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Characterization of peptidic and carbohydrate cross-reactive determinants in pollen polysensitization

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Clinical and Experimental Allergy

Summary

Background Cross-reactivity may be due to protein sequence or domain homologies and/or the existence of cross-reactive carbohydrate determinants (CCDs). The clinical relevance of peptidic cross-reactivities is well known, whereas that of CCDs is still a question of debate. The aim of this study is to characterize the IgE specificity of various patients suffering from pollen polysensitization to identify both peptidic and carbohydrate cross-reactive determinants.

Material and methods Rapeseed, grass and *Arabidopsis* proteins were separated by isoelectric focusing, followed by SDS-PAGE, and transferred to a nitrocellulose sheet. The sheets were incubated either with an individual serum from a birch+grass-sensitive patient, followed by anti-human IgE, or with labelled Concanavalin A (ConA). Binding inhibition was tested by incubation of the sera with a mixture of sugar residues.

Results The results showed two different patterns of cross-reacting sera: a pattern that implies few proteins, not always glycosylated and known as allergens, and a pattern that implies numerous proteins with molecular masses over 30 kDa. This second pattern was very close to the ConA-binding pattern. The IgE binding was abolished by pre-incubation with sugar residues only in the case of the second pattern.

Discussion This study shows that multiple pollen sensitizations could result from multiple sensitizations to specific proteins or from a cross-sensitization to a wide range of glycoproteins. Two-D blots allow to characterize a cross-sensitization due to carbohydrate determinants, and thus to improve the diagnosis of allergy and its medical treatment.

Keywords cross-reaction, cross-reactive carbohydrate determinants, 2-D blots, diagnosis, pollen allergy

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Introduction

Polysensitization to various allergenic substances may be due to either multiple independent sensitizations or cross-reactivity between proteins or glycoproteins. Cross-reactivity may be due to protein sequence or domain homologies and/or the existence of cross-reactive carbohydrate determinants (CCDs). Protein sequence/domain homologies are now widely described, and it was shown that approximately 65% of the plant food allergens were related to only four protein families (prolamin, Bet v 1, cupin and profilin) [1]. It is generally known that at least a 70% sequence identity is required to induce a cross-reactivity, and that under 50% identity, the risk of cross-reactivity is very low [2, 3]. However, an epitope is

determined by a short linear amino acid sequence or a tri-dimensional structure that is restricted to a part of the protein (e.g. the binding site or the catalytic domain of an enzyme). Thus, cross-reactive epitopes may be found for only short sequences with high identity, when these sequences determined a characteristic domain. The clinical implication of protein cross-reactivity is well known and several syndromes induced by related food and pollen allergens have been described [4–6].

It is now well established that carbohydrate determinants may be recognized by the IgEs of allergic patients, even if their clinical relevance is still a matter of debate. A unique carbohydrate determinant is unable to induce the cross-linking of the IgEs bound to mast cells and/or basophils because multivalent allergens are requested for

IgE cross linking [7]. The polyvalency of an antigen may be due to the existence of multiple peptidic epitopes, of a mixture of peptidic and carbohydrate epitopes or of multiple carbohydrate epitopes, even in the case of a unique carbohydrate determinant associated several times with a protein. However, the biological activity of anti-carbohydrate IgEs probably may not be assessed by a general rule, but mostly depends on the type of the glycan (e.g. N- or O-linked or even more subtle differences in structure that may not be evidenced by classical immunochemical analysis) and the affinity of the antibodies [2].

Checking cross-reactivity among allergens is an important goal, even very practical, when the avoidance of the allergen is the rule of the treatment. Carbohydrate determinants may be of particular interest as they are shared by phylogenetically unrelated species, including plants and invertebrates [8]. Various tools were developed to investigate the implication of sugar residues in IgE binding, mostly based on ELISA inhibition by various monovalent glycoconjugates or periodate degradation of the allergen [9, 10]. The aim of the present study is to characterize the IgE response to peptidic and carbohydrate cross-reactive determinants. For this purpose, we selected several patients suffering from pollen polysensitization and analysed their IgE response to pollen allergens by two-dimensional (2-D) immunoblots. Oilseed rape is a good candidate to study cross sensitization because its cultivation has increased in France and its pollinic season overlaps those of grasses and trees.

Material and methods

Human sera

One hundred and forty-five human sera were collected from biological analysis laboratories and represented residues of IgE titre determinations. The sera were selected on the basis of positive values for serum-specific IgE to birch and grass pollen allergens (Phadia CAP system ≥ 5 kU/L, Uppsala, Sweden). These sera have been used as immunochemical reagents to identify allergens as molecules recognized by IgE.

