Molecular characterization of human IgG monoclonal antibodies specific for the major birch pollen allergen Bet v 1. Anti-allergen IgG can enhance the anaphylactic reaction

Stéphane Denépoux[a,1], Petra B. Eibensteiner[b,1], Peter Steinberger[b], Susanne Vrtala[b], Vincenzo Visco[a], Anne Weyer[c], Dietrich Kraft[b], Jacques Banchereau[d], Rudolf Valenta[b,*], Serge Lebecque[a]

[a] *Laboratory for Immunological Research, Schering-Plough, Dardilly, France*
[b] *Institute of General and Experimental Pathology, AKH, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria*
[c] *Unit of Immuno-Allergy Pasteur Institute, Paris, France*
[d] *Baylor Institute for Immunology Research, Dallas, TX, USA*

Received 4 October 1999; received in revised form 15 November 1999

Edited by Masayuki Miyasaka

Abstract We report the molecular characterization of five human monoclonal antibodies, BAB1–5 (BAB1: IgG_1; BAB4: IgG_2; BAB2, 3, 5: IgG_4), with specificity for the major birch pollen allergen, Bet v 1. BAB1–5 were obtained after immunotherapy and contained a high degree of somatic mutations indicative of an antigen-driven affinity maturation process. While BAB1 inhibited the binding of patients IgE to Bet v 1, BAB2 increased IgE recognition of Bet v 1, and, even as *Escherichia coli*-expressed Fab, augmented Bet v 1-induced immediate type skin reactions. The demonstration that IgG antibodies can enhance allergen-induced allergic reactions is likely to explain the unpredictability of specific immunotherapy.

Key words: Allergy; Antibody; Antigen-peptide-epitope; Molecular biology; Immunotherapy

1. Introduction

How antigen recognition by antibodies of different isotypes can result in completely different effects is exemplified by Type I allergy, an immune disorder affecting almost 20% of the population world wide [1,2]. The immediate type symptoms of Type I allergy (e.g. allergic rhinitis, conjunctivitis, asthma) result from the recognition of minute amounts of per se innocuous environmental antigens (e.g. proteins from pollen, mites, animals, fungi) by IgE antibodies and subsequent high affinity Fcε-receptor-mediated activation of allergic effector cells (e.g. mast cells, basophils) [3]. While the IgE antibody-mediated release of biological mediators (e.g. histamine, leukotrienes) constitutes the immunopathological basis for the immediate symptoms observed in allergic patients, much less is known concerning the role of allergen-specific IgG antibodies in atopy.

In 1935 Robert A. Cooke and his colleagues cured allergic rhinitis by transfusing to an allergic recipient the blood obtained from a patient who had received specific immunotherapy with ragweed extract [4]. It was later shown that the transferable factor represents a thermostable antibody which according to its ability to 'block' the skin response to injected allergen was termed 'blocking antibody' [5]. Next it was demonstrated that all of the antibodies that blocked allergen-induced histamine release from human leukocytes belonged to the IgG isotype [6] and that allergen-specific IgG_4 antibody levels increased during allergen-specific immunotherapy [7]. However, the hypothesis that successful immunotherapy may be based on the induction of blocking IgG antibodies was questioned by studies indicating that efficacy does not always correlate with the induction of allergen-specific IgG antibodies [8] and there is nowadays great controversy on the beneficial role of IgG in atopy. Moreover, immunotherapy of allergy may cause not only immune deviations, but also anergy and deletions [9].

In an effort to analyze allergen-specific IgG antibodies at a molecular level, to directly compare antigen recognition by IgE and IgG antibodies, and to study the interference of IgE and IgG antibodies, we cloned five human monoclonal IgG antibodies (BAB1–5) with high affinity for the major birch pollen allergen Bet v 1 [10]. We chose Bet v 1 as a model allergen because it is recognized by IgE antibodies of 95% of tree pollen allergic patients and almost 60% of them react exclusively to Bet v 1 [11]. It binds most of the tree pollen-specific IgE antibodies [12] and shares epitopes with the major allergens present in *Fagales* (e.g. alder, hazel, hornbeam, oak) pollen [13] and plant-derived food (fruits, vegetables, spices) [14]. Recombinant Bet v 1 possesses equal immunological and biological properties as natural Bet v 1 and has been expressed and purified [15]. Lastly, Bet v 1's three-dimensional structure has been determined [16] and the expression of recombinant Bet v 1 fragments indicates that IgE epitopes of Bet v 1 belong to the discontinuous/conformational type [17].

Here we report the molecular characterization of five Bet v 1-specific human monoclonal IgG antibodies, BAB1–5, and the remarkable property of the antibody BAB2 to enhance the binding of Bet v 1-specific IgE to Bet v 1. The interaction of BAB2-Bet v 1-IgE is discussed as a paradigm for a immunomodulatory network, in which binding of one antibody to its antigen can augment antigen-recognition by disease-elicit-

*Corresponding author. Fax: (43)-1-40400 5130.
E-mail: rudolf.valenta@akh-wien.ac.at

[1] Both authors contributed equally and are listed in alphabetical order.

PII: S0014-5793(99)01703-2

ing antibodies of another class and thus can lead to disease aggravation.

2. Materials and methods

2.1. Biological materials, E. coli strains and plasmids

Pollen from birch, alder, hazel and hornbeam were purchased from Allergon (Välinge, Sweden). Plant food (apple, celery, hazelnuts) was freshly obtained in a local retail store. Birch pollen-allergic patients and non-allergic individuals were characterized by case history, skin prick testing and serology using natural as well as recombinant allergens as described [12]. *E. coli* strain XL1 Blue was purchased from Stratagene (La Jolla, CA, USA). Plasmid pComb3HSS was provided by Dr. Carlos F. Barbas, Scripps Research Institute, La Jolla, CA, USA [18].

The recombinant Bet v 1 utilized in our study is encoded by the cDNA originally isolated by IgE immunoscreening of a birch pollen cDNA library [15]. Recombinant Bet v 1 fragments (amino acids (aa) 1–74; aa 75–160) were purified as described [17]. Recombinant Phl p 2, a major timothy grass pollen allergen which does not share epitopes with Bet v 1, was expressed and purified as described [19].

