

Characterization of the T cell response to the major hazelnut allergen, Cor a 1.04: evidence for a relevant T cell epitope not cross-reactive with homologous pollen allergens

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Summary

Background IgE antibodies specific for the major birch-pollen allergen, Bet v 1, cross-react with homologous allergens in particular foods, e.g. apples, carrots and hazelnuts. In a high number of tree pollen-allergic individuals, this cross-reactivity causes clinical symptoms, commonly known as the 'birch-fruit-syndrome'.

Objective To characterize the T cell response to the Bet v 1-related major allergen in hazelnuts, Cor a 1.04, and its cellular cross-reactivity with Bet v 1 and the homologous hazel pollen allergen, Cor a 1.

Methods Using recombinant Cor a 1.04, T cell lines (TCL) and T cell clones (TCC) were established from peripheral blood mononuclear cells of tree pollen-allergic patients with associated food allergy. T cell epitopes were determined using overlapping synthetic peptides in Cor a 1.04-reactive TCL and TCC. In parallel, reactivity to Bet v 1 and Cor a 1 was tested.

Results In total, 20 distinct T cell epitopes on the hazelnut allergen were identified. Several Cor a 1.04-specific TCL and TCC reacted with pollen allergens albeit less pronounced than with the hazelnut allergen. Several Cor a 1.04-specific TCC did not react with pollen allergens. Interestingly, these clones were found to react with the Bet v 1-related major allergen in carrots, Dau c 1. The cellular cross-reactivity between both food allergens could be associated with the most frequently recognized T cell epitope of Cor a 1.04, Cor a 1.04_{142–153}.

Conclusions The major hazelnut allergen cross-reacts with the major allergens of birch and hazel pollen but apparently contains a relevant T cell epitope not shared with pollen allergens. Our finding may have important implications for the specific immunotherapy of tree pollen-allergic patients suffering from concomitant hazelnut allergy.

Keywords Bet v 1, food allergy, hazelnut, human, recombinant allergens, T lymphocytes

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Introduction

Birch-pollen allergy is one of the main causes of allergic rhinitis from spring to early summer in central Europe, and its prevalence has tremendously increased during the last two decades [1, 2]. Most patients sensitized to birch pollen also experience respiratory symptoms during the pollination of other trees from the order Fagales, e.g. hazel, which is because of the high structural homology between Bet v 1, the major allergen of birch pollen, and Cor a 1, the major allergen of hazel pollen [3–5]. Both proteins were shown to share IgE-binding epitopes [6, 7]. Furthermore, both proteins share relevant T cell epitopes, as Bet v 1-specific T cell clones (TCC) also proliferated in response to stimulation with Cor a 1 [8, 9].

Birch pollen-allergic individuals frequently develop food allergy to hazelnuts, which is also because of cross-reactive IgE antibodies [10, 11]. Cor a 1.04, the major allergen of hazelnuts, belongs to the pathogenesis-related (PR) protein family 10 that comprises proteins sharing a high amino acid (aa) sequence identity with Bet v 1 [12, 13]. PR-10 like allergens were also found in other foods, e.g. in apples (Mal d 1), cherries (Pru av 1), pears (Pyr c 1), celery tuber (Api g 1) and carrots (Dau c 1) [14–18]. In most cases, IgE reactivity to these proteins causes an 'oral allergy syndrome' (OAS) characterized by the immediate onset of oral itching, irritation of the oral mucosa and sometimes angioedema after consumption of the respective foods [19–21]. However, PR-10-like proteins contained in soybean (Gly m 4) and peanuts (Ara h 8) frequently induce systemic reactions ranging from urticaria to anaphylaxis [22, 23]. Other allergens contained in hazelnuts are Cor a 2 (profilin), Cor a 8 (lipid transfer protein), Cor a 9 (11S globulin), Cor a 11 (vicilin-like protein), a 2S albumin and a thaumatin-like protein [12, 21, 24–26]. In particular, Cor a 8 and Cor a 9 were

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associated with the induction of systemic and more severe symptoms than OAS after ingestion of hazelnuts [21, 25].

