

Identification of HLA-DR and -DQ alleles conferring susceptibility to pollen allergy and pollen associated food allergy

W.-H. BOEHNCKE, C. LOELIGER*, P. KUEHNLE*, H. KALBACHER†, B. O. BÖHM‡ and H. GALL§

Department of Dermatology, University of Frankfurt, *Department of Transfusion Medicine and Transplantation Immunology, University of Hamburg, †Medical and Natural Sciences Research Centre, University of Tübingen, Departments of ‡Internal Medicine I and §Dermatology, University of Ulm, Germany

Summary

Background Allergenic crossreactivity of pollen and foods due to the antigenic similarity of oligopeptides is a well established clinical phenomenon.

Objective To determine the immunopathological relevance of antigen presentation, we analysed the HLA class-II genotype of patients with either pollen allergy or pollen associated food allergy.

Methods One hundred and twenty patients with pollen allergy and 80 patients with pollen associated food allergy were evaluated by skin-prick tests, RAST, and HLA class-II genotyping. The control population comprised 4251 healthy blood and bone marrow donors.

Results Monovalent pollen allergy was observed in 57% ($n=68$) of patients with pollinosis (57x grass pollen, 11x birch pollen), but only in 15% ($n=12$) of patients with food allergy (9x grass pollen, 3x birch pollen). Hazelnut (71%), almond (65%), walnut (44%) and apple (41%) were the most common food allergens and frequently associated with birch pollen allergy. Grass pollen allergy was associated with an increased frequency of HLA-DQB1*0301 ($RR=2.3$; $EF=0.4$; $P=0.0016$) when compared with the control population. HLA-DRB1*08 conferred a sixfold higher risk for peanut allergy ($EF=0.3$; $P=0.0013$) and -DRB1*12 a 13-fold higher risk for carrot allergy ($EF=0.3$; $P<0.000001$). The differences on allele frequencies detected among patients with food allergies diminished or turned statistically insignificant when their genotypes were directly compared to those of patients with the corresponding pollen allergies. This was found in the case of birch pollen associated hazelnut allergy for the extended haplotype HLA-DRB1*01, -DQA1*0101, -DQB1*0501 as well as in grass pollen associated peanut allergy for HLA-DRB1*08 (from $RR=6$, $P=0.0013$ to insignificant) and in birch pollen associated carrot allergy for HLA-DRB1*12 (from $RR=13$, $P<0.000001$ to insignificant).

Conclusion We were able to identify HLA class-II alleles associated with some allergies thus indicating that these alleles might confer susceptibility to the respective allergens. Similarities at the level of the HLA class-II genotype parallel the empirical finding of distinct cross-reactivity patterns thus complementing investigations of IgE specificities. Our observations provide evidence for the major importance of antigen presentation on the manifestation of distinct crossreactivity patterns.

Keywords: allergy, antigen presentation, carrot, crossreactivity, food, grass, HLA, peanut, pollen, risk factor

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Correspondence: Dr W.-H. Boehncke, Department of Dermatology, Frankfurt University School of Medicine, Theodor-Stern-Kai 7, D – 60590 Frankfurt/Main, Germany.

Table 1. Patterns of pollen associated food allergy (adapted from [13]). Foods frequently found in this study are highlighted in *italic*

Pollen	Food
Birch	<i>Hazelnut, walnut, almond, apple, kiwi</i> , cherry, peach, pear, potato
Birch/mugwort	<i>Carrot</i> , celery
Grass	<i>Peanut</i> , tomato, melon, watermelon, Swiss chard
Ragweed	Melon, banana

Introduction

The specific IgE mediated type I hypersensitivity reaction initially depends on recognition of allergen derived peptides bound to antigen presenting MHC class II molecules by T helper lymphocytes [1]. In recent years it became evident that peptide binding to MHC class II molecules representing the restriction elements of T helper lymphocytes is promiscuous and that a high degree of variation within an antigen is tolerable to retain functional T-cell epitopes [2–4]. Thus, cross-allergenicity is an expected phenomenon. A clinical manifestation of this constellation, pollen-associated food allergy, has been appreciated more than 50 years ago and patterns of clinically relevant crossreactivities were deduced (Table 1) [5–13]. The molecular basis of pollen-associated food allergy is currently best understood at the level of B-cell epitopes. Crossreactive IgE antibodies have been demonstrated to recognize epitopes on profilins, a family of plant pan-allergens and many plant-derived food agents containing highly homologous proteins [14,15].

