Curr Allergy Asthma Rep (2015) 15:8
DOI 10.1007/s11882-014-0506-9

IMMUNOLOGIC/DIAGNOSTIC TESTS IN ALLERGY (M CHAPMAN AND A POMÉS, SECTION EDITORS)

Interfaces Between Allergen Structure and Diagnosis: Know Your Epitopes

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Abstract Allergy diagnosis is based on the patient's clinical history and can be strengthened by tests that confirm the origin of sensitization. In the past 25 years, these tests have evolved from the exclusive in vivo or in vitro use of allergen extracts, to complementary molecular-based diagnostics that rely on in vitro measurements of IgE reactivity to individual allergens. For this to occur, an increase in our understanding of the molecular structure of allergens, largely due to the development of technologies such as molecular cloning and expression of recombinant allergens, X-ray crystallography, or nuclear magnetic resonance (NMR), has been essential. New in vitro microarray or multiplex systems are now available to measure IgE against a selected panel of purified natural or recombinant allergens. The determination of the three-dimensional structure of allergens has facilitated detailed molecular studies, including the analysis of antigenic determinants for diagnostic purposes.

Keywords Allergy · Diagnosis · Allergen structure · Cross-reactivity · Linear and conformational epitopes

This article is part of the Topical Collection on *Immunologic/Diagnostic Tests in Allergy*

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Published online: 07 March 2015

Introduction: When Allergen Extracts Are Not Sufficient for Allergy Diagnosis

Allergy diagnosis begins with an analysis of the patient's clinical history and physical examination [1]. A confirmation of IgE reactivity to allergens is performed either in vivo by skin tests using allergen extracts or by provocation tests, which are the gold standard for allergy diagnosis, or in vitro by serological analysis. However, the variability in allergen composition and content of commercial allergen extracts can affect their in vivo allergenic activity [2, 3]. Food challenges, specifically double-blind placebo-controlled food challenges, represent the most reliable way to diagnose food allergies, but it cannot always be performed if patients are very sensitive to a certain food [4]. In vitro tests, using extracts or purified allergens, are advantageous for patients who do not have a normal skin, cannot discontinue interfering medications, are opposed to undergo skin test or have high sensitivity to allergens judging by clinical history, which indicates that anaphylaxis is possible [5]. Nevertheless, in vitro assays need to be always evaluated in the context of the patient's clinical history, because positive IgE reactivity in vitro, which is indicative of allergen sensitization, does not necessarily lead to clinical responsiveness.

Tests based exclusively on allergen extracts do not always reveal the source of IgE sensitization, especially when cross-reactive allergens are involved and patients may be sensitized to multiple sources of homologous allergens. In the last 20 years, molecular cloning, expression, and analysis of the molecular structure of allergens have allowed improving in vitro diagnosis by using panels of purified individual allergens instead of extracts. This approach, called molecular allergy diagnosis, relies on the availability of properly folded purified allergens [6]. The panels of allergens to be tested should be selected based on careful considerations of sensitizing allergens, patterns of sensitization, prevalence of IgE sensitivity, and cross-reactivities among homologous allergens

present in a specific area. For this, knowledge of the structural features shared by homologous allergens and of molecular structures of antigenic determinants is essential. In addition, the stability of the allergens used for molecular diagnosis may be improved by avoiding degradation by proteolytic enzymes, often present in extracts. Molecular diagnosis, used within the context of the patient's clinical history, is an effective approach to identify patient's IgE sensitization profiles, which can be highly heterogeneous or show geographical variability [7, 8••]. Molecular diagnosis has proven beneficial and able to improve allergy diagnosis based solely on allergen extracts [7, 8••, 9–11]. In this review, recent progress on defining molecular features of allergens that are relevant for allergy diagnosis will be evaluated.

Update of Allergen Nomenclature and Recently Identified Allergens

The identification of new allergens, name assignment, and assessment of their allergenic relevance is necessary for the selection of panels of allergens for molecular diagnosis. The Allergen Nomenclature Sub-committee from the World Health Organization and International Union of Immunological Societies (WHO/IUIS) maintains a systematic nomenclature of allergenic proteins and publishes the official database of approved allergen names (www.allergen.org). The Sub-committee recently revised the current nomenclature to reflect progress in identification, cloning, and sequencing of allergens, while increasing consistency in the classification of allergens. Names were updated for respiratory allergens from birch and ragweed pollen, midge larvae, and horse dander; food allergens from peanut, cow's milk, and tomato; and cereal grain allergens [12].

