

Human IgG Monoclonal Antibodies That Modulate the Binding of Specific IgE to Birch Pollen Bet v 1¹

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Birch pollen allergy is a very frequent pathology in Europe and North America. More than 95% of the tree pollen allergic patients display IgE reactivity against Bet v 1, the major birch pollen allergen. Starting with PBL from a patient desensitized by immunotherapy, we have generated five B cell lines (BAB1 to BAB5) that secrete human IgG mAbs of high affinity for Bet v 1. Although competition studies indicated that these human IgG mAb recognized different epitopes, broad cross-reactivity was found with Bet v 1 homologous allergens present in tree pollens and plant-derived foods. When tested for interference with allergic patients' IgE, BAB1 inhibited (by 80–100%) the binding of IgE to nitrocellulose-blotted Bet v 1, while BAB2 enhanced it. The biologic significance of the ability of BAB1 to interfere with patients' IgE binding is indicated by the finding that BAB1 completely inhibited Bet v 1-induced histamine release from allergic patients' basophils. Allergen-specific human IgG mAbs such as BAB1, which presents high blocking activity in both immunochemical and cellular IgE competition experiments, might have therapeutical application. *The Journal of Immunology*, 1996, 157: 956–962.

Clinical manifestations of allergy are triggered when allergens cross-link effector cell-bound IgE and aggregate the underlying high affinity FcεRI (1). This leads to the liberation of biologic mediators, including histamine, leukotrienes, and PGs, which then cause the symptoms of allergy, such as rhinitis, conjunctivitis, and asthma. Besides IgE, sera from both allergic and nonallergic individuals contain allergen-specific IgG and IgA Abs (2), for which a blocking role remains controversial (3). In this context, the recent demonstration that anaphylactic reaction can occur in IgE-KO mice (4) and the regulatory role of FcγRII on the FcεRI-mediated mast cell activation (5) suggest a broader spectrum of activities for allergen-specific non-IgE Abs.

The generation of human IgG mAbs (IgG HumAbs)⁴ of a given specificity has represented a long sought after goal (6), inasmuch as they could have considerable medical applications. Recently, technologic advances in two domains have changed the perspectives. First, as a consequence of progresses in molecular biology, combinatorial libraries have provided several HumAbs displaying a high affinity for selected Ags (7–9). Second, improvement of

human B cell cultures, based on the cross-linking of the CD-40 Ag (10) has allowed the efficient immortalization of memory B lymphocytes into stable cell lines secreting high affinity Abs (9–11).

In our attempt to understand the influence of IgG on IgE-allergen interaction, we have characterized Bet v 1-specific HumAbs, that were generated from a patient who had been successfully desensitized to birch pollen. Allergy to birch pollen was selected because 1) of the prominent role of a single 17-kDa glycoprotein, Bet v 1, which is exclusively responsible for all the symptoms in >65% of the birch pollen-sensitive patients (12); 2) the complete Bet v 1 cDNA sequence was determined (13) and recombinant *Escherichia coli*-expressed Bet v 1 was found to behave in a manner immunologically equivalent to that of the natural protein (14); 3) cross-sensitization with several other tree pollens and fruit allergens has been documented (15–19). Thus, five monoclonal B cell lines secreting high affinity Bet v 1-specific IgG have been generated. One HumAb (BAB1) was able to inhibit the binding of allergic patients' IgE to Bet v 1, whereas another HumAb (BAB2) enhanced IgE binding. The possible physiologic role played by an allergen-specific IgG HumAb such as BAB1 and its potential usefulness in the treatment of allergy are supported by its ability to inhibit the Bet v 1-induced histamine release from allergic patients' basophils.

Materials and Methods

Selection of patients

Birch pollen allergic patients were selected based on the occurrence of allergic symptoms (rhinitis, conjunctivitis, and asthma) in early spring. The total serum IgE level was measured by PRIST (Pharmacia, Uppsala, Sweden), and the presence of birch pollen-specific IgE Abs was confirmed by skin prick testing and CAPS (Pharmacia). For generation of the mAbs, a Bet v 1 allergic patient (H.D.) who attended the Allergy Clinic of the University Hospital Saint Luc, Brussels, Belgium (Dr. Saint-Remy), was selected. To determine the ability of anti-Bet v 1 HumAbs to interfere with the binding of Bet v 1 to basophil-bound IgE, blood basophils from six patients who attended the Allergy Clinic from the Pasteur Institute of Paris (Dr. Guinnepain) were tested for the release of histamine in the presence of increasing concentrations of purified natural Bet v 1 (from 0.001–100

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⁴ Abbreviations used in this paper: HumAb, human monoclonal antibody; nBet v 1, natural Bet v 1; rBet v 1, recombinant Bet v 1; mAb, murine antibody; MNC, mononuclear cells; Tris-ACM, 25 mM Tris, pH 7.6; 5 mM KCl; 130 mM NaCl; and human serum albumin at 0.33 mg/ml supplemented with 0.6 mM Ca²⁺ and 1 mM Mg²⁺.

$\mu\text{g/ml}$ of Bet v 1). The sera from two nonallergic individuals (C1 and C2) were used as a control in Western blots.