2.2. Isolation and molecular characterization of human Bet v 1-specific IgG antibodies BAB1–5

Cell lines secreting BAB1–5 Bet v 1-specific IgG antibodies were established from a birch pollen allergic patient who had received hyposensitization therapy with natural birch pollen extract as described [10]. RNA was isolated from B-cell clones according to a single step method described [20]. BAB1–5 cDNAs were obtained by reverse transcription using the Superscript Reverse Transcriptase kit (BRL, Gaithersburg, MD, USA) and oligo dT_{12-18} primers (Pharmacia, Uppsala, Sweden). The cDNAs were used as templates for PCR amplification using a PCR amplification core kit (Perkin Elmer, Norwalk, CT, USA). For amplification of the heavy chain fragment-encoding cDNAs, individual PCRs were carried with primers with specificity for the six different human V_H families in conjunction with a primer located at the 3′-end of the gamma constant region. Heavy chain primers: V_{H1} 5′ CCC GAA TTC ATG GAC TGG ACC TGG AGG 3′; V_{H2} 5′ CCC GAA TTC ATG GAC ATA CTT TGT ACC AC 3′; V_{H3} 5′ CCC GAA TTC ATG GAG TTT GGG CTG AGC 3′; V_{H4} 5′ CCC GAA TTC ATG AAA CAC CTG TGG TTC TT 3′; V_{H5} 5′ CCC GAA TTC ATG GGG TCA ACC GCC ATC CT 3′; V_{H6} 5′ CCC GAA TTC ATG TCT GTC TCC TTC CTC AT 3′ Cg 5′ AGC TTC CAC CAA GGG CCC ATG GGT CTT 3′. cDNAs encoding the light chains were amplified by individual PCRs with κ or λ chain primers with specificity for the 3′-end in conjunction with primers complementary to the different V_κ and V_λ gene families. Light chain primers: V_{K1} 5′ CCC GAA TTC ATG GAC ATG AGG GTC CCC GCT CAG CTC 3′; V_{K2} 5′ CCC GAA TTC ATG AGG CTC CCT GCT CAG CTC CTG 3′; V_{K3} 5′ CCC GAA TTC ATG GAA ACC CCA GCG CAG 3′; V_{K4} 5′ CCC GAA TTC ATG GGG TCC CAG GTT CAC CTC 3′; C_k 5′ GAG AGA GCT CTT TGT GAC GGG CGA GGG CGA GCT CAG G 3′; V_{L1} 5′ CCC GAA TTC ATG ACC TGC TCC CCT CTC CT 3′; V_{L2} 5′ CCC GAA TTC ATG GCC TGG GCT CTG CTG CTC 3′; V_{L3a} 5′ CCC GAA TTC ATG GCC TGG TCT CCT CTC CTC 3′; V_{L3b} 5′ CCC GAA TTC ATG GCA TGG ATC CCT CTC CTC 3′; V_{L3c} 5′ CCC GAA TTC ATG GCC TGG ACT CCT CTC TTT CTG 3′; C_l 5′ GAG AGA GCG GCC GCC TAT GAA CAT TCT GTA GGG GCC AC 3′. Two PCR products obtained independently for each of the heavy chain fragments and light chains were purified by preparative agarose gel electrophoresis using GELase (Epicentre, WI, USA) and used as templates for double stranded DNA sequencing in a 373DNA sequencer with a TaqDyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The BAB DNA and deduced aa sequences were compared with germline sequences deposited in the V Base Sequence Directors (Tomlinson et al., MRC Center for Protein Engineering, Cambridge, UK) using the DNASTAR software (Madison, WI, USA) and were submitted to GenBank.

2.3. Expression of recombinant BAB2 Fabs in E. coli

RNA was isolated from EBV-transformed human B-cells secreting BAB2 antibody by lysis of the cells in 4.7 M guanidine isothiocyanate and subsequent CsCl density centrifugation [21]. The light chain and heavy chain Fd encoding cDNA was obtained by reverse transcription and PCR amplification using the following primer pairs: heavy chain: 5′ GCA TGA ACT AGT TGG GGG ACC ATA TTT GGA 3′; 5′ CGC GGG ATC CCG CAG GTG CAC CTG CTC GAG TCT GG 3′; light chain: 5′ T CCT TCT AGA TTA CTA ACA CTC TCC CCT GTT GAA GCT CTT TGT GAC GGG CGA ACT C 3′; 5′ GAA GTT GAG CTC AGT CAG TCT CCA 3′. The cDNAs encoding the light chain and heavy chain fragment were purified by preparative agarose gel electrophoresis, cut with *Sac*I/*Xba*I or *Spe*I/*Xho*I, respectively. Purified fragments were subcloned into plasmid pComb3HSS to yield plasmid pComb3HBAB2 and transformed into *E. coli* XL1 Blue. The sequences of the BAB2 light chain and heavy chain fragment-encoding cDNAs of one Bet v 1-reactive clone (clone 28) were confirmed by double stranded sequencing. Clone 28, containing plasmid pComb3HBAB2, was modified for the production of soluble BAB2 Fabs by removal of the gene III-containing *Spe*I/*Nhe*I fragment. *E. coli* XL1 Blue were transformed with the modified pComb3HBAB2 plasmid and induced with IPTG to produce soluble recombinant BAB2 Fabs in their periplasmic space.

2.4. Antibody, Fab binding assays

Plant extracts (pollen, plant-derived food) were prepared as described [14]. Total protein extracts containing natural Bet v 1/Bet v 1-homologous allergens, purified recombinant Bet v 1 and rBet v 1 halves, each at approximately 5 μg/cm gel, were separated by SDS–PAGE [22] and blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) [23]. Human IgG antibodies were detected with 1:1000 diluted rabbit anti-human IgG antibodies (Dako, Glostrup, Denmark) and a 1:1000 diluted ^{125}I-labeled donkey anti-rabbit Ig antiserum (Amersham, Buckinghamshire, UK). The mouse monoclonal antibody (#15) with specificity for rBet v 1 (aa 1–74) [24] was detected with a 1:500 diluted ^{125}I-labeled sheep anti-mouse Ig antiserum (Amersham). Human IgE antibodies were detected with ^{125}I-labeled anti-human IgE antibodies (RAST, Pharmacia, Uppsala, Sweden).

E. coli XL1 Blue colonies expressing Bet v 1-reactive BAB2 Fabs were identified by ELISA. rBet v 1 was coated to ELISA plates (Nunc, Roskilde, Denmark) at a concentration of 10 μg/ml and blocked as described [12]. Plates were then probed with the periplasmic extracts of pComb3HBAB2-transformed bacteria. Bound Bet v 1-specific recombinant Fabs were detected with an alkaline phosphatase-conjugated goat anti-human Ig $(Fab)_2$ antiserum (Sigma, St. Louis, MO, USA), diluted 1:5000 in TBST-buffer (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% v/v Tween 20, 1% BSA). The goat anti-human Ig $(Fab)_2$ antiserum was also used to demonstrate the binding of recombinant BAB2 Fabs to nitrocellulose-blotted Bet v 1 and Bet v 1-homologous allergens.