In the last years, new allergens were identified that may contribute to improved allergy diagnosis, including panels of inhaled allergens (i.e., olive pollen) and food allergens (i.e., kiwi) [13, 14]. New allergens originating from domestic animals, such as small mammals and rodents which have become popular pets in the USA and Europe, have been reported. These include Fel d 1-like allergens from dogs and rabbits [15, 16], a major dog allergen Can f 5 which is a prostatic kallikrein [17], and two guinea pig lipocalins, Cav p 2 and Cav p 3. The latter are major allergens, proven to be valuable for diagnosis of guinea pig allergy when combined with serum albumin Cav p 4 [18]. Several new allergens, classified in up to 33 groups, have been identified in mite. They include two major allergens: Der p 23, a peritrophin-like protein, and Der f 24, an ubiquinol-cytochrome *c* reductase-binding protein homolog [19•, 20]. Both allergens show high prevalence of IgE sensitization, comparable with the one reported for Der p 1 (93 %) and Der p 2 (77 %), currently used to diagnose mite-allergic patients [21]. The need to add new allergens for improving diagnosis of mite allergy will need to be evaluated, and may not be as critical as it is for other allergen sources, such as kiwi fruit and honeybee venom. The use of larger allergen panels can improve the diagnostic sensitivity in some cases and has revealed the importance of allergens underrepresented in commercial therapeutic extracts [8••, 22••].

Three-Dimensional Structures of Allergens

The WHO/IUIS official database of systematic allergen nomenclature (www.allergen.org) currently contains over 780 allergens. In the past 15 years, the three-dimensional structures of just over 100 allergens have been determined thanks to the development of X-ray crystallography and nuclear magnetic resonance technologies (Tables 1 and 2). The availability of recombinant allergens has also contributed to the determination of their three-dimensional structure when: (1) the natural allergens were not available in sufficient amounts required for crystallography, (2) natural polymorphisms led to a lack of molecular homogeneity required for crystallization, (3) degradation or proteolytic cleavage of the natural allergen occurred, or (4) the natural allergens underwent post-translational modifications that impaired crystallization (i.e., glycosylation). Recombinant allergens can be engineered for high-level expression of homogeneous whole molecules or stable structural fragments, with mutations that prevent undesired N-glycosylation. They are usually expressed in vitro in the prokaryotic system *Escherichia coli* or in eukaryotic systems. Examples include yeasts such as *Pichia pastoris* or, less commonly, tobacco plants or Chinese hamster ovary cells [23, 24]. Allergens used for in vitro molecular diagnosis need to be properly folded and meet high standards of quality. Usually, mass spectrometry is used to confirm the amino acid sequence, and spectroscopic and/or NMR analysis are used to confirm the secondary and/or tertiary structures, respectively [25–28]. A recent study applied high-throughput NMR technology to assess the molecular fold of food allergens utilized for diagnosis [29]. The structural conformation of an allergen preferentially recognized by IgE needs to be also taken into consideration for diagnostic purposes. Some allergens have regulatory functions resulting from major conformational changes upon calcium binding to EF-hand motifs. A recently determined solution structure of Phl p 7 showed three different conformations of the allergen [30]. Although most calcium-binding allergens have been described for

Table 1 Tertiary structures of inhaled allergens

Allergen	Species of origin	Function/structure	X-ray crystal structure											NMR structure		
Inhaled (indoor)																
Bla g 1	*Blattella germanica*	Gut protein that carries lipids	4JRB													
Bla g 2	*B. germanica*	Inactive aspartic protease	1YG9 (m)	2NR6 (m)[a]	3LIZ (m)[a]	4RLD (m)										
Bla g 4	*B. germanica*	Lipocalin	3EBK	4N7D												
Bla g 5	*B. germanica*	Glutathione S-transferase	5Q5R													
Blo t 5	*Blomia tropicalis*	Structural-three-helical bundle												2JMH	2JRK	2MEY
Blo t 8	*B. tropicalis*	Glutathione S-transferase	4Q5N													
Blo t 12	*B. tropicalis*	Chitin-binding protein												2MFK		
Blo t 19	*B. tropicalis*	Anti-microbial peptide homolog												2MFJ		
Blo t 21	*B. tropicalis*	Structural-three-helical bundle												2LM9		
Bos d 2	*Bos domesticus*	Lipocalin	1BJ7	4WFU	4WFV											
Can f 2	*Canis familiaris*	Lipocalin	3L4R													
Can f 4	*C. familiaris*	Lipocalin	4ODD													
Der f 1	*Dermatophagoides farinae*	Cysteine protease	3D6S	3RVV[a]												
Der f 2	*D. farinae*	Lipid binding protein	1XWV	2F08										1AHK	1AHM	1WRF
Der f 7	*D. farinae*	LPS-binding protein-like	3UV1													
Der f 13	*D. farinae*	Fatty acid binding protein												2A0A		
Der p 1	*D. pteronyssinus*	Cysteine protease	1XKG	2AS8 (m)	3F5V	3RVW[a]	3RVX[a]	4PP1[a]	4PP2[a]							
Der p 2	*D. pteronyssinus*	Lipid binding protein	1KTJ (m)											1A9V (m)		
Der p 5	*D. pteronyssinus*	Structural-three-helical bundle	3MQ1													
Der p 7	*D. pteronyssinus*	LPS-binding protein-like	3H4Z													
Der p 8	*D. pteronyssinus*	Glutathione S-transferase	4Q5Q													
Fel d 1	*Felis domesticus*	Uteroglobin	1PUO (m)	1ZKR (m)	2EJN (m)											
Mus m 1	*Mus musculus*	Lipocalin	1MUP	1JV4										1DF3		
Per a 4	*Periplaneta americana*	Lipocalin	3EBW													
Rat n 1	*Rattus norvegicus*	Lipocalin	2A2G	2A2U												
Inhaled (outdoor)																
Alt a 1	*Alternaria alternata*	Unknown	3V0R	4AUD												
Amb t 5	*Ambrosia trifida*	Unknown												1BBG	2BBG	3BBG
Ara t 8	*Arabidopsis thaliana*	Profilin	1A0K	3NUL												
Art v 1	*Artemisia vulgaris*	Defensin fold with polyproline domain												2KPY		
Asp f 1	*Aspergillus fumigatus*	Mitogillin	1AQZ													
Asp f 6	*A. fumigatus*	Manganese superoxide dimustase	1KKC													
Asp f 11	*A. fumigatus*	Cyclophilin: peptidyl-propyl isomerase	2C3B													
Asp o 21	*Aspergillus oryzae*	TAKA-amylase A	2TAA	6TAA	7TAA											
Bet v 1	*Betula verrucosa*	Pathogenesis-related protein (PR-10)	1BV1	1QMR (m)	1FM4	1LLT (m)	1FSK[a]	3K78	4QIP	4MNS	4BTZ	4BK7	4BKC	4BKD	1BTV	1B6F	

