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Molecular characterization of a human immunoglobulin G4 antibody specific for the major birch pollen allergen, Bet v 1

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Clinical and Experimental Allergy

Summary

Background Allergen-specific IgG4 antibodies induced by specific immunotherapy are thought to represent a protective immune response.

Objective Our aim was the molecular characterization of a human IgG4 antibody (BAB5) specific for the major birch pollen allergen Bet v 1 that was derived from an immunotherapy-treated patient.

Methods The cDNA coding for BAB5 was obtained by reverse transcriptase-PCR from the BAB5-producing cell line, compared with the germ line sequences and was expressed as a soluble antibody fragment in *Escherichia coli*. The epitope specificity and cross-reactivity of BAB5 were investigated with recombinant and synthetic Bet v 1 fragments and Bet v 1 homologous allergens from pollen. The ability of BAB5 to block allergic patients IgE was determined by competition experiments and sandwich ELISA.

Results BAB5 is an affinity-matured Bet v 1-specific IgG4 antibody that reacts exclusively with Bet v 1 but not with Bet v 1-related allergens. Unlike an earlier-described monoclonal IgG1-blocking antibody, BAB1, which had been isolated from the same patient, BAB5 did not block allergic patients' IgE reactivity to Bet v 1.

Conclusion Our study demonstrates that not all allergen-specific IgG antibodies inhibit IgE recognition of allergens and can contribute to the success of immunotherapy. The epitope specificity and affinity of IgG antibodies but not their isotype are decisive for their protective activity.

Keywords allergen, Bet v 1, birch pollen allergy, immunotherapy, monoclonal human IgG antibodies, recombinant antibody fragment

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Introduction

Allergen-specific immunotherapy is based on the administration of allergens to allergic patients in order to induce a state of antigen-specific 'non-responsiveness' [1]. It has been shown to be effective especially in seasonal rhinitis and allergic asthma [2]. Specific immunotherapy induces several immunological alterations that include the induction of allergen-specific IgG antibody responses, alteration of the balance of T-helper (Th) cells towards Th1 and the development of regulatory T cells [3–5].

First evidence that the induction of allergen-specific IgG antibodies may be an important mechanism underlying allergen-specific immunotherapy came from the

observation that transfer of serum from patients with successful immunotherapy into untreated patients could prevent allergen-induced skin inflammation [6]. The concept that the induction of allergen-specific IgG antibodies that can block IgE recognition of allergens is a major mechanism underlying specific immunotherapy gained further support by studies characterizing these antibodies and their blocking activity [7, 8]. Besides allergen-specific IgG1, IgG4 has been identified as the major isotype induced by allergen-specific immunotherapy [9, 10]. The pronounced induction of allergen-specific IgG4 responses during the course of specific immunotherapy [11–13] may be related partly to the fact that this isotype is regulated by Th2-related mechanisms that dominate in allergic

patients [14]. It is equally possible that allergens through intrinsic properties, components in allergen extracts and some of the adjuvants commonly used in immunotherapy (e.g. aluminium hydroxide) induce preferential Th2 responses favouring the formation of IgG4 [15–17].

The pronounced induction of allergen-specific IgG4 during immunotherapy even gave rise to the idea that a major part of the protective blocking antibody response resides within the allergen-specific IgG4 isotype, and therefore allergen-specific IgG4 was used to monitor immune responses during the course of immunotherapy. However, it has been reported that therapy-induced IgG4 responses are not always associated with clinical success [18]. There are several possible explanations for the previously reported poor correlation between clinical success and induction of IgG4 antibodies. They may include the lack of purified allergens/epitopes for the serological and clinical assessment, the possibility that in addition isotypes other than IgG4 may be important for blocking the IgE reactivity to allergens, titres and affinities of the antibodies and other antibody-mediated mechanisms, such as silencing or enhancing of effector cells by co-crosslinking mechanisms [19–24]. Finally, it is possible that other immunoregulatory mechanisms may contribute to the clinical success of immunotherapy [3].

In this study, we report the molecular characterization of a human IgG4 antibody, designated BAB5, which has been isolated from a birch pollen-allergic patient who had received birch pollen-specific immunotherapy. BAB5 exhibited classical features of an affinity-matured antibody but did not inhibit IgE binding to Bet v 1 because it bound to epitopes on Bet v 1 that were different from those recognized by IgE antibodies. Our results provide one possible explanation for the partly controversial results regarding the beneficial effect of allergen-specific IgG antibodies. They show that not the isotype but rather the epitope specificity, titre and affinity of IgG antibodies are decisive for their ability to block IgE recognition of allergens.