Recently, Cor a 1.04 was cloned, sequenced and expressed as a recombinant protein [27]. The major hazelnut allergen shares 67% aa sequence identity (79% similarity) with Bet v 1 and 60% (71%) with Cor a 1 in hazel pollen. Accordingly, IgE-inhibition experiments indicated that Cor a 1.04 shared more IgE-binding epitopes with its homologue in birch than in hazel pollen [27].

This study focused on the characterization of the T cell response to the major hazelnut allergen and its cellular cross-reactivity with Bet v 1 and pollen Cor a 1 to further elucidate the immunological relationship between these three important allergens. Cor a 1.04-reactive T cell lines (TCL) and clones (TCC) were established with recombinant (r) Cor a 1.04 and used for T cell epitope mapping experiments, with synthetic peptides representing the entire hazelnut protein. To determine cellular cross-reactivity, Cor a 1.04-reactive TCL and TCC were stimulated with Bet v 1 and Cor a 1. As some Cor a 1.04-specific TCC reacted with neither pollen antigen, these cultures were tested for cross-reactivity with other food allergens of the PR-10 protein family.

Material and methods

Allergic patients

Allergic individuals ($n = 20$) had a history of hayfever during early spring, a specific IgE RAST/CAP class > 3 to birch and hazel pollen (Pharmacia Diagnostics, Uppsala, Sweden) and positive skin prick reactions (weal diameter > 5 mm) to birch pollen, hazel pollen and hazelnut extract (Soluprick; ALK Abello, Hørsholm, Denmark). In addition, each patient displayed typical weal-and-flare reactions when tested with hazelnuts in a prick-to-prick test. All patients reported experiencing OAS after ingestion of hazelnuts, and none had developed more severe symptoms than OAS. Nine individuals had OAS to carrots and all but one to apples. The sera of all allergic patients contained IgE antibodies specific for the major allergens Cor a 1.04, Bet v 1 and Cor a 1, which was determined by ELISA and immunoblotting using recombinant allergens and hazelnut and pollen extracts (data not shown). Non-allergic individuals ($n = 6$) were non-atopic and not sensitized to any allergen under investigation.

Allergens

Recombinant allergens were purchased from Biomay (Vienna, Austria) except rCor a 1.04, which was produced as described [27]. Bacterial endotoxin contents were detected using a Limulus Amoebocyte Lysate assay (BioWhittaker, Walkersville, MD, USA). Endotoxin levels were below 2.5 EU/ μ g for each allergen. Natural Bet v 1 and hazel pollen extract were purified as described previously [12, 28].

Cor a 1.04-specific T cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia Diagnostics). PBMC (2×10^5) were cultured in triplicate in 96-well plates (Nunc, Nunc,

Denmark) in 200 μ L of serum-free Ultra Culture Medium (BioWhittaker) supplemented with 2 mM glutamine and 2×10^{-5} M 2-mercaptoethanol with and without allergen for 6 days. During the last 16 h of culture, 0.5 μ Ci [^{3} H]-thymidine was added and the incorporated radioactivity was measured by scintillation counting. TCL were established by culturing 1.5×10^6 PBMC in 24-well flat-bottomed culture plates (3524 Costar, Cambridge, MA, USA) with or without 10 μ g/mL rCor a 1.04. After 5 days, 10 U/mL of human rIL-2 (Boehringer, Mannheim, Germany) was added. At day 7, T cell blasts were harvested by density gradient centrifugation and subjected to limiting dilution to isolate TCC. The remaining T cell blasts of TCL established with and without Cor a 1.04 were expanded using irradiated PBMC and rIL-2. Ten days after the last feeding, TCL were stimulated with rCor a 1.04 (5 μ g/mL), rBet v 1 (5 μ g/mL) or rCor a 1 (5 μ g/mL) in the presence of 1×10^5 irradiated (60 Gy) autologous PBMC. After 48 h, [^{3} H]-thymidine was added and proliferation was evaluated as described above. To identify T cell epitopes, Cor a 1.04-reactive TCL were stimulated with a panel of 50 dodeca peptides representing the complete aa sequence of Cor a 1.04 (5 μ g/mL of each peptide; Mimotopes/Biotrend, Köln, Germany) for 48 h [29]. The stimulation index (SI) was calculated as a ratio between cpm obtained in cultures with TCL plus autologous PBMC plus stimulus and cpm obtained in cultures containing TCL and PBMC.