Allergen extracts, natural and recombinant Bet v 1 (rBet v 1)

Screening of the HumAbs-producing cells was performed using crude natural birch pollen extract. We extracted the proteins from *Betula alba* (purchased from Sigma Chemical Co., St. Louis, MO) and also used a pollen extract from *Betula pendula* (Smith Kline Beecham Pharmaceuticals, London, U.K.). Alder and hazel pollen extracts as well as apple extract were obtained as previously described (14, 20). Aliquots of pollen extracts diluted 1/2 with glycerol were stored at -20°C . Natural Bet v 1 was purified from birch pollen extract by affinity chromatography, using a Bet v 1-specific mouse mAb (moAb7) coupled to Sepharose as described by others (S. Lebecque, C. Dolecek, V. Visco, S. Denépoux, J. J. Pin, C. Guret, G. Boltz-Nitulescu, A. Weyer, and R. Valenta, manuscript in preparation). *E. coli*-derived rBet v 1, which is immunologically equivalent to the natural allergen, has been previously described (20). Quantification of natural Bet v 1 (nBet v 1) in pollen extract was performed with the sandwich ELISA described below, using purified rBet v 1 as a reference.

Bet v 1-specific murine monoclonal antibodies and HumAb negative control

Generation and characterization of the Bet v 1-specific murine mAbs (moAb) are described in a separate paper (S. Lebecque et al., manuscript in preparation). A human IgG mAb X3A3 (IgG4, κ), which recognizes human IL-1 α (a gift from P. Garrone), was used as a negative control.

Bet v 1-specific direct and sandwich ELISAs

For direct ELISA, the *B. pendula* pollen extract was coated onto 96-microwell ELISA plates (Immunoplate Maxisorp F96 certified, purchased from Nunc, Roskilde, Denmark). The plates were then washed manually once. One hundred microliters of culture supernatants or of purified mAbs diluted 1/100 in dilution buffer was added per well and incubated for 2 h at 37°C , then washed once. One hundred-microliter aliquots of sheep anti-murine IgG coupled to horseradish peroxidase (Biosys, Compiègne, France) were added to each well and incubated for 2 h at 37°C . After three washes, 100 μl of peroxidase substrate, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.) diluted to 1 mg/ml in citrate/phosphate buffer was added to each well, and the colorimetric reaction was read at 450 nm (Molecular Devices Co., Menlo Park, CA). Background was determined as the OD of control wells without Ab. The protocol for the sandwich ELISA was the same, except that a Bet v 1-specific mAb (moAb13) was first adsorbed onto microtiter plates. Plates were next incubated with birch pollen extract, washed, and incubated with culture supernatants or purified mAbs as described above.

Generation of human Bet v 1-specific monoclonal antibodies

EBV transformation of mononuclear cells (MNC). Heparinized blood (50 ml) was collected from birch pollen allergic patients, diluted 1/1 with RPMI 1640 medium, loaded onto Ficoll/Hypaque (Pharmacia, Uppsala, Sweden), and centrifuged (30 min, $600 \times g$) according to manufacturer's instructions. PBMC were harvested at the interface. The upper phase was recovered, aliquoted, and frozen at -20°C to be used later for ELISA and Western blot screening. The PBMC were washed four times in PBS and frozen at a concentration of 1×10^6 cells/ml in liquid nitrogen. PBMC from a patient with a high serum level of birch pollen-specific IgG and strong IgE and IgG reactivity to Bet v 1 (as determined by immunoblotting) were used for transformation. PBMC (14×10^6) from this patient were thawed and washed once in RPMI 1640. Viability, as estimated by trypan blue exclusion, was $>90\%$. The cells were pelleted and resuspended in 1 ml of RPMI 1640. Two hundred microliters of concentrated EBV suspension (strain B95.8, produced by culturing transformed marmosets leukocytes, essentially as described by Miller and Lipman (21)) was added, and this mixture was incubated for 2 h at 37°C in a humidified incubator. The cells were then washed twice in RPMI 1640 complete medium, and the pellet was resuspended at 5×10^4 cells/ml in Linolea complete medium, which consisted of Yssel's modified Iscove's medium (22) supplemented with 15% heat-inactivated FCS, 2 mM L-glutamine, and 50 $\mu\text{g/ml}$ gentamicin. Irradiated L cells (7000 rad) stably expressing the Fc γ receptor II for human IgG (CDw32) were added at a final concentration of 5×10^4 cells/ml, together with the murine monoclonal anti-human CD40 Ab mAb 89, used at a final concentration of 0.5 $\mu\text{g/ml}$. Aliquots (100 μl each) of this mixture were then distributed in Microwells 96U round-bottom plates (Nunc), which were incubated at 37°C in a 5% CO_2 humidified atmosphere. After 5 days of incubation, 125 μl of fresh Linolea complete medium containing 0.5 $\mu\text{g/ml}$ anti-CD40 mAb 89 was added to each well.

Selection of Bet v 1-specific B cell lines. On day 12, the supernatants were first screened by indirect ELISA (see above) for the presence of Abs against birch pollen proteins. One hundred microliters was collected from each well and replaced by the same volume of fresh Linolea complete medium. On day 19, the same screening was repeated for every well to confirm the first assay and to identify lines that became lately positive. A total of 51 positive lines were thus identified, all of which were transferred in 2 ml into 24-well multidishes (Nunc) for amplification. After expansion, 5×10^6 cells from each line were frozen and kept in liquid nitrogen. Twenty-five lines were kept in culture for further expansion, characterization of antigenic specificity, and cloning. Supernatants from five cell lines were found to immunoprecipitate purified radiolabeled Bet v 1 (see below). These five cell lines were cloned by limiting dilution in Microtest III Tissue Culture Plates (Becton Dickinson, Franklin Lakes, NJ) flat-bottom microwells. One clone was derived from each of those five lines, i.e., clones BAB1 to BAB5.

