

Pollen-Cross Allergenicity Mediated by Panallergens: A Clue to the Pathogenesis of Multiple Sensitizations

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Abstract: Allergens are proteins capable of raising in predisposed (atopic) individuals an IgE-dependent type I hypersensitivity reaction, supporting allergic symptoms such as anaphylactic shock, asthma and rhinitis. Panallergens are evolutionarily conserved, ubiquitous components of several complex sources of allergens, which usually act as minor allergens, i.e., they do not react with the majority of sera from patients allergic to a given allergen source. However, their presence has important clinical implications in establishing the phenomenon of food-pollen cross-reactivity, in the interpretation of diagnostic tests and in the preparation of immunotherapy extracts. A T-lymphocyte component is necessary to support the panallergen-specific humoral IgE antibody response.

While several excellent reviews are available regarding allergen cross-reactivity from the IgE-perspective, data on cross-allergenicity from the T-lymphocyte standpoint are quite limited. Indeed, this is a crucial issue in the comprehension of polysensitization, since it is the initial exposure to conserved panallergens that may subsequently drive the allergic immune response towards major allergenic components, which at first proved non-sensitizing to a given patient, through a mechanism of intermolecular epitope spreading.

Here, we will discuss data showing that a functionally relevant T-cell response to molecularly defined, conserved regions of panallergens can support cross-allergenicity in allergic patients.

This subject is relevant to the comprehension of the natural history and to the clinical management of the majority of allergic patients, who suffer from multiple allergies.

Keywords: Allergy, allergen, cross-reactivity, T-lymphocytes.

INTRODUCTION

The specificity of the allergic response is dictated by immunoglobulins of the IgE isotype, produced in predisposed (atopic) individuals, towards innocuous glycoprotein antigens, which are referred to as "allergens". Allergen sources are complex mixtures of single allergenic proteins. They are generally identified as "major" or "minor" allergens on an epidemiological basis, i.e. based on the frequency of individuals in the population who are allergic (sensitized) to that given allergen source (e.g., grass pollen) and display IgE antibody to each single component. Major allergens are those components in which the majority of allergic subjects are sensitized (e.g., group 1 and 5 allergens in grass pollen allergic subjects), whereas minor allergens are recognized by IgE antibodies only in a limited portion of allergic subjects (e.g., group 12 allergens in grass pollen allergic individuals). This intra-allergenic heterogeneity is usually not measured in common clinical settings: therefore, subjects allergic to grass are considered homogeneous in immunological terms. On this basis, each one of them can receive, if needed, a unique form of immunotherapy, with extracts containing defined amounts of major allergens, but lower and relatively variable amounts of minor allergens, depending on the manufacturer and other technical factors. This situation poses an initial problem for subjects with important sensitizations to minor allergens, since they may not fully exploit the potential of

immunotherapy and even face the risk of being exposed to components they were not allergic to. Component-resolved diagnosis and preparation of patient-tailored allergen extracts faithfully reflecting the allergenic profile of each vaccinated subject are the rationale, which have been proposed to resolve this problem [1].

With presently available assays using complex allergen extracts, a consistent but relatively minor proportion of allergic subjects appear mono-sensitized to a single allergen source (e.g., either grass or birch, etc.), whereas most allergic individuals simultaneously display multiple sensitizations [2, 3]. Polysensitization depends mainly on the concurrent presence of sensitizations to multiple allergen sources. However, a different possibility has been highlighted by the analysis of IgE reactivity to single allergenic components, which have recently become available also to the clinical laboratory, for patients sensitized to grass, birch, mites and pellitory [4-6]. By applying such a molecular dissection of the IgE profile, it appears that a proportion of polysensitized patients are indeed allergic to minor allergens defined "panallergens". These are relatively homogeneous components of different allergen sources, which are tightly preserved throughout the evolutionary tree from plants to animals, likely due to the fact that they perform vital functions. Panallergens belong to relatively broad molecular families, such as profilins, lipid transfer proteins and pathogenesis-related proteins [7]. On this basis, polysensitization can be explained with the recognition by cross-reacting IgE of highly homologous panallergens within each different allergens source [8].

