ANIMAL ALLERGENS: COMMON PROTEIN CHARACTERISTICS FEATURING THEIR ALLERGENICITY

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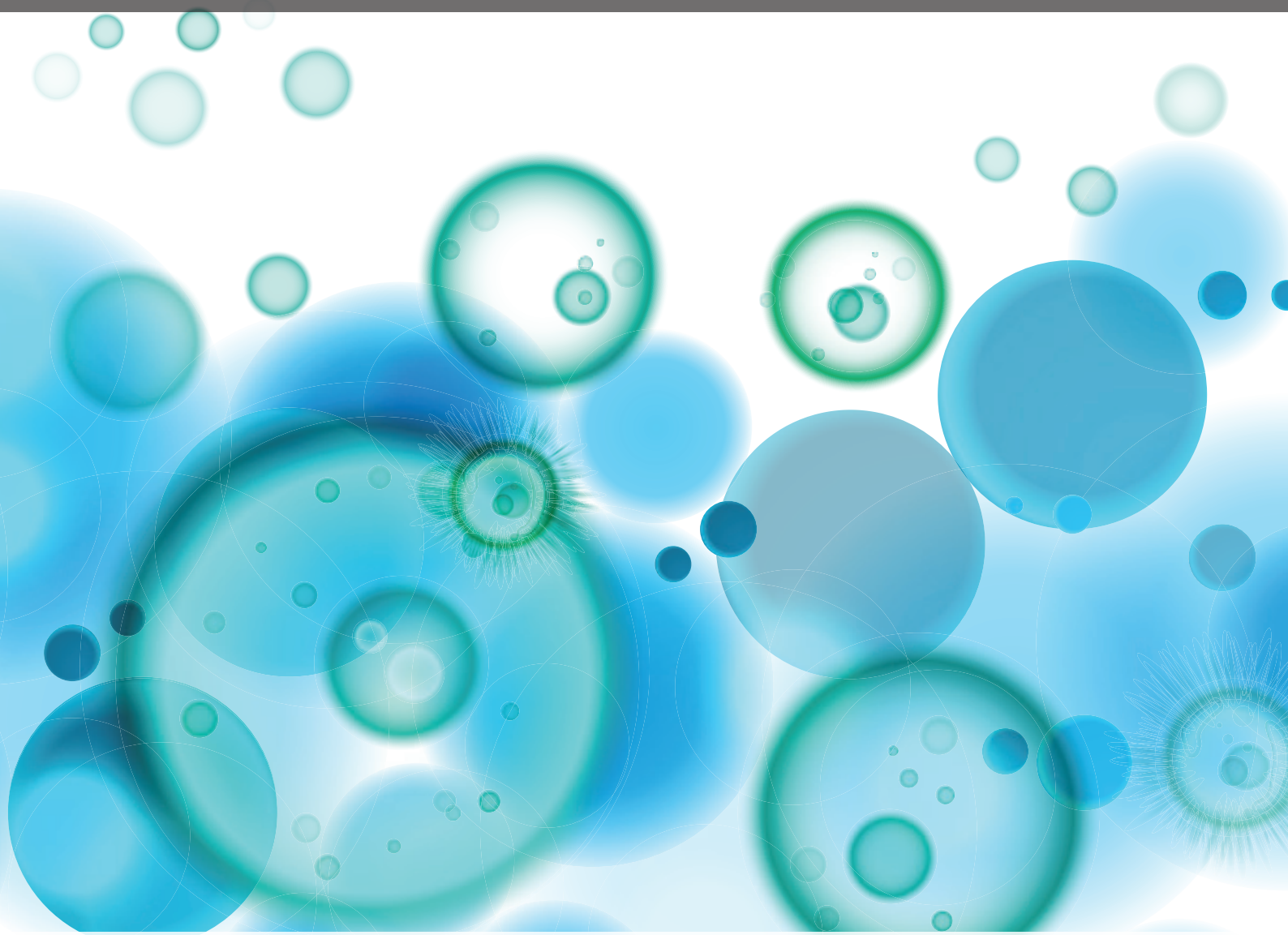

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ANIMAL ALLERGENS: COMMON PROTEIN CHARACTERISTICS FEATURING THEIR ALLERGENICITY

Topic Editors:
Christiane Hilger, Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg
Annette Kuehn, Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg

Among the many molecules present in our environment, some have the property to induce allergic sensitization and IgE-mediated reactions. The analysis of known major animal allergens has shown that most belong to single protein families: lipocalins and serum albumins for inhalant allergens, EF-hand proteins, tropomyosins and caseins for the digestive allergens. The finding that allergens are often clustered in large families may be related to the fact that common structural, biochemical or functional features contribute to their allergenicity, in addition to external adjuvant factors. Currently, there is no curative treatment for animal allergy available. In order to lower allergic reactions to respiratory allergens in daily life and to food allergens upon accidental exposure, it is important to desensitize concerned patients. Tolerance induction by allergen-specific immunotherapy is in the current focus of an ambitious research.

This Research Topic aims to provide a comprehensive view of the basic and recent insights on the allergenicity of animal allergens in view of their structural and functional aspects as well as allergen-specific immunotherapy.

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Animal allergens: common protein characteristics featuring their allergenicity

Annette Kuehn* and Christiane Hilger

Department of Infection & Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg
**Correspondence: annette.kuehn@lih.lu*

Edited and reviewed by:
Kendall Arthur Smith, Weill Medical College of Cornell University, USA

Keywords: animal allergen exposure, allergenicity, allergy, biological function, cross-reactivity, immune response, isoallergens, immunotherapy

Allergy to proteins and glycoproteins from animal sources is an important public health problem affecting both children and adults (1–3). Exposure to animal allergens is a key factor for the development of allergy, particularly for the development of allergic airway diseases such as asthma and rhinitis (4, 5). Among the many particles and molecules of animal origin present in our environment, some have the property to induce allergic sensitization and IgE-mediated reactions. Major animal allergens share common protein characteristics as they belong to specific protein families: a majority of inhalant mammalian allergens belong to the lipocalin and serum albumin families. Many mite allergens are proteases, food allergens are often EF-hand proteins, tropomyosins, or caseins (6–8). These protein family clusters observed in allergens suggest that common structural, biochemical, or functional features in addition to external adjuvant factors contribute to their allergenicity. The understanding of the nature their allergenicity is of high importance as it contributes to the development of future diagnostic tools and therapeutic strategies (9–12).

The present compilation of review articles illustrates that animal allergens are ubiquitous. The human immune system is exposed to these proteins not only via the respiratory tract such as for pet and house dust mite allergens via the intestine such as for food and helminth allergens but also via the skin such as for insect allergens.

The extensive review of Zahradnik and Raulf focuses on animal allergens from furry animals in indoor (cat, dog) and outdoor environment (horse, cattle). Allergies to these animal proteins are a public problem as exposure is not limited to the original source but as these molecules become airborne and are carried on clothing to public buildings, the general population is exposed to these allergens. Allergen detection in the private or occupational context is therefore an important first step to develop avoidance strategies for patients at risk.

Díaz-Perales et al. present an overview of uncommon allergenic sources such as exotic pets, which are on the rise as elicitors of animal allergies. These sources are to be considered as relevant because on one hand, their numbers are progressively increasing in households and on the other hand, cross-reactivity to common companion animals via molecules of conserved protein families might occur.

Hentges and colleagues approach the topic of animal allergens from another point of view by focusing on common features of the human immune response to three allergen families, namely, secretoglobins, lipocalins, and serum albumins. Mammalian allergens initiate a broad variety of immunological processes in sensitized patients. The understanding of these mechanisms is crucial for defining strategies of future immunotherapies.

Dumez et al. refer to the most frequent animal elicitors of allergy, which are house dust mites. Among the spectrum of identified mite allergens, they focus on the allergens' biological function in the context of activation pathways initiating the underlying immune response during sensitization. The answer to the crucial question "why is an allergen an allergen?" seems to be well advanced in this context. This highlights the need for such functional analysis for further animal allergens.

Fitzsimmons, Falcone, and Dunne report on allergens from helminths, which provoke strongly skewed Th2 responses as a normal physiological response upon parasitic infection. This review touches another important aspect: a reduced allergy prevalence has been associated with helminth endemic areas. But on the other hand, many environmental animal allergens have homologs in metazoan parasites, which may lead to cross-reactivity and increase of allergy prevalence. As a perspective, further elucidation of the immunological processes during parasite infection may improve insights for the development of therapeutic strategies in allergy.

Kuehn and colleagues target another important source of animal allergens from food origin, namely fish allergens, which enter the human body by ingestion, inhalation, or skin contact. Molecular and immunological characteristic of the major fish allergens, parvalbumins, as well as the recently described enolases and aldolases, are in the focus while highlighting the variable allergenicity of closely related isoallergens and providing a basis for further studies of the mechanism of sensitization and allergy.

Spillner, Blank, and Jakob detail the recent rapid advances in the characterization of hymenoptera venom allergens. The availability of a substantial number of isolated and recombinant allergens allowed to define individual patient IgE-profiles, to predict cross-reactivity and clinical cross-sensitization. The use of an increasing molecular allergen panel will ultimately allow to monitor and assess therapeutic outcome.

Salazar and Ghaemmaghami report on the role of dendritic and epithelial cells in the process of allergic sensitization. They focus on how different receptors such as C-type lectin, toll-like and

protease activated receptors, and the associated signaling pathways contribute to the sensitization process. The elucidation of the molecular mechanisms of dendritic and epithelial cell cross-talk in the process of sensitization will hopefully lead to the development of new therapeutic applications.

Van Hage and Pauli finally summarize the recent advances in immunotherapy for mammalian allergy. Most studies focused on Fel d 1, the major cat allergen, as it is a well characterized allergen in terms of B- and T-cell epitopes. Different molecular design strategies, the use of the molecules in mouse models as well as in clinical trials and therapeutic outcomes are presented. The field is evolving rapidly and further studies are needed to assess safety and efficacy of the new molecules.

We are thankful to all colleagues who contributed to this Research Topic, without their highly valuable expertise in different fields of research on animal allergen research it would not have been possible to realize this issue.

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Animal allergens and their presence in the environment

Eva Zahradnik and Monika Raulf*

Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr-Universität Bochum (IPA), Bochum, Germany

Edited by:
Christiane Hilger, Centre de Recherche Public Santé, Luxembourg

Reviewed by:
Mark A. Suckow, University of Notre Dame, USA
Inge M. Wouters, Institute for Risk Assessment Sciences, Netherlands

****Correspondence:***
Monika Raulf, Center of Allergology/Immunology, Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr-Universität Bochum (IPA), Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany
e-mail: raulf@ipa-dguv.de

Exposure to animal allergens is a major risk factor for sensitization and allergic diseases. Besides mites and cockroaches, the most important animal allergens are derived from mammals. Cat and dog allergies affect the general population; whereas, allergies to rodents or cattle is an occupational problem. Exposure to animal allergens is not limited to direct contact to animals. Based on their aerodynamic properties, mammalian allergens easily become airborne, attach to clothing and hair, and can be spread from one environment to another. For example, the major cat allergen Fel d 1 was frequently found in homes without pets and in public buildings, including schools, day-care centers, and hospitals. Allergen concentrations in a particular environment showed high variability depending on numerous factors. Assessment of allergen exposure levels is a stepwise process that involves dust collection, allergen quantification, and data analysis. Whereas a number of different dust sampling strategies are used, ELISA assays have prevailed in the last years as the standard technique for quantification of allergen concentrations. This review focuses on allergens arising from domestic, farm, and laboratory animals and describes the ubiquity of mammalian allergens in the human environment. It includes an overview of exposure assessment studies carried out in different indoor settings (homes, schools, workplaces) using numerous sampling and analytical methods and summarizes significant factors influencing exposure levels. However, methodological differences among studies have contributed to the variability of the findings and make comparisons between studies difficult. Therefore, a general standardization of methods is needed and recommended.

Keywords: animal allergens, allergen exposure, environmental monitoring, cats, dogs, rodents, cattle, horses

INTRODUCTION

Exposure to animal allergens is a major risk factor for the development of sensitization and allergic diseases such as asthma, allergic rhinitis/conjunctivitis, and atopic dermatitis (1, 2). Generally, intensive contact with any animal, including diverse arthropods, reptiles, birds, and mammals, can induce allergic reactions (3, 4). Apart from ubiquitous mites and cockroaches, the most frequent allergies developed are those to domesticated mammals with fur that are typically kept as pets or farm animals (1, 5). Contact with animals arises via many different occupations and activities. Cats, dogs, guinea pigs, hamsters, and rabbits are all very popular pets in industrialized countries, where the percentage of pet ownership continues to increase (6). Horses, whose use has decreased in agriculture, are today widely owned for recreational riding and show activities. Besides pigs, cows are the most common farm animals used for dairy and meat production. Another important source of occupational animal allergies is the handling of laboratory animals (7). Rodents, especially mice and rats, are kept in large numbers in research facilities of universities and pharmaceutical industries. In addition to these rodents housed in laboratories or occasionally kept as pets, mice, and rats can infest human urban and agricultural environments, where they find food supplies and have few predators.

During the past few decades, the distribution of various mammalian allergens has been extensively studied. Based on this research, it can be stated that animal allergens are present ubiquitously in the human environment, even though concentrations differ considerably. Numerous studies of animal allergen exposure levels in different locations and geographical regions have been published, with variable results that have been attributed to the use of different study designs (e.g., kind and number of samples, exposure grouping, data analysis). A mandatory requirement to assess allergen exposure is the availability of a reliable assay for allergen quantification. Therefore, this article focuses on well-characterized mammalian allergens derived from cats, dogs, mice, rats, cows, and horses for which very sensitive and specific ELISA assays have been validated. Some of these assays are currently commercially available (Indoor Biotechnologies, Charlottesville, VA, USA). Although, several allergens from other mammals (e.g., rabbit, guinea pig, ferret, hamster, chinchilla) have also been identified [summarized by Díaz-Perales et al. (6)], no assays for these species have been developed or published so far.

As a background, a short overview of characteristics of major allergens and sensitization rates in different population groups are presented in this report. The main focus of this review is a summary of exposure assessment studies conducted in recent years. Reported allergen levels and relevant factors associated with concentration differences are described for different exposure settings. Examples of studies were selected based on the fact that they were carried out in different environments (homes, workplaces, public

buildings) using various dust sampling methods. Recommendations with regard to standardization of methods are being made for future research.

COMMON FEATURES OF ANIMAL ALLERGENS INFLUENCING THEIR UBIQUITY

Inhalant allergens derived from mammals comprise a large and complex group. With the exception of the cat allergen Fel d 1, the majority of all animal major allergens belong to the lipocalin protein family (8). Typically, lipocalins from mammals are small extracellular proteins composed of approximately 150–170 amino acids with a molecular mass of about 20 kDa. Lipocalins share common biological functions that are predominantly related to the transport of small hydrophobic molecules such as steroids, odorants, and pheromones. Although, the overall amino acid identity between lipocalins is low (usually between 20 and 30%), this protein family is characterized by conserved sequence motifs and a common tertiary structure. Albumins represent another large protein family containing several respiratory allergens. They are components of serum and regulate osmotic pressure of blood. Compared to lipocalins, albumins are of minor allergic importance, but they are often responsible for allergic cross-reactions between animal dander of different species due to the high sequence identity among family members (about 80%) (9, 10). A partial cross-reactivity also exists between several lipocalins; however, the clinical relevance of this feature needs to be assessed (11).

Animal allergens are mainly produced in the liver or secretory glands and localized in animal skin and body fluids, such as urine, saliva, blood, milk, and sweat. These proteins adhere to fur and other surfaces. The allergens can be efficiently dispersed into the environment as animals shed hair and dander, and secrete and excrete fluids. Indoors, the allergens accumulate primarily in different textiles, including carpets, upholstery, mattresses, and curtains where they are detectable for a long time, even after removal of the animal (12–14). In addition, the aerodynamic properties of animal allergens influence their environmental distribution and human exposure. Especially, lipocalins tend to be carried on a diverse range of small dust particles, from <1 to 20 μm. Some proportion of allergens (<5 μm) can stay suspended in the air for extended periods of time (15). In contrast, mite allergens are primarily associated with large-sized (>20 μm) dust particles that settle rapidly (16). Based on their aerodynamic characteristics, animal allergens can be transferred to environments that were never occupied by the animals, such as public buildings, including schools, day-care centers, hospitals, and offices (17). Although, the concentrations of the allergens are low in these environments, they may be high enough to cause symptoms in sensitized individuals (18, 19).

There is strong evidence that clothing is the primary transfer mechanism of allergens. Significantly higher allergen levels have been found in dust collected from pet owners' clothes than from the clothes of non-pet owners (20, 21). Furthermore, allergen levels have been shown to be dependent on clothing type and washing frequency (22). Egmar et al. (23) studied the accumulation of animal allergens in furniture stores by comparing the dust from factory-new mattresses to used ones. Allergen concentration correlated to the period of time that the mattresses were used by customers. In addition to clothing, human hair is also an important vehicle for transfer, and thus a source of animal allergens (24, 25).

MEASUREMENT OF ALLERGEN EXPOSURE

Measuring airborne allergens is necessary to detect allergen sources, to assess the relationship between allergen exposure, sensitization, and symptoms and to generate preventive measures. Allergen exposure assessment comprises two essential procedures: collection of dust samples and quantification of allergen levels. For both steps, a large variety of sampling strategies and analytical tests are available. However, the different methods used have made it difficult to compare the results among the different studies. Although, a general standardization would be preferred, in practice the variations cannot be avoided. The choice of sampling method is often influenced by the size of the study, available budget, practical performance, aerodynamics of the relevant allergens, and relevance to the personal exposure. Each method has advantages and disadvantages depending on the aims and technical limitations of the investigation (26).

DUST SAMPLING

Reservoir or *settled dust* sampling by vacuuming of surfaces is the most common method used for determining domestic exposure, e.g., pet allergens at home. It is inexpensive, easy, and fast to perform and therefore, widely applied in large-scale studies. Dust is collected on filters or in nylon bags mounted in special sampling devices that are commercially available (27). Several surfaces can be vacuumed, including floors (smooth or carpeted), beds (mattress or bedding), and furniture (sofas, chairs, desks). Apart from the collector and surface type, differences occur in the power of vacuum cleaner, size of the area sampled, sampling time, and sampling location (living room, bedroom, or kitchen). Collected dust may be sieved by some researchers to separate coarse particles. All the variations described influence the amount and composition of dust, and ultimately the results of the allergen analysis. Results are expressed as allergen per unit weight or per square meter, and although significant relationships have been shown between allergen levels in reservoir dust and allergic diseases, the relevance to personal exposure is still questionable. Many collected dust particles never become airborne and are therefore never inhaled.

Airborne dust sampling using pumps is commonly used in occupational settings, for example to measure mouse allergens in laboratories. According to the aerodynamic properties of animal allergens, airborne levels might be more suitable for defining exposure to pets. This method requires time, expensive equipment, and trained staff. The pumps are noisy and need recharging and calibration. Dust is sampled using several types of filters and sampling heads constructed for collection of particles with defined size (e.g., inhalable, respirable dust). Air may be sampled stationary by low (2–20 L/min) or high volume (60–1100 L/min) pumps, or using person-carried pumps often at flow rates of 2 or 3.5 L/min. Different sample volumes and collection times directly affect the lower detection limits, and dust amounts are strongly dependent on activities in the room causing air disturbance. Results are expressed as allergen per cubic meter of air. In comparison to reservoir dust,

airborne dust may be considered a more representative measure of inhaled allergen. Personal sampling in the breathing zone used for task and shift measurements is regarded as a gold standard in occupational settings.

Another method that can be used to collect airborne dust is the ion-charging device (28). This technique is based on a commercial air cleaner, where particles in the air are loaded with a positive charge, which allows them to attract and bind to the negatively charged collector plates. In general, airborne measurements are rarely performed in home environments because of high logistic costs.

Settling or passive airborne dust sampling is an alternative or a complement to the other two sampling techniques. This method collects airborne dust that has settled over a period of time (e.g., 2 weeks) on a certain sampling height (e.g., 1.5 m above the floor). In recent years, several sampling devices have been used such as Petri dishes (29), aluminum foil-covered boxes (30), or electrostatic cloths (31). Results are expressed in allergen per square meter (and per day). Settling dust seems to correlate moderately with both airborne dust as well as reservoir dust. Due to its low cost, ease of use, and simple transport, this method is suitable for large-scale exposure studies.

ALLERGEN ANALYSIS

Standard methods for allergen quantification are immunoassays based on specific antibodies directed against the allergens. A broad panel of assays has been developed (32), but some methods such as quantitative immunelectrophoresis or ELISA inhibition are hardly used. In the past two decades, ELISAs (enzyme linked immunosorbent assay) in "sandwich" design have been established as the gold standard for allergen analysis (33). The antibodies may be either monoclonal or polyclonal against either allergen mixtures or single allergens. Variations occur according to the source, specificity and purity of antibodies. In particular, the choice of standard (e.g., animal dander extract or purified or recombinant major allergens) and its protein determination method (Bradford-, BCA-, Lowry-assay, amino acid analysis) may influence the resulting values up to several orders of magnitude. Furthermore, several detection (conjugates) and visualization (substrates) methods are available.

The increasing interest and need to quantify allergens in different environments in recent years has led to the development of multiplex arrays for indoor allergens (MARIA) (34). Multiplex technology uses the same (or equivalent) antibody combinations used in ELISAs. Capture antibodies are covalently coupled to polystyrene beads that are internally labeled with fluorophores. Combining different bead types with different antibodies allows simultaneous measurement of several allergens in a single test.

CATS AND DOGS

The popularity of cats and dogs as pets means that allergy to both animals affects the general population. In Europe, the frequency of pet ownership is highly variable. Cat ownership ranged from 7.2 to 35% (average 14.9%) and dog ownership from 5.4 to 35% (average 12%) across 12 European birth cohorts with a total of 25,056 subjects (35). In the United States, according to the American Pet Products Manufacturers Association, nearly 40 and 33% of households own dogs and cats, respectively (36). Equally, the prevalence of sensitization also varies in different countries because of cultural differences and environmental factors. A large patient-based study GA^{2}LEN (The Global Asthma and Allergy European Network) investigated the sensitization patterns for diverse inhalant indoor and outdoor allergens across 14 European countries using skin prick testing (5). The overall European sensitization frequency to cats and dogs were very similar, but regional differences were found. Cat sensitization rate was 26.3%, ranging from 16.8 to 49.3%. The rate of sensitization to dog allergens was 27.2%, ranging from 16.1 to 56%. Sensitization rates to both allergens were particularly high in Nordic countries (e.g., Denmark and Finland) and lower in Central/Western and Mediterranean countries (e.g., Austria, Belgium, Italy). In the US Inner City Asthma Study, skin test sensitivity among asthmatic children was 41% to cat allergens and 21% to dog allergens (37). Naturally, in the general population, the sensitization frequencies are much lower compared with those detected in patient populations (5). In a recent survey, performed in Germany (38), 7% of 7025 adult participants were sensitized to cat as well as to dog allergens (detection of specific IgE). Allergy to pets may also occur in some professions where workers are heavily exposed to animal dander during most of their working time. Work-related allergic symptoms have been reported in animal laboratory workers (30% to cats, 25% to dogs) and veterinarians (26% to cats, 19% to dogs) when handling animals (39, 40). However, it has to be considered that these persons may also handle animals before and/or outside of their respective careers.

CAT (*FELIS DOMESTICUS*)

Cat dander contains several allergens. The major cat allergen Fel d 1 is the most extensively studied animal allergen with regard to its structure, aerodynamic properties, environmental distribution, and the relationship between allergen exposure and the development of allergic disease (41, 42). Fel d 1 is a tetrameric glycoprotein formed by two heterodimers and has an apparent molecular weight of about 38 kDa (43). It reacts with IgE from over 90% of cat-sensitized individuals (44). In contrast to other animal major allergens, Fel d 1 is not a lipocalin. The three dimensional structure of Fel d 1 is very similar to that of uteroglobins, anti-inflammatory proteins, but its biological function is still unknown (45). Fel d 1 is primarily found in cat skin and hair follicles and is produced in sebaceous, anal, and salivary glands (46–48). It is transferred to the fur by licking and grooming. Male cats produce a larger amount of Fel d 1 than female cats (49). Fel d 1 was mainly associated with particles $>9\,\mu m$, representing approximately 49% of the total allergen recovered. About 23% of airborne Fel d 1 was carried on small particles $<4.7\,\mu m$ (12). Other cat allergens are: Fel d 2 (67 kDa), serum albumin; Fel d 3 (11 kDa), cystatin; the second major cat allergen Fel d 4 (20 kDa), lipocalin; Fel d 5, immunoglobulin A; Fel d 6, immunoglobulin M; Fel d 7 (18 kDa), von Ebner gland protein; and Fel d 8 (24 kDa), latherin (11, 50).

DOG (*CANIS FAMILIARIS*)

Hair/dander and saliva are the main sources of dog allergens. The major dog allergen, Can f 1 (about 22–24 kDa) belongs to the lipocalin family of proteins and is produced in tongue epithelial tissue (51). About, 70% of dog allergic subjects have been shown to have IgE directed to Can f 1 (51, 52). Similar to Fel d 1 in cats,

males produce more Can f 1 than females (53). The particle size distribution of Can f 1 is similar to that of Fel d 1 as well (54). Although, differences in Can f 1 allergen production have been found between different dog breeds, the variability between individuals is very large and a hypoallergenic breed does not exist (53, 55). Further dog allergens are: Can f 2 (24–27 kDa), lipocalin; Can f 3 (67 kDa), serum albumin; Can f 4 (16 kDa), lipocalin; Can f 5 (28 kDa), prostatic kallikrein; and Can f 6 (27–29 kDa), lipocalin (11, 50).

PET EXPOSURES

There are an overwhelming number of publications concerning the quantitative measurements of cat and dog allergens. Studies have been conducted worldwide but the research has been most active in the United States and in European countries. Study results are presented in **Table 1** for cat allergens and in **Table 2** for dog allergens. Most of the investigations chosen have estimated the levels of both cat and dog allergens in parallel. A further common feature of these studies is the usage of the same (or very similar) quantification method. The sandwich ELISA for cat allergens is based on two monoclonal antibodies, 6F9 and 3E4 against Fel d 1 (56). The sandwich ELISA for dog allergens is a combination of a monoclonal capture antibody 6E9 and polyclonal detection antibody against Can f 1 (57). Both assays are commercially available by Indoor Biotechnologies. Currently, Fel d 1 and Can f 1 can also be quantified with the multiplex array MARIA (Indoor Biotechnologies). In this system, the original Can f 1 ELISA was modified to use two monoclonal antibodies, 10D4 for capture and biotinylated 6E9 for detection. Commercial Fel d 1 and Can f 1 assays use purified natural single allergens as the standard quantified by amino acid analysis, but variations can occur in the protein standard used, which is not always specified in the relevant publications.

The most important finding of all studies is that cat and dog allergens are ubiquitously found in every type of human indoor environment, regardless of the presence of pets, most likely due to passive transfer via clothing. Exposure levels vary widely between different environments and geographical regions. For example, a multicenter cross-sectional study measured Fel d 1 levels in mattress dust from 2800 households in 22 municipal areas across Europe (61). European regions showed substantial differences with respect to allergen levels, with the highest concentrations of Fel d 1 found in central European countries followed by the northern and finally southern countries. The overall geometric mean was 0.94 μg/g, ranging from the lowest measured value of 0.12 μg/g in Huelva, Spain to the highest of 3.76 μg/g in Antwerp, Belgium. The major strength of this study was the good comparability among different regions because the identical dust collection protocol was used, and Fel d 1 measurements were performed in one single laboratory using identical batches of ELISA kits.

Apart from the high variability, exposure intensity/degree is primarily related to the presence (past or present) of a pet in the home. Not surprisingly, cat and dog allergen concentrations were found to be much higher in homes with pets than in homes without pets, as shown using both reservoir dust samples (58, 60–62, 64, 73) and air samples (58, 59). In settled dust, there was an 80- to 250-fold difference for Fel d 1, and 25- to 120-fold difference for Can f 1 between houses with and without pets. Higher geometric mean of cat allergens has been also estimated in homes where cats were once present compared with those that never housed cats (61). Homes with outdoor dogs had significantly higher dog allergen levels than homes without any dogs, but significantly lower levels than homes with indoor dogs (73). Another multicenter cross-sectional survey from the United States investigated the distribution of Can f 1 and Fel d 1 within households according to the sampling site and vacuumed surface (60). Independent of the presence of pets, the highest concentrations of both allergens were found in living room sofas. In homes with pets, such high concentrations generally indicated the favorite indoor location of the animals; whereas, in homes without pets, such sites were those that came into most contact with clothing. Additionally, sofas are generally less frequently cleaned than floors or bedding.

Different infrastructural characteristics (urban, suburban, rural) also appear to influence the allergen exposure intensity. Suburban homes contained higher levels of cat and dog allergens than inner city homes, probably reflecting the higher rate of pet ownership in these households (62). In rural homes, the median concentrations of cat and dog allergens in mattresses were significantly lower than in those from urban houses, probably reflecting the different habits of pet owners (75). In rural areas, animals are usually kept outdoors, whereas more indoors pets are found in cities. Moreover, a study from Norway has reported that gender-specific differences can occur in pet allergen exposure among children (63). Girls had higher concentrations of cat and dog allergens in their mattresses compared with boys, also after adjustment for pet ownership. This was most likely caused by differences in behavior, such as a greater affinity among girls to cuddle pets or the tendency to decorate their rooms with soft toys, which may act as reservoirs for allergens.

To explore the differences in allergen distribution among different dog breeds, allergen levels in settled airborne dust (electrostatic cloths) were measured in homes of various so-called hypoallergenic (Labradoodle, Poodle, Spanish Waterdog, Airedale terrier) and non-hypoallergenic dogs (Labrador retriever, control heterogeneous group). Despite Can f 1 differences in hair samples between hypoallergenic and non-hypoallergenic dogs (with enormous variability between individual dogs of the same breed), no differences were observed in environmental levels. Surprisingly, airborne Can f 1 concentrations in homes were similar across different breeds and no evidence was found for a reduced production of allergen by hypoallergenic dogs (55).

Besides domestic settings, many studies on indoor allergens have focused on schools and day-care centers due to lengthy periods that children spend in these locations. A comparison of settled dust samples between these two types of environments suggests that cat and dog allergen levels in schools are higher than in homes where no pets are present (64, 68). These results clearly demonstrate that educational facilities may be the most important site of persistent exposure for some children and thus a risk factor, especially for those who are allergic or asthmatic. Higher allergen concentrations were found in furniture compared to floors (64, 66), consistent with what was described above for personal home environments. Furthermore, carpeted floors contained higher allergen levels than smooth floors (65, 66). Fel d 1 and Can f 1 were

Table 1 | Studies related to exposure assessment to cat allergen.

Study	Environment/ country	Assay	Sampling method	Allergen level	
Custovic et al. (58)	Homes, UK	Fel d 1 Sandwich ELISA (mAB)	Air samples (high volume pumps)		Range
			Homes with cats, $n=34$		0.4–22.3 ng/m^3
			Homes without cats, $n=62$		<LOD–1.8 ng/m^3
			Reservoir dust from living room floor	GM	95% CI
			Homes with cats, $n=34$	204.5 μg/g	108.5–385.3 μg/g
			Homes without cats, $n=62$	0.8 μg/g	0.61–1.06 μg/g
Custis et al. (59)	Homes, USA	Fel d 1 Sandwich ELISA (mAB)	Air samples (ion-charging device)	GM	
			Homes with cats		
			Living room, $n=27$	1.52 μg/day	
			Bedroom, $n=16$	1.12 μg/day	
			Homes without cats		
			Living room, $n=17$	0.049 μg/day	
			Bedroom, $n=13$	0.042 μg/day	
Arbes et al. (60)	Homes, USA	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust	GM	
			House index[a], $n=825$	4.73 μg/g	
			Bedroom bed, $n=728$	2.74 μg/g	
			Bedroom floor, $n=736$	2.13 μg/g	
			Living room floor, $n=759$	2.14 μg/g	
			Living room sofa, $n=717$	6.17 μg/g	
			Homes with cats, $n=187$	199.7 μg/g	
			Homes without cats, $n=630$	1.47 μg/g	
Heinrich et al. (61)	Homes, across Europe (several countries)	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from mattresses	GM	95% Range[b]
			Total, $n=2800$	0.94 μg/g	0.84–1.05 μg/g
			Central Europe $n=1023$	1.79 μg/g	1.48–2.15 μg/g
			Northern Europe, $n=767$	1.45 μg/g	1.17–1.79 μg/g
			Southern Europe, $n=1010$	0.35 μg/g	0.30–0.41 μg/g
			Never cat owners, $n=2044$	0.29 μg/g	0.01–8.3 μg/g
			Past cat owners, $n=192$	1.37 μg/g	0.01–149.3 μg/g
			Current cat owners, $n=555$	61.40 μg/g	0.22–17,072 μg/g
Simons et al. (62)	Homes, USA	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from bedroom	GM	
			Inner city, $n=98$	0.75 μg/g	
			Suburban, $n=19$	2.4 μg/g	
			Homes with cats	16.9 μg/g	
			Homes without cats	0.43 μg/g	
Bertelsen et al. (63)	Homes, Norway	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from mattresses	GM	95% CI
			All homes, $n=797$	1.32 μg/g	1.14–1.54 μg/g
			Girls, $n=360$	1.93 μg/g	1.50–2.47 μg/g
			Boys, $n=437$	0.97 μg/g	0.81–1.17 μg/g
			Homes without cats, $n=640$	0.62 μg/g	0.54–0.86 μg/g
			Girls, $n=276$	0.74 μg/g	0.63–0.86 μg/g
			Boys, $n=364$	0.55 μg/g	0.48–0.62 μg/g
Wardzynska et al. (75)	Homes, Poland	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from mattresses	Median	IQR
			Homes with cats		
			Rural, $n=23$	0.62 μg/g	0.42–0.77 μg/g
			Urban, $n=12$	0.80 μg/g	0.65–1.07 μg/g
			Homes without cats		
			Rural, $n=168$	0.42 μg/g	0.26–0.61 μg/g
			Urban, $n=129$	0.59 μg/g	0.26–0.83 μg/g

(Continued)

Table 1 | Continued

Study	Environment/ country	Assay	Sampling method	Allergen level	
Perzanowski et al. (64)	Schools and homes, Sweden	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust	GM	Range
			Schools, $n = 176$	0.76 μg/g	<LOD–13 μg/g
			Desk and chairs, $n = 64$	2.6 μg/g	<LOD–13 μg/g
			Floors, $n = 64$	0.31 μg/g	<LOD–8.8 μg/g
			Homes without cats, $n = 74$	0.42 μg/g	<LOD–9.4 μg/g
			Homes with cats, $n = 9$	33 μg/g	1.8–950 μg/g
Karlsson et al. (29)	Schools, Sweden	Fel d 1 Sandwich ELISA (mAB)	Petri dishes (5 days)	Median	
			Many cat owners in class, $n = 22$	81.0 ng/m^2/day	
			Few cat owners in class, $n = 22$	14.2 ng/m^2/day	
			Air personal samples (pumps)		
			Many cat owners in class, $n = 22$	1.6 ng/m^3	
			Few cat owners in class, $n = 22$	0.3 ng/m^3	
Arbes et al. (65)	Day-care centers, USA	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from floors	GM	
			89 Day-care centers	1.43 μg/g	
			20 Day-care centers		
			Carpet	2.28 μg/g	
			Smooth floor	0.39 μg/g	
Tranter et al. (66)	Schools and day-care centers, USA	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from floors	Median	IQR
			Schools		
			Carpet, $n = 79$	0.28 μg/m^2	0.14–0.77 μg/m^2
			Tile, $n = 65$	0.014 μg/m^2	0.006–0.027 μg/m^2
			Day-care centers		
			Carpet, $n = 42$	0.42 μg/m^2	0.14–0.9 μg/m^2
			Furniture, $n = 18$	1.6 μg/m^2	0.74–4.0 μg/m^2
Cai et al. (67)	Day-care centers, Sweden	Fel d 1 Sandwich ELISA (mAB)	Petri dishes (30–40 days)	GM	Range
			Diverse rooms, $n = 97$	9.4 ng/m^2/day	0.9–78.6 ng/m^2/day
Permaul et al. (68)	Schools and homes, USA	Fel d 1 MARIA (mAB)	Reservoir dust	GM	Range
			Schools, $n = 229$	0.19 μg/g	0.004–285.8 μg/g
			Homes (bedroom), $n = 118$	0.06 μg/g	0.004–392.3 μg/g
			Air samples (ion-charging device)		
			Schools, $n = 196$	1.82 ng/m^3	
Custovic et al. (69)	Hospitals, UK	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from upholstered chairs, $n = 42$	GM 22.9 μg/g	Range 4.5–58 μg/g
			Air samples (high volume pumps)		<LOD–0.22 ng/m^3
Partti-Pellinen et al. (70)	Public transport vehicles, Finland	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust	Median	Range
			Seats, $n = 8$	0.87 μg/g	0.003–2.6 μg/g
			Floors, $n = 10$	0.01 μg/g	0.002–0.08 μg/g
Macher et al. (71)	Offices, USA	Fel d 1 Sandwich ELISA (mAB)	Reservoir dust from floors	Median	Range
			92 offices, $n = 251$	0.3 μg/g	<LOD–19 μg/g
Samadi et al. (72)	Animal hospital, Netherlands	Fel d 1 Sandwich ELISA + MARIA (mAB)	Different locations	GM	Range
			Air personal samples (pumps), $n = 67$	0.3 ng/m^3	<LOD–9.4 ng/m^3
			Electrostatic cloths (14 days), $n = 30$	56 ng/m^2	<LOD–579 ng/m^2
			Reservoir dust from floors, $n = 110$	11 ng/m^2	0.2–183 ng/m^2

mAB, monoclonal antibodies; GM, geometric mean; IQR, interquartile range; CI, confidence interval; LOD, limit of detection.

