

Four recombinant isoforms of *Cor a 1*, the major allergen of hazel pollen, show different reactivities with allergen-specific T-lymphocyte clones

Siegfried SCHENK¹, Karin HOFFMANN-SOMMERGRUBER¹, Heimo BREITENEDER¹, Fatima FERREIRA², Gottfried FISCHER³, Otto SCHEINER¹, Dietrich KRAFT¹ and Christof EBNER¹

¹ Institute of General and Experimental Pathology, University of Vienna, Austria

² Department of Genetics and Developmental Biology, University Salzburg, Austria

³ Institute for Blood Group Serology, General Hospital, Vienna, Austria

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Purified preparations of allergenic proteins from plants, and in particular from pollens, consist of multiple closely related isoforms. These isoforms are highly similar in their amino acid sequences, yet they display different properties with respect to antibody binding. In this study we report of differential potencies of cross-reacting tree pollen allergens and cloned isoforms of these allergens to activate allergen-specific T-lymphocyte clones (T-cell clones; TCC). Six TCC with specificity for *Bet v 1*, a representative tree pollen major allergen, were established from peripheral blood of five birch-pollen-allergic donors. All TCC displayed the helper-cell phenotype. Five TCC reacted with distinct epitopes present on natural (n) and on recombinant (r) *Bet v 1*. One TCC could not be stimulated with r *Bet v 1*, in spite of strong reactivity with purified natural *Bet v 1*. The TCC were tested in proliferation assays using purified n *Bet v 1*, n *Cor a 1* (the homologous major allergen of hazel pollen), r *Bet v 1*, four recombinant isoforms of *Cor a 1* and peptides representing corresponding T-cell stimulating regions (isoepitopes) on these proteins. The clones showed different patterns of reactivity in response to stimulation with the five recombinant molecules and the corresponding peptides. Certain exchanges of amino acids within stimulating peptides correlated with a lack of proliferation of the TCC tested. These findings are important with respect to the use of broadly cross-reactive recombinant allergens or allergen-derived peptides for immunotherapy of type I allergy.

Type I allergy to tree pollens is very common in the northern hemisphere. In this respect, pollens from trees of the order Fagales, in particular birch, hazel and alder, represent the most important sources of allergens [1, 2]. The major allergens of these pollens (*Bet v 1*, *Cor a 1* and *Aln g 1*) show a high degree of similarity at the nucleic acid [3] and at the amino acid levels [4], and cross-reactivity was described for IgE antibodies [5] and for T-cell clones (TCC) [6]. This fits well with the observation that tree pollen-allergic patients develop symptoms when exposed to pollens from all these tree species.

Natural allergens consist of multiple isoallergens [7–9]. Recently, four isoforms of *Cor a 1*, the major allergen of hazel pollen, were cloned, sequenced and expressed as recombinant non-fusion proteins [8]. Comparison of the amino acid sequences of these *Cor a 1* isoforms revealed identities

of 96–99%, the amino acid sequence identities between the recombinant (r) *Cor a 1* isoforms and r *Bet v 1* were 71–73% (89.3–95% similarity). The *Cor a 1* isoforms showed different IgE-binding patterns indicating different allergenic potencies [8].

In recent years a substantial role for allergen-specific T helper cells could be demonstrated in type I allergy [10, 11]. Information about these cells was obtained by investigation of TCC established by special culture techniques [12, 13]. It could be shown that the majority of allergen-specific TCC derived from atopic patients displayed a Th2-like type of cytokine production pattern, i.e. a high ratio of secreted interleukin (IL)-4 versus interferon (IFN) γ [14–16]. Molecular cloning techniques and the knowledge about amino acid sequences [7, 17–19] of atopic allergens made it possible to identify T-cell epitopes [6, 20, 21].