Methods

Antibodies, sera, allergens

A cell line secreting the monoclonal Bet v 1-specific IgG4 antibody BAB5 was established from a patient who had been treated with birch pollen-specific immunotherapy [25]. The molecular characterization of the mouse IgG1 antibody specific for Bet v 1, Bip 1, was reported [26]. Sera were collected from birch pollen-allergic patients who were characterized by clinical history, skin prick testing, specific IgE detection and by testing with recombinant birch pollen allergens as described [27]. A rabbit anti-serum was obtained by immunization of a rabbit with purified rBet v 1 using complete and incomplete Freud's

adjuvant (CFA and IFA), respectively (Charles River, Kislegg, Germany). Pollen from birch, alder, hazel, hornbeam and oak were purchased from Allergon (Välinge, Sweden). Purified recombinant allergens (rBet v 1, rAln g 1, rCor a 1, rMal d 1, rPhl p 5) were purchased from Biomay (Vienna, Austria). Recombinant Bet v 1 fragments, synthetic rBet v 1 peptides and rBet v 1 peptide-specific rabbit anti-sera were described [28, 29].

Isolation of the cDNAs coding for the heavy and light chain of BAB5 and sequence analysis

The DNA sequences coding for the variable regions of the heavy-chain fragment and the light chain of BAB5 were obtained by direct PCR-based sequencing and have been deposited in GenBank under the following accession numbers: AJ131060 and AJ131061. The heavy-chain and light-chain DNA sequences were compared with germ line sequences deposited in the IMGT/V-QUEST (<http://imgt.cines.fr>). IMGT/V-QUEST identifies the V, D and J genes and alleles by alignment with the germline immunoglobulin gene and allele sequences of the IMGT reference directory [30].

Expression of recombinant BAB5 antibody fragments in Escherichia coli

The cDNAs coding for the BAB5 heavy-chain fragments and the light-chain fragments were isolated from the BAB5-secreting cell line by reverse transcriptase-PCR using the following primers pairs: heavy-chain fragment: 5' CGC GGG ATC CCG CAG GTG CAC CTG CTC GAG TCT GG 3' 5' GCA TGA ACT AGT TGG GGG ACC ATA TTT GGA 3'; light-chain fragment: 5' GAA ATT GAG CTC ACG CAG TCT CCA 3'; 5' GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G 3'. The restriction sites used for subcloning into the pComb3H plasmid are indicated as follows: heavy chain: *Xho*I, underlined; *Spe*I, italics; light chain: *Sac*I, underlined; *Xba*I, italics.

Phage clones expressing functional BAB5 antibody fragments (Fabs) were isolated by panning using ELISA plate-coupled Bet v 1, and a plasmid construct expressing soluble BAB5 Fabs was generated by *E. coli* XL1-Blue cells containing the pComb3H plasmid expressing soluble BAB5 Fabs [31] were grown in SB medium containing 50 µg/mL ampicillin at 37 °C up to optical density (OD at 600 nm) 0.6–1. After induction with 3 mM isopropyl β-D-thiogalactoside, cells were incubated for 5 h at 32 °C. Induced *E. coli* were harvested by centrifugation at 1700 g for 15 min at 4 °C. The supernatants containing culture medium and BAB5 Fabs were kept for analysis at –20 °C. Cells were re-suspended in phosphate-buffered saline containing 100 µg/mL polymyxin B sulphate (Sigma, Steinheim, Germany), incubated for 2 min at room

temperature and centrifuged at 1700 g for 15 min at 4 °C to obtain the periplasmic fraction. Again, cells were resuspended in phosphate-buffered saline. Soluble *E. coli* extracts were obtained by freezing and thawing of the resuspended cells three times after centrifugation (1700 g for 15 min at 4 °C). The insoluble pellet fraction was also kept for analysis at -20 °C. Aliquots of each fraction (culture medium, periplasmic extract, soluble *E. coli* extract and insoluble pellet fraction) were analysed by 12.5% SDS-PAGE under reducing conditions [32] and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) [33]. Nitrocellulose sheets were blocked twice for 5 min and once for 30 min with tris-buffered saline tween-20 (TBST) (50 mM Tris, 150 mM NaCl, pH 7.5, containing 0.05% v/v Tween 20) containing 0.5% w/v bovine serum albumin (BSA) at room temperature and were then incubated for 2 h with an alkaline phosphatase (AP)-coupled goat anti-human IgG F(ab)₂ anti-serum (Pierce, Rockford, IL, USA) diluted 1 : 2500 in TBST/0.5% w/v BSA. The nitrocellulose sheets were washed as described for blocking. Bound Fabs were visualized by addition of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt) diluted in AP-buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) as described [26].