[a] *House index represents the mean of the sample location concentrations.*

[b] *95% Range calculated as log mean plus and minus two standard deviations and back-transformed.*

Table 2 | Studies related to exposure assessment to dog allergen.

Study	Environment/ country	Assay	Sampling method	Allergen level	
Custovic et al. (58)	Homes, UK	Can f 1 Sandwich ELISA (mAB/pAB)	Air samples (high volume pumps)		Range
			Homes with dogs, $n=31$		0.5–99 ng/m^3
			Homes without dogs, $n=62$		<LOD–12.4 ng/m^3
			Reservoir dust from living room floor	GM	95%CI
			Homes with dogs, $n=31$	181.3 μg/g	102.0–322.3 μg/g
			Homes without dogs, $n=62$	1.56 μg/g	1.17–2.08 μg/g
Custis et al. (59)	Homes, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Air samples (ion-charging device)	GM	
			Homes with dogs		
			Living room, $n=17$	1.48 μg/day	
			Bedroom, $n=10$	2.80 μg/day	
			Homes without dogs		
			Living room, $n=27$	<LOD	
			Bedroom, $n=19$	0.116 μg/day	
Arbes et al. (60)	Homes, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust	GM	
			House index[a], $n=825$	4.69 μg/g	
			Bedroom bed, $n=682$	2.48 μg/g	
			Bedroom floor, $n=718$	2.99 μg/g	
			Living room floor, $n=731$	3.61 μg/g	
			Living room sofa, $n=690$	5.49 μg/g	
			Homes with dogs, $n=247$	69.23 μg/g	
			Homes without dogs, $n=570$	1.33 μg/g	
Simons et al. (62)	Homes, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from bedroom	GM	
			Inner city, $n=98$	0.38 μg/g	
			Suburban, $n=19$	5.5 μg/g	
			Homes with dogs	13.4 μg/g	
			Homes without dogs	0.17 μg/g	
Nicholas et al. (73)	Homes, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from bedroom floor	GM	95% CI
			Homes with dogs, $n=254$	1.24 μg/g	0.91–1.71 μg/g
			Dogs indoors, $n=219$	1.59 μg/g	1.14–2.21 μg/g
			Dogs outdoors only, $n=30$	0.13 μg/g	0.07–0.23 μg/g
			Homes without dogs, $n=738$	0.055 μg/g	0.048–0.063 μg/g
Bertelsen et al. (63)	Homes, Norway	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from mattresses	GM	95% CI
			All homes, $n=797$	0.61 μg/g	0.53–0.73 μg/g
			Girls, $n=360$	0.78 μg/g	0.62–0.98 μg/g
			Boys, $n=437$	0.50 μg/g	0.41–0.62 μg/g
			Homes without dogs, $n=674$	0.31 μg/g	0.27–0.34μg/g
			Girls, $n=297$	0.37 μg/g	0.31–0.44 μg/g
			Boys, $n=374$	0.26 μg/g	0.23–0.30 μg/g
Wardzynska et al. (75)	Homes, Poland	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from mattresses	Median	IQR
			Homes with dogs		
			Rural, $n=13$	0.55 μg/g	0.17–2.47 μg/g
			Urban, $n=57$	5.0 μg/g	3.25–7.07μg/g
			Homes without dogs		
			Rural, $n=176$	0.35 μg/g	0.22–0.64 μg/g
			Urban, $n=84$	1.55 μg/g	0.85–2.44μg/g
Vredegoor et al. (55)	Homes, Netherlands	Can f 1 Sandwich ELISA (mAB/pAB)	Electrostatic cloths (28 days)	GM	
			Homes of dog owners, $n=168$		
			Labradoodle, $n=54$	5.22 μg/m^2	

(Continued)

Table 2 | Continued

Study	Environment/ country	Assay	Sampling method	Allergen level	
			Labrador retriever, $n = 25$	5.04 $\mu g/m^2$	
			Poodle, $n = 23$	6.32 $\mu g/m^2$	
			Spanish waterdog, $n = 13$	9.05 $\mu g/m^2$	
			Airedale terrier, $n = 22$	8.44 $\mu g/m^2$	
			Rest, $n = 31$	7.18 $\mu g/m^2$	
Perzanowski et al. (64)	Schools and homes, Sweden	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust	GM	Range
			Schools, $n = 176$	3.3 $\mu g/g$	<LOD–176 $\mu g/g$
			Desk and chairs, $n = 64$	15 $\mu g/g$	0.45–176 $\mu g/g$
			Floors, $n = 64$	1.1 $\mu g/g$	<LOD–60 $\mu g/g$
			Homes without pets, $n = 74$	2.0 $\mu g/g$	<LOD–22 $\mu g/g$
			Homes with dogs, $n = 29$	79 $\mu g/g$	13–625 $\mu g/g$
Arbes et al. (65)	Day-care centers, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from floors	GM	
			89 Day-care centers	2.06 $\mu g/g$	
			20 Day-care centers		
			Carpet	2.13 $\mu g/g$	
			Smooth floor	0.29 $\mu g/g$	
Tranter et al. (66)	Schools and day-care centers, USA	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust	Median	IQR
			Schools		
			Carpet, $n = 79$	0.45 $\mu g/m^2$	0.23–1.4 $\mu g/m^2$
			Smooth floor, $n = 65$	0.03 $\mu g/m^2$	0.014–0.053 $\mu g/m^2$
			Day-care centers		
			Carpet, $n = 42$	0.44 $\mu g/m^2$	0.18–0.96 $\mu g/m^2$
			Furniture, $n = 18$	1.1 $\mu g/m^2$	0.40–2.2 $\mu g/m^2$
Cai et al. (67)	Day-care centers, Sweden	Can f 1 Sandwich ELISA (mAB/pAB)	Petri dishes (30–40 days)	GM	Range
			Diverse rooms, $n = 97$	7.2 ng/m^2/day	1.2–72.5 ng/m^2/day
Permaul et al. (68)	Schools and homes, USA	Can f 1 MARIA (mAB)	Reservoir dust	GM	Range
			Schools, $n = 229$	0.08 $\mu g/g$	0.004–285.8 $\mu g/g$
			Homes (bedroom), $n = 118$	0.03 $\mu g/g$	0.004–392.3 $\mu g/g$
			Air samples (ion-charging device)		
			Schools, $n = 196$	1.17 ng/m^3	
Custovic et al. (74)	Public places, UK	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust		Range
			Total (5 schools, 6 hotels, 4 cinemas, 6 pubs,		0.2–52.5 $\mu g/g$
			3 buses, 2 trains)	GM	95% CI
			Upholstered seats	9.4 $\mu g/g$	6.4–13.9 $\mu g/g$
			Carpets	1.5 $\mu g/g$	1.3–1.7 $\mu g/g$
Custovic et al. (69)	Hospitals, UK	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust from upholstered	GM	Range
			chairs, $n = 42$	21.6 $\mu g/g$	4.0–63 $\mu g/g$
			Air stationary samples (pumps), $n = 10$		0.12–0.56 ng/m^3
Partti-Pellinen et al. (70)	Public transport vehicles, Finland	Can f 1 Sandwich ELISA (mAB/pAB)	Reservoir dust	Median	Range
			Seats, $n = 8$	2.4 $\mu g/g$	0.02–8.5 $\mu g/g$
			Floors, $n = 10$	0.2 $\mu g/g$	0.004–0.86 $\mu g/g$
Samadi et al. (72)	Animal hospital, Netherlands	Can f 1 Sandwich ELISA + MARIA (mAB)	Different locations	GM	Range
			Air personal samples (pumps), $n = 67$	3.6 ng/m^3	<LOD–73.3 ng/m^3
			Electrostatic cloths (14 days), $n = 30$	720 ng/m^2	<LOD–12,105 ng/m^2
			Reservoir dust from floors, $n = 110$	7.1 ng/m^2	<LOD–13,644 ng/m^2

mAB, monoclonal antibodies; pAB, polyclonal antibodies; GM, geometric mean; IQR, interquartile range; CI, confidence interval; LOD, limit of detection.

[a] *House index represents the mean of the sample location concentrations.*

also detectable in active airborne dust (ion-charging device) from schools (68) and passive airborne dust (Petri dishes) from day-care centers (67). Karlsson et al. (29) have shown that airborne Fel d 1 concentrations in schools were strongly dependent on the number of children with cats at home. Median cat allergen levels in both Petri dishes and personal air samples were approximately fivefold higher in classes with many cat owners (>20%) compared to classes with few cat owners (<10%).

The widespread distribution of cat and dog allergens is further demonstrated by several studies carried out in other public indoor environments. Fel d 1 and Can f 1 have been measured in reservoir dust from seats and floors inside public buildings such as hotels, cinemas and pubs (74), hospitals (69), office buildings (71), and in public transport vehicles such as buses, trams, and trains (70, 74). In general, seats were more contaminated with pet allergens compared to floors, presumably because they come in direct contact with clothing.

Reports on measuring exposure to pet allergens in occupational settings are rare. One study investigated the allergen levels in veterinary medicine students, and workers in a companion animal hospital using various dust collection methods throughout different locations (72). In personal airborne samples, significant differences in exposure levels were found for Fel d 1 and Can f 1 between the different performed tasks. The highest exposure was observed in the intensive care unit and during practical animal courses for students. Allergen concentrations varied greatly between different locations, with the highest levels measured in the examination and waiting rooms (reservoir dust) and in the intensive care unit (electrostatic cloths) and the lowest in the operating room (both methods). Exposure levels in dust captured by diverse sampling methods only correlated moderately with each other.

RODENTS

Allergy to mice and rats is an important occupational health problem, primarily because these animals are the most widely used in medical research. Rodent allergy is commonly observed among technicians, animal caretakers, physicians, and scientists who work in pharmaceutical industries, university laboratories, and animal breeding facilities. In occupational settings, the prevalence rates of rodent allergies vary from 11 to 44% depending on the diagnostic methods (questionnaire or laboratory testing) used (76). The prevalence of mouse and rat allergy is very similar. In a large cross-sectional survey from Japan that included over 5000 laboratory animal workers, 26% reported allergic symptoms to mice and 25% to rats (39). Sensitization and work-related symptoms occur at the latest, 2–3 years after the initial exposure to laboratory animals (77). Besides an atopic background, the most important risk factor for the development of an allergy to rodents is the level of exposure to laboratory animal allergens. Children of parents who are occupationally exposed to laboratory animals were shown to have more frequently positive skin prick tests against mice, rats, and hamsters compared to children of non-exposed parents (78). Studies from the United States have demonstrated that rodent allergen exposure in domestic environments is also clinically relevant. In inner city children with asthma, the prevalence of mouse sensitization (skin test sensitivity) ranged from 11 to 46% (79), and the prevalence of rat sensitization was estimated to be 21% (80). A recent study from Europe has reported a very low sensitization prevalence (1.6% for mouse and 0.6% for rat) in urban atopic populations without occupational exposure (81).

MOUSE (*MUS MUSCULUS*)

Rodent allergens can be found in dander, hair, urine, saliva, and serum. Urine is the main source of allergenic proteins in both mice and rats. The major mouse allergen, Mus m 1 (about 19 kDa) is a prealbumin and lipocalin–odorant binding protein (82) belonging to the rodent family of major urinary proteins (MUP) (83). MUPs are produced in the liver and other exocrine glands under hormonal control. Mouse MUPs are encoded by 35 genes, with 15 forms detectable in urine. The expression of MUPs varies according to species, strains, sexes, and individuals. MUPs seem to play a complex role in chemosensory signaling among rodents. Study of particle size distribution revealed that airborne Mus m 1 is carried on particles with aerodynamic diameter ranging from 0.4 to >10 μm, with the majority on particles between 3.3 and 10 μm (84).

To quantify mouse allergens, sandwich ELISAs were developed by several groups using polyclonal antibodies produced in rabbit or sheep (84–86). In these studies, either the purified Mus m 1 or the whole protein from mouse urine was used as the immunogen or standard. Therefore, some assays are known in the literature as mouse urinary allergen (MUA) or MUP. A polyclonal antibody-based assay is commercially available as the Mus m 1 ELISA kit or as a component of the MARIA (Indoor Biotechnologies).

Numerous studies of mouse allergen exposure levels were conducted in occupational settings such as laboratory animal facilities of universities, research institutes, and pharmaceutical companies (**Table 3**). Ohman et al. (84) demonstrated that Mus m 1 is widely distributed in the air of a major mouse breeding facility, even in rooms that do not house mice (e.g., offices). This was shown for stationary as well as for personal air samples. Direct contact to mice was associated with the highest Mus m 1 levels (up to 560 ng/m^3). Within mouse rooms, airborne Mus m 1 levels were strongly correlated with the number of mice in the room. Another large-scale study (seven different facilities) investigated the personal allergen exposure intensity according to the type of job and the type of tasks performed (85). Animal technicians and caretakers had elevated median MUA exposure levels compared with the scientific staff and supervisors. Removal of contaminated bedding from cages and moving animals into new cages were associated with the highest personal exposure; lower exposures were seen with feeding or handling the animals. However, mouse allergen concentrations during tasks varied enormously among different facilities (<LOD–2000 ng/m^3), probably due to the differences in cleaning practices and feeding technologies, or the local ventilation equipment.

A recent study from the Jackson Laboratory assessed mouse allergen exposure across a range of jobs, including non-mouse handlers (87). Although mouse handlers had significantly higher median levels of mouse allergen than non-handlers (4.13 vs. 0.21 ng/m^3), 71% of administrative/support personnel and 68% of materials/supplies handlers had detectable mouse allergen levels, in some cases in concentrations similar to those measured in animal caretakers. Among mouse handlers, those involved

Table 3 | Studies related to exposure assessment to mouse allergen.

Study	Environment/ country	Assay	Sampling method	Allergen level	
Ohman et al. (84)	Mouse facility, USA	Mus m 1 Sandwich ELISA (pAB)	Air samples		
			Stationary	Range of AM	
			Mouse rooms	0.5–15.1 ng/m^3	
			Offices and lunch room	0.2–1.5 ng/m^3	
			Personal		
			Mouse rooms	16.6–563 ng/m^3	
			Offices and lunch room	1.2–2.7 ng/m^3	
Hollander et al. (85)	Laboratory animal facilities, Netherlands	MUA Sandwich ELISA (pAB)	Air samples (personal)		
			Job categories, $n = 171$	Median	
			Animal caretaker, $n = 63$	12.1 ng/m^3	
			Animal technicians, $n = 94$	6.4 ng/m^3	
			Scientists, $n = 2$	2.7 ng/m^3	
			Supervisor, $n = 12$	0.58 ng/m^3	
			Task categories, $n = 123$	GM	Range
			Cage emptying, $n = 25$	74.8 ng/m^3	<LOD–2700 ng/m^3
			Changing animals, $n = 33$	22.8 ng/m^3	<LOD–501 ng/m^3
			Feeding animals, $n = 19$	19.6 ng/m^3	<LOD–542 ng/m^3
			Handling animals, $n = 8$	16.0 ng/m^3	<LOD–209 ng/m^3
			Experiments, $n = 2$	33.5 ng/m^3	8–140 ng/m^3
			Biotechnical work, $n = 11$	5.4 ng/m^3	<LOD–51 ng/m^3
			Cage wash, $n = 8$	2.6 ng/m^3	<LOD–89 ng/m^3
			Cleaning rooms, $n = 17$	2.1 ng/m^3	<LOD–151 ng/m^3
Curtin-Brosnan et al. (87)	Mouse facility, USA	Mus m 1 Sandwich ELISA (pAB)	Air samples (personal)	Median	IQR
			Mouse handlers, $n = 97$	4.13 ng/m^3	0.70–12.12 ng/m^3
			Non-mouse handlers, $n = 71$	0.21 ng/m^3	<LOD–0.63 ng/m^3
			Job categories		Range
			Animal caretaker, $n = 57$	9.6 ng/m^3	0.58–220.9 ng/m^3
			Administrative personnel, $n = 34$	0.23 ng/m^3	<LOD–30.94 ng/m^3
			Supplies/Material handler, $n = 19$	0.63 ng/m^3	<LOD–423.9 ng/m^3
			Task categories (mouse handlers)		IQR
			Animal care, $n = 42$	8.73 ng/m^3	3.56–18.68 ng/m^3
			Husbandry, $n = 26$	5.83 ng/m^3	3.26–14.95 ng/m^3
			Experiments, $n = 25$	0.36 ng/m^3	0.07–1.77 ng/m^3
Renström et al. (86)	Laboratory animal facility, Sweden	MUA Sandwich ELISA (pAB)	Air samples (stationary)	Median	IQR
			Open cages, $n = 11$	44 ng/m^3	36–45 ng/m^3
			IVC 1, $n = 13$	0.62 ng/m^3	<LOD–2.4 ng/m^3
			IVC 2, $n = 15$	4.3 ng/m^3	2.7–8.8 ng/m^3
Thulin et al. (88)	Laboratory animal facility, Sweden	MUA Sandwich ELISA (pAB)	Air samples (personal)		
			Cage changing	GM	Range
			Unventilated table, $n = 5$	77.3 ng/m^3	65.1–88 ng/m^3
			Ventilated changing wagon, $n = 5$	17.2 ng/m^3	14.0–20.8 ng/m^3
			Handling animals		
			Outside ventilated bench, $n = 9$	87.2 ng/m^3	34.8–220 ng/m^3
			Ventilated bench, $n = 6$	2.1 ng/m^3	0.6–9.8 ng/m^3
Krop et al. (25)	Homes of animal caretakers, Netherlands	MUA Sandwich ELISA (pAB)	Reservoir dust from mattresses	GM	95% CI
			Laboratory animal workers, $n = 15$	29.5 ng/g	11.7–74.6 ng/g
			Controls (no contact to animals), $n = 15$	8.8 ng/g	4.6–16.8 ng/g

(Continued)

Table 3 | Continued

Study	Environment/ country	Assay	Sampling method	Allergen level	
Phipatanakul et al. (89)	Homes, USA	Mus m 1 Sandwich ELISA (pAB)	Reservoir dust from bed, furniture, floor	Median	Range
			Bedroom, $n = 506$	0.52 μg/g	<LOD–294 μg/g
			Living room, $n = 608$	0.57 μg/g	<LOD–203 μg/g
			Kitchen, $n = 559$	1.60 μg/g	<LOD–618 μg/g
Matsui et al. (90)	Homes, USA	Mus m 1 Sandwich ELISA (pAB)	Reservoir dust from bed, furniture, floor		
			Inner city	Median	IQR
			Bedroom, $n = 78$	0.76 μg/g	0.16–3.21 μg/g
			Living room, $n = 77$	0.99 μg/g	0.18–5.59 μg/g
			Kitchen, $n = 75$	2.48 μg/g	0.27–18.95 μg/g
			Suburban		
			Bedroom, $n = 257$	0.012 μg/g	<LOD–0.048 μg/g
			Living room, $n = 250$	0.016 μg/g	<LOD–0.044 μg/g
			Kitchen, $n = 250$	0.007 μg/g	<LOD–0.050 μg/g
Simons et al. (62)	Homes, USA	Mus m 1 Sandwich ELISA (pAB)	Reservoir dust from beds and floors		
			Bedroom	GM	
			Inner city, $n = 98$	3.2 μg/g	
			Suburban, $n = 19$	0.013 μg/g	
			Air samples		
			Bedroom		
			Inner city, $n = 98$	0.055 ng/m^3	
			Suburban, $n = 19$	0.016 ng/m^3	
Chew et al. (91)	Schools, USA	MUP Sandwich ELISA (pAB)	Reservoir dust from floors	Range of GM	Range of samples
			11 Schools, 87 classrooms	0.21–133 μg/g	<LOD–1125 μg/g
Arbes et al. (65)	Day-care centers, USA	Mus m 1 Sandwich ELISA (pAB)	Reservoir dust from floors	GM	
			89 Day-care centers	0.01 μg/g	
			20 Day-care centers		
			Carpet	0.008 μg/g	
			Smooth floor	0.004 μg/g	
Sheehan et al. (92)	Schools and homes, USA	MUP Sandwich ELISA (pAB)	Reservoir dust	GM	Range
			Schools (floor, desks, chairs), $n = 46$	1.66 μg/g	<LOD–238 μg/g
			Homes (bedroom), $n = 38$	0.41 μg/g	<LOD–6.97 μg/g
Permaul et al. (68)	Schools and homes, USA	Mus m 1 MARIA (pAB)	Reservoir dust	GM	Range
			Schools (floor, desks, chairs), $n = 229$	0.65 μg/g	0.001–544.4 μg/g
			Homes (bedroom), $n = 118$	0.10 μg/g	0.002–82.6 μg/g
			Air samples		
			Schools, $n = 196$	1.80 ng/m^3	

MUA, mouse urinary allergens; pAB, polyclonal antibodies; AM, arithmetic mean; GM, geometric mean; IQR, interquartile range; CI, confidence interval; LOD, limit of detection.

in animal care or husbandry had higher allergen levels than those conducting laboratory experiments. Several studies examined the effects of caging systems and various ventilation and automation measures in reducing allergen levels. For example, in undisturbed animal rooms mouse allergen levels were much lower using individually ventilated cage (IVC) system compared to open cages (86). Using a ventilated cage changing wagon or handling animals on ventilated benches also resulted in lower exposure levels (88). Krop et al. (25) investigated the spreading of laboratory animal allergens outside the animal facilities. The authors found that levels of rodent allergens were significantly higher in mattress dust of laboratory animal workers compared with those of unexposed controls. In addition, high amounts of mouse allergens were recovered from hair-covering cups (not routinely used by laboratory animal workers), and therefore the authors concluded that the transfer of allergens via uncovered hair was the most likely cause for the spread of these allergens to the home.

In the past decade, the importance of rodent allergens outside of the workplace has been demonstrated in several studies (**Table 3**). Mouse allergen seems to be widespread in US communities and may be regarded as an environmental allergen. Phipatanakul et al. (89) analyzed house dust samples (bedroom, living room, and kitchen) from 608 homes of major inner city areas (New York, Baltimore, Chicago, Cleveland, Detroit, St Louis, Washington). Ninety-five percent of all homes had detectable Mus m 1 in at least one room, with the highest levels found in kitchens. Matsui et al. (90) compared the distribution of mouse allergen between inner city and suburban homes (surroundings of Baltimore). The prevalence of Mus m 1 was lower in suburban homes (e.g., bedrooms: 69 vs. 94%) and at approximately 100-fold lower concentrations (e.g., living room: 0.99 vs. 0.016 μg/g), compared with homes in the inner city. These differences were confirmed by another study based in Baltimore that analyzed both air samples and settled dust from bedrooms (62). The inner city homes also had significantly higher airborne mouse allergen levels than suburban homes, reflecting the higher rate of reported mouse infestation (80 vs. 5%) in cities.

Quantification of mouse allergen was also performed in school and day-care settings. Mouse allergen was detectable in 81% of settled dust samples collected from 11 schools in a major metropolitan area in the northeastern USA (91). Mouse allergen levels varied greatly between schools with geometric means (GM) ranging from 0.21 to 133 μg/g. In contrast, very low Mus m 1 (GM: 0.01 μg/g) concentrations were found in 86 day-care facilities in North Carolina, with a prevalence of 83% of all samples collected (65). The aim of two other studies was to investigate allergen exposure in schools compared with homes (68, 92). Both studies reported significantly higher levels of mouse allergen in classrooms vs. students' bedrooms (settled dust samples). Mus m 1 was also detectable in airborne samples from schools using a very sensitive MARIA multiplex array. Airborne and settled dust Mus m 1 levels in classrooms were moderately correlated ($r = 0.48$; $p < 0.0001$).

RAT (*RATTUS NORVEGICUS*)

Analogous to mouse allergens, the major rat allergen Rat n 1 (about 17 kDa) is a prealbumin or α-2u-globulin that belongs to the lipocalin group and to the family of MUPs. The amino acid identity between mouse and rat MUPs is approximately 65% (83, 93). A major difference between species is that MUPs are glycosylated in rats but not in mice. Furthermore, the urine of male rats contains much larger quantities of Rat n 1 than urine collected from female rats. Airborne rat allergen was detected on particles raging from >0.5 to 20 μm with the majority on particles larger than 8 μm (94).

The ELISA assays for rat allergen measurements are based on polyclonal antibodies against whole protein isolated from rat urine (85), or monoclonal antibodies against the major allergen Rat n 1 (80, 95). One assay based on the monoclonal antibodies RUP1 and RUP6 is commercially available by Indoor Biotechnologies as the Rat n 1 ELISA kit or as a component of the MARIA.

As for mice, occupational exposure to rat allergens is well documented in the literature, but with fewer published studies. For many studies, exposure to rodent allergens in the workplace was assessed in parallel, with the same findings (**Table 4**). Briefly, rat allergen levels in laboratory animal facilities are dependent on room, job, and task (85, 96). More specifically, airborne Rat n 1 was significantly higher in rat rooms than in experimental rooms, and the highest personal exposure was measured for animal technicians and caretakers, compared with students and scientists. Cage cleaning resulted in much higher rat allergen levels than animal handling. In addition, exposure varied strongly with facility. One study assessed the individual exposure to rodent allergens using nasal air samplers and demonstrated the effectiveness of personal respiratory protection equipment (97). This unique sampling device is worn inside the nostrils and controlled by the wearer's breathing. The inhaled particles are collected by impaction on adhesive tape within the samplers. Using this method, clear differences in allergen levels (2.6 vs. 0.1 ng/h) were seen between high exposure tasks (manual cage emptying, animal handling) and low exposure tasks (automated cage changing, supervision). In addition, nasal air sampling correlated well with conventional sampling using air pumps ($r = 0.8$). The study also clearly showed that the use of face masks decreased the amount of inhaled allergen by about 90%. Rat allergen was also detected in dust collected from mattresses of laboratory animal workers (25).

In contrast to mice, the distribution of rat allergens outside of occupational settings is not well studied, probably due to much lower prevalence of rats indoors. Thus far, only one study has examined rat allergen exposure in the home environment (80) and reported Rat n 1 in 33% of inner city homes (settle dust samples). This is in contrast to mouse allergen, which was detectable in 95% of the homes. Rat allergen was more common in the TV/living room (27%) than in the kitchen (19%) and bedroom (21%). The median level for all rooms was below the limit of detection, and there was no correlation between rat and mouse allergens in dust samples. The authors attributed this dissimilarity to the differences in nesting habits between the both rodents. Rats do not nest in buildings, but rather build their nests in underground burrows in close proximity to water. Mice on the other hand are more likely to live indoors and nest near food stores. These territorial differences were compatible with reported rodent infestations, where 51% of families reported mice infestation, whereas only 8% reported problems with rats. Another possible explanation for the disparity between the prevalence of mouse and rat allergen levels is that the rat allergen assay was not sensitive enough to detect allergens in these samples. A second study focusing on allergen exposure in urban schools and homes assessed Rat n 1 concentrations in settled and airborne dust using the very sensitive MARIA technology (68). Again, no significant differences in rat allergen levels were detected in this sample set, suggesting that rat allergen exposure may occur primarily outdoors.

HORSE (*EQUUS CABALLUS*)

Horse allergy mainly affects people who are in direct contact to horses, either occupationally or for recreational purposes. Exposed subjects are farmers, stable-workers, breeders, veterinarians, and horse owners or riders. The prevalence of horse sensitization in occupational settings varies between 3.6 and 16.5% (98). However, the sensitization rate in the general population is not well known. Some cases of horse allergy, despite a lack of regular exposure, have been described in children (99) and adults (100). A

Table 4 | Studies related to exposure assessment to rat allergen.

Study	Environment/ Country	Assay	Sampling method	Allergen level	
Hollander et al. (85)	Laboratory animal facilities, Netherlands	RUA Sandwich ELISA (pAB)	Air samples (personal)	Median	
			Job categories, $n = 251$		
			Animal caretaker, $n = 90$	1.6 ng/m^3	
			Animal technicians, $n = 87$	0.77 ng/m^3	
			Supervisor, $n = 14$	0.65 ng/m^3	
			Scientists, $n = 51$	<LOD	
			Scientific assistant, $n = 9$	<LOD	
			Task categories, $n = 196$	GM	Range
			Cage emptying, $n = 29$	5.6 ng/m^3	<LOD–1600 ng/m^3
			Changing animals, $n = 35$	5.3 ng/m^3	<LOD–127 ng/m^3
			Feeding animals, $n = 20$	2.4 ng/m^3	<LOD–60 ng/m^3
			Handling animals, $n = 34$	1.2 ng/m^3	<LOD–94 ng/m^3
			Experiments, $n = 25$	0.85 ng/m^3	<LOD–9.0 ng/m^3 s
			Biotechnical work, $n = 23$	0.83 ng/m^3	<LOD–34 ng/m^3
			Cage wash, $n = 8$	0.81 ng/m^3	<LOD–4.9 ng/m^3
			Cleaning rooms, $n = 15$	0.80 ng/m^3	<LOD–14 ng/m^3
Lieutier-Colas et al. (96)	Laboratory animal facilities, France	Rat n 1 Sandwich ELISA (mAB)	Air samples	Range of GM	
			12 Facilities (personal), $n = 113$	0.49–48.96 ng/m^3	
			12 Facilities (stationary), $n = 128$	0.43–27.36 ng/m^3	
			Room categories (stationary)	AM	
			Rat rooms, $n = 65$	53.1 ng/m^3	
			Experimental rooms, $n = 56$	9.7 ng/m^3	
			Job categories (personal)	AM	
			Animal technicians, $n = 27$	72.3 ng/m^3	
			Laboratory technicians, $n = 26$	21.2 ng/m^3	
			Students, $n = 33$	33.7 ng/m^3	
			Scientist, $n = 27$	24.0 ng/m^3	
			Task categories (personal)		
			Cage cleaning and rat feeding, $n = 34$	91.1 ng/m^3	
			Handling rats, $n = 31$	5.4 ng/m^3	
Renström et al. (97)	Laboratory animal facility, Sweden	Rat n 1 Sandwich ELISA (mAB)	Nasal air samplers	Median	IQR
			High exposure		
			No mask, $n = 11$	2.6 ng/h	0.6–5.0 ng/h
			P2-mask, $n = 10$	0.06 ng/h	<LOD–0.7 ng/h
			Low exposure		
			No mask, $n = 25$	0.1 ng/h	<LOD–0.2 ng/h
			P2-mask, $n = 10$	<LOD	<LOD–<LOD
Krop et al. (25)	Homes of laboratory animal workers, Netherlands	RUA Sandwich ELISA (pAB)	Reservoir dust from mattresses	GM	95% CI
			Laboratory animal workers, $n = 15$	39.3 ng/g	19.8–78.0 ng/g
			Controls (no contact to animals), $n = 15$	7.6 ng/g	4.7–12.2 ng/g
Perry et al. (80)	Homes (inner city), USA	Rat n 1 Sandwich ELISA (mAB)	Reservoir dust from bed, furniture, floor	Median	Range
			Bedroom, $n = 602$	<LOD	<LOD–1413 ng/g
			Living room, $n = 603$	<LOD	<LOD–3380 ng/g
			Kitchen, $n = 556$	<LOD	<LOD–4620 ng/g

RUA, rat urinary allergens; pAB, polyclonal antibodies; AM, arithmetic mean; GM, geometric mean; IQR, interquartile range; CI, confidence interval; LOD, limit of detection.

retrospective study of horse allergy revealed a sensitization rate of 2.7% in a population of 23,460 children who underwent skin prick testing (101). A recent Italian multicenter study in an urban population with respiratory allergy reported that the prevalence of sensitization to horse dander was 5.38% among atopic subjects (102). Only 27% of horse-sensitized patients reported direct

contact with the animals. Therefore, the authors of both studies recommended that the horse allergen be included in the standard panel for the diagnosis of respiratory allergy.

Several horse allergens have been identified and characterized, with Equ c 1 reported as the most important. Equ c 1 is found at high concentrations in dander and saliva, as well as in small quantities in urine (103). The 22-kDa glycoprotein belongs to the lipocalin family (104) and has surfactant properties (105). The other proteins, Equ c 2, lipocalin (16 kDa); Equ c 3, horse serum albumin (65 kDa); Equ c 4 (18.7 kDa); and Equ c 5 (16.7 kDa), both latherins, were classified as minor allergens (11, 50). The analysis of the allergenic composition of horse dander from several breeds showed considerable inter-breed and within-breed variations but no breed-specific allergens (106). Moreover, all dander extracts contained the most important allergens.

The dispersion of horse allergens have been studied in different environments, including stables and their immediate surroundings as well as in public places such as schools and day-care centers (**Table 5**). Most of these studies were performed in Sweden, where the rate of horse ownership is high and horseback riding has become increasingly popular. To detect horse allergen in environmental samples, a sandwich ELISA based on monoclonal antibodies was developed and established by Emenius et al. (107). The two monoclonal antibodies, 103 and 14G4, were produced against horse dander extract, and recognize different epitopes of the same molecule, but the target protein was not further characterized. Immunoblotting analysis demonstrated antibody binding to a protein of around 16 kDa (108). However, the horse allergen could not be identified because Equ c 2, Equ c 4, and Equ c 5 are within similar molecular weight range. Therefore, the ELISA assay is often referred to as Equ c x in the literature. In 2009, Emenius et al. (109) assumed that the antibodies recognize the allergen Equ c 4, but further identification is necessary. The horse allergen assay is in the meantime, commercially available and provided as Equ c 4 ELISA (Indoor Biotechnologies) with horse hair extract as the calibration standard. All the studies described below used this monoclonal assay with minor modifications (different standards and detection limits). Allergen concentrations are expressed as units per milliliter, where 1 unit is equal to 1 ng protein of the horse standard.

The first study performed to detect horse allergen in the environment assessed airborne allergen levels at different distances from the stable (107). As expected, the highest allergen concentrations were measured inside the stable, and were approximately 500-fold higher than levels measured outdoors at the stable entrance. The levels of horse allergen declined rapidly with increasing distance from the stable and were not detected in air samples collected 40 m from the stable. A similar dispersal pattern was obtained with settled dust samples collected from hard surfaces wiped with compresses (allergen levels not stated). The dispersion of airborne horse allergen around the stable was also analyzed by Elfman et al. (108) during different seasons. The authors additionally investigated the influence of weather conditions such as temperature, relative and absolute humidity, wind speed and direction, and reported that horse allergen generally spread about 50 m from the stable and outdoor areas where horses were kept (e.g., pastures, riding grounds = source area). Depending on wind speed and direction, low levels of horse allergen (2–4 U/m^3) were sometimes detected at distances up to 500 m from the source area. Allergen levels did not correlate to air temperature or humidity, but were influenced by the seasons; concentrations in winter were lower than in summer. At the stable entrance, the median level in summer was 316 and 123 U/m^3 in winter, and in the source area 16 and 8.3 U/m^3, respectively. More rain in autumn and a frozen ground in winter were mentioned as possible explanations for the reduced levels of airborne allergen. The rapid decrease in horse allergen with increasing distance from the stable was confirmed by analyzing settled dust collected with electrostatic cloths. All but one sample collected 100 m from the source area were below the detection limit.

Further research by Emenius et al. (109) examined the transfer of horse allergen into homes located near the stables. In apartments, Petri dishes were placed indoors (living rooms) or outdoors (balconies). Only 6 out of 45 indoor samples had detectable horse allergen levels (three families with horse contact) and 16 out of 26 outdoor samples were positive. Indoor levels were about 1–2% of the outdoor levels. In the second part of this study, the dispersion of allergens was investigated using a very unusual method. At different distances from a horse track, aspen leaves were collected and then extracted. The allergen level at 1 m from the track was set to 100%. At a distance of 25 m from the track, <10% of the original allergen concentration was found. In conclusion, horse allergens seem to disperse poorly through the air as allergen levels drop quickly with increasing distance from the source.