The aim of this study was to investigate the T-cell response to different isoforms of allergens. Six TCC were tested with r *Bet v 1* and four recombinant isoforms of *Cor a 1*. Moreover, dodecapeptides representing T-cell epitopes on these recombinant allergens were used in proliferation assays. The knowledge of cross-reacting T-cell epitopes of allergens will certainly have impact on the choice of immunodominant isoforms of allergens for immunotherapy. Moreover, in the future, this information will be essential for the design of peptides which represent relevant T-cell epitopes

Correspondence to C. Ebner, Institute of General and Experimental Pathology, Department of Immunopathology, University of Vienna, AKH-EBO, Währinger Gürtel 18–20, A-1090 Vienna, Austria

Abbreviations. TCC, T-cell (T lymphocyte) clone; *Bet v 1*, the major pollen allergen of birch (*betula verrucosa*); TCR, T-cell receptor; n, natural; r, recombinant; *Cor a 1*, the major pollen allergen of hazel (*Corylus avellana*); PBMC, peripheral blood mononuclear cells; APC, antigen-presenting cells; IL, interleukin; IFN, interferon.

and are capable of modulating the T-cell response of allergic patients [22, 23].

MATERIALS AND METHODS

Patients

Peripheral blood was collected from five tree pollen allergic patients. HLA typing was performed as described [24]. type I-allergy to birch and hazel pollen was proven by typical case history, positive skin tests, and positive radio-allergosorbent test (RAST class 2.5-6).

Allergen protein preparations

Purification of natural (n) Bet v 1 and n Cor a 1

Hazel pollen and birch pollen were purchased from Allergon AB. Aqueous extracts of these pollens were prepared as previously described [2] and resolved in double-distilled water. Biocryl BPA 1000 (TosoHaas) was added (2500 parts/million) followed by centrifugation in an Eppendorf centrifuge for 10 min. The clear supernatants were subjected to HPLC employing a linear gradient of 2-propanol created over 60 min at room temperature (C₈ Hypersil WP 300, 10 µm, 8 mm×250 mm; solvent A, 0.1% trifluoroacetic acid in water; solvent B, 90% 2-propanol, 0.1% trifluoroacetic acid; gradient from 0 to 80% B; flow rate, 1.8 ml/min; monitoring absorbance at 280 nm). The purity of n *Bet v 1* and n *Cor a 1* samples was established in immunoblots and on silver stained polyacrylamide gels [25]. Protein concentrations of purified protein samples were determined by micro-Kjedahl [26] using glycine as standard.

Expression and purification of the recombinant non-fusion proteins

R *Bet v 1* and the four r *Cor a 1* isoforms (*Cor a 1/5*, *Cor a 1/6*, *Cor a 1/11* and *Cor a 1/16*) were expressed as non-fusion proteins in *Escherichia Coli* JM 105. r *Bet v 1* was cloned into the expression plasmid pKK 233-2 and r *Cor a 1* isoforms were inserted into pKK 223-3 (Pharmacia) [8, 27]. Expression of the recombinant allergens was induced by adding isopropyl-thio-β-D-galactoside (1.5 mM) to the culture medium. After 3 h, cells were harvested and recombinant proteins purified from bacterial protein extracts as described [8, 27]. The protein concentration of all purified samples was determined by the micro-Kjedahl method [26]. Aliquots were analyzed by SDS/PAGE using silver staining and immunoblotting. Proteins were dissolved in tissue culture medium, sterile filtered, then used for cell-culture.

IgE immunoblots

IgE immunoblots were performed according to procedures described previously [2]. Protein estimation was performed before loading identical protein amounts onto SDS/polyacrylamide gels in order to make comparison of the IgE-binding capacities of *Cor a 1* isoforms possible [8]. Plasma of five patients was incubated in 1:5 dilution with nitrocellulose strips coated with r *Bet v 1*, r *Cor a 1/5*, r *Cor a 1/6*, r *Cor a 1/11* and r *Cor a 1/16*. A serum pool of 13 non-allergic donors and buffer were used as controls. IgE binding to proteins was detected with a 125I-labelled polyclonal rabbit anti-human IgE antibody (Pharmacia).