Cor a 1.04-specific T cell clones

TCC were established according to published protocols [29]. Briefly, T cell blasts from Cor a 1.04-reactive TCL were seeded by limiting the dilution technique in 96-well round-bottom plates (Nunc) in the presence of 2×10^5 irradiated PBMC, 0.5% v/v PHA (Gibco, Grand Island, NY, USA) and rIL-2 (4 U/well) in the medium described above. The specificity of grown microcultures was assessed in proliferation assays using 5 μ g/mL rCor a 1.04. Thereafter, Cor a 1.04-reactive TCC were expanded by alternating turns of stimulation with irradiated PBMC plus allergen or rIL-2. The expression of CD3, CD4, CD8 and T cell receptor (TCR) α/β at the surface of TCC was analysed by flow cytometry as described previously [30]. Epitope mapping experiments were performed as described above. For induction of cytokine secretion, TCC were washed and incubated with irradiated autologous PBMC in the presence of rCor a 1.04 (5 μ g/mL) for 48 h. Cultures containing TCC and PBMC in medium alone served as negative controls. Cytokine levels in supernatants were measured by ELISA using matched antibody pairs (Endogen, Woburn, MA, USA) according to the manufacturers' instructions (sensitivity limits: IL-4: 9 pg/mL; IFN- γ : 9.5 pg/mL). TCC were assigned to T helper (Th) subsets as follows: Th2: IL-4/IFN- γ > 10 ; Th1: IFN- γ /IL-4 > 10 and Th0: IFN- γ /IL-4 = 0.1–10. The TCR was analyzed by RT-PCR as described, and revealed that TCC were monoclonal [31].

Results

T cell epitopes of rCor a 1.04

To evaluate the optimum concentration of rCor a 1.04, PBMC of 20 allergic and six non-allergic individuals were