Table 1 (continued)

Allergen	Species of origin	Function/structure	X-ray crystal structure						NMR structure		
Bet v 2	*B. verrucosa*	Profilin	1CQA								
Bet v 4	*B. verrucosa*	Polcalcin (Calcium binding protein)							1H4B		
Che a 3	*Chenopodium album*	Polcalcin (Calcium binding protein)	2OPO								
Chi t 1	*Chironomus thummi thummi*	Hemoglobin	1ECO								
Cla h 8	*Cladosporium herbarum*	Mannitol dehydrogenase	3GDF	3GDG							
Equ c 1	*Equus caballus*	Lipocalin	1EW3								
Equ c 3	*E. caballus*	Serum albumin	3V08								
Heb v 2	*Hevea brasiliensis*	Beta-1,3-glucanase	4HPG	4IIS							
Hev b 6	*H. brasiliensis*	Hevein precursor	1Q9B	1WKX					1T0W	1HEV	
Hev b 8	*H. brasiliensis*	Latex profilin	1G5U								
Jun a 1	*Juniperus ashei*	Pectate lyase	1PXZ								
Ole e 6	*Olea europaea*	Unknown							1SS3		
Ole e 9	*O. europaea*	Beta-1,3-glucanase (C-terminus)							2JON		
Phl p 1	*Phleum pratense*	Expansin	1N10								
Phl p 2	*P. pratense*	Grass group II/III	1WHO	1WHP	2VXQ[a]				1BMW		
Phl p 3	*P. pratense*	Grass group II/III	3FT1	3FT9					2JNZ		
Phl p 4	*P. pratense*	Oxidoreductase	3TSJ	3TSH	4PVE	4PVJ	4PVK	4PWB	4PWC		
Phl p 5	*P. pratense*	Unknown-Phl p 5b	1L3P								
Phl p 6	*P. pratense*	Unknown	1NLX								
Phl p 7	*P. pratense*	Polcalcin (Ca binding protein)	1K9U						2LVK	2LVJ	2LVI
Zea m 1	*Zea mays*	Beta expansin	2HCZ								

The codes refer to the structure of molecules that are not necessarily the same polymorphism reported in the WHO/IUIS Allergen Nomenclature database

