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**Mass Spectrometry to complement standardization of house dust mite and other complex
allergenic extracts**

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“The contributions of Jelena Spiric, Ronald L. Rabin, and Andreas Reuter *are an informal communication and represent our best judgment. These comments do not bind or obligate FDA and PEI, respectively.*”

Abstract

In the United States, the Center for Biologics Evaluation and Research (CBER) of the US Food and Drug Administration regulates biologics used for diagnosis and treatment of allergic diseases. The Code of Federal Regulations 21CFR680.3(e) states that when measured, the potency of an allergenic extract is assessed according to its allergenic activity. As of 2016, 19 allergenic extracts are standardized for potency in the United States. While these standardized extracts constitute a minority of those available, they treat the most prevalent allergies (e.g. grass and ragweed pollens, dust mites and cat) and those that induce life-threatening anaphylaxis (e.g. Hymenoptera venom). Standardization for potency enhances safety and efficacy of immunotherapy by minimizing the risks of variations in allergen dosing when switching from one lot of manufactured extract to another, and by providing an objective measure of stability of each lot of allergenic extract over time. Allergenic extracts that have multiple immunodominant allergenic proteins are standardized with little or no information about compositional differences among extracts. Here we propose application of mass spectrometry towards measurement of compositional differences among extracts that may affect the efficacy and safety of allergen immunotherapy. In addition, we discuss of house dust mite allergen extracts as a prototypical complex extract that may be standardized by mass spectrometry.

House Dust Mites and HDM allergenic extract

Allergic diseases such as allergic rhinitis, atopic dermatitis, and asthma have a major impact on quality of life and healthcare costs. Since the 1970s, the incidence of allergic diseases has been increasing; allergic rhinitis affects between 30% and 15% of the population, while 300 million

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individuals worldwide have asthma (1). House dust mites (HDM) are often the first environmental allergens to elicit hypersensitivity in susceptible individuals, and may initiate the “allergic march” towards multiple allergies and allergic asthma. Currently, up to 85% of asthmatics from highly populated areas of Asia, Australia, Europe, North and South America are allergic to HDM (2-4). HDM normally feed on human skin scales and other organic debris, and are ubiquitous, as they can be found in homes (upholstery of couches and chairs, pillowcases, mattresses, carpets, and clothing), schools and daycare centers, work places, on public transportation, and agricultural environments including stored grains (5). HDM allergens are compartmentalized mostly to the mite bodies (live and dead) or fecal pellets that accumulate in house dust (6, 7), although they may be found in salivary secretions, cuticle debris, molting enzymes, and cells that are left behind as mites molt from one life stage to the next. *Dermatophyoides pteronyssinus* and *Dermatophyoides farinae* are the predominant HDM species Europe and North America (6, 8). While these two HDM species cohabitate all geographical regions, one of the two species may be dominant. For example, *D. pteronyssinus* is most common in Australia and the UK (4, 9), but *D. farinae* is most common in Thailand (10).

Allergenic extracts are unselective aqueous extractions produced from natural source materials that contain various amounts of major and minor allergens, isoallergens, as well as other non-allergenic compounds. Diagnosis and treatment of allergy depend entirely on the quality of allergen preparations being used, and manufacturers are challenged to produce a high quality allergenic extracts with consistent composition that are intended for diagnosis and treatment of allergy. HDM allergenic extracts are produced from cultured HDM, which vary in allergen composition due to variations in methods of culture and harvest. For example, extract composition will be affected by whether the extracts are derived from mite bodies (predominant source of Groups 2, 10, 11, 14 and 18) or whole mite cultures that include fecal pellets (predominant source of Groups 1, 5, 7, 21 and 23) (11, 12). The European Medical Agency (13) recommends consistency among consecutive batches of the source material that will enhance consistency in the final product and historically, the

US Food and Drug Administration has required the source material to consist of mite bodies in order to limit exposure to ingredients in the growth media. It should not be surprising, therefore, that HDM allergen extracts produced in different countries vary in allergen composition and concentration (14-16). For example, Casset et al. demonstrated that that levels of Der p 1 and Der p 2 vary among extracts, and that some HDM extracts lack allergens from groups 5, 7, 10, and 21. In addition, because proteases may degrade allergens at any point during manufacture, variation in methods of extraction and storage may also qualitatively affect the final product (17, 18).