2.5. Modulation of human IgE binding to rBet v 1 by BAB antibodies and rBAB2 Fabs

200 ng rBet v 1/well were coated to ELISA plates (Nunc) and blocked as described [12]. In experiments investigating IgE binding to Bet v 1 depending on the ratios of blocking (BAB1) or enhancing (BAB2) BAB antibodies, plates were incubated with complete BAB1 or BAB2 antibodies (50 μg/well) as well as with mixtures consisting of 75% BAB1:25% BAB2, 50% BAB1:50% BAB2 and 25% BAB1:75% BAB2. The effect of rBAB2 Fabs on human IgE binding to rBet v 1 was studied by incubating plates with periplasmic extracts of pComb3HBAB2- or, for comparison, with that of pComb3H-transformed bacteria diluted 1:1 in TBST-buffer overnight at 4°C. Plates were washed and incubated with 1:5 in TBST-buffer diluted sera from Bet v 1 allergic patients and, for control purposes, with serum from an atopic individual without Bet v 1-specific IgE, with serum from a non-atopic individual or with buffer alone. Bound serum IgE was detected with an 1:1000 diluted alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody (Pharmingen, San Diego, CA, USA). Results were obtained as mean of duplicate determinations (error of $< 10\%$) after subtraction of the background (i.e. values obtained with serum from a non-atopic individual) and were expressed as % of IgE binding to Bet v 1 without BAB antibodies or as % enhancement of IgE binding to Bet v 1 (rBAB2 Fabs). Statistical analysis of the data was performed with two-tailed paired *t*-tests using Microsoft Excel.

The presence of potential IgE anti-BAB2 immunoreactivity in sera was also investigated by ELISA. 200 ng/ml of purified BAB2 anti-

bodies and, for control purposes, rBet v 1, were coupled to ELISA plates. Sera were tested for IgE reactivity to plate-bound BAB2 antibodies and rBet v 1 as described above. The successful coupling of BAB2 antibodies to the ELISA plate was demonstrated with an alkaline phosphatase-conjugated goat anti-human Ig $(Fab)_2$ antiserum (Sigma, St. Louis, MO, USA).

2.6. Purification of recombinant BAB2 Fabs

5 mg of purified recombinant Bet v 1 were coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The column was stripped with 100 mM Glycin–HCl pH 2.7 and washed with PBS to remove unbound Bet v 1. *E. coli* extracts containing recombinant BAB2 Fabs were prepared by homogenizing cells in PBS and removal of insoluble particles by centrifugation. After passing the *E. coli* lysate twice over the Bet v 1 affinity column, the matrix was washed with PBS until the baseline ($OD_{280\,nm}$) was reached. Bound BAB2 Fabs were eluted with 100 mM Glycin–HCl pH 2.7 and immediately neutralized with 3 M Tris–HCl pH 9. The purity of eluted BAB2 Fabs was confirmed by SDS–PAGE and silver-staining.

2.7. Skin testing

Skin prick tests were performed on the forearms of four birch pollen allergic and two non-allergic individuals as described [17]. Individuals were pricked with 20 μl of PBS solution containing either 100 ng or 20 ng of purified rBet v 1 or rPhl p 2, with or without 2 μg of purified BAB2 Fabs. Birch pollen extract (ALK, Horsholm, Denmark), PBS (phosphate buffered saline), and histamine hydrochloride (ALK) were used as controls. The skin reactions were recorded after 20 min by photography and by determining the wheal surface area as follows: Surface = $[D1+D2)/2]^2$. *D*1 represents the maximal longitudinal and *D*2 the maximal transverse diameter. Statistical analyses of the BAB2 induced increases of wheal surfaces were performed with Student's paired *t*-test.

3. Results

3.1. BAB1–5 are five different human IgG monoclonal antibodies that recognize different epitopes of Bet v 1

The sequences of the BAB1–5 heavy and light chains have been submitted to GenBank under the following accession numbers: AJ131062: BAB1 hc; AJ131063: BAB1 lc; AJ131064: BAB2 hc; AJ131055: BAB2 lc; AJ131056: BAB3 hc; AJ131057: BAB3 lc; AJ131058: BAB4 hc; AJ131059: BAB4 lc; AJ131060: BAB5 hc; AJ131061: BAB5 lc. BAB1 belongs to the γ-1 subclass, BAB4 to the γ-2 subclass and BAB2, 3 and 5 to the γ-4 subclass (Fig. 1; GenBank). The clonality of the BAB cell lines was confirmed by sequencing V_H and V_L of two independently obtained cDNAs. Each of the variable region sequences contained many somatic mutations (ranging from 13 to 47, average: 23) (data not shown; GenBank). Mutations have preferen-