The presence of horse allergens in schools was first investigated by Kim et al. (110). Settled dust from desks, chairs, and floors was collected from 8 primary schools and 23 classrooms ($n = 92$) in Sweden. Horse allergens were found at high levels in most classrooms (median 945 U/g), and asthma and respiratory symptoms were more common in schools where higher levels of horse allergens were measured. No information on the prevalence of horse ownership in the families was available, but common horse contact was expected due to the geographical region (rural suburb of Uppsala). These data were used for two further projects to compare the school environment in China (111) and Korea (112) using the same sampling strategy (settled dust) and analysis method (Equ c × ELISA). In contrast to Swedish classrooms, none of the Chinese samples ($n = 78$, 39 classrooms) contained horse allergen. In Korea, horse allergen was only detected in one sample ($n = 68$, 34 classrooms).

The aim of another study was to investigate the correlation between horse allergen levels in schools and the number of children, who came into contact with horses in their leisure time (113). Petri dish and vacuumed dust samples were collected in 116 classrooms from 35 primary and secondary schools situated in inner-cities and in rural locations (county of Uppsala). In classrooms where many children had regular horse contact (>12%), the levels of horse allergen were significantly higher (for both sampling methods) than in classrooms where children reported less or no contact. Furthermore, weekly measurements with Petri dishes were performed in 20 classrooms during a 10-week period. The results showed that horse allergen levels were strongly variable during the 10-weeks and the authors recommended repeated measurements when assessing indoor allergen exposure. Horse allergen contamination was also common in Swedish day-care

Table 5 | Studies related to exposure assessment to horse allergen.

Study	Environment/ country	Assay	Sampling method	Allergen level	
Emenius et al. (107)	Inside and outside a stable, Sweden	Equ c x Sandwich ELISA (mAB)	Air samples, $n = 17$	AM	
			Inside the stable	439000 U/m^3	
			Stable entrance	1140 U/m^3	
			10–20 m from stable	150 U/m^3	
			40–500 m from stable	<LOD	
Elfman et al. (108)	Inside and outside a stable, Sweden	Equ c x Sandwich ELISA (mAB)	Air samples (stationary)	Median	Range
			Inside the stable, $n = 12$	4300 U/m^3	1926–6272 U/m^3
			Stable entrance, $n = 10$	316 U/m^3	169–706 U/m^3
			Source area, $n = 49$	16 U/m^3	<LOD–203 U/m^3
			50 m from stable, $n = 27$	<LOD	<LOD–41 U/m^3
			100–500 m from stable, $n = 91$	<LOD	<LOD–24 U/m^3
			Electrostatic cloths, $n = 29$	Median	Range
			Source area	27000 U/m^2	3000–97000 U/m^2
			25–50 m from stable	30000 U/m^2	5000–195000 U/m^2
			>100 m from stable	<LOD	<LOD–3000 kU/m^2
Emenius et al. (109)	Homes nearby stables, vicinity of a horse track, Sweden	Equ c x Sandwich ELISA (mAB)	Petri dishes (14 days)	Positive samples n	
			Indoors (living rooms), $n = 45$	6 (Apartments < 20 m from stable or families with horse contact)	
			Outdoors (balconies), $n = 26$	16 (15 Apartments < 250 m from stable)	
			Aspen leaves	Allergen level	
			1 m from horse track	100%	
			10 m from horse track	31–37%	
			25 m from horse track	8–9%	
			No horse track	None	
Kim et al. (110)	Schools, Sweden	Equ c x Sandwich ELISA (mAB)	Reservoir dust from desks, chairs and floor, $n = 92$	Median	Range
				945 U/g	<LOD–31000 U/g
Merrit et al. (113)	Schools, Sweden	Equ c x Sandwich ELISA (mAB)	Reservoir dust from furniture and floor	GM	
			Total, $n = 116$	1343 U/g	
			Classes > 12% horse contact	2051 U/g	
			Classes < 12% horse contact	880 U/g	
			Petri dishes (14 days)	GM	
			Total, $n = 116$	73.9 U/m^2/day	
			Classes > 12% horse contact	96.2 U/m^2/day	
			Classes < 12% horse contact	65.7 U/m^2/day	
Cai et al. (67)	Day-care centers, Sweden	Equ c x Sandwich ELISA (mAB)	Petri dishes (30–40 days)	GM	Range
			Diverse rooms, $n = 97$	5 U/m^2/day	<LOD–208.7 U/m^2/day

mAB, monoclonal antibodies; GM, geometric mean; LOD, limit of detection; AM, arithmetic mean.

centers (67), where allergen was detected in 63% of Petri dish dust samples with geometric mean of 5 ng/m^2/day.

CATTLE (*BOS DOMESTICUS*)

Cattle allergy is almost exclusively associated with occupational exposure and occurs primarily in cattle farmers. Studies in Scandinavian countries found that 5–20% of farmers are sensitized to cattle allergens (114–116). Moreover, the German Cattle Allergy Study has indicated that 9.1% of 5627 farmers with occupational airway diseases were due to cattle allergies (117). The effect of the farming environment on sensitization to different allergens was investigated in children (118) and adults (119). For both, no significant differences were observed with respect to sensitization against most of the common allergens (e.g., house dust mites, pets, pollens). Only cattle sensitization was more prevalent among subjects living on farms compared with those not living on farms.

The main sources of bovine allergens are cow hair and dander, but allergens are also found in urine, saliva, whey, amniotic fluid, and beef (120). Early investigations of bovine materials have found 17 different antigenic components, several of which have been currently identified and characterized as allergens. The lipocalin Bos d 2 is the major respiratory allergen in cow dander (121, 122), with several isoforms in existence (123). It is produced in the sweat glands and transported to the skin surface as a carrier of pheromones (124). Other bovine allergens are: Bos d 3 (11 kDa), calcium-binding protein; Bos d 4 (14 kDa), α-lactoglobulin; Bos d 5 (18 kDa), β-lactoglobulin; Bos d 6 (67 kDa), serum albumin; Bos d 7 (150 kDa), immunoglobulin; and Bos d 8 (19–25 kDa), caseins (11, 50). Bos d 4, 5, and 8 are major allergens in cow's milk and play an important role in food allergy (125).

Measurements of epithelial bovine allergens have been carried out mainly in occupational settings, stables and homes of farmers (**Table 6**). To quantify allergen concentrations, several different methods have been developed including: (1) ELISA inhibition with polyclonal antibodies against bovine skin scrape (126), (2) Rocket immunoelectrophoresis with rabbit antiserum against the major allergen Bos d 2 (127), (3) sandwich ELISA with polyclonal antibodies against an extract prepared from hair of different cattle breeds (128), and (4) sandwich ELISA with anti-Bos d 2 monoclonal antibodies 3D4 and mAB1 (129). Recently, the last method with recombinant Bos d 2 as a reference standard was made commercially available (Indoor Biotechnologies).

In cow stables, levels of bovine allergen were estimated in airborne dust (129, 130) as well as in settled dust samples (128, 131). All studies reported very high and strongly variable allergen concentrations. Allergen levels differed up to 200-fold between stables and were about 1000-fold higher than in homes. Virtanen et al. (133) examined the long-term variability in airborne allergen concentrations and found that in some cow stables, bovine allergen levels tend to be low, whereas in others the levels are consistently high. These variations are likely explained by factors associated with stable characteristics, such as size, heating, ventilation, and construction details of the building. Measurements of bovine allergens were also performed in stables of other animals (128). Whereas, only trace amounts of allergens were detectable in horse, sheep, pig, and chicken stables, goat stables had slightly increased allergen levels, most likely due to cross-reactivity between cow and goat epithelia.

The presence of cattle allergens in homes of dairy farmers was initially investigated by Hinze et al. (127). Floor dust samples were collected in different rooms of homes from patients with cow hair asthma and analyzed for Bos d 2 using Rocket immunelectrophoresis. The quantities of Bos d 2 detected were dependent on the architectural setup and floor cover. The separation of barn and living quarters (not in the same building) led to a marked reduction in Bos d 2 levels in house dust. Bos d 2 concentrations were also considerably lower in carpets than on tiles or linoleum. Furthermore, high indoor Bos d 2 levels were shown to correlate with the degree of IgE sensitization.

Using the same analytical methods, allergen levels in homes were analyzed in relation to the exposure intensity of cattle farmers (131). Farmers with occupational asthma or rhinitis caused by cow dander were divided into three groups: (1) no contact with cattle (giving-up the cattle husbandry for at least 2 years), (2) indirect exposure through family members, and (3) regular contact with cattle. The results showed a highly significant association between level of exposure and level of allergen. The terminating or limiting contact to cows reduced Bos d 2 concentrations in both living room and mattress dust. The aim of a further study was to assess bovine allergen exposure in homes of cattle farmers by sampling settled airborne dust using electrostatic cloths (128). Cow hair allergens measured by polyclonal antibody-based ELISA were detected with a wide variation among the individual samples (0.3–900 $\mu g/m^2$). The results, categorized by room type, showed significantly higher allergen concentrations in changing rooms compared with living rooms, bedrooms, or kitchens. As a control, dust sampling was also performed in urban dwellings. Interestingly, although none of the household members had any contact with cattle farms, the majority of urban samples were positive in the assay, though at very low concentrations. The median of 0.2 $\mu g/m^2$ was 100-fold lower in comparison to farmer homes. Because, the dispersal of cattle allergens from rural to urban environments through the ambient air was quite implausible, the authors supposed that the positive results were caused by the cross-reactivity between human and pet hair or by the presence of bovine allergens derived from foods such as milk and beef. Follow-up analysis using monoclonal antibody-based ELISA (Indoor Biotechnologies) confirmed the presence of the major respiratory allergen Bos d 2 in these dust samples and extracts from foods [(134), EAACI abstract].

Finally, the distribution of cattle allergens was assessed at different distances to dairy facilities (132). The study was conducted in the Yakima Valley, Washington State, USA, where over 60 industrial scale dairies operate. Airborne samples were collected inside and outside homes and analyzed using Bos d 2 ELISA (Indoor Biotechnologies). Homes with resident dairy facility workers or cows on the premises were excluded to minimize the influence of occupational exposures on indoor environments. Similar to studies of dispersion of horse allergen, an allergen concentration gradient was observed. Outdoor and indoor results for airborne Bos d 2 showed the highest concentrations at proximal homes closest to dairies (within a 1/4 mile, 0.4 km), and lowest concentrations in distal homes farthest from dairies (>3 miles, 4.8 km). Median outdoor levels of Bos d 2 were significantly higher at proximal and intermediate homes compared with indoor levels.

SUMMARY

Measurements of animal allergens have been extensively performed during the past few decades. Allergen exposure to animal allergens occurs in a wide range of indoor environments including homes, educational facilities, workplaces, and different kind of public buildings and modes of public transportation. Mostly, settled dust and airborne dust samples were collected to measure animal allergen levels. The variability of allergen concentrations in a particular environment is high and dependent on numerous factors, the most important being the presence of animals. Highest allergen levels have generally been found in homes with pets, laboratory animal facilities housing mice or rats, and cow or horse stables. However, high allergen levels have also been frequently detected in locations where no animals reside (e.g., schools and public places), most likely due to passive transfer via human

Table 6 | Studies related to exposure assessment to cattle allergens.

Study	Environment/country	Assay	Sampling method	Allergen level	
Virtanen et al. (130)	Stables, Finland	Bovine epithelial antigen ELISA inhibition (pAB)	Air samples (stationary)	AM	Range
			Feeding passage, $n = 18$	350 ng/m^3	40–2700 ng/m^3
			Manure passage, $n = 18$	730 ng/m^3	40–9500 ng/m^3
Ylönen et al. (129)	Stables, Finland	Bos d 2 Sandwich ELISA (mAB)	Air samples (stationary)	AM	Range
			Stables, $n = 19$	280 ng/m^3	54–804 ng/m^3
Hinze et al. (127)	Homes of farmers, Germany	Bos d 2 Rocket immunelectrophoresis (pAB)	Reservoir dust from floor	AM	
			Barn and living quarters		
			Separated		
			Corridor, $n = 17$	40.6 μg/g	
			Living room, $n = 17$	82.4 μg/g	
			Bedroom, $n = 16$	56.3 μg/g	
			In the same building		
			Corridor, $n = 13$	103.6 μg/g	
			Living room, $n = 13$	112.1 μg/g	
			Bedroom, $n = 13$	150.4 μg/g	
Berger et al. (131)	Stables and homes of farmers (three groups with different cattle exposure), Germany	Bos d 2 Rocket immunelectrophoresis (pAB)	Reservoir dust	Median	Range
			Stables, $n = 36$	20,400 μg/g	680–55,400 μg/g
			Homes (living room floor)		
			Former contact, $n = 10$	13 μg/g	3–43 μg/g
			Indirect contact, $n = 13$	148 μg/g	34–2929 μg/g
			Direct contact, $n = 23$	316 μg/g	46–4209 μg/g
			Homes (mattress)		
			Former contact, $n = 10$	12 μg/g	4–381 μg/g
			Indirect contact, $n = 13$	195 μg/g	15–403μg/g
			Direct contact, $n = 23$	265 μg/g	31–1268 μg/g
Zahradnik et al. (128)	Stables and homes of farmers and controls, Germany	Cow hair allergen Sandwich ELISA (pAB)	Electrostatic cloths (14 days)	Median	Range
			Stables		
			Cow, $n = 37$	51,700 μg/m^2	4760–559,000 μg/m^2
			Goat, $n = 6$	315.7 μg/m^2	91–701.4 μg/m^2
			Other, $n = 14$	1.2 μg/m^2	<LOD–6.5 μg/m^2
			Homes		
			Cattle farmers, $n = 128$	22.6 μg/m^2	0.3–900 μg/m^2
			Urban dwellers, $n = 32$	0.2 μg/m^2	<LOD–2.7 μg/m^2
Williams et al. (132)	Inside and outside of homes nearby dairy facilities, USA	Bos d 2 Sandwich ELISA (mAB)	Air samples (stationary)	Median	Maximum
			Indoor		
			Proximal, $n = 16$	0.12 μg/m^3	0.97 μg/m^3
			Intermediate, $n = 5$	0.01 μg/m^3	0.12 μg/m^3
			Distal, $n = 12$	0.01 μg/m^3	0.03 μg/m^3
			Outdoor		
			Proximal, $n = 19$	0.66 μg/m^3	1.87 μg/m^3
			Intermediate, $n = 6$	0.17 μg/m^3	0.29 μg/m^3
			Distal, $n = 12$	0.01 μg/m^3	0.10 μg/m^3

mAB, monoclonal antibodies; pAB, polyclonal antibodies; LOD, limit of detection; AM, arithmetic mean.

clothing or hair. Some studies have demonstrated that animal allergen levels in these mostly public environments can be significantly higher than in domestic areas without animals. The number of pet owners is one of the strongest predictors of increased allergen levels in these settings. Apart from the presence of animals or number of individuals with direct and frequent contact to animals, differences in allergen concentrations are associated with various building-related factors such as size and type of room, type of flooring, and furniture, cleaning frequency, ventilation system, and also the distance to animal rooms or stables. For example, carpets, mattresses, and upholstery are consistently found to have much higher concentrations of animal allergens than smooth surfaces.

Allergen levels can also vary in different parts of the world, which appears to be primarily influenced by regional and cultural differences in pet ownership or livestock farming. Rodent infestation is another factor that is strongly associated with increased mouse allergen levels. In contrast to mites, mammalian allergens seem to be independent of climate or seasonal variations.

Besides environmental factors, sample collection strategies and analytic methods enormously influence the results of exposure measurements. A variety of commercially available and well-characterized sampling equipment have been used in the studies. For each sampling method, differences exist regarding features, such as sampling pump, air flow rate, vacuum power, collection device, filter type, sampling duration and number, and size and type of surfaces sampled. Concerning the ELISA method, which was used to quantify allergen concentrations, the variations comprise the type of antibodies, calibration standard and its protein determination, replicate precision, and detection/visualization methods. Differences in data analysis for example, median, arithmetic, or geometric mean and calculation of the results as nanograms per gram or nanograms per meter square impede the direct comparison of the data produced in different studies.

CONCLUSION AND RECOMMENDATIONS

Environmental allergen exposure plays a significant role in the development of asthma and allergy. Allergic diseases are important public health concerns because of severity of symptoms, reduced quality of life, and limited productivity of the affected persons and high healthcare costs. Therefore, the identification of major sites of exposure and factors influencing allergen levels is essential to prevent allergic health effects. Specific measures to reduce or to avoid exposure to allergens can be initiated. Moreover, the knowledge of exposure levels is helpful to estimate the risk of sensitization or induction of symptoms in occupational or environmental settings. There is still lack of information on risk limits. One reason is the complexity of allergen monitoring, which is a multi-step task requiring various tools and techniques. Although, more allergen exposure data and more accurate methods are becoming available, a general standardization of sampling, and analytical procedures is much needed. The development of consensus protocols can be advantageous in the future for a better comparison of data from different studies. In the case of animal allergens, one basic requirement for standardization is fulfilled through commercial availability of monoclonal sandwich ELISA/MARIA kits for the detection of major allergens. Some assays, e.g., Fel d 1 and Can f 1 ELISA, have already reached global dissemination.

Apart from methodological issues, the estimation of "general" risk levels is complex. In contrast to toxic substances, which affect more or less all exposed individuals, the reactivity to the same allergen can vary extremely between people. Some persons will never become sensitized even at high exposure. Allergen levels associated with an increased risk of disease and/or sensitization are certainly different for healthy, sensitized and allergic persons. Finally, the determination of risk levels should also include the type of environment (workplace, home, school) because the circumstances of personal exposure are different. Therefore, a clear definition of strategy which provides the best proxy of allergen burden for different exposure scenarios is needed. The definition should include the type of dust, dust sampling procedure with validated protocol, type of allergenic substances (allergen mix or single allergen) and standardized immunoassay.

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Allergy to uncommon pets: new allergies but the same allergens

Araceli Díaz-Perales[1†], David González-de-Olano[2†], Marina Pérez-Gordo[3†] and Carlos Pastor-Vargas[4*†]

[1] *Center for Plant Biotechnology and Genomics (UPM-INIA), Pozuelo de Alarcón, Madrid, Spain*
[2] *Allergy Unit, Hospital Universitario Fuenlabrada, Madrid, Spain*
[3] *Institute of Molecular Applied Medicine (IMMA), CEU San Pablo School of Medicine, Madrid, Spain*
[4] *Department of Immunology, IIS-Fundación Jiménez Díaz, Madrid, Spain*

Edited by:
Annette Kuehn, Centre de Recherche Public de la Santé, Luxembourg

Reviewed by:
Beatrice Jahn-Schmid, Medical University of Vienna, Austria
Christiane Hilger, Centre de Recherche Public de la Santé, Luxembourg

****Correspondence:***
Carlos Pastor-Vargas, Department of Immunology, Hospital Fundación Jiménez Díaz, IIS-Fundación Jiménez Díaz, Avda. Reyes Católicos 2, 28040 Madrid, Spain
e-mail: cpastor@fjd.es

[†]Araceli Díaz-Perales, David González-de-Olano, Marina Pérez-Gordo and Carlos Pastor-Vargas have contributed equally to this work.

The prevalence of exotic pet allergies has been increasing over the last decade. Years ago, the main allergy-causing domestic animals were dogs and cats, although nowadays there is an increasing number of allergic diseases related to insects, rodents, amphibians, fish, and birds, among others. The current socio-economic situation, in which more and more people have to live in small apartments, might be related to this tendency. The main allergic symptoms related to exotic pets are the same as those described for dog and cat allergy: respiratory symptoms. Animal allergens are therefore, important sensitizing agents and an important risk factor for asthma. There are three main protein families implicated in these allergies, which are the lipocalin superfamily, serum albumin family, and secretoglobin superfamily. Detailed knowledge of the characteristics of allergens is crucial to improvement treatment of uncommon-pet allergies.

Keywords: allergens, albumins, lipocalins, pet allergy, uncommon pet

Especially in urban areas, daily exposure to domestic animals, has been described as a potential risk factor for the development of respiratory symptoms and allergic disease, and is an increasingly common problem (1). The most frequent pet allergy is allergy to cats and dogs. However, in recent years it has become more and more popular to have other animals as pets, so that the risk of exposure to new and unknown potential allergens increased. The incidence of allergy to uncommon pets – that is, pets other than cats, dogs, birds, or fish – is unknown because descriptions in the literature include only isolated cases or small series. Nevertheless, the number of scientific publications has increased significantly over the last 10 years (2). Despite the lack of statistics providing the total number of households with exotic or non-traditional pets, such pets are certainly kept in a significant percentage of households. The ever-growing list of exotic pets includes various animals such as rodents (mice, rats, hamsters, guinea pigs, chinchillas, gerbils, jerboas, etc.), other mammals (ferrets, pigs, monkeys), spiders (tarantulas), reptiles (snakes), and exotic birds (3).

Most animal allergens are spread through airborne particles, and these particles have been detected in some animal-free environments (4). Despite the large number of animal allergens described, most of them belong to a small number of families, which is important for the study of their allergenicity and cross-reactivity. Most of the major mammalian allergens belong to one of three families: the lipocalin superfamily, the secretoglobin superfamily, or the serum albumin (SA) family. Within these families, the most widely studied allergens are lipocalin-like proteins and SAs.

This review summarizes the knowledge of the most common exotic animals used as pets, the allergic symptoms they might cause, and the new allergens responsible for those reactions.

EPIDEMIOLOGY AND CLINICAL FEATURES

The number of households with pets is progressively increasing[1]. Six out of every 10 Spanish households – 8.5 million homes – keeps at least one pet, according to a study carried out in 2009 by the Propet pet professional fair[2]. There are around 20 million pets in Spain: 5.5 million dogs, 4 million cats, more than 7 million birds, and around 4.5 million fish. Uncommon animals also taken as pets, such as small mammals, amphibians, and reptiles, form a group of approximately two million. These data are proportionally similar to those described by the American Pet Products Association's (APPA) 2011–2012 National Pet Owner Survey. This increase is even bigger within the group of exotic or uncommon animals; indeed, until 2008 – the year the financial crisis began – the number of animals imported to our country to later be sold increased by more than 100% relative to the previous decade, according to the convention on international trade in endangered species of wild fauna and flora[3]. This considerable increase in ownership of uncommon pets might be due to trends in consumption or to a larger proportion of people living in small apartments, where having large animals is usually not permitted. As happens with

[1] http://www.americanpetproducts.org/press_industrytrends.asp
[2] http://www.ifema.es/ferias/propet/default.html
[3] http://www.cites.org

traditional pets such as dogs and cats, sustained contact with these exotic animals can sometimes lead to the development of allergic symptoms.

The most frequently reported clinical features of allergy to uncommon animals are usually the result of inhalation, contact, or bites.

MAMMALS

Exotic mammals are the largest group of uncommon pets. The most frequent symptoms presented after exposure to these animals – i.e., rhinitis, conjunctivitis, and asthma – affect the upper and lower respiratory tract and have been reported in relation to prairie dog (5), chinchilla (6), guinea pig (7, 8), ferret (9, 10), gerbil (11, 12), hamster (13–17), hedgehog (18), rabbit (19–21), hare (22), and monkey (23–25). Contact urticaria has also been reported with chinchilla (6), ferret (10, 26), and hedgehog (18). In cases the owner was bitten by the animal, the subsequent symptoms reported varied from urticaria (15, 27) or respiratory discomfort (28) to anaphylactic shock, as described with gerbil (28), hamster (14, 29, 30), sunda slow loris (2), and mouse (31).

REPTILES

The main symptoms developing after exposure to these animals also affect the upper and lower respiratory tract; indeed, asthma, rhinitis, and conjunctivitis after exposure to iguana (25, 32–34) and lizard (2) have been documented. The symptoms reported after reptile bites range from eruption of crusty pruriginous papules after an iguana bite (35) to anaphylaxis with clear predominance of vascular symptoms after bites by lizard (36–38), crotalus (39), and king cobra (40), but the mechanism underlying these reactions was not studied.

BIRDS

To a greater or lesser extent, mostly all the symptoms prompted by exposure to birds affect the respiratory tract. Besides rhinitis and/or asthma (41, 42), the inhalation of allergens related to exotic birds, and as it happens in cases of exposure to birds not considered as exotic, might sometimes cause hypersensitivity pneumonitis as described after exposure to lovebirds (43, 44), cockatiel (45), pheasants (46), canaries (47), parakeets (48, 49), parrots (50), geese (51), and owls (52). Other respiratory diseases, such as bronchiolitis obliterans due to parakeets, have also been described (53). Patients with bird-egg syndrome may present respiratory symptoms induced by bird antigens and gastrointestinal symptoms after the intake of bird egg (44, 54, 55).

OTHERS

Arachnids have recently come to be regarded as pets as well. Some arachnids, including tarantulas, have hairs that produce urticaria that is not immune-mediated and can result in persistent papular dermatitis, or, when the hairs come into contact with the cornea and conjunctiva, ophthalmia nodosa (56). Generalized rash and hypotension after a spider bite has also been reported (57), but no allergic study was carried out.

ALLERGEN SOURCES

Contrary to popular belief, dander, and not hair, is the main cause of allergy to animals. Dander contains allergens formed in the sebaceous gland secretions and saliva. In animals, as in humans, the skin sheds gradually as microscopic scales. Secretions containing allergens are adhered to hair and stratum corneum of the skin. Small particles are able to remain floating in the air for long periods of time and, when inhaled, cause allergic symptoms in the nose, eyes, and respiratory tract. These particles settle slowly on the floor or furniture and are re-dispersed in the air so that allergens can be inhaled although the pet is not present at the time. For this reason, patients notice animal-allergy symptoms just entering homes or places where there are, although not present at the time. While less frequent, hair can also cause allergy, though animal hair stays at the floor and is not present in ambient air. Few allergens have been described in uncommon pets, and most are homologous to the lipocalin family, the secretoglobins family, the family of immunoglobulins, and SAs.

ALLERGENS TO UNCOMMON MAMMALIAN PETS

Although there is a growing number of reports on allergens mediated by uncommon pets, there is little information about the allergens involved. We will now describe only the allergens already characterized within this family of pets. Not all of them are fully characterized and some of the allergens presented in **Table 1** are only partially described and tentatively named. Rodents and other small domestic and laboratory animals have a high sensitizing potential (58). Allergenic proteins of small rodents have been found in hair, urine, and salivary gland extracts. In certain rodents, 10 or more allergens have been identified. The molecular weight of these allergens ranges from 8 kDa to more than 80 kDa. The major allergens of mouse (Mus m 1, Mus m 2), rat (Rat n 1A, Rat n 1B), and guinea pig (Cav p 1, Cav p 2) have all been identified, and extracts are commercially available for each animal. Rabbit allergens are not well described, but at least three individual glycoproteins, Ory c 1, Ory c 2, and Ory c 3 are identified in hair, dander, and urine (21, 59). Most of them are included in the lipocalin family (**Table 1**). For the Siberian hamster, no allergen has been officially named, although one has been recently sequenced (deposited in GenBank, accession number GI: KF148615). By contrast, IgE immunoblotting revealed three IgE-binding bands of about 18, 21, and 23 kDa which correspond to isoforms of a single allergen which has been identified as a lipocalin (29).

Other popular uncommon pets are small pigs, mini pigs, or teapot pigs. Pig allergy was studied from the point of view of food sensitization and occupational allergy. Pig hair and dander are important inducers of occupational allergies in cattle-exposed farmers (65, 66). More than 10 allergens have been associated with allergic events in patients who are caretakers of this animal. The most prevalent allergens include lipocalin proteins and albumins (**Table 1**)[4].

The domestic ferret (*Mustela putorius furo*) is the third most common furred pet in US households. Some case reports have appeared in the literature. A 66-kDa and a novel 17 kDa protein were characterized as putative allergens in ferret extract prepared from fur, urine, feces, and bedding material (9, 10). The 66-kDa protein was assumed to represent ferret albumin because of its *in vitro* cross-reactivity with cat albumin. The novel 17 kDa showed molecular weight that was similar to lipocalin.

[4]www.allergome.com

Table 1 | Uncommon pets allergens.

Common name	Species	Source	Allergen	Family	Reference
Chinchilla	*Chincilla lanigera*	Epithelial, saliva, urine	Chi La	Protein kinase inhibitor	(6)
			Chi Lb	Lipocalin	
Guinea pig	*Cavia porcellus*	Epithelial, saliva, urine	Cav p 1[a]	Lipocalin	
			Cav p 2[a]	Lipocalin	
			Cav p 3[a]	Lipocalin	
			Cav p 4[a]	Serum albumin	
			Cav p 6[a]	Lipocalin	
Gerbil	*Meriones unguiculatus*	Epithelial, saliva, urine, sleep bed	Mer un 23kDa	Lipocalin	(12)
			Mer un 4	Serum albumin	(60)
Hamster	*Phodopus sungorus*	Epithelial, saliva, urine	Phos 21 kDa	Lipocalin	(29)
Rat	*Rattus norvegicus*	Epithelial, saliva, urine	Rat n 1[a]	Lipocalin	
			Rat n 4	Serum albumin	(61)
			Rat n 7	Immunoglobulin	(62)
Mouse	*Mus musculus*	Epithelial, saliva, urine	Mus m 1[a]	Lipocalin	
			Mus m 2	*Unknown*	(61)
			Mus m 4	Serum albumin	(61)
			Mus m 7	Immunoglobulin	(62)
Rabbit	*Oryctolagus cuniculus*	Epithelial, saliva, urine	Ory c 1[a]	Lipocalin	
			Ory c 2	Lipocalin	(59)
			Ory c 3[a]	Secretoglobin	
			Ory c 4[a]	Lipocalin	
Ferret	*Mustela putorius*	Epithelial, saliva, urine	Mus p 17	*Unknown*	(9)
			Mus p 66	Serum albumin	(9)
Pig	*Sus scrofa*	Meat	Sus s 5	Lipocalin	(63)
			Sus s 6	Serum albumin	(64)

The allergen names delivered by IUIS Allergen Nomenclature Sub-Committee have been marked by [a].

OTHER ALLERGENS IN UNCOMMON PETS

Spiders have become pets because of their small size and ease of care. Most of the reported events regarding these animals are produced by toxicity. Little has been published on environmental spider allergy or allergy to spider bite. However, Bobolea and colleagues identified two new allergens in *Holocnemus pluchei*: hemocyanin and arginine kinase (67).

In reptiles, the primary IgE-binding proteins are present in the venom, urine, and epithelial cells. The principal allergens have been described among 59–63 and 8–15 kDa but have not been identified yet (68).

Although many allergens have been described in birds, all of them have been associated with ingestion and are not related to the role of birds as pets. Only in some reports which refer to allergy by inhalation, the molecular weights of allergens have been described but without their identification (44).

MAJOR ALLERGENS IN UNCOMMON PETS

Most of the allergens reported in cases of allergy to uncommon pets have been characterized by homology to other allergens previously described in meat or milk animals. Here we present a descriptive summary of the most relevant protein families identified as allergens such as the lipocalin family, SAs, secretoglobins, and other allergens, as described in **Table 1**. Within these families, the most widely studied allergens are lipocalin-like proteins and SAs.

LIPOCALINS

Most of the important animal-derived allergens belong to the lipocalin protein family (**Table 1**). Lipocalin allergens are found in dander, saliva, and urine. These allergens disperse effectively and are widely present in indoor environments. Initially, lipocalins were characterized as transport proteins for principally hydrophobic molecules such as retinol, odorants, steroids, and pheromones, but now they are known to be involved in many other biological functions (69).

Lipocalins are a large group comprising proteins from vertebrate and invertebrate animals, plants, and bacteria. The family is part of a larger[5] superfamily, calycins (70).

The amino acid sequences of lipocalins compromise 160–230 residues with an average predicted molecular mass of about 20,000 Da (without post-translational modifications) (70). They

[5]http://prosite.expasy.org/PS00213

can be *N*- and/or *O*-glycosylated. The overall amino acid identity between lipocalins is 20–30%, but it can be considerably higher. For example, human lipocalin-9 is more than 50% identical to its rodent homologs, and identities of about 40% are found with Mus m 1, Rat n 1, Equ c 1, and Fel d 4. The amino acid identity of dog Can f 1 with human tear lipocalin is about 60%. Although the sequential identity among lipocalins is low in general, they share a common three-dimensional structure (70). The central β-barrel of lipocalins, which is composed of eight anti-parallel β-strands, encloses an internal ligand-binding site. Most lipocalins contain one or more intramolecular disulfide bonds.

The arrangement of lipocalin molecules in a multisubunit complex (oligomerization) is variable (71). In all, the physicochemical and structural features of the characterized lipocalin allergens are not known to account for their allergenic capacity or to distinguish them from other lipocalin proteins (72). However, they induce IgE production in a large proportion of atopic individuals exposed to the allergen source.

As lipocalins are known to carry small hydrophobic ligands in their internal ligand-binding site, recent studies finding that pollen extracts from birch and several other plants contain E1-phytoprostanes and possibly other Th2-deviating lipid mediators are of interest (73, 74). It has even been suggested that lipid binding can be a key characteristic for many allergens because lipids can directly activate innate immunity (73). Although there are no data supporting the idea that lipocalin allergens would carry immunomodulatory substances favoring allergy, the hypothesis is no doubt worth further examination.

There are only a few T cell epitopes reported for lipocalin allergens, and those examined have proved to be suboptimal. Moreover, the frequency of lipocalin allergen-specific CD4+ T cells is very low in the peripheral blood. Importantly, recent research suggests that the lipocalin allergen-specific T cell repertoires differ considerably between allergic and healthy subjects. These observations are compatible with the hypothesis that the way CD4+ T-helper cells recognize the epitopes of lipocalin allergens may be implicated in the severity of the symptoms (75).

SERUM ALBUMIN

Serum albumins, characterized by a molecular weight of 67 kDa and a tendency to participate in IgE-mediated cross-reactions, are recognized by the serum of 20–30% of patients with some pet allergy (**Table 1**) (76).

Serum albumin, often referred to simply as albumin, is a globular protein that in humans is encoded by the ALB gene (77). SA is the most abundant plasma protein in mammals. Albumin is essential for maintaining the oncotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones and as a transport protein for heme groups and fatty acids. Too much SA in the body can be harmful.

Allergic sensitization to SA can occur by inhalation as well as ingestion. SAs are found in dander and saliva of pets and are important inhalant allergens.

The best characterized member of this family is the bovine serum albumin (BSA). Its covalent bonds maintain its tertiary structure under denaturing conditions (e.g., low pH or heating). The protein is organized in three homologous domains (I–III) and consists of nine loops (three loops/each) connected by 17 covalent disulfide bridges. Most of the disulfide bonds are well protected in the core of the protein and are not readily accessible to the solvent (78). Interestingly, members of this family are important food allergens in bird and mammal species. However, there are no reports of sensitization to SA by inhalation in birds.

SECRETOGLOBIN ALLERGENS

Secretoglobins are the most potent allergens in cat and there have been described as allergens in other pets (21). These proteins show unknown function, and they are produced by the skin and by salivary and lacrimal glands of pets (79). Secretoglobins are transferred to the pelt by licking and grooming. Dried saliva and dandruff are spread from the hair to the surrounding environment as small airborne particles possibly causing sensitization in susceptible individuals (79).

This family consists of two allergic relevant members to pets such as Fel d 1 and Ory c 3. Little is known about rabbit allergen, although on Fel d 1 there is more information. Fel d 1 is a protein produced largely in cat saliva and sebaceous glands. The complete quaternary structure of Fel d 1 has been determined. The allergen is a tetrameric glycoprotein consisting of two disulfide-linked heterodimers. Both chains share an all alpha-helical structure (80).

OTHER ALLERGENS

Other allergens have been described in domestic animals (**Table 1**), and are included in the family of the caseins, immunoglobulins, gelatins, and transferrins. These are all minor allergens that are present in secretions (e.g., saliva, urine, and semen) and flaking of the animals.

CONCLUDING REMARKS

Exotic pet allergy and their associated respiratory symptoms have increased in recent years. Nowadays, avoidance therapy is the best measure for the prevention of any pet allergic reaction. Biomolecular characterization of allergens remains essential to the development of emerging therapeutic modalities to treat respiratory symptoms, such as attenuated allergy vaccines.

This review compiles the existing descriptions of the main exotic or uncommon pets that cause allergy in our environment and the main allergens implicated. Most of the animal allergens described belong to a small number of families. Furthermore, it would be reasonable to study the allergenicity and cross-reactivity of these major pet allergens to improve specific treatment of patients with allergy to animals.

AUTHOR CONTRIBUTIONS

All author have contributed to the conception, design, and drafting of the paper.