Besides studies focusing on B- and T-cell epitopes of allergens considerable efforts have also been made regarding the genetic basis of the respective allergies with emphasis on the patients' immunogenetic background. HLA class II alleles, almost exclusively of the HLA-DR type, were found to be associated with a number of pollen allergens and plant-derived food allergens [16].

Since some but not all patients with certain pollinoses eventually develop allergies to plant-derived food agents with IgE antibodies recognizing highly homologous peptides we were interested in analysing the impact of the patients' HLA genotype on the manifestation of well-defined patterns of pollen-associated food allergy. HLA typing was performed at the genomic level for -DRB1, -DQA1, and -DQB1 alleles utilizing a novel procedure [17]. We found several HLA class II alleles to be associated with distinct pollen associated allergies. Moreover, association of grass pollen allergy with an HLA-DQ allele was observed.

Materials and methods

Patients

Between October 1995 and September 1996 patients with a history of pollinosis and/or adverse reactions to plant-derived food agents referred to the outpatient clinic of the Department of Dermatology at Ulm University were investigated. One hundred and twenty patients with the diagnosis of pollinosis and 80 patients with pollen-associated food allergy based on the criteria described below were recruited in the study. A total of 4251 unrelated subjects (2268 men, 1983 women) of the same ethnic origin (white subjects) were included as controls. These subjects were healthy blood donors and healthy volunteers for bone marrow donation. The control population has not been screened for presence of pollen or food allergy or manifestation of atopic diseases. Given the high prevalence of atopic symptoms in the German caucasian population exceeding 10% [18] it needs to be taken into account that also among this group pollen allergy and pollen associated food allergy is present.

Allergy test procedure

Skin-prick testing

In all patients a standardized skin-prick test comprising seven pollen allergens (grass, rye, birch, alder, hazel, mugwort, plantain) was performed utilizing purified allergen extracts from Bencard, Worthing, UK, registered by the Paul-Ehrlich-Institut (German regulatory authority for sera and allergen extracts). In patients with a history of adverse reactions to plant-derived food agents a skin-prick test was performed with the respective agents in native form.

RAST

To analyse serum levels of specific IgE antibodies directed against the allergens indicated by the patients' history commercial RAST tests were performed (Pharmacia Diagnostics AB, Uppsala, Sweden).

Establishment of diagnosis

A patient was considered allergic to pollen in case of (i) a history of rhinitis and conjunctivitis through peak season of the pollen along with exposition for at least two consecutive years prior to testing and (ii) the skin-prick test was clearly positive (histamine equivalent reaction). The diagnosis of pollen-associated food allergy was based on (i) the established diagnosis of pollinosis and at least oral allergy syndrome, (ii) a clearly positive skin-prick test (histamine equivalent reaction), and (iii) the food agent in question described as being associated with the pollinosis found in the respective patient [5–13]. A positive RAST (>1) was

considered confirmatory but not necessary for establishing this diagnosis. Patients suffering from food allergy without accompanying pollinosis or with the food agent not yet described as being associated with pollinosis were excluded from the study.

