

Dimerization of the Major Birch Pollen Allergen Bet v 1 Is Important for its In Vivo IgE-Cross-Linking Potential in Mice¹

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In type I allergy, the cross-linking of membrane IgE on B lymphocytes and of cytophilic IgE on effector cells by their respective allergens are key events. For cross-linking two IgE molecules, allergens need at least two epitopes. On large molecules, these could be different epitopes in a multivalent, or identical epitopes in a symmetrical, fashion. However, the availability of epitopes may be limited on small allergens such as Bet v 1, the major birch pollen allergen. The present work analyzes whether dimerization is required for the cross-linking capacity of this allergen. In immunoblots, murine monoclonal and polyclonal human Bet v 1-specific Abs detected, besides a Bet v 1 monomer of 17 kDa, a dimer of 34 kDa. In dynamic light scattering, Bet v 1 appeared as dimers and even multimers, but a single condition could be defined where it behaved exclusively monomerically. Small-angle x-ray scattering of the monomeric and dimeric samples resulted in diagrams agreeing with the calculated models. Circular dichroism measurements indicated that the structure of Bet v 1 was preserved under monomeric conditions. Skin tests in Bet v 1-allergic mice were positive with Bet v 1 dimer, but remained negative using the monomer. Furthermore, in contrast to dimeric Bet v 1, the monomer was less capable of activating murine memory B cells for IgE production in vivo. Our data indicate that the presentation of two identical epitopes by dimerized allergens is a precondition for cross-linking of IgE on mast cells and B lymphocytes. *The Journal of Immunology*, 2005, 175: 6645–6650.

The cross-linking of IgE Abs by allergens is a key event in the sensitization phase as well as the effector phase of type I allergy. Only upon cross-linking is the BCR able to enter lipid rafts and initiate signaling in mature B cells (1), and after cross-linking of at least two FcεRI-bound IgE molecules, mast cells and basophils are triggered to release mediators responsible for the allergic reactions (2–4). Successful cross-linking depends on at least two critical requirements. First, most allergens are nonsymmetrical molecules, probably representing different conformational epitopes in a multivalent manner, even though there are some examples of allergens presenting repetitive epitopes (5, 6). Therefore, they can only cross-link IgE Abs harboring the correct complementary paratopes. In this model the presence and vicinity of different IgE specificities bound to FcεRI on effector cells have to coincide with the respective fitting epitopes on the allergen. Second, there is a discrepancy in the sizes of allergens

and Abs. The IgE Ab has a M_r of 188 kDa, thus by far exceeding that of allergens, for instance of the major birch pollen allergen Bet v 1 (17 kDa), the panallergen profilin (14 kDa), some lipid transferase proteins (9 kDa), the ragweed pollen allergens Amb a 5 and Amb t 5 (5 and 4.4 kDa, respectively), and bee venom melittin Api m 4 (3 kDa). This means that these small molecules have to present at least two epitopes for cross-linking the IgE, which could be difficult from a stereochemical point of view. Moreover, through the absence of a hinge region, IgE is rather rigid, and its flexibility is limited when bound to FcεRI, where IgE is fixed in a bent shape (7, 8). The cross-linking situation is even more stringent in the case of B lymphocyte activation, because each B cell expresses only one type of Ig with one epitope specificity.

From these arguments the question arises, if there is an additional mechanism for making cross-linking effective, such as dimerization. Thereby, even epitopes on small allergens could achieve cross-linking capacity. The occurrence of Bet v 1-dimers in pollen extracts as well as in recombinant allergen preparations has previously been observed in SDS-PAGE gels and Western blots (9–13). Furthermore, many important allergens are known to appear as homodimers or -oligomers in nature, e.g., Phl p 1 (14), Phl p 5b (15), Phl p 7 (16), and Phl p 11 (17) from grass pollen; Fel d 1 from cat dander (18); parvalbumin from cod (19); the panallergen tropomyosin (20, 21); Api m 4 from bee venom (22); Equ c 1 from horse (23, 24); Sol i II from fire ant (25); Ara h 1 (26, 27) and Ara h 2 (28) from peanut; ABA-1 from *Ascaris* (helminth) (29, 30); bovine β-lactoglobulin (31–34) and bovine dander allergen (35); BGP-2 from Bermuda grass pollen (36); or Ves v 5 from wasp venom (37).