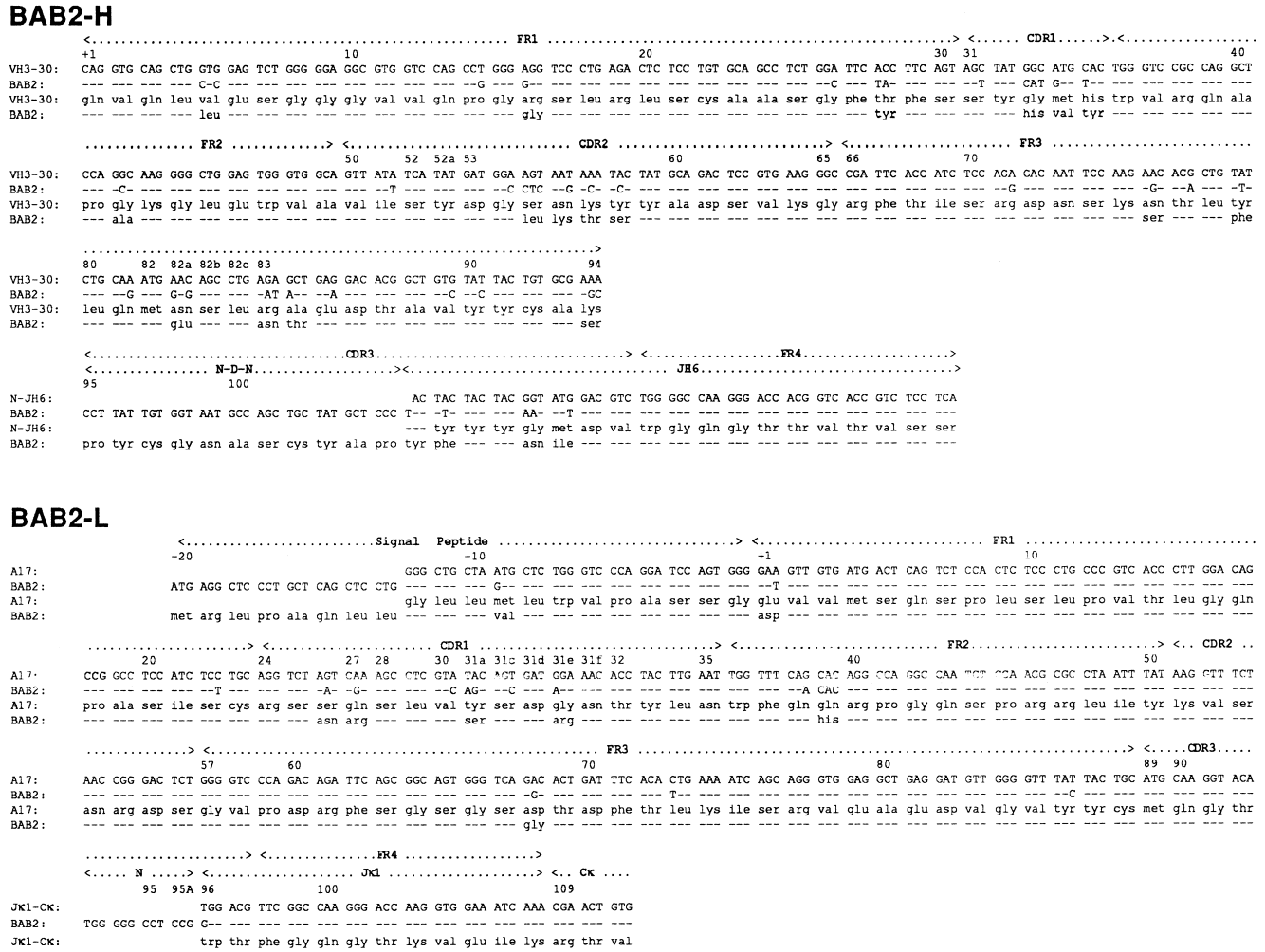


Fig. 1. Comparison of the BAB2 heavy chain (H) and light chain (L) DNA and aa sequence with their putative germline homologues. The positions of the complementary determining regions (CDRs) and frameworks (FRs) are indicated. Numbering and CDRs are according to Kabat. Identical nucleotides or aa are displayed by dashes.

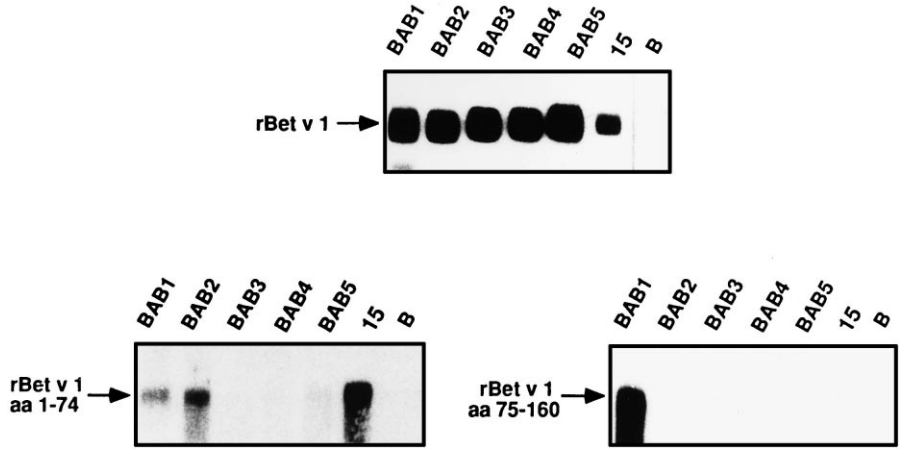


Fig. 2. Binding of BAB antibodies to nitrocellulose-blotted recombinant Bet v 1 and recombinant Bet v 1 fragments. rBet v 1, the N-terminal (rBet v 1 aa 1–74), and the C-terminal (rBet v 1 aa 75–160) Bet v 1 fragment were probed with the BAB antibodies (BAB1–5), a mouse monoclonal anti-Bet v 1 antibody (#15) and buffer (B).

tially accumulated in CDR1 or CDR2. Sixty nine out of a total of 196 somatic mutations (34%) were present in the CDRs which only represented 22% of the total size of the V fragments, and the ratio of replacement versus silent mutations in CDR1 and CDR2 was high (5.7) compared with that of the FWRs (1.2). (Fig. 1; GenBank).

BAB3 and BAB4, which are of different isotype (BAB3: IgG_4; BAB4: IgG_2), rearranged the same V_{H2-5} gene with JH5 and JH1, respectively ([25]; GenBank). BAB3 and BAB4 heavy chains have recombined with two different light chains (BAB3: Vl1e; BAB4: L12A) [26] but shared four identical replacement mutations in the heavy chain CDRs (Ser-31a → Arg, Met-32 → Val, Gly-60 → Ser, Lys-64 → Arg) (GenBank).

All BAB antibodies recognized native [10] as well as recombinant Bet v 1 (Fig. 2). Because none of the human monoclonal antibodies reacted with synthetic dodecapeptides spanning the complete Bet v 1 sequence, the epitopes recognized by BAB1–5 were determined using the N-terminal (aa 1–74) and C-terminal (aa 75–160) fragments of Bet v 1 [17]. BAB1 bound strongly to the C-terminal Bet v 1 fragment and showed a very weak reactivity with the N-terminal fragment, suggesting that it predominantly recognizes an epitope on the C-terminal portion of Bet v 1. BAB2 bound strongly and BAB5 weakly to the N-terminal Bet v 1 fragment but not at all with the C-terminal fragment. Neither BAB3 nor BAB4 bound to any of the two recombinant Bet v 1 fragments (Fig. 2).