Allergen extracts

Oilseed rape (*Brassica napus*) pollen and grass (*Dactylis glomerata*) pollen were purchased from Allergon, Angelholm, Sweden. Mature *Arabidopsis thaliana* flowers (*wassilewskija*) were provided by J. J. Leguay *et al.* (CEA Cadarache, France).

Allergen extractions were performed routinely as published previously [11–13]. Briefly, *B. napus* pollen, *D. glomerata* pollen and *Arabidopsis* flowers were incubated for 1 h at 22 °C in distilled water under gentle agitation. All three water-soluble extracts were recovered by centrifugation (16 000 g), followed by glass wool filtration.

Western blotting

The allergen extracts were submitted to 2-D gel electrophoresis as described previously [11]. Briefly, each extract was separated by a unique isoelectric focusing separation (IEF) performed in a polyacrylamide gel containing 5% v/v Servalyt[®] pH 2–11 (Serva, Heidelberg, Germany), and then several strips of the IEF separation were submitted to a SDS-PAGE separation on a pre-cast commercially standardized 8–18% gradient gel (ExcelGel, GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). One 2-D separation was silver stained according to Blum *et al.* [14], and the others were used for Western blots. After the 2-D electrophoretic separation, the proteins were electroblotted onto a nitrocellulose (NC) sheet with a Novablot apparatus (GE Healthcare) following the manufacturer's instructions (1 h, 1 mA/cm²). After blotting, the NC sheet was dried and blocked with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (PBS-BSA-Tw) for 1 h at 22 °C. Then, each NC sheet was incubated with individual serum (300 μ L diluted 1 : 10 in PBS-BSA-Tw, overnight, 22 °C), followed by alkaline phosphatase-conjugated goat anti-human IgE (1 : 700 in PBS-BSA-Tw, 2 h, 22 °C, Sigma Biochemicals, USA). The enzymatic activity was detected using 5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitro Blue Tetrazolium (NBT-BCIP, Sigma Biochemicals) in 0.1 M Tris buffer pH 9.5.

To assess the implication of sugar residues in the IgE-binding specificity, individual human sera were pre-incubated with a mixture of 300 mM methyl α -D glucopyranoside and 10 mM methyl α -D mannopyranoside (Sigma Biochemicals).

The carbohydrate pattern was also evidenced by incubating the saturated NC sheet with biotinylated Concanavalin A (ConA) (1 : 80 000 in PBS-Tw 0.3, 1 h, 22 °C, Biosys SA, Compiègne, France), followed by alkaline phosphatase-conjugated streptavidine (1 : 5000 in PBS-Tw 0.3, 1 h, GE Healthcare, USA) and NBT-BCIP as described above.

Results

The silver-stained 2-D gel electrophoresis of the oilseed rape pollen extract showed the classical proteic pattern of this extract with some allergens identified previously by amino acid sequencing and/or mass spectrometry [11, 12] (Fig. 1a).

Out of the 145 birch- and grass-positive sera, 45 were IgE-positive to rapeseed proteins in 1-D IEF immunoblots. Fourteen of these 45 positive sera were further analysed in 2-D blots to characterize the molecular specificity of their IgEs. These 14 sera were selected according to the specificity of their IgE in 1D (non-redundant specificities) and the intensity of the 1-D IEF staining.

The results of the 2-D blot analysis showed that two types of recognition profiles could be evidenced. The first one corresponded to sera that recognized a restricted