Production and typing of human monoclonal antibodies. To increase the yield of HumAbs, cells were also cultured in a bioreactor (Tecnomouse, Integra Bioscience, Woburn, MA) in serum-free medium according to manufacturer's instructions. Purification of the HumAbs was performed by ammonium sulfate precipitation and anion exchange chromatography on a Q HyperD F column (IBF Sepracor, Villeneuve la Garenne, France) equilibrated with 20 mM Tris, pH 8, buffer. Proteins were eluted with a NaCl gradient (range, 0–1 M NaCl). Two-milliliter fractions were collected and tested by ELISA for the presence of anti-Bet v 1 Ab. The fractions reacting with Bet v 1 were pooled, dialyzed, and frozen. Aliquots of purified mAb were peroxidase labeled according to the method of Wilson (23). Peroxidase-labeled mAb were diluted in 50% glycerol and stored at -20°C . Supernatant from each clone was tested in an ELISA specific for human IgM, IgG, or IgA (24); for human IgG1, IgG2, IgG3, or IgG4 subclasses; and for κ and λ light chains (24).

Cross-reactivity of HumAbs with nitrocellulose-blotted Bet v 1 homologous allergens

Approximately 50 ng/cm of proteins from different sources of allergens (aqueous extract from birch pollen, alder pollen, hazel pollen, or hazel nut and apple) or from rBet v 1 were immunoblotted. Nitrocellulose strips containing the major allergen of each extract or rBet v 1 were cut (each row represents strips from a single sheet), washed three times in buffer A (50 mM Na/phosphate, pH 7.5; 0.5% BSA; 0.5% Tween-20; and 0.05% $\text{Na}_2\text{S}_2\text{O}_3$), and subsequently incubated with purified HumAb diluted 1/100 in buffer A overnight at 4°C . After three washings, strips were incubated with 1/1000 diluted rabbit anti-human IgG antiserum (Dako, Glostrup, Denmark) and ^{125}I -labeled donkey anti-rabbit antiserum (Amersham, Aylesbury, U.K.) for 2 h at room temperature. Bands were visualized by exposure of the dried strips to Kodak X-OMAT S films using intensifying screens (Kodak, Heidelberg, Germany) at -70°C .

Immunoprecipitation of radio-iodinated Bet v 1 with HumAbs and analysis of the precipitate on SDS-PAGE

Birch pollen extract (and purified Bet v 1 for Scatchard analysis) were iodinated with the Iodobeads kit (Pierce Chemical Co., Rockford, IL) as described (S. Lebecque et al., manuscript in preparation). Fifty microliters of purified HumAb diluted in PBS/1% BSA were incubated for 45 min at room temperature with 100,000 cpm of [^{125}I]Bet v 1 (diluted in PBS/1% BSA) in a 5-ml polypropylene tube (Nunc). Nonspecific binding controls were performed by the addition of 250 pM of unlabeled Bet v 1. Fifty microliters of protein G-coupled Sepharose 4B (Sigma Chemical Co.) was added to each tube and incubated for 45 min at room temperature. The Sepharose was then centrifuged for 5 min at $2500 \times g$ and washed three times with 1 ml of PBS. After the last centrifugation, the pellet was resuspended in O'Farrell/Laemmli Tris-glycine-SDS buffer and loaded on a 15% SDS-PAGE gel, which was dried after completion of the run and exposed to x-ray film (Kodak).

Measurement of the affinities of the HumAbs for Bet v 1

To determine the binding affinities of the human mAbs to Bet v 1, the association constants of HumAb-Bet v 1 complexes were measured. Purified HumAbs were incubated with increasing concentrations of natural [^{125}I]Bet v 1 (0.15–2.5 pM) in a final volume of 250 μl PBS-1% Tween. Each condition was performed in triplicate. Nonspecific binding controls were determined by the addition of 250 pM unlabeled Bet v 1. After 4-h incubation at 4°C , 200 μl of each sample was distributed in one well of a 96-well special filtration plate (0.45 μm , MultiScreen-HA, Millipore Corp., Bedford, MA) containing 50 μl of protein G-coupled Sepharose (Sigma Chemical Co.). After 1-h incubation at 4°C , plates were washed four times

Table I. Immunochemical properties of the five Bet v 1 specific HumAbs

HumAb	BAB1	BAB2	BAB3	BAB4	BAB5
Heavy Chain	IgG1	IgG4	IgG4	IgG2	IgG4
Light Chain	kappa	kappa	lambda	kappa	kappa
Binding to nBet v 1 in direct ELISA ^a	+	+	+	+	+
in sandwich ELISA ^b	—	+	+	+	+
Binding to nBet v 1 in Western blot	+	+	+	+	+
Binding to rBet v 1 in Western blot	+	+	+	+	+
Precipitation of nBet v 1	+	+	+	+	+
Affinity (K_a) $\times 10^{-9}$ M ^c	1.4–7.9	1.3–9.8	0.16–0.18	0.25–0.40	1.5–3.0

^a Binding to nBet v 1 is directly adsorbed in microtiter wells.

^b Binding to nBet v 1 presented by a mouse mAb specific for Bet v 1.

^c Results from two independent experiments.

with PBS, and dried membranes were collected from each well. Radioactivity corresponding to the complexes [¹²⁵I]Bet v 1/HumAb/protein G-beads retained on the membranes was counted using a Wizard gamma counter (Wallac Oy, Turku, Finland). Specific binding of [¹²⁵I]Bet v 1 was calculated, plotted vs free [¹²⁵I]Bet v 1 concentration, and subjected to Scatchard analysis.

Competition ELISA between murine and human monoclonal antibodies

In this ELISA wells were preincubated with a 100-fold molar excess of competitor-purified unlabeled HumAb before revelation with peroxidase-labeled Bet v 1-specific murine or human mAbs. These conditions were selected after preliminary experiments established that this 100-fold molar excess ratio resulted in maximal inhibition for each Ab: % inhibition = $[(M - i)/(M - b)] \times 100$, where M is the OD measured in the absence of inhibitor, i is the OD measured in the presence of mAb competitor, and b is the OD measured in the absence of peroxidase-labeled mAb.