(m) modified or mutated molecule

[a] Allergen in complex with an antibody fragment

Table 2 Tertiary structures of food, venom, and contact allergens

Allergen	Species of origin	Function/structure	X-ray crystal structure								NMR structure	
Ingested (food)												
"Act c 1"	*Actinidia chinensis*	Cysteine protease (Act d 1-homolog)	2ACT	1AEC								
Act c 5	*A. chinensis*	Kiwellin	4PMK									
Act d 2	*A. deliciosa*	Thaumatin-like protein	4BCT									
Act d 11	*A. deliciosa*	Kirola	4IGV	4IHR	4IGW	4IGX	4IGY	4IH0	4IH2			
Ani s 5	*Anisakis simplex*	SXP/RAL-2 protein									2MAR	
Api g 1	*Apium graveolens*	Pathogenesis related protein (PR-10)	2BK0									
Ara h 1	*Arachis hypogaea*	Cupin (Vicillin-type, 7S globulin)	3S7E	3S7I	3SMH							
Ara h 2	*A. hypogaea*	Conglutin (2S albumin)	3OB4									
Ara h 3	*A. hypogaea*	Cupin (11S globulin, Glycinin)	3C3V									
Ara h 5	*A. hypogaea*	Profilin	4ESP									
Ara h 6	*A. hypogaea*	Conglutin (2S albumin)									1W2Q	
Ara h 8	*A. hypogaea*	Pathogenesis related protein (PR-10)	4MAP	4MA6	4M9W	4M9B						
Ber e 1	*Bertholletia excelsa*	2S sulfur-rich seed storage albumin									2LVF	
Bos d 4	*Bos domesticus*	Alpha-lactalbumin	1F6R	1F6S	2G4N	1HFZ	1HFX	1HFY				
Bos d 5	*B. domesticus*	Beta-lactoglobulin	1GX8	1GX9	1GXA	2AKQ	1BSO	1UZ2 (m)	2R56[a]			
Bos d 6	*B. domesticus*	Serum albumin	3V03	4F5S	4JK4							
Bra n 1	*Brassica napus*	2S seed storage albumin	1PNB									
Cyp c 1	*Cyprinus carpio*	Carp beta-parvalbumin	4CPV	5CPV								
Dau c 1	*Daucus carota*	Pathogenesis-related protein (PR-10)	2WQL									
Fra a 1	*Fragaria ananassa*	Pathogenesis-related protein (PR-10)									2LPX	
Gad m 1	*Gadus morhua*	Cod beta-parvalbumin									2MBX	
Gal d 2	*Gallus domesticus*	Ovalbumin	1JTI (m)	1OVA	1UHG (m)							
Gal d 3	*G. domesticus*	Ovotransferrin	1RYX	2D3I	1OVT	1AIV	1TFA	1IEJ	1N04			
Gal d 4	*G. domesticus*	Lysozyme[b]	1LYZ	1H6M (m)	1YQV[a]	1FDL[a]	1MLC[a]	3HFM[a]	2A2Y[a]	1DQJ[a]	1GXV	1GXX
Gly m 4	*Glycine max*	Pathogenesis-related protein (PR-10)									2K7H	
Gly m 5	*G. max*	Beta-conglycinin (vicilin, 7S globulin)	1IPJ	1IPK	1UIJ (m)							
Gly m 6	*G. max*	Glycinin (legumin 11S globulin)	1FXZ	1OD5	2D5H	2D5F						
Mal d 2	*Malus domestica*	Thaumatin-like protein	3ZS3									
Mus a 4	*Musa acuminata*	Thaumatin-like protein	1Z3Q									

Table 2 (continued)

Allergen	Species of origin	Function/structure	X-ray crystal structure										NMR structure	
Mus a 5	*M. acuminata*	Beta-1,3-glucanase	2CYG											
Pis s 1	*Pisum sativum*	Legumin 11S globulin	1PNB	3KSC										
Pru av 1	*Prunus avium*	Pathogenesis-related protein (PR-10)											1H2O (m)	1E09
Pru av 2	*P. avium*	Thaumatin-like protein	2AHN											
Pru du 6	*Prunus dulcis*	Amandin, 11S globulin l egumin-like protein	3FZ3											
Pru p 3	*Prunus persica*	Non-specific lipid transfer protein	2ALG	2B5S										
Ric c 1	*Ricinus communis*	2S albumin storage protein											1PSY	
Tri a 14	*Triticum aestivum*	Non-specific lipid transfer protein	1BWO										1CZ2	1GH1
Tria a 18	*T. aestivum*	Agglutinin isolectin-1	4AML	2X3T	2UVO	2CWG	1WGC	2WGC	7WGA	9WGA				
Vig r 6	*Vigna radiata*	Cytokinin-specific binding protein (CSBP), Bet v 1 family member	2FLH	3C0V										
Zea m 14	*Zea mays*	Non-specific lipid ransfer protein	1FK0	1FK1	1FK2	1FK3	1FK4	1FK5	1FK6	1FK7	1MZL	1MZM	1AFH	
Injected														
Aed a 2	*Aedes aegypti*	Salivary antigen	3DXL	3DY9	3DYE	3DZT								
Api m 1	*Apis mellifera*	Phospholipase A2	1POC											
Api m 2	*A. mellifera*	Hyaluronidase	1FCQ	1FCU	1FCV	2J88[a]								
Api m 4	*A. mellifera*	Melittin	2MLT										1BH1 (m)	
Arg r 1	*Argas reflexus*	Histamine-binding lipocalin	2X45	2X46										
Sol i 2	*Solenopsis invicta*	Transport of hydrophobic ligands	2YGU											
Sol i 3	*S. invicta*	Ves v 5-like	2VZN											
Ves v 2	*Vespula vulgaris*	Hyaluronidase	2ATM											
Ves v 5	*V. vulgaris*	Antigen 5	1QNX											
Through skin														
Mala s 1	*Malassezia sympodialis*	Beta-propeller fold	2P9W											
Mala s 6	*M. sympodialis*	Cyclophilin	2CFE											
Mala s 13	*M. sympodialis*	Thioredoxin	2J23											

The codes refer to the structure of molecules that are not necessarily the same polymorphism reported in the WHO/IUIS Allergen Nomenclature database.