Standardization of allergenic extracts

Classically, standardization for potency has been performed with quantitative *in-vivo* assays performed on allergic subjects such as ID₅₀EAL intradermal skin testing in the United States (19), or the “Nordic system” of percutaneous testing in Europe (20). While *in-vivo* tests may best reflect the true allergenic potency of an extract, they are obviously not practical for testing the potency of each lot of extract. For this purpose, surrogate measurements of potency that vary according to characteristics of the allergens have been developed and validated. For example, cat and ragweed pollen extracts are standardized according to the concentration of the proteins that are most reactive with IgE—Fel d 1 and Amb a 1, respectively. Alternatively, potencies of honeybee and other hymenoptera venoms are reported according to activities of hyaluronidase and phospholipase A2. Because of their relative complexity, potencies of grass pollen and HDM extracts are not represented by the concentration of one or two proteins, but are measured with a competitive enzyme-linked immunosorbent assay (cELISA) in which allergenic proteins from a lot of manufactured extract compete with a reference lot for binding to specific IgE present in sera pooled from 10-15 highly allergic donors.

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The cELISA provides a useful index of overall allergenic potency of these complex extracts, but its value is limited because does not detect compositional differences among extracts. Since patients vary in their sensitivity (i.e. IgE reactivity) to different HDM allergenic proteins, compositional differences among the extracts may determine which extracts may be effective for particular subsets of allergic patients (Figure 1). An additional limitation of the cELISA is that its reproducibility may be adversely affected by potential instability of human sera over time, and variations in pooled sera that are collected to replace depleted or expired lots. Because of these limitations, regulatory scientists are exploring alternatives for measurement of potency of complex allergen extracts that are quantitative and less subject to qualitative or quantitative drift.

Alternatively, complex natural allergenic extracts may be standardized with multiplex immunoassays, such as that recently developed for German cockroach (21). As has been described for Der p 2 (22) and other HDM group 2 allergens (23), mAbs to detect specific allergens may not detect uncommon or poorly described isoforms. For example, the CREATE research consortium reported a collaborative study that commenced in 2001, in which a mAb did not detect the isoform Phl p 5b in Timothy grass pollen extracts, and isoforms of Der p 2 and Bet v 1 were not detected in HDM and birch pollen extracts, respectively (24). In addition, they reported on a set of reference mAb that were five-fold less sensitive to the reference allergen Bet v 1.0101 than to Bet v 1 in natural extracts. At the Paul-Ehrlich Institute, we demonstrated that natural birch pollen extract includes a mixture of isoforms, at least 18 different sequence variants of Bet v 1, including an isoallergen that had not been previously been detected at the protein level (25). Having identified these issues, the ELISA assays for Bet v 1 were fully characterized in 2012 (26) and validated in 2016 (27). In addition, an ELISA for standardization requires reference standards, which was demonstrated for Phl p 5.0109 (28) that must be comprehensively characterized to assess identity, quantity, homogeneity, folding, thermal degradation, aggregation and biological activity, and stability. Clearly, as proposed by Briza and Chapman, allergen standardization would benefit by the application of new technologies such as mass spectrometry (29).

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While compositionally defining complex allergenic extracts by mass spectrometry is initially expensive and technically sophisticated, it is ultimately cost effective compared to repetitive development of biological tests and reference materials. It is well known that the outcome of potency tests, skin prick tests, or oral provocations strongly depends on the quality of the protein extracts and on the content of individual allergens (30). For example, Ballmer Weber and co-workers demonstrated that an isoform of the carrot allergen Dau c 1.0202 is more predictive of clinically relevant sensitization than Dau c 1.0104 (31), and that combining rDau c 1.0104 with rDauc 1.0201 increases diagnostic sensitivity (32). It is also known that extracts may vary among manufacturers. For example, Nolte et al. reported significant variations in the ratio of Der p 1 and Der p 2 among HDM extracts that are not detected by the competitive ELISA used to define potency in the US (33). Similarly, Benitez and colleagues reported large variations in ratios of Der f 1 & 2 and Der p1 & 2 among five commercial products used for SLIT in Europe (34). While the critical step in determining allergen content is usually protein extraction (35), methods used to culture and harvest the mites may also affect allergen content. Since there are few studies that compare clinical efficacy with variations in a single allergen (and none with complex extracts), the impact of these variations on the diagnosis and treatment of allergic diseases is unknown. Thus, using methods such as those reported by Spiric et al. to quantify the allergen profile of birch pollen extract offers the unique opportunity to reproducibly link comprehensive allergen profiles to diagnostic or clinical performance of an allergenic extract (submitted; (36)).