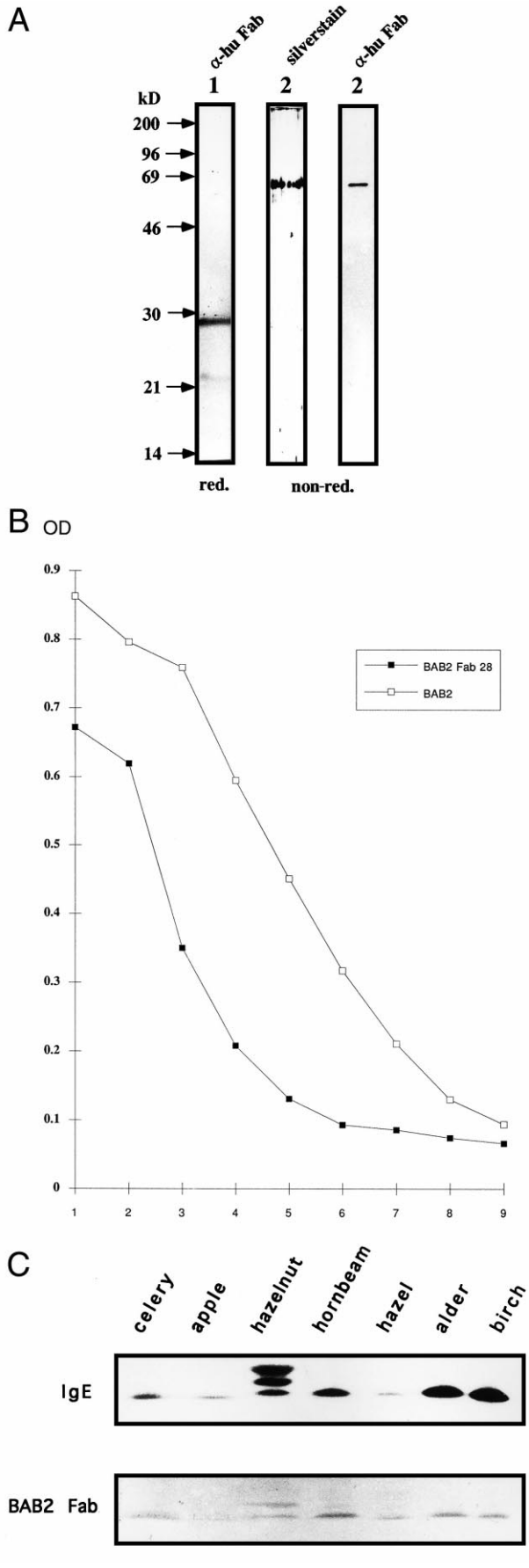
3.2. Recombinant BAB2 Fabs bound to Bet v 1 and cross-reacted with Bet v 1-homologous allergens in various pollen and plant-derived food

The cDNAs encoding the BAB2 heavy chain fragment (variable region plus a portion of the first constant domain) and

Table 1
Modulation of IgE binding to Bet v1 after preincubation with BAB antibodies

Patients sera	IgE binding after preincubation (%)				
	100% BAB2	75% BAB2 25% BAB1	50% BAB2 50% BAB1	25% BAB2 75% BAB1	100% BAB1
A	130	114	102	80	51
B	113	108	98	82	71
C	117	104	93	70	41
D	177	154	141	83	23
E	154	146	122	106	58
F	141	146	120	111	70
G	123	123	104	92	57
H	145	144	124	108	51
I	161	144	124	105	38
J	149	143	121	980	45
K	165	147	137	100	35
L	161	143	146	114	37
M	151	145	135	120	56
N	165	150	140	106	33
O	229	235	218	185	91
mean	152	143	128	104	50

Modulation of IgE binding to rBet v 1 depends on the ratios of blocking (BAB1) or enhancing (BAB2) IgG antibodies. ELISA plate-bound rBet v 1 was preincubated with buffer, BAB1 or BAB2 alone, and with mixtures (75% BAB2:25% BAB1; 50% BAB2:50% BAB1; 25%BAB2:75% BAb1) of BAB1 and BAB2. IgE binding of sera from 15 birch pollen allergic patients (patients A–O) is displayed as percentage binding where 100% represents the IgE binding yielded after allergen preincubation with buffer alone. The mean modulation of IgE binding is displayed in the bottom line.



the complete BAB2 light chain (Fig. 1) were co-expressed as pComb3HBAB2 construct. The assembly of the recombinant heavy chain fragment and light chain is confirmed by our finding that under non-reducing conditions, purified recombinant BAB2 Fabs migrate as disulphide bond-linked heterodimers at approximately 60 kDa in SDS–PAGE (Fig. 3A; lanes 2: silverstain, α-hu Fab). Both chains are disrupted under reducing conditions and thus appear as double band of approximately 26–27 kDa (Fig. 3A; lane 1: α-hu Fab).

The binding of recombinant BAB2 Fabs and complete natural antibodies to ELISA plate-bound rBet v 1 were compared at different antibody/Fab dilutions (Fig. 3B). The similar shape of the binding curves recorded for the different antibody/Fab dilutions suggests a comparable ability of complete BAB2 antibodies and rBAB2 Fabs to bind to rBet v 1. Furthermore, like serum IgE from Bet v 1 allergic patients, rBAB2 Fabs cross-reacted identically with Bet v 1-homologous allergens present in various tree pollen (hornbeam, hazel, alder) as well as in plant-derived food (celery, apple, hazelnut) (Fig. 3C).

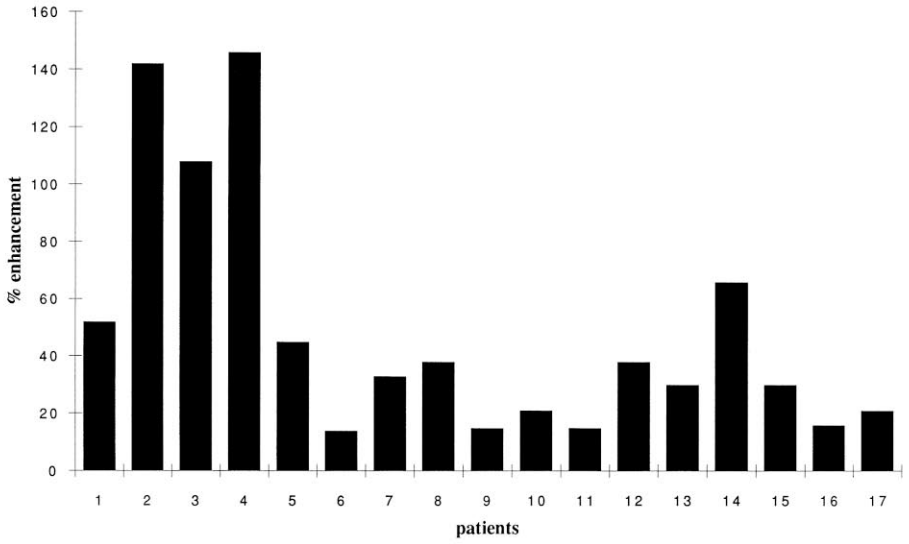
3.3. *BAB1 inhibits the binding of IgE from atopic patients to Bet v 1 while BAB2 enhances it*