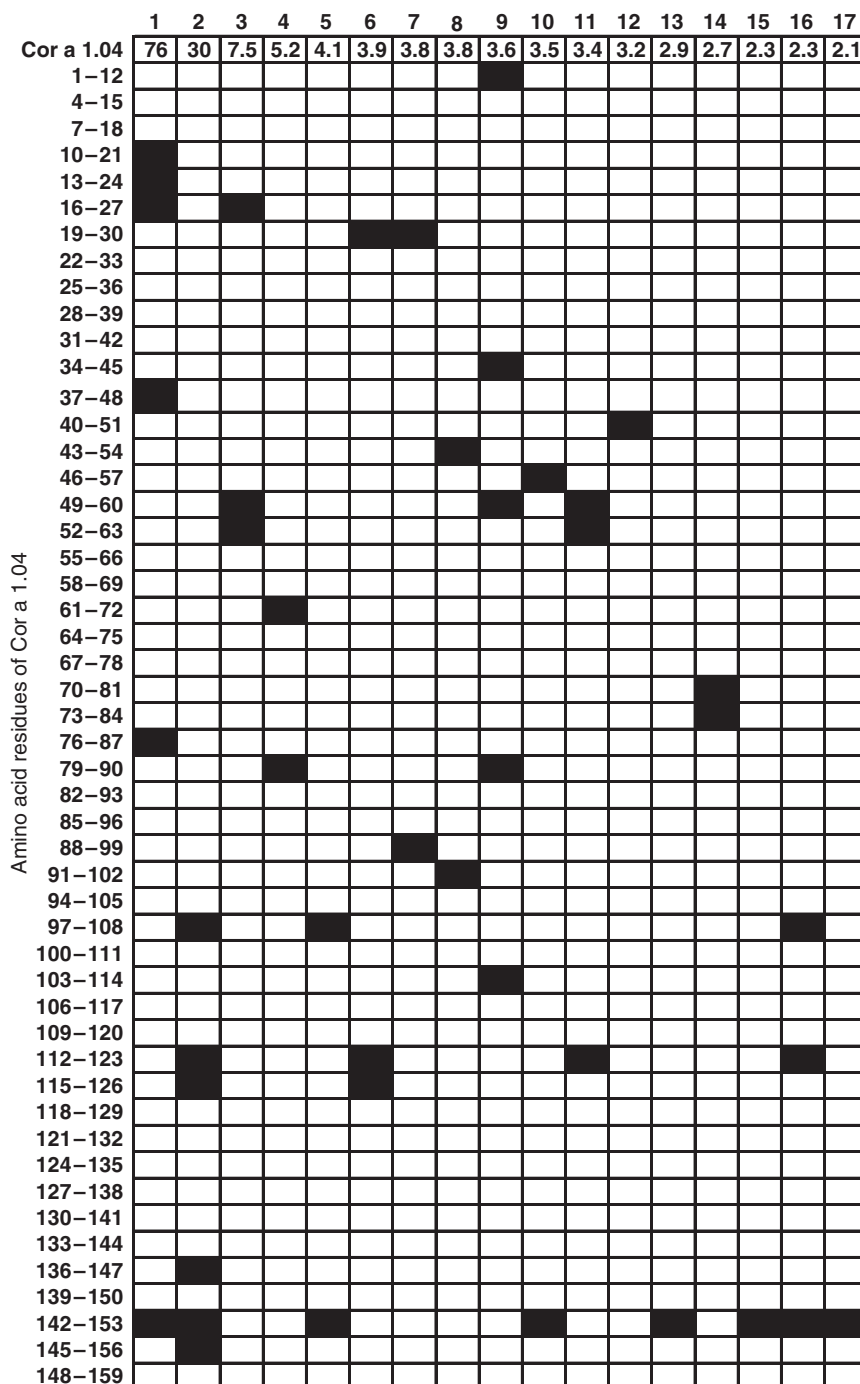


Fig. 1. T cell epitope mapping of Cor a 1.04. Cor a 1.04-induced T cell lines (TCL) from 17 patients were tested for proliferation with 50 overlapping peptides spanning the entire Cor a 1.04 protein sequence. In the first line SI induced by rCor a 1.04 are shown. Proliferative responses to peptides (SI > 3) are shown as filled boxes. Background levels with medium alone ranged from 215 to 6245 cpm.

stimulated with different concentrations of rCor a 1.04 (range 3.18–25 µg/mL). In both groups, 12.5 µg/mL of rCor a 1.04 induced the highest proliferative responses. The median values of allergic vs. non-allergic individuals in response to rCor a 1.04 were 10 542 and 6604 cpm. Background levels with medium alone were 4496 and 4349 cpm, respectively. Next, TCL were established with rCor a 1.04 from 17 different allergic individuals and cultures responding with an SI > 2 to the hazelnut allergen were tested with 50 12-mer

peptides representing the complete aa sequence of the protein (Fig. 1). For peptides that usually induce stronger proliferation than the entire allergen, an SI > 3 was defined as a cut-off for positivity. Multiple T cell epitopes distributed over the entire hazelnut molecule were identified. Peptides Cor a 1.04₁₋₁₂, Cor a 1.04₃₄₋₄₅, Cor a 1.04₃₇₋₄₈, Cor a 1.04₄₀₋₅₁, Cor a 1.04₄₃₋₅₄, Cor a 1.04₄₆₋₅₇, Cor a 1.04₆₁₋₇₂, Cor a 1.04₇₆₋₈₇, Cor a 1.04₈₈₋₉₉, Cor a 1.04₉₁₋₁₀₂, Cor a 1.04₁₀₃₋₁₁₄ and Cor a 1.04₁₃₆₋₁₄₇ were recognized by one individual each. Peptides