As stated above, HDM extracts may vary according to relative concentrations of the allergenic proteins and their variants depending on source materials or method of preparation, and HDM allergic patients are heterogeneously reactive to the large number of HDM allergenic proteins. Consequently, it is possible that despite identical overall potencies, different patients may respond differently to two nominally identical products (Figure 1). Therefore, quantitation of all allergenic proteins and characterization of their variants may reveal important differences among the preparations that can inform patient care and benefit public health. Given the large number of

allergenic proteins and sequence variants in HDM extracts (6, 8), assessing the concentration of all allergens by immunoassay may not be feasible. Rather than individual immunoassays, we propose that using mass spectrometry (MS) to compositionally defining HDM allergenic extracts will complement overall potency measurements by cELISA or measurements of dominant allergens by standard sandwich ELISA (sELISA). In addition, while we have focused on HDM extracts in this report, we propose that MS may serve as a benchmark for compositionally defining any complex allergenic extract for which there is no single dominant allergen, and in which there may be variants or isoforms of allergenic proteins.

Mass spectrometry for quality control and standardization of allergenic extracts

MS has been used as a tool to identify novel allergens or to prove identity and enhance the quality control of recombinant allergens for over twenty years. For example, the structural integrity of Bet v 1, the dominant allergen of birch pollen, was confirmed by high resolution MS in 2007 (37), and detailed characterizations of GMP-produced recombinant Bet v 1 and Phl p 5 (a major allergen of Timothy grass pollen), were published in 2009 and 2016, respectively (28, 38). The first studies describing the potential of MS as a tool for standardization of allergenic extracts were published in 2009, when Carnes et al. used high resolution MS to confirm the presence of allergens in depigmented allergoids of *Betula alba* (39), and Fenaille et al. (40) measured the variability of the concentration of species-specific peptides in HDM extracts. . Both studies were valuable for regulatory purposes because they described methodologies to record allergen and isoallergen patterns in the context of natural extracts. However, neither study included quantitative information of individual allergen content, which is necessary for a complete description of these complex biologics (41, 42).

In addition to identifying proteins, MS may accurately quantify selected proteins, including those that are present in complex samples such as plasma (43). This approach utilizes a MS technique known as multiple-reaction-monitoring (MRM) that is often performed on a triple-quadrupole (QqQ)

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or triple-quadrupole ion trap mass spectrometer, because they have a short duty cycle and a wide linear quantification range (44). Absolute quantification of proteins using MRM is accomplished by adding known concentrations of synthetic isotope-labeled peptides to the protein digest of the extract. The concentration of the natural peptide is then determined by comparing the peak areas between the isotope-labeled and natural peptides that have the same amino acid sequence but different masses. Comparison of the peak areas from the natural peptide to its isotope-labeled standard yields a ratio, which is used to calculate the concentration of the natural peptide. The concentration of the protein is then extrapolated from the concentrations of one or more peptides (Figure 2).

MRM-MS was first applied towards quantitative measurement of peptides in 1991 to quantify natural peptides from human pituitary extracts (45). In 2002, Helsper et al. demonstrated that quadrupole time-of-flight (qTOF) MS can be used for semi-quantitative measurement of natural isoforms of Mal d 1 from Granny Smith apples (46). In 2011, Seppälä and colleagues reported the use of High Resolution Accurate Mass Spectrometry (HRAM) to qualitatively and quantitatively characterize Phl p 1 and Phl p 5 in Timothy grass pollen extracts (47). These investigators were the first to combine qualitative and quantitative information on allergens from complex samples, and the first to use synthetic, stable isotope-labelled peptides as calibrants. Synthesis of isotope-labeled peptides is a well-established method and does not require extensive characterization as compared to biological reference preparations such as recombinant allergens. To further support and emphasize the use of synthetic peptides as calibrants, we recently published the first label-free MS method that facilitates parallel identification and quantification of all IUIS approved allergens of one allergen source, including information on the non-allergenic part of the protein profile (submitted; (36)). Our method uses a single synthetic calibrant to reliably identify and quantify Bet v 1, 2, 4, 6 and 7 and provides unambiguous data on isoforms and variants of Bet v 1.