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On a clinical perspective, respiratory allergies to inhalant allergens are rarely affected by IgE to panallergens. This likely depends on the quantitatively limited relative amount of IgE for these components in most allergic patients. Correspondingly, panallergens represent a minor constituent of native allergen sources patients are exposed to [9]. However, cross-reactivity *via* panallergens certainly poses a well recognized problem in the relationship between pollen sensitization and food allergy, especially as manifested in the oral allergy syndrome, i.e. in the phenomenon of food-pollen cross-reactivity [10]. In fact, due to their conservation in widely divergent plant species, they can trigger immediate-type oral symptoms in subjects allergic to pollens who consume several different fruits. Moreover, panallergens have obvious clinical implications not only in the interpretation of diagnostic tests but also in the preparation of immunotherapy extracts, and this will hold true at least until component-resolved diagnosis will achieve widespread clinical use. Here, we will discuss a further potential critical issue raised by panallergens, i.e., the possibility that they may have an until now unrecognized role in the spreading of the allergic response from components of one to components of multiple allergen sources.

Several excellent reviews are available of allergen cross-reactivity from the IgE-standpoint, based both on pollen cross-allergenicity and on plant taxonomy [11-13]. Here, we will discuss cross-allergenicity specifically focusing on the T-lymphocyte perspective. Data will be highlighted suggesting that inter-molecular epitope spreading *via* panallergens may explain the pathogenesis of polyreactivity. We will include data from a work from our group showing directly that a functionally relevant T-cell response to molecularly defined, conserved regions of a panallergen underlie cross-allergenicity in a subset of allergic patients, and discuss how this may reflect to a general pathogenic mechanism of polysensitization.

BOTANICAL VERSUS MOLECULAR CLASSIFICATION OF POLLEN CROSS-ALLERGENICITY

Pollen aeroallergens belonging to a given group of plants are cross-reactive at the level of major allergen, due to the homology of the latter in different species. This intra-group cross-reactivity has a facilitating impact on diagnosis and therapy of allergy. In fact, positive skin tests of non-endemic plants may surely depend not only on prior exposure in a mobile human population but also on cross-reactivity with endemic plants. Thus, the decision to include a non-endemic species in allergen immunotherapy depends on whether or not related plants provide adequate immunological coverage. For instance, in the case of grass allergen, it has been demonstrated that, although around twenty different species can provide pollens useful to diagnosis in prick testing, one grass species is sufficient for *in vitro* diagnosis of all grass pollen allergic individuals [14, 15], due to the high level of humoral (IgE) cross-reactivity within group 1 and group 5 allergens, respectively [16]. In the last two decades, a great deal of information has become available on pollen allergenicity and plants systematic [11, 12, 17] with cloning of recombinant allergens. In this context, molecular classification, rather than botanical taxonomy has emerged as a superior way to address the cross-reactivity issue [18]. Along this line, Fagales pollen allergy (birch, alder, hazel, hornbeam, oak,

chestnut) can be identified with the only Bet v 1 recombinant molecule as a marker allergen [18]. Similarly, Ole e 1 can serve as a marker molecule for allergy to Scrophulariales (olive, ash, plantain, privet, iliac)[18]. Thus, by switching from the use of allergen source to that of recombinant allergens, apparently complex sensitization profiles may be narrowed down to a very limited number of marker allergens. Cross-reactivity among major allergens of different allergenic sources has also been demonstrated at the T-cell level with grass [19, 20] and cedar [21] allergens.

At the same time, however, molecular dissection of the sensitization pattern by recombinant allergens allows us to identify subjects with relevant levels of IgE to allergens (panallergens), which are highly conserved through widely divergent species. In particular, profilins represent the most cross-reactive allergens described to date [22, 23]. Profilins, such as Bet v 2, are ubiquitous eukaryotic 14-kDa proteins with acting-binding properties [24]. A high level of cross-reactivity has been demonstrated among profilins, including those from pollens of botanically unrelated plants (trees, grasses, weeds), from plant food (fruit, vegetables, nuts, spices), from latex and even from humans [24-32]. The high degree of cross-reactivity among profilins can be attributed to their highly conserved three-dimensional structures [33].