Genomic HLA class II typing

The HLA-DRB1, -DQA1, and -DQB1 alleles were determined by use of a locus specific amplification procedure as previously described [19]. In brief, 1 µg of genomic DNA, which had been extracted from peripheral blood leucocytes, was added to 10 µL of prepacked biotinylated primer mixes each of which corresponded to a given HLA class II locus, for example, HLA-DRB1, -DQA1, and -DQB1, respectively. After 35 cycles of polymerase chain reaction (PCR) amplification in an automatic thermocycler (Thermocycler 9600, Perkin Elmer Cetus) the amplificate was denatured by sodium hydroxide and hybridized to sequence specific oligonucleotides, which were fixed after oligo-dT tailing to a nylon membrane (Hybond N, Biotryne B, Pall Filtration Ltd, Portsmouth, UK). Allele specific hybridization of the locus specific amplificate was detected by adding streptavidine coupled peroxidase and a colourimetric reaction of tetra-methyl-benzidine. As a control of the reverse dot blot protocol the standard DNA typing procedure of the 11th International HLA Workshop was used in randomly collected samples [20,21]. In all typing procedures, DNA from homozygous typing cell lines (10th International HLA Workshop panel) were also included as internal controls.

The following HLA-DRB1 alleles could be determined with the HLA genotyping procedures: HLA-DRB1*01, *15, *16, *03, *04, *11, *12, *13, *14, *07, *08, *09, and *10 (13 alleles). Very rare HLA-DRB1 alleles or splits have not been considered. The following HLA-DQA1 alleles were determined: HLA-DQA1*0101 to *0103, *0201, *0301, *0401, and *0501 (seven alleles). The following HLA-DQB1 alleles were determined: HLA-DQB1*0501, *0502, *0503, *0601 to *0605, *0201, *0301 to *0303 (12 alleles).

Statistical analysis

The chi-square test was used as described [22]. Relative risk (RR) was calculated using Woolf's formula, the aetiological fraction (EF), and the preventive fraction (PF), which provide an estimate of the relative contribution of a marker to the disease, were calculated as described [23–25]. The Bonferoni inequality method was used to correct for each antigen comparison [26]. The threshold for significance of a deviation for the HLA-DRB1 and HLA-DQB1 alleles was given by a corrected *p*-value of <0.0038, and for the HLA-DQA1 alleles by a corrected *p*-value of <0.0071. With regard to interpretation of the data it

needs to be taken into account that a considerable proportion of the control population also may exhibit pollen allergy or pollen associated food allergy [see above and ref. 18]. Thus, any significant difference found would become more significant if these individuals were to be eliminated from the control population (which cannot be achieved practically due to the large number). As a consequence there is an error in this investigation towards the differences observed being even more pronounced than it appears on the basis of the statistical analyses.

Results

Frequencies of allergies

Among the 120 patients diagnosed as suffering from pollinosis 57% (*n* = 68) exhibited a monovalent allergy with grass pollen clearly dominating over birch (57 patients vs 11 patients) (Table 2). Allergy to all three main pollen involved in crossreactivity — grass, birch, mugwort — was present in 12%. In contrast, those patients suffering from pollen-associated food allergy (*n* = 80) rarely exhibited monovalent pollen allergies (15%); again grass pollen were more frequent than birch pollen (9x grass pollen, 3x birch pollen). However, allergy to all three main pollen involved in crossreactivity was common (43%). In either group isolated allergy to mugwort pollen was not observed at all.

By far the most common food agents causing allergy were hazel nut (71%) and almond (65%), followed by walnut (44%) and apple (41%). Other foods accounting for the allergic symptoms in >10% of the patients were carrot (16%), kiwi (15%), cherry (14%), and peanut (11%) (Table 3).

Hazelnut belongs to those foods associated with birch pollen allergy [10]. Besides, combined allergy against hazel pollen and hazelnuts is a well recognized clinical phenomenon [27]. We therefore analysed this constellation in our study population. Allergy to hazel pollen was found to be more frequent among patients with pollinosis and food

Table 2. Number of pollen involved in cross-reactivity (grass, birch, mugwort) to which a given patient exhibited a clinically manifest allergy. The percentages refer to percent of patients with pollen allergy or pollen associated food allergy having reactivity to 1, 2 or 3 different allergens

Number of allergies	Pollen (%)	Pollen-associated food allergy (%)
1	57	15
2	31	42
3	12	43

Table 3. Food agents accounting for > 10% of allergies in the cohort with pollen associated food allergy