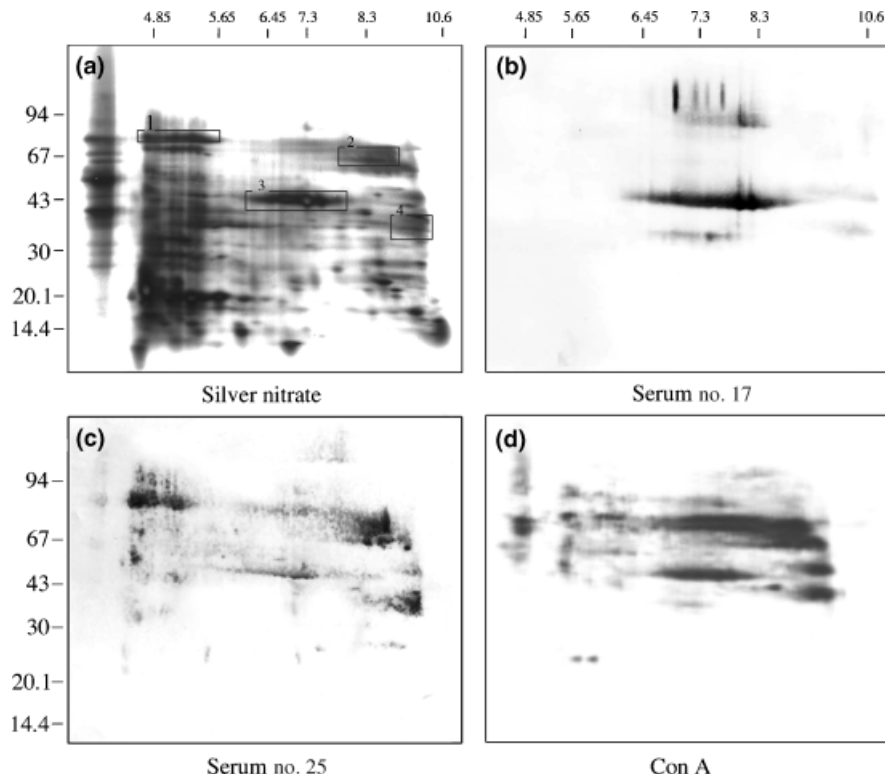


Fig. 1. Two-dimensional (2-D) blot analysis of the IgE specificity of human sera to oilseed rape pollen proteins. Towards the various proteins evidenced on a silver-stained 2-D gel (a), two characteristic IgE recognition patterns may be evidenced. One pattern is restricted to a limited number of proteins, as shown with serum no. 17. (b) The second pattern corresponds to the recognition of various proteins with molecular masses (MM) of more than 30 kDa. (c) This pattern is very close to the one obtained after Concanavalin A (ConA) binding (d). MM are indicated on the left and isoelectric points (pI) on the top. 1, cobalamine-independent methionine synthase; 2, Berberin bridge protein; 3, pectinase; 4, receptor-like protein kinase.

diversity of proteins compared with the global protein content evidenced by silver staining. Each serum of this type recognized only a few spots, differing in isoelectric points and molecular masses from one serum to another. A representative example of such a profile is shown with serum no. 17 (Fig. 1b), which has been found previously to recognize the rapeseed pectinase [11]. The second type of recognition profile corresponded to sera that recognized numerous proteins with molecular masses >30 kDa. A representative example of such profiles is shown with serum no. 25 (Fig. 1c). This multispot pattern was observed in 20–30% of the positive sera. All the multispot patterns were very similar among them and also to the staining observed with ConA binding (Fig. 1d). Thus, these patterns will be further referenced as 'ConA-like' patterns.

Incubation of the sera with methyl- α -2 glucopyranoside and methyl α -D mannopyranoside did not modify the staining in the case of serum no. 17 (Fig. 2a), or in any case of a serum recognizing a restricted set of proteins, suggesting that IgEs of these sera recognized only peptidic epitopes. On the contrary, the staining with serum no. 25 was completely abolished by pre-incubation with sugar residues (Fig. 2b). A similar inhibition was also observed with the ConA binding (data not shown). This complete inhibitory effect was observed for all the ConA-like

sera, suggesting that all these sera recognized oilseed rape allergens only by cross-reacting carbohydrate determinants. Similar results were also observed after a meta-periodate oxidation of the blotted proteins (data not shown).

Patient no. 17 was known to be clinically allergic to grass pollen [15]. When tested against a grass pollen extract, IgEs from serum no. 17 recognized several grass pollen proteins very strongly (Fig. 3a). Almost all of them have been characterized as known allergens [16–18] (www.allergen.org). Some of these allergens are glycosylated and others are not. In contrast, when serum no. 25 was tested to an extract from *Arabidopsis* flowers, it displayed a typical ConA-like pattern (Fig. 3b) of anti-carbohydrate IgEs that was very similar to the one observed with rapeseed pollen (Fig. 1c). Only a few of the numerous spots recognized by these IgEs corresponded to known allergens. Furthermore, a closer look at the blots showed a fluffy pattern that is frequently associated with the detection of IgEs to carbohydrates.