Influence of HumAbs on IgE binding of allergic patients to rBet v 1

Nitrocellulose strips containing rBet v 1, which were cut from the same preparative sheet, were blocked in buffer A (50 mM Na/phosphate, pH 7.5; 0.5% Tween-20; 0.5% BSA; and Na₂S₂O₃) and preincubated with purified BAB1 or with buffer. After washing, the strips were incubated with 1/5 diluted sera from birch pollen allergic patients. Bound IgE was detected with ¹²⁵I-labeled anti-human IgE Abs (RAST, Pharmacia). After washing, strips were exposed to x-ray films (Kodak), and signals were analyzed by scanning using a densitometer (Hirschmann, Unterhaching, Germany).

Influence of HumAbs on Bet v 1-induced histamine release assay

The procedures for the cell preparation and histamine release assays were previously described by May et al. (25). Tris-albumin buffer containing 25 mM Tris, pH 7.6; 5 mM KCl; 130 mM NaCl; and human serum albumin at 0.33 mg/ml was used for washing the cells. The same buffer supplemented with 0.6 mM Ca²⁺ and 1 mM Mg²⁺ (Tris-ACM) was used in the histamine release experiments.

Thirty milliliters of peripheral blood from four patients was drawn in syringes containing 120 μ l of heparin. Six milliliters of glucose-dextran was added, and most of the erythrocytes and platelets were allowed to sediment for 45 min at room temperature. The peripheral blood leukocytes, including basophils, were recovered in the plasma supernatant. After centrifugation, the cells were washed twice in Tris-albumin buffer and resuspended in Tris-ACM buffer. Fifty microliters of nBet v 1 was incubated with 2×10^6 cells for 40 min at 37°C in a final volume of 500 μ l. In all experiments a control for the determination of spontaneous histamine release was included. The incubation was stopped by the addition of 500 μ l of cold PBS.

Aliquots (850 μ l) of the cell suspensions (for the determination of total histamine content by cell lysis) or of the supernatants were recovered and acidified with 100 μ l of 4 N perchloric acid. After 20-min centrifugation at 4000 $\times$ g, the supernatants were transferred to new tubes for histamine quantification.

For the inhibition assays, the concentration of nBet v 1 that induced between 50 and 100% of the maximal histamine release was first established in preliminary experiments for each individual patient: M.M., 1 μ g/ml; J.C., 3 μ g/ml; H.L., 0.3 μ g/ml; and E.B., 1 μ g/ml. Fifty microliters of

nBet v 1 at the appropriate concentration was preincubated for 90 min at 37°C with 50 μ l of a mixture of the human anti-Bet v 1 mAbs (BAB1 to BAB5) or with the individual BAB. The BAB were used at concentrations varying between 0.4 and 1.5 mg/ml in the preincubation mixture and then diluted 1/5 when the cell suspension was added. Control experiments were performed by preincubation with Tris-ACM buffer or with X3A3, an IgG human mAb of unrelated specificity (at 1.3 mg/ml in the preincubation mixture). Preincubation of Bet v 1 was also performed with the mixture of BAB2, BAB3, BAB4, and BAB5, starting at 0.1 mg/ml each. In addition, each BAB was tested, in the absence of the allergen, for its individual capacity to induce histamine release. Four hundred microliters of the cell suspension was then added to the preincubated Bet v 1 allergen, and the histamine release assay proceeded as described above. Each experiment was performed in triplicate.

The histamine content was quantified by an automated fluorometric method (26). The results were expressed as the percentage of the total histamine content as follows: $100(a - b)/(c - b)$, where a is the mean of the fluorometric readings for experimental supernatants, b is the mean for spontaneous histamine release, and c is the mean for total histamine. Inhibition of histamine release was expressed as $(1 - \text{HRp}/\text{HRC})100\%$, where HRp and HRC are the percentage of histamine release observed when the allergen was preincubated with the human mAbs or with Tris-ACM buffer, respectively.

Results

Generation of five human monoclonal antibodies with specificity for the major birch pollen allergen Bet v 1

PBMC of an allergic patient (H.D.) who had undergone successful immunotherapy were immortalized with EBV in the presence of anti-CD40 Ab. After two runs of limiting dilution culture, five B cell clones secreting IgG specific for Bet v 1 were isolated, which were designated BAB1 to BAB5. These clones had a doubling time of about 16 h and proved to be very stable, inasmuch as they have been kept in culture for >1 yr. Each produced from 5 to 10 μ g of Ab/ml of conventional culture medium. When grown in a continuous flow bioreactor, the yield of Ab ranged from 1 to 10 mg/ml.

Immunologic characterization of the five HumAbs

For a detailed analysis of their specificity, the five HumAbs were purified from the culture supernatants by affinity chromatography using protein G-Sepharose. The properties of the five HumAbs are summarized in Table I. The clonality of each Ab was established by 1) a class-specific ELISA showing that BAB2, BAB3, and BAB5 belong to the IgG4 subclass, while BAB1 and BAB4 are IgG1 and IgG2, respectively; 2) a light chain-specific ELISA demonstrating that BAB4 expresses only λ light chain, while the four others HumAbs use only κ light chain; and 3) the amplification by PCR of a single V_H gene family member using primers specific for the γ heavy chain and for the leader sequence of the different V_H families (V_H1 to V_H6) and of either κ or λ light chain from the mRNA of each HumAb (data not shown). Moreover, the sequences obtained from two independent PCR for each heavy and