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Immune responses to inhalant mammalian allergens

François Hentges[1], Cathy Léonard[1], Karthik Arumugam[2] and Christiane Hilger[1]*

[1] *Laboratory of Immunogenetics and Allergology, CRP-Santé, Luxembourg, Luxembourg*
[2] *Laboratory of Retrovirology, CRP-Santé, Luxembourg, Luxembourg*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Beatrice Jahn-Schmid, Medical University of Vienna, Austria
Markus Ollert, Technische Universität München, Germany

***Correspondence:**
Christiane Hilger, Laboratory of Immunogenetics and Allergology, CRP-Santé, 84 Val Fleuri, Luxembourg L-1526, Luxembourg
e-mail: christiane.hilger@crp-sante.lu

In Europe and the USA, at least one person in four is exposed every day to inhalant allergens of mammalian origin, a considerable number is regularly exposed for professional reasons and almost everyone is occasionally exposed to inhalant allergens from pets or domestic animals. The production of IgE to these inhalant allergens, often complicated by asthma and rhinitis, defines the atopic status. However, the immune response to these allergens largely imprints the cellular immune compartment and also drives non-IgE humoral immune responses in the allergic and non-allergic population. During the recent years, it has become clear that IgE antibodies recognize mammalian allergens that belong to three protein or glycoprotein families: the secretoglobins, the lipocalins, and the serum albumins. In this article, we review the humoral and cellular immune responses to the major members of these families and try to define common characteristics and also distinctive features.

Keywords: secretoglobin, lipocalin, albumin, allergen, cellular response, IgE response, cross-reactivity

INTRODUCTION

The immune system of the respiratory tract of children and adults is continuously being exposed to inhaled particles of inorganic and organic origin. Some particles or molecules have potential adjuvant activities, some have allergenic potential such as mite allergens, other molecules for instance of human origin are devoid of immunogenic or allergenic properties. In their daily environment, most people are also exposed to allergens of mammalian origin. The exposure rate can be high for people having pets at home, for farmers who keep or raise domestic animals, and for persons who have other types of professional contact with animals, for instance veterinarians or animal workers in a laboratory setting. However, even people that do not have direct contact with animals may have contact with inhalant allergens that have been shed by animals or that have been carried to public places by animal owners, as documented by the presence of cat allergens in schools and other public places (1). Indeed, allergic sensitization to animal allergens is common in persons who do not have pet animals at home. Data from a large pan-European study show that about 27% of the patients referred to an allergy center for allergic reactions to inhalant allergens were sensitized to cat and/or dog (2). Sensitization was particularly high in Nordic countries, the highest sensitization rate for dogs reaching 56% in Denmark.

As dendritic cells combine antigen presentation capacities with sensing of signals of innate immunity, they are the master players in all types of adaptive immunity. The important role of airway epithelial cells in alarming dendritic cells for allergic sensitization has more recently been highlighted (3, 4). In contrast to certain other inhalant allergens (Der p 1, Der p 2), an inherent capacity of mammalian allergens to trigger the pathways of innate immunity has not been convincingly shown, although a recent publication argues for an enhancing activity of some mammalian allergens on toll-like receptor (TLR) activation by lipid ligands (5). Allergens of mammalian origin do not only induce IgE antibodies but also IgG isotypes and different T cell responses (Th2, Th1, Th17, and regulatory T cells) in allergic and non-allergic persons. We will review the immune responses to three major mammalian allergen families: secretoglobins, lipocalins, and serum albumins. We will focus on the most prominent of their members and try to distinguish common and specific characteristics based on published data.

SECRETOGLOBINS

Fel d 1, the major cat allergen, is a 35-kDa tetrameric glycoprotein formed of two non-covalently linked heterodimers (6, 7). Each heterodimer comprises a light alpha-chain (or chain 1) and a heavy beta-chain (or chain 2) containing an *N*-linked oligosaccharide (8). Until the recent description of rabbit lipophilin Ory c 3 (9), Fel d 1 was the only known allergen of the secretoglobin family. Both molecules display little sequence identity (24%) despite a high structural identity. There are no protein stretches of more than three consecutive identical amino acids which are common to the two molecules and which could form identical linear epitopes. Surface representation of Fel d 1 overlaid with the sequence of Ory c 3 does not show evidence for significant common discontinuous epitopes (**Figure 1**). Indeed, IgE cross-reactivity between Fel d 1 and Ory c 3 could not be shown (9).

EXPOSURE AND EFFECT ON THE HUMORAL IMMUNE RESPONSE

Cats are present in 24% of European Union and up to 37% of United States households (11). The level of airborne Fel d 1 in homes with a cat was found to be in the range of 1.8–578 ng/m^3, a comparable level of airborne Fel d 1 ranging from 2.8 to 88.5 ng/m^3 was also measured in 25% of homes without a cat (12). The quantity of Fel d 1 inhaled on airborne particles by children in homes with a cat has been estimated to be 1 μg/day, which is about 100 times the quantity of mite or pollen allergens inhaled in 1 day (13, 14). At the age of 4, already 5.8% of children of a U.K. birth cohort study were sensitized to cat (15) while 16.9% of adults were sensitized in another study (16). In a recent study including

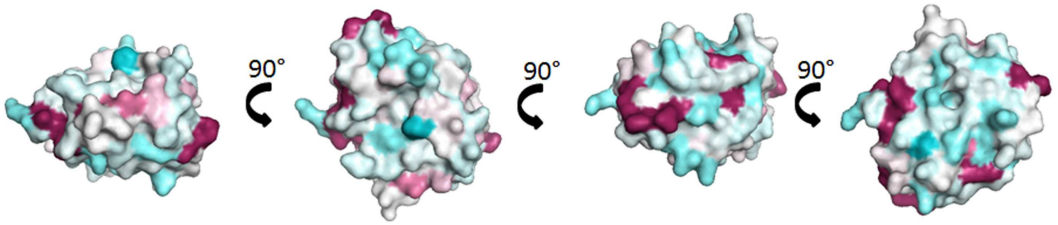

FIGURE 1 | Comparison of Fel d 1 and Ory c 3 composing the secretoglobin group. Surface representation of Fel d 1 (2EJN) (7) colored by sequence conservation with Ory c 3 (Q9GK63; Q9GK67) (9). Sequence conservation was determined through Consurf (10), the highly variable sequence conservation was identified as deep blue, the average was in white, and the conserved sequences are denoted in deep red.

96 cat-allergic patients, IgE antibodies to Fel d 1 represent on average 55% of a cat-specific IgE response, however, with a range from 0 to 100%. Fel d 1 was the cat allergen that bound the highest amount of IgE in 65% of the patients (17). Surprisingly, children exposed to cat during the first year of life are less often skin prick test positive to cat at 12–13 years than controls (18). Exposure to high concentrations of Fel d 1 was found to be associated with lower sensitization in terms of specific IgE but increased levels of IgG (and IgG4) antibodies to Fel d 1 which has led to the concept of "modified Th2 response" (19). Exposure to cat and Fel d 1 was associated with three patterns of humoral responses: an allergic response characterized by the presence of IgE and IgG (often IgG4) to Fel d 1, a modified Th2 response characterized by presence of IgG (mostly IgG4) antibodies but absence of IgE response to Fel d 1, and thirdly an absence of humoral response to Fel d 1 characterized by failure to produce specific IgE or IgG to Fel d 1, even though exposure to Fel d 1 took place (20). However, the analysis of IgE, IgG, and IgG4 antibodies to Fel d 1 in relation to wheezing in two large birth cohorts showed that allergen-specific IgG but not IgG4 antibody levels were related to improved wheezing in children with Fel d 1-specific IgE (21). At this point, it might be interesting to note that a phase I/II a clinical trial using intralymphatic immunotherapy with a technology called modular antigen translocation (MAT) linking Fel d 1 to a construct enhancing its presentation by the MHC class II pathway, increased Fel d 1-specific IgG4 production (22). Thus, exposure to high concentrations of Fel d 1 in the daily environment is associated with clinical improvement in parallel to increased levels of Fel d 1-specific IgG and in particular IgG4 antibodies (19). A similar modified Th2 response can be achieved by high Fel d 1 loading of the antigen-presenting pathway by intralymphatic immunotherapy and MAT technology (22). This is in line with the concept that a modified Th2 response is associated with high antigen exposure.

T CELL RESPONSE AS MEASURED *EX VIVO*

The frequency of Fel d 1-peptide-specific T cells in peripheral blood as assessed by tetramer technology using a chain 1 peptide (aa 32–48) – DRB1*01:01 tetramer complex in a population of HLA – DRB1*01:01 positive cat-sensitized patients with atopic dermatitis and controls gave the following results. Atopic dermatitis patients had 3–53:100.000 and controls about 3:100.000 tetramer-positive CD4 T cells in their peripheral blood (*ex vivo*). The majority of about 80% expressed a central memory phenotype (with high surface expression of CCR7, CD62L, CD27, and CD28) (23). Comparable results were obtained for Fel d 1-specific CD4 cells with tetramers containing six different peptides bound to six different HLA class II molecules (24). Peptide-specific CD4 cells ranged from 1:7000 to 1:300000 in allergic subjects. In subjects without allergy, tetramer-positive CD4 T cells were barely detectable. Nearly all cells exhibited a central memory phenotype, however, CCR7 expression was heterogeneous. A relevant percentage of cells were $CCR4^{+}$, interpreted as a commitment to migrate to non-lymphoid sites. In comparison, in birch-allergic persons, the percentage of CD4 T cells recognizing MHC class II tetramers containing an immunodominant peptide was about 500:100.000 in allergic and 300:100.000 in non-allergic persons during the peak pollen season (25). This is more than one order of magnitude higher than for Fel d 1 in cat-allergic persons. In birch-allergic persons, the cells were mainly of an effector memory phenotype (IL-5 and some IL-10). The cells of non-allergic persons were of central memory phenotype (secreting IFN-γ and IL-10) in response to the allergen. Peptide-positive cells could not be detected directly *ex vivo* outside the pollen season. Their number was estimated to be at that time point 2–3 logs lower than during the peak pollen season (25). One possible explanation for this difference in peptide-specific T cells could be a smaller initial peripheral T cell repertoire against Fel d 1 due to a stronger thymic deletion of T cells recognizing epitopes of mammalian origin (phylogenetically closer to human epitopes) than T cell recognizing epitopes on molecules of plant origin. It is, however, more likely that the greater number of CD4 T cells recognizing MHC class II tetramers loaded with immunodominant pollen-derived peptides is due to the seasonal boosting of cellular immune response linked to pollen exposure.

PEPTIDES AND PEPTIDE IMMUNOTHERAPY

By T cell epitope mapping with peptides, amino acid positions 1–10 and 16–24 of Fel d 1 chain 2 were found to be associated with a HLA-DR7-restricted secretion of high IL-10, respectively IFN-γ in PBMC cultures of persons with a modified Th2 response (20). Previous work had defined Fel d 1 T cell epitopes mainly on chain 1, by means of cell proliferation assays of T cell lines established from persons allergic to cats (26). Intradermal administration of short overlapping peptides derived from chain 1 of Fel d 1 that did not cross-link IgE, did not elicit a visible early or late cutaneous response, but caused late asthmatic reactions

in 9/40 cat-allergic asthmatics (27). The individual peptides were able to induce proliferation and IL-5 secretion in a HLA class II restricted manner from T cell lines established from asthmatic subjects, indicating IgE-independent, T cell-dependent allergic reaction. Determination of the binding affinities of Fel d 1 peptides to 10 commonly expressed HLA-DR molecules, combined with their proliferative and cytokine responses (IFN-γ, IL-10, and IL-13) in cat-allergic persons allowed a comprehensive identification of immune-dominant sequences including those on chain 2 (28). A short peptide immunotherapy course with a combination of promiscuous peptides (serving as restriction element to different HLA-DR molecules) improved the ocular and nasal components of rhino-conjunctivitis symptoms in subjects with cat allergy, with a treatment effect persisting 1 year after the start of treatment (29). This approach uses only short peptides (12–16 amino acids long) which are not recognized by IgE antibodies able to trigger an early asthmatic response through mediator release by basophils and mast cells. These short peptides are also not likely to be recognized by surface-bound IgM and thus interfere at the immature B cell level. Their effect is rather due to a dampening of the effector T cells for instance IL-5-secreting T cells implicated in the late allergic asthmatic response. The impact could be due to changes of the helper or regulatory cellular functions. Of course, an immune system modified by this approach at the cellular level could secondarily be susceptible to changes at the humoral level after later inhalation of Fel d 1 molecules present in the environment.

LIPOCALINS

Lipocalins represent the largest group of mammalian inhalant allergens. They are major allergens from dog, horse, cattle, guinea pig, rat, mouse, rabbit, and hamster (30). Lipocalins have a common tertiary structure composed of a central β-barrel formed of eight anti-parallel β-strands (31). Lipocalins were shown to carry small hydrophobic molecules such as retinol, steroids, odorants, and pheromones in their internal binding pocket. Despite a highly conserved structural similarity, lipocalins generally have a very low amino acid identity, which for some of them can be lower than 20% (32), a fact that makes IgE cross-reactivity among these lipocalins unlikely. Until recently, it was assumed that IgE cross-reactivity between lipocalins would be limited to isolated epitopes with great amino acid identity between lipocalins (33). However, besides lipocalins with very low amino acid identity, a group with greater homologies and IgE cross-reactivity has been individualized. It comprises the following allergens: Fel d 4 (cat), Can f 6 (dog), Equ c 1 (horse), Ory c 4 (rabbit), Mus m 1 (mouse), and Rat n 1 (rat) (**Table 1**). Pairwise sequence comparisons show identities in the range of 47–67% whereas other cat and dog lipocalins have only weak identities. There is also another lipocalin pair namely Fel d 7 and Can f 1 that shares 63% identity at the amino acid level and which might give rise to cross-sensitization. Concerning the main group, IgE cross-reactivity was first shown between mouse and rat urinary lipocalins using sIgE inhibition (34). Indeed at the amino acid level, there exists an identity of 64% between the major rat lipocalin Rat n 1 and mouse lipocalin Mus m 1. More recently considerable IgE cross-reactivity was shown between dog lipocalin Can f 6 and cat Fel d 4 (amino acid identity 67%) and between these lipocalins and horse lipocalin Equ c 1 (35–37). IgE cross-reactivity between Equ c 1 and Can f 6 was shown to be clinically relevant in a horse- and dog-allergic patient who showed no specific IgE to known dog allergens except Can f 6 (36). IgE reactivity of this patient to Can f 6 could be completely inhibited by Equ c 1. Can f 6 and Equ c 1 share 57% amino acid identity. **Table 1** gives a summary of amino acid identity and cross-reactivity between members of the cross-reactive lipocalin group. Structural identity between Equ c 1, Fel d 4, and Can f 6 are visualized in **Figure 2**, structural identities between Mus m 1, Rat n 1, and Ory c 4 are shown in **Figure 3**.

Table 1 | Amino acid identities (%) and IgE cross-reactivity between members of a mammalian lipocalin subgroup.

Fel d 4		67 (35, 37)[a]	67 (37)[a]	63	49	55
Can f 6	67 (35, 37)[a]		57 (37)[a]	58	47	52
Equ c 1	67 (37)[a]	57 (36)[b] (37)[a]		52	46 (33)[a]	47
Ory c 4	63	58	52		51	54
Mus m 1	49	47	46	51		64 (34)[a]
Rat n 1	55	52	47	54	64 (34)[a]	
	Fel d 4	Can f 6	Equ c 1	Ory c 4	Mus m 1	Rat n 1

Amino acid identities between lipocalins are given as %.

[a] *Documented IgE cross-reactivity (allergen-specific inhibition).*

[b] *Documented IgE cross-reactivity with clinical history of cross-reactivity.*

Literature references are given in brackets.

Vertical: sensitizing allergen. Horizontal: cross-reactive allergen.

EXPOSURE AND EFFECT ON THE HUMORAL IMMUNE RESPONSE

Lipocalins are shed into the environment by animal dander and secretions. Dog allergen Can f 1 is ubiquitously present in human residential environment. The most probable mechanism of allergen transfer to public places is clothing (1). Lipocalins are found both in airborne and settled dust. Although Fel d 1 and Can f 1 belong to different protein families, a number of studies have shown that allergen levels found in airborne or settled dust are in the same range of magnitude for both molecules (38).

The importance of mouse allergens was initially demonstrated in the occupational setting (39). However, the role of mouse allergen exposure in domestic environments has also gained attention. Mus m 1, the major mouse allergen, is prevalent in US urban and suburban residential environments. It has been shown to be related to asthma morbidity (40–42). Importantly, airborne and settled dust mouse allergen levels were shown to vary over time in a given home, implying that environmental conditions of sensitized patients may change from high to low exposure and vice versa. Conditions of high exposure reached values up to 5.68 ng/m^3, which are comparable to measurements obtained in animal facilities.

The effect of allergen concentration on the immune response has been addressed in a prospective study analyzing the immune response of newly hired employes of a mouse facility over time. The concentrations of Mus m 1 in the air ranged from 0.09 to 9.88 ng/m^3, the median of the allergen concentrations over time being 0.69 ng/m^3. By 24 months, 23% of the participants had developed a positive SPT. Interestingly, the risk of becoming positive was not linear, increasing from low to moderate levels of

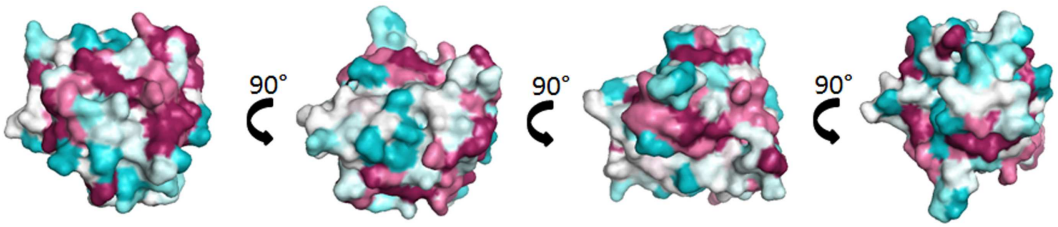

FIGURE 2 | Comparison of the cross-reactive lipocalin group from horse (Equ c 1), cat (Fel d 4), and dog (Can f 6). Surface representation of Equ c 1 (1EW3) (69) colored by sequence conservation with Fel d 4 (Q5VFH6) (70), Can f 6 (H2B3G5) (35).

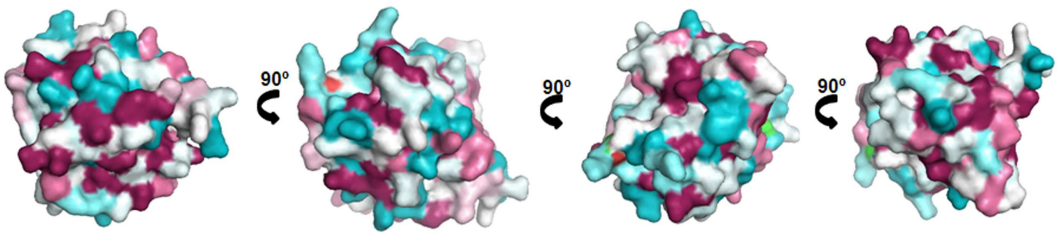

FIGURE 3 | Comparison of the cross-reactive lipocalin group from rat (Rat n 1), mouse (Mus m 1), and rabbit (Ory c 4). Surface representation of Rat n 1 (1MUP) (71) colored by sequence conservation with Ory c 4 (U6C8D6) (72) and Mus m 1 (P02762) (73).

exposure, peaking at approximately 1.2 ng/m^3, and then decreasing from moderate to high levels of exposure (43). Eight percent had developed mouse-specific IgG4, the incidence increasing with increasing levels of mouse allergen exposure. Ten percent of the participants had developed mouse-specific IgG1–3 with a non-significant association with higher exposure. A previous cross-sectional occupational study had shown that high exposure to rats was associated with lower rates of symptoms and specific IgE to rat urine allergen (containing Rat n 1) but an increased frequency of highly specific IgG and IgG4 (44).

A recent study addressing specific IgE, IgG1, IgG4 levels and the peripheral blood mononuclear cytokine responses to eight separate cat allergens in cat-allergic and in cat non-allergic persons showed IgG4 antibodies to Fel d 4 in 12% of allergic persons and in only 3% of non-allergic persons (17). Although IgG1 antibodies to Fel d 1 were found in allergic and non-allergic persons, there were none detected against Fel d 4. Another recent study analyzing on a microarray system of IgE and IgG antibodies to a series of dog, cat, and horse allergens showed that almost all the patients but also controls had IgG antibodies to the cross-reacting lipocalin group (Fel d 4, Can f 6, and Equ c 1) (45).

T CELL RESPONSES TO LIPOCALIN ALLERGENS

Rat n 1 allergen purified from rat urine and pools of overlapping peptides spanning Rat n 1 were tested for the proliferative responses of PBMCs of rat-allergic individuals, rat-exposed but non-allergic individuals and non-exposed, non-allergic individuals (46). The proliferative responses to Rat n 1 of the three groups were similar and weak with a median stimulation index below 2 (but with an extremely great range between 0.01 and 22.2). Nevertheless, four peptide pools induced with high frequency weak positive responses in allergic individuals in comparison to non-allergic referents (46). Interestingly, the levels of IL-5 were significantly increased in supernatants of PBMCs stimulated with rat urinary protein from allergic subjects, compared to non-allergic control subjects and even more so when compared to non-exposed controls when stimulated with rat urinary protein. Four similar epitope areas had previously been defined in the cow dander allergen Bos d 2 in cow-asthmatic individuals (47). Here also, the proliferative response of PBMCs to native Bos d 2 had been weak and the four epitopes concentrated on the conserved regions of the molecule had been most clearly defined by the proliferative response of a number of T cell clones established from five cow-allergic patients. According to their cytokine-producing pattern, 37% of the clones were classified as Th0-like (IL-5, IL-4, IFN-γ), 7.9% were Th1-like (IFN-γ), and 55% were Th2-like (IL-4, IL-5). Using HLA class II-peptide tetramer technology, naïve Bos d 2-specific T cells of PBMC cultures of individuals with or without allergy were of similar frequency, whereas the frequency of CD4$^+$, CD45RO$^+$ memory cells appeared to be higher in subjects with allergy (48). These findings were confirmed by a recent study on the CD4 T cell response to Equ c 1, the major horse lipocalin allergen (49). Allergic and non-allergic subjects had a similar low frequency of Equ c 1-specific CD4 T cells, but the cells from the allergic subjects had a stronger proliferative response, were predominantly Th2 biased and originated mostly from memory CD4 T cells.

T cell epitopes have also been mapped for the major allergens of dog and horse, Can f 1 and Equ c 1, respectively (50, 51). Seven epitope regions were defined for Can f 1 and on average, patients recognized three epitopes. T cell lines from allergic patients produced more IL-4 than those from healthy controls. However,

depending on the peptide used, they produced also more IL-10 or more IFN-γ (52). A comparison of Can f 1-specific T cell lines generated from dog-allergic and non-allergic but exposed persons showed an absence of IL-4 secreting T cell lines in non-allergic persons, while IL-5, IL-10, IFN-γ, and IL-17 lines were found in both groups although at different frequencies (53). Specific T cell lines established from 10 horse-allergic patients determined 8 epitope regions and 1 dominant epitope in the C-terminal region of Equ c 1 (51). Similarly to the findings on Rat n 1 and Bos d 2, epitopes are clustered in a few regions and they elicit only weak T cell responses in PBMCs which are enhanced in the T cell lines obtained after several stimulation cycles (51).

SERUM ALBUMINS

Serum albumins represent the major protein component in the circulatory system of mammals. They are produced by hepatocytes and have a molecular weight in the range of 66–69 kDa. They contribute significantly to colloid osmotic blood pressure and aid in the transport of many endogenous and exogenous ligands. The albumin molecule is very flexible, it has an α-helical structure stabilized by several disulfide bridges and is divided into three domains (54). Serum albumins are also present in body fluids and on dander. In house dust samples, concentrations of human serum albumin (HSA) have been measured in the range of 40–301 μg/g dust (55). Again, this is the range of the settled dust amounts measured for Fel d 1 and Can f 1, two molecules representative of a different allergen family. There are no commercial tools available up to date for the specific measurement of animal albumins in dust samples.

IMMUNE RESPONSE TO SERUM ALBUMINS

The immune response to serum albumins is well-documented at the antibody level, however, there are few data on the cellular response to serum albumins. Specific IgE to dog serum albumin (DSA) were first described in dog dander asthmatic children who were prick test positive to DSA (9 out of 80) (56). The titers of anti-DSA IgG measured did not correlate with specific IgE titers. Lymphocyte transformation tests in anti-DSA IgE-positive patients were weak except for one patient. In subsequent reports, the importance of DSA as a cross-reactive allergen was established. In a study with 110 dog-allergic patients, 35% were shown to have IgE against DSA (57). IgE antibodies from several selected patients bound also to albumins from other species such as cat, mouse, and rat. Histamine release with the different albumins was shown for one patient. A more extensive analysis of cross-reactivity was performed on a sample of 200 patients allergic to animal dander (58). Thirty percent of these patients presented IgE reactivity to albumins in animal hair/dander extracts and were further tested in dot-blot experiments for cross-reactivity with 11 different mammalian albumins. The majority of the patients' IgE recognized a large spectrum of albumins; some, however, displayed a highly selective reactivity.

IgE CROSS-REACTIVITY BETWEEN INHALED AND INGESTED OR SYSTEMICALLY ADMINISTERED SERUM ALBUMINS

Despite their high level of cross-reactivity, albumins were considered minor allergens without documented clinical significance. This was challenged by a case report of a severe anaphylactic reaction after artificial insemination in a patient sensitized to animal dander (59). Bovine serum albumin (BSA), a compound of the medium used, could be identified as trigger for the reaction. A first report on cat-allergic patients experiencing anaphylactic reactions upon consumption of pork meat coined the term pork–cat syndrome (60). Another study investigated the role of serum albumin in this syndrome. The sensitization of cat-allergic patients to cat serum albumin was analyzed and possible cross-sensitization profiles to pork albumin were determined by inhibition assays (61). The frequency of sensitization to cat albumin ranged between 14 and 23%, depending on the cohort, while sensitization to porcine serum albumin ranged from 3 to 10%, respectively. About 1/3 of the patients sensitized to porcine serum albumin are likely to experience adverse reaction by the consumption of pork, especially ham or sausages, as albumins are heat-labile proteins. Statistically, about 1–3% of cat-allergic patients would be at risk for adverse reactions to pork (61). The described cases all originated from Europe, but recently several cases with immediate type allergic reactions upon pork consumption have been reported in the US (62). Testing for serum IgE to cat and pork serum albumin allows discriminating this syndrome from the reactions of delayed food allergy related to the presence of IgE directed to alpha-gal sugar determinants on meat (63). The high level of IgE cross-reactivity between serum albumins hampers the determination of the sensitizing molecule. The clinical history of sensitization, the level of specific anti-albumin IgE titers as well as IgE inhibition data have to be taken into account to establish a correct diagnosis. Although IgE cross-reactivity is most frequent between mammalian albumins, cross-reactivity may also occur between cat and chicken albumin which share only 46% identical amino acids (64). BSA is an important allergen of meat and milk. IgG and IgA responses to BSA and different fragments thereof have been analyzed in three cohorts: unselected persons, new-onset insulin-dependent diabetes mellitus patients, and atopic patients (65). IgG and IgA antibodies to BSA were inversely correlated with age in the normal population. In all three cohorts, IgG antibodies recognized all three BSA domains with an equivalent frequency, however, only 31–46% of the subjects' IgA antibodies were able to bind to the N-terminal part of the BSA molecule. This finding correlated with the fact that the N-terminal domain was also the first to be degraded in simulated gastric fluid experiments, suggesting that systemic IgG antibody responses and gut-associated lymphoid tissue IgA responses to the food allergen are independent.

Mammalian serum albumins display a very high amino acid identity (72–82%) to HSA (66). This leaves very little space for the discrimination of self from non-self. It is interesting to note that up to now no clear autoimmune reaction to HSA has been proven. The role of the so-called ABBOS peptide, an epitope present on the BSA molecule, has been controversially discussed in the development of insulin-dependent diabetes mellitus (67).

It has been hypothesized that, at least for food allergens, molecules with a high degree of similarity to human homologs would be poorly immunogenic (68). Above a threshold of 62% sequence identity, proteins were found to be rarely allergenic. BSA, an allergen of cow's milk is an exception as it shares 75.6% identity with HSA. The respiratory allergenic albumins present even

identities of 81.7% (cat), 79.8% (dog), and 76.1% (horse) to HSA. The structural features of mammalian and avian serum albumins have been addressed in a recent review by Chruszcz et al. (66). Their three-dimensional structure is crucial for antibody binding and allergenicity. Albumins are sensitive to heat treatment and thoroughly cooked food is generally tolerated by allergic patients.

CONCLUSION

Inhalant mammalian allergens are capable of eliciting a large variety of immune responses, of which production of specific IgE is only one. At the humoral level, a particular aspect is the high IgE cross-reactivity mainly within the serum albumins but also a cross-reactive lipocalin group. At the T cell level, the overall proliferative response to mammalian allergens is rather low, with nevertheless important variations. Allergen-specific CD4 T cells in peripheral blood of allergic persons are slightly more frequent than in non-allergic persons and predominantly have a Th2 central memory profile. The studies with Fel d 1 peptides have clearly established the existence of an effector cellular immune response, which has a pathogenic potential which is independent of the humoral IgE response. High allergen exposure, whether natural or immunotherapy-induced, is correlated to clinical benefit in parallel to the production of high titers of allergen-specific IgG4. A causal relationship between the two observations needs still to be proven. The issue of an autonomous immunogenicity and allergenicity of mammalian allergens is still elusive, the possibility of an allergenic bystander effect of other inhaled particles with adjuvant properties is a realistic option. At last, mammalian allergens, especially those whose amino acid sequence is close to that of their human homologs, are unique tools to study the immune response at the frontier between self and non-self.

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Orchestration of an uncommon maturation cascade of the house dust mite protease allergen quartet

Marie-Eve Dumez[1,2], Julie Herman[2], Vincenzo Campizi[1,2], Moreno Galleni[2], Alain Jacquet[3] and Andy Chevigné[1]*

[1] *Laboratory of Retrovirology, Department of Infection and Immunity, Centre de Recherche Public Santé, Luxembourg, Luxembourg*
[2] *Macromolécules Biologiques, Department of Life Sciences, Centre for Protein Engineering, University of Liège, Liège, Belgium*
[3] *Faculty of Medicine, Department of Medicine, Division of Allergy and Clinical Immunology, Chulalongkorn University, Bangkok, Thailand*

Edited by:
Christiane Hilger, Centre de Recherche Public Santé, Luxembourg

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Beatrice Jahn-Schmid, Medical University of Vienna, Austria
Enrique Fernandez-Caldas, Inmunotek, Spain

****Correspondence:***
Andy Chevigné, Laboratory of Retrovirology, Centre de Recherche Public Santé, 84 Val Fleuri, Luxembourg L-1526, Luxembourg
e-mail: andy.chevigne@crp-sante.lu

In more than 20% of the world population, sensitization to house dust mite allergens triggers typical allergic diseases such as allergic rhinitis and asthma. Amongst the 23 mite allergen groups hitherto identified, group 1 is cysteine proteases belonging to the papain-like family whereas groups 3, 6, and 9 are serine proteases displaying trypsin, chymotrypsin, and collagenolytic activities, respectively. While these proteases are more likely to be involved in the mite digestive system, they also play critical roles in the initiation and in the chronicity of the allergic response notably through the activation of innate immune pathways. All these allergenic proteases are expressed in mite as inactive precursor form. Until recently, the exact mechanisms of their maturation into active proteases remained to be fully elucidated. Recent breakthroughs in the understanding of the activation mechanisms of mite allergenic protease precursors have highlighted an uncommon and unique maturation pathway orchestrated by group 1 proteases that tightly regulates the proteolytic activities of groups 1, 3, 6, and 9 through complex intra- or inter-molecular mechanisms. This review presents and discusses the currently available knowledge of the activation mechanisms of group 1, 3, 6, and 9 allergens of *Dermatophagoides pteronyssinus* laying special emphasis on their localization, regulation, and interconnection.

Keywords: mite, proteases, Der p 1, allergen, activation cascade, localization, interaction

INTRODUCTION

House dust mites (HDMs; *Dermatophagoides* spp.) are common reservoirs of potent airborne allergens, which induce Th2-biased inflammatory diseases such as allergic asthma, perennial rhinitis as well as atopic dermatitis in sensitized patients (1). To date, over 23 different HDM allergen groups inducing the production of allergen-specific IgE in humans have been referenced (2).

A growing amount of literature suggests that HDM allergens can stimulate numerous innate immune activation pathways to initiate the Th2 allergic response (3). Although HDM allergens can induce lung inflammation by protease-independent mechanisms, the proteolytic activities of HDM allergens trigger key innate signaling to initiate the allergic response through, among others, the disruption of the airway/skin epithelial barrier, the protease-activated receptor-2 (PAR-2) activation, and other cell-surface receptor cleavages (3–5). These proteolytic attacks facilitate the uptake of the allergens by dendritic cells (DCs) in subepithelial tissues and lead to the release of numerous proinflammatory (IL-6, IL-8, and IL-1β) as well as innate Th2 (IL-25, IL-33, and TSLP) cytokines from the target cells. While the crystal structure of Der p 1 demonstrated that this allergen is a papain-like cysteine protease, sequence homologies, and protease inhibition assays proved that Der p 3, Der p 6, and Der p 9 belong to the trypsin-like, chymotrypsin-like, and collagenolytic-like serine protease families, respectively (6). Although the biological roles of these proteases in mites have not hitherto been completely unraveled, these allergens could more probably play a digestive function for the mite as they were detected in the gut as well as in mite feces. The four HDM allergen proteases are all synthesized as pre-zymogens formed by a signal peptide essential for the secretion, an N-terminal propeptide followed by the mature protease domain. Each corresponding prosequence inhibits the respective protease to prevent cellular toxicity during their expression. Considering the critical role of proteolytically active HDM allergens in the initiation of the allergic response, the elucidation of the pathways for the maturation of these allergens offers opportunities to deeply characterize their proteolytic specificities allowing the identification of their corresponding protein substrates on the target innate and adaptive immune cells.

The present minireview will update the information about the inter- and intra-molecular maturation mechanisms of the protease allergens from *Dermatophagoides pteronyssinus* with special emphasis on particular features of propeptides and protease interactions. We will highlight that the HDM protease quartet processing follows an uncommon and interconnected maturation pathway, which is uniquely orchestrated by Der p 1.

THE MATURATION OF ALLERGEN PROTEASES FROM *DERMATOPHAGOIDES PTERONYSSINUS*

proDer p 1

Mite cysteine protease Der p 1 (group 1) belongs to the papain-like protease family (CA1) and is considered one of the most

potent HDM allergens on the basis of the high frequency (70–100%) of specific IgE in HDM allergic patients (7, 8) as well as of its capacity to proteotically trigger innate immune activation (3). Through the removal of its signal peptide (18 residues), Der p 1 is secreted as an inactive zymogen, proDer p 1, composed of a catalytic domain of 222 residues and an N-terminal propeptide of 80 residues, which acts as an internal chaperone during protease folding and then locks the protease active site (9, 10). The crystallographic structure of proDer p 1 revealed that the propeptide of Der p 1 adopts a unique fold within the CA1 protease family (10). The propeptide of Der p 1 notably displays an intermediate size (80 residues) and is devoid of the canonical ERFNIN motif in its N-terminal globular domain (**Table 1**). The propeptide of Der p 1 is also characterized by the presence of an additional fourth α-helix replacing the unstructured C-terminal tail normally found in the other propeptide subfamilies (60 or 100 residues).