(m) modified or mutated molecule

[a] Allergen in complex with an antibody fragment

[b] Structures selected from multiple PDB entries (>540 for hen egg lysozyme)

pollens, they are also present in animals such as cockroach [31]. IgE antibody binding to the calcium-bound allergen is usually higher, suggesting that sensitization occurs preferably against that allergen form [31]. The allergen in the conformation that best binds IgE should be selected for diagnosis.

What We Learnt from Allergen Structures that Contributes to Diagnosis

Allergen Structure and Standardization

Currently, the potency of allergen extracts is determined as total allergenic activity, regardless of the allergen content, and is measured in units that differ among manufacturers. Allergen extracts contain a mix of allergenic and non-allergenic molecules and often include proteases that may reduce potency over time. Since 2001, the CREATE project funded by the European Union developed certified international standards with verifiable allergen content expressed in mass units [32, 33]. Nine recombinant major allergens from birch, timothy grass, olive pollen, and dust mite were measured by amino acid analysis, and their IgE reactivity was assessed by direct RAST, RAST inhibition, immunoblotting, and basophil histamine release. The recombinant allergens rBet v 1, rPhl p 5a, and rDer p 2 were found to be equivalent to the natural molecules in terms of structure and IgE antibody reactivity. As a follow-up project, rBet v 1 and Phl p 5a were selected and produced under GMP conditions for their establishment as European Pharmacopoeia (Ph. Eur.) Reference Standards through a project run by the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines and HealthCare (EDQM) [34]. These standardization programs are made possible thanks to the availability of properly folded allergens whose molecular structure was tested by SDS-PAGE, mass spectrometry, circular dichroism spectroscopy, and small-angle X-ray scattering. Similar efforts will facilitate standardization of allergen extracts for diagnosis and immunotherapy, by using homogeneous preparations of properly folded allergen (natural or recombinant) with IgE reactivity comparable with the native counterpart. Recently, a single multi-allergen standard was prepared with eight natural allergens following the CREATE principles, for assessment of allergen exposure [35].

Such homogeneous solutions are easily obtained when molecules have a relatively stable structure. However, allergen degradation may occur due to the presence of proteolytic enzymes in extracts or the labile nature of certain allergenic molecules. The cockroach allergen Bla g 1 illustrates an example of a molecule that is difficult to standardize, because it naturally breaks down into fragments and has always been measured in arbitrary units. This allergen is formed by multiple consecutive amino acid repeats resulting from gene duplication of a ~100 amino acid domain [36]. The basic structural unit of Bla g 1 was recently determined and facilitated standardization of assays in absolute units [37••]. This study showed that the structural integrity of either the whole allergen or a fragment is important for standardization. Finally, diagnostic products based on purified allergens should facilitate standardization and increase batch-to-batch consistency [38].

Allergen Stability and Association with Disease

Allergenicity of food proteins has largely been associated with their lability, despite a lack of absolute correlation between digestibility measured in vitro and protein allergenicity [39]. Patients with oral allergy syndrome have IgE reactive to pepsin-sensitive allergens, whereas IgE from patients suffering systemic reactions recognize pepsin-resistant allergens. A study confirmed a difference in the lability of kiwi fruit allergens recognized by both kinds of patients [40]. A reduction in acidity reduced pepsin digestion and presumably increased the sensitizing capacity of the food. Interestingly, kiwi digestion resulted in the creation of new epitopes, either by aggregation, dissociation, or unmasking of allergenic protein digest products that were recognized by patients showing systemic reactions. The lability of some food allergens (i.e., Bet v 1-homologs from the Rosaceae family) is also the main reason why most commercial food extracts for SPTs (particularly those of the Rosaceae) are not reliable, and SPTs with fresh fruits and vegetables remain the best way to diagnose food allergy in vivo in these patients [41]. In contrast, lipid transfer proteins (LTP) are highly resistant to both heat treatment and proteolytic digestion. The stability of LTP has been associated with the induction of severe systemic reactions [42, 43]. These characteristics were attributed to the LTP three-dimensional structure composed of four α-helices, held by four disulfide bridges, with a large internal hydrophobic cavity that can harbor lipids. Similarly, digestion-resistant fragments of 2S albumins from cashew, Ana o 3, and peanut Ara h 6, retained IgE-binding epitopes, and disruption of disulfide bonds eliminated their IgE-binding capacity [44, 45•]. These studies highlight the protective effect of the three-dimensional structure of the allergen against digestion. Given the complexity of the human gastrointestinal tract, the use of physiologically relevant in vitro systems to evaluate digestibility of allergens has been evaluated [46]. These would involve not only the use of pepsin but also the simulation of the stomach and small intestine environment with addition of surfactants (i.e., phospholipids) and bile salts, considering the effect of food matrices on allergen digestion. In general, differences in molecular stability, which is determined by the three-dimensional structure of the allergen, can influence the variability in allergen composition of extracts used for diagnosis and immunotherapy. Also,

conventional extracts may be deficient in significant IgE-binding components, due to differences in protein extractability [47]. These are factors to be considered for the production of diagnostic products.