Food agent	Frequency (%)	Association (x/y)*
Hazel nut	71	Birch (55/57)
Almond	65	Birch (51/52)
Walnut	44	Birch (30/35)
Apple	41	Birch (31/33)
Carrot	16	Birch (6/13)
		Mugwort (2/13)
		Birch + mugw. (5/13)
Kiwi	15	Birch (5/12)
Cherry	14	Birch (11/11)
Peanut	11	Grass (7/9)

* Absolute number of patients showing the association indicated/ absolute number of all patients exhibiting the respective food allergy.

allergy than in the pollinosis group (94% vs 30%). In 76% the food associated with hazel pollen allergy was hazel nut; and only two patients were observed where allergy to hazelnut was associated with pollen other than hazel (data not shown).

Besides hazel nut, kiwi is a food agent possibly associated with birch pollen allergy [13]. When investigating this relation we found that all 12 individuals allergic to kiwi had positive skin prick tests and positive RAST tests for birch pollen. In five patients these tests were clinically relevant and the respective patients suffered from pollinosis, the remaining seven patients did not exhibit clinical symptoms related to birch pollen (Table 3). Generally, the crossreactivity patterns deduced from clinical observations

Table 4. Sensitivity of RAST test in patients with a clinically manifest allergy as diagnosed by the patient's history and skin prick test. The absolute case numbers are given

Agent	Skin prick +/RAST +	Skin prick +/RAST –
Grass pollen	123	0
Birch pollen	62	4
Mugwort pollen	45	3
Hazel nut	40	6
Walnut	14	14
Almond	18	25
Peanut	6	0
Apple	21	1
Kiwi	2	10
Cherry	0	2
Carrot	10	1

were also demonstrable in the patient population studied here (Tables 1 and 3).

Correlation between RAST and skin-prick test

To test the potential use of RAST analyses in the diagnosis of pollinosis and pollen-associated food allergy the results of these analyses were compared with the outcome of skin-prick tests. We found a very good correlation between these methods regarding pollen allergens (Table 4). This was true for both clinically manifest allergy as well as symptom-less sensitization. Moreover, apple and carrot were adequately diagnosed with both techniques. However, most other food agents could not reliably be detected by RAST analyses (Table 4).

HLA genotype of patients with pollinosis

Initially, we focused on analysis of gross genotypic differences between the three major cohorts of individuals studied, healthy controls, patients with pollinosis, and subjects suffering from pollen-associated food allergy. In our study population we did not observe distinct HLA class-II alleles being associated with a significantly increased risk of either pollinosis or pollen-associated food allergy in general. However, when analysing different pollen allergies separately, we found that HLA-DQB1*0301 was associated with allergy to grass pollen ($P = 0.0016$; $RR = 2.3$). The aetiological fraction (EF) measuring the population-attributable risk was 0.39 (Table 5).

HLA genotype of patients with birch pollen-associated food allergy

Among the food allergens frequently found in our study population and known to show association with birch pollen allergy were hazel nut, almond, apple, and kiwi [9–11,13]. Significant differences were noted when patients with birch pollen-associated hazel nut allergy were analysed. Alleles HLA-DRB1*01, -DQA1*0101, and -DQB1*0501 were significantly decreased when compared to the normal control population; the preventive fraction (PF) measuring the population-attributable protection were 0.25, 0.34, and 0.20, respectively (Table 5). There was a general tendency of the differences of these three alleles to be smaller in comparison with birch pollen allergic patients than with pollen allergic patients in general. This finding is in line with the interpretation of hazel nut allergy being a birch pollen associated food allergy. The associations of the respective HLA alleles are not independent since they form an extended haplotype, i.e. they are in linkage disequilibrium [28]. In our patient series, the extended haplotype comprised all three alleles in 24 individuals, in 10

Table 5. HLA genotypes exhibiting statistically significant associations to distinct allergens