Another highly cross-reactive family of allergens is represented by calcium-binding proteins [18]. Bet v 3 and Bet v 4 were the first calcium-binding pollen allergens identified according to sequence comparison and functional calcium-binding assays [34]. Bet v 3 contains three typical calcium-binding domains and is highly expressed in mature pollen [34]. Bet v 4 contains 2 calcium-binding domains and shares IgE epitopes with the structurally-related two-EF-hand calcium binding allergens present in grass pollens (Cyn 7 from Bermuda grass, Phl p 7 from timothy grass) [35, 36], weed pollen (Bra 1 and Bra 2 from rape)[37] and tree pollens (Aln g 4 from alder, Ole e 3 from olive)[38, 39]. All EF-hand containing plant allergens are primarily expressed in mature pollen and exhibit extensive immunological cross-reactivity [40].

Thus, classification of allergen cross-reactivity, including its implication in explaining the oral allergy syndrome, in the preparation of extracts for immunotherapy and in the interpretation of results obtained with diagnostics extracts, can be correctly organized only through information obtained with single, molecularly cloned allergens.

T-LYMPHOCYTES AND THE IMMUNOLOGICAL BASIS FOR IGE PRODUCTION

For any humoral memory immune response to be generated towards an antigen, T-cell help needs to be provided to B-lymphocytes by antigen-specific helper T cells. In the case of allergens, IgE production is dictated by T cells with a typical effector phenotype described as Th2 and characterized by the prevalent production of cytokine such as IL-4, IL-5, IL-13 [41]. Reaginic (i.e., immediate type hypersensitivity inducing-) antibodies are produced following switch of naive B lymphocyte from IgM to IgE isotype production. The molecular basis of this event is the triggering of CD40 ligand on the membrane of T cells by membrane-bound CD40 on antigen presenting cells (APC), in the presence of a specific soluble factor, IL-4 [42]. In contrast to B cells, T

cells do not recognize native allergens, but the product of allergen processing, generated by the action of a complex proteolytic intracellular machinery in APC (mainly professional APC, such as dendritic cells) [43]. After processing, allergen peptides are presented to T lymphocytes on Major Histocompatibility Antigen (MHC) molecules of type II. T cells can recognize the MHC class II-peptide complex through a specific, membrane bound antigen receptor, the T cell receptor [44]. These T cells are the elements which, by cell-to-cell contacts associated with the release of soluble mediators, can help B cells expressing a receptor specific for the original native allergen on their membrane to differentiate and mutate their membrane-bound immunoglobulin to a secretory form [45]. B cells matured to immunoglobulin secreting cells (plasma cells) are phenotypically characterized by an impressive secretory apparatus. During the process of selection of allergen-specific B cells, affinity maturation also takes place through the somatic mutation of the variable parts of immunoglobulin genes [46]. This allows the generation by selection of B cells with membrane immunoglobulins, whose variable part suitably fits with specific conformational epitopes on the native antigen. Thus, T cells orchestrate the humoral immune response not only by helping B cells to mature to plasma cells and to produce high affinity antibody, but also by determining the immunoglobulin isotypes that B cells will produce. This latter characteristic is crucial in the determination of the quality of the humoral immune response that will follow antigen encounter and is the result of selective pressures generated to counteract invader microorganisms, requiring functionally specific countermeasures. In the case of the allergic immune response, IgE antibodies are the unwanted effect of the immunoglobulin switch driven on B cells by T lymphocytes with the same allergen specificity as the B cells. Thus, B cell epitopes require the conservation of higher level (secondary, tertiary and quaternary) protein structure, whereas T cell epitopes are totally defined by the primary structure dictated by their amino acid sequence. One of the most promising perspectives for future immunotherapy is based on the search of selected T cell epitopes. These crucial reagents can prove to be capable of generating a response by changing the phenotype of the allergic response without presenting the risk of immediate type hypersensitivity reactions [47]. T cell epitopes determining the allergen specific T cell response in one individual depend on the intrinsic characteristics of their sequence, which allows their binding to the MHC class II groove on APC through hydrophobic anchor residues in the context of protein motifs, which have been extensively characterized for several MHC alleles [48]. Flanking sequences can also influence loading [49] and stability [50] of MHC class II peptides. A limited number of T cell epitopes can be identified for each allergen, depending on the length and type of its sequence, out of much more numerous theoretical peptides generated by antigen processing. Typically, this analysis is called epitope scanning and is performed by *in vitro* experiments where T cells are reacted in the presence of APC with each of the potential peptides of an allergen with a known sequence. Usually peptides in the 12 to 15 amino acid length range are used, facilitating the reaction of the MHC class II groove. The end-point measured is usually T cell proliferation. Immunodominant epitopes may be defined as immune determinants expressed by naturally proc-