Non-protein Molecules Involved in the Induction of Allergic Responses or IgE Recognition: Implications for Diagnosis

Carbohydrates N-glycans from plant and insect glycoproteins are common in allergens, and their IgE recognition in in vitro diagnostic tests may cause false-positive results. These sugars are known as cross-reactive carbohydrate determinants (CCD) and display a wide variety of structures that range from oligomannosidic type to complex Le(a)-carrying glycans [48, 49]. The most important CCD molecules are α1,3-fucose in insect glycoproteins or this fucose plus β1,2-xylose in plant glycoproteins [50, 51]. Two types of O-glycans have been identified in Art v1, and one of them, a mono-β-arabinosylated hydroxyproline, was found to constitute a new, potentially cross-reactive, carbohydrate determinant in plant proteins [52].

The clinical relevance of carbohydrates as antigenic determinants has been a source of controversy for a long time. With some exceptions, cross-reactive carbohydrate determinants are mostly considered not to be clinically relevant. One third of the CCD-positive sera from patients with tomato allergy were reported to have biologically relevant CCD-specific IgE antibodies [53]. In this case, the use of natural allergens versus recombinant allergens expressed in prokaryotic systems would be preferred for diagnostic purposes. Conversely, the use of recombinant allergens expressed in *E. coli*, which are not glycosylated, would be preferred to allergens expressed in *P. pastoris* which may contain O- and N-linked glycans that decrease the specificity of diagnostic tests [54]. Another option would be to express the allergen in *P. pastoris* with substitutions of specific sites involved in glycosylation. Recently, the use of a semi-synthetic CCD blocker has been suggested to inhibit IgE binding to CCD and enhance diagnostic selectivity [55•].

Lipids and Other Small Ligands An increasing number of allergens are known to bind lipids or small ligands. The identity of some allergen ligands was revealed in part thanks to determination of the three-dimensional structure of the allergen [37••]. The lipocalin allergen Bla g 4 binds tyramine and octopamine in solution, as shown by NMR and isothermal titration calorimetry [56]. Ara h 8 is a Bet v 1-like allergen that binds the isoflavones quercetin and apigenin, as well as resveratrol [57]. Some studies suggest that lipidic ligands from allergens could possess immunomodulatory properties. Der p 2 was reported to mimic the function of MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signaling complex, involved in activation of the innate immune system. This function was identified as a consequence of determining the immunoglobulin-like fold of Der p 2 [58]. Similarly, Fel d 1, Can f 6, and Par j 1 were proposed to bind LPS and had immunomodulatory activity [59, 60]. Bla g 1 is formed by capsules with an internal cavity that contains lipids, such as palmitic, oleic, and stearic acids. Lipidic ligands could also increase molecular stability, a desired quality for allergens to be used for diagnosis. The addition of phosphatidylcholine (which is a natural ligand of LTPs) to grape LTP contributed to the resistance of the protein to digestion [43]. The possible influence of allergen-associated lipids in diagnosis has not been investigated, as it has for carbohydrates.

Diagnosis and Molecular Determinants of Cross-Reactivity

The identification of cross-reactive allergens from different sources has improved the capacity to correctly diagnose and understand allergic reactions. The diagnostic procedure and therapeutic regimen can be simplified by selecting representative molecules out of clusters of cross-reactive allergens [61]. Species-specific allergens can be added to the panel for molecular diagnosis to contribute to the identification of the source of sensitization.

Clinical cross-reactivity indicates sensitization to cross-reactive allergens (whereas the opposite is not always true). Therefore, an understanding of the molecular determinants of cross-reactivity is important for diagnostic purposes. In general, for cross-reactivity to occur, a high degree of amino acid identity throughout the entire protein is required (>70 %), whereas cross-reactivity is rare below 50 % identity [62]. The main determinant of cross-reactivity is the presence of identical amino acids at the molecular surface accessible to the antibodies.

Recently, unusual cases of cross-reactivity or lack thereof have been described that question the criterion to assess the allergenic risk of novel proteins according to the WHO/FAO/EFSA/Codex [63]. This criterion is a value of 35 % or more amino acid identity over a sliding window of 80 amino acid residues. Despite a high sequence identity of 91 % between bovine and caprine β-caseins, there are cow's milk-tolerant patients that recognize the caprine allergen, without cross-reacting with bovine β-casein [64]. Conversely, the kiwi allergen Act d 11 showed cross-reactivity with Bet v 1-like allergens, despite sharing low sequence identity (under 21 %) [65]. Recently, an unexpected IgE cross-reactivity, attributed to similar surface-exposed peptides, was found between the major peanut allergen Ara h 2 and the

nonhomologous allergens Ara h 1 and Ara h 3, despite structural and sequence differences [66••]. This study is an exception to the general rule that cross-reactivity mainly occurs among homologous proteins that share similar structural features. Overall, these results demonstrate that the presence of conserved residues that define IgE antibody recognition of similar epitopes is responsible for IgE cross-reactivity. Therefore, criteria for prediction of allergenicity based on amino acid sequence homology are useful as guidelines, especially when dealing with new proteins of unknown structure, but they are not an accurate way to predict the presence of IgE antibody-binding epitopes and cross-reactivity.