MRM assays overcome major limitations of antibody-based assays because they can simultaneously measure the concentration of multiple proteins in mass units without the need to create complex reagents for each, and specifically measure isoallergens and variants of an allergenic protein. This ability to measure variants may be critical because allergenic extracts may vary in their content of clinically relevant allergens, isoallergens, or variants and may therefore be incomplete for accurate diagnosis or effective therapy of allergic disease (14, 48). While MRM assays provide absolute quantities of peptides, their main limitation is that protein concentration is inferred from peptide concentration. Therefore, MRM assay cannot detect loss in potency due to denatured proteins or partial digestion that leaves the allergenic peptides intact. By contrast, a sELISA provides relative concentrations of an allergen that depend on the reference extract or the individual recombinant protein that is used as a calibrant, and a cELISA provides the sum of the antigenicity or biological activity rather than concentration of any one allergen. Therefore, the outputs of ELISA and MRM assays are complementary and together provide a more complete characterization of an extract than either alone. MRM assays measures mass units of allergens and their variants, while antibody-based assays demonstrate biological activity or antigenicity.

In conclusion, MRM assay offers an opportunity to apply 21st century regulatory science to define and characterize allergenic extracts. The MRM targeted approach that monitors multiple peptide transitions in parallel offers the high specificity and throughput to measure the allergen content, which is among the most important parameters in quality control of allergenic extracts. Critically, MRM quantifies multiple allergens isoallergens and variants in the same analysis in a sequence specific manner, and compared with developing new antibodies or antisera, developing new reagents for MRM is reasonably straightforward. Rather than using MRM assay to develop specifications for lot release, we suggest that MRM assay may complement overall potency measurements or quantitation of allergenic proteins by ELISA and to inform practitioners of compositional differences among HDM products, and of variations in composition among lots from each manufacturer. In addition, MRM assay may also facilitate a personalized approach towards

HDM immunotherapy, in which the compositional profile of HDM extracts may be matched to the individual patient's reactivity. Finally, we propose that establishing this assay for house dust mite (HDM) extracts will provide a methodological template towards compositionally defining other complex allergenic extracts and revision of labeling of standardized extracts.

Appendix: Allergen repertoire and biochemical characterization of HDM

HDM allergens are extremely diverse and numerous studies over the last several decades have been conducted to understand the biological, chemical, and structural properties of HDM allergens. Currently, 84 distinct allergens, excluding sequence variants, from suborder Astigmata that have been accepted by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (49). Of these, 50 allergens are from *Dermatophagoides* species. Allergens from different *Dermatophagoides* species are segregated into groups, each of which is structurally homologous, with similar physiochemical properties, amino acid sequences, and molecular weights. Conversely, each allergen group is structurally and sequentially distinct from the other groups. HDM allergens are named according to the systematic nomenclature developed by the IUIS Allergen Nomenclature Sub-Committee, which uses the first three letters for the genus of the allergen source, followed by single letter for the species, and then by an Arabic numeral corresponding to the canonical order of allergen. For example, Der p 1 and Der f 1 are the Group 1 allergens from *D. pteronyssinus* and *D. farinae*, respectively. Isoallergens are identified by an additional two numbers from 01 to 99 (e.g. Der p 1.01). Variants of an isoallergen are distinguished by two additional numbers following the isoform denomination (e.g. Der p 1.0101) (50, 51). Table 1 summarizes the biochemical and IgE antibody binding characteristics of known HDM allergens that have been accepted by IUIS Allergen Nomenclature Sub-Committee.

Group 1 allergens

The group 1 allergens are cysteine proteases with MW ~25 kDa that belong to the same family as papain and actinidin (52-54). Der p 1 was the first major allergen described for *D. pteronyssinus* (55). The group 1 allergens have amino acid sequence homology of 80%, an N-glycosylation site at residues 53-55, and a calcium binding domain (56, 57). The group 1 allergens have a high degree of polymorphism whose prevalence can differ in different geographical regions, which is attributed to allelic variations of single genes, rather than gene families, as is the case for pollen allergens (58-60). For Der p 1, 24 sequence variants are included in the IUIS database; nevertheless, Der p 1.0102 and Der p 1.0105 are the most frequently observed sequence variants in published literature. By contrast, ten sequence variants are reported for Der f 1.