Allergen-specific TCC

Allergen-specific TCC were established according to previous publications [11, 12]. Briefly, 10⁶ peripheral blood mononuclear cells (PBMC) were stimulated with individual optimal *Bet v 1* concentrations (5–20 µg/ml) in Ultra Culture medium (BioWhittaker) supplemented with 2 mM/l glutamine and 20 µM 2-mercaptoethanol in 24-well, flat-bottomed culture plates (3524 Costar). After 5 days of culturing human r IL-2 (5 U/ml, Boehringer) was added and the culture continued for an additional 7 days. Viable T-cell blasts were then separated on Ficoll/Hypaque (Pharmacia), and *Bet v 1* specificity was assessed by an [³H]thymidine (Amersham) incorporation assay. Thereafter, T-cell blasts were seeded in limiting dilution (0.5 cells/well) in 96-well round-bottomed plates (Nunc, Nunc) in the presence of 2×10⁵ irradiated (50 Gy) allogeneic PBMC as feeder cells, 1% phytohemagglutinin (by vol., Gibco) and r IL-2 (4 U/well) in the above described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and r IL-2. The specificity of TCC was assessed as previously described [12]. Briefly, 2×10⁴ T-cell blasts were incubated in triplicate cultures in 96 well plates in the presence of 10⁵ autologous PBMC, as antigen-presenting cells (APC), purified *Bet v 1* and whole birch pollen extract. Microtiter plates were then incubated for 48 h at 37°C, 5% CO₂, humidified atmosphere. After treatment for 16 h with 0.5 µCi/well of [³H]thymidine, cells were harvested and the incorporated radioactivity was measured by scintillation counting. When the stimulation index (ratio between radioactivity obtained in cultures with TCC plus autologous irradiated PBMC, plus antigen and radioactivity obtained in cultures containing TCC and PBMC alone) was greater than 10, responses were considered positive.

Phenotyping and specific stimulation of TCC for cytokine production

The phenotype of TCC was analyzed by Flow cytometry using a FACScan (Becton-Dickinson). For analyses the fluorescein-isothiocyanate-labelled mAb anti-(Leu 4/CD3), anti-(Leu 3a/CD4), anti-(Leu 2a/CD8), anti-(TCR α/β WT 31) and anti-(TCR γ/δ I) (TCR, T-cell receptor; Becton-Dickinson) were used [13].

For cytokine production, 10⁶ clone cells were incubated with 5×10⁵ irradiated (50 Gy) autologous APC (PBMC or Epstein-Barr-virus-transformed B cells) and n *Bet v 1* (10 µg/ml) in 1 ml of the culture medium described above. Supernatants were collected after 24 h of incubation. Cytokine levels in the supernatants were measured using ELISA systems provided by Quantakine R&D systems, Minneapolis MN (IL-4); Medgenix Diagnostix, Fleurus, The Netherlands (IFN-γ, IL-2) and BioSource International, Camarillo CA (IL-10). Cultures containing APC alone and TCC plus APC without allergen were used as negative controls.

TCR analysis

Analysis of TCR Vβ region was performed using the polymerase chain reaction (PCR) as described [28–30].

Peptides

Epitope mapping of TCC was performed using dodecapeptides overlapping for two residues (neighbours

