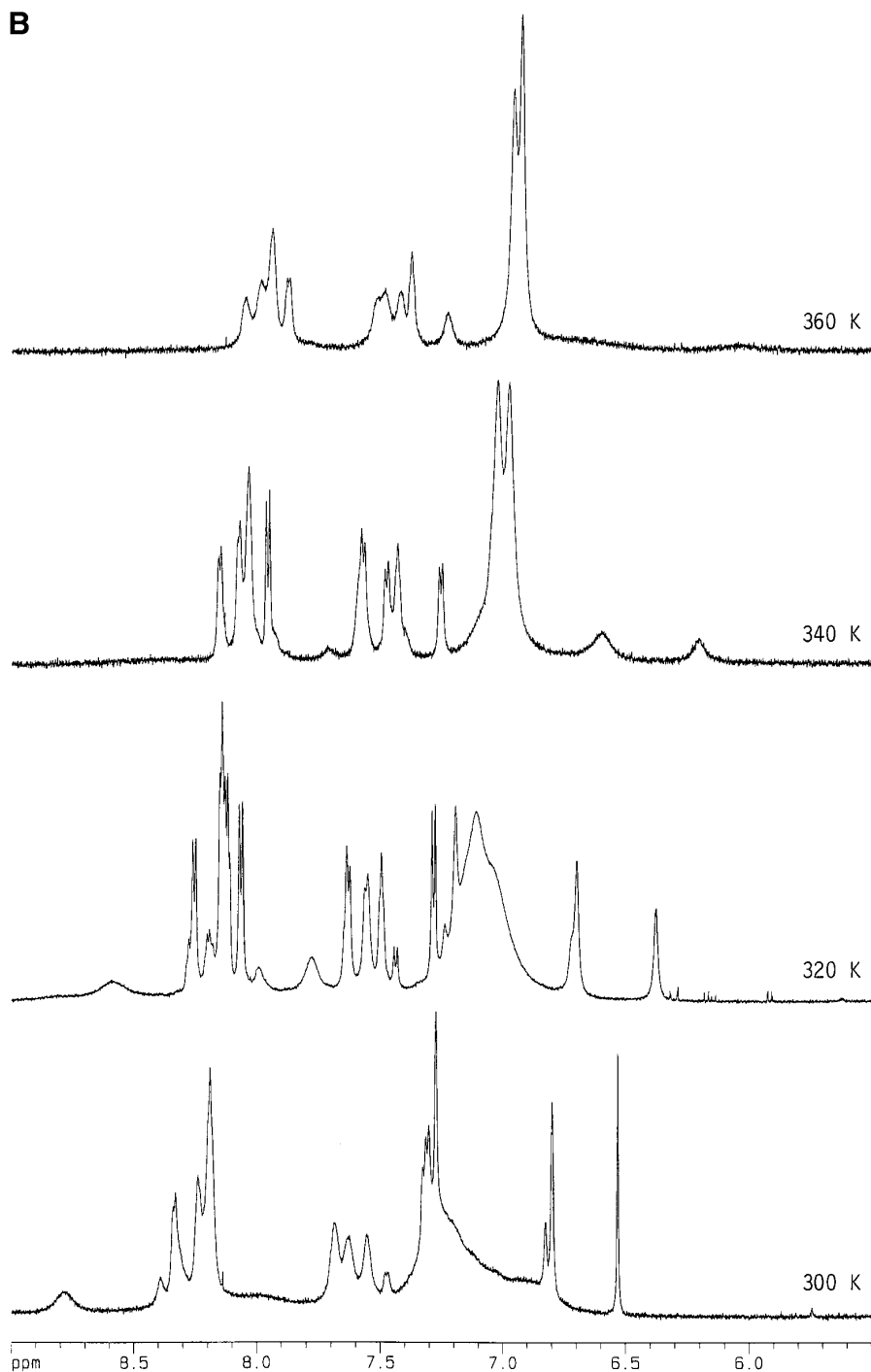


Figure 2. ^1H -NMR spectroscopic analysis of synthetic BIP1 and BIP4 oligopeptides defined by phage display. A) Aromatic region of the 600 MHz ^1H -NMR spectrum of BIP1 oligopeptide (CFPYCYPSESA, 1–5 cyclo) in DMSO-d_6 at 300 K; ^1H -NMR exhibits severe line broadening for some of the amide protons and at least two sets of signals in the aromatic region of the spectrum of the BIP1 oligopeptide. B) Amide region of the 600 MHz ^1H -NMR spectrum of BIP4 oligopeptide (CRQTRTMPGC) in DMSO-d_6 at various temperatures. Spectra were recorded in the region from 300 to 360 K. The observed differences indicate conformational changes. The spectra remain complex even at high temperatures and cannot be interpreted in a straightforward manner.

neva, Switzerland). On the exposed loop between beta-strands 3 and 4, i.e., between aa F⁵⁸ and Y⁶⁶ (. . .FPEGFPFKY. . .), we found similarities to the sequence of the BIP1 ligand. However, no alignments were possible for the BIP4 ligand.