Approximately 80% of HDM-allergic patients have IgE antibodies specific for group 1 mite allergens (55, 61). Consistent with their sequence and structural homology, Der p 1 and Der f 1 allergens are cross-reactive, as each can compete for with other for the group 1 specific-IgE antibodies (62, 63). Still, species-specific monoclonal antibodies have been cloned for Der p 1 and Der f 1 (61, 63). The amino acid sequences and IgE binding frequencies have also been studied for Blo t 1 (62%) (64) from *B. tropicalis*, Der m 1 (31%) (65) from *D. microceras*, while no published information was found on IgE binding for Eur m 1 from *E. maynei* mites.

Group 2 allergens

The group 2 allergens are also considered major HDM allergens. They are classified as Niemann-Pick type C2 family (NPC2) of proteins, and are present in *B. tropicalis* (Blo t 2(66)), *D. farinae* (Der f 2(67)), *D. pteronyssinus* (Der p 2(68, 69)), *E. maynei* (Eur m 2(70)), *G. domesticus* (Gly d 2(71)), *L. destructor* (Lep d 2(72)), and *T. putrescentiae* (Tyr p 2(73)). Furthermore, there is evidence showing that both Der f 2 and Der p 2 can bind lipopolysaccharide (74, 75).

The group 2 allergens have molecular masses of 15 – 16 kDa and no N-glycosylation sites. Der f 2 and Der p 2 have a sequence identity of 88%, and are highly cross-reactive (76). Single gene

polymorphism and sequence variations have also been observed for Der f 2 (60, 77) and Der p 2 (58, 60); 17 and 15 cDNA sequence variants have been identified for each allergen, respectively.

The IgE binding frequency to the group 2 allergens can range from 70% to >90% in HDM allergic patients (68, 69). In addition, the IgE binding to an allergen can vary from one geographical region to another and among different populations of allergic patients. For example, Choopong et al. showed that only 40% of patients had IgE antibodies against Der f 2; thus, Der f 2 is a less predominant allergen in this Thai cohort than many western cohorts (10).

Group 3 allergens

The group 3 allergens are serine proteases with trypsin-like function (78, 79), and have a MW of ~30 kDa with no N-glycosylation sites (80). Der f 3, Der p 3 and Eur m 3 have 73% sequence identity, while Blo t 3 and Tyr p 3 share 45 and 35% sequence identity with both *Dermatophagoides* species, respectively. The frequency of binding of in sera from allergic patients to group 3 allergens ranges from 15 to 97% (78, 81-84). Although there are reports suggesting polymorphism among group 3 allergens, only one variant is included in the IUIS database for each species (80, 85).

Group 4 allergens

The group 4 allergens belong to the α -amylase protein family, and have a MW of approximately 56-60 kDa. Der p 4 (86) contains an N-glycosylation site that appears to differ from N-glycosylation sites of Blo t 4 (87) and Eur m 4 (88). Cui et al. recently cloned and characterized Der f 4, and reported that 40% of subjects in their Chinese pediatric cohort were reactive (89). This is consistent with reports of western cohorts of IgE binding frequency among western HDM allergic cohorts to the group 4 allergens that range between 4 and 46% (86-88). Der p 4 appears to be a major allergen in the Australian Aboriginal community, who have high IgE titers to Der p 4 but not to Der p 1 or Der p 2 (90).

Group 5 allergens

The function of group 5 allergens (14 kDa) is unknown. Der p 5 is regarded as a mid-range allergen with IgE binding frequency between 30 and 60% (68, 91). Two sequence variants from Der p 5 clones are included in the IUIS database (91). Recombinant Blo t 5 elicits an IgE serologic response from 20-45% IgE of patients from tropical and subtropical regions (e.g. the southern states of the US, Central and South America). The amino acid sequence of Blo t 5 is 43% identical to Der p 5, but in most cases these two allergens group 5 allergens are not serologically cross-reactive (92). In addition, a variant clone from *L. destructor* (Lep d 5) is recognized by 9% of *L. destructor*-sensitized subjects (93).

Group 6 allergens

The group 6 allergens of *D. farinae* (Der f 6) and *D. pteronyssinus* (Der p 6) are 25 kDa proteins described as having characteristics of serine proteases with the substrate specificity of chymotrypsin (94). The group 6 allergens are not cross-reactive with the group 3 allergens, which are also serine proteases. Der f 6 and Der p 6 bind to IgE from 40% of allergic subjects, and have 75% sequence homology (94). The amino acid sequence of Blo t 6 is included in the IUIS database, but to our knowledge, there are no published data on IgE binding by allergic subjects.