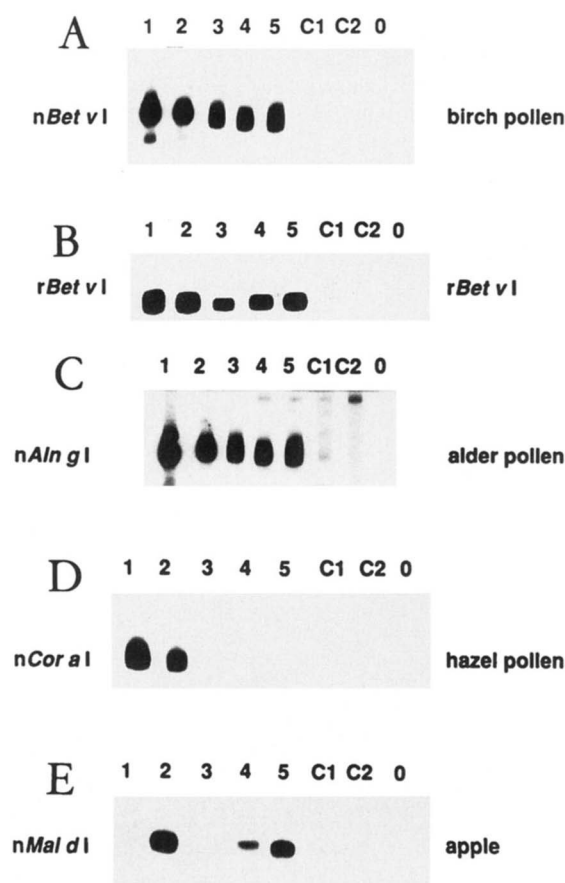


FIGURE 1. Reactivity of the five HumAbs with nitrocellulose-blotted natural and recombinant Bet v 1 as well as Bet v 1 homologous plant allergens. Protein extracts from birch pollen (A), alder pollen (C), hazel pollen (D), apple pollen (E), and *E. coli*-derived recombinant Bet v 1 (B) were loaded on SDS-PAGE gels and, after separation, blotted on nitrocellulose. Nitrocellulose strips containing approximately 50 ng/cm of each allergen were cut and preincubated with each HumAb (lanes 1–5), with a 1/100 dilution of sera from two nonallergic individuals (lanes C1 and C2), or with buffer alone (lane 0). Bound human IgG was detected with a rabbit anti-human IgG antiserum and an ^{125} I-labeled donkey anti-rabbit antiserum, followed by autoradiography.

light chain of each HumAb were identical and will be reported separately. The five HumAbs were able to bind to Bet v 1 directly coated on the microtiter well plastic as well as to nitrocellulose-blotted natural (Fig. 1A) and recombinant (Fig. 1B) Bet v 1. Finally, BAB2, BAB3, BAB4, and BAB5, but not BAB1, were able to bind equally well to Bet v 1 presented by the Bet v 1-specific murine mAb moAb13 (sandwich ELISA). When incubated with ^{125} I-labeled birch pollen extract, the HumAb immunoprecipitated a single 17-kDa protein (Fig. 2). Preincubation of the HumAbs with an excess of unlabeled purified natural Bet v 1 strongly reduced the binding of the labeled protein, thereby confirming the specificity of the reaction. AK6A3, an anti-*Der p* I HumAb used as a control, did not precipitate significant amounts of radioactivity. It is noteworthy that the K_a values of the five HumAbs for Bet v 1 were high, ranging from 10^{-9} to 10^{-10} M.

Bet v 1-specific HumAbs cross-react with homologous plant allergens

IgE Abs from Bet v 1 allergic patients frequently cross-react with homologous allergens from other vegetals and are hence responsible for the food intolerance in pollen allergic patients. We, there-

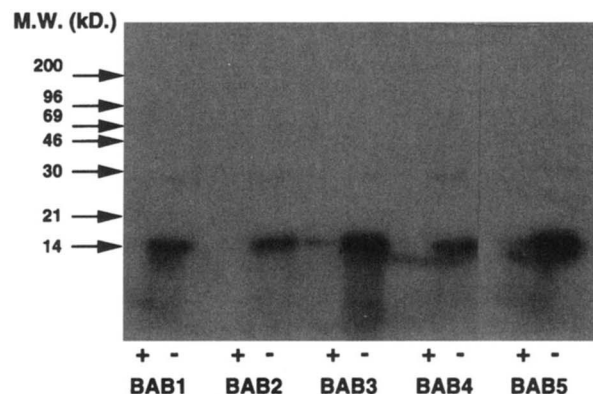


FIGURE 2. The five HumAbs immunoprecipitate ^{125} I-nBet v 1. Each purified HumAb (1 mg/ml) was incubated with ^{125} I-labeled birch pollen extract in the presence (lanes +) or the absence (lanes -) of a 100-fold excess of unlabeled purified natural Bet v 1, followed by the addition of protein G-coupled Sepharose. After centrifugation, the precipitates were loaded on 15% reducing SDS-PAGE gels. After completion of the run, the gel was dried and exposed on x-ray film for 2 days. MW, M_r markers.

fore, tested the reactivity of the five HumAbs with the major allergens from alder (nAln g I), hazel (nCor a I), and apple (nMal d I) (16, 20, 27, 28). As shown in Figure 1C, the five HumAbs bound to nitrocellulose-blotted alder nAln g I as well as to the natural and rBet v 1. BAB1 and BAB2 recognized blotted hazel nCor a I (Fig. 1D), while BAB2, BAB4, and BAB5 cross-reacted with the apple nMal d I (Fig. 1E).