Using an ELISA assay we found that BAB3, 4 and 5 had no significant influence on IgE binding to Bet v 1. In contrast, BAB1 strongly inhibited the binding of IgE in 15/15 atopic sera ($P = 4.9 \times 10^{-6}$) (Table 1). On the other hand, BAB2 stimulated the binding of IgE to Bet v 1 in 15/15 atopic sera ($P = 6 \times 10^{-6}$) (Table 1). Both antibodies antagonized each other, however the BAB2 enhancing activity seemed to dominate over the inhibitory capacity of BAB1 because preincubation of Bet v 1 with an equimolar mixture of both antibodies enhanced IgE binding in 13 out of 15 sera (mean binding 128%) and even a ratio of 25% BAB2:75% BAB1 was sufficient to increase IgE binding to Bet v 1 in 8 out of 15 sera (Table 1). That the increased IgE reactivity to BAB2-bound Bet v 1 resulted from the presence of IgE anti-BAB2 immunoreactivity is unlikely because no IgE antibodies against purified BAB2 antibodies could be detected by ELISA in the sera (data not shown).

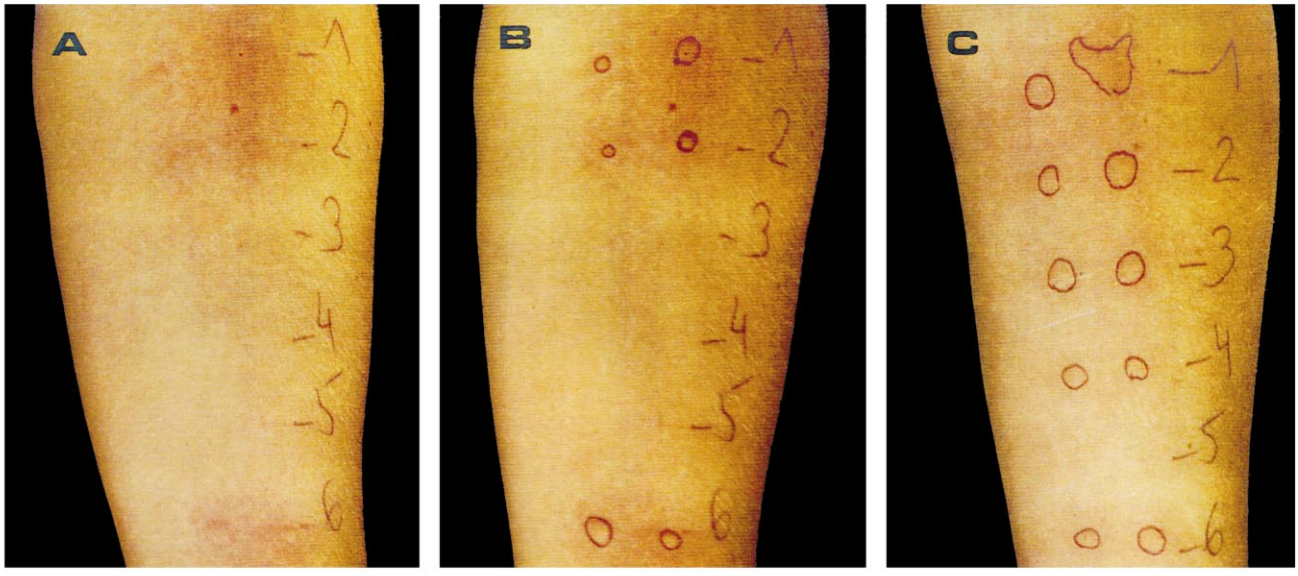
←

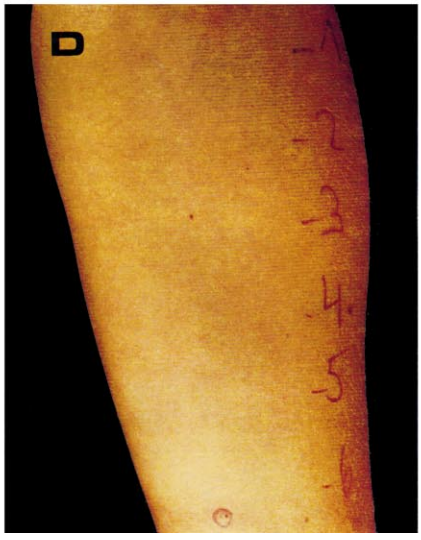
Fig. 3. A: *E. coli* expression and purification of BAB2 Fabs. Lane 1 shows nitrocellulose-blotted *E. coli* periplasmic extracts containing recombinant BAB2 Fabs separated by SDS–PAGE under reducing conditions. The comigrating recombinant heavy chain fragment and light chain were detected with a goat anti-human Fab antiserum (α-hu Fab). Lanes 2 show affinity purified BAB2 Fabs separated by SDS–PAGE under non-reducing conditions visualized by silverstaining (silverstain) and after blotting to nitrocellulose detected by the goat anti-human Fab antiserum (α-hu Fab). B: Comparison of the binding of natural BAB2 (white squares) and recombinant BAB2 Fab clone 28 (filled squares) to ELISA plate-bound recombinant Bet v 1. One:two dilutions (*x*-axis) of natural BAB 2 or recombinant BAB2 Fabs were exposed to constant amounts of ELISA plate-bound rBet v 1. The optical density (OD) values at the *y*-axis represent means of duplicate determinations and correspond to the bound antibodies or rFabs. C: Cross-reactivity of IgE antibodies from a birch pollen allergic patient (IgE) and recombinant BAB2 Fabs with nitrocellulose-blotted Bet v 1 and Bet v 1-homologous allergens. from alder, hazel, hornbeam, hazelnut, apple and celery.