Binding of phage ligands to BIP1 or BIP4 inhibited by natural Bet v 1 or rBet v 1 in competitive ELISA

BIP1- and BIP4-specific phage were captured by the respective mAb's in ELISA experiments and detected

by a rabbit anti-phage Ab (**Fig. 1**). Maximal reactivity was observed when using 10^7 phage pfu/ml. This reactivity could be inhibited by addition of rBet v 1 as well as natural Bet v 1 (nBet v 1), representing a mixture of isoforms, in a dose-dependent manner. Incubation of antibodies with phages prior to application of Bet v 1 or the reversed order of application did not influence the pattern of reactivity. At equimolarity of BIP1 mAb with rBet v 1 or with nBet v 1, phage-Ab interactions were abolished. No inhibition was achieved when using recombinant birch profilin

(Bet v 2) for control experiments. Moreover, using synthetic oligopeptide mimotopes CFPYCYPSESA for BIP1, or CRQTRTMPGC for BIP4, we were not able to show inhibition in ELISA.

Using BIP4 as catching Ab a specific and significant reduction of phage-Ab interaction by rBet v 1 and nBet v 1 could be observed (Fig. 1). However, neither the addition of equimolar doses nor a 10-fold molar excess of rBet v 1 or nBet v 1 was capable of blocking phage/Ab interactions completely.

NMR spectroscopy of synthetic peptides CFPYCYPSESA (BIP1) and CFPYCYPSESA (BIP4)

As judged by their ^1H -NMR spectra, the oligopeptides in question were not conformationally homogeneous at 300 K in DMSO-d_6 solution. A severe line broadening for some of the amide protons and at least two sets of signals in the aromatic region of the spectrum of BIP1 oligopeptides were observed (Fig. 2A, B).

In the case of CFPYCYPSESA (BIP1), two AB systems (Y^4 and Y^6 , 4 protons each) as well as a system of higher order for the monosubstituted aromatic moiety of F^2 (5 protons) had to be expected. The corresponding resonances could be found in the region between 6.4 and 7.4 ppm, and the respective connectivities were easily established by means of a COSY experiment. However, in addition to the main component, a second conformation was present according to the small doublets centered at about 6.66 and 6.96 ppm. The 2-dimensional technique mentioned above proved these signals to be connected via scalar couplings and thus to represent the AB system of one of the tyrosine residues of the minor conformation. The resonances of the remaining aromatic signals of the minor component were obscured by those of the major conformation, as could be seen by inspection of the integral values. From a rough estimation, the minor component was present in an amount of approximately 10–20% (Fig. 2A).

The situation was even more obscure with CRQTRTMPGC (BIP4) from an NMR spectroscopic point of view owing to the lack of aromatic signals and the presence of many amino acids containing additional NH and OH groups (R^2 , R^5 , Q^3 , T^4 , T^6). This led to a spectrum completely uninterpretable in terms of amide signal counting. The integral values, however, again indicated the presence of more than one species in solution. To explore the effect of variation of the temperature upon the behavior of CFPYCYPSESA (BIP4), spectra were recorded in the region from 300 to 360 K (Fig. 2B). The differences observed indicated conformational changes, but the spectra remained complex even at high temperatures and could not be interpreted in a straightforward manner. The fact that the ^1H -NMR spectrum of CRQTRTMPGC (BIP4) returned to its original shape

after recooling to 300 K also supported the assumption that no defined tertiary structure was present that would most likely be destroyed irreversibly upon heating to 360 K.

Induction of IgG-response to Bet v 1 in Balb/c mice by intragastral administration of BIP1-phages (CFPYCYPSESA)

Two of the four Balb/c mice orally immunized with BIP1-specific phages (CFPYCYPSESA) developed an IgG response to the 17 kDa protein (natural Bet v 1) from birch pollen extract (Fig. 3, lane 2). Reactivity was weaker as compared to the binding of mouse IgG raised in Balb/c mice by conventional i.p. immunization with rBet v 1 (Fig. 3, lane 3). Sera of mice from the wild-type phage or PBS control groups did not show reactivity (data not shown). Assuming a maximum number of 2700 peptide copies per phage, only nanogram quantities of the mimotope were fed to each mouse.