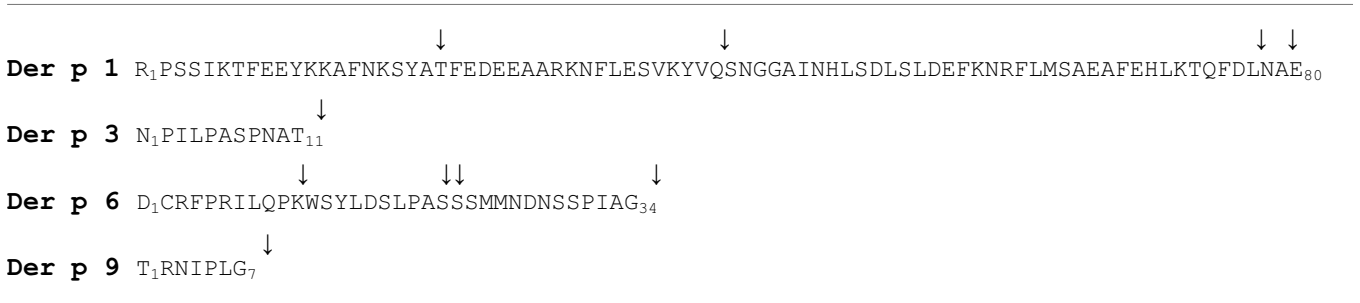
In vitro activation of proDer p 1 produced by the yeast *Pichia pastoris* or by S2 insect cells was shown to be induced under acidic conditions (i.e., pH 4) (11–14). Biophysical studies demonstrated that under acidic conditions, the propeptide of Der p 1 partly unfolds, leading to a considerable increase in the solvent accessibility and flexibility of the residues located in the N-terminal globular domain. Under these conditions, the propeptide loses its inhibitory ability and becomes a substrate for Der p 1 and most probably for other mite allergen proteases (11, 15–17). *In vitro*, the auto-activation of proDer p 1 at pH 4 leads to the formation of intermediates, which correspond to the successive loss of the first and second N-terminal α-helices following cleavages at the -NKSY_{19}-A_{20}TFE- and -KYVQ_{40}-S_{41}NGG- sites, respectively, considering the first residue of the zymogen as residue 1 (11, 13, 18) (**Table 1**). Generation of fully active Der p 1 with or without two additional residues (AE_{80}) is then achieved through a final cleave at overlapping cleavage sites (-FDLN_{78}-A_{79}ETN- or -LNAE_{80}-T_{81}NAC-) located at the propeptide C-terminus (11, 13, 18–20). It is noteworthy that these cleavages take place in propeptide regions that correspond to solvent exposed coil connecting the different α-helices and at sequences corresponding to Der p 1 proteolytic specificity (21). Activation of proDer p 1 was also shown to occur through inter-molecular cleavages of the precursor by active Der p 1 protease (14, 15). The proDer p 1 sequence contains two N-glycosylation sites, one within the propeptide (-N_{16}KS-) and one within the catalytic domain (-N_{132}QS-) the latter being glycosylated in the recombinant and natural forms of Der p 1 (11, 13, 14, 22, 23). Surprisingly, while pH is the major factor triggering proDerp 1 maturation, the glycosylation of the Der p 1 propeptide by the yeast *P. pastoris* at Asn16, which is N-terminally located to the -N_{16}KSY_{19}-A_{20}TFE- cleavage site, was shown to decelerate the activation rate of the zymogen (11, 24). Although the glycosylation pattern of the Der p 1 precursor in mites is most probably different, one cannot rule out that such interference might also be observed in mites and could consequently constitute a regulation system for allergen maturation.

proDer p 3

Based on the high percentage of its sequence identity with trypsin-like enzymes but also on its proteolytic specificity (i.e., preference for an Arg or a Lys residue in P1 position), Der p 3 (group 3) was classified into the S1A serine protease family (25–27). To date, the binding of IgE from sera of allergic patients to Der p 3 appears controversial and varies between 10 and 100% (7, 25, 27–30). Although the protein substrates targeted by Der p 3 still need to be fully elucidated, the proteolytic activation of PAR-2 by Der p 3 was clearly demonstrated (31, 32). Moreover, the enzymatic activity of a recombinant form of Der p 3 toward the QAR-AMC fluorescent peptide substrate was demonstrated to be 50 times higher than that of Der p 1, thereby indicating that although present in low quantity in the HDM extracts, Der p 3 greatly contributes to the total proteolytic activity of the HDM extracts (33).

Der p 3 is synthesized in mites as a pre-zymogen constituted of a signal peptide (18 residues), a propeptide of 11 residues, and a serine protease domain of 232 residues (**Table 1**) (25). In contrast to the Der p 1 propeptide, the Der p 3 prosequence was shown as not involved in the correct folding of the zymogen (34). Although it shows poor inhibitory capacity toward the mature protease, the Der p 3 propeptide is essential to block the Ile_{12} residue of Der p 3 and to maintain the allergen in less active conformation as previously observed for trypsinogen, the precursor of trypsin (33–35). Trypsinogen is commonly activated through inter-molecular cleavages by the membrane serine protease enterokinase, following the recognition of a conserved poly-aspartyl lysine motif [(D)DDDK] located at the end of the propeptide (36, 37). Alternatively, trypsinogen can be activated through autocatalytic cleavage occurring after neutralization of

Table 1 | Propeptides of *Dermatophagoides pteronyssinus* proteases.



The arrows indicate the identified Der p 1 cleavage sites.

the negative charges of the poly-aspartyl lysine motif by calcium ions (36, 38, 39). Compared with other trypsin proteases, the propeptide of proDer p 3 (N*P*IL*P*AS*P*NAT_{11}-) shows some distinct features such as the presence of a Thr instead of an Arg or a Lys residue in P1 position. Consequently and in contrast to trypsinogen, no auto-activation of recombinant proDer p 3 was observed (33). The activation mechanism of proDer p 3 produced in *P. pastoris* was shown to be inter-molecular and led by cysteine protease Der p 1 (33) (**Table 1**). It is noteworthy that the N-glycosylation at -N_9AT_{11}- site within the propeptide decreased the maturation rate as observed for proDer p 1 (33). The maturation of proDer p 3 was also shown to depend on the interactions between the polyproline motif (P_2ILP_5ASP_8) of the propeptide and Der p 1, since mutation or deletion in this motif, especially of Pro_5 and Pro_8, drastically reduced the activation rate of the zymogen (33, 34). This uncommon polyproline motif within the protease propeptide was also demonstrated to protect proDer p 3 against undesired hydrolysis (33, 34). Indeed, as observed for trypsin, mature Der p 3 undergoes rapid autolysis through cleavages at the -GGEK_{17}-A_{18}LAG- and -KNAK_{115}-A_{116}VGL- sites, explaining more probably the low amount of Der p 3 detected in HDM extracts (25, 27, 33, 34).

proDer p 6

Der p 6 (group 6) is a chymotrypsin-like serine protease (S1 family) that preferentially cleaves peptide bonds preceded by an aromatic residue (i.e., Phe, Tyr, and Trp) (30). The precursor of Der p 6 is composed of a signal peptide of 16 residues, a propeptide of 34 amino acids, and a catalytic domain of 231 amino acids (**Table 1**) (40). The propeptide of Der p 6 has recently been shown to act as an inhibitor of the cognate catalytic domain (16). This suggested that as for Der p 1 and Der p 3, the propeptide of Der p 6 regulates the spatio-temporal activation of the protease zymogen in mites (16). Similarly to Der p 3, the propeptide of Der p 6 was shown as not required for the correct folding of recombinant Der p 6 expressed in *P. pastoris* (16).

Surprisingly, while chymotrypsinogen displays an Arg at the C-terminus of its propeptide for the recognition and cleavage by trypsin (41, 42), the C-terminal extremity of the Der p 6 propeptide (-P_{31}IA_{33}G-) is highly similar to that of the Der p 3 propeptide (-P_8NA_{10}T-). In line with this observation, we recently showed that as for proDer p 3, proDer p 6 can be activated by Der p 1 providing a fully active Der p 6 protease presenting the expected mature N-terminal extremity (V_{35}IGG-) (**Table 1**) (16).

proDer p 9

Although very poorly characterized, Der p 9 (group 9) is classified as a collagenolytic-like serine protease on the basis of its ability to hydrolyze collagen (43). Interestingly, its high percentage of identity with Der p 3 (76%) together with the conservation of the residues corresponding to the catalytic triad (His_{48}–Asp_{88}–Ser_{200}) as well as those related to the specificity pocket all suggest that Der p 9 could be a trypsin-like protease. Moreover, like Der p 3 and trypsin, Der p 9 was shown to activate PAR-2 through a proteolytic cleavage occurring at the -SKGR_{36}-S_{37}LIG- site of the receptor (32).

The Der p 9 pre-zymogen is composed of a signal peptide of 17 residues, a propeptide of 7 amino acids (TRNIPLG_{7-}) preceding a 220-residue catalytic domain (**Table 1**) (43) (Uniprot: Q8MWR5). The role of the propeptide and the activation mechanism leading to fully active protease Der p 9 remain to be fully elucidated. By using fluorescence resonance energy transfer (FRET) substrates, we have recently demonstrated that recombinant and natural active Der p 1 cleave the peptide mimicking the junction between the propeptide and the mature form of Der p 9 (Dnp-IPLG_7-V_8IGG-AMC), which suggests that Der p 1 could also be critical for the maturation of proDer p 9 (**Table 1**) (16). Nevertheless, the isolation of another cDNA coding for a Der p 9 related serine protease with an alternative putative extended propeptide sequence (Uniprot: Q7Z163, Q8MWR4) would require additional experiments.

UNCOMMON AND UNIQUE ACTIVATION PATHWAY

Taken together, the *in vitro* results generated using recombinant forms of the different zymogens and FRET substrates clearly demonstrate the major role of Der p 1 in the activation process of the *D. pteronyssinus* mite allergen proteases (11, 13, 14, 16, 18, 33). Following its auto-activation under acidic conditions, Der p 1 remarkably orchestrates the inter-molecular maturation of its own precursor (proDer p 1) but also of serine protease precursors proDer p 3, proDer p 6, and most probably proDer p 9 (**Figure 1**) (11, 13, 14, 16, 18, 33).

Although the exact location where the maturation of the allergen proteases takes place in the mite remains unknown, different hypotheses can be considered. Mite proteases Der f 1, Der p 1, Der f 3, and Der p 6 were all immuno-localized in the digestive tract of the *Dermatophagoides farinae* and *D. pteronyssinus* species (16, 44–46). In particular, Der p 1 was localized in the cells lining the anterior midgut (AMg) corresponding to an acidic environment (pH 4), in the posterior midgut (PMg) (pH 5) as well as in the hindgut (Hg) where the pH was shown to reach a value of 6 (16, 44, 45, 47, 48). It is therefore plausible that proDer p 1 is secreted in the anterior gut and activated in the acidic lumen. Alternatively, it is worth noticing that the Der p 1 propeptide contains a highly conserved two-lysine motif (Lys_{37} and Lys_{72}) that might be involved in the targeting of the zymogen to the acidic vesicles of the anterior gut cells to initiate its intracellular maturation before its release in the lumen (49, 50). Serine proteases Der f 3 and Der p 6 were observed in the Hg (16, 46) and Der p 1 was co-localized with Der p 6 in the Hg of *D. pteronyssinus* sections indicating that mature protease Der p 1 could activate the secreted serine protease zymogens in the Hg where pH corresponds to its maximum activity (i.e., pH 6.5) (16, 48).

The activation mechanisms of the serine protease zymogens of the trypsin-like family (proDer p 3 and proDer p 6) by a cysteine protease (Der p 1) appear to be very uncommon for such protease families and are most probably related to the presence of specific residues at the C-termini of the propeptides. Noticeably, the P4–P3–P2–P1 residues (Schechter and Berger nomenclature) N-terminally located to the cleavage sites of the Der p 1 (*LNAE*$_{80}$-), Der p 3 (*P*NAT_{11}-), Der p 6 (*PIAG*$_{34}$-), and Der p 9 (*I*PLG_7-) proteases are all similar and perfectly match Der p 1 specificity (21). It is noteworthy that the propeptides of homologous zymogens from other dust mites such as the *D. farinae* and *Euroglyphus maynei* species exhibit a high degree

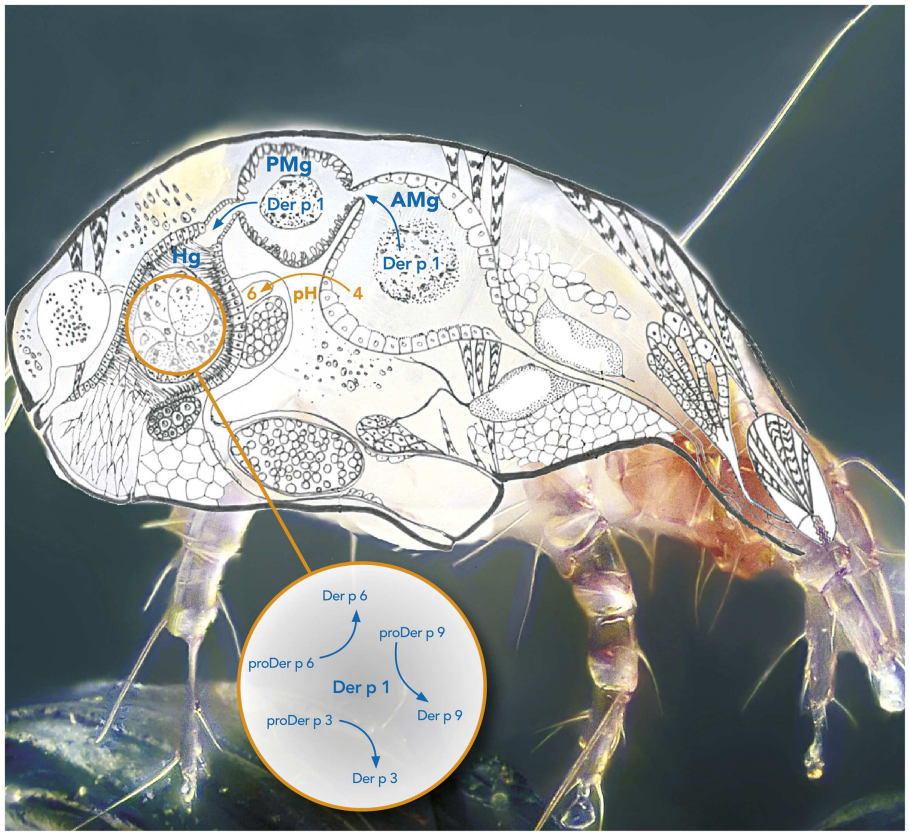


FIGURE 1 | Protease activation cascade in the digestive tract of the mite Dermatophagoides pteronyssinus. The box represents the activation cascade in the hindgut. AMg, Anterior midgut; PMg, Posterior midgut; Hg, Hindgut.

of similarity to those from *D. pteronyssinus* suggesting that a similar proteolytic pathway might also occur in these organisms (**Table 2**) (16).

CONCLUSION

During the last decade, we and others have unraveled the *in vitro* activation mechanisms of the mite cysteine (Der p 1) and serine (Der p 3, Der p 6, and Der p 9) protease precursors. All the generated data highlighted the role of Der p 1 as the "maestro" in the maturation processes of the different HDM protease allergens. This orchestration which appears rather uncommon among the protease world depends on specific sequences present at the C-terminus of the different propeptides.

Although it remains to be demonstrated that *in vivo* HDM protease allergen maturation is similar to the *in vitro* observations, the elucidation of the present activation cascade firstly provides key information for the design of new potent specific inhibitors to these clinically relevant allergens. Such molecules represent potential novel acaricidal compounds to control the HDM population by impairing their digestive function. The critical role of PAR-2 activation in HDM allergy and the effective PAR-2 cleavage by at least Der p 3 and Der p 9 demonstrates the interest in the blockage of the proteolytic activity to modulate the HDM allergic response (31, 32, 51, 52). It must be pointed out that the first preclinical results generated with inhaled Der p 1-specific allergen delivery inhibitors also provide clear evidence for the interest of such therapeutics in the treatment of HDM allergy (53).

Secondly, consistent productions of highly pure and fully active recombinant mature HDM protease allergens could open the way

Table 2 | Activation sites of zymogens from *Dermatophagoides pteronyssinus, Dermatophagoides farinae,* and *Euroglyphus maynei* species.

Groups	Proteins	Sequences
		↓
Group 1	Der p 1	..H_{70}LKTQFDLNAE$_{80}$-**T_{81}NACSINGNA$_{90}$**..
	Der f 1	..Q_{70}LKTQFDLNAE$_{80}$-**T_{81}SACRINSVN$_{90}$**..
	Eur m 1	..Q_{70}LKTQFDLNAE$_{80}$-**T_{81}YACSINSVS$_{90}$**..
Group 3	Der p 3	N_1PILPASPNAT$_{11}$-**I_{12}VGGEKALAG$_{21}$**..
	Der f 3	T_1PILPSSPNAT$_{11}$-**I_{12}VGGVKAQAG$_{21}$**..
	Eur m 3	N_1PILPSSPNAT$_{11}$-**I_{12}VGGQKAKAG$_{21}$**..
Group 6	Der p 6	..M_{24}MNDNSSPIAG$_{34}$-**V_{35}IGGQDAAEA$_{44}$**..
	Der f 6	..R_{20}SKIGDSPIAG$_{30}$-**V_{31}VGGQDADLA$_{40}$**..
	Eur m 6	/
Group 9	Der p 9	T_1RNIPLG$_7$-**I_8VGGSNASPG$_{17}$**..
	Der f 9	/

The arrow indicates the putative cleavage sites between the propeptide and the mature protease sequences which are in bold. (/) Unknown sequences.

for further characterization of their proteolytic specificities, for better definition of their respective interplay with the innate and adaptive immune system and for analysis of their IgE reactivity. Finally, the mapping of their corresponding IgE-binding epitopes, in the absence of any propeptide interference (epitope masking), could initiate the development of hypoallergenic variants for novel immunotherapeutic treatments.

AUTHOR CONTRIBUTIONS

Marie-Eve Dumez and Andy Chevigné designed the content and supervised the writing of the publication. Marie-Eve Dumez, Julie Herman, Vincenzo Campisi, Alain Jacquet, and Andy Chevigné wrote the minireview. Moreno Galleni critically revised the intellectual content of the publication. All authors gave their final approval to the version to be published.

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Helminth allergens, parasite-specific IgE, and its protective role in human immunity

***Colin Matthew Fitzsimmons*[1†], *Franco Harald Falcone*[2*†] and *David William Dunne*[1]**

[1] *Department of Pathology, University of Cambridge, Cambridge, UK*
[2] *School of Pharmacy, University of Nottingham, Nottingham, UK*

Edited by:
Annette Kuehn, Centre de Recherche Public de la Santé, Luxembourg

Reviewed by:
Tracey Lamb, Emory University School of Medicine, USA
Adriano Mari, Centri Associati di Allergologia Molecolare, Italy

****Correspondence:***
Franco Harald Falcone, Division of Molecular and Cellular Science, School of Pharmacy, University of Nottingham, Boots Science Building, Science Road, Nottingham NG7 2RD, UK
e-mail: franco.falcone@notttingham.ac.uk

[†] Colin Matthew Fitzsimmons and Franco Harald Falcone have contributed equally to this work.

The Th2 immune response, culminating in eosinophilia and IgE production, is not only characteristic of allergy but also of infection by parasitic worms (helminths). Anti-parasite IgE has been associated with immunity against a range of helminth infections and many believe that IgE and its receptors evolved to help counter metazoan parasites. Allergens (IgE-antigens) are present in only a small minority of protein families and known IgE targets in helminths belong to these same families (e.g., EF-hand proteins, tropomyosin, and PR-1 proteins). During some helminth infection, especially with the well adapted hookworm, the Th2 response is moderated by parasite-expressed molecules. This has been associated with reduced allergy in helminth endemic areas and worm infection or products have been proposed as treatments for allergic conditions. However, some infections (especially *Ascaris*) are associated with increased allergy and this has been linked to cross-reactivity between worm proteins (e.g., tropomyosins) and highly similar molecules in dust-mites and insects. The overlap between allergy and helminth infection is best illustrated in *Anisakis simplex,* a nematode that when consumed in under-cooked fish can be both an infective helminth and a food allergen. Nearly 20 molecular allergens have been isolated from this species, including tropomyosin (Ani s 3) and the EF-hand protein, Ani s troponin. In this review, we highlight aspects of the biology and biochemistry of helminths that may have influenced the evolution of the IgE response. We compare dominant IgE-antigens in worms with clinically important environmental allergens and suggest that arrays of such molecules will provide important information on anti-worm immunity as well as allergy.

Keywords: helminth, allergen, *Schistosoma mansoni,* protective role, IgE

THE IgE RESPONSE IS A PHYSIOLOGICAL IMMUNE RESPONSE TO HELMINTH INFECTION

The parallels between allergy and the immune response to parasitic worms (helminths) have been noted for some time. Unlike most other inflammatory/infectious conditions, allergy, and helminths induce strongly Th2-skewed responses associated with cytokines such as IL-4, IL-5, and IL-13, with mastocytosis, eosinophilia, and antibody class-switching to produce IgE [reviewed in Ref. (1)]. This normally rare, tightly controlled antibody isotype is greatly elevated in helminth infection. It is widely accepted that IgE, its receptors and distinctive cellular responses did not evolve to target harmless molecules occurring in plant pollen, dust-mites, or animal dander. Instead many believe that the IgE axis evolved to counter metazoan parasites (worms and parasitic arthropods) which are too large to be phagocytosed, and that allergy is a misdirected anti-parasite response in hypersensitive people (2). The symptoms of allergic responses; lachrymation, rhinitis, coughing, increased mucus production, and itching in response to histamine release are all responses likely to dislodge, trap, or flush out large parasites from skin or mucosa, e.g., by scratching.

There are however critical differences between the two conditions. Allergy occurs in people with atopy; defined as "a genetic predisposition toward the development of immediate hypersensitivity reactions against common environmental antigens" (3). It is a polygenic disorder linked to polymorphisms in genes of cytokine, cytokine receptors, and transcription factors associated with Th2 immune responses and with the expression of IgE and its receptors (4–7). In contrast, the elevated Th2 cytokines, IgE and eosinophilia during helminth infection are normal physiological responses to these pathogens. Furthermore, helminths actively moderate the inflammatory Th2 response of the host, inducing regulatory T and B cells, alternatively activated macrophages and production of immunoregulatory cytokines, such as IL-10 and TGFß, as well as IgG4 antibodies that counteract IgE [reviewed in Ref. (8)].

Recently Medzhitov and colleagues (9) re-appraised the toxin hypothesis of allergy (10), proposing that the IgE-mediated hypersensitivity response evolved to counter venoms and other noxious substances rather than macro-parasites. They argued that (1) immediate hypersensitivity is very rapid and worms are slow, (2) IgE is not required for worm immunity in mice, and (3) allergens do not have any obvious relationship with worms. Instead they proposed that it is toxins and venoms that need to be rapidly neutralized and that unpleasant allergic symptoms provoke toxin-avoidance behavior. This "toxin hypothesis" of allergy can in fact be traced back to the original discovery of anaphylaxis by Portier and Richet (11) [reviewed in (12)]. However, we would argue that (1) defense against invading helminth larvae also requires very fast responses – as elegantly demonstrated in the film of

Schistosoma mansoni cercariae penetrating and moving rapidly through skin tissue (13). Most recently, work by Obata-Ninomiya in Karasuyama's group (14) has demonstrated the importance of IgE (via ablation of the high affinity receptor) on basophils (but not mast cells) in trapping invading *Nippostrongylus brasiliensis* larvae in the skin of mice.

While it can be shown that IgE is not strictly necessary for anti-worm immunity in mice [argument (2) above], it needs to be stressed that there are other immunity mechanisms operating as well; IgE is a late mammalian additional mechanism to the Th2-mediated mechanisms of lower vertebrates (which are nonetheless still present in mammals), thus IgE-immunity is not the only mechanism of immunity against metazoan parasites available to mammals. This is exemplified by the occurrence of Th2-like immune responses to helminth infection in avian hosts in the absence of IgE (15). Finally [argument (3) above], we propose here that nearly all known allergens have equivalents (of widely varying structure) in metazoan parasites.

Most of the evidence relating IgE to anti-helminth immunity comes from epidemiological data. In a number of studies on human schistosomiasis, levels of anti-parasite IgE have been correlated with resistance to infection (16–22). Anti-parasite IgE responses have also been associated with immunity in human infections with hookworms (23, 24), *Trichuris* (25), and *Ascaris* (26, 27). Human experimental infection with a single, low dose of *Necator americanus* larvae in the context of helminth immunotherapy trials has shown that peripheral blood basophils become sensitized to parasitic allergens within 6 weeks of exposure, and remain fully responsive to stimulation with hookworm allergens years after this single infection (28). Thus, it appears that helminths are indeed powerful inducers of an IgE response, but how does this response relate to allergy?

EFFECTS OF HELMINTHS ON ALLERGY

Paradoxically, the global increase in allergy especially in urban areas (29) has led researchers to propose a modified hygiene hypothesis in which the decline in helminth infections is associated with an increase in allergic diseases (30). A number of studies show that communities with helminth infections have reduced rates of allergy (31–33) and the evidence that people with hookworm have less asthma (34–36) has inspired researchers to use experimental infections on asthma patients (37). It is proposed that the active suppression of Th2 responses by helminths has a bystander effect on concurrent allergic responses [reviewed in Ref. (8)]. In a study on Gabonese children, van den Biggelaar et al. (31) showed that the increased IL-10 levels induced by schistosome infection were negatively correlated with dust-mite sensitivity. The other side of these phenomena is that anti-helminth treatment programs risk increased rates of allergic disease and this has already been demonstrated in a number of intervention studies (38–40).

Under some circumstances helminth infection can actually increase prevalence of atopic disease and asthma (41, 42). A meta-analysis of 30 clinical studies on intestinal nematodes, concluded that while hookworm reduced the incidence of asthma, *Ascaris lumbricoides* increased the risk (34). It is likely that cross-reactivity between *Ascaris* and environmental allergens is involved.

The concept of cross-reactivity between helminth and environmental allergens is central to this review. We suggest that most if not all environmental allergens can be related to helminth counterparts and that the IgE response against these allergens is associated with host protection.

ARE ALL ALLERGENS PROTEINS WITH HOMOLOGS IN METAZOAN PARASITES?

Work in the allergy field has shown that very few protein families contain allergens (43) and importantly, the molecules targeted by IgE in helminths appear to be in these known allergen families (see **Tables 1** and **2**). Certain domains are highly represented in the list of known molecular allergens with the 10 most common allergen families containing approximately 40% of all know allergens. In the following section, we review the relationship between known helminth allergens and the structural allergen classification in the allergen database AllFam (http://www.meduniwien.ac.at/allergens/allfam).

For example, the muscle protein tropomyosin (AllFam code AF054) is an important IgE target in a number of nematode infections; *Onchocerca volvulus* (76, 77); *Ascaris lumbricoides* (78); *Anisakis simplex* [Ani s 3, (46)]; and tropomyosin from the blood fluke *Schistosoma mansoni* is also a human IgE antigen (Fitzsimmons, unpublished data). Tropomyosin is highly conserved across many invertebrates and is responsible for much of the IgE cross-reactivity between *Ascaris* and dust-mites (63). Cockroach tropomyosin is a major allergen (Bla g 7) that also shows strong IgE cross-reactivity with the highly similar *Ascaris* molecule (78). Santiago and co-authors (77) showed that tropomyosin from filarial nematodes is recognized by IgE against dust-mite tropomyosin (Der p 10), which can be absorbed completely using the nematode molecule. More importantly, they showed that the IgE response to Der p 10 was stronger in filarial-infected than in uninfected individuals.

Paramyosin is another allergen family (AF100) from invertebrate muscle targeted in IgE responses against *Schistosoma japonicum* (20), *Ascaris lumbricoides* (79), *Anisakis simplex* [Ani s 2 (45, 80)], and *Onchocerca volvulus* (81). There is evidence that *Ascaris* paramyosin shows IgE cross-reactivity with the tropical dust-mite paramyosin and allergen Blo t 11 (79). Cross-reactivity between helminths and environmental allergens has clear implications. Not only may some helminth infections increase sensitivity to mites and insects, but also high degrees of homology between parasite and allergic orthologs could lead to false diagnosis. Human helminth infections are not restricted to tropical regions (82). Ani s 2 and Ani s 3 are thought to be responsible for much of the cross-reactivity between *Anisakis* and other invertebrate species (83).

The helminth venom-allergen-like (VAL) proteins are another family targeted by IgE. Hookworms secrete a VAL-like molecule, called *Ancylostoma* Secreted Protein-2 (ASP-2), which was shown to be a potent IgE antigen in human studies in China and Brazil (24, 84). An IgE response to this molecule has been correlated with immunity (24). ASP-2 belongs to the Pathogen related-1 (PR-1) allergen family (AF044) characterized by the presence of the SCP/TAPS domain (Pfam, PF00188). The family contains group 3 and 5 insect venom allergens and VAL molecules from filarial

Table 1 | Summary of helminthic allergens.

Helminth allergen	Common name	Gene ontology (biological process)	Related common allergen	Conserved domains	UniProt accession number	AllFam	Reference
***Anisakis simplex* (HERRING WORM)**							
Ani s 1	Serine protease inhibitor (Kunitz type)	Serine protease inhibitor	Aprotinin	BPTI/Kunitz family of serine protease inhibitor cd00109	L7V3Q3	AF003	Moneo et al. (44)
Ani s 2	Paramyosin	Motor activity	Panallergen	Myosin tail PF01576	L7V1I9	AF100	Pérez-Pérez et al. (45)
Ani s 3	Tropomyosin	Troponin T binding	Panallergen	Tropomyosin PF00261	Q9NAS5	AF054	Asturias et al. (46)
Ani s 4	Cystatin	Cysteine type endoprotein type inhibitor	Minor cat allergen (Fel d3)	Cystatin-like domain cd00042	Q14QT4	AF005	Moneo et al. (47)
Ani s 5	SXP/RAL-2	Unknown	Unknown	PF02520/DUF148	A1IKL2	AF137	Kobayashi et al. (48)
Ani s 6	Trypsin inhibitor like cysteine rich domain	Trypsin inhibitor like cysteine rich domain	Minor latex allergen (Hev b SPI)	Trypsin inhibitor like cysteine rich domain PF01826	A1IKL3	n/a	Kobayashi et al. (48)
Ani s 7	n/a	Unknown	Unknown	None	A9XBJ8	n/a	Rodríguez et al. (49)
Ani s 8	SXP/RAL-2	Unknown	Unknown	DUF148 PF02520	A7M6S9	AF137	Kobayashi et al. (48)
Ani s 9	SXP/RAL-2	Unknown	Unknown (As14 ascaris allergen)	DUF148 PF02520	B2XCP1	AF137	Rodriguez-Perez et al. (50)
Ani s 10	Unknown	Unknown	Unknown	Unknown	D2K835	n/a	Caballero et al. (51)
Ani s 11	Unknown	Unknown	Unknown	Unknown	E9RFF3	n/a	Kobayashi et al. (52)
Ani s 12	Unknown	Unknown	Unknown	Unknown	L7V0K0	n/a	Kobayashi et al. (52)
Ani s CCOS3	Cytochrome *c* oxidase subunit 3	Aerobic electron transport chain	Bermuda grass pollen allergen 46 kDa (Cyn d Bd46k)	Cytochrome *c* oxidase subunit III cd01665	Q1 × 6K9	n/a	López and Pardo (53)
Ani s Cyt B	Cytochrome *b*	Aerobic electron transport chain	Unknown	Cytochrome *b* (N-terminus)/b6/petB cd00284	Q1 × 6L0	n/a	López and Pardo (53)
Ani s FBPP	Fructose 1,6-bisphosphatase	Phosphatase activity	Unknown	n/a	n/a	n/a	López and Pardo (53)
Ani s NADHDS4L	NADH dehydrogenase subunit 4L	NADH dehydrogenase	Unknown	ND4L cl10160	Q1 × 6K2	n/a	López and Pardo (53)
Ani s NARaS	Nicotinic acetylcholine receptor alpha-subunit	Unknown (nicotinic acetylcholine receptor)	Unknown	n/a	n/a	n/a	López and Pardo (53)
Ani s PEPB	(Phosphatidyl-ethanolamine-binding Protein)	Unknown (phosphatidyl-ethanolamine-binding)	Unknown	n/a	n/a	n/a	López and Pardo (53)
Ani s Troponin	Troponin C	Calcium ion binding	German cockroach allergen (Bla g 6)	EF-hand Ca^{2+} binding motif PF00036	Q9U3U5	AF007	Arrieta et al. (54)

(Continued)

Table 1 | Continued

Helminth allergen	Common name	Gene ontology (biological process)	Related common allergen	Conserved domains	UniProt accession number	AllFam	Reference
***Schistosoma mansoni* (BLOOD FLUKE)**							
Sch ma PM	Paramyosin	Motor activity	Panallergen	Myosin tail, PF01576	P06198	AF100	Webster et al. (55)
Sch ma Sm20	CBP, Sm20.8, Sm20	Calcium ion binding	Unknown	EF-hand Ca^{2+} binding motif, PF00036	P91804	n/a	Fitzsimmons et al. (56)
Sch ma Sm21	SmTAL2, Sm21.7	Calcium ion binding	Unknown	EF-hand Ca^{2+} binding motif PF00036	P32070	n/a	Fitzsimmons et al. (56)
Sch ma Sm22	SmTAL1, CBP	Calcium ion binding	Unknown	EF-hand Ca^{2+} binding motif, PF00036	P14202	n/a	Webster et al. (57)
Sch ma Sm31	Sm31, SmCB1, cathepsin B-like cysteine proteinase	Proteolysis, regulation of catalytic activity	Papain	Papain family cysteine protease, PF00112	P25792, Q8MNY2, G4V5C2, Q8MNY1, G4V5C1, G4V5D0	n/a	de Oliveira Fraga et al. (58)
Kappa-5	k-5	Unknown	Unknown	Unknown	AAX83114.1	n/a	Schramm et al. (59)
***Necator americanus* (HOOKWORM)**							
Nec a ASP-2	ASP-2	Unknown	Unknown	SCP-like extracellular protein domain, cd00168	Q7Z1H1	n/a	Zhan et al. (60)
Nec a calreticulin	Calreticulin	Calcium ion binding	Unknown	Calreticulin superfamily, PF00262	O76961	n/a	Pritchard et al. (61)
***Ascaris suum* (PIG ROUNDWORM) AND *Ascaris lumbricoides* (HUMAN ROUNDWORM)**							
Asc s 1	ABA-1, nematode polyprotein allergens	Fatty acid and retinoid binding	Unknown	n/a	Q06811	n/a	Christie et al. (62)
Asc s3	Tropomyosin	Troponin T binding	Panallergen	Tropomyosin, PF00261	F1L5K1, F1L3V2, F1KVZ5, F1L218	n/a	Acevedo et al. (63)
GSTA	Glutathione *S*-transferase 1	Transferase	Dust-mite allergen, Der p 8	GST_C_Sigma_like, cd03039, PF13417, GST_N_Sigma_like, cd03192, PF02798	P46436	n/a	Acevedo et al. (64)
***Echinococcus granulosus* (DOG TAPEWORM)**							
AgB	Antigen B	n/a	Unknown	n/a	n/a (multigene family)	n/a	Vuitton, (65)

(Continued)

Table 1 | Continued

Helminth allergen	Common name	Gene ontology (biological process)	Related common allergen	Conserved domains	UniProt accession number	AllFam	Reference
Ag5	Antigen 5	Proteolysis	Unknown	Trypsin-like serine protease, PF00089, cd00190	A2MJI2, I1WXU1	n/a	Khabiri et al. (66)
EA21	Cyclophilin	Protein folding	*Malassezia furfur* allergen, Mal f 6	Cyclophilin_ABH_like, cd01926	P14088	AF038	Ortona et al. (67)
HSP70	Heat shock protein 70	Response to stress	Dust-mite allergen Hsp70	Hsp70 PF00012	Q24789	AF002	Ortona et al. (68)
EF-1 beta/delta	EF-1	Translation elongation factor	Unknown	Elongation factor 1 beta (EF1B) guanine nucleotide exchange domain	Q9U8D5, Q9NGP3, Q0PWC5	n/a	Ortona et al. (69)
***Brugia malayi* (MALAYAN FILARIA)**							
Bru m 3	Tropomyosin	Troponin T binding	Panallergen	Tropomyosin, PF00261	A8NGJ2	n/a	Sereda et al. (70)
Bru m 13	GST, glutathione *S*-transferase	Metabolic process	House dust-mite allergen Der p 8	GST_N family cd03076, GST C-terminal domain family cd03210	A8PTL9, O02636	n/a	Rathaur et al. (71)
Bru m Bm33	Aspartic protease inhibitor, Bm33	Unknown	Unknown	Ascaris pepsin inhibitor-3 (API3) cl11634	A8Q4E4	n/a	Krushna et al. (72)

This table was compiled mainly from data extracted from the Allergome database (73) in combination with published literature. Conserved domain annotation is from conserved domain database (CDD) (74) and Pfam (PF; DUF, domain of unknown function) (75). AllFam numbers (AF) are from the database of allergen families AllFam (43). As can be seen from this table, not all helminth allergens currently have related common (non-helminthic) allergens. For example, there are currently no known common environmental allergens structurally related to the nematode polyprotein allergens.

nematodes, *Onchocerca volvulus* (85), and *Brugia malayi* (86), as well as trematodes *S. mansoni* (87) and *S. japonicum* (88). Furthermore, the presence of VAL molecules is also predicted in tapeworms (89). One of the *S. mansoni* homologs (SmVAL4) has been recently shown to be an IgE antigen in mice (90), but requires confirmation in the natural human host.