Discussion

In the present work, we chose several sera from polysensitized allergic patients to analyse the cross-reactivity

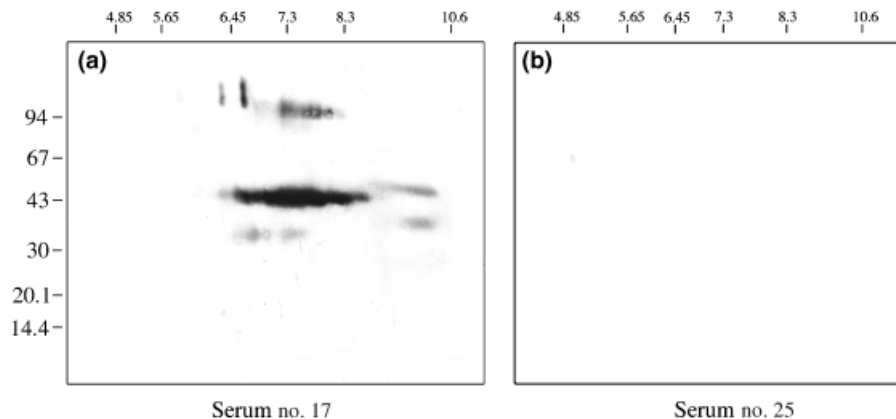


Fig. 2. Two-dimensional blot analysis of the effect of incubation with methyl α 2 glucopyranoside and methyl α -D mannopyranoside on the IgE-binding pattern. Incubation with methyl α 2 glucopyranoside and methyl α -D mannopyranoside does not modify the IgE-binding pattern of serum no. 17 (a), whereas it completely abolishes the binding of the IgE of serum no. 25 (b). Molecular masses are indicated on the left and isoelectric points on the top.

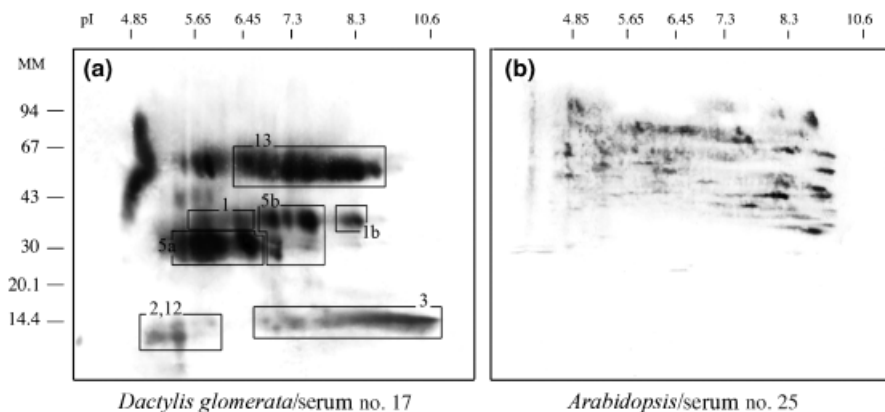


Fig. 3. Two-dimensional blot analysis of the IgE specificity to other plant proteins. When tested for grass pollen proteins, serum no. 17 IgEs (a) recognized several proteins known as allergens, whereas serum no. 25 IgEs (b) recognized the typical ConA-like pattern. Molecular masses are indicated on the left and isoelectric points on the top. 1, Dac g 1; 1b, basic Dac g 1; 2, Dac g 2; 3, Dac g 3; 5a, acidic Dac g 5; 5b, basic Dac g 5; 12, Dac g 12; 13, Dac g 13.

patterns of proteins and carbohydrates recognized by the IgEs. The results clearly show that cross-reactivity due to peptidic epitopes presents a typical 2-D blot pattern with a limited and variable number of spots, whereas carbohydrate cross-reactive sera display a typical uniform ConA-like pattern. However, a cross-reactivity by only CCDs does not mean that the sera do not possess IgE to proteins. It only means that IgEs to proteins are not cross-reactive, most probably because the same peptidic epitopes are not shared by both plants.

Cross-reactivity to peptidic determinants

The peptidic cross-reactivity that we illustrated in this study is due to pectinase (polygalacturonase). Pectinase is a glycosylated molecule, evidenced by the ConA binding in this study. This glycoprotein may be recognized by IgE directed to peptidic epitopes and/or by IgE to CCDs. The 2-D blot analysis of serum no. 17 shows that this patient has IgE only directed to peptidic epitopes of the oilseed