essed and presented peptides that elicit T cell proliferation. The immunodominance concept can be extended from the individual to the general population, if the majority of human MHC are included in this analysis [51]. Thus, the experimentally observed T-cell response to a given peptide can be modulated at any of the several steps: antigen processing, peptide presentation, or T-cell receptor recognition.

T CELL CROSS REACTIVITY IN THE CASE OF PANALLERGENS

Recently, we observed that Bet v 2, the birch profilin, has immunodominant epitopes in proximity of homologous sites of the grass profilin, Phl p 12 [52]. In this study, proliferation of T lymphocytes to birch and grass allergen, respectively, was assessed in patients with sensitization to these two allergen sources. Six out of 31 patients were identified, where T-cell lines specific to one allergen also recognized the other, as assessed by two independent end-points, i.e. proliferation and molecularly-determined clonal size following *in vitro* stimulation. These cross-reacting T cells were then used to derive T cell clones, which were subjected to epitope mapping with a combination of *in silico* and *in vitro* analysis. In fact, the rule for defining anchor residues and the contiguous protein motifs for binding to MHC have been quite extensively characterized and a bioinformatic tool has been developed, which assisted in the finding of peptides of an antigen which are predicted to bind human MHC alleles, thus being as immunodominant in the general population [53]. We identified T-cell clones in patients with a measurable grass-birch T-cell cross-reactivity, which promiscuously recognized homologous regions on birch and grass profilins. Namely, two peptides were recognized by Bet v 2 specific T cell clones (Bet₄₀₋₅₄ and Bet₆₅₋₇₉), which also proliferated when the homologous Phl p 12 peptides (Phl₃₈₋₅₂ and Phl₆₃₋₇₇) were presented by syngeneic APC. An improved prediction of helper T-cell epitopes was provided by the combined application of sequence conservation and MHC class II protein binding motifs. In fact, these peptides corresponded to phylogenetically conserved region on profilins, which are highly homologous across different plants.

The mechanism of cross-recognition by T-cell of determinants belonging to a different allergen has been described by other investigators in studies focused on the pathogenesis of pollen-food cross-reactivity. Jahn-Schimid *et al.* reported that a dominant Bet v 1 epitope plays an important role in the cellular cross-reactivity between this birch allergen and several related food allergens (apple, cherry, hazelnut, celery, carrot, and soybean) [54]. Bohle *et al.* found that Bet v 1 specific T cell clones recognize homologous determinants from Api g 1, the major celery allergen, which is responsible for hypersensitivity reactions to celery in birch allergic individuals [55]. Fritsch *et al.* reported that cross-reactivity between apple and birch pollen leading to the clinical oral allergy syndrome occurs not only at the serological, but also at the cellular level [56].

By applying *in silico* analysis to another class of panallergens (calcium-binding proteins, CBP), a similar result, presently waiting for *in vitro* validation, is predicted to be obtained. In Table 1, alignment of CBP from white birch, common timothy, short ragweed, common olive and prickly juniper are shown. Two different programs were used: T-

These observations have important implications on the comprehension of the generation of allergen cross-reactivity. In fact, our data support the notion that IgE cross-reactivity can result from the T cell help provided to B lymphocytes by T cells specific to a panallergen, which is a component of a different allergen source. Thus, the natural exposure to, or the immunotherapy with allergen sources containing "third party" panallergens has the potential to directly modulate the clonal size and, possibly, the functional phenotype of T lymphocytes specific to a given sensitizing panallergen.