share 10 amino acids) as described previously [12]. Peptides representing homologue isoepitopes on the four *Cor a 1* isoallergens were synthesized (Cambridge Research Biochemicals Limited). According to the mapped *Bet v 1* epitopes, TCC were tested with following peptides. TCC SS 10, VIPAARLFKAFI (r *Bet v 1*, amino acids 12–23), VI-SAARLFKSYV (r *Cor a 1/16*, amino acids 12–23), VI-PAARLFKSYV (r *Cor a 1/5*, r *Cor a 1/6*, r *Cor a 1/11*, amino acids 12–23); TCC HC 31, TNFKYNSVIEG (r *Bet v 1*, amino acids 77–88), TNFTYSYTVIEG (r *Cor a 1/5*, r *Cor a 1/11*, amino acids 77–88), TNFKYSYTVIEG (r *Cor a 1/6*, r *Cor a 1–16*, amino acids 77–88); TCC MS 2 and HC 4, KISNEIKIVATP (r *Bet v 1*, amino acids 97–108), KVCHQLKIVAAP (r *Cor a 1/5*, r *Cor a 1/11*, amino acids 97–108), KVCSQLKIVAAP (r *Cor a 1/6*, r *Cor a 1/16*, amino acids 97–108); TCC AA 4, GSILKISNKYHT (r *Bet v 1*, amino acids 111–122), GSILKISSKFHA (r *Cor a 1/5*, r *Cor a 1/6*, r *Cor a 1/11*, amino acids 111–122), GSTLKISSKFHA (r *Cor a 1/16*, amino acids 111–122); TCC BE 15, LRAVESYLLAHS (r *Bet v 1*, amino acids 144–155); LRAVETYLLAHS (r *Cor a 1/5*, r *Cor a 1/6*, r *Cor a 1/11*, r *Cor a 1/16*, amino acids 144–155).

RESULTS

Purification of recombinant and natural allergens

All proteins (r *Bet v 1*, r *Cor a 1/5*, r *Cor a 1/6*, r *Cor a 1/11*, r *Cor a 1/16*, n *Bet v 1*, n *Cor a 1*) were purified to homogeneity. Silver staining revealed single bands in the molecular-mass range described [8, 27] for these allergens (data not shown).

IgE immunoblots

All patients displayed strong IgE binding to recombinant *Bet v 1* (Fig. 1) and exhibited specific IgE antibodies to the four recombinant isoforms of *Cor a 1* (Fig. 2). Differential IgE reactivity of the plasma samples with each isoform could be observed. Controls did not show IgE binding to any of the recombinant proteins.

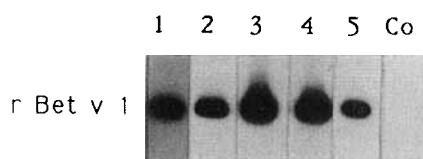


Fig. 1. Reactivity of patients' IgE with purified r *Bet v 1*. 1–5, patients' plasma, SS, HC, MS, AA, BE. Co, negative control, a serum pool of 13 non-allergic donors.

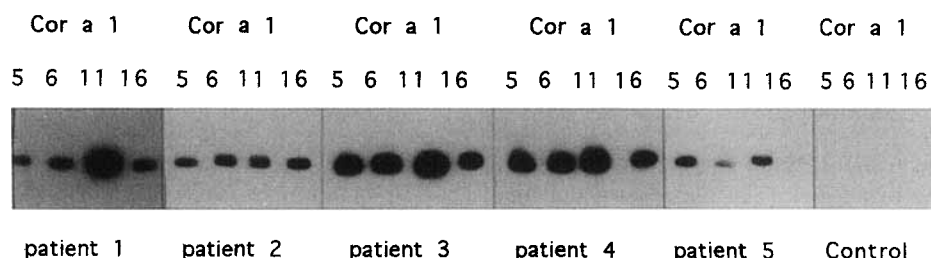


Fig. 2. The reactivity of patients' IgE with four r *Cor a 1* isoforms. Patients 1–5, plasma of patients SS, HC, MS, AA, BE. Control, a serum pool of 13 non-allergic donors.

Phenotyping, cytokine production and TCR analysis of the TCC

According to HLA typing, patients revealed different MHC class II phenotypes. Six TCC (SS 10, HC 31, MS 2, HC 4, amino acids 4, BE 15), established from *Bet-v-1*-specific T-cell lines of five patients were investigated. Flow cytometry analysis revealed the Th phenotype (CD 3+, CD 4+, CD 8-) for all clones. TCC were stimulated with 10 µg/ml n *Bet v 1*. Cytokine production in response to specific stimulation is summarized in Table 1. TCR could in all cases be identified as TCR α/β . PCR analysis of the TCR V β region revealed monoclonality for all TCC. Results of Phenotype, cytokine production and TCR analysis are summarized in Table 1.