DISCUSSION

To our knowledge, this is the first report on the use of phage display techniques in molecular allergology. Our results provide evidence that filamentous phages can serve as vectors for the induction of a B cell re-

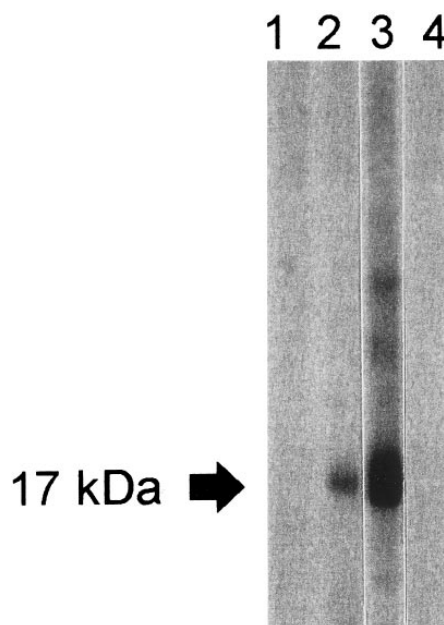


Figure 3. Immunoblot on separated birch pollen extract with mouse sera. Lane 1: preimmune serum, lane 2: mouse IgG directed against birch pollen major allergen Bet v 1 (17 kDa) after intragastral immunization with BIP1 phages expressing a mimotope for Bet v 1 (serum 1:250), lane 3: mouse IgG after i.p. immunization with rBet v 1 (1:1000). Lane 4: buffer. Bound IgG was detected by ^{125}I -labeled sheep anti-mouse IgG; the autoradiogram is shown.

sponse to conformational epitopes on the major birch pollen allergen Bet v 1. We show here that this type of application is superior to the use of synthetic peptides, since synthetic peptides are conformationally unstable in solution, as shown by NMR spectroscopy. By phage display technique, we selected ligands for the anti-Bet v 1 mAb's BIP1 and BIP4 (11), that react with distinct epitopes on the Bet v 1 molecule (12, 13). Neither BIP1- nor BIP4-specific ligands were recognized by IgE from a pool of birch pollen-allergic patients' sera highly reactive with Bet v 1 (data not shown), indicating that the mimotopes selected did not represent IgE epitopes.

The deduced aa-sequences (CFPYCYPSESA for BIP1; CRQTRTMPGC for BIP4), showed that both mimotopes were derived from the 'pVIII 9aa.Cys' library expressing peptides constrained by a disulfide bond (27), and most likely represented discontinuous epitopes. Sequence variations as seen for the peptide selected by BIP1 may occur during the construction of the library (F. Felici, personal communication). Sequence similarities to Bet v 1 were found only for the BIP1 peptide in the area between F⁵⁸ and Y⁶⁶, which forms an accessible loop between beta-strands 3 and 4 (9).

Specific inhibition of interactions between mAb's (BIP1 and BIP4) and rBet v 1 or nBet v 1, respectively, was achieved in a dose-dependent manner in ELISA. At equimolar concentration of BIP1 and Bet v 1, the mAb's showed higher affinity to the allergen than to specific phages. However, the number of copies of peptides on the phage surface is not precisely known (Dr. Franco Felici, personal communication), and therefore we could not determine binding affinities. Thus, it seemed reasonable to approach the question by use of synthetic oligopeptides for BIP1 and BIP4. However, sequence identical peptides were unable to inhibit antibody-allergen interactions due to the adoption of different conformations in solution, as shown by NMR spectroscopy. These analyses showed that the oligopeptides were conformationally inhomogeneous. The reason for the internal mobility of peptides CFPYCYPSESA (BIP1) and CRQTRTMPGC (BIP4) obviously resulted from the combination of small cycles that prevent random coil behavior and the presence of proline residues being prone to *cis/trans* isomerization. Similar situations have long been known in connection with small cyclic peptides (34, 35). The presence of several species of peptides might explain their ineffectiveness in inhibition experiments. Only the peptides together with the phage surface represented perfect mimics of the natural epitope. We conclude that the peptides fused to pVIII might, in connection with the hydrophobic virion surface, adopt a rigid conformation resembling the epitope, thereby enhancing affinities.

Another strong indication for correct mimicking of the Bet v 1 epitopes by the phage-fused peptides was

obtained by immunization experiments. Immunizations with phagotypes have been performed previously i.p., leading to an immune response to the natural antigen as has been shown, for example, for a hepatitis B virus antigen (32). Recently the first report on oral administration of phages for immunization was published (36). We could show that intragastric administration of phages expressing only nanogram amounts of a mimotope for Bet v 1 could induce IgG response to the natural allergen in birch pollen extract. This could be achieved without the use of mucosal adjuvants in two of four mice (Fig. 3). The mechanism of presentation of the peptide fused to the phages from the pVIII 9aa.Cys library remains, however, little understood.

Mimotopes for immunogenic determinants on allergens may therefore be candidates for new therapeutic concepts in allergy. Allergen peptides fused to the phage surface are more stable, and are most likely resistant to the gastrointestinal environment. Moreover, we demonstrate here that phages expressing mimotopes for Bet v 1 are capable of inducing IgG responses to the natural allergen. This suggests a possible application of filamentous phages as vectors for induction of nonanaphylactogenic 'blocking antibodies' by oral immunization in humans. **[F]**

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