Purification of soluble BAB5 Fabs and BAB5 immunoglobulin G antibodies

BAB5 Fabs were purified by affinity chromatography to rBet v 1 as described [34]. rBAB5 Fabs were then tested for their solubility. For this purpose 50 µL of rBAB5 Fabs were centrifuged at 10 000 g for 5 min. The pellet and supernatant were analysed by 12.5% SDS-PAGE under reducing and non-reducing conditions and Coomassie-Blue staining.

BAB5 IgG antibodies were purified via protein G affinity according to the manufacturer's protocol (Pierce).

Preparation of natural pollen extracts; identification of Bet v 1-homologous allergens by immunoblotting

The preparation of extracts from pollen is described [34]. For detection of Bet v 1 and Bet v 1-homologous allergens, nitrocellulose membranes containing comparable amounts of blotted extracts from the different sources were blocked twice for 5 min and once for 30 min with TBST/0.5% w/v BSA and incubated overnight with purified BAB5 IgG4 antibodies (20 µg/mL) and, for control purposes, with Bet v 1-specific rabbit antibodies and rabbit's pre-immune serum (1 : 1000 diluted in TBST/0.5% BSA). The BAB5 IgG4 antibodies were detected with AP-coupled goat anti-human anti-serum (Pierce). Bound rabbit's antibodies were visualized by AP-conjugated goat

anti-rabbit IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA).

Reactivity with Bet v 1-homologous allergens

ELISA plates (Nunc-Immuno, Maxisorp, Roskilde, Denmark) were coated with 100 µL (*c* = 5 µg/mL) rBet v 1, rAln g 1, rCor a 1, rMal d 1 and for control purposes with 100 µL (*c* = 5 µg/mL) rPhl p 5 in 0.1 M bicarbonate buffer, pH 9.6, at 4 °C overnight. Plates were washed twice with TBST containing 0.5% w/v BSA and blocked with TBST containing 3% w/v BSA at 37 °C for 3 h. After blocking, plates were incubated in duplicate overnight at 4 °C with 20 µg/mL purified BAB5 IgG4 antibodies or serum (diluted 1 : 50 in TBST/0.5% w/v BSA) from a birch pollen-allergic patient who had developed Bet v 1-specific IgG4 antibodies during immunotherapy, with culture supernatants from *E. coli* transformed with empty plasmid pComb3H or pComb3H expressing rBAB5 Fabs, or buffer alone. After washing five times with TBST/0.5% w/v BSA, bound human IgG antibodies were incubated with mouse monoclonal anti-human IgG4 antibodies (PharMingen, San Diego, CA) diluted 1 : 1000 in TBST/0.5% w/v BSA. Bound rFabs were detected with an AP-labelled goat anti-human IgG F(ab)₂ (Pierce) diluted 1 : 5000 in the same buffer [31]. After additional five washes with TBST/0.5% w/v BSA, mouse IgG antibodies were detected with a horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare, Little Chalfont, UK) diluted 1 : 2000 in TBST/0.5% w/v BSA. OD at 405 nm were determined with an ELISA reader (Dynatech, Denkendorf, Germany or Spectramax PLUS, Molecular Devices, Sunnyvale, CA, USA). Results represented means of duplicate determinations with variations of < 10%.

Enzyme-linked immuno sorbent assay competition experiments

The ability of BAB5 IgG4 antibodies and BAB5 IgG Fabs to inhibit the binding of birch pollen-allergic patients' IgE antibodies to rBet v 1 was studied by ELISA competition experiments. ELISA plates (Nunc-Immuno, Maxisorp) were coated with rBet v 1 (100 µL; *c* = 1 µg/mL) in 0.1 M sodium bicarbonate, pH 9.6, at 4 °C overnight. Plates were washed twice with TBST containing 0.5% w/v BSA and blocked with TBST containing 3% w/v BSA at 37 °C for 3 h. After blocking, the following incubations were performed in duplicate overnight at 4 °C:

- (1) Incubation with 100 µg/mL purified BAB5 IgG4 antibodies in TBST/0.5% w/v BSA.
- (2) Incubation with culture supernatants from *E. coli* transformed with pComb3H expressing rBAB5 Fabs diluted 1 : 1 in phosphate-buffered saline tween (PBST)/0.5% w/v BSA.