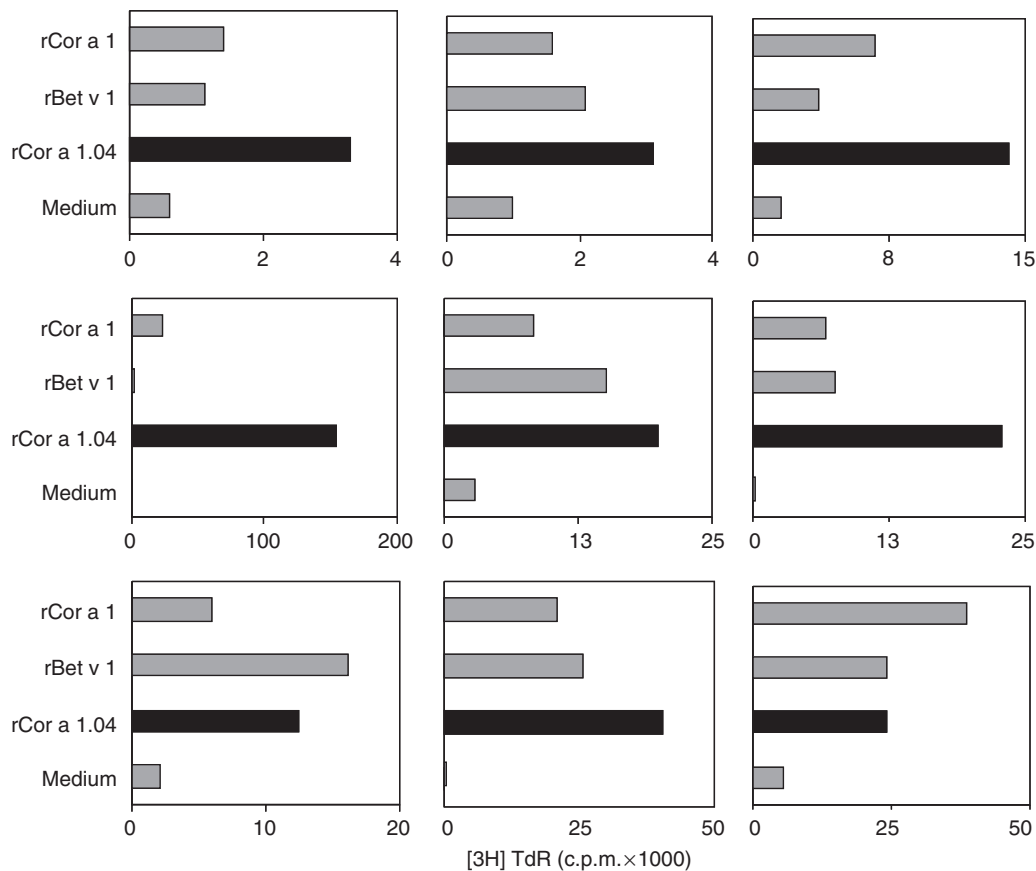


Fig. 2. Cross-reactivity of Cor a 1.04-specific T cell lines with homologous birch and hazel pollen allergens. Cor a 1.04-initiated TCL from nine different allergic patients were stimulated with rCor a 1.04, rBet v 1 and pollen rCor a 1, respectively. Mean cpm of duplicates are shown.

Cor a 1.04_{10–27}, Cor a 1.04_{19–30}, Cor a 1.04_{79–90} and Cor a 1.04_{97–108} were recognized by two individuals each. Three patients harboured T cells specific for an epitope located at Cor a 1.04_{49–63}, and four individuals reacted with Cor a 1.04_{112–126}. In eight of 17 TCL the peptide Cor a 1.04_{142–153} was recognized. Twelve patients harboured T cells specific for more than one epitope. Three individuals recognized Cor a 1.04_{142–153} as the sole peptide. In summary, Cor a 1.04_{142–153} represented the most prominent epitope of the hazelnut allergen.

Cross-reactivity of rCor a 1.04-induced T cell lines

Nine rCor a 1.04-reactive TCL from different allergic individuals were tested for their reactivity with rBet v 1 and rCor a 1, respectively (Fig. 2). In seven of nine TCL, rCor a 1.04 induced a higher lymphoproliferation as compared with either pollen antigen. Nevertheless, all TCL established with the food allergen proliferated in response to at least one pollen allergen. TCL of each individual that were initiated simultaneously without rCor a 1.04 did not proliferate to any of the allergens (data not shown).

Cross-reactivity of rCor a 1.04-specific T cell clones

In total, 18 Cor a 1.04-specific TCC were isolated from nine different allergic individuals (Table 1). All TCC were CD3⁺CD4⁺TCR α/β ⁺ and were analysed for their cytokine

secretion pattern after stimulation with rCor a 1.04. Eight TCC were defined as Th2, six as Th0 and four TCC as Th1. In addition, all TCC were tested for their reactivity with rBet v 1 (Table 1). Considering an SI > 3 as positive, eight of 18 TCC cross-reacted with the birch-pollen allergen and all but one TCC responded clearly more vigorously to rCor a 1.04. Ten of 18 TCC did not proliferate in response to rBet v 1 despite a strong reactivity with rCor a 1.04. Considering that the major allergens of tree pollens consist of a mixture of different isoforms, these TCC were additionally stimulated with natural Bet v 1 and complete hazel pollen extract [5, 32, 33]. Furthermore, the Bet v 1-homologue in carrot, Dau c 1, was included as recent observations in our laboratory indicated a cellular cross-reactivity between the major allergens of carrot and hazelnut. Three TCC responded to the natural pollen antigens. Seven TCC reacted with rDau c 1 (Table 1).