Applying human polyclonal Bet v 1-specific IgE for B cell epitope mapping using a phage display library, we isolated mimotopes of one predominant Bet v 1 epitope. This could mean that a polyclonal B cell response might also be restricted due to a small

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Received for publication November 8, 2004. Accepted for publication August 25, 2005.

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¹ This work was supported by Austrian Science Fund Grant F01808-B04 and in part by Grants F01808-B05 and 14339-B13. The DLS investigations were supported by grants from RiNA (Berlin, Germany) and Deutsche Forschungsgemeinschaft (BE1443/9-1).

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number of available epitopes on the allergen. In accordance, purified polyclonal Bet v 1-specific IgE behaves like a mAb in affinity determination using the surface plasmon resonance technique (38).

Our working hypothesis in this study was that dimerization or oligomerization could be an additional explanation for the cross-linking of cell-bound IgE Abs. Taking the birch pollen major allergen Bet v 1 as a model, we analyze in this study whether monomeric or dimeric conditions can be defined for this allergen and whether the monomer possesses any cross-linking capacity.

Materials and Methods

SDS-PAGE and Western blotting

Western blotting was performed as described previously (39). Briefly, rBet v 1 was separated on 12% SDS-PAGE gels under nonreducing conditions and silver stained. For immunoblots, all blocking, diluting, and washing steps were performed with blotting buffer (50 mM sodium phosphate buffer (pH 7.5), 0.5% Tween 20, and 0.5% BSA). Mouse anti-Bet v 1 mAb BIP1 (40) was diluted 1/100, human sera was diluted 1/20, and they were incubated overnight at 4°C. Bound Ig was detected with ¹²⁵I-labeled sheep anti-mouse Ig or with ¹²⁵I-labeled anti-human IgE. A control immunoblot was developed using ECL detection (ECL detection reagents; Amersham Biosciences) according to the manufacturer's instructions. Reactions were visualized by exposing blots to Biomax-MS films (Eastman Kodak).

Dynamic light scattering (DLS)³

Recombinant Bet v 1a (lot 18; Biomay) was analyzed by DLS measurements at 20°C. All reagents were filtered using 0.22-μm pore size cutoff filters, and the protein solutions were passed through 0.1-μm mesh size microcentrifuge filters. The sample solution (25 μl) was carefully transferred to a cylindrical cuvette, taking care to avoid bubble formation in the solution. A protein concentration of 4 mg/ml was used. Equal volumes of samples were adjusted to a range of pH from 1.2 to 9.2 (in steps of 1.0) by mixing it with buffer (50 mM Tris-HCl). A series of measurements with a sampling time of 20 s and a wait time of 1 s was conducted using a Spectroscatter 201 (RiNA). A diode laser of wavelength 680 nm was used as the source. The scattered light was collected at a fixed angle of 90° by a lens system and directed through a glass fiber to a photomultiplier. Its output pulses were processed by the autocorrelator. The resulting autocorrelation functions were analyzed with the program CONTIN to obtain hydrodynamic radius (R_H) distributions. The R_H is related to the diffusion coefficient by the Einstein-Stokes equation: $DT = k_B T / 6\pi\eta R_H$, where k_B is the Boltzmann constant, T is the temperature in Kelvin, and η is the solvent viscosity.

Small-angle x-ray scattering (SAXS)

SAXS measurements were performed with Bet v 1 stock solution (20 mM PBS (pH 6.5) and 2.5 mg/ml) using a SAXSess system (Anton Paar) with slit collimation and an elliptically bent multilayer mirror combined with a PW3830 x-ray generator and a Fuji BAS 1800 image plate detector. The experimental scattering functions were evaluated by the generalized indirect Fourier transformation. The resulting pair distance distribution functions (41) were compared with the theoretical functions of the monomer and the dimer calculated from the crystal structure data (42) using a recent upgrade of the program MULTIBODY.