Group 7 allergens

The functional properties of the group 7 allergens (22 kDa) are unknown. Proteins in this group have been identified for *D. farinae* (Der f 7), *D. pteronyssinus* (Der p 7) and *L. destructor* (Lep d 7) mites. Der f 7 and Der p 7 share 85% sequence homology, while Lep d 7 has 27-29% sequence homology to both Der f/p 7 proteins. Naturally occurring allergens are glycosylated and are structurally related to the lipopolysaccharide binding bacterial permeability increasing protein family of proteins (95, 96). The group 7 allergens were recognized by 46% (Der f 7), 30% (Der p 7), and 62% (Lep d 7) of sera from sensitized subjects (93, 97).

Group 8 allergens

The group 8 allergens belong to glutathione S-transferase protein family, and have molecular masses of 27-32 kDa. Der f 8 (98) and Der p 8 (99) have 64% sequence homology, and 40% of mite-allergic subjects recognize the mite glutathione S-transferase allergen(100), while unpublished studies revealed that 25% of individuals in an asthmatics cohort reacted positively in a skin prick test with the purified natural Blo t 8 (49).

Group 9 allergens

Like the group 3 and group 6 allergens to which it has ~40% amino acid identity, Der p 9 (29 kDa) is a serine protease. Der p 9 has collagenolytic activity and is reported to bind to IgE from 92% of allergic subjects (83).

Group 10 allergens

The group 10 allergens are tropomyosins, which are involved in regulating muscle contraction and cell mobility with molecular masses of 33-37 kDa. The group 10 allergens have highly conserved amino acid sequences (75% identity of mite tropomyosins to other arthropods), and are highly cross-reactive (101). Der f 10 and Der p 10 exhibit 98% sequence identity with only five amino acid differences in the sequence. While tropomyosins are predominant shellfish allergens, Batard et al. showed that the prevalence of IgE binding to HDM tropomyosins is below 11%, irrespective of the population (i.e. children vs adults or European population vs US individuals or Japanese subjects) (12). In addition to *Dermatophagoides* species (Der f/p 10) (102, 103), the group 10 allergens have been identified for *B. tropicalis* (Blo t 10) (104), *C. arcuatus* (Cho a 10) (105), *L. destructor* (Lep d 10) (106), and *T. putrescentiae* (Tyr p 10) (107).

Group 11 allergens

The paramyosins are also structural muscle proteins of invertebrates with molecular masses of 98-110 kDa and four potential N-glycosylation sites. Amino acid sequence alignment showed that Der p 11 shares 89 and 97% identity with Blo t 11 and truncated Der f 11. The prevalence of serum

IgE binding for the group 11 allergens ranges between 40 and 90% (108-110). Clinical relevance and IgE binding frequency of Der p 11 seemed to be more prominent in patients with HDM-associated atopic dermatitis (~60%) than in patients with a respiratory form of HDM allergy (5%) (111).

Group 12 allergens

The group 12 allergens are low molecular weight (14 kDa) proteins with unknown functional properties and no predicted N-glycosylation sites that have been described only for *B. tropicalis* storage mites (Blo t 12) (112). Blo t 12 was recognized by 50% tested sera of patients sensitized to *B. tropicalis* mites.

Group 13 allergens

The group 13 allergens are characterized as fatty acid binding proteins that are mostly responsible for a transport of fatty acids and other lipophilic substances across cell membranes. The tertiary structure of Der f 13 demonstrated a typical fold of fatty acid binding families of proteins (113). The group 13 allergens have a molecular mass of 15 kDa, and sequence identity of 45% between five allergens described for *A. siro* (Aca s 13), *B. tropicalis* (Blo t 13), *D. farinae* (Der f 13), *D. pteronyssinus* (Der p 13), *L. destructor* (Lep d 13), and *T. putrescentiae* (Tyr p 13). Prevalence studies have shown that the group 13 allergens have a minor role in IgE binding in sensitized patients, 23% for Aca s 13 (114), 13% for Blo t 13 (115), 7% for Der p 13 (unpublished study reported on IUIS Allergen Nomenclature Sub-Committee (49) , 13% for Lep d 13 (93), and 6% for Tyr p 13 (116).