Allele	Pat. cohort	Compared to	<i>P</i> value	RR	EF	PF
-DQB1*0301	Grass pollen	Normal	0.0016	2.31	0.39	
-DRB1*01	Hazel nut	Normal	0.0014	0.08		0.25
		Pollen	0.0005	0.10		0.20
		Birch pollen	0.0006	0.14		0.19
-DQA1*0101	Hazel nut	Normal	0.0021	0.24		0.34
		Pollen	0.0004	0.25		0.32
		Birch pollen	0.0006	0.28		0.29
-DQB1*0501	Hazel nut	Normal	0.0026	0.10		0.20
		Pollen	0.00094	0.11		0.18
		Birch pollen	0.0011	0.12		0.16
-DRB1*08	Peanut	Normal	0.0013	6.04	0.28	
		Pollen	0.00027	6.86	0.29	
		Grass pollen	not significant			
-DRB1*12	Carrot	Normal	<0.000001	13.01	0.34	
		Pollen	0.0035	4.96	0.29	
		Birch pollen	not significant			
		Mugwort p.	0.0011	5.45	0.30	

patients HLA-DRB1*01 and -DQA1*0101 were present and the -DQB1 allele was -DQB1*0503.

HLA genotype of patients with grass pollen-associated food allergy

The only food allergy frequently observed in our study population which is known for its association with grass pollen allergy was allergy to peanut [7]. Patients with peanut allergy showed a strong association with HLA-DRB1*08 when compared with the control population ($P = 0.0013$; $RR = 6.0$) (Table 5). This association lost significance when patients with peanut allergy were compared with patients with grass pollen allergy thus confirming the concept of peanut allergy being associated with this pollinosis.

HLA genotype in patients with pollen-associated carrot allergy

Carrot allergy is described as being associated with allergy to both birch pollen and mugwort pollen [12]. Among our patients allergy to carrot showed a significant association with HLA-DRB1*12 when compared with the control population ($P < 0.000001$; $RR = 13.0$) (Table 5). When compared to patients with pollinosis, this association remained significant ($P = 0.0035$; $RR = 5.0$). Interestingly, this association was stronger when it was analysed in comparison to patients with mugwort pollen allergy, and it

turned insignificant in comparison with birch pollen allergy. Thus, our data point towards a closer association of carrot allergy with allergy to birch pollen rather than mugwort pollen.

Discussion

In recent years the molecular basis for cross-allergenicity became increasingly clear. The identification of IgE reactions with epitopes on profilins, a family of plant pan-allergens as well as plant-derived food agents, was most significant in this regard [14,15]. Since agents triggering type I hypersensitivity reactions need to be recognized by HLA class II restricted T helper lymphocytes [1] studying the HLA class II genotype of patients should yield important insight in the genetics of type I allergy in general and the molecular basis of crossreactivity in particular.

Currently, there is evidence for at least four 'atopy loci' located at chromosome 5q31–32 [29,30], chromosome 11q13 [31], and chromosome 14q11 [32]. In addition, Mao *et al.* reported on an association between a genetic marker at chromosome 14q and atopic eczema [33]. An influence of the HLA genotype (chromosome 6) has been described [16,34]. The HLA class II alleles exhibiting associations with pollen associated food allergies in this study are distinct from genetic loci discussed in the context of atopy as referenced above. Thus, the possibility that the associations we observed simply mirror a bias to analysing

atopic subjects (who exhibit a much higher prevalence of type I allergy) can be excluded based on current knowledge of the genetic basis of atopy.

Crossreactivity and HLA restriction are expected to parallel structural homology of the respective allergens. Thus, if homologies exist between peptide binding sites of HLA genotypes from patients with different allergy patterns, one would also expect structural similarities of the respective allergens. One corollary of this concept is that significant HLA associations among patients with pollen-associated food allergies compared with the control population should turn insignificant when compared with a cohort exhibiting allergy to the corresponding pollen. This tendency is documented here for two food agents, hazel nut and peanut. Another interesting food agent in this regard is carrot previously described as being associated with allergies to both birch pollen and mugwort pollen [12]. We, too, observed patients exhibiting either constellation. On the basis of the HLA class II genotype the bias towards an increase of -DRB1*12 seen in carrot allergic patients with pollinosis was smallest — and lost statistical significance — when compared with the cohort of birch pollen allergic patients without those individuals suffering from mugwort allergy. Therefore, carrot in this study falls into the category of birch pollen associated food allergens. Taken together, the data on the HLA class II genotypes presented here support the concept of crossreactivity patterns originally deduced clinically and provide evidence for the influence of antigen presenting molecules on the manifestation of these patterns.