Table 1. The DNA sequences of the heavy chain (H) and light chain (L) variable sequences of BAB5 were compared with the closest-related germline IG gene sequences

	IGHV	<i>n</i>	%	IGHD	<i>n</i>	%	IGHJ	<i>n</i>	%
BAB5H	IGHV5-51*03	270/285 bp	94.73	IGHD2-8*01	9/14 bp	64.28	IGHJ4*02	43/47 bp	91.48
	IGKV	<i>n</i>	%				IGKJ	<i>n</i>	%
BAB5L	IGKV3-15*01	271/279 bp	97.13				IGKJ1*01	34/38 bp	89.47

The numbers (*n*) and the percentages (%) of identical nucleotides are displayed for the IGHV, IGHD, IGHJ and IGK/LV, IGK/LJ, respectively. Gene names and alleles are indicated.

- (3) Incubation with culture supernatants from *E. coli* transformed with empty pComb3H diluted 1 : 1 in PBST/0.5% w/v BSA.
- (4) Incubation with Bet v 1-specific rabbit immune serum diluted 1 : 100 in TBST/0.5% w/v BSA.
- (5) Incubation with rabbit's pre-immune serum diluted 1 : 100 in TBST/0.5% w/v BSA.

After washing five times with TBST/0.5% w/v BSA, plates were incubated in duplicate with sera from birch pollen-allergic patients diluted 1 : 5 in TBST/0.5% w/v BSA and, for control purposes, with serum from a non-allergic individual overnight at 4 °C. Experiments were also performed by mixing directly the competing antibody or control reagent with the serum dilution instead of performing pre-incubation. Bound IgE antibodies were detected with AP-conjugated mouse monoclonal anti-human IgE antibodies (PharMingen) diluted 1 : 1000 in TBST/0.5% w/v BSA. To avoid plate-to-plate variabilities, experiments were performed for each patient's serum on the same plate. Results represented means of duplicate determinations with variations of <10%. The percentage inhibition of IgE binding was calculated as follows: percentage inhibition = $OD_{\text{competitor}} \times 100 / OD_{\text{control}}$. A sandwich ELISA for the detection of Bet v 1-specific IgE antibodies was established and interpreted as described [34].

Results

BAB5 is an affinity-matured human immunoglobulin G4 monoclonal antibody with a κ light chain

The DNA sequences of the variable regions of BAB5 have been submitted to GenBank under the following accession numbers: AJ131060 for a heavy chain and AJ131061 for a light chain. Compared with the germline IG genes available at IMGT/V-QUEST, the V-GENE of the variable region of the heavy chain of BAB5 showed the highest similarity (94.73%) to VH5-51*03 (X56368) [35]. The alignment of the D-GENE identified 64.28% identity to IGHD2-8*01 (X13972) [36]. The J-GENE was most closely related (91.48%) to germline J-region IGHJ4*02 91 (X86355) [37] (Table 1). The variable region of the BAB5 heavy