A



B





rBet v1 (5μg/ml) + PBS	rBet v1 (5μg/ml) + BAB2 Fab	- 1
rBet v1 (1μg/ml) + PBS	rBet v1 (1μg/ml) + BAB2 Fab	- 2
rPhl p2 (5μg/ml) + PBS	rPhl p2 (5μg/ml) + BAB2 Fab	- 3
rPhl p2 (1μg/ml) + PBS	rPhl p2 (1μg/ml) + BAB2 Fab	- 4
PBS	BAB2 Fab	- 5
Histamine	Birch	- 6

Table 2
Effect of recombinant BAB2 Fabs on Bet v 1-induced immediate type skin reactions

Individual	Wheal surface in mm^2 after testing with									
	rBet v 1 1 μg/ml+ PBS	rBet v 1 1 μg/ml+ BAB2	rBet v 1 5 μg/ml+ PBS	rBet v 1 5 μg/ml+ BAB2	rPhl p 2 1 μg/ml+ PBS	rPhl p 2 1 μg/ml+ BAB2	rPhl p 2 5 μg/ml+PBS	rPhl p 2 5 μg/ml+ BAB2	birch	histamine
O.S.	6.25	25	12.25	49	0	0	0	0	25	56.25
T.C.	42.25	81	64	289	42.25	36	49	49	52.56	36
S.S.	6.25	36	56.25	81	0	0	0	0	64	30.25
F.S.	12.25	56.25	76.56	110.25	0	0	0	0	45.56	45.56
V.R.	0	0	0	0	0	0	0	0	0	36
E.P.	0	0	0	0	0	0	0	0	0	20.25

Influence of recombinant BAB2 Fabs on Bet v 1-induced immediate type skin reactions. Individuals in Table 2 correspond to Fig. 4B as follows: O.S.: A = B, T.C.: C and E.P.: D.

3.4. Recombinant BAB2 Fabs enhance IgE binding to Bet v 1 and Bet v 1-induced immediate type skin reactions

Preincubation of rBet v 1 with recombinant BAB2 Fabs substantially increased IgE binding of all 17 birch pollen allergic patients' sera to rBet v 1 (Fig. 4A). Five sera showed a more than 50% and eight sera a 20–50% increase of IgE binding to Bet v 1. Only four sera showed a lower than 20% enhancement of IgE binding to Bet v 1. No significant IgE binding to Bet v 1 was detected when serum from an atopic patient without Bet v 1-specific IgE, a non-atopic individual or buffer were tested (data not shown).

Using in vivo skin test experiments, preincubation of rBet v 1 with rBAB2 Fabs augmented the Bet v 1-induced skin reactions (wheal area) in all four Bet v 1-allergic patients tested (Table 2; Fig. 4B). We observed a two-threefold increase of the Bet v 1-induced wheal area at each of the two different Bet v 1 concentrations (wheal areas in Table 2; Fig. 4B). The results were statistically significant ($P = 0.01$) when analyzed in a Student's paired *t*-test. Preincubation of rBet v 1 with rBAB2 Fabs also enlarged the erythema area in Bet v 1-sensitized patients (Fig. 4B). The specificity of the BAB2-induced increase of skin reactivity was supported by the following controls:

1. Preincubation of a Bet v 1-unrelated grass pollen allergen, Phl p 2, with rBAB2 Fabs did not increase rPhl p 2-induced skin reactions (Table 2; Fig. 4B).
2. rBAB2 Fabs alone failed to induce skin reactions in Bet v 1-allergic and in non-allergic individuals (Table 2; Fig. 4B).
3. A mixture of rBAB2 Fabs and rPhl p 2 did not cause immediate type skin reactions in patients who were not sensitized to Phl p 2 or in non-allergic individuals (Table 2; Fig. 4B). Additionally, all Bet v 1-allergic patients mounted immediate type skin reactions to birch pollen extract and allergic and non-atopic persons presented a wheal reaction in response to histamine (Table 2; Fig. 4B).

4. Discussion

The role of allergen-specific IgG antibodies in atopy has been a matter of debate for more than 50 years. Herein, we report the molecular characterization of five human IgG monoclonal antibodies, BAB1–5, with specificity for the major birch pollen allergen, Bet v 1, one of the most frequently recognized environmental allergens [11]. Three (BAB2, BAB3, BAB5) of the five Bet v 1-specific IgG antibodies belonged to the IgG_4 subclass, what may reflect either the typical Th2-biased cytokine environment in allergic patients that favors isotype switching towards IgE and IgG_4 [27] or the increase of allergen-specific IgG_4 antibody levels induced by immunotherapy [7]. The high frequency of somatic mutations indicates that the five BAB antibodies derived from memory B-cells as previously suggested by these antibodies high affinity [10]. BAB3 (γ4-subclass) and BAB4 (γ2-subclass) rearranged the same V_{H2-5} gene with JH5 and JH1, respectively, but expressed different light chains (Vλ1e and L12A, respectively), which suggests that the V_{H2-5} peptide may play an important role in the contact/binding to Bet v 1.

In contrast to IgE, three out of the five BAB antibodies (BAB1, BAB2 and BAB5) were able to bind to the recombinant Bet v 1 halves, indicating that the epitopes recognized by these BABs are different from the IgE binding sites. This finding exemplifies that allergen-specific IgE and IgG antibodies can recognize different epitopes. When tested for their ability to influence IgE binding to Bet v 1, three of the five BAB antibodies (BAB3, 4 and 5) had little or no effect and only BAB1 blocked IgE anti-Bet v 1 reactivity. This provides one explanation why immunotherapy does not always lead to reduction of symptoms: some therapy-induced IgG antibodies directed to epitopes other than IgE epitopes are unable to block the IgE-allergen interaction.