Cross-reactivity between rCor a 1.04 and rDau c 1 is associated with the C-terminal epitope Cor 1.04_{142–153}

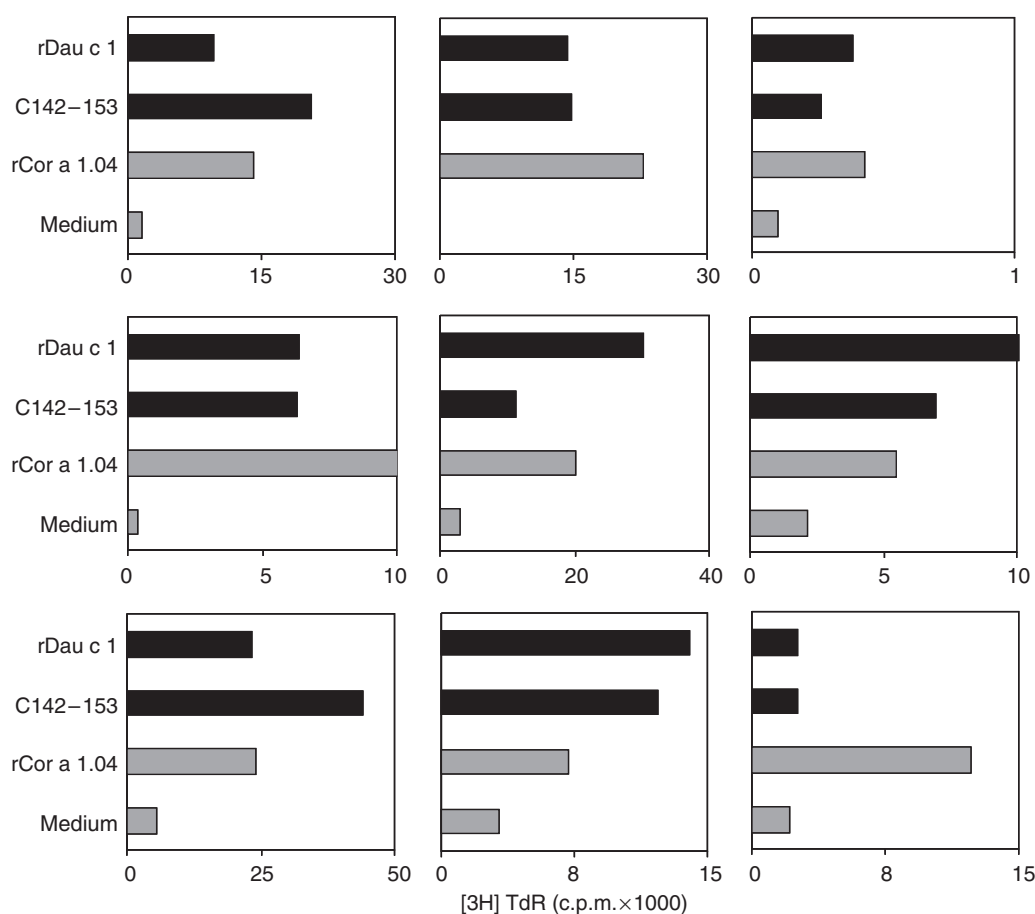
Stimulation of rCor a 1.04-specific TCC with 50 Cor a 1.04-derived dodeca peptides revealed that all rDau c 1-reactive TCC were exclusively specific for Cor a 1.04_{142–153} (AGLF KAVEAYLL). To further confirm the involvement of this epitope in the cellular cross-reactivity between both food allergens, 9 rCor a 1.04-specific TCL were stimulated with rDau c 1 and the peptide Cor 1.04_{142–153} (Fig. 3). Eight TCL