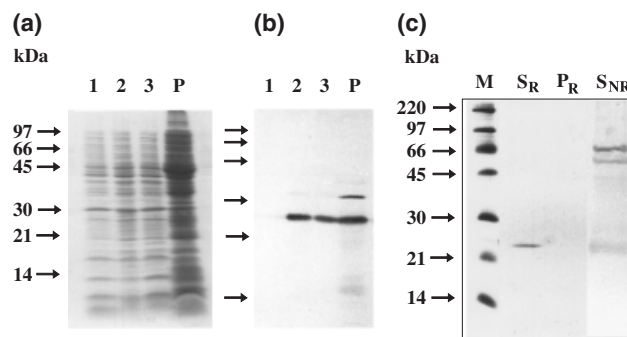


Fig. 1. (a) Expression of recombinant BAB5 Fabs in *Escherichia coli*. rBAB5 IgG Fab-containing extracts [1: culture medium obtained after induction; 2: periplasmic *E. coli* fraction; 3: soluble *E. coli* extract obtained after freeze/thawing; insoluble pellet fraction (P)] obtained after induction were separated under reducing conditions on SDS-PAGE and stained with Coomassie Brilliant-blue. The positions of the molecular weights are indicated on the left side. (b) Nitrocellulose-blotted extracts as above (1, 2, 3, P) were reacted with an alkaline phosphatase-coupled goat anti-Fab anti-serum to identify recombinant Fabs. (c) rBAB5 IgG Fabs purified by affinity chromatography are soluble. Supernatant (S) and P obtained from purified rBAB5 IgG Fabs which had been subjected to centrifugation were separated by SDS-PAGE under reducing (S_R , P_R) and non-reducing (S_{NR} , P_{NR}) conditions and stained with Coomassie Brilliant-blue. The molecular weight marker (M) is shown on the left margin.

chain contained 15 somatic mutations, of which 10 [six in the complementarity-determining regions (CDRs), four in the framework regions (FWRs)] led to replacements. The V-GENE of the light chain showed 97.13% identity to the IGKV3-15*01 (M23090) [38]. The alignment of the J-GENE revealed 89.47% similarity to IGKJ1*01 (J00242) [39] (Table 1). The BAB5 variable region of the light chain contained eight nucleotide exchanges, of which seven (four in the CDRs, three in the FWRs) led to replacement.

Expression and purification of recombinant BAB5 Fabs

We searched for the presence of rBAB5 Fabs in culture medium, periplasmic extract, soluble *E. coli* extract and the insoluble pellet fraction of bacteria that had been induced for 5 h (Figs 1a and b). Little or no BAB5 Fabs were found in the culture medium (Figs 1a and b, lanes 1). Most of the BAB5 Fabs occurred in the periplasmic fraction that had been obtained by treatment of the

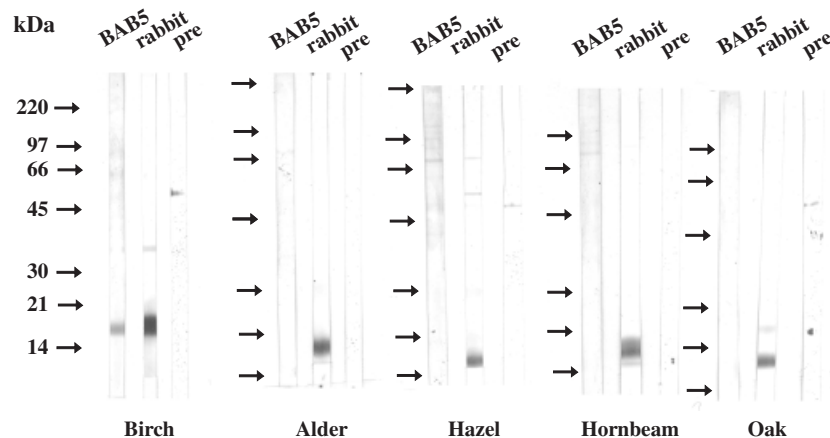


Fig. 2. Cross-reactivity of BAB5. Nitrocellulose-blotted birch, alder, hazel, hornbeam and oak pollen extracts were exposed to BAB5, rabbit anti-Bet v 1 antibodies or the pre-immune serum. Bound antibodies were visualized by alkaline phosphatase (AP)-coupled goat anti-human anti-serum or AP-conjugated goat anti-rabbit IgG (H+L), respectively. The positions of the molecular weights are indicated on the left side.

bacteria with polymyxin B sulphate and in the soluble and insoluble fraction of an extract obtained by freeze thawing of *E. coli* cells (Figs 1a and b; lanes 2, 3 and P). Mechanical homogenization of bacteria yielded soluble cellular extracts rich in functional Fabs (data not shown).

Purified rBAB5 Fabs were obtained by affinity chromatography using a column containing sepharose-bound rBet v 1 allergen. Approximately 0.8 mg of soluble rBAB5 Fabs could be purified from 120 mL culture. Purified rBAB5 Fabs were obtained as a soluble heavy-chain fragment and a light chain co-migrating at 25 kDa (lane S_R) and did not occur in the pellet fraction obtained after centrifugation of the protein preparation (lane P_R) (Fig. 1c). Under non-reducing conditions, we obtained a double band between 50 and 66 kDa, presumably representing disulphide bond-linked heavy-chain fragments and light chains and a weaker 25 kDa band of non-linked chains (Fig. 1c, lane S_{NR}).