The observation made about panallergens is likely to reflect a general rule of immunodominance. For instance, T cell epitopes of the major dog allergen Can f 1 [60] localize in proximity of those found in corresponding Bos d 2 [61] and Rat n 1 [62] major bovine and rat allergens, which are all proteins of the lipocalin family.

Thus, also in this case, homologous allergens from different allergen sources tend to display peptides immunologically dominant in relatively homogeneous sites of the protein, which usually correspond to the most conserved regions of the allergen. Along this line, it has been reported that homologous antigens of different sources, such as the surface M proteins of group A streptococci, are immunodominant in conserved part of the antigenic molecule [63]. Taken together, these observations suggest that the localization of immunodominance in phylogenetically conserved portions of antigens may be a general rule of the immune system, which holds true also for non-allergenic proteins. The simplest explanation of this may be the added value that the individual acquires by encountering a given invader's antigen. In fact, the relative degeneracy of recognition applied to conserved regions of the antigen, may allow to achieve protection also against different invaders. Different invaders may well be on the whole, different in antigenic terms, but they may be subjected to recognition by a pre-existing memory immune response targeted to the most conserved parts of their antigenic repertoire. The observation that sequence conservation appears to be particularly useful as a predictor

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-----10-----20-----30-----40-----50-----
Bet v 4   Q394199 -----MADDHPQDKAERERIFKRFDANGDGKISAAELGEALKTLG-SITP
Phl p 7   o82040 -----MADDMERIFFKRFDTNGDGKISLSELTDALRTLG-STSA
Amb a 10  AY894659 -----MAPENNKHSIFPTDKEEVTKIFNRFDTNGDGQISEDELFAILKSLGSDTSP
Ole e 3   081092 -----MADDPQEVAEHERIFFKRFDANGDGKISSSELGETLKTLG-SVTP
Jun o 2   064943 MDEVPSDDGSKSACSGEVVMEQSVHELEEVFKKKFDANGDGKISGSELADILRLSLGSDVGE
          : .*:***:** ** *:** .

60-----70-----80-----90-----100-----110-----
Bet v 4   DEVKHMAEIDTDGDGFISFQEFTDFGRANRGLLKDVAKIF-----
Phl p 7   DEVQRMMAEIDTDGDGFIDFNEFISFCNANPGLMKDVAKVF-----
Amb a 10  DEVKRVM AEIDADGDGFISLDEFILFCKGIESEGDEINDLKEAFKFYDQNNNGVISANEL
Ole e 3   EEIQRMMAEIDTDGDGFISFEETVTFARANRGLVKDVAKIF-----
Jun o 2   AEVKAMMEADADGDGYVSLQEFVDLNNKG----ASVKDLKNNAFKVFDRCNGSISAAEL
          *:::* * *:***:~::~* : . .: .:

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Alignment of calcium binding protein sequences from pollen allergens. The accession number according to the GeneBank/EMBL/DBJ database is shown in the first block of alignments. Allergen nomenclature is according to IUIS/WHO Allergen Nomenclature Subcommittee (Bull World Health Organ 1994; 72: 797-806). Alignment was performed with the T-COFFEE Version 1.41 software and with the Nomad (Neighborhood Optimization for Multiple Alignment Discovery) program. The symbols, * and :, indicate identity and homology, respectively, according to the T-COFFEE, whereas the bold residues show the occurrences found with NOMAD (objective score: 27.8). Anchor residues for most common MHC class-II alleles, as predicted by the TEPITOPE algorithm, are underlined.

of immuno dominance was previously deduced on a theoretical ground by Landry [50] in the case of Hen Egg Lysozyme and HIV gp120. This author highlighted that this may be a consequence of the fact that conformationally labile antigen segments tend to be excluded from MHC binding by the proteolytic degradation, which precedes MHC loading of peptides discharged from the proteasome complex. Indeed, local antigen instability is likely to be an important determinant of endoproteolytic cleavage of antigens. Lysosomal enzymes available to carry out antigen processing exhibit only modest amino acid sequence specificity [64-67]. Along this line, the tendency for proteases to nick between well-folded domains has been employed by biochemists for many years as standard immunochemistry procedures for the preparation of immunoglobulin fragments [68]. In particular, papain, a member of the family of proteases that contains several cathepsins, selectively cleaves between domains of proteins, such as immunoglobulins and myosin. Protein processing probably also depends on the resistance of well-folded domains to the action of redundant processing enzymes [67, 69]. A relationship between the locations of T-cell epitopes and structurally unstable sites in proteins has been reported [70].