Proliferation assays using r *Bet v 1*, four r *Cor a 1* isoforms, and natural allergen preparations

The specificity of six TCC (SS 10, HC 31, MS 2, AA 4, BE 15) for *Bet v 1* was proved by proliferation assays using n *Bet v 1*. All TCC except TCC HC 4, could be stimulated with recombinant *Bet v 1*. Upon stimulation with four r *Cor a 1* isoforms and with n *Cor a 1*, the clones showed distinct responses (Table 2): BE 15 and AA 4 could be stimulated with n *Cor a 1* and with all four r *Cor a 1* isoforms. TCC HC 31 showed proliferation upon stimulation with n *Cor a 1*, r *Cor a 1/6* and r *Cor a 1/16*, but failed to react with r *Cor a 1/5* and r *Cor a 1/11*. TCC SS 10 reacted with n *Cor a 1*, r *Cor a 1/5*, r *Cor a 1/6* and r *Cor a 1/11*, but could not be stimulated with r *Cor a 1/16*. MS 2 showed no reaction with n *Cor a 1* or any r *Cor a 1* isoform. TCC HC 4 did not proliferate upon stimulation with r *Bet v 1*, n *Cor a 1*, r *Cor a 1/5* or r *Cor a 1/11*, but n *Bet v 1*, r *Cor a 1/6* and r *Cor a 1/16* induced proliferation (Table 2).

Proliferation assays using epitope-representing peptides

Each TCC was tested with peptides representing its epitope on r *Bet v 1* and the homologous regions (isoepitopes) on the r *Cor a 1* isoforms. These dodecapeptides differed in one to six amino acids. Proliferation assays with peptides confirmed the results obtained with complete recombinant molecules (Fig. 3).

DISCUSSION

Pollens from birch and hazel (both of the order *Fagales*) contain major allergens with highly similar amino acid sequences [4, 8]. Previous studies demonstrated that these major allergens, proteins with a molecular mass of about

Table 1. Characterization of allergen-specific TCC for phenotype, cytokine production upon specific stimulation, TCR V β gene usage and HLA phenotype. n.d., not determined.

TCC	Phenotype	Production				TCR V β	Patients' HLA phenotype	
		IL-2	IL-4	IFN- γ	IL-10		DR β	DQ β
		U/ml	pg/ml	U/ml	pg/ml			
SS 10	CD 3+, CD 4+, CD 8-	>10	300	4.6	n.d.	n.d.	1, 13; 52	5, 6
HC 31	CD 3+, CD 4+, CD 8-	< 1.0	980	0.5	60	5.2/3	7, 11; 52, 53	2, 7
MS 2	CD 3+, CD 4+, CD 8-	1	<3	5	<5	12	4, 13; 52, 53	3, 6
HC 4	CD 3+, CD 4+, CD 8-	< 0.1	34	26	117	2	7, 11; 52, 53	2, 7
AA 4	CD 3+, CD 4+, CD 8-	< 0.1	526	27	243	14	13; 52	3
BE 15	CD 3+, CD 4+, CD 8-	12	4635	< 0.1	<5	24	7; 53	2, 3

Table 2. Proliferative responses of TCC upon stimulation with natural and recombinant allergens. Stimulation indices of greater than 10 were considered as positive. TCC were SS 10, HC 31, MS 2, HC 4, AA 4, BE 15.