BAB5 immunoglobulin G4 as well as BAB5 immunoglobulin G Fabs react exclusively with Bet v 1 but not with Bet v 1-related pollen or plant food allergens

The cross-reactivity of BAB5 with Bet v 1-homologous plant allergens was tested by immunoblotting. Monoclonal BAB5 IgG4 antibodies and rBAB5 Fabs (data not shown) reacted with Bet v 1 at 17 kDa in birch pollen extract but not with nitrocellulose-blotted Aln g 1 from alder, Cor a 1 from hazel, Car b 1 from hornbeam and Que a 1 from oak (Fig. 2; lanes BAB5). Bet v 1 and each of the Bet v 1-related allergens were detected with a rabbit anti-Bet v 1 anti-serum. The rabbit's pre-immune serum gave almost no background reactivity with blotted birch, alder, hazel, hornbeam and oak extract (Fig. 2).

Next, we tested BAB5 IgG4 for reactivity to ELISA plate-bound purified and non-denatured allergens, re-

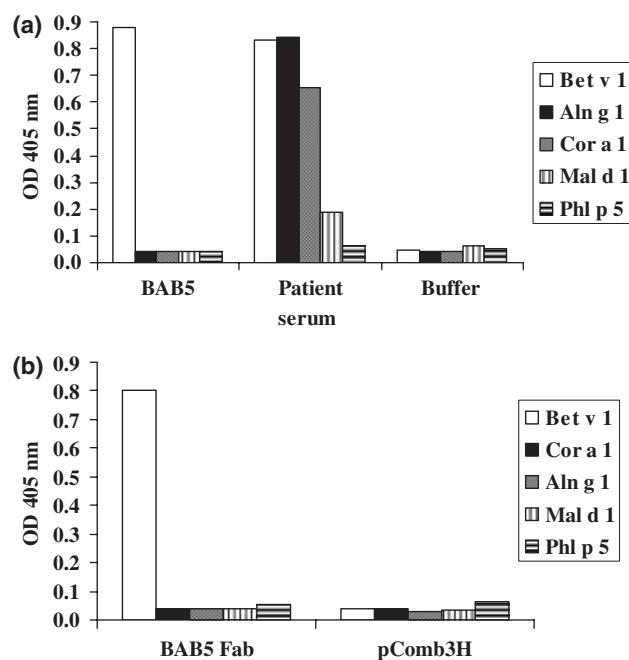


Fig. 3. Reactivity of BAB5 IgG4 (a) and recombinant BAB5 IgG Fabs (b) with purified recombinant allergens. ELISA plate-coated rBet v 1 (birch), rAln g 1 (alder), rCor a 1 (hazel), rMal d 1 (apple) and rPhl p 5 (timothy grass) were incubated with BAB5 IgG4, serum from a patient who had received birch pollen-specific immunotherapy or buffer (a), recombinant BAB5 Fabs or supernatants from *Escherichia coli* transformed with the empty plasmid pComb3H (b). OD levels (y-axis) correspond to bound IgG4 antibodies or Fabs.

combinant Bet v 1 fragments and peptides. We again found that BAB5 IgG4 reacted exclusively with rBet v 1 but did not react with rAln g 1, rCor a 1 or rMal d 1 (Fig. 3a). Recombinant BAB5 Fabs exhibited the same binding pattern and reacted exclusively with Bet v 1 whereas no binding was found when the culture supernatant for *E. coli* transformed with an empty plasmid

pComb3H plasmid was tested (Fig. 3b). There was also no reactivity of BAB5 with the timothy grass pollen allergen, Phl p 5 (Figs 3a and b). Polyclonal serum IgG4 from a patient who had received birch pollen-specific immunotherapy cross-reacted with Bet v 1 and Bet v 1-homologous allergens (Fig. 3a). BAB5 did not react with recombinant Bet v 1 fragments comprising aa 1–74 and 75–160 [29] nor with any of the Bet v 1-derived synthetic peptides [28] (data not shown).