rape pectinase, as the IgE binding is not inhibited by competitive binding with sugar residues. This polygalacturonase has been identified previously as a rapeseed allergen [11] and belongs to the glycosyl hydrolase 28 family. Several polygalacturonases have already been identified as pollen allergens: group 13 from grass [19], Pla a 2 from *Platanus acerifolia* from the order of Proteales [20], and Cha o 2 from *Chamaecypranus obtusa* [21], Cry j 2 from *Cryptomeria japonica* [22] and Jun a 2 from *Juniperus ashei* [23] from the order of Coniferales. As shown in this study, serum no. 17 recognizes the rapeseed pectinase, but also Dac g 13, which is a group 13 grass allergen. Group 13 grass allergens Phl p 13, Lol p 13, Fes e 13, Poa p 13, Dac g 13 and Zea m 13 have been shown to be cross-reactive [19], and we can confirm that serum no. 17 recognizes both Dac g 13 (Fig. 3a) and Phl p 13 (data not shown). Thus, the IgE binding to the rapeseed pectinase may be due to cross-reactivity between the grass and oilseed rape pollen proteins of the glycosyl hydrolase 28 family.

Patient no. 17 was found to be allergic to *Dactylis* pollen [15]. His sensitivity to rapeseed pollen was not known as being not clinically tested.

Cross-reactivity to carbohydrate determinants

More than providing basic information on the presence and the amount of IgEs to CCDs, as ELISA or any other related techniques will do, the 2-D analysis allows excellent qualitative characterization of the cross-reactive glycoproteins. All the proteins that share CCDs have a molecular mass over 30 kDa, but display pIs varying from the acidic to the basic zone. Some of the rapeseed glycoproteins are already well identified as the berberine bridge enzyme, the pectinase, the cobalamin-independent methionine synthase and a receptor-like protein kinase [11, 12]. The differences observed between rapeseed and *Arabidopsis* patterns recognized by serum no. 25 are mainly due to the origin of the extracts (pollen vs. flowers), but the similarities (numerous spots, molecular masses over 30 kDa, wide range of pIs, fluffy pattern ...) are characteristic of anti-carbohydrate IgEs.

The CCDs evidenced in this study are shared by most of the glycoproteins in rapeseed or in a phylogenetically related plant, *A. thaliana*. Most of the reports on pollen allergen glycosylation [7, 24] focused on the N-linked carbohydrates and show that these structures are constituted by a chain of *N*-acetylglucosamine and mannose residues, branched with fucose, xylose and mannose residues. ConA is known to bind strongly to carbohydrates with terminal mannose residues [25], and thus to these glycosylated allergens. Both $\alpha(1-3)$ fucose and $\beta(1-2)$ xylose are recognized as major determinants in IgE binding [7, 24]. However, our study indicates that mannose residues participate in the epitope because IgE binding may be inhibited by pre-incubation of the serum with an excess of methyl α -D mannopyranoside. Such a role of mannose residues in the epitope structure of CCDs has been suggested previously [26].

Carbohydrate cross-reactive determinants may be shared by the pollen of taxonomically unrelated species [27], by pollens and food proteins [28], as well as by plants and invertebrates [3]. Recently, O-glycans from the mugwort pollen allergen Art v 1 were characterized, and their role in antibody binding was suggested [29].

According to Mari [30], IgE to carbohydrates are mostly prevalent in patients with multiple pollen sensitizations, varying from 31% of the pollen-allergic patients to 71% in the case of multiple pollen sensitizations. However, the prevalence of IgEs to carbohydrates varies from 16% to 55% in food-allergic patients and from 56% to 79% among patients allergic to hymenoptera venoms [7]. In the present work, 20–30% of the positive sera displayed a typical anti-carbohydrate ConA-like pattern.

The question of the biological importance of IgE to cross-reactive carbohydrates is still a matter of debate, but recent reports have demonstrated the biological activity of these IgEs in the case of tomato and celery allergies [31, 32], and in the birch–mugwort–celery–spice syndrome [33]. These recent results showed the importance of carbohydrate determinants in some cases of IgE binding, and raised the question of the use of unglycosylated recombinant proteins in the diagnosis of certain cases of allergic diseases [29, 32]. It is obvious that recombinant proteins will be very useful tools in the diagnosis of allergic diseases, especially with the development of micro- and nano-technologies. However, especially in the case of polysensitization, the use of natural allergen isoforms with various levels of glycosylation will allow the detection of anti-carbohydrate antibodies, which is also a very important goal in allergy diagnosis.

In conclusion, the present work shows that, for research purposes, 2-D blots are useful tools to evidence and characterize cross-reactivity either due to peptidic or carbohydrate determinants among various allergen sources. The characterization of CCDs is an important goal to determine the role of carbohydrates in the diagnosis and therapy of allergy.

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