Frequently, HLA restriction of allergens is assessed *in vitro* by blocking the stimulation of allergen-specific T-cell clones using anti-HLA antibodies. These experiments along with studies focusing on the allelic characterization of the HLA restriction clearly pointed towards HLA-DR molecules as the prime restriction elements, e. g. for birch pollen [35,36], grass pollen [37], ragweed pollen [38] or foods, e. g. peanuts [39]. In contrast, HLA-DQ restriction is seemingly rare [40]. Moreover, the cohort described by Cordaba *et al.* to show an HLA-DQ association also exhibit an association with an HLA-DR allele [40].

Our data are in line with these reports in as much as we found increased frequencies of HLA-DR alleles in patients exhibiting peanut allergy and carrot allergy. With regard to the HLA restriction of pollen allergies, however, we got different results. In our cohort of birch pollen allergic patients we could not confirm the association with HLA-DR alleles described previously [35,36]. The study by Fischer *et al.* was able to show that only in subjects exhibiting immunoreactivity exclusively to Bet v I an association with -DRB3 alleles was found, whereas this was not the case among patients with a broader immunoreactivity [35]. As our study population of birch pollen

allergic patients is heterogeneous concerning the specificity of their IgE response lack in HLA association is in accordance with Fischer's observations.

Interestingly, we found a significant association with HLA-DQB1*0301 but not with -DR alleles among patients suffering from grass pollen allergy. The fact that this association reached statistical significance in a cohort not further selected on the basis of their IgE specificity might indicate a more selective immunoreactivity to grass pollen when compared with birch pollen, possibly caused by the immunodominance of one or very few closely related allergenic peptides. Müller *et al.* described the exclusive presentation of group V grass pollen allergens by HLA-DR [37]. This discrepancy may be due to technical reasons, since Müller *et al.* analysed the gross restriction of T-cell clones *in vitro* and focused on group V grass pollen allergens. Additionally, since HLA associations never reach 100% it is generally likely that more than one restriction element is relevant for each given allergen.

Hazel nut allergy is known to exhibit an association with birch pollen allergy [10]. Three HLA class II alleles were found to be significantly decreased in comparison to the control population, namely -DRB1*01, -DQA1*0101, and DRB1*0501. However, these alleles are unlikely to be independently associated with protection from hazel nut allergy, since they form an extended haplotype, i.e. they are in linkage disequilibrium [28]. Therefore, it cannot be stated which of these alleles may be of functional significance in the context of hazel nut allergy.

In our analyses we found highly significant *p*-values for the association of some allergens with distinct HLA alleles. Moreover, we found relatively high values for the relative risk and the aetiologic or preventive fraction, respectively. This indicates the major importance of the HLA genotype for the development of a given allergy or pattern of allergies as well as the clinical relevance of these findings. Moreover, it needs to be taken into account that a considerable proportion of the control population also may exhibit pollen allergy or pollen associated food allergy [see above and ref. 18]. Thus, any significant difference found would become more significant if these individuals were to be eliminated from the control population (which cannot be achieved practically due to the large number). As a consequence there is an error in this investigation towards the differences observed being even more pronounced than it appears on the basis of the statistical analyses. In fact, some weaker HLA associations may have been overlooked since statistical significance could be obscured by the symptomatic individuals in the control population.

To summarize, we were able to identify HLA class-II alleles associated with some allergies thus indicating that these alleles might confer susceptibility to the respective allergens. Similarities at the level of the HLA class-II

genotype parallel the empirical finding of distinct cross-reactivity patterns thus complementing investigations of IgE crossreactivities. Our observations provide evidence for the major importance of antigen presentation on the manifestation of distinct crossreactivity patterns.

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