BAB5 recognizes epitopes on Bet v 1 that are distinct from those recognized by immunoglobulin E antibodies and does not inhibit allergic patients' immunoglobulin E binding to Bet v 1

The question of whether BAB5 IgG4 and rBAB5 IgG Fabs bind to epitopes on Bet v 1 that are also recognized by IgE antibodies was addressed by two types of experiments. First, we immobilized Bet v 1 to ELISA plates, added BAB5 IgG4 in excess and after washing allowed IgE from 10 different allergic patients to bind. BAB5 did not inhibit IgE reactivity to Bet v 1 in a relevant manner (mean inhibition 3.4%) whereas polyclonal Bet v 1-specific rabbit antibodies caused a mean inhibition of IgE binding of 55.9% (Table 2). On adding the antibodies in the solution phase, comparable results were obtained as with the pre-incubation protocol. Rabbit anti-Bet v 1 antibodies added in solution inhibited on an average (patients sera: $n = 6$) 47.8% of IgE reactivity to Bet v 1 whereas the pre-incubation protocol yielded a 47.6% inhibition (data not shown). No inhibition was obtained with the rBAB5 Fabs under each of these conditions similar as to when the

Table 2. Influence of BAB5 on patients' IgE binding to rBet v 1

Inhibition of IgE binding to rBet v 1 after pre-incubation with					
Patient	Pre-OD _{405 nm}	Rabbit		BAB5	
		OD _{405 nm}	%	OD _{405 nm}	%
1	1.083	0.241	77.7	1.005	7.2
2	0.362	0.294	18.8	0.350	3.3
3	0.341	0.156	50.8	0.357	–
4	0.588	0.381	35.2	0.518	11.9
5	0.604	0.231	61.7	0.685	–
6	0.301	0.126	58.2	0.286	5.0
7	0.459	0.243	47.1	0.448	2.4
8	0.862	0.319	63.0	0.836	3.0
9	0.747	0.266	64.4	0.757	–
10	0.859	0.156	81.8	0.847	1.4
Mean	0.621	0.241	55.9	0.609	3.4

OD values corresponding to Bet v 1-specific IgE levels and the percentages of inhibition of IgE binding to rBet v 1 after pre-incubation with BAB5 antibodies or Bet v 1-specific rabbit antibodies are displayed for sera from 10 birch pollen allergic patients (1–10). Mean OD values and mean percentages of inhibition are shown in the bottom line. OD, optical density.

Table 3. BAB5 presents Bet v 1 to allergic patients IgE

Patient	Mean OD _{405 nm} Presentation of			
	Bet v 1 via		Phl p 5 via	
	BAB5	Bip1	BAB5	Bip1
1	0.271	1.288	0.053	0.073
2	0.190	0.521	0.060	0.063
3	0.184	0.320	0.053	0.050
Non-allergic	0.051	0.050	0.043	0.050

BAB5 and Bip 1 were coated to ELISA plates and allowed to present Bet v 1 or Phl p 5 to patients (1, 2, 3) IgE or to serum from a non-allergic patient. OD values (y-axis) correspond to Bet v 1-specific IgE levels.

complete natural BAB5 antibody was used in the pre-incubation protocol (data not shown).

This experiment indicated that BAB5 does not inhibit IgE reactivity to Bet v 1. Next, we were interested in studying whether immobilized BAB5 can present Bet v 1 to patients' IgE antibodies and indeed reacts with epitopes distinct from those recognized by IgE. We found that ELISA plate-coupled BAB5-bound Bet v 1 and allowed IgE to react with Bet v 1 whereas no IgE reactivity was observed when the unrelated grass pollen allergen, Phl p 5, was used. IgE reactivity to BAB5-bound Bet v 1 was weaker than IgE reactivity to Bet v 1 bound to Bip 1, a mouse monoclonal antibody that even enhances patients' IgE reactivity to Bet v 1 [26] (Table 3).

Discussion

Several recent studies that have been conducted with purified allergens suggest that the induction of allergen-specific IgG antibodies capable of inhibiting IgE-mediated processes is a major mechanism underlying allergen-specific immunotherapy [3, 4, 19–21, 40–45]. In this context, it has been demonstrated that therapy-induced allergen-specific IgG blocks IgE-dependent histamine release from basophils and mast cells as well as IgE-mediated antigen presentation to T cells [40–43, 21]. Furthermore, there is evidence that blocking IgG antibodies prevents boosting of IgE memory responses by allergens [41, 42, 44]. The observation that allergen-specific IgG antibodies induced in the nasal mucosa by vaccination were associated with reduced nasal allergen sensitivity [45] even contradicts earlier reports [18] that did not find correlations between clinical improvement and the induction of allergen-specific IgG antibodies during immunotherapy.