EPITOPE SPREADING AND THE GENERATION OF POLYSENSITIZATION

The mechanism by which the immune response to a given epitope of an antigen extends to more epitopes of the same antigen is indicated as "intra-molecular epitope [or determinant] spreading". Indeed, inter-molecular spreading is also a well-recognized immunological mechanism, whereby the original recognition of one component of an antigen can drive the immune response towards other components, which simultaneously reach the same immune site (e.g., the organized lymphoid tissue of a lymph node). These mechanisms have been extensively investigated in different autoimmune diseases, both in humans and in animal models [71], and several excellent reviews are available on this matter for multiple sclerosis [72], Graves's hyperthyroidism [73], systemic lupus erythematosus [74], type I diabetes [75] and myasthenia gravis [76]. In these autoimmune conditions, epitope spreading has been correlated with the shift of reactivity to a single determinant to several different specificities, as a consequence of different possible triggers, mostly viral infections, and of other co-factors. However, epitope spreading can occur in the case of any antigen, including non-self antigens, whose processing can be influenced by protection or exposure to proteolysis by bound MHC class II [77, 78], immunoglobulin [79, 80] or proteins working as physiological receptor of a viral antigens [81]. In the case of the allergic response to pollen allergens, the close proximity of the different protein components within the same allergen source can support the possibility for inter-molecular help to occur [82].

In the generation of epitope spreading, B and T lymphocytes exhibit a concurrent activity. B cells bind to native antigens with membrane-bound immunoglobulin (B-cell receptors) and assist in the differentiation of plasma cells secreting the soluble form of that immunoglobulin, or derivatives with somatically generated higher affinity, by antigen specific T cells [83]. Data on cross-reactivity reported

here indicate that panallergens may have a crucial role in the spreading of the allergic response across conserved epitopes of different allergenic sources, including major allergenic proteins. In this scenario, an established (memory) T-cell response specific towards one panallergen could act as a driver [84] capable of rapidly expanding the T cell repertoire to allergenic proteins from a different allergen source. The latter could provide totally new allergenic molecules to this pre-activated micro-environment and trigger inter-molecular help sustaining the generation of IgE to major (non-panallergenic) proteins. The IgE-facilitation of allergen presentation *via* CD23 (the low affinity receptor for IgE) on the membrane of B cells may very well play a crucial role in this phenomenon [85, 86]. In short, the spreading of the allergic response can derive from the original T cell, help provided by cross-reacting panallergen specific T-cells to naive B lymphocytes specific to allergens of the new allergenic source.

B cell reactivity to panallergens, epidemiologically and quantitatively represents in most cases a secondary reactivity in allergic individuals [4, 6]. This may depend on the fact that in contrast to group 1 and 5 major allergens, panallergens do not have isoforms. Isoallergens are highly homologous protein variants which, by continuously repeating the same antigenic array, contribute to the generation of the prevalent pattern of IgE reactivity against each allergen source [87]. Moreover, a role in the relatively poor immunogenicity of panallergens may be derived from homology extended to their human counterparts, which make them subjected to a yet to be defined level of immune tolerance [24]. Despite this, in allergic individuals, panallergens could act as behind-the-scene drivers of inter-molecular epitope spreading, which could facilitate the generation of polysensitization, a condition by far more common than monosensitization. Obviously, patient specific factors and actual exposure to new allergens are compulsory co-factors in establishing this phenomenon.

CONCLUSION

In allergic subjects, a reciprocal modulation of the response to one sensitizing allergen can occur following natural exposure to, or immunotherapy with another allergen, through the intervention of a panallergen. This issue is relevant in the definition of the natural history and in the clinical management of most allergic patients, who suffer from multiple allergies.

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