The tegumental allergen-like (TAL) proteins are some of the most dominant IgE-antigens in *S. mansoni* and an IgE response to some members of the TAL family has been associated with resistance to re-infection with the parasite (18, 19, 22). These molecules are EF-hand proteins (see **Figure 1A**), one of the biggest groups of molecular allergens (AF007). Other known allergenic helminth EF-hand proteins include *Anisakis simplex* troponin C (54) and the *Fasciola* calcium-binding protein, FgCaBP (91).

The glutathione *S*-transferase (GST) is another source of IgE cross-reactivity. GST of nematode species is targeted by IgE during infection (92). This enzyme is homologous with other members of the GST allergen family (AllFam, AF010) including major allergens in dust-mite (Der p 8) and cockroach (Bla g 5) as well as IgE-antigens in grass and fungi. GST from the filarial nematode *Wuchereria bancrofti* binds IgE against Bla g 5 (77).

Probably, the most potent helminth allergens are the nematode-polyprotein-antigens (NPA). These are large multimeric proteins that are cleaved into smaller fatty acid binding subunits (93) with functional but not structural similarity to the lipocalin allergens (AF015). The best characterized example is the ABA-1 protein from *Ascaris* species. Highly abundant in the body fluid of the adult worm, it provokes a strong IgE response in many infected individuals (93) and this has been associated with resistance to infection (27). The filarial nematode NPA termed gp15/400 has also been shown to be an IgE antigen (94). Interestingly, the non-NPA lipocalin-like fatty acid binding protein from filarial nematodes, BmA1.1, is an IgE antigen which can induce wheal and flare response in sensitized dogs (95).

While some of the Top 10 allergen families (tropomyosins, EF-hand proteins, PR-1, and lipocalins) have members in helminth

Table 2 | Examples of known allergens, compiled from AllFam (43) and published literature, illustrating that nearly all families of allergens in animals, plants, or fungi have corresponding allergens in helminths.

Structural motif (AllFam Acc.)	Parasite allergens	Plant allergens	Animal allergens (non-helminth)	Fungal allergens
Tropomyosin (AF054)	Ani s 3, Asc s 3, Bru m 3, Onc v 3, Onc o 3	–	Bla g 7, Blo t 10	–
Paramyosin (AF100)	Ani s 2, Sch j PM, Sch ma PM	–	Blo t 11, Der f 11, Der p 11	–
CRISP/PR-1/venom group 5 (AF044)	Na ASP-2, SmVAL4 (?)	Art v 2, Cyn d 24	Dol a 5, Pol a 5, Pol d 5, Ves g 5, Vesp m 5	–
EF-hand (AF007)	Sm TAL1, Ani s Troponin	Bet v 3, Bet v 4, Art v 5, Par j 4, Phl p 7	Cyp c 1, Gad m 1, Sal s 1, Thu a 1	–
Glutathione *S*-transferase (AF010)	Wb GST[a], Bru m 13, Onc v 13, Asc l 13, Asc s 13	Tri a GST	Bla g 5, Der p 8, Blo t 8	Asp f GST, Pen c 24
Nematode Polyproteins (n/a)	ABA-1 (Asc s 1) Gp 15/400	–	–	–
Cyclophilin (AF038)	EA21 (*E. granulosus*)	Bet v 7, Cat r 1	–	Asp f 11, Mala s 6
Hsp70 (AF002)	Hsp70 (*E. granulosus*)	Cor a 10	Der f HSP70	Alt a 3, Cla h HSP70
Calreticulin (AF055)	Na Calreticulin	–	–	Pen ch 31
Kunitz Trypsin inhibitor (AF003)	Ani s 1	Gly m TI, Sola t2, Sola t3, Sola t4	Bos d 3, Bos d TI (aprotinin)	–

As can be seen from this table, there currently appear to be no known non-helminth allergens corresponding to the nematode polyprotein family, although similar biological lipid binding functions are found, e.g., in the lipocalin allergen family (AF015).

[a] *N-terminal domain similar to C-terminal domain of glutathione S-transferase (AF010).*

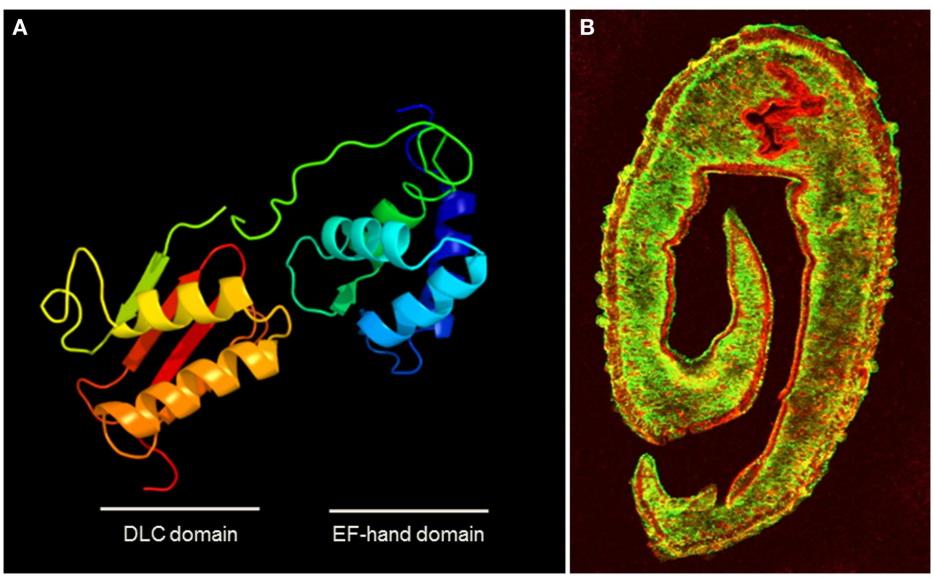

FIGURE 1 | (A) Homology modeling of the structure of the dominant SmTAL1 allergen in *S. mansoni* generated using protein homology/analogy recognition engine 2 (PHYRE2) (132), showing the two helix-loop-helix Ca^{2+}-binding motifs within the EF-hand domain. **(B)** Transverse section of male *S. mansoni* worm stained for the surface protein SmCD59 (green) and under that in the tegument layer, the EF-hand protein SmTAL1 (red) (courtesy of Prof. Alan Wilson University of York). The walls of the gut also stain for SmTAL1. The location illustrates how this sub-surface allergen in inaccessible to host IgE, unless the tegument layer is damaged, but its physiological function and role in host protection remain to be elucidated.

species that are known to be targeted by IgE during infection, other common allergen families (profilin, trypsin-like serine proteases, and lipocalin) have been identified in helminths, but their IgE binding has not yet been tested (96). Furthermore, the plant prolamins (AF050) and expansins (AF093 and AF094) are Top 10 allergen families that do not contain helminth equivalents. However, this assessment is made on sequence alignment and it is possible that these plant proteins share conformational motifs formed by non-homologous sequences (mimetopes) in un-related proteins from metazoan parasites. There is some evidence for this in that the plant expansin Php p 1 has no sequence homology with the mite allergen Der p 2, but Phl p 1, and Der p 2 have domains that share function (carbohydrate-binding) and close 3D conformational homology (97). While dust-mites are not metazoan parasites, they have close relatives that are (e.g., the scabies mite, *Sarcoptes scabiei*). Interestingly, the IgE response to *Sarcoptes scabiei* is thought to be involved in protection against repeat infestation (98).

WHAT MAKES AN ANTIGEN AN ALLERGEN?

Perhaps the greatest unanswered question in allergy is why only a small minority of antigens has allergenic properties. As stated previously, most proteins are not allergens. Thus, there are currently almost 15,000 protein domain families in the Pfam database (http://pfam.sanger.ac.uk/) of which only 255 have been identified in allergens (http://www.meduniwien.ac.at/allergens/allfam). The debate about which functional and molecular properties make a protein an allergen has continued for some time (99–101). Some functional properties give environmental and food proteins, a greater chance of sensitizing susceptible individuals. For example, high thermal stability allows allergens to persist in the environment or survive cooking and digestion. This is well illustrated by the example of plant chitinases, which are members of the pathogenesis-related family of proteins 4 (PR-4). Plant *chitinases* (AF041) have been described as panallergens in latex-fruit syndrome and are contained in a multitude of plants, such as Heveine [in latex, (102), kiwi fruit (103), in avocado (Pers a1, (104)] or grapes (105) and are related to dust-mite allergens Der p 15 and Der p 18 (106). Consistently with the hypothesis of thermal stability, despite the ubiquitousness of such PR-4 group proteins across the plant kingdom, allergenicity is only reported in foods that are consumed uncooked, as type I chitinases are inactivated by heating (107). While chitinases are also well represented in non-parasitic as well as parasitic helminths, to the best of our knowledge, no helminthic chitinases have yet been reported as allergens. The reasons for this are not understood.

In relation to food allergens and cooking, the special case of *Anisakis simplex* (*A. simplex*) deserves to be mentioned. *Anisakis* is the only currently known case of an organism being both a helminth parasite and a food allergen. The L3 larvae of the marine nematode *A. simplex* infect fish and cephalopods and consequently people that consume under-cooked seafood, however humans are a non-permissive host and the parasites cannot continue their life-cycle in man. Exposure to this helminth through food has been associated with allergic symptoms; asthma, rhinitis, dermatitis, and conjunctivitis (80), and in the case of uncooked fish, epigastric pain, erythema wheals, and pruritus ("gastroallergic anisakiasis"). It is not clear whether initial sensitization requires live parasite infection (anisakiasis) but it has been shown that sensitized patients can respond to heated or frozen *Anisakis* antigens in their food (108) or to small quantities by other exposure routes (109), such as skin contact, inhalation, or during skin prick testing. That the immune system responds to *Anisakis* as an invading helminth and as an allergen suggests that these are two aspects of the same response.

A feature of a relatively small subset of allergens is their proteolytic activity, which may permit penetration of mucosal barriers (110), for example, by cleaving proteins involved in tight junction formation (111). Many helminthic parasites rely on production of proteases during tissue migration, and we have previously argued that such proteases may be a factor underlying the parasites' intrinsic allergenicity (112).

However, such biological properties are not always present in allergens and the small percentage of protein domains that are targeted by IgE overall, in the absence of common biological activities, suggests they contain structures that are inherently allergenic. These structures vary widely and appear to have little in common overall. Given the probable evolution of the IgE system, we have proposed that proteins have inherent allergenicity because they have structural similarity to dominant antigens in metazoan parasites (96). However, it still remains unclear how such intrinsic structural features selectively enable a subset of antigens to induce, or become the object of, an IgE response.

Another consideration seems necessary. Many of the allergen families described above are also present in humans, but are not the target of an IgE response. Following *in silico* analysis of animal food proteins and their IgE responses, Jenkins and colleagues proposed that proteins with a sequence identity to a human homolog of >62% were rarely allergenic (113). We believe the IgE system evolved to target Th2 responses at large multi-cellular parasites, organisms that are much more closely related to us that bacterial, fungal, or viral pathogens. This means the evolved molecular targets had to be restricted if foreign metazoan antigens were to be targeted without inducing tolerance or risking auto-reactivity, and that non-parasitic proteins are allergenic because of their homology with metazoan parasites.

The hypothesis was examined by Santiago et al. (114). Using a bioinformatic approach, they compared the sequences of 499 allergens against the predicted proteomes of four helminths (including *Schistosoma mansoni*), four bacterial, and three fungal species. Their analysis supported previous work by Emanuelsson and Spangfort (115) finding little homology between bacterial proteins and allergens and the work by Jenkins et al. (113) who showed a drop in allergenicity as homology with human equivalents increased. While they reported that over 200 allergens had homologs in helminths, this was the minority, and indeed those with the greatest homology were the least allergenic. They concluded that allergenicity does not depend on similarity with parasite proteins, but on dissimilarity with human proteins. It should be remembered however, that most IgE epitopes are probably conformational (discontinuous) (116, 117) and would not be identified in such primary sequence comparisons.

LIFE-CYCLE EXPRESSION OF HELMINTH ALLERGENS AND THE HOST RESPONSE

Clinically important helminths often have complex life-cycles. Many involve a definitive host (man) and one or more intermediate hosts. The life-cycle expression profile of allergen-like molecules influences the host response. For example, trematodes (flukes) such as schistosomes undergo asexual reproduction in snail species before releasing larvae that infect humans, which then develop into adult worms that produce eggs following sexual reproduction. Some of the schistosome allergen-like TAL proteins are developmentally transcribed (22). SmTAL1 is sequestered inside the adult worms (**Figure 1B**) and is only exposed on the rare occasions when the adults die (56). Typically, *S. mansoni* worms live for 7–9 years (118). As individuals are usually infected more than once, resulting in asynchronous development and death of the parasite, this resembles seasonal allergen stimulation and infected people in areas endemic for *S. mansoni* have high levels of IgE to SmTAL1. SmTAL2 is expressed in schistosome eggs. In chronic infection hundreds of parasite eggs are trapped and die in the tissue every day. In a process that resembles specific allergen immunotherapy (SIT), the IgE response to allergen-like SmTAL2 appears to be desensitized by the continuous exposure to small doses of the antigen, while the specific IgG4 response becomes pronounced (56). SmTAL6 is only expressed in the snail stage and has no effect on the human response (119).

Adult tapeworms live in the lumen of the gut shedding eggs for excretion. If these eggs are ingested by a secondary host, they hatch and larvae encyst in the soft tissue. The contents of these structures are highly allergenic and can cause anaphylaxis if they burst. People carrying cysts of *Echinococcus granulosus* (echinococcosis) have IgE to parasite antigens AgB, a protease inhibitor, Ag5, a serine protease, and EA21 (65, 67). EA21 is a cyclophilin that shares close homology with allergenic yeast cyclophilin (Mal f 6) and may be cross-reactive with allergenic birch cyclophilin Bet v 7 (65). Infected individuals also produce IgE to the C-terminal region of *E. granulosus* Heat Shock Protein 70 an antigen with close homology to the dust-mite allergen, Der f HSP70 (68).

Hookworm eggs hatch in the soil where the larvae undergo several molts before becoming the infectious L3 form that penetrates the skin of the foot. The larvae then migrate to the lung and are coughed up, swallowed, and hence taken to their niche in the small intestine. It is the skin-penetrating L3 form that expresses and secretes the VAL protein ASP-2 (120). Since an antibody response to the molecule was associated with reduced infection ASP-2 has been tested as a vaccine candidate (24). Unfortunately, clinical trials in a hookworm endemic region of Brazil had to be stopped when vaccinated volunteers with a probable previous history of infection (as judged by the levels of pre-vaccination parasite-specific IgE) developed symptoms of generalized urticaria (84). The relationship between the protective effects of parasite-specific IgE and the hazards of vaccinating a sensitized population with an allergen present a major conundrum which is currently hampering the development of anti-helminthic vaccinations.

These three examples were chosen to illustrate the concept that allergen expression in helminth parasites is not a generalized feature of parasitic worms but a specific property of distinct developmental phases in the human host which is tightly linked to host protective mechanisms. Anti-protein IgE responses and host defense are two sides of the same coin which in our opinion are inseparable from each other.

However, while the link between the presence of parasite-specific IgE and resistance to infection is well supported by epidemiological and experimental evidence, the detailed molecular basis underlying such resistance is less well understood.

Specifically, one of the great unanswered questions is whether the presence of IgE on FcεRI-carrying cells (mainly basophils, mast cells, eosinophils) and subsequent receptor cross-linking by parasitic allergens is needed for host protection. Is the activation of basophils, mast cells, and other IgE-bearing effector cells necessary for protection?

It is well know that activation of mast cells and eosinophils can release proteases and toxic proteins (chymase, tryptase, major basic protein, eosinophil-derived neurotoxin, eosinophils cationic protein, etc.), some of which have been shown to directly kill larval stages of parasites (121).

Similarly, it could be speculated that IgE-dependent activation of basophils, which can result in the release of preformed or *de novo* produced highly toxic polypeptides such as Granzyme B (122) and possibly defensins (Falcone, unpublished data), also may result in parasite killing. While host-derived defensins have been shown to be effective against several unicellular parasites such as *Plasmodium* (123), *Toxoplasma* (124), *Babesia* (125), or *Trypanosoma* (126), their role in anti-helminthic immunity has only recently begun to be explored (127).

A LOOK INTO THE FUTURE: "MOLECULE-BASED" ANALYSIS OF ANTI-PARASITE HOST IMMUNE RESPONSES?

Traditionally, immunoparasitological research has relied on the use of complex antigenic mixtures such as somatic extracts of larval or adult stages, of eggs or of the tegument, or excretory/secretory materials collected *in vitro*, which all contain a multitude of antigens, allergens, and other un-related components. This can result in a low signal to noise ratio, for example caused by the presence of highly cross-reactive carbohydrate moieties, masking specific interactions at the individual protein level.

Due to the widespread use of complex water-soluble extracts obtained from parasitic materials in the past decades of parasitology research, several questions still remain to be answered. What are the individual molecular targets of the protective IgE response? Are certain patterns of IgE reactivity (rather than against a single determinant) associated with host protection? Do different IgE reactivity patterns correlate with various degrees of resistance to infection or post treatment re-infection?

This is reminiscent of the situation previously encountered in allergy research, which relied on water-soluble extracts which are difficult to standardize for diagnostic purposes (128), and may contain interfering components. Major impulses in the past years have come from introducing component resolved diagnosis (CRD) to the study of human allergy. In CRD, individual recombinant or purified allergens are used for measurement of immunoglobulin responses in allergic individuals (129). This frequently takes advantage of the availability of protein microarrays

(130). One of the key advantages of CRD is that it may enable distinction between genuine IgE reactivity and cross-reactive IgE (131).

While the use of CRD in allergy diagnosis is conceptually slightly different (it is used to identify the allergen source when cross-reactive allergen components are present), we suggest that a similar "molecule-based" approach would allow a better understanding of host resistance against helminths at the molecular level and, from a practical point of view, point the way to safer or more effective multi-target anti-helminthic vaccinations.

CONCLUDING REMARKS

If the IgE axis evolved to protect mammals against multi-cellular parasites, studying host responses to these organisms may teach us much about other IgE-mediated phenomena such as allergy. For example, characterizing parasite structures targeted by IgE may identify homologous molecules and potential allergens in novel foods and genetically modified organisms. The relationship between allergy and helminth infection brings costs and benefits. Elucidation of the molecular mechanisms by which some parasites moderate Th2 response in their hosts, may yield improved therapy for allergic conditions. On the other hand, treatment for the same worms in the developing world may inadvertently increase the prevalence of atopic disease. Moreover as a consequence of cross-reactivity between parasite and environmental allergens certain helminths can actually sensitize and aggravate allergy. Parasitic worm infections are a serious health problem in many countries and high resolution molecular techniques developed in the allergy field may help us to understand better the anti-parasite responses that are associated with immunity.

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Fish allergens at a glance: variable allergenicity of parvalbumins, the major fish allergens

Annette Kuehn[1]*, Ines Swoboda[2], Karthik Arumugam[1], Christiane Hilger[1] and François Hentges[1,3]

[1] *Laboratory of Immunogenetics and Allergology, Public Research Centre for Health (CRP-Santé), Luxembourg, Luxembourg*
[2] *Molecular Biotechnology Section, University of Applied Sciences, Vienna, Austria*
[3] *Unit of Immunology and Allergology, Centre Hospitalier de Luxembourg, Luxembourg, Luxembourg*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Richard E. Goodman, University of Nebraska-Lincoln, USA
Habib Chabane, Centre Hospitalier Delafontaine, France

***Correspondence:**
Annette Kuehn, Laboratory of Immunogenetics and Allergology, Public Research Centre for Health (CRP-Santé), 84 Val Fleuri, Luxembourg L-1526, Luxembourg
e-mail: annette.kuehn@crp-sante.lu

Fish is a common trigger of severe, food-allergic reactions. Only a limited number of proteins induce specific IgE-mediated immune reactions. The major fish allergens are the parvalbumins. They are members of the calcium-binding EF-hand protein family characterized by a conserved protein structure. They represent highly cross-reactive allergens for patients with specific IgE to conserved epitopes. These patients might experience clinical reactions with various fish species. On the other hand, some individuals have IgE antibodies directed against unique, species-specific parvalbumin epitopes, and these patients show clinical symptoms only with certain fish species. Furthermore, different parvalbumin isoforms and isoallergens are present in the same fish and might display variable allergenicity. This was shown for salmon homologs, where only a single parvalbumin (beta-1) isoform was identified as allergen in specific patients. In addition to the parvalbumins, several other fish proteins, enolases, aldolases, and fish gelatin, seem to be important allergens. New clinical and molecular insights advanced the knowledge and understanding of fish allergy in the last years. These findings were useful for the advancement of the IgE-based diagnosis and also for the management of fish allergies consisting of advice and treatment of fish-allergic patients.

Keywords: allergenicity, fish allergy, fish gelatin, food allergy, isoallergens, isoforms, monosensitivity, parvalbumin

FISH ALLERGY

PREVALENCE

In human diet, fish is a valuable source of essential amino acids, polyunsaturated fatty acids, and lipid-soluble vitamins. Although the fish consumption in European countries is quite stable, the global demand for fish and fish products is increasing still steadily (1). While many literature sources indicate that allergies to fish are on the rise, the actual prevalence is not well established as most studies are based on self-reported food allergies (2). It is estimated that up to 0.2% of the general population is affected by fish allergy (3, 4). However, the exposure to fish is an important factor determining the reported prevalence of fish allergy. Therefore, the prevalence of fish allergy is greater in countries with high fish consumption and fish-processing industries (5).

CLINICAL FEATURES

Fish allergy is a pathophysiological, IgE-mediated immune response to specific fish proteins. Patients become sensitized by allergen exposure via the gastro-intestinal tract during ingestion, which is the major route of sensitization, or via the respiratory system by fish aeroallergens or skin contact (6–9). Common clinical manifestations are oral allergy syndrome, rhinitis, abdominal pain, diarrhea, urticaria, angioedema, asthma, and in severe cases, even life-threatening anaphylaxis (10–12). Fish aeroallergens can be important triggers of atopic dermatitis (13). There are only a few clinical studies addressing minimal eliciting doses of fish allergy. However, already low milligram amounts of fish seem to be sufficient to trigger allergic symptoms in sensitized patients (14). Cross-reactivity among fish species has been commonly reported for fish allergy (11, 15, 16). This clinical cross-reactivity seems to be even more pronounced between closely related fishes. On the other hand, it has been shown that even highly fish-sensitized patients can tolerate certain fish species, such as tuna (17, 18). In addition, in case reports, clinical monosensitivity to single fishes has been proven for sole, swordfish, pangasius/tilapia, tuna/marlin, and more recently, for salmon and salmonid fishes (19–26). So far, no cross-reactivity has been shown for fish and other seafood allergens while in some cases, a co-sensitization to both allergen sources might occur (27).

DIAGNOSIS AND PATIENT CARE

The diagnostic procedure is based on four main pillars: the patient's clinical history, *in vivo* analysis of skin reactivity, *in vitro* quantification of specific serum IgE and, in selected cases, oral provocation challenges. A broad diversity of fishes is globally consumed but only a limited number of commercial extracts are available for skin testing. Therefore, fresh or processed fish is commonly used for this *in vivo* analysis. However, the predictive value of skin tests is low (28). For *in vitro* analysis of specific IgE levels, the ImmunoCAP system (ThermoScientific) offers a wide panel of fish extracts. Meanwhile, two recombinant parvalbumins from carp and cod are available for this diagnostic assay. The predictive value of fish extract-specific IgE measurements is not well

established, but a high titer of specific IgE (20 kU_A/L) was reported to predict an allergy to cod with a likelihood of 95% (29).

Other adverse reactions might be misdiagnosed as fish allergy (30). Allergy-like symptoms occur upon ingestion of histamine-contaminated, spoiled fish ("scombroid fish poisoning") (31). Also, consumption of fish contaminated with the parasite *Anisakis simplex* (herring worm) provokes acute allergic manifestations caused by IgE-mediated sensitization to *Anisakis* allergens (27, 32).

To avoid severe reactions, the management of fish allergy relies on the elimination of each fish product from the diet of the sensitized patient. In some cases, it has been reported that patients may lose their sensitivity upon eliminating diet (33, 34). The therapeutic desensitization to fish has been reported only for a single case (35). The development of specific immunotherapeutics based on hypoallergenic variants of parvalbumins, the major fish allergens, is the focus of ongoing studies (36, 37). Research on a primary strategy for the prevention of fish allergy is very limited. The current recommendations of the American Academy of Allergy, Asthma & Immunology also do not suggest a general, delayed introduction of fish in the diet of children (38).

FISH ALLERGENS

FOOD ALLERGENS

The few foods that are responsible for causing most allergic reactions are milk, eggs, peanuts, tree nuts, fish, shellfish, soy, and wheat. They contain potent food allergens (39). Their allergenic potency has been related to specific protein features. These allergens are highly abundant in the food sources, and moreover, they possess a high stability toward food processing and digestion (40). The structural stability has been allocated to different protein characteristics such as intrinsic ligand binding and intramolecular disulfide bonds (41). Some food allergens form protein aggregates of high stability. Although some food allergens are sensitive to gastric and intestinal digestion, degradation fragments are still recognized by specific IgE antibodies (42). Food allergens of animal origin are mainly grouped into three protein superfamilies such as caseins, tropomyosins, and EF-hand proteins (43).

FISH PARVALBUMINS

Most fish parvalbumins belong to the beta-subtype while the alpha-subtype is predominantly found in other organisms. Beta-parvalbumin has first been identified as fish allergen in Baltic cod (44). Later on, the importance of this protein as the fish panallergen was confirmed for a wide range of commonly consumed species such as salmon, carp, mackerel, tuna, and pilchard (45–49). Parvalbumins are highly stable, low-molecular-weight proteins (10–12 kDa), which are very common in fish muscle (5). The muscle of bony fishes is composed of two tissues, the light and dark muscle differing by their physiological function and composition (50). The parvalbumin expression is considerably higher in light than in dark muscle tissue (51). In contrast to most bony fishes, pelagic fishes such as tuna have mainly dark muscles of low parvalbumin content (48). This complies with the low allergenicity reported for tuna which is even used in canned preparations as matrix for food challenges (17). Overall, the parvalbumin content differs considerably in fish species and the different amount of parvalbumins correlates with the variable allergenicity of fishes (52–54). It has been shown that the parvalbumin level is up to 100-times higher in carp than in mackerel or tuna muscle. In addition, physical and chemical effects of food processing may alter the allergenicity of fish in food preparations by parvalbumin degradation or oligomerization which may decrease or increase the number of IgE epitopes (55).

Parvalbumins belong to the family of EF-hand proteins. As such, they contain specific EF-hand motifs composed of 12 residues of long loops which are involved in the binding of divalent metal ions. The N-terminal EF-hand site is a non-functional domain while the two other EF-hand motifs are binding calcium and magnesium ions (56). The physiological role of muscle parvalbumins is related to the regulation of the intracellular calcium concentration during muscle relaxation (57). Upon ion binding and ion release, the beta-parvalbumin structure is subjected to a global rearrangement indicating a general flexibility of the EF-hand domains (58). The apo-protein, which is calcium-depleted parvalbumin, has a reduced ability to bind IgE antibodies from fish-allergic patients (59). Consequently, these calcium-binding protein regions were attributed to conformational B-cell epitopes (60).

As of today, the official database of allergens contains 21 parvalbumins from 12 fish species[1] (**Table 1**) (61). The Allergome database lists far more than 100 entries for fish parvalbumins and their isoforms/isoallergens[2] (62). This much higher number arises from a compilation of homolog, potentially allergenic molecules, in addition to proteins of proven allergenicity. Parvalbumin has been defined as the major fish allergen as a majority of fish-allergic patients have IgE antibodies reacting to this muscle protein (63–65). However, the prevalence of parvalbumin-specific IgE antibodies seems to vary across different patient populations. For example, fish-allergic patients with sensitization to tropical fishes react mostly to allergens other than parvalbumins (66, 67). Generally, the high clinical cross-reactivity among fishes has been attributed to cross-reacting IgE antibodies recognizing parvalbumins from several species (68). Recently, clinical monosensitivity to salmonid fishes has been linked to salmonid parvalbumin-specific IgE antibodies suggesting that cross-reactivity among fish parvalbumins may be restricted to these closely related fishes (23, 25).

FISH ENOLASES AND ALDOLASES

Initially, IgE reactivity to fish beta-enolase and aldolase had been described in single fishes (69–71). In 2013, 50 kDa enolases and 40 kDa aldolases were identified as important fish allergens in cod, salmon, and tuna (**Table 1**) (72). Both enzymes are abundant in the fish muscle as they are involved in metabolic glycolysis, the sugar degradation during physiological production of energy.

In a recent study, IgE binding to enolases and aldolases was detected in a high number of fish-allergic patients (72). Most of the enolase/aldolase-positive patients also had IgE antibodies to parvalbumin. Both allergens were positive in the mediator release assay using rat basophilic leukemia cells expressing the human high-affinity IgE receptor passively sensitized with patient sera. They were found to be sensitive to heat treatment. Their

[1] www.allergen.org
[2] www.allergome.org

Table 1 | Entries of official fish allergens by the International Union of Immunological Societies Allergen Nomenclature Subcommittee database (www.allergen.org).

Order	Fish	Allergen name	Protein identity
Clupeiformes	*Clupea harengus* (Atlantic herring)	Clu h 1.0101 Clu h 1.0201 Clu h 1.0301	Parvalbumin
	Sardinops sagax (Pacific pilchard)	Sar sa 1.0101	Parvalbumin
Cypriniformes	*Cyprinus carpio* (common carp)	Cyp c 1.0101 Cyp c 1.0201	Parvalbumin
Gadiformes	*Gadus callarias* (Baltic cod)	Gad c 1.0101 Gad m 1.0101 Gad m 1.0102 Gad m 1.0201	Parvalbumin Parvalbumin
	Gadus morhua (Atlantic cod)	Gad m 1.0202 Gad m 2.0101 Gad m 3.0101	Enolase Aldolase
Perciformes	*Lates calcarifer* (barramundi)	Lat c 1.0101 Lat c 1.0201	Parvalbumin
	Oreochromis mossambicus (tilapia)	Ore m 4.0101	Tropomyosin
	Thunnus albacares (yellowfin tuna)	Thu a 1.0101 Thu a 2.0101 Thu a 3.0101	Parvalbumin Enolase Aldolase
	Xiphias gladius (swordfish)	Xip g 1.0101	Parvalbumin
Pleuronectiformes	*Lepidorhombus whiffiagonis* (megrim)	Lep w 1.0101	Parvalbumin
Salmoniformes	*Oncorhynchus keta* (Pacific salmon)	Onc k 5.0101	Vitellogenin
	Oncorhynchus mykiss (rainbow trout)	Onc m 1.0101 Onc m 1.0201	Parvalbumin
	Salmo salar (Atlantic salmon)	Sal s 1.0101 Sal s 2.0101 Sal s 3.0101	Parvalbumin Enolase Aldolase
Scorpaeniformes	*Sebastes marinus* (redfish)	Seb m 1.0101 Seb m 1.0201	Parvalbumin

relevance as food allergens is still not well understood especially in parvalbumin-negative patients.

FISH GELATIN

The relevance of fish gelatin as a food allergen has been controversially discussed for many years (4). Fish gelatin is commonly used in food and pharmaceutical products replacing mammalian gelatins (73).

Originally, IgE binding to fish collagen had been shown in fish-allergic patients (74, 75). Later, the prevalence of fish gelatin allergy was addressed in single clinical studies (76, 77). In one study, skin prick tests were positive in 3 of 30 cod-allergic patients, while food challenges were positive in one of the three tested patients. Also, its potency as a food allergen was shown in a case report of severe anaphylaxis upon ingestion of sweets containing several grams of fish gelatin (78). No cross-reactivity of mammalian and fish gelatin has been reported so far. Another risk factor of fish gelatin is a potential contamination with fish parvalbumin as it is produced from fish as a natural source. In fact, parvalbumin traces were detected in isinglass, a fish collagen-based food supplement (79).

OTHER IgE-REACTIVE FISH PROTEINS

In the past decades, numerous studies reported allergens different from parvalbumin which were specified by their molecular weight or identified by other methods. Although most studies showed IgE reactivity to these proteins, the relevance of these potential allergens was not addressed. Several allergens (63 kDa protein, further IgE-reactive proteins) were described for cod while some were identified as parvalbumin oligomers (80). Furthermore, allergens were identified in swordfish (25 kDa), eel/eelpout (40 kDa), snapper (35–90 kDa), tuna/marlin (94–105 kDa), scad (46–50 kDa), tropical fishes (29–54 kDa), and pangasius/tilapia (18–45 kDa) (20–22, 64, 66, 81, 82). Potential allergens of known identity were found in cod (aldehyde phosphate isomerase), salmon (triose-phosphate isomerase, fructose-bis-phosphate isomerase, serum albumin), and tuna (creatine kinase, beta-enolase) (70, 83, 84).

Two further fish allergens have been registered by the IUIS-allergen database (**Table 1**). Food allergy to fish roe has been addressed in a few studies so far. Vitellogenin, a fish yolk protein, has been identified as a food allergen in caviar from different fishes (85, 86). In 2013, tropomyosin, a filamentous muscle protein, was purified, cloned, and identified as a fish allergen in tilapia-sensitized patients (87). This tilapia allergen showed high homology (88%) to the human homolog. It was suggested that it may be involved in autoimmune reaction of inflammatory bowel disease as a significant proportion of the patients with IgE to tropomyosin suffered from this autoimmune disease.

ALLERGENICITY OF PARVALBUMINS

During last decades, parvalbumins, enolases, aldolases, and fish gelatin have been identified as allergenic fish proteins. Specific properties have been attributed to common food allergens such as resistance toward conditions of the gastrointestinal tract and influences by food processing conditions (88). So far, only the allergenicity of parvalbumins has been characterized in more detail as to their allergenicity while consolidated findings still need to be generated for the other allergens.

PARVALBUMINS OF THE ALPHA- AND BETA-LINEAGE

Parvalbumins have been classified into two phylogenetic lineages, alpha and beta subtype. Their isoelectric points (alpha, $pI > 5.0$; beta, $pI < 4.5$) and the other multiple characteristics of the primary protein structure are different (89, 90). Generally,

parvalbumins occur in various organs such as the central nervous system and the muscles (56). In muscles, both subtypes have been detected in amphibian tissues while only the alpha-subtype has been reported in mammalian and avian muscles (91, 92). Cartilaginous fish muscles express alpha-parvalbumins whereas the beta-homolog is found in muscle tissue of bony fishes (93). So far, the potency as important food allergens has been shown only for fish beta-parvalbumins. Despite their overall structural similarity to beta-homologs, alpha-parvalbumins are generally considered as non-allergenic proteins (43). Indeed, parvalbumins of the alpha-lineage have been described only as frog meat allergens (94). However, IgE cross-reactivity has also been reported between homologs from fish and frog in a population of fish-sensitized patients (95). In 2011, chicken alpha-parvalbumin has been reported as a poultry allergen in chicken meat allergy (96). Interestingly, IgE recognition of both fish and avian homologs was found in one case study while cross-reactivity was not detected in another clinical case (97, 98). However, the sequence identity between fish parvalbumins and homologs from mammals and birds is low (<55%) so that IgE cross-reactivity seems to be unlikely.

Recent classification by structural, biochemical, and phylogenetic analysis assigned alpha-parvalbumins into a common cluster together with higher vertebrate, including human proteins while beta-subtypes form a separate, only remotely related cluster of parvalbumins which might explain the variable allergenicity of both lineages (99, 100).