Circular dichroism (CD) experiments

The lyophilized Bet v 1 protein (~1.0 mg) was dissolved in 100 μl of MilliQ water. Aliquots of 8 μl were added to 392 μl of PBS with pH 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, and 8.2. CD measurements were conducted on a Jasco J-715 spectropolarimeter using a 0.1-cm path length. The sample was investigated at 22°C, using the following parameters. Spectra were recorded at a wavelength range from 185 to 260 nm with 0.2 nm resolution at a scan speed of 50 nm/min and resulted from averaging three scans per pH point. For all pH values, except pH 1.2 and 2.2, the samples were mixed and incubated at 4°C for at least 15 min before CD measurement. The samples at pH 1.2 and 2.2 were measured immediately after mixing.

Sensitization of mice

Four- to 6-wk-old female BALB/c mice (Institute for Laboratory Animal Science and Genetics, University of Vienna) were used and treated according to European Community rules of animal care (43) with the permission of the Austrian Ministry of Science. Mice ($n = 8$) were sensitized to Bet v 1 by two i.p. injections of 2 μg of rBet v 1 in 50 μl of PBS adsorbed to 100 μl of Al(OH)₃ (Serva) on days 0 and 14. Blood samples were taken on days 0 (preimmune), 7, 21, and 35 and were screened for Bet v 1-specific IgG1 and IgE (sera diluted 1/100 and 1/10, respectively) by ELISA, as described previously (39).

Skin tests of mice

On day 42, 100 μl of Evans Blue (5 mg/ml PBS; Merck) was injected i.v. into the tail vein. Subsequently, 30 μl of the following test substances (filtered through 0.22-μm pore size sterile filters; Corning) were administered intradermally into the shaved abdominal skin: compound 48/80 (20 μg/ml; Sigma-Aldrich) as the positive control, PBS as the negative control, rBet v 1 (3 μg/ml Tris-HCl (pH 6.2) and 4% glycerol), rBet v 1 (3 μg/ml PBS), and Tris-HCl plus 4% glycerol. As another positive control, we first injected Tris-HCl and 4% glycerol, then after ~15 min administered rBet v 1 in PBS at exactly the same test point. After 20 min, mice were killed and skinned. The diameter of color reaction was measured on the inside of the abdominal skin. The color intensity was determined using a hand-held reflection densitometer (Vipdens). Skin reactivity indices were calculated from the diameter of skin reactivity × densitometric signal intensity, as described previously (44, 45).

Treatment of sensitized mice with monomeric or dimeric Bet v 1

For the treatment experiments with monomer and dimer, mice were sensitized as described above ($n = 5$ mice/group). Thereafter, treatment was performed by two i.p. applications of monomeric or dimeric Bet v 1 without adjuvant (each 3 μg of Bet v 1/100 μl) on days 28 and 35. Sera were taken before (preimmune) and at 7-day intervals after each immunization and evaluated for Bet v 1-specific IgE in ELISA.

Statistical analysis

Statistical comparison of the IgE values from the treatment groups was performed by Mann-Whitney U test with the use of SPSS software version 11.5 for Windows (SPSS). A value of $p \leq 0.05$ was considered statistically significant.

Results

Immunoblot experiments

To demonstrate whether Bet v 1 monomers and oligomers are present in our rBet v 1 preparation, we performed SDS-PAGE experiments. Silver staining of immunoblots revealed bands at 17, 34, and 68 kDa. In immunoblots, these bands were recognized by patients' IgE and mAb BIP1, identifying these proteins as Bet v 1 monomer, dimer, and tetramer (Fig. 1).