Moreover, BAB2, a human IgG_4 antibody, was found to increase the binding of IgE to Bet v 1, and thus can be defined

←
Fig. 4. A: Increased IgE recognition of ELISA-plate-bound rBet v 1 after preincubation with rBAB2 Fabs. The percentage enhancement of IgE binding to rBet v 1 (*y*-axis) is displayed for sera from 17 birch pollen allergic patients (*x*-axis). Results represent mean values of duplicate measurements with an error of $< 10\%$. B: Enhancement of Bet v 1-induced immediate type skin reactions by recombinant BAB2 Fabs. Two birch pollen allergic patients (B: A = B, C) and a non-allergic individual (B: D) were skin tested on their forearms with two concentrations of rBet v 1 and rPhl p 2 mixed with PBS or rBAB2 Fabs, with PBS, rBAB2 Fabs alone, birch pollen extract and histamine. The application order of the test substances is displayed in the inset. Skin reactions in the first birch pollen-allergic patient are shown without (B,A) and with (B,B) surrounding of the wheal area. Wheal areas in individuals C and D are surrounded with a ball point pen (Fig. 4B).

as an 'enhancing antibody'. This in vitro finding is further supported by in vivo studies where rBAB2 Fabs were shown to increase Bet v 1-induced immediate skin reactions in Bet v 1-allergic patients. The existence of stimulatory anti-allergen IgG antibodies provides another explanation for the failures of immunotherapy with respect to the reduction of allergic symptoms. The balance between 'stimulatory' versus 'blocking' anti-allergen IgG antibodies may thus dictate the outcome of a course of allergen immunotherapy.

rBAB2 Fabs cannot interfere with allergic effector cells (e.g. mast cells, basophils [28] because they lack Fc regions. BAB2 Fabs may however display an enhancing activity by directly exposing IgE epitopes through conformational changes of the allergen. In line with this hypothesis, IgG antibodies like BAB2 may directly act on the selection of IgE epitopes during the early stage of allergen sensitization as IgG-complexed allergens presented on germinal center follicular dendritic cells (FDCs) may contribute to the positive selection of B-cells expressing Bet v 1-specific IgE antibodies.

In summary, BAB2 represents an example of a human IgG antibody whose binding to its antigen further enhances the binding of anaphylactic IgE and thus contributes to disease aggravation. Therefore, the analysis of the BAB human monoclonal IgG antibodies may help to explain the unpredictability of allergen-based immunotherapy and, perhaps, help to learn how to orient the production of IgG antibodies towards the blocking type to improve the outcome of specific immunotherapy.

Acknowledgements: The excellent technical assistance of Ute Vollmann regarding the culture of BAB cell lines is acknowledged. We thank Dr. Tyler Curial for statistical analysis. Skin prick experiments with recombinant allergens were performed with permission of the local ethics committee and after informed consent was obtained from the individuals. This study was supported by a grant from the Austrian Ministry of Research and Transports and by Grants Y078GEN and F0506 of the Austrian Science Foundation and by the ICP Programme of the Austrian Ministry of Research and Transports.

References

[1] Casolaro, V., Georas, S.N., Song, Z. and Ono, S.J. (1996) Curr. Opin. Immunol. 8, 796–803.
[2] A.B. Kay, A.B. (Ed.), (1997) Allergy and Allergic Diseases, Blackwell Science, Oxford.
[3] Segal, D.M., Taurog, J.D. and Metzger, H. (1977) Proc. Natl. Acad. Sci. USA 41, 457–461.
[4] Cooke, R.A., Bernhard, J.H., Hebald, S. and Stull, A. (1935) J. Exp. Med. 62, 733–750.
[5] Loveless, M.H. (1940) J. Immunol. 38, 25–50.
[6] Lichtenstein, L.M., Holtzman, N.A. and Burnett, L.S. (1968) J. Immunol. 101, 317–324.
[7] Aalberse, R.C., Gaag, R. and Leeuwen, J. (1983) J. Immunol. 130, 722–726.
[8] Birkner, T., Rumpold, H., Jarolim, E., Ebner, H., Breitenbach, M., Skvaril, T., Scheiner, O. and Kraft, D. (1990) Allergy 41, 418–426.
[9] Rolland, J. and O'Hehir, R. (1998) Curr. Opin. Immunol. 10, 640–645.
[10] Visco, V., Dolecek, C., Denépoux, S., Le Mao, J., Guret, C., Roussett, F., Guinnepain, M.T., Kraft, D., Valenta, R., Weyer, A., Banchereau, J. and Lebecque, S. (1996) J. Immunol. 157, 956–962.
[11] Jarolim, E., Rumpold, H., Endler, A.T., Ebner, H., Breitenbach, M., Scheiner, O. and Kraft, D. (1989) Allergy 44, 385–395.
[12] Niederberger, V., Pauli, G., Grönlund, H., Fröschl, R., Rumpold, H., Kraft, D., Valenta, R. and Spitzauer, S. (1998) J. Allergy Clin. Immunol. 102, 579–591.
[13] Rohac, M., Birkner, T., Reimitzer, I., Bohle, B., Steiner, R., Breitenbach, M., Kraft, D., Scheiner, O., Gabl, F. and Rumpold, H. (1991) Mol. Immunol. 28, 897–906.
[14] Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D. and Scheiner, O. (1995) J. Allergy Clin. Immunol. 95, 962–969.
[15] Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach, M. (1989) EMBO J. 8, 1935–1938.
[16] Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., Joost van Neerven, R.J., Schou, C., Lowenstein, H. and Spangfort, M.D. (1996) Nat. Struct. Biol. 3, 1040–1045.
[17] Vrtala, S., Hirtenlehner, K., Vangelista, L., Pastore, A., Eichler, H.G., Sperr, W.R., Valent, P., Ebner, C., Kraft, D. and Valenta, R. (1997) J. Clin. Invest. 99, 673–681.
[18] Barbas III, C.F., Kang, A.S, Lerner, R.A. and Benkovic, S.J. (1991) Proc. Natl. Acad. Sci. USA 88, 7978–7982.
[19] Vrtala, S., Susani, M., Sperr, W.R., Valent, P., Laffer, S., Dolecek, C., Kraft, D. and Valenta, R. (1996) J. Allergy Clin. Immunol. 97, 781–787.
[20] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[21] Davis, L.G., Dibner, M.D. and Battey, J.F. (Eds.), (1986) Basic Methods in Molecular Biology. Elsevier, New York.
[22] Fling, S.P. and Gregerson, D.S. (1986) Anal. Biochem. 155, 83–88.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[24] Lebecque, S., Dolecek, C., Laffer, S., Visco, V., Denépoux, S., Pin, J.J., Guret, C., Boltz-Nitulescu, G., Weyer, A. and Valenta, R. (1997) J. Allergy Clin. Immunol. 99, 374–384.
[25] Matsuda, F., Shin, E.K., Nagaoka, H., Matsumura, R., Haino, M. and Fukita, Y. et al. (1993) Nat. Genet. 3, 88–94.
[26] Williams, S.C., Frippiat, J.P., Tomlinson, I.M., Ignatovich, O., Lefranc, M.P. and Winter, G. (1996) J. Mol. Biol. 264, 220–232.
[27] Romagnani, S. (1997) Immunol. Today 18, 263–266.
[28] Daeron, M. (1997) Int. Arch. Allergy Immunol. 113, 138–141.