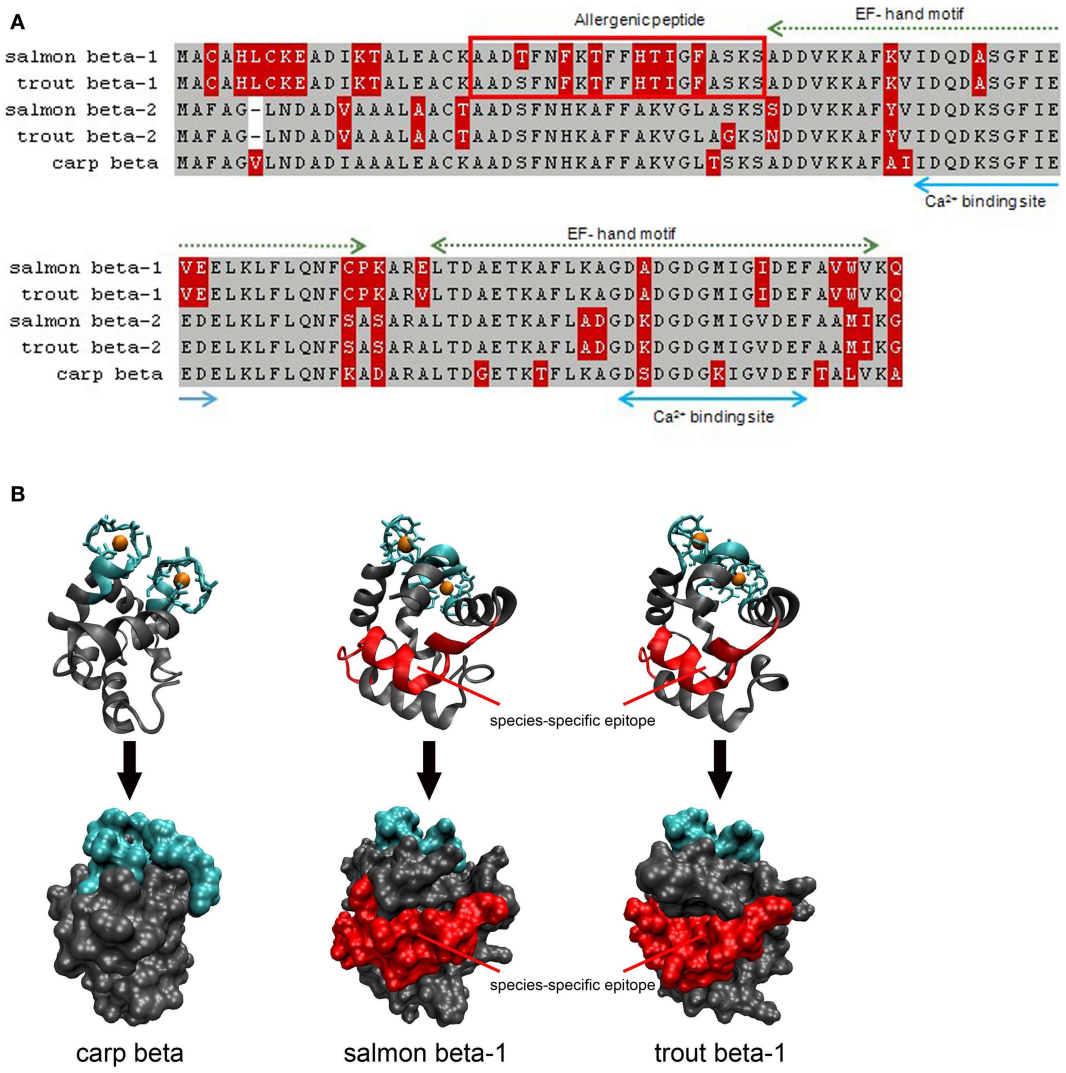

FIGURE 1 | Comparison of salmon (X97824, X97825), trout (FN544258, FN544259), and carp (P02618; 4cpv) parvalbumins. **(A)** Both salmonid beta-1 parvalbumins sequences differ from their homologs in the N-terminal third of the protein which is not involved in calcium binding. The allergenic peptide is specifically recognized by IgE from a patient monosensitized to salmonid fishes. Gray, identical residues; red, variable residues. **(B)** Both Ribbon and surface models show that the allergenic peptide is localized on the surface of salmon and trout beta-1 parvalbumins. Blue, calcium-binding regions; red, IgE epitope.

ANTIGENIC DETERMINANTS OF PARVALBUMIN

The amino acid sequence identities of fish parvalbumins vary substantially (55–95%) but high structural similarity has been reported as a common protein characteristic (101). IgE-binding epitopes were suggested to be located in highly conserved, calcium-binding regions of the molecule. Indeed, reduced IgE binding was found for parvalbumin mutants with single amino acid modifications in calcium-binding motifs (60). The analysis of human B-cell epitopes has only been addressed so far in a few parvalbumins namely allergens from cod, carp, mackerel, and salmon (25, 102–104).

Both linear and conformational epitopes were found in these studies but overall, non-plausible peptide pattern was found to define a common IgE-binding region of fish parvalbumins. It seems that B-cell epitopes are distributed over the whole parvalbumin primary structure. A possible explanation might be the polyclonal B-cell response in fish-allergic patients, which is even more stimulated by individual's eating habits (e.g., canned, fried, smoked fish) leading to a high variety of exposed allergen forms such as native, modified, or degraded parvalbumins from different species.

PARVALBUMIN ISOFORMS OF VARIABLE ALLERGENICITY

For most patients, clinical cross-reactivity among fish species was postulated as a specific feature of fish allergy. Although single clinical reports showed true monosensitivity to single fishes, there was no allergen-based explanation for these observations. Only recently, the species-specific sensitization was attributed to the presence of IgE antibodies selectively recognizing a single salmonid parvalbumin isoallergen (beta-1 parvalbumin) (25). These findings may allow two important conclusions concerning the allergenicity of parvalbumins.

First, parvalbumins from different fishes might vary by their allergenic potential. This might be explained by the specific sensitization of the individual patient, which results from the clinical history (eating habits, age of onset of fish allergy).

Second, the allergenicity of parvalbumin isoforms/isoallergens from the same fish might be variable, as reported for salmon allergens. In fact, fishes express often a high number of parvalbumins such as that reported for carp and catfish (105, 106). These isoforms seem to play a physiological role in the fish muscle adaptation to developmental and environmental changes. In the same fish, parvalbumins differing by sequence microheterogeneity (sequence identity >90%) have been reported (99). It is conceivable that these highly identical isoforms might be of variable allergenicity. For Bet v 1, the major birch pollen allergen, a number of isoforms with different allergenic properties were isolated (107). Several allergenic parvalbumins have been identified in commonly consumed fish, cod (beta-1, beta-2), salmon (beta-1, beta-2), and herring (beta-1, beta-2, beta-3) (5). During studies of monosensitivity to salmonid fishes, different antigenic regions were assumed but only a single epitope was defined as a species-specific allergy marker (23, 25). This unique epitope was localized on a single isoform, the beta-1 salmon parvalbumin. For a patient with documented monosensitivity to salmonid fishes, we could confirm a single region from salmon and trout as species-specific parvalbumin epitope ("allergenic peptide"; **Figure 1**). This antigenic region was located on the parvalbumin surface and was unique when compared with different homologs from other fish species. The allergenic region matched with the IgE-binding epitope identified by a subsequent study using a peptide-based microarray assay (25). Studies with further sera from patients with specific sensitizations are required to demonstrate the existence of antigenic parvalbumin regions linked to specific clinical phenotypes. A recent study showed that 9 out of 62 fish-allergic patients (15%) experienced clinical reactions with salmonid fishes only (72). This suggests the conclusions that the prevalence of clinically salmon-monosensitized patients is higher than previously assumed.

CONCLUSION

During recent decades, important insights into clinical and allergen-based features were gained for fish allergy: the variable allergenicity among salmon parvalbumins (beta-1, beta-2) was shown, in addition, important new fish allergens (enolase, aldolase, fish gelatin) were identified. These findings will help to develop new immunotherapeutic strategies, but they have also shown that the clinical picture of fish allergy is more complex than anticipated. New molecules need to be implemented in IgE-based routine assays to advance patient diagnosis and advice.

AUTHOR CONTRIBUTIONS

All authors have contributed to the conception, design, and drafting of the paper.

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Hymenoptera allergens: from venom to "venome"

Edzard Spillner[1]*, Simon Blank[2] and Thilo Jakob[3]*

[1] *Immunological Engineering, Department of Engineering, Aarhus University, Aarhus, Denmark*
[2] *Center of Allergy and Environment (ZAUM), Helmholtz Center Munich, Technical University, Munich, Germany*
[3] *Allergy Research Group, Department of Dermatology, Medical Center, University of Freiburg, Freiburg, Germany*

Edited by:
Christiane Hilger, Centre de Recherche Public Santé, Luxembourg

Reviewed by:
Robert T. Wheeler, University of Maine, USA
Andreas Kerstan, University Hospital of Würzburg, Germany

****Correspondence:***
Edzard Spillner, Immunological Engineering, Department of Engineering, Aarhus University, Gustav Wieds Vej 10, Aarhus C, Aarhus 8000, Denmark
e-mail: e.spillner@eng.au.dk;
Thilo Jakob, Allergy Research Group, Department of Dermatology, Medical Center, University of Freiburg, Hauptstr. 7, Freiburg 79104, Germany
e-mail: thilo.jakob@uniklinik-freiburg.de

In Western Europe, Hymenoptera venom allergy (HVA) primarily relates to venoms of the honeybee and the common yellow jacket. In contrast to other allergen sources, only a few major components of Hymenoptera venoms had been characterized until recently. Improved expression systems and proteomic detection strategies have allowed the identification and characterization of a wide range of additional allergens. The field of HVA research has moved rapidly from focusing on venom extract and single major allergens to a molecular understanding of the entire "venome" as a system of unique and characteristic components. An increasing number of such components has been identified, characterized regarding function, and assessed for allergenic potential. Moreover, advanced expression strategies for recombinant production of venom allergens allow selective modification of molecules and provide insight into different types of immunoglobulin E reactivities and sensitization patterns. The obtained information contributes to an increased diagnostic precision in HVA and may serve for monitoring, re-evaluation, and improvement of current therapeutic strategies.

Keywords: allergen components, allergy, cross-reactivity, insect venom, recombinant allergens, sensitization

HYMENOPTERA VENOM ALLERGY

Hymenoptera venom allergy (HVA) is defined as systemic allergic or anaphylactic reactions that occur in response to stings of insects of the Hymenoptera order. In central and western Europe, this involves most commonly stings by yellow jackets and honey bees, and less frequently stings by hornets or bumble bees. In southern parts of Europe, paper wasps (Polistinae) also play a relevant role. The prevalence of systemic allergic reactions to Hymenoptera stings ranges from 0.3 to 3.4% in the general population. The lowest occurrence is reported in children and the highest in beekeepers (1). Data extrapolated from hospital admissions and emergency department visits (2–5) as well as from a national register for anaphylaxis (6) suggest that HVA may account for up to one third of all anaphylactic reactions. The diagnosis of HVA is routinely based on the clinical history and detection of immunoglobulin E (IgE)-mediated sensitization by skin testing and/or by *in vitro* detection of venom-specific IgE. In addition, cellular test such as basophil activation test or histamine release test are used to identify the sensitizing venom in cases in which the routine testing is not conclusive. Once the diagnosis is confirmed, immunotherapy with the culprit venom offers a high degree of protection from future anaphylactic sting reactions ranging from 80 to 84% in bee venom allergy and 90–95% in yellow jacket venom (YJV) allergy (1).

For diagnostic as well as for therapeutic purposes, whole venom preparations are generally used (**Figure 1A**). All diagnostic systems that use whole venom preparations are potentially hampered by IgE cross-reactivity that does not allow a precise distinction between true double sensitization and cross-reactivity between different venoms. This cross-reactivity may be based on IgE-reactivity to homologous single venom allergens present in venom of different families or on IgE-reactivity to cross-reactive carbohydrate determinants (CCD) (7).

Until recently, the limited information on single venom allergens and their unavailability for diagnostic and therapeutic purposes rendered HVA an outmoded field, particularly when compared to the progress made in the molecular understanding of other forms of allergies.

Recent advances in expression systems and proteomic detection strategies have allowed the identification and characterization of a wide range of additional Hymenoptera allergens and have moved the field rapidly from focusing on whole venoms and single major allergens to a molecular understanding of the entire "venome" as a system of unique and characteristic components. Here, we review the current information on venom allergens in different Hymenoptera species, their use for reliable diagnostic detection of HVA as well as their potential role in therapeutic intervention.

HYMENOPTERA VENOM ALLERGENS

Understanding hypersensitivity reactions to venom allergens is often hampered by complexity of the source material (**Figure 1A**).

Abbreviations: BAT, basophil activation test; CCD, cross-reactive carbohydrate determinant; CRP, carbohydrate-rich protein; DPP IV, dipeptidyl peptidase IV; DW, dry weight; ELISA, enzyme-linked immunosorbent assay; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; HRP, horseradish peroxidase; IgE, immunoglobulin E; IgG, immunoglobulin G; Man, mannose; MRJP, major royal jelly protein; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor; sIgE, specific immunoglobulin E; VEGF, vascular endothelial growth factor.

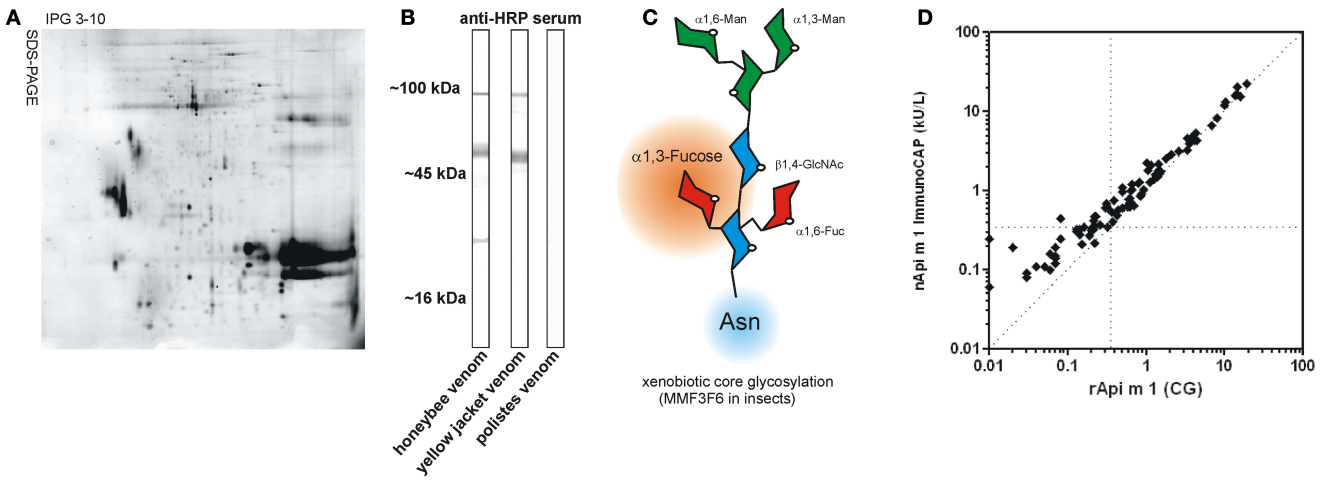

FIGURE 1 | Venom components and CCD. **(A)** Representative 2D-gel of the honeybee venom stained with Coomassie Brilliant Blue G-250 demonstrating the complexity of the venome (kindly provided by Dr. Nico Peiren, Laboratory of Zoophysiology, Ghent University, Belgium). **(B)** CCD sIgE-reactivity of rabbit anti-HRP serum with different Hymenoptera venoms in immunoblotting. Note the lack of CCD-reactivity in *Polistes* venom. **(C)** Schematic representation of xenobiotic core glycosylation as found in insects. This carries an additional α 1,3-linked fucose residue compared to plants having an additional β 1,2-linked xylose residue. (GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose). **(D)** Comparison of IgE antibody levels to glycosylated rApi m 1 (CG) and native purified Api m 1 in CCD negative HBV allergic patients ($n = 89$). Hatched horizontal and vertical lines indicate the 0.35 kUA/L cut-off and the hatched diagonal line represents a 1:1 ratio.

This is best exemplified by the venom allergen components of the honeybee (*Apis mellifera*) and the common yellow jacket (*Vespula vulgaris*), as they are known to date (**Table 1**).

The most prominent honeybee venom (HBV) allergens include phospholipase A2, hyaluronidase, and the basic 26 amino acid peptide melittin (8), all of which constitute higher abundance proteins with estimated amounts of 12, 2, and 50% of the venom dry weight (DW), respectively (9). Classical YJV allergens are phospholipase A1, hyaluronidase, and antigen 5 (10), the function of which remains unknown. These two sets of proteins are found with modifications throughout most Hymenoptera species and by far most identified allergens correspond to these protein classes.

In recent years, however significant progress has been made in identification of novel molecules of lower abundance. For some the allergic potential had already been described, such as the acid phosphatase of HBV (Api m 3), however the gene was identified and recombinantly expressed only recently (8, 11). Moreover, with the identification of the 100 kDa allergen C of HBV and its YJV homolog as dipeptidyl peptidase s IV, a novel class of Hymenoptera venom enzymes could be described (12, 13). In YJV in addition to the classical hyaluronidase (Ves v 2.0101), an inactive isoform (Ves v 2.0201), was identified, which seems to be the dominating isoform in the venom (14). Furthermore, it was demonstrated that Api m 10 represents a novel major allergen of HBV with potentially high impact for diagnostic and therapeutic applications (15, 16). Other IgE-reactive proteins of HBV include a putative protease inhibitor (17, 18), a protease (19), an esterase, and a peptidase whose relevance is currently investigated. The newest allergens are the two major royal jelly proteins (MRJP) 8 and 9 (two isoforms of Api m 11) in HBV (20) as well as novel pan-allergens, the vitellogenins Api m 12, and Ves v 6 (21).

In addition to these components with documented allergenic nature, recently some other components such as a C1q-like protein (22), a platelet derived growth factor (PDGF)/vascular endothelial growth factor (VEGF)-like protein (23), and hexamerin (24) were identified, the allergenic nature of which still has to be evaluated.

Transcriptomics very recently suggested the presence of an antigen 5 like protein in the venom of winter bees (25). Even the season (and most likely the climate and geographic region) seem to have profound impact on the venome. Proteomics revealed the presence of the antimicrobial peptide apidaecin (25) further demonstrating that the complexity of the venome is not restricted to larger proteins. The lower molecular weight (MW) fraction of the venom contains a variety of peptidic components with unique biophysical and clinical characteristics. Their contributions to the sting reaction beyond IgE-reactivity however still need to be addressed.

By increasing application of advanced proteomic, peptidomic, and genomic approaches, the venome and thereby the number of allergens, certainly will significantly increase in the future. The most recent proteomic analysis of honey bee venom (**Figure 1A**) revealed >100 different components (26). Furthermore, another level of complexity is achieved by the generation of additional isoforms and post-translational modification. All available data however suggest that the apparent plasticity of the venome makes its final definition a never ending story.

As HBV and YJV can be considered prototypic for other Hymenoptera venoms, their composition is reflected in other species including the bumble bee (*Bombus terrestris* and the American *B. pennsylvanicus*), the venom composition of which closely resembles that of the honeybee. Bumble bees gained particular importance for pollination industry workers.

Table 1 | Overview about the presently known Hymenoptera venom allergens.

Allergen	Name/function	MW (kDa)	% DW	Potential N-glycosylation	Eukaryotic expression
BEES (*Apis mellifera, A. cerana, A. dorsata*)					
Api m 1, Api c 1, Api d 1	Phospholipase A2	17	12	1	+
Api m 2	Hyaluronidase	45	2	3	+
Api m 3	Acid phosphatase	49	1–2	2	+
Api m 4	Melittin	3	50	0	–
Api m 5	Allergen C/DPP IV	100	<1	6	+
Api m 6	Protease inhibitor	8	1–2	0	+
Api m 7	Protease	39	?	3	+
Api m 8	Carboxylesterase	70	?	4	+
Api m 9	Carboxypeptidase	60	?	4	+
Api m 10	CRP/icarapin	55	<1	2	+
Api m 11.0101	MRJP 8	65	?	6	+
Api m 11.0201	MRJP 9	60	?	3	+
Api m 12	Vitellogenin	200	?	1	+
BUMBLEBEE (*Bombus pennsylvanicus, B. terrestris*)					
Bom p 1, Bom t 1	Phospholipase A2	16		1	–
Bom p 4, Bom t 4	Protease	27		0, 1	–
YELLOW JACKETS (*Vespula vulgaris, V. flavopilosa, V. germanica, V. maculifrons, V. pensylvanica, V. squamosa, V. vidua*)					
Ves v 1, Ves m 1, Ves s 1	Phospholipase A1	35	6–14	0, 0, 2	+
Ves v 2.0101, Ves m 2	Hyaluronidase	45	1–3	4	+
Ves v 2.0201	Hyaluronidase[a]	45	?	2	+
Ves v 3	DPP IV	100	?	6	+
Ves v 5, Ves f 5, Ves g 5, Ves m 5, Ves p 5, Ves s 5, Ves vi 5	Antigen 5	25	5–10	0	+
Ves v 6	Vitellogenin	200	?	4	+
WHITE-FACED HORNET, YELLOW HORNET (*Dolichovespula maculate, D. arenaria*)					
Dol m 1	Phospholipase A1	34		2	–
Dol m 2	Hyaluronidase	42		2	–
Dol m 5, Dol a 5	Antigen 5	23		0	+
HORNETS (*Vespa crabro, V. magnifica, V. mandarinia*)					
Vesp c 1, Vesp m 1	Phospholipase A1	34		0	–
Vesp ma 2	Hyaluronidase	35		4	
Vesp c 5, Vesp ma 5, Vesp m 5	Antigen 5	23		0	–
EUROPEAN PAPER WASPS (*Polistes dominula, P. gallicus*)					
Pol d 1, Pol g 1	Phospholipase A1	34		1	–
Pol d 4	Protease	33		6	–
Pol d 5, Pol g 5	Antigen 5	23		0	–
AMERICAN PAPER WASPS (*Polistes annularis, P. exclamans, P. fuscatus, P. metricus*)					
Pol a 1, Pol e 1	Phospholipase A1	34		0	–
Pol a 2	Hyaluronidase	38		2	–
Pol e 4	Protease	?			
Pol a 5, Pol e 5, Pol f 5, Pol m 5	Antigen 5	23		0	+
FIRE ANTS (*Solenopsis invicta, S. geminata, S. richteri, S. saevissima*)					
Sol i 1	Phospholipase A1	35	<1	3	–
Sol i 2, Sol g 2, Sol r 2, Sol s 2		14		0	+
Sol i 3, Sol g 3, Sol r 3, Sol s 3	Antigen 5	26		2	+
Sol i 4, Sol g 4		12		0	–

CRP, carbohydrate-rich protein; DPP IV, dipeptidyl peptidase IV; DW, dry weight; MRJP, major royal jelly protein.

[a] *inactive isoform.*

In analogy, venom allergens of diverse other *Vespidae* species such as white-faced hornet (*Dolichovespula maculata*) or the European hornet (*Vespa crabro*) are fairly similar to those of the yellow jacket. Allergy to venom of the phylogenetic more distant paper wasps (Polistinae) is common in North America as well as in Europe, especially in Mediterranean areas. Important

Polistes species in Europe are *P. dominula* and *P. gallicus*, whereas in Northern America other species such as *P. annularis*, *P. apachus*, *P. exclamans*, *P. fuscatus*, and *P. metricus* are dominant. In the last decades, *P. dominula* has increasingly spread across the North American continent and central and northern parts of Europe. The IgE cross-reactivity between European and American *Polistes* species is described as rather low because they belong to different subgenera. In contrast, cross-reactivity between Polistinae and Vespinae (*Vespula*, *Dolichovespula*, and *Vespa*) venoms and purified venom proteins (27) is frequently observed, especially for *Vespula* and both American and European *Polistes* venom (28).

For all these species, only a limited set of allergens has been identified so far although it is quite likely that all venoms will contain conserved allergens such as hyaluronidases, dipeptidyl peptidases, and vitellogenins that in part contribute to molecular cross-reactivity. Other protein families such as proteases (Api m 7, Pol d 4) show clear molecular differences and it remains open if these proteases will be found in all Hymenoptera venoms.

Moreover, it is widely accepted that IgE cross-reactivity between different insect venoms can be attributed to CCD that are present on a large number of venom allergens (**Figure 1B**). The only exceptions are apparently venoms of *Polistes* species that seem to lack the alpha 1,3-linked fucose (Fuc) residue that is responsible for IgE-reactivity to CCDs (29).

RECOMBINANT ALLERGENS FOR THE DIAGNOSIS OF HVA

The above mentioned considerations and the entomological diversity of potential culprit insects demand a careful diagnostic algorithm prior to immunotherapeutic intervention. Diagnosis of HVA is based on a history of anaphylactic sting reactions, positive skin test responses, and/or detection of specific IgE to Hymenoptera venom. Positive results in skin and serological tests with conventional venom extracts, however, do not always reflect genuine sensitizations and are frequently caused by clinically irrelevant cross-reactive antibodies. Treatment modalities therefore often include different venoms, resulting in higher costs, increased risk of side effects, and possible *de novo* sensitizations. Molecular approaches are increasingly recognized as elegant way to obtain reliable and detailed diagnostic information. Until recently, only a very limited number of venom allergens such as Api m 1, Api m 4, and Ves v 5 was available either as native or recombinant proteins (30, 31). Their use and the possibility to perform analyses on a molecular level resulted in a clear improvement of diagnostic precision (32, 33). Inherent problems and general considerations however apply for the isolation and production of venom allergens. Even with isolation of high abundance allergens you run the risk of having contaminating residual components in the preparation that may distort the picture at a molecular level.

Applying recombinant technologies, this particular problem does not exist but difficulties rather lie in the establishment of an adequate and efficient production system. The efficiency of the prokaryotic approach is often compromised by the need of extensive folding steps limiting its use to structurally relatively simple and small molecules. In contrast, eukaryotes such as yeast and in particular insect and mammalian cells add oligosaccharides, which are similar but not identical to the glycan of the native allergen and which influence the folding and the immunoreactivity (34). Although early recognized (34), in the last few years expression in insect cells was established as appropriate system for insect venom allergens. The functionality of proteins, the epitope authenticity, and the correct folding of resulting proteins could be demonstrated for a large number of allergens (**Table 1**) (12, 34, 35).

A common problem of *in vitro* diagnosis of insect venom allergy using venom extracts are patients with double positive test results for HBV and YJV that in our HVA patient cohort from the south west of Germany constitute approximately 45–50% of all cases. This double positivity may reflect true double sensitization to HBV and YJV, or may be based on IgE cross-reactivity.

Immunoglobulin E cross-reactivity may be based on common protein epitopes of homologous allergens of both venoms as described for hyaluronidases, dipeptidyl peptidases, and the new 200 kDa vitellogenin allergens. Alternatively, cross-reactivities can be attributed to IgE antibodies directed against cross-reactive glyco-epitopes of the allergens (7, 36, 37). This is of particular importance, since most HBV and YJV allergens are glycoproteins with one or more of such carbohydrate structures (**Table 1**).

Causative for the phenomenon of cross-reactivity are IgE antibodies that are directed against an alpha 1,3-linked fuc residue of the *N*-glycan core established by insects and plants (**Figure 1C**). In plants, additionally a beta 1,2-xylose residue is found at the core glycan to which IgE also can be directed. Such xenobiotic modifications represent highly immunogenic epitopes, which can induce specific immunoglobulin G (IgG) as well as IgE antibodies (38). CCD-specific IgE antibodies have been reported to be responsible for more than 50% of double sensitizations to HBV and YJV (37), complicating the choice of the appropriate therapeutic intervention. The clinical relevance of CCD-reactive IgE antibodies is controversially discussed, but in the case of insect venom allergy appears to be low or non-existing. Accordingly, CCD-carrying glycoproteins can effect mediator release from basophils but do not provoke significant responses in individuals with CCD-specific IgE (39). Nevertheless, anti-CCD IgE represent an undoubted pitfall of *in vitro* allergy diagnostics, since they cause multiple reactivities with any glycosylated plant (food, pollen) or insect venom allergen and thereby interfere with the detection of clinically relevant sensitization to protein epitopes. A prominent example of CCD-based interference with diagnostic precision is the honey bee venom major allergen Api m 1 that carries in its natural form an alpha 1,3-linked fuc on a *N*-glycan core structure and thus is reactive with IgE directed against CCDs. Generation of recombinant forms of Api m 1 that either lack the entire core glycan or only the 1,3 fuc residue, demonstrated a high reliability to detect sensitization to the species-specific protein epitopes as compared to nApi m 1 (**Figure 1D**) (40).

Molecular diagnosis applying non-glycosylated species-specific allergens such as Api m 1 and Ves v 5 (41–43) and strategies to circumvent the presence of CCDs led to a significant advance in the dissection of true double sensitization versus irrelevant cross-reactivity. The use of Sf9 insect cells from *Spodoptera frugiperda* as expression system results in allergens with functional glycosylation, proper folding, and complete epitope spectrum but not showing any immunologically detectable CCD-reactivity (**Figure 1D**). This phenomenon is obviously based on the specific absence of

alpha 1,3-core fucosylation (35). Resulting CCD-free engineered and correctly folded allergens allow for the first time the assessment of their relevance regardless of their natural glycosylation bypassing complex inhibition analyses. Using CCD-free, correctly folded Ves v 2.0101 and Ves v 2.0201, we were able to clearly demonstrate that hyaluronidases – contrary to previous assumptions – do not play a significant role as major allergens of YJV (35), a fact that was corroborated by findings of others (44, 45). In contrast, even for highly glycosylated proteins such as Api m 5, Api m 10, and Api m 11, a pronounced IgE-reactivity beyond CCDs with clinical relevance was demonstrated (12, 15, 16, 20).

Another problem of *in vitro* diagnosis of HVA using venom extracts are patients with a documented history but negative test results. A possible reason might be that venom extracts represent heterogeneous mixtures in which the components are present in widely varying concentrations and that particular allergens can be lost or degraded during the processing (15). Alternatively, coupling behavior within the assay system or accessibility of individual allergens within the extract may be different from the isolated protein. An excellent example for this kind of discrepancies has been reported quite recently in patients with YJV allergy (46). Among patients with a well-documented history of yellow jacket sting anaphylaxis but negative IgE test results to YJV extract, 84% could be diagnosed by using recombinant Ves v 5 as allergen. Subsequent analysis of a large cohort of YJV allergic patients confirmed that IgE-reactivity to Ves v 5 was under-represented in the whole venom extract. This discrepancy could be solved by spiking the venom extract with rVes v 5, a procedure that was adopted by one of the manufacturers of these diagnostic extracts (Thermo Fisher Scientific). These and many other problems can be bypassed using molecular diagnostics in form of recombinant allergens, which additionally are available in unlimited amounts and thus analytically better accessible.

VENOM IMMUNOTHERAPY IN HVA

Specific immunotherapy with the culprit venom (VIT) offers a high degree of protection from future anaphylactic sting reactions ranging from 80 to 84% in bee venom allergy and 90–95% in YJV allergy (1).

The degree of protection induced by VIT is either extrapolated from patient information on the occurrence and tolerance of field stings or obtained from clinical data in which patients who are receiving maintenance VIT undergo a sting challenge in a controlled clinical setting. A most recent study by Rueff et al. that included more than 1500 patients that had received a sting challenge, observed a protection rate of 84% for bee VIT and 96% for yellow jacket VIT (47). Using a logistic regression model to assess relative risk factors for not being protected, they identified among others VIT with HBV as one of the highest risk factors (as compared to VIT with YJV) with an odds ratio of 5. This difference in honeybee versus yellow jacket VIT has been known for decades and has been suggested to be related to differences in quantities and qualities of venoms that are injected during the sting. The recent progress in the molecular characterization of relevant venoms has demonstrated that in particular in HBV low abundance proteins such as Api m 3, Api m 5, and Api m 10 play an important and until then underestimated role as allergens (11, 12, 15).

Despite the fact that these allergens are present only in low quantities, they must be regarded as major allergens since more that 50% of HBV allergic patients display IgE-reactivity to them (16). Interestingly, two of these allergens, Api m 3 and Api m 10, while present in the crude venom abstract, are absent or under-represented in therapeutic venom preparations (15). When analyzing sensitization profiles in HBV allergic patients, IgE to Api m 3 and/or Api m 10 was detected in up to 68% and in 5% of the patients IgE was directed against Api m 3 and/or Api m 10 only (16). The under-representation of Api m 3 and Api m 10 in therapeutic venom preparations was additionally confirmed indirectly by analyzing allergen sIgG4 in patients under VIT. While VIT induced a robust sIgG4 response to Api m 1, Api m 2, and Api m 4, no or only very little IgG4 induction could be observed to Api m 3 and Api m 10.

Based on these findings, it is tempting to speculate that the relative lack of these two allergens in therapeutic venom preparation may account for the reduced efficacy of VIT in bee venom-allergic patients, a hypothesis that is currently under investigation. Provided that indeed different sensitization profiles to bee venom allergens are associated with an increased risk of not being protected by VIT, one could envision different strategies to improve to efficacy of VIT in these patients. Here improved methods of generating the therapeutic venom preparation may be developed to circumvent the loss of individual allergens. Alternatively, existing venom preparations could be enhanced by spiking with recombinant allergens that are under-represented or lacking. Finally, generation of individual allergen cocktails for patient tailored immunotherapy would be conceivable. The latter two options, however, are highly unlikely, given the relatively small group of patients that would benefit and the high regulatory requirements for registrations of individualized immunotherapy products.

CONCLUSION

Advances in the characterization of Hymenoptera venoms offer detailed insights into the molecular basis of toxicology and allergic sensitization potential of individual venom components. Recombinant access and the capability to define the allergen glycosylation allows for advanced strategies for differentiation of genuine double sensitization and cross-reactivity as well as for avoidance of a reactivity bias toward less relevant allergens in extracts. Applying a growing panel of CCD-free species-specific as well as homologous recombinant allergens, molecular diagnosis increasingly allows for establishment of individual sensitization profiles. Such profiles also include the potential to follow the course of therapy, to diagnose therapy-induced *de novo* sensitizations, opportunities to adapt therapeutic intervention, and possibly to develop prognostic markers for therapeutic success.

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Allergen recognition by innate immune cells: critical role of dendritic and epithelial cells

Fabián Salazar and Amir M. Ghaemmaghami*

Division of Immunology, Faculty of Medicine and Health Sciences, The University of Nottingham, Nottingham, UK

Edited by:
Christiane Hilger, Centre de Recherche Public de la Santé, Luxembourg

Reviewed by:
María Marcela Barrio, Fundación Cáncer, Argentina
François Hentges, Centre de Recherche Public de la Santé, Luxembourg

***Correspondence:**
Amir M. Ghaemmaghami, Division of Immunology, West Block, A Floor, Queen's Medical Centre, The University of Nottingham, Nottingham NG7 2UH, UK
e-mail: amg@nottingham.ac.uk

Allergy is an exacerbated response of the immune system against non-self-proteins called allergens and is typically characterized by biased type-2 T helper cell and deleterious IgE mediated immune responses. The allergic cascade starts with the recognition of allergens by antigen presenting cells, mainly dendritic cells (DCs), leading to Th2 polarization, switching to IgE production by B cells, culminating in mast cell sensitization and triggering. DCs have been demonstrated to play a crucial role in orchestrating allergic diseases. Using different C-type lectin receptors DCs are able to recognize and internalize a number of allergens from diverse sources leading to sensitization. Furthermore, there is increasing evidence highlighting the role of epithelial cells in triggering and modulating immune responses to allergens. As well as providing a physical barrier, epithelial cells can interact with allergens and influence DCs behavior through the release of a number of Th2 promoting cytokines. In this review we will summarize current understanding of how allergens are recognized by DCs and epithelial cells and what are the consequences of such interaction in the context of allergic sensitization and downstream events leading to allergic inflammation. Better understanding of the molecular mechanisms of allergen recognition and associated signaling pathways could enable developing more effective therapeutic strategies that target the initial steps of allergic sensitization hence hindering development or progression of allergic diseases.

Keywords: dendritic cell, epithelial cell, asthma, allergy, type-I hypersensitivity, house dust mite, pattern recognition receptor, TSLP

INTRODUCTION

Asthma is a chronic disease of the lung characterized by inflammation and airway hyper-responsiveness. Allergic asthma is probably the most common form of asthma and is classified as a type-I hypersensitivity reaction. Most pathologies that are associated with allergic asthma are the consequence of an exacerbated immune response to specific proteins known as allergens in genetically susceptible individuals (1, 2). An allergic reaction is characterized by the synthesis of allergen-specific immunoglobulin of the IgE class and Th2 cytokines (e.g., IL-4, IL-5, and IL-13), which lead to recruitment and sensitization of effector cells such as eosinophils, basophils, and mast cells (1, 3). During allergen re-exposure, the crosslinking of IgE molecules bounded to high-affinity Fcε receptors (FcεR) on the surface of mast cells and basophils results in an immediate release of the soluble mediators, such as histamine, leukotriene, and prostaglandins, which are responsible for the allergic reaction (1, 4).

Antigen recognition and uptake by innate immune cells is the first step in the process of antigen presentation that could lead to initiation of adaptive immune responses. Using a diverse set of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors different types of immune and non-immune cells are able to sense conserved motifs on antigens. Dendritic cells (DCs) have been demonstrated to play a pivotal role in this process; however, the molecular mechanisms of how Th2-driven allergic immune responses are initiated and amplified have remained elusive (3–5). Recently, the role of epithelial cells as key modulators of DC behavior has been highlighted (6, 7). Specifically, airway epithelial cells (AECs) have been demonstrated to be able to recognize diverse allergens leading to the release of chemokines, cytokines, and danger signals that activate and recruit other immune cells to the site of inflammation (6, 7).

In this review, we will discuss the role of dendritic and epithelial cells in allergen recognition and how the cross-talk between DCs and AEC could affect Th2-mediated allergic diseases.