Group 14 allergens

The group 14 allergens are homologous to apolipophorins that belong to a larger family of proteins known as large lipid transfer proteins, which are responsible for the transport of retinoids and other lipids. They are high molecular mass proteins (177 kDa) whose complete amino acid sequence for the group was first determined for a cDNA clone *E. maynei* (117). Initially, the group 14 allergens were represented by two cDNA clones (Mag 1 and Mag 3) of Der f 14 from *D. farinae* (117). Fragment peptides of Der f 14 and Der p 14 share 89% sequence identity. IgE binding studies have

only been conducted with the peptides as the storage of HDM extracts led to the proteolytic breakdown of the allergen (118). Prevalence of IgE binding of Der p 14 in patients with atopic dermatitis was 67%(119), while an earlier study by Fujikawa et al. showed that the allergenic activity of peptide fragments of Der f 14 was greater than of the intact allergen. In this particular study, 84% of mite allergic patients had specific IgE against the peptide fragment and 66% were positive for the intact protein (118).

Group 15 allergens

The group 15 allergens represent a chitinase protein family and they have been described for *D. farinae* and *D. pteronyssinus* mites. These are high molecular mass proteins (98/109 kDa) that were determined to be major allergens in dogs (120). Der f 15 and Der p 15 have sequence identity of 86%. The group 15 allergens are highly glycosylated, and specific IgE antibodies to the group 15 allergens were detected in 70% of mite allergic sera (120, 121).

Group 16 & 17 allergens

The group 16 and 17 allergens have been described only for *D. farinae* mites. They are characterized as gelsolin, i.e., a group of actin cytoskeleton-regulatory proteins, and calcium-binding proteins, respectively. Recombinant Der f 16 bound IgE antibodies in 47% of sera, while recombinant Der f 17 bound IgE antibodies in 35% of sera from mite allergic patients (122, 123).

Group 18 allergens

Chitin-binding proteins are also represented as the group 18 allergens, which are distinct from the group 15 allergens. They have a molecular weight of 60 kDa and are known to be present in *D. farinae* and *D. pteronyssinus* mites. The group 15 and 18 allergens share only a 25% sequence identity, while Der f/p 18 are 89% identical in amino acid sequence. Natural Der f 18 bound IgE antibodies in 57% of mite allergic patients and 77% of mite sensitized dogs (124), while recombinant Der p 18 bound IgE antibodies in 63% of mite-allergic patients (121).

Group 19 allergens

The group 19 allergens described only for *B. tropicalis* mites, which are homologous to poorly characterized antimicrobial peptides. They have a molecular mass of 7 kDa with an IgE binding frequency of 10% in mite allergic patients (80).

Group 20 allergens

The group 20 allergens are in the arginine kinase protein family with molecular masses of 40 kDa. Der f 20 and Der p 20 have 96% sequence similarity, and it has been demonstrated to have overall IgE binding frequency of 40% in mite allergic patients (98, 125).

Group 21 allergens

The functional properties of the group 21 allergens (13-14 kDa) are unknown; although, proteins have been characterized as allergenic in *B. tropicalis* (Blo t 21), *D. farinae* (Der f 21), *D. pteronyssinus* (Der p 21) mites. Der f/p 21 share 66% sequence identity, while Blo t 21 has sequence identity of 38 and 37% with Der f 21 and Der p 21, respectively. Allergenicity of Blo t 21 varied between 58-95% in allergic patient depending on which assay system and techniques were used for the assessment, i.e. SPT, ELISA, or immune-blotting (126). Even though biochemical characterization of Der f 21 has been studied (127), there are no published data available of IgE binding frequency in mite-allergic patients. On the other hand, Der p 21 was found to bind IgE antibodies in 30% of the sera of mite allergic patients (12, 128).

Group 22 allergens

The group 22 allergens only include Der f 22, which is a 17 kDa protein of unknown functional properties. It has 32% sequence identity with Der f 2 (Niemann-Pick C2 family), but to IgE cross-reactivity to Der f 2. IgE binding frequency was observed in 50% of mite allergic patients (129).

Group 23 allergens

This peritrophin-like protein domain protein has only been found in *D. pteronyssinus*. Der p 23 has a molecular mass of 14 kDa, and IgE binding frequency of 46-74% (12, 130).

Group 24 allergens

The group 24 allergens are found in *D. farinae* and *D. pteronyssinus* mites. They have a molecular weight of 13 kDa and 97% sequence identity. The group 24 allergens are identified as Ubiquinol-cytochrome c reductase binding proteins, having high IgE response in patients with HDM allergy. All tested sera had IgE antibodies against rDer f 24 and 5 out of 10 patients had positive SPT (131). While unpublished studies revealed that 50-100% of individuals reacted positively with the Der p 24 using both in-vitro and in-vivo testing, i.e., 5 out of 5 samples had positive IgE-immunoblot; 20 out of 24 were IgE-ELISA positive; and 5 out of 10 patients has positive STP (49).