Epitope mapping of Bet v 1-specific HumAbs

No binding of BAB1-BAB5 could be detected when tested with synthetic dodecapeptides (with three-amino acid overlap), which spanned the complete Bet v 1 molecule (data not shown). This is consistent with the observation that human IgE and IgG Abs of Bet v 1 allergic patients were unable to bind to proteolytic fragments of Bet v 1 or to recombinant Bet v 1 fragments (S. Vrtala et al., unpublished observation). Assuming that B cell epitopes of Bet v 1 are of the conformational type, we analyzed Bet v 1 epitopes by cross-competition experiments. Each HumAb and nine Bet v 1-specific murine mAbs previously characterized as recognizing independent epitopes (S. Lebecque et al., manuscript in preparation) were labeled with peroxidase, and the binding of the conjugates to adsorbed purified nBet v 1 was tested without or with a 100-fold excess of unlabeled HumAbs. The results, which are summarized in Table II, showed that the five HumAbs are directed against different epitopes, as they each displayed a very distinct pattern of cross-competition. Noticeably, BAB1 competed efficiently with all the human and mouse mAbs, while BAB4 and BAB5 enhanced the binding to Bet v 1 of BAB2 and BAB3.

HumAbs BAB1 and BAB2 modulate patients' IgE binding to Bet v 1

To investigate whether the HumAbs would influence human IgE binding to Bet v 1, competition experiments were performed. Nitrocellulose-blotted rBet v 1 was preincubated with or without HumAbs before analyzing binding of patient's IgE. Bound IgE was detected with ^{125} I-labeled anti-human IgE Abs without cross-reactivity to IgG and visualized by autoradiography. Densitometric analysis showed that BAB1 significantly inhibited IgE binding to Bet v 1, whereas BAB2 enhanced it. BAB3, BAB4, and BAB5 only weakly influenced IgE binding (data not shown). When sera

Table II. Cross-competition between the five HumAbs and with Bet v 1 specific murine mAbs for the binding to Bet v 1 in ELISA

inhibitors	Peroxidase-labeled mAbs													
	HumAbs					Bet v 1 specific mouse mAbs								
	BAB1	BAB2	BAB3	BAB4	BAB5	mAb1	mAb10	mAb2	mAb11	mAb3	mAb4	mAb7	mAb8	mAb9
BAB1	100	78	73	82	89	85	50	60	100	90	90	77	65	52
BAB2	—	100	50	53	—	—	—	—	—	70	70	—	—	—
BAB3	66	—	100	—	—	—	—	—	—	75	72	63	50	—
BAB4	—	+	+	100	—	—	—	—	—	57	50	—	—	—
BAB5	—	+	+	50	100	—	—	—	—	63	62	50	52	—

Results are expressed as percent of inhibition of the binding of conjugated mAbs to Bet v 1. + = Enhancement of the binding. — = No significant inhibition.

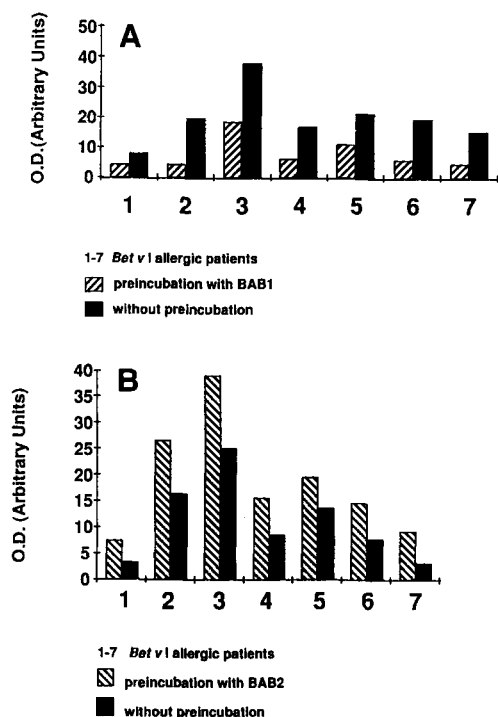


FIGURE 3. BAB1 inhibits (A) and BAB2 enhances (B) the binding of patients' IgE to nitrocellulose-blotted Bet v 1. *E. coli*-derived rBet v 1 was loaded on SDS-PAGE gels and, after completion of the run, blotted on nitrocellulose. Nitrocellulose strips containing equal amounts of recombinant Bet v 1 were preincubated with purified BAB1 (A), purified BAB2 (B), or buffer before incubation with sera from seven allergic patients. Bound IgE was detected with ^{125} I-labeled mouse anti-HumAbs. X-ray film was exposed to the nitrocellulose, and the bound radioactivity was quantified in arbitrary OD units by scanning the film.

from seven patients were analyzed, reproducible and consistent inhibition of IgE reactivity to Bet v 1 (between 47–77%) could be demonstrated for BAB1 (Fig. 3A). On the other hand, preincubation with BAB2 consistently increased (ranging from two- to eightfold) the binding of IgE from the same seven sera (Fig. 3B). Comparable results were obtained in dot blot assays using not denatured Bet v 1 (data not shown).

Bet v 1-specific HumAbs interfere with Bet v 1-induced histamine release from allergic patients' basophils

The biologic activities of the HumAbs were addressed with allergen-induced histamine release inhibition experiments that better reproduce in vitro the allergic challenge of the patient. Preincubation of the Bet v 1 with the mixture of the five HumAbs completely inhibited degranulation of the basophils of one birch-sensitive pa-

tient (data not shown). We, therefore, tested the effect of preincubation with single HumAbs. Basophils from two patients (M.M. and J.C.) were challenged with Bet v 1, preincubated or not with BAB1, BAB2, and BAB3, and with Bet v 1, preincubated or not with BAB4 and BAB5, respectively. As shown in Figure 4A, preincubation of Bet v 1 with BAB1 almost entirely suppressed the allergen-induced basophil degranulation, while the other four HumAbs had no effect on histamine release. The efficient inhibition of histamine release by BAB1 was further confirmed with the basophils of three more patients (inhibition ranging from 86–100%; Fig. 4B). The specificity of the inhibition of Bet v 1-induced histamine release by BAB1 is suggested by 1) the absence of nonspecific blocking activity of BAB2, BAB3, BAB4, and BAB5 when tested separately (Fig. 4A); and 2) the absence of blocking activity of X3A3, an unrelated IgG1 HumAb that specifically recognizes human IL-1 α (Fig. 4C). As illustrated in Figure 4C, the level of inhibition of histamine release was dose dependent, as it increased with higher concentrations of BAB1. Interestingly, the mixture of BAB2 to BAB5 was a potent inhibitor of Bet v 1-induced histamine release (Fig. 4C), in contrast to the absence of effect of these HumAbs used separately (Fig. 4A).