Antigenic Structure for Diagnosis of Allergy and Design of Immunotherapy

Linear Epitopes: Markers of Severity or Persistence in Foods

Most studies for mapping IgE antibody-binding epitopes in allergens focus on the identification of linear epitopes. Synthetic peptides derived from the allergen amino acid sequence or allergen fragments are tested for IgE antibody binding in immunoblot or microarray assays, to identify linear IgE antibody-binding epitopes. This experimental approach has revealed interesting associations between IgE recognition patterns of linear epitopes and allergic disease, specifically for foods, including milk, peanut, egg, and lentil. Initial studies reported that the IgE recognition of at least one of three epitopes in caseins identified patients with persistence to milk allergy [67, 68]. The development of microarray technology allowed further exploration of the significance of linear epitopes in food allergens. Different IgE recognition patterns of sequential epitopes of four caseins and β-lactoglobulin were observed between reactive and tolerant milk-allergic patients [69]. Severity of cow's milk allergy was associated with a greater IgE epitope diversity (i.e., number of epitopes recognized) and higher IgE antibody affinity [70]. Regarding peanut, highly heterogeneous patient's IgE profiles to epitopes from Ara h 1, Ara h 2, and Ara h 3 were found, with no dominant epitopes in Ara h 2 [71, 72]. The clinical sensitivity to peanut allergens, determined by double-blind, placebo-controlled peanut challenge, was positively related to the IgE (but not IgG4) epitope diversity. The epitope-recognition patterns remained stable over time, although no specific epitopes were associated with severe reactions to peanut [73]. Another study found a significantly greater IgE binding and broader epitope diversity in peanut-allergic patients compared with peanut-tolerant individuals, with no significant difference in IgG4 binding between groups [74••]. Regarding egg allergy, four linear epitopes from ovomucoid were identified as markers of persistent egg allergy [75]. A positive correlation between epitope diversity in the lentil allergen Len c 1, lentil-specific IgE levels, and respiratory symptoms was found [76]. These studies provided a detailed analysis of the IgE reactivity profiles of allergic patients to peptides (therefore linear epitopes) using microarray technology. However, the usefulness of this approach for clinical practice needs to be further evaluated [77].

Conformational Epitopes for Diagnosis and Immunotherapy

Most epitopes in inhalant allergens are conformational, since these proteins do not undergo digestion and transformation processes typical of foods [78]. Increasing evidence shows that, in addition to linear epitopes, conformational epitopes are also important in food allergens if sensitization occurs to intact or partially digested allergens. Jarvinen et al. showed that persistent hen's egg allergy was associated with IgE recognition of not only linear but also conformational epitopes in ovomucoid and ovalbumin [75]. The IgE reactivity and allergenic potential of Pru p 3 was shown by using a reduced and alkylated form of recombinant Pru p 3 to depend mainly on conformational epitopes [79]. Another study proved that peptides identified as major linear epitopes on Pen a 1 and Ara h 2 had no relevant capacity to inhibit the IgE binding to the native allergen. Overall, these results reveal that conformational epitopes resulting from the three-dimensional structures of allergens need also to be considered to evaluate IgE responses to food allergens [80].

Conformational epitopes are formed by amino acids that are brought close in space upon protein folding but are not necessarily contiguous in the sequence of the allergen. Most published evidence about the existence of conformational epitopes in allergens is indirect, derived from reduction of IgE antibody binding to modified allergens. These can be obtained by reduction of disulfide bonds that hold their three-dimensional structure, fragmentation or expression of recombinant fragments, or mutagenesis or alteration of allergen structure by calcium depletion [31, 44, 45•, 81]. The identification of the exact location of conformational epitopes has only been possible with the development of technologies that elucidate the three-dimensional structure of the proteins. Lysozyme was the first allergen to have antigenic determinants mapped by X-ray crystallography in the 1980s [82]. Since then, the structures of 11 allergen-antibody complexes have been determined. Two were complexes of Fab fragments of IgG antibodies with the birch pollen allergen Bet v 1 and the bee venom allergen Api m 2 (hyaluronidase) [83, 84]. The

epitope in Bet v 1 was proven to be important for IgE antibody binding and for IgE cross-reactivity among homolog allergens [85]. Two complexes were formed using IgE antibody Fab obtained from combinatorial libraries from allergic patients. The allergens involved were β-lactoglobulin from bovine milk and timothy grass pollen allergen Phl p 2 [86, 87]. These studies were especially interesting given the fact that IgE is polyclonal, and native IgE is unavailable as a monoclonal antibody in large amounts required for X-ray crystallography. Thus, recombinant IgE represents the closest molecule representing a native antibody.