Abbreviations: AEC, airway epithelial cell; APC, antigen presenting cell; CLR, C-type lectin receptor; CRD, carbohydrate recognition domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin; FcεR, Fcε receptor; GM-CSF, granulocyte macrophage colony-stimulating factor; HDM, house dust mite; IDO, indoleamine 2,3 dioxygenase; LPS, lipopolysaccharide; MR, mannose receptor; NF-κB, nuclear factor κB; PAMP, pathogen-associated molecular pattern; PAR, protease activated receptor, PRR, pattern recognition receptor; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

ALLERGEN RECOGNITION BY DENDRITIC CELLS

Immature DCs reside in the peripheral tissues and can efficiently sample the microenvironment for antigens. Once taken up by DCs such antigens are processed into peptides and appear on the

surface of DCs in the context of MHC molecules. Antigen bearing DC migrate to the local lymph nodes where through expression of MHCII-peptide complex, cytokines, and co-stimulatory molecules they can stimulate naïve T cells toward distinct effector T cell subsets (e.g., Th1, Th2, Th17) (8, 9) or induce tolerance through induction of regulatory T cells (10), depending on the nature of the antigen and other microenvironmental factors (11). DCs serve as sentinels of the mucosal surfaces, where they constantly sample antigens at the interface between external and internal environments using different PRRs (**Figure 1**). Even intraepithelial DCs are able to form tight junctions with epithelial cells through expression of proteins like occludin and claudin, which can further facilitate antigen/allergen recognition and uptake by these cells (7, 8). In addition, some allergens can gain access to DCs by disrupting the tight junctions (9–15); different mechanisms of allergen recognition and uptake by DCs will be further described in the following sections.

C-TYPE LECTIN RECEPTORS

C-type lectin receptors are mainly involved in the recognition of glyco-allergens. Diverse C-type lectin receptors (CLRs), such as dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and mannose receptor (MR) on human DCs have been shown to be able to recognize and internalize allergens.

MANNOSE RECEPTOR

This type-I integral transmembrane protein is primarily expressed by myeloid cells such as macrophages and DCs (4, 16). The extracellular portion of MR consists of three regions: a cysteine-rich domain, a fibronectin type II-like domain, and eight carbohydrate recognition domains (CRDs). Interestingly, DCs from patients with house dust mite (HDM) allergy have been shown to express higher levels of MR and to be more efficient in allergen uptake than DCs from non-atopic donors (17). More recently, it was reported that bronchoalveolar lavage fluid from patients with asthma and/or allergic rhinitis contains higher numbers of MR expressing myeloid-DCs compared to healthy controls (18). In terms of allergen uptake, *in vitro* studies have found that MR expressed on human monocyte-derived DCs is the main receptor for major allergens from HDM (*Der p* 1), dog (*Can f* 1), cockroach (*Bla g* 2), peanut (*Ara h* 1), and cat (*Fel d* 1) (19, 20). Similar studies have also highlighted MR's role in allergen-induced Th2 cell differentiation where MR-deficient (MR^-), as opposed to MR expressing (MR+), DCs failed to induce Th2 cell differentiation in response to Der p 1 in DC-T cell co-cultures from HDM atopic individuals. The bias toward Th1 cell polarization by MR^- DCs was shown to be partially mediated through the up-regulation of indoleamine 2,3-dioxygenase (IDO) activity in DCs (19). IDO is an enzyme that participates in tryptophan metabolism and is involved in many immune-regulatory processes in health and

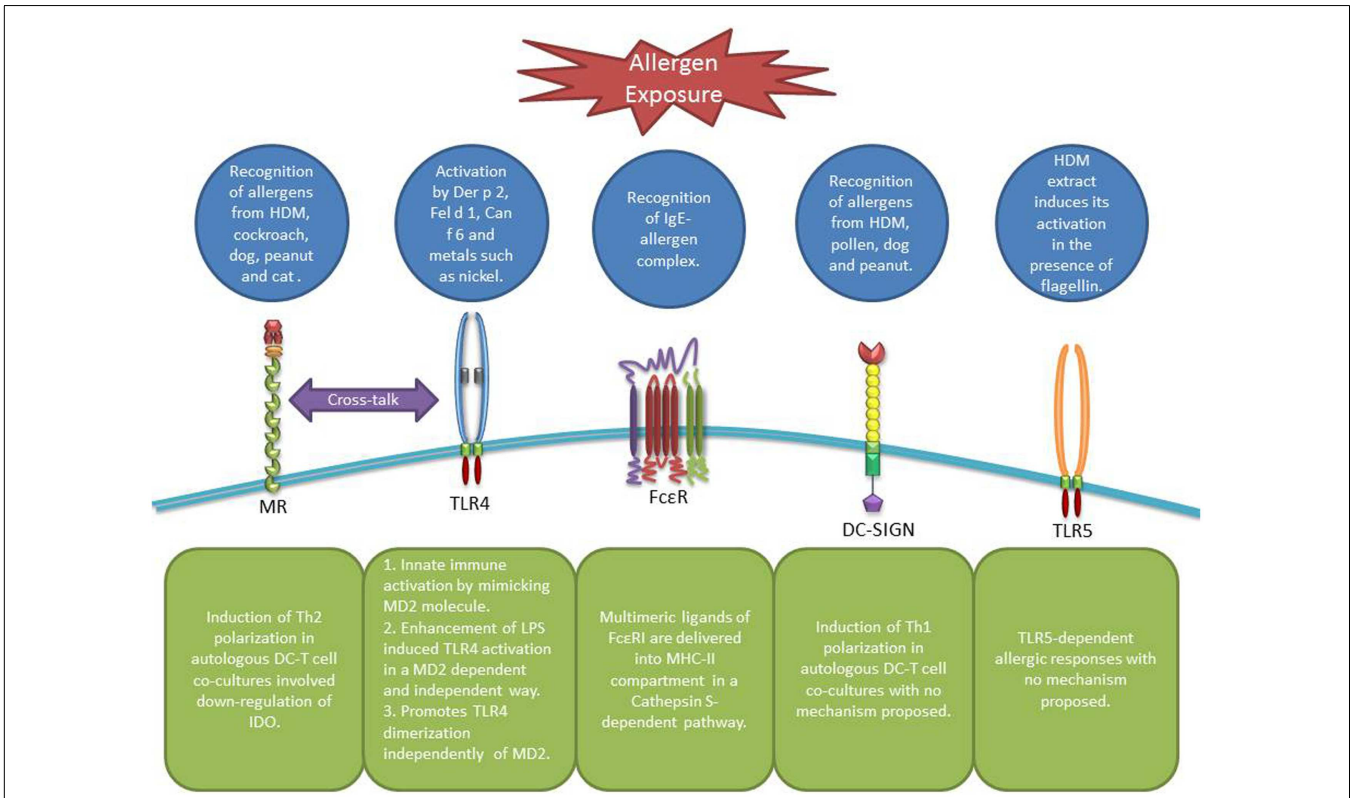

FIGURE 1 | Receptors involved in allergen recognition and uptake by DCs. MR can recognize allergens from diverse sources leading to Th2 polarization and IDO down-regulation in DCs involving a possible cross-talk with TLR4 (19). TLR4 itself can be activated by airborne allergens and diverse metals in an MD2 dependent or independent way (33, 35–37). FcεR facilitate allergen uptake through the recognition of the IgE-allergen complex (41, 46). DC-SIGN can recognize diverse allergens as well, however, leading to a Th1 polarization. Finally, TLR5 can be activated by HDM extracts containing flagellin (34).

disease (21, 22). Further studies by the same group showed that MR recognition of major cat allergen *Fel d* 1 mediated the production of specific IgE and IgG1 antibodies in a mouse model of allergy (20). Other studies have shown that omega-1, a glycosylated T2 ribonuclease secreted by *Schistosoma mansoni* eggs, is recognized and internalized by DCs through MR and subsequently interferes with proteins synthesis and conditions DCs for Th2 priming (23). Collectively these data highlight MR's role in allergen recognition and promotion of Th2-mediated immune responses.

DENDRITIC CELL-SPECIFIC INTRACELLULAR ADHESION MOLECULE 3-GRABBING NON-INTEGRIN

Dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin is a type-II integral transmembrane protein that consists of four regions: a CRD, a hinge domain, and a transmembrane region connected to a cytoplasmic signaling domain (4, 24). DC-SIGN is mainly expressed by antigen presenting cells (APCs) and has been demonstrated to participate in the recognition of allergens from different sources, such as peanut, HDM, pollen, and dog (25–27). *In vitro* studies have been shown that DC-SIGN recognition and uptake of Der p 1 induces Th1 cell differentiation. On the contrary, DC-SIGN deficient DCs bias the response toward a Th2 profile (27). This is opposite to previous observations in MR^{-} DCs which seem to support Th1 differentiation (19). Interestingly, it has also been shown that Der p 1, using its enzymatic activity, can cleave (28) and induce the down-regulation (29) of cell surface DC-SIGN but not MR (28). In this context, we have previously proposed that the Th1/Th2 balance in response to allergen exposure can be determined by the cross-talk between these receptors and the level of their expression on DCs (4, 27). Accordingly, it is important to note that DCs from asthmatic patients show lower expression of DC-SIGN (29), which is in contrast to the high levels of MR expression reported in atopic individuals (17, 18).

TOLL-LIKE RECEPTORS

The TLRs are type-I integral membrane receptors, each with an N-terminal ligand recognition domain constructed of tandem copies of a leucine-rich repeat motif, a single transmembrane helix, which participates in nucleic acid pathogen-associated molecular patterns (PAMPs) recognition, and a C-terminal cytoplasmic signaling domain known as Toll IL-1 receptor domain (30). There are more than 10 different TLRs identified in humans so far (31), with some of them being involved in allergen recognition or pathways that induces allergic responses. Within this context, mainly three different mechanisms have been proposed. Der p 2, a major allergen from HDM with lipid-binding activity, has been shown to induce signaling through TLR2 (32) and TLR4 (33) depending on the cell type involved. Due to its high homology with MD2, which participates in the recognition of lipopolysaccharide (LPS) by TLR4, Der p 2 forms a complex with TLR4 that signals similarly to MD2/TLR4 complex inducing innate immune activation (33). In addition, HDM extracts contaminated with flagellin can induce TLR5-dependent allergic responses in mice, however, the mechanism is still unclear (34). The second mechanism involves sensitization by nickel, which may not be relevant in the context of airway sensitization but highlights the importance of TLR4 on DCs in allergic reactions. Nickel and other bivalent metals such as cobalt induce a lipid-independent activation of TLR4, which is dependent on the presence of two histidine residues, promoting TLR4 dimerization and subsequent receptor activation independently of MD2 (35, 36). Finally, the last mechanism involves allergens such as Fel d 1 and Can f 6 that belong to lipocalin family and cause enhanced LPS-induced TLR4 activation in an MD2 dependent and independent manner respectively (37).

HIGH-AFFINITY IGE RECEPTOR

FcεRI is a multimeric cell surface receptor that binds IgE with high-affinity. In humans, this receptor can be expressed by mast cells, basophils, eosinophils, platelets, monocytes, and DCs; however, in the last four cell types it adopts a trimeric ($\alpha\gamma2$) structure instead of the classical tetrameric ($\alpha\beta\gamma2$) structure (38–43). It has been previously suggested that cell-bound IgE participates in the presentation of aero-allergens by Langerhans cells (44). In addition, the presence of FcεRI on Langerhans cells maximizes antigen uptake via specific IgE and subsequent presentation to T cells (45). In monocytes and peripheral blood DCs, FcεRI has been shown to mediate IgE-dependent allergen presentation (41, 46). Further studies in DCs demonstrated that multimeric ligands of FcεRI are delivered into a major histocompatibility complex class II compartment in a Cathepsin S-dependent pathway (47). *In vivo* experiments with a transgenic mouse model with human-like FcεRI expression in DCs showed that after allergen capture DCs instructed naïve T cells to differentiate into allergen-specific Th2 cells at the site of allergen exposure (48). Taking into account the fact that higher expression of FcεRI has been detected in atopic individuals (40, 42), this can lower the atopic individual's threshold to mount allergen-specific T cell responses. All this highlights the fact that the high-affinity IgE receptor could play an important role in capturing allergens by DCs and subsequent presentation to T cell particularly in previously sensitized individuals with high levels of specific IgE; however, the precise role of IgE receptors and the role of other stimulatory and inhibitory Fc receptors (49, 50) in allergen presentation still need to be fully understood.

ALLERGEN RECOGNITION BY EPITHELIAL CELLS

Airway epithelial cells constitute the first line of defense against pathogens and allergens by not only forming a physical barrier, but also through expressing a wide range of PRR, such as TLRs, CLRs, and protease activated receptor (PARs) (**Figure 2**). These receptors enable AECs to recognize microbial motifs and environmental allergens which lead to a cascade of events culminating in the release of cytokines, chemokine ligands, and danger signals which recruit and activate other immune cells.

PROTEASE ACTIVATED RECEPTORS

Protease activated receptors are G-protein coupled receptors characterized by a self-activation mechanism following proteolytic cleavage of their extracellular N-terminal domain. There are four PAR members identified to date. PAR-1,-3,-4 respond to the protease thrombin, expressed primarily by cells in the vasculature; and are mainly involved in homeostasis and thrombosis. Conversely, PAR-2 is activated by trypsin-like serine proteases but not by trypsin and can be found in airways, vascular, skin, and intestinal cells, and mediates proliferative and inflammatory responses

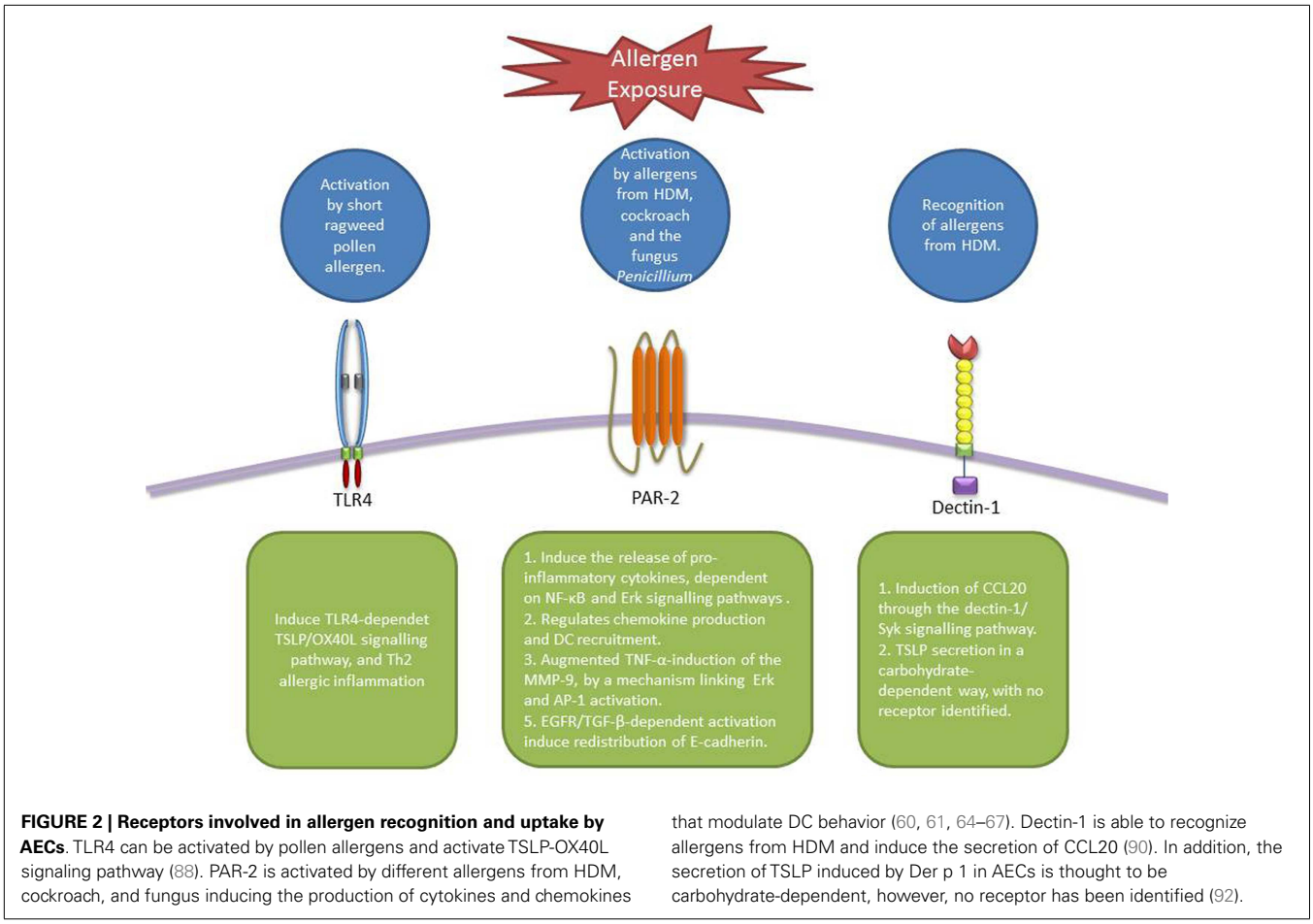

FIGURE 2 | Receptors involved in allergen recognition and uptake by AECs. TLR4 can be activated by pollen allergens and activate TSLP-OX40L signaling pathway (88). PAR-2 is activated by different allergens from HDM, cockroach, and fungus inducing the production of cytokines and chemokines that modulate DC behavior (60, 61, 64–67). Dectin-1 is able to recognize allergens from HDM and induce the secretion of CCL20 (90). In addition, the secretion of TSLP induced by Der p 1 in AECs is thought to be carbohydrate-dependent, however, no receptor has been identified (92).

linked to tissue damage (51, 52). In addition, endothelial cells, epithelial cells, fibroblasts, and immune cells such as lymphocytes, monocytes, mast cells, neutrophils, eosinophils, macrophages, and DCs have been shown to express functional PAR-2 (53, 54). Due to its ability to respond to serine proteases, proteolytic allergens from diverse sources such as HDM, cockroach, pollen, or mold can act as exogenous activators of PAR-2 with implications in allergy and asthma.

Different cysteine and serine proteases from HDM, pollen, and the fungus *Penicillium* have been shown to increase epithelial permeability and disrupt the tight junctions by mainly targeting the transmembrane adhesion proteins occludin and zonula occludens-1 (9–15). In addition, it has been demonstrated that allergen-induced cytokine production, cell detachment and morphological changes in AECs is largely dependent on allergens' protease activity (13, 55–58). Protease-dependant induction of IL-25 and thymic stromal lymphopoietin (TSLP) have been shown to be mediated by Erk and p38 MAPK pathways (58). More recently, it was demonstrated, in an *in vivo* model, that IL-33 also contributes to protease-dependent allergic airway inflammation (59).

Main allergens from HDM can activate PAR-2 and induce the release of pro-inflammatory cytokines (60, 61). However, there is some contradictory results showing that Der p 1-induction of IL-8 and IL-6 is independent of PAR-2 activation and dependent on nuclear factor κB (NF-κB) and Erk signaling pathways (62, 63). In the case of allergens from the German cockroach and the fungus *Penicillium*, this effect has been demonstrated to be mediated by the activation of Erk (64, 65). In an *in vivo* model of allergy, only when the allergens are administered through the mucosa, cockroach proteases regulate chemokine production and DC recruitment in a PAR-2-dependent way (66, 67). Furthermore, it was demonstrated that cockroach proteases augmented tumor necrosis factor (TNF)-α-induction of the matrix metalloproteinases-9, an enzyme that has been implicated in the pathogenesis of bronchial asthma (68, 69), by a mechanism linking PAR-2, Erk, and AP-1 activation (70). More recently, PAR-2-mediated allergic sensitization was shown to be associated with TNF signaling pathways (71).

In addition to the proteolytic activity of some allergens, one of the main soluble mediators that accounts for the increase in epithelial permeability is the vascular endothelial growth factor (VEGF). It has been shown that extract from cockroach increases bronchial airway epithelial permeability by inducing the release of VEGF (72). Besides, HDM extract can induce the secretion of VEGF by human pulmonary epithelial cells (73). Recently, it was demonstrated that HDM-induced redistribution of E-cadherin was mediated via epidermal growth factor receptor-dependent activation of PAR-2 and transforming growth factor-β (TGF-β) enhanced this signaling (74). Nevertheless, not only PAR-2 is directly involved

in allergen-mediated cytokine production, it has also been shown that IL-8 production by AECs in response to allergens from the fungus *Penicillium* is dependent on PAR-1 and PAR-2 via activation of ERK1/2 (65). In addition, proteases from different fungal allergens induce the release of pro-inflammatory cytokines from human nasal polyp epithelial cells, leading to eosinophil and neutrophil migration, in a mechanism that could involve PAR-2 and PAR-3 (75).

TOLL-LIKE RECEPTOR-4

As previously described, TLRs are widely expressed by both APCs and epithelial cells and recognize conserved microbial structures and as such play a key role in controlling adaptive immune responses. LPS is recognized by TLR4 with the participation of the accessory proteins including CD14, LPS binding protein, and MD2. This leads to the recruitment of the signaling adapter protein MyD88, the activation of the transcription NF-κB among others, and the expression of pro-inflammatory cytokines (76). TLR4 (77–80), MyD88 (79, 81, 82), and NF-κB (83–86) have been shown to be crucial in the elicitation of allergic Th2 immune responses. Different studies using knock-out mice have demonstrated the importance of TLR4 expression in both hematopoietic radiosensitive and structural radioresistant cells in the induction of TLR4-dependent Th2 responses to intranasal allergens in the presence of endotoxin (77–80). In addition, it has been shown that the LPS dosage is crucial in driving either Th1 or Th2 responses, with lower levels of LPS inducing Th2 responses to inhaled allergens in a mouse model of allergic sensitization (87). Recently, it was shown that short ragweed pollen acts as a TLR4 agonist, initiating TLR4-dependet TSLP/OX40L signaling pathway, triggering Th2 allergic inflammation (88).

C-TYPE LECTIN RECEPTORS

C-Type Lectin Receptors are receptors that recognize oligosaccharide moieties among other molecular patterns on antigens including allergens (89). CLR's role in allergen recognition and uptake by DCs is well established (4); moreover, they have also been shown to participate in allergen recognition by AECs. It was demonstrated that HDM induction of CCL20 by AECs was not protease or TLR4/2 dependant; however, it was mediated by β-glycan moieties within HDM extract. This effect was specific for HDM because other allergens, such as cockroach and ragweed, failed to induce this response (90). The authors suggest the involvement of the dectin-1/Syk signaling pathway, since Syk inhibition abrogated the HDM-induced CCL20 production (91). More recently, our group demonstrated that TSLP secretion by AECs upon stimulation with Der p 1 was at least partly carbohydrate-dependent. In addition, DC uptake of deglycosylated Der p 1 was considerably decreased compared with its natural (glycosylated) counterpart, indicating that glycosylation of allergens plays a crucial role in their recognition by immune and non-immune cells (92).

CROSS-TALK BETWEEN DENDRITIC AND EPITHELIAL CELLS

Due to their strategic location at the interface of external-internal environments, AECs are able to modulate and coordinate immune responses. Ample data have shown that the cross-talk between DCs and AECs is crucial in driving allergen-induced Th2 responses (6, 7). As previously described, the ligation of different PRR on AECs results in the secretion of chemokines (93–95), that attract DCs, and cytokines that induce DC maturation and activation. However, there are some contradictory results indicating that inducible signals driven by LPS in non-hematopoietic tissues such as AECs do not play an essential role in DCs activation (96). On the other hand other studies have demonstrated that AECs can induce DC maturation after LPS inhalation (81). Nevertheless a range of cytokines including TSLP, IL-33, IL-25, IL-1β, and granulocyte macrophage colony-stimulating factor (GM-CSF) are known to be secreted by AECs after allergen challenge (6, 7) which are able to modulate DCs function (**Figure 3**). Here we focus on TSLP as a key cytokine in initiation and maintenance of allergic responses.

ROLE OF TSLP

Thymic stromal lymphopoietin is a 140-amino acid four-helix-bundle cytokine that belongs to the IL-2 family of cytokines. This cytokine is mainly produced by AECs and is able to modulate DC by binding to its receptor complex composed by the TSLP receptor (TSLPR) and the IL-7 receptor (IL-7R) (97). This induces the production of Th2-attracting chemokines such as CCL22 and CCL17, and primes naïve T cells to produce IL-5, IL-4, TNF-α, IL-13, whereas down-regulate IL-10 and IFN-γ (98, 99) even in the absence of DCs in an IL-4 dependant way (100). TSLP induces the expression of OX40L in DCs which in turn has been shown to trigger Th2 cell polarization in the absence of IL-12 (101). Conversely, TSLP is also able to induce IL-12 secretion after CD40 ligation in DCs however still maintaining its Th2-polarizing effect (102). Furthermore, DCs activated with TSLP were able to induce the expansion of Th2 memory cells and help to maintain their phenotype (103).

Human epithelial cells can produce TSLP in response to diverse stimuli, such as microbial products, physical injury, ambient particulate matter, protease allergens, and either pro-inflammatory or Th2-polarizing cytokines (58, 104–108). Protease allergens induce TSLP in a PAR-2-dependent way (109) with the involving of MAPK signaling pathway (58) however inflammatory cytokines induction of TSLP is NF-κB signaling dependent (104, 106). In addition to DCs, TSLP can also activate mast cells and CD34+ blood hematopoietic progenitor cells to produce Th2 cytokines and in that way induce the innate phase of allergic immune responses (105, 110). Finally, TSLP can interfere with regulatory T cell development impairing the balance between tolerance and inflammation (111). Keratinocytes too can secrete functional TSLP after stimulation with pro-inflammatory or Th2-driven cytokines, and induces DC activation in human skin lesions of atopic dermatitis (112). Recently, it was demonstrated that DCs can also produce TSLP in response to TLR stimulation. Moreover, interestingly DCs from mice challenged with HDM extract express higher mRNA levels of TSLP than epithelial cells (113).

In vivo experiments have shown that TSLPR knock-out mice exhibited strong Th1 responses while Th2 responses were impaired and they failed to develop an inflammatory response to allergen challenge to lung (114) or skin (115). In addition, skin and lung over-expression of TSLP induces atopic dermatitis (116) and airway inflammation respectively (117). In an experimental model of

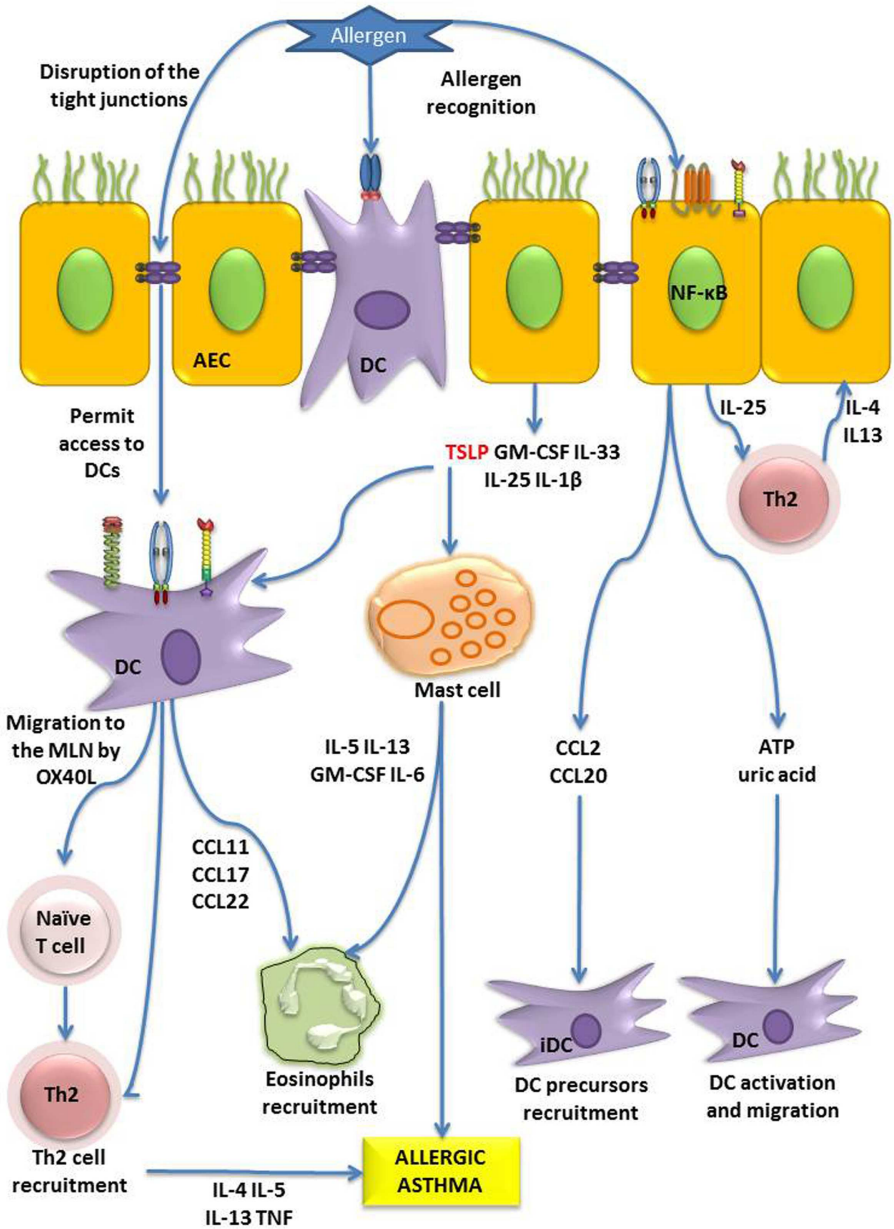

FIGURE 3 | Role of AECs in allergen sensitization. AECs can directly recognize allergens by PARs, TLRs or CLRs, and through NF-κB signaling, they induce the production of the cytokines (TSLP, GM-CSF, IL-33, IL-25, IL-1β), chemokine ligands (CCL2, CCL20), and danger signals (adenosine triphosphate, uric acid). TSLP has diverse effects on DCs and mast cells, which in absence of the cytokine IL-12 can lead to recruitment of Th2 cells and (eosinophils, Th2 polarization, and the onset of allergy or allergic asthma. Moreover, IL-25 can activate Th2 cells to produce pro-allergenic cytokines. On the other hand, DCs are able to form tight junction with AECs, and allergen can digest the tight junction and both phenomena contribute to facilitate the allergen sensitization process (6, 7).

allergic conjunctivitis it was demonstrated that after topical allergen challenge mucosal epithelial cells produce high levels of TSLP compared with controls leading to induction of allergic inflammation through the TSLP-OX40L signaling pathway (118). More recently, it was shown that the soluble TSLPS antagonist, comprised of the extracellular domain of the murine TSLPR and an IgG2a Fc tail, reduced the severity of airway inflammation by regulating DC function (119).

Furthermore, it has been demonstrated that TSLP and Th2-attracting chemokines are increased in airways of asthmatic subjects compared with normal controls (120). In addition, different studies have shown an association between genetic polymorphisms in the human IL-7Rα chain and TSLP genes with allergy, allergic rhinitis, and bronchial asthma further highlighting a possible link between these proteins and allergy (121–123).

CONCLUSION

Dendritic cells are professional APCs and sentinels of the immune system that efficiently sample allergens in the airways leading to a cascade of events that culminates in the induction of Th2 type immune responses. DCs are able to recognize and internalize allergens from diverse sources through expression of a plethora of receptors such as CLRs, TLRs, and FcεR. Recently, the role of AECs as key players in the modulation and induction of DCs in the airways has been highlighted. Like DCs, AECs are able to recognize allergens through several PRRs including PARs, CLRs, and TLRs. This further leads to the production of different cytokines, chemokines, and danger signals with the ability to initiate and propagate immune responses to allergens. Allergens have diverse molecular features such as specific oligosaccharide moieties, protease activity, lipid-binding properties, among others that can facilitate their recognition by immune and non-immune cells and contributes to their "allergenicity." Better understanding of the molecular basis of early events at the interface of allergens and their receptors and the key soluble mediators/signaling pathways involved could lead to development of more effective therapeutic strategies for allergic diseases including allergic asthma. For instance, due to the contribution of TLRs and CLRs in the recognition of allergen by both DCs and AECs, agonist and antagonists to those receptors may provide new therapeutic targets to modulate allergic responses. In addition, different studies have highlighted the role of sugar moieties on allergens in their recognition and internalization by immune cells. Accordingly, different "glycoforms" of allergens with immunoregulatory properties could be developed and used in allergen-specific immunotherapy strategies. Finally, diverse intracellular and extracellular molecules have been implicated in the process of allergen recognition and sensitization. Further studies to decipher these mechanisms could pave the way for the rational design of more effective therapeutic entities for the treatment of allergic diseases.

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New vaccines for mammalian allergy using molecular approaches

Marianne van Hage*[1] *and Gabrielle Pauli*[2]*

[1] *Clinical Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet and University Hospital, Stockholm, Sweden*
[2] *Faculty of Medicine, Strasbourg University, Strasbourg, France*

Edited by:
Christiane Hilger, Centre de Recherche Public de la Santé, Luxembourg

Reviewed by:
Mark Larché, McMaster University, Canada
Margarete Focke-Tejkl, Medical University of Vienna, Austria

****Correspondence:***
Gabrielle Pauli, Hôpitaux Universitaires de Strasbourg, BP 426, Strasbourg Cédex 67091, France
e-mail: gabrielle.pauli@chru-strasbourg.fr

Allergen-specific immunotherapy (SIT) offers a disease specific causative treatment by modifying the allergen-specific immune response allowing tolerance to higher doses of allergen and preventing progression of allergic diseases. It may be considered in patients allergic to furry animals. Current mammalian allergy vaccines are still prepared from relatively poorly defined allergen extracts and may induce immediate and late phase side effects. Although the mechanisms of SIT are still not fully understood, the more recent approaches report different strategies to reduce both allergen-specific IgE as well as T cell reactivity. The availability of recombinant allergens and synthetic peptides from the mammalian species has contributed to formulating new allergy vaccines to improve SIT for furry animal allergy. The majority of studies have focused on the major cat allergen Fel d 1 due to its extensive characterization in terms of IgE and T cell epitopes and to its dominant role in cat allergy. Here we review the most recent approaches, e.g., synthetic peptides, recombinant allergen derivatives, different hypoallergenic molecules, and recombinant allergens coupled to virus-like particles or immunomodulatory substances as well as strategies targeting the allergen to Fcγ receptors and the MHC class II pathway using a new route for administration. Many of the new vaccines hold promise but only a few of them have been investigated in clinical trials which will be the gold standard for evaluation of safety and efficacy in allergic patients.

Keywords: allergen-specific immunotherapy, Fel d 1, mammalian allergens, recombinant allergens, T cell peptides, vaccines

INTRODUCTION

Allergen-specific immunotherapy (SIT) offers a disease specific causative treatment by inducing tolerance to the allergen and preventing progression of allergic diseases, for example development of asthma (1). Hence, patients who suffer from allergies where avoidance is not possible such as house-dust mite respiratory allergies are the prime candidates for SIT. Allergy to cats, dogs, or other furry animals is common in society and the prevalence of sensitization to pets has increased (2). Although the best treatment is avoidance, it is often difficult to realize as well as not being well accepted by the patients. Moreover allergens from furry animals, e.g., the major cat allergen Fel d 1 is equally present in houses without cats, in public places, public transport, day care centers, and school rooms. So the question of SIT may be considered. Allergen-specific immunotherapy for cat allergy has shown to be more favorable than for, e.g., dog allergy (3). This may depend on the standardization of cat extracts to Fel d 1, whereas dog extracts may vary greatly in terms of allergen content (4).

The mechanisms of SIT are still not fully understood. The clinical efficacy is associated with the production of allergen-specific blocking immunoglobulin (Ig)G antibodies and the generation of T regulatory cell (T regs) responses and secretion of immunoregulatory cytokines such as interleukin 10 (5, 6); however, current SIT involves drawbacks such as treatment persistence, due to the long treatment duration both with subcutaneous SIT and sublingual immunotherapy (7). Furthermore, SIT of today can induce adverse reactions ranging from mild symptoms to life-threatening anaphylaxis and even death (8).

Allergy vaccines of today are still made from crude allergen extracts, which are not well-defined. Since the structures of the most common allergen molecules are now available, well-defined recombinant and synthetic allergy vaccines can be generated allowing targeting specific mechanisms of allergic disease without increasing their allergenicity (9–11). Many of these new molecular approaches concern pollen allergens. It has been shown that SIT with the recombinant major birch allergen Bet v 1 is equally effective as SIT with birch pollen extract or natural purified Bet v 1 (12). In this review, we describe different approaches for new vaccines, e.g., recombinant allergen derivatives, different hypoallergenic molecules, and recombinant allergens coupled to virus-like particles or to immunomodulatory substances. Studies being available concern principally evaluation of the cat major allergen, either in animal models or in allergic patients.

GENERAL CONCEPTS TO INCREASE