One possible explanation for this discrepancy may be the fact that the earlier studies were conducted with allergen extracts that represent crude mixtures of allergens and non-allergenic materials and hence did not allow the measurement of true allergen-specific clinical

and immune alterations. Another explanation for the discrepancy between the clinical results and the induction of allergen-specific IgG is given here. The BAB5 antibody specific for the major birch pollen allergen Bet v 1 described in our study could be regarded as a classical therapy-induced IgG4 antibody. The cell line secreting BAB5 was isolated from a birch pollen-allergic patient after birch pollen-specific immunotherapy [25]. The frequency of somatic mutations especially in the V-region of the heavy chain of BAB5 antibodies indicated affinity maturation typically caused by allergen contact and was confirmed by the high affinity of this Bet v 1-specific antibody (K_D $1.5\text{--}3.0 \times 10^{-9}$ M) [25]. Interestingly, BAB5 as well as recombinant BAB5 Fabs were monospecific for Bet v 1 and did not cross-react with Bet v 1-homologous allergens from pollens and plant food as did natural IgE and IgG antibodies present in sera from untreated allergic patients. It cannot be proven that BAB5 was indeed induced by the injection of a Bet v 1-containing birch pollen extract but the high specificity for Bet v 1 and the presence of somatic mutations argue for its induction through specific immunotherapy. Only one cDNA coding for a heavy chain and one for a light chain could be isolated from the BAB5-secreting cell line and we could not provide evidence for the claimed bi-specificity of allergen-specific IgG4 antibodies [46, 47] in the case of BAB5. BAB5 reacted only with Bet v 1 but no other antigens in birch pollen extract or unrelated allergens such as the grass pollen allergen, Phl p 5.

Furthermore, BAB5 did not inhibit IgE binding to Bet v 1, although the antibody reacted specifically with Bet v 1. Using a sandwich ELISA in which BAB5 was immobilized to the plate, we could show that the antibody bound Bet v 1 and even presented the allergen to patients' IgE antibodies. This experiment rules out the possibility that BAB5 recognized the same epitope as patients' IgE antibodies. The fact that an allergen-specific IgG4 antibody isolated from a patient after immunotherapy did not compete with IgE demonstrates that epitope specificity, affinity and titre but not the isotype are relevant for the blocking activity of therapy-induced allergen-specific IgG. This assumption is also supported by a study demonstrating that the blocking activity of therapy-induced IgG antibodies does not reside exclusively in the IgG4 fraction because IgG4-depleted sera retained their blocking activity [48].

At present, we cannot exclude that antibodies such as BAB5 that do not interfere with the IgE recognition of allergens play a role in the allergic immune responses in patients. It is possible that IgG antibodies recognizing epitopes different from those recognized by IgE may up- or down-regulate mast cell or basophil degranulation [49, 22]. In this context, it was found that IgG antibodies may enhance histamine release by mechanisms of super-cross-linking [22] but this was not found for BAB5. Another possibility would have been that BAB5 down-regulates

effector cell degranulation by co-crosslinking of allergens but this mechanism was not yet found to be operative in allergen-specific immunotherapy [23] and we also have no evidence for such an activity in the case of BAB5. Possibly, antibodies such as BAB5 simply contribute to the elimination of allergens via binding to antigen-presenting cells or other Fc γ receptor-bearing cells or simply by forming small immune complexes, but we have no experimental data for this.

In conclusion, our study demonstrates that even the measurement of allergen-specific antibody responses cannot predict with absolute certainty whether each of the allergen-specific antibodies can block IgE binding and has protective activity. It will therefore be necessary to develop allergen-specific assays that directly allow to assess the blocking of allergen-specific IgE by therapy-induced antibodies or other protective functions. Furthermore, it indicates that allergen-specific immunotherapy may be improved by the use of vaccines containing exactly the desired allergens or derivatives that have been engineered to foster the production of IgG antibodies that inhibit the IgE recognition of allergens.

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Note added in proof: That the recently reported dynamic Fab arm exchange of human IgG4 results in the formation of relevant amounts of bispecific IgG4 in humans seems unlikely, because it has been shown that a 20-fold excess of irrelevant IgG4, as is easily present in humans, prevents this process [50].

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