Dynamic light scattering

To determine the behavior of rBet v 1 in solution, we performed DLS measurements. In a wide pH range (Table I), Bet v 1 was present as a mixture of monomers, dimers, and oligomers, at least at the protein concentrations required for DLS. For example, diluting Bet v 1 at a pH of 6.2 rendered a peak at the calculated dimer size and, in addition, a higher M_r fraction (Fig. 2A). The addition of glycerol and filtering through 0.22-μm pore size meshes defined the unique condition, where Bet v 1 behaved monomerically (Fig. 2B). Fig. 2C demonstrates that a relatively slight pH shift to 7.2 in the same buffer already changed the spectrum dramatically, rendering higher M_r aggregates. Thus, in this experimental setting, Bet v 1 occurred as a monomer only under very restricted conditions.

SAXS

To control whether Bet v 1 truly behaved monomerically under the experimental conditions defined by DLS, we performed SAXS experiments. For comparison, the theoretical curves of a Bet v 1 monomer and dimer were calculated based on the x-ray crystallographic data for Bet v 1. The angular distribution of scattered

³ Abbreviations used in this paper: CD, circular dichroism; DLS, dynamic light scattering; R_H , hydrodynamic radius; SAXS, small angle x-ray scattering.

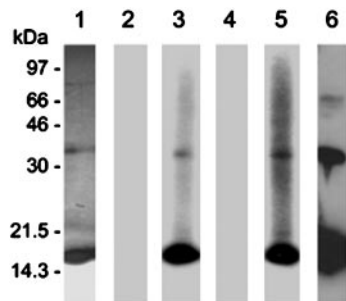


FIGURE 1. Mouse monoclonal IgG1 as well as human polyclonal IgE Abs recognize Bet v 1 oligomers in immunoblots. Recombinant Bet v 1 was separated in SDS-PAGE. Part of the gel was silver stained (*lane 1*). The other part was blotted onto nitrocellulose and incubated with a pool of human sera from nonallergic individuals (*lane 2*), a pool of human sera from Bet v 1-allergic patients (*lane 3*), buffer (*lane 4*), and the anti-Bet v 1 mAb BIP1 (*lane 5*). Murine IgG was detected by radiolabeled anti-mouse Ig Abs, and human IgE was detected by radiolabeled anti-human IgE Abs. Using more sensitive ECL (*lane 6*), mouse mAb BIP1 detected protein bands at 17 kDa (monomer), 34 kDa (dimer), and ~68 kDa (tetramer).

intensity was transformed into the pair distance distribution function $p(r)$. Fig. 3 shows that diluting Bet v 1 in Tris-HCl with glycerol at pH 6.2 indeed resulted in a monomeric sample; otherwise, the $p(r)$ agreed with the theoretical curve for a dimer (Bet v 1/HR, Bet v 1 in histamine release buffer; Bet v 1/PBS, Bet v 1 diluted in PBS).

CD experiments

In CD experiments we examined whether pH 6.2 and the addition of glycerol affect the secondary structure of Bet v 1. The protein turned out to be very stable at a wide pH range; between 2.9 and 8.2, no significant change in its secondary structure (dominated by

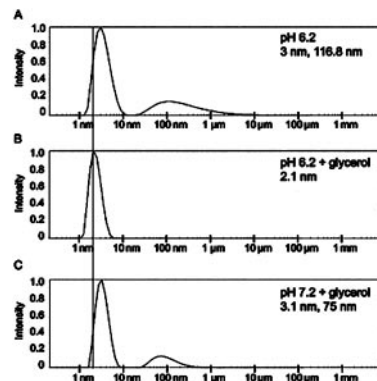


FIGURE 2. Determination of monomeric conditions for Bet v 1 by DLS. Recombinant Bet v 1 was examined in Tris-HCl buffer at different pH values. The solution at pH 6.2 without glycerol contained monomers, dimers, and aggregates (A). At pH 6.2 with the addition of 4% glycerol and after filtering through 0.22- μ m pore size cutoff filters, Bet v 1 was present as a monomer only (B). A slight shift of the pH to 7.2 of the same solution again resulted in the formation of oligomers (C). Monomers are suggested to have a hydrodynamic radius of ~2.0 nm (indicated by the dotted line); the cutoff for dimers is 2.5 nm. The determined size of Bet v 1 molecules in the solutions is indicated in the panels (x-axis, size in nanometers; y-axis, intensity of light scattered by the molecules in the solution).