Allergen	Proliferative response of					
	SS 10	HC 31	MS 2	HC 4	AA 4	BE 15
n <i>Bet v 1</i>	+	+	+	+	+	+
r <i>Bet v 1</i>	+	+	+	-	+	+
r <i>Cor a 1/5</i>	+	-	-	-	+	+
r <i>Cor a 1/6</i>	+	+	-	+	+	+
r <i>Cor a 1/11</i>	+	-	-	-	+	+
r <i>Cor a 1/16</i>	-	+	-	+	+	+
n <i>Cor a 1</i>	+	+	-	-	+	+

17 kDa, share IgE-binding epitopes [5]. Recently, it has been reported that cross-reactivity between *Bet v 1* and *Cor a 1* also exists at the T-cell level, as was demonstrated by activation of *Bet-v-1*-reactive TCC by purified pollen-derived *Cor a 1* [6]. Like many other plant-derived proteins, natural allergens consist of different closely related isoforms (isoallergens; [7–9, 18]). Moreover, we recently demonstrated that natural preparations of an allergen contain isoforms which are highly similar, yet possess distinct immunogenic properties in terms of IgE binding [8]. Regarding pollen allergens, the quantitative ratio of different isoforms present in an allergen extract may vary depending on environmental factors and genetic background of the individual plant. From an allergologic point of view, a certain immunodominant isoform of a natural allergen would be of special interest. Moreover, if that isoform was cloned, expressed as a recombinant protein and used for diagnosis of type I allergies, reproducibility and comparability of routine tests would be increased [31, 32]. Concerning tree pollen allergy, r *Bet v 1* seems to be suited for that purpose [31]. Other than the *Cor a 1* isoforms (Fig. 2; [8]), it reacts strongly with IgE antibodies of every patient sensitized to the major allergens of tree pollens (Fig. 1; [31]). Moreover, binding of patients' IgE to the major allergens of other trees of the order *Fagales* can be blocked by prior incubation of patients' sera with r *Bet v 1* [33].

The sera collected from our tree-pollen-allergic patients displayed IgE binding to r *Bet v 1*. In the case of the four r *Cor a 1* isoforms, different intensities of IgE reactivity were found, as described previously [8], within single individuals (Figs 1 and 2). To find out whether different cross-reacting allergens and isoforms of allergens [8] also showed distinct

potencies in inducing T-cell activation we tested six allergen-specific TCC. All clones were known to be reactive with different epitopes on the homologous proteins. Individual patterns of reactivity in response to stimulation with recombinant isoforms of *Cor a 1* and with r *Bet v 1* occurred; these differences could be correlated with amino acid exchanges within the TCC-stimulating epitopes [8]. For example, TCC SS 10 reacted with the epitope amino acids 12–23 on r *Bet v 1* and on r *Cor a 1/5*, r *Cor a 1/6* and r *Cor a 1/11*. The amino acid exchange at position 14, from proline to serine, in r *Cor a 1/16* correlated with non-recognition of the peptide by the clone (Fig. 3). Within the epitope of HC 31, the substitution of lysine (r *Bet v 1*, r *Cor a 1/6* and r *Cor a 1/16*) by threonine (r *Cor a 1/5* and r *Cor a 1/11*) at position 80 led to non-reactivity of the respective clone (Fig. 3). The replacement of serine (r *Cor a 1/6* and r *Cor a 1/16*) by histidine (r *Cor a 1/5* and r *Cor a 1/11*) at position 100 caused a lack of proliferation of HC 4, which reacted with amino acids 97–108 on r *Cor a 1/6* and r *Cor a 1/16* (Fig. 3). Other amino acid exchanges between the *Cor a 1* isoforms and r *Bet v 1* did not influence the proliferative responses of TCC [6], even if amino acids of different groups were exchanged. (Fig. 3; SS 10, serine/alanine; AA 4, isoleucine/threonine, threonine/alanine).

Most of the TCC raised with natural, pollen-derived *Bet v 1* react with preparations of n *Bet v 1* and purified r *Bet v 1* with comparable intensity [13, 34]. However, the fact that some natural *Bet v 1*-reactive TCC do not recognize recombinant *Bet v 1* [6, 13, 34] led us to the assumption that such clones were specific for *Bet v 1* isoforms which are present in natural preparations but obviously not represented by r *Bet v 1*. In this respect TCC HC 4 is of special interest: it reacted strongly with n *Bet v 1* but did not respond to stimulation with r *Bet v 1*. However, the clone could be stimulated with two of the r *Cor a 1* isoforms, indicating the presence of corresponding epitopes in n *Bet v 1*, which represents a mixture of isoforms [9]. Recently three new *Bet v 1* isoforms were cloned and sequenced in our laboratory (EMBL data bank accession numbers 77200, 77265, 77272) displaying cysteine at position 99 like the r *Cor a 1* isoforms 5 and 11 within the HC 4 epitope.