Group 25-36 allergens

The 25-36 groups represent allergens of HDM whose allergenic importance is unknown. They have been described in *D. farinae* extract by 2-D immunoblotting that revealed IgE binding of allergic sera to Der f 25 (triosephosphate isomerase), Der f 26 (myosin alkali light chain), Der f 27(132) (serpin), Der f 28 (heat shock protein), Der f 29 (cyclophilin), Der f 30 (ferritin) proteins (98). The amino acid sequences of Der f 31 (cofilin), Der f 32 (pyrophosphatase), and Der f 33 (α -tubulin) and Der f 35 (unknown function) are included in the IUIS database; however, there are no published IgE-binding data available on these particular proteins. Recently published Der f 34 (enamine/imine deaminase) is another novel mite allergen with IgE-binding frequency of 60-70% (133). Troponin C (Tyr p 34) from *T. putrescentiae* is a new mite allergen with 10% IgE binding reactivity in a study population from Korea (134). Unpublished study on Tyr p 36 (profilin) reported that 65% of HDM allergic subjects had a weak IgE binding to this protein by western blot analysis (49).

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Figure legends

Figure 1. Overall potency may not reflect compositional differences or correlate with efficacy of immunotherapy.

A and B: Hypothetical sets of extracts in which two extracts with identical overall potencies are compositionally identical (A) or different (B).

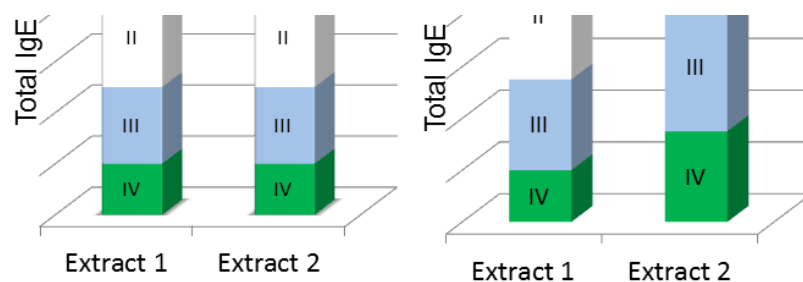
C and D: Profiles of allergen reactivity. (C) An “inclusive profile,” in which all patients are allergic to an immunodominant Group 1 allergen, whose concentration may therefore represent the potency of the allergenic extract. Therapeutic potencies cat hair and ragweed pollen extracts are represented by concentrations of Fel d 1 and Amb a 1, respectively. (D) A hypothetical profile of “variable reactivity,” in which there is no immunodominant allergen. For these extracts, compositional differences among extracts shown in (B) may be therapeutically relevant.

Figure 2. Principles of multiple reaction monitoring (MRM) and absolute quantification of a protein using isotope-labeled peptide as standard. Cartoon depiction in blue and green represents natural peptides generated after enzyme digestion of Der p 1. Cartoon depiction in red represents an isotope-labeled peptide standard. 3D structure of Der p 1 was adopted from Chruszcz et al. (135).

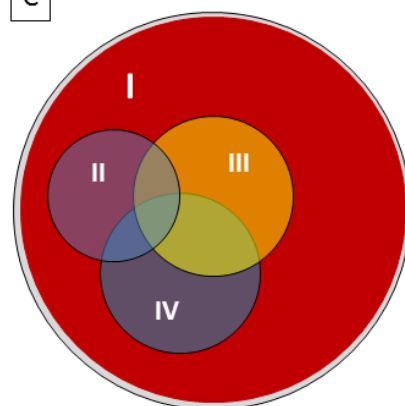
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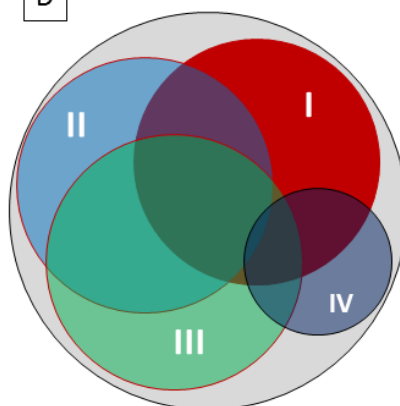


C



"Inclusive" allergen reactivity

D



Variable allergic reactivity