In recent years, an extensive analysis of antigenic determinants in cockroach and mite allergens has been performed following the determination of the crystal structures of antibody fragments in complex with Bla g 2, Der p 1, and Der f 1 (Table 1) [88, 89••, 90••]. A mechanism of antibody recognition involving protein plus a carbohydrate was found for Bla g 2 co-crystallized with a monoclonal antibody [89••] (Fig. 1, top). The epitopes recognized by the IgG mAb were also involved in IgE antibody binding to Bla g 2 and group 1 mite allergens, as proven by detailed antibody binding analysis of allergen epitope mutants [90••, 91]. Structural analysis of these epitopes combined with site-directed mutagenesis and antibody-binding analysis revealed determinants of specificity and cross-reactivity for Der p 1 and Der f 1 (Fig. 1, bottom).

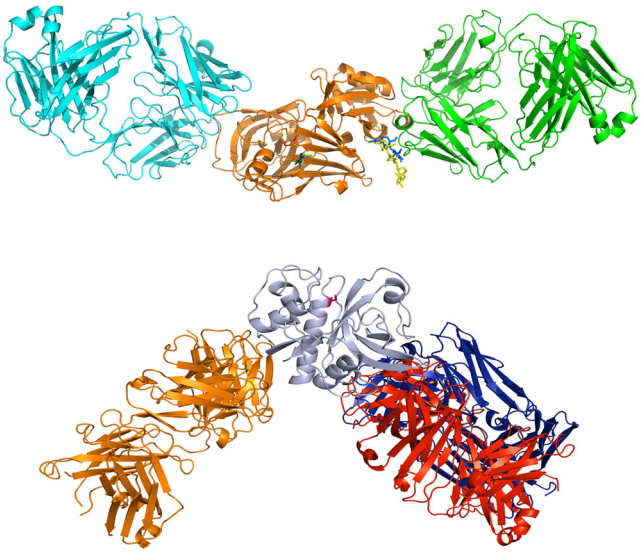

Fig. 1 Composite diagram showing binding of monoclonal antibody (mAb) Fab/Fab′ fragments to Bla g 2 (*top*) and Der p 1 (*bottom*). *Top*, binding of mAb 7C11 (PDB ID 2NR6; *cyan*) and mAb 4C3 (PDB ID 3LIZ; *green*). Carbohydrate residues bound to Asn268 of free Bla g 2 are *yellow* and repositioned in the complex with mAb 4C3 are *blue*. These sugars are involved in the antibody interactions with Bla g 2 (*orange*), extending the mAb 4C3 epitope. A carbohydrate moiety bound to Asn317 is located far from the epitope. *Bottom*, binding of Der p 1 (*gray*) with Fab fragments of mAb 4C1 (PDB ID 3RVW; *blue*), 10B9 (PDB ID 4PP2; *red*), and 5H8 (PDB ID 4PP1; *orange*). Epitopes recognized by the mAb 4C1 (cross-reactive with Der f 1), and the Der p 1-specific mAb 10B9 partially overlap. Catalytic Cys34 is shown in *pink*. Figure was prepared with Pymol (www.pymol.org)

Recently, the structures of isolated antibody constructs have also been solved. These include an anti-Bla g 1 IgG scFv, an anti-Bet v 1 IgE scFv and three anti-Der p 1 IgG Fab (mAb 4C1: 3RVT, 3RVU; mAb 10B9: 4POZ) [90••, 92, 93]. The use of antibodies specific for Blag 1 and Bet v 1 in mutant or peptide-binding experiments, respectively, led to the molecular location of species-specific epitopes. The identification of conformational epitopes involved in IgE antibody binding contributes to our understanding of antigenic relationships among molecules. This information is useful for diagnostic purposes, especially if dominant cross-reactive epitopes are found among homologous allergens from different sources, as described for Bet v 1 and Der p 1 [85, 90••]. Most interestingly, the allergen structure and/or residues involved in IgE antibody binding can be specifically modified to produce hypoallergens for future use in immunotherapy. These low-IgE-binding molecules are expected to reduce side-effects due to increasing allergen doses administered during immunotherapy.

Conclusions

Correctly folded molecules are needed for diagnostic purposes, given the importance of the allergen fold as a determinant of allergenicity. The three-dimensional structure of allergens provides relevant information for diagnosis by facilitating structural and functional classification of allergens, allergen standardization, evaluation of the molecular stability of allergens for diagnostic products, and the analysis of proteinaceous and non-proteinaceous molecules that may influence diagnosis. In recent years, detailed analyses of the antigenic structure of allergens have led to the identification of common structural features and molecular determinants of specificity and cross-reactivity, including linear and conformational antibody binding epitopes relevant for allergic disease. Overall, this information is the basis for the design of molecules for diagnosis and immunotherapy.

Acknowledgments Research reported in this publication was supported in part by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI077653, and in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Compliance with Ethics Guidelines

Conflict of Interest Anna Pomés reports grants from NIH/NIAID and Maksymilian Chruszcz, Alla Gustchina, and Alexander Wlodawer declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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