Our data adds new information to the described situation: T cells specific for an allergen recognize different linear epitopes on the protein [13, 35]. Moreover, natural allergens consist of multiple isoallergens, T-cell-stimulating epitopes and non-stimulating regions can be found, at corresponding positions, on these isoforms and on other cross-reacting allergens. There is evidence that successful allergen-specific immunotherapy modulates the immune response at the T-cell

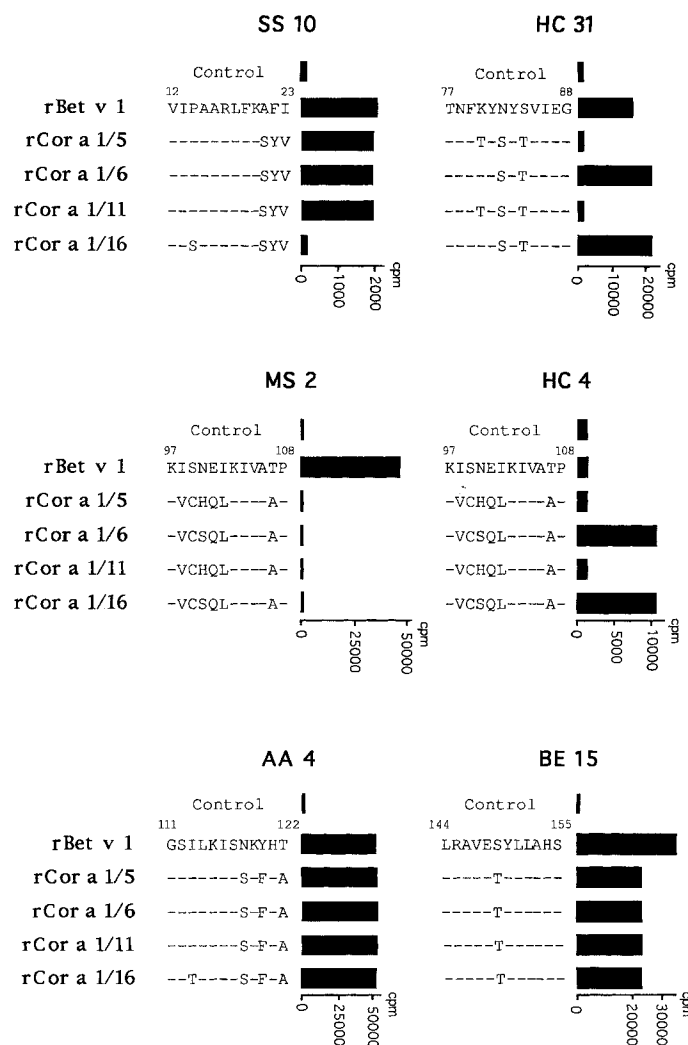


Fig. 3. Response of the TCC to stimulation with peptides (I–V) synthesized according to the sequences of the recombinant allergens. I, r *Bet v* 1; II, r *Cor a* 1/5; III, r *Cor a* 1/6; IV, r *Cor a* 1/11; V, r *Cor a* 1/16. Co, background control (TCC + APC, no peptide).

level [36] which then leads to a decrease of clinical symptoms. Considering the use of recombinant allergens for immunotherapy it would certainly be necessary to identify immunodominant isoforms. Moreover, hyposensitization with a mixture of highly antigenic recombinant isoforms of one allergenic protein, or multiple allergen-derived peptides [22, 23] with the characteristics of T-cell but not B-cell epitopes, should be considered as another approach to affect T-cell reactivity.

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