Table 1. Characterization of Cor a 1.04-specific TCC

TCC	Subset	Medium	rCor a 1.04	RBet v 1	TCC	Subset	Medium	rCor a 1.04	rBet v 1	nBet v 1	HPE	rDau c 1
B-C70	Th2	2.6*	48.1	8.1	B-C2	Th1	0.6	13.0	0.6	1.5	4.8	0.8
B-C87	Th2	2.2	51.1	27.9	B-C55	Th0	1.4	27.0	1.2	50.5	6.1	0.6
B-C193	Th0	0.4	41.4	13.5	H-C32	Th2	0.7	28.6	1.0	ND	ND	23.4
K-C18	Th2	0.5	109.8	6.4	O-C32	Th2	7.9	188.3	5.3	5.5	ND	ND
O-C2	Th0	0.2	10.8	106.6	P-C4	Th2	0.4	23.2	0.6	0.7	0.6	68.5
O-C67	Th2	3.4	21.5	19.0	P-C30	Th0	2.4	25.1	2.7	2.0	2.7	15.7
R-C185	Th1	0.7	2.8	2.2	RA-C98	Th1	3.6	131.6	1.8	12.3	ND	54.7
W-C42	Th2	0.7	7.1	2.5	R-C134	Th1	1.3	27.9	1.5	1.5	2.7	16.7
					S-C16	Th0	0.2	52.4	0.2	0.2	0.2	49.3
					S-C28	Th0	0.2	11.3	0.2	0.2	0.2	16.9

*cpm $\times$ 1000.

nBet v 1, natural Bet v 1; HPE, hazel-pollen extract; TCC, T cell clones; ND, not defined. T helper (Th) subsets were defined as described in Materials and methods.

**Fig. 3.** Cross-reactivity of Cor a 1.04-specific T cell lines with the major carrot allergen. Cor a 1.04-initiated TCL from nine different allergic patients were stimulated with rCor a 1.04, the peptide Cor a 1.04₁₄₂₋₁₅₃ and rDau c 1, respectively. Mean cpm of duplicates are shown.

cross-reacted with the carrot allergen and Cor 1.04₁₄₂₋₁₅₃ was recognized in all of these TCL. One TCL reacted neither with the carrot allergen nor with the peptide.

Discussion

The cellular response to the major hazelnut allergen was characterized and compared with the response to homologous

allergens in birch and hazel pollen. Twenty distinct T cell epitopes on Cor a 1.04 were identified, among which the C-terminal peptide Cor a 1.04₁₄₂₋₁₅₃ represented the most prominent epitope (Fig. 1). All epitopes were found in regions that correspond to T cell-activating regions of the major birch-pollen allergen, which is obviously because of the high aa sequence homology between Cor a 1.04 and Bet v 1 [34]. Consequently, Cor a 1.04-induced TCL cross-reacted with the homologous pollen proteins. Interestingly, when investigating

the T cell response at a clonal level, we found that the majority of TCC specific for Cor a 1.04_{142–153} do not cross-react with pollen proteins but with the major carrot allergen, Dau c 1.

In a high percentage of patients with birch pollinosis, primary respiratory allergy is followed by the onset of OAS to particular foods, most frequently apples and hazelnuts. It is assumed that the major birch-pollen allergen initiates the sensitization to its homologous food proteins. Our previous characterization of the T cell response to the Bet v 1-related major allergens in apple and celeriac substantiated this hypothesis as T cell cultures established with rMal d 1 or rApi g 1 predominantly contained rBet v 1-specific T lymphocytes [29, 35]. We applied a similar approach to investigate the cross-reactivity between the major hazelnut allergen and Bet v 1. In contrast to T cell cultures established with Mal d 1 or Api g 1 [29, 35], Bet v 1-reactive Cor a 1.04-established TCL and TCC responded far more vigorously to the food than the pollen allergen. It has to be emphasized that a predominance of pollen-reactive T lymphocytes in the cultures could have been expected as in the present study all blood samples were collected during and shortly after the tree pollen season. Furthermore, 10/18 Cor a 1.04-specific TCC did not react with rBet v 1, whereas only a minority of Api g 1-specific (two of 13) and Mal d 1-specific (three of 19) TCC had not been reactive with rBet v 1 in previous studies [29, 35]. Together, these results suggest that – in contrast to the major apple and celery allergen – the cellular reactivity to the major hazelnut allergen does not exclusively depend on cross-reactivity with the major birch pollen allergen.