Discussion

A direct analysis of the binding of allergen by both IgE and non-IgE human Abs is required to understand their roles and mutual interactions in allergic reactions. Therefore, combining CD40 activation and EBV transformation, we have cloned from the PBL of an allergic patient five HumAbs of the IgG isotype (BAB1 to BAB5) directed against Bet v 1, the major birch pollen allergen. Bet v 1 was selected as a model allergen because 95% of tree pollen allergic patients display IgE reactivity to Bet v 1, and almost 65% of the tree pollen allergic patients react exclusively to Bet v 1. The five BAB specifically recognized immobilized as well as soluble Bet v 1 in ELISA, Western blot, and immunoprecipitation assays. Interestingly, three of them belong to the IgG4 subclass. This overrepresentation of IgG4 probably reflects the predominance of this subclass among allergen-specific Abs after prolonged desensitization (29, 30), rather than any bias introduced in vitro in our selection procedure. Indeed, IgG4 was the most abundant anti-Bet v 1 γ subclass present in the serum of the allergic donor, as judged by Western blot analysis (data not shown). The high affinity of the five BAB for Bet v 1 and their IgG isotype suggested that they originated from memory B cells selected by the allergen.

Competition experiments with the HumAbs and with epitope-specific anti-Bet v 1 murine mAbs as well as the pattern of binding to homologous allergens demonstrated that each of the five BAB bound to a different site of the Bet v 1 molecule. No sequential epitope could be directly identified for any BAB using a series of overlapping dodecapeptides spanning the entire Bet v 1 sequence.

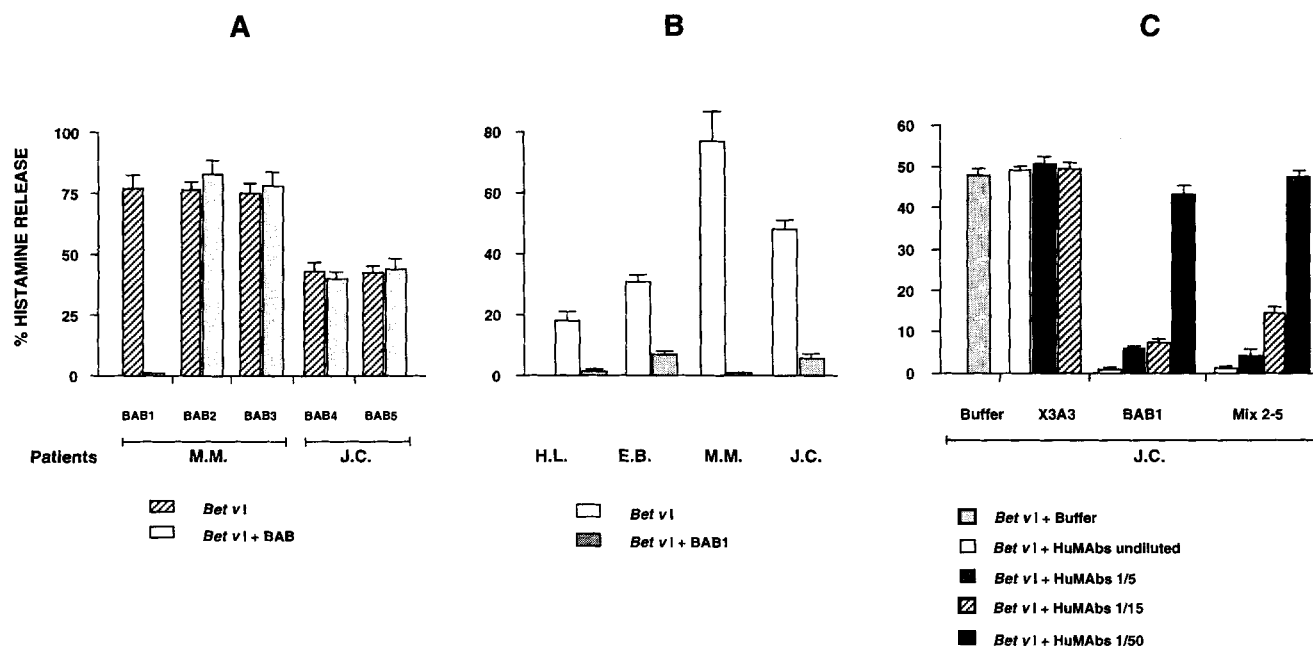


FIGURE 4. Enriched basophils from birch pollen allergic patients were incubated with concentrations of nBet v 1 inducing from 50 to 100% of the maximal histamine release in the presence or the absence of the mentioned HumAbs. The histamine released was expressed as a percentage of maximal histamine release. The mean and SD of three measurements are presented. *A*, When tested individually, only BAB1 inhibits the Bet v 1-induced histamine release. The five HumAbs were individually preincubated, at concentrations ranging from 0.4 to 1.5 mg/ml (see in the text), with nBet v 1 before challenging exposure of the basophils of two allergic patients (M.M. and J.C.). *B*, BAB1 preincubated at 1.5 mg/ml with nBet v 1 inhibits the histamine release from the basophils of the four patients tested (H.L., E.B., M.M., and J.C.). *C*, The Bet v 1-induced histamine release inhibition is specific and dose dependent. nBet v 1 was preincubated with successive dilutions of an anti-IL1 α HumAb X3A3 (starting from 1.3 mg/ml), of BAB1 (starting from 1.5 mg/ml), or of a mixture of BAB2 to BAB5 (starting at 0.1 mg/ml for each) and then exposed to the basophils from patient J.C.