β sheets) was observed (Fig. 4). The far-UV CD spectra of Bet v 1 at these conditions showed a broad minimum at 215 nm and a maximum <196 nm. Only below pH 2.9 did the protein undergo an unfolding process toward a random coil structure. At pH 2.2 the unfolding process depended on the incubation time, whereas at pH 1.2 the protein adopted the random coil structure immediately. Far-UV CD spectra of the monomeric sample of Bet v 1 (pH 6.2 in 50 mM Tris-HCl buffer with 4% glycerol) indicated that the secondary structure was intact.

Skin tests of allergic mice

The in vivo relevance of our in vitro data was examined in Bet v 1-sensitized mice ($n = 8$). Their allergic status was confirmed by high Bet v 1-specific IgG1 and IgE titers in ELISA. We subjected them to type I skin tests using monomeric Bet v 1, or dimeric Bet v 1 diluted in PBS. Whereas all mice showed positive reactions to Bet v 1 in PBS, none of them reacted with the monomeric sample

Table I. DLS of rBet v 1 at different pH values

Concentration of Bet v 1	pH	R_H in nm (SD) ^a	Mode
4 mg/ml (50 mM Tris buffer)	1.2		Polydisperse
	2.2		Polydisperse
	3.2		Polydisperse
	4.2		Polydisperse
	5.2	3–4 ($\leq 15\%$)	Broad monomodal
		75 ($\leq 8\%$)	
	6.2	3–4 ($\leq 21\%$)	Broad monomodal
		77 ($\leq 17\%$)	
	7.2	2–3 ($\leq 23\%$)	Broad monomodal
		72 ($\leq 22\%$)	
4 mg/ml (in deionized water)	8.2	3–4 ($\leq 20\%$)	Broad monomodal
		102 ($\leq 29\%$)	
	9.2	2.2 ($\leq 12\%$)	Narrow monomodal
	9.7	2.0 ($\leq 7\%$)	Narrow monomodal
3.33 mg/ml, 4% glycerol	6.2	2.0 ($\leq 9\%$)	Narrow monomodal
1.25 mg/ml, 12.5% glycerol	6.2	3–4 ($\leq 12\%$)	Broad monomodal
		74 ($\leq 20\%$)	
	7.2		Polydisperse
	8.2		Polydisperse

^a The R_H is considered to be 2.0 nm for monomer; the cut-off for the dimer is at 2.5 nm. A relative SD of the R_H of $\leq 15\%$ is considered to show negligible polydispersity in the solution with narrow monomodal size distribution. If the SD is $\leq 30\%$, the solution is broad monomodal containing monomers, monomer-dimer mixtures, and/or oligomers. Bold font indicates monomeric conditions chosen for in vivo testing.

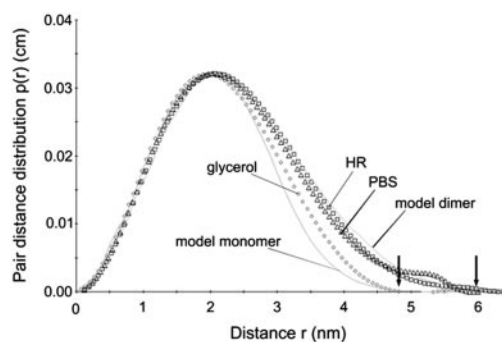


FIGURE 3. SAXS was performed with rBet v 1. The functions of a theoretical monomer and dimer were calculated from the crystal structure data of Bet v 1. The solution in Tris buffer (pH 6.2) with glycerol (glycerol) stabilized the monomeric state of Bet v 1, and the protein behaved like the calculated model (model monomer, with a maximum dimension of ~4.8 nm). In contrast, the same concentration of Bet v 1 in histamine release buffer (HR) or in PBS displayed the same distance radius as the model for the dimer (model dimer, with a maximum dimension of ~6 nm).

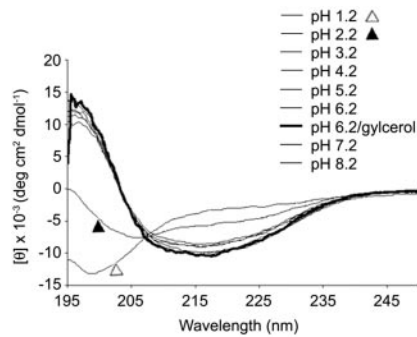


FIGURE 4. CD measurements were performed with rBet v 1 at different pH values. A low pH of 1.2 destroyed the secondary structure immediately (Δ), whereas the damage by pH 2.2 was less harmful ($\blacktriangle$). At all other conditions, including the monomer solution at pH 6.2 with 4% glycerol (pH 6.2/glycerol; bold line), Bet v 1 showed no change in structure. Spectra were recorded at a wavelength range from 185 to 260 nm with 0.2-nm resolution at a scan speed of 50 nm/min; the data resulted from averaging three scans. Results are expressed as the mean residue ellipticity (y-axis) at a given wavelength (x-axis).

of Bet v 1 in buffer at pH 6.2 containing glycerol (Fig. 5). For the control, skin sites were pretreated with Tris-HCl (pH 6.2) containing 4% glycerol, followed by injection of dimeric Bet v 1. In this setting, the allergen effectively triggered mediator release, indicating that the negative tests using the monomer samples were due to the monomeric nature of Bet v 1 and not to the buffer.

Treatment of allergic mice with monomeric or dimeric Bet v 1

To evaluate whether monomeric or dimeric Bet v 1 may modulate an ongoing allergic response in BALB/c mice, we performed immunization experiments. Mice were sensitized twice with recombinant Bet v 1 and $\text{Al}(\text{OH})_3$ i.p. Thereafter, mice were treated with monomeric or dimeric Bet v 1 i.p. without adjuvant. The results of these experiments are depicted in Fig. 6. Interestingly, only treatments with dimeric al-

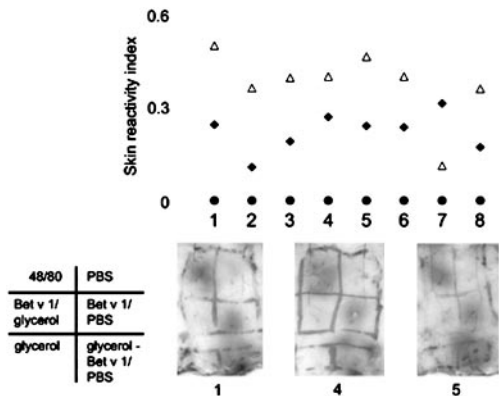


FIGURE 5. Skin tests of Bet v 1-allergic mice. Mice ($n = 8$) were sensitized to Bet v 1 by immunization with rBet v 1 in $\text{Al}(\text{OH})_3$ as a Th2-promoting adjuvant. The animals were tested with monomeric Bet v 1 in Tris-HCl (pH 6.2) containing 4% glycerol (Bet v 1/glycerol) and with Bet v 1 in PBS (Bet v 1/PBS). None of the mice reacted to monomeric Bet v 1 ($\bullet$), whereas all animals showed positive reactions to Bet v 1 in PBS ($\blacklozenge$). As a control experiment, buffer and glycerol were administered first, followed by Bet v 1 in PBS (glycerol-Bet v 1/PBS), and all mice showed a positive reaction (Δ). In contrast, tests with buffer and glycerol without Bet v 1 (glycerol) remained negative. PBS, negative control; 48/80, positive control. The upper panel displays the skin reactivity indices (densitometric reflection intensity $\times$ diameter of color reaction) of all mice ($n = 8$). The lower panel shows representative skin tests of mice 1, 4, and 5.

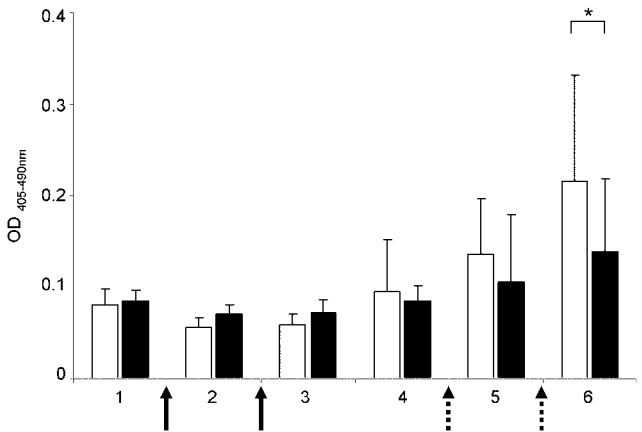


FIGURE 6. The treatment of sensitized mice with dimeric, but not monomeric, Bet v 1 boosts Bet v 1-specific IgE production. BALB/c mice ($n = 5$) were sensitized against Bet v 1 by two i.p. immunizations of Bet v 1 adsorbed to $\text{Al}(\text{OH})_3$ (black arrows) and thereafter boosted twice (dotted arrows) with either dimeric ($\square$) or monomeric ($\blacksquare$) Bet v 1 without adjuvant. Blood samples were taken from preimmune mice and at 7-day intervals after each immunization and were examined by ELISA. The difference between the resulting IgE levels was significant (*, $p = 0.019$).

lergen resulted in an additional increase in the Bet v 1-specific IgE response. In contrast, the monomeric molecule was less effective in supporting further IgE secretion. The difference between the groups was statistically significant ($p = 0.019$).

Discussion

For cross-linking of IgEs expressed by B lymphocytes (BCR) or of IgE passively bound to $\text{Fc}\epsilon\text{RI}$ on mast cells and basophils, allergens have to display several characteristics. First, they require at least two antigenic determinants per molecule to cross-link two IgE molecules, which means that they have to be di- or multivalent (2, 3). In contrast, it was early recognized that univalent haptens inhibit rather than evoke reactions (46). Second, the IgE Abs with the right specificity for the different epitopes on an allergenic molecule have to be within close vicinity, which could be achieved by lateral diffusion of the $\text{Fc}\epsilon\text{RI}$. In multisensitized patients, however, the effectiveness of this mechanism would be weakened; due to the multiple IgE specificities present on the effector cells, cross-linking would very much rely on coincidence. With respect to B lymphocytes, the presentation of two different epitopes to the BCR would even prevent cross-linking, because each B cell expresses Igs with only one epitope specificity. In this respect, it seems interesting that during epitope mapping, using mimotopes or cocrystallization of allergens with mAb, single dominating epitopes were found, for instance on the major birch pollen allergen Bet v 1 (47, 48). Sequential epitope mapping based on overlapping peptides may render several sections exhibiting IgE subepitopes (49), leading to an overestimation of the numbers of epitopes. However, aligning them with the surface of allergens, they may together form few patches of discontinuous conformational epitopes (50–52). In agreement, on Amb t 5 of ragweed pollen only one dominant B cell epitope was detected, at least for human IgG binding (53). Thus, we believe that the number of allergen epitopes relevant for the individual patient may be much more restricted than previously assumed. For these reasons, cross-linking of cell-bound IgE by multivalent allergens seems less feasible.

In the present study we examined whether dimerization or oligomerization of pollen allergens could be the key in the course of IgE cross-linking, taking Bet v 1 as an example. This allergen was

previously described to be a monomer (54). Interestingly, the dimer of Bet v 1, like the monomer, has been shown to possess RNase activity (55). Also, in the macromolecular structure data base (www.ebi.ac.uk/msd-srv/msdlite/atlas/assembly/1bv1.html) a homodimeric assembly of Bet v 1 is described, probably representing the biological unit of the protein (42).

In this study we reinvestigated the behavior of Bet v 1 in vitro as well as in an in vivo system. In SDS-PAGE gels, rBet v 1 forms bands at 17, 34, and 68 kDa, identified as Bet v 1 monomers, dimers, and tetramers by murine mAb as well as by human IgE Abs in our study and by others (10, 55–57).

To exclude experimental artifacts from electrophoresis gels, we subjected Bet v 1 in solution to DLS measurements. The overall charge of a protein may vary depending on the pH of the solution. This can have a significant impact on its ability to form molecular interactions. Therefore, we tested rBet v 1 at different pH values and analyzed the size distribution of molecules in the solutions. In a wide pH range, Bet v 1 was present as a mixture of monomers, dimers, and oligomers. We have to admit that the concentrations used in DLS (1.25–4 mg/ml) seem rather high for a protein solution. However, in a recent study it has been shown that the allergen content in pollen may vary considerably and, depending on the ozone exposure, may reach a concentration of 6 mg/ml (58).

As we intended to analyze the cross-linking capabilities of monomer and dimer in biological tests, we had to find conditions close to neutral pH, where Bet v 1 could exist only as a monomer, even at the high concentration used in the study of DLS. Finally, a dilution of Bet v 1 in Tris-HCl buffer at pH 6.2 with the addition of 4% (v/v) glycerol resulted in an exclusive monomer form. As confirmation of these results, we analyzed monomeric Bet v 1 and Bet v 1 dissolved in PBS by SAXS. The agreement with the simulated curves for the monomer and the dimer, respectively, suggests that under the above-defined conditions Bet v 1 actually persisted as a monomer, whereas the solution in PBS corresponded to the theoretical dimer curve. Using the same technique, it has recently been shown that natural Bet v 1 contains ~10% dimers (unpublished observation by Prof. F. Ferreira, University of Salzburg, Salzburg, Austria). The buffer conditions necessary for monomer formation did not alter the secondary structure of Bet v 1, as demonstrated in CD experiments. Therefore, our monomeric Bet v 1 could be compared with dimeric Bet v 1 in biological tests.

Our original aim was to test the cross-linking capacity of Bet v 1 monomers and Bet v 1 dimers in histamine release tests of human PBMC. Unfortunately, due to methodological problems, the monomeric nature of Bet v 1 could not be controlled by DLS when diluted in histamine release buffer. Furthermore, in SAXS it seemed that Bet v 1 in histamine release buffer behaved rather dimerically. For these reasons, we decided to test the different states of Bet v 1 in vivo in skin tests of Bet v 1-sensitized mice. As expected, dimeric Bet v 1 revealed positive reactions at the application sites when simply dissolved in PBS. Impressingly, skin tests with monomeric Bet v 1 remained negative in all mice. In this experiment the concentration of the allergen could be scaled down to 3 µg/ml without affecting the results. The possibility that IgE Abs had been stripped from the cells by the slightly acidic pH of our monomer solution was excluded by a simultaneous control experiment.

To evaluate the influence of monomer or dimer on an ongoing allergic response, we performed immunization experiments of sensitized mice. The results indicated that dimeric, but not monomeric, Bet v 1 has the capability of activating specific B cells for IgE secretion in a secondary immune response, an effect likely to be independent of T cell help (59). We suggest that monomeric Bet v 1 is not capable of cross-linking the BCR, which is a precondition

for activation of mature B cells (1). Therefore, a monomeric allergen could act hypoallergenicly by two mechanisms: 1) lack of IgE cross-linking on mast cells in a sensitized individual and therefore enhanced safety, and 2) lack of B cell cross-linking and therefore no boost of IgE production in the onset of allergen immunotherapy.

From our in vitro measurements and in vivo data we suggest that dimerization of Bet v 1 is an essential feature for its cross-linking ability of effector cells as well as B lymphocytes. We suggest that dimerization and oligomerization of proteins could be crucial common features of allergens.

Acknowledgments

We thank Ing Magdolna Vermes for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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