In this study, we used rBet v 1a, the most prominent isoform of the birch-pollen allergen. To include additional isoforms of Bet v 1 for the evaluation of cellular cross-reactivity, rBet v 1a-non-reactive TCC were tested with natural pollen allergens (Table 1) [32, 33]. A few TCC appreciably responded to natural pollen proteins, e.g. TCC B-C55. Epitope mapping of this TCC revealed exclusive specificity for the peptide Cor a 1.04_{49–63} (GPGTIKKITFAEGNE, SI = 82). When tested with different recombinant Bet v 1 isoforms, TCC B-C55 proliferated in response to rBet v 1b (GPGTIKKITFPEGSP, SI = 55), rBet v 1l (GPGTIKKINFPEGFP, SI = 19) and rBet v 1d (GPGTIKKINFPEGFP, SI = 17). No reactivity was found after stimulation with rBet v 1e (GPGTIKKISFPEGIP, SI = 1.2), which shares the highest sequence identity with the respective peptide of rBet v 1a (GPGTIKKISFPEGFP), confirming its non-reactivity. However, we cannot exclude that the natural pollen allergen preparations used in this study lacked particular isoforms that might have been cross-reactive. Nevertheless, it is striking that most Cor a 1.04-specific TCC did not respond to stimulation with natural tree-pollen allergens.

Ongoing studies in our laboratory indicated a cellular cross-reactivity between the major allergens in hazelnut and carrot. Therefore, we stimulated pollen-non-reactive TCC also with Dau c 1 and observed pronounced proliferation (Table 1). This cross-reactivity could be associated with the C-terminal epitope Cor a 1.04_{142–153} as all Dau c 1-reactive T cell cultures were specific for this peptide (Table 1 and Fig. 3). Cor a 1.04_{142–153} is present on all described variants of the hazelnut allergen and 67% identical (83% similar) to the corresponding region of Dau c 1 [18, 27]. In contrast, this epitope shows

only 58% aa identity with the respective parts of both, Bet v 1 and Cor a 1, which are identical among all known isoforms of these allergens [5, 32]. Thus, Cor a 1.04_{142–153} appears to be a food-specific T cell epitope shared by the major allergens in hazelnut and carrot but not with homologous birch and hazel-pollen proteins. Similar to hazelnut allergy, carrot allergy represents a typical birch pollen-related phenomenon mainly based on IgE cross-reactivity between Dau c 1 and Bet v 1 [18, 36–38]. In contrast to other Bet v 1-related food allergens, Dau c 1 was proposed to possibly initiate a clinically relevant sensitization independent of the birch-pollen allergen because it contains B cell epitopes that are not shared with Bet v 1 [39]. In addition, some carrot-allergic patients have been described who display serum IgE specific for Dau c 1 but lack IgE specific for Bet v 1 [40]. All individuals included in this study from whom Cor a 1.04_{142–153}-specific TCC were derived also suffered from carrot allergy. Therefore, our observations could support the hypothesis of a potentially sensitizing capacity of the major carrot allergen.

There is an ongoing debate regarding whether or not specific immunotherapy (SIT) with pollen allergens also has a curing effect on pollen-related food allergy. Whereas some studies described an improvement of concomitant food allergy in successfully treated birch-pollen-allergic individuals [41–43], other studies reported no effects and even the induction of hypersensitivity reactions to apples during the course of birch-pollen SIT [44, 45]. Successful SIT has been associated with a down-regulation of allergen-specific Th2 cells [46, 47]. Our data indicate that the major birch-pollen allergen lacks the most frequently recognized T cell-activating peptide of the hazelnut allergen, Cor a 1.04_{142–153}. Consequently, T cells specific for this epitope would not be targeted by SIT with birch-pollen extract. This assumption may provide an explanation at the cellular level for the limited efficacy of pollen therapy for food allergy treatment. Hence, in particular, for tree pollen-allergic patients with concomitant hazelnut allergy, treatment strategies in addition to a therapy with pollen extracts should be considered.

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