Competition assays revealed the ability of BAB1 to strongly interfere with the binding of all the human and murine mAbs tested. Moreover, BAB1 significantly blocked the binding to blotted rBet v 1 of human IgE from seven allergic patients. In contrast, BAB2, enhanced up to eightfold the binding of the same patients' IgE to blotted rBet v 1.

An indication of the possible biologic significance of the capacity of BAB1 to inhibit IgE binding to Bet v 1 was provided by testing the effect of BAB1 on Bet v 1-induced histamine release from allergic patients' blood basophils. The basophil degranulation assay was chosen because it allows estimation of the blocking capacity of BAB1 on the allergic effector reaction and closely mimics the in vivo allergen challenge (31, 32). Preincubation of Bet v 1 with the mixture of the five HumAbs suppressed degranulation of the basophils of one birch-sensitive patient (data not shown). Furthermore, one of the five HumAbs, BAB1, was able by itself to inhibit the Bet v 1-induced histamine release. The blocking activity of BAB1 was not restricted to a particular patient, inasmuch as strong inhibition of histamine release was observed in all four of the patients tested. The concentration ratio between BAB1 and Bet v 1 in those inhibition experiments varied from 1/1000 to 1/100, depending on the concentration of Bet v 1 used for the different patients. Therefore, maximal blocking activity was found when BAB1 ($M_r = 150$ kDa) was in 10- to 100-fold molar excess to Bet v 1 ($M_r = 17$ kDa). The requirement for this high ratio of BAB1 over Bet v 1 might indicate that part of the HumAb was bound to the Fc γ R on the basophil membrane and was, therefore, unable to compete with IgE for allergen binding (5) (see below). The absence of blocking activity of BAB2, BAB3, BAB4, and BAB5 tested separately makes it unlikely that a nonspecific component present in our preparations of purified Abs is responsible for the inhibition of basophil degranulation observed with BAB1

alone. Nevertheless, covering four distinct epitopes of Bet v 1 with the mixture of BAB2 to BAB5 suppressed histamine release, probably through steric hindrance. The absence of inhibition of Bet v 1-induced basophil degranulation by the control hIL-1 α -specific HumAb X3A3 established that the effect of BAB1 is dependent on allergen-specific recognition, as confirmed by the inability of any BAB to affect the anti-IgE induced basophil degranulation (data not shown).

The inhibitory capacity of BAB1 cannot be attributed to a higher binding affinity for Bet v 1; its K_a (10^{-9}) is similar to or lower than the K_a (10^{-9} – 10^{-10}) of the other HumAbs. On the other hand, the role of the Fc portion of BAB1, an IgG1, cannot be directly addressed because the four other HumAbs are either IgG2 (BAB4) or IgG4 (BAB2, BAB3, and BAB5). In theory, BAB1 could recognize an epitope identical or closely related to the major IgE binding site of Bet v 1. The surface density of membrane-bound IgE specific for the other Bet v 1 epitopes would be too low to permit their cross-linking by Bet v 1, therefore explaining the complete inhibition of histamine release by BAB1. However, cross-inhibition experiments by Bet v 1 of the binding of human IgE to apple allergen (15) to *Aln g* 1 (27, 28) and to *Cor a* 1 (16) have established that several important IgE epitopes are present on Bet v 1. It therefore seems unlikely that BAB1 could physically cover all the epitopes recognized by the IgE of different patients. Alternatively, upon binding to Bet v 1, BAB1 might modify the conformation of the allergen so that the IgE epitopes are no longer present at the surface of the molecule. Influence of the binding of an mAb on the conformation of a peptide epitope (through either the stabilization or the induction of a tertiary structure) has been demonstrated when crystals of free peptide vs Fab'-complexed

peptide were compared (33). Moreover, the enhancing effect of BAB2 on the binding of patients IgE to blotted Bet v 1 might suggest that the conformation of this allergen is modified when complexed with this Ab. Definitive assessment of the mechanisms of interference of BAB1 and BAB2 with human IgE, however, will await the comparison by x-ray crystallography of free vs Ab-complexed Bet v 1. While competition between IgE and BAB1 for the recognition of Bet v 1 could explain the histamine release inhibition observed, an alternative and not mutually exclusive explanation may relate to the recently described down-regulation of FcεRI-mediated mast cell activation by cross-linking the murine FcεRI and FcγRII with IgG-allergen immune complexes (5).

In conclusion, BAB1, a Bet v 1-specific HumAb, represents the ideal tool to further investigate the complex interplay between allergen-specific IgG and IgE Abs in the context of allergy. Moreover, BAB1 seems to display a desirable profile for a blocking Ab of therapeutic interest as 1) it binds with high affinity to a major allergen, Bet v 1, which is responsible for most symptoms in birch pollen-sensitive patients; 2) it efficiently cross-reacts with Bet v 1 homologous allergens present in different tree pollens and plant-derived foods, therefore extending its range of potential application; and 3) it significantly reduces the binding of polyclonal human IgE to immobilized Bet v 1 and specifically prevents the allergen-induced basophil histamine release. In view of the rather low amounts of specific IgE in allergic patients, it could be expected that local application of a blocking Ab such as BAB1 might prevent the degranulation of tissue mast cells and help to control the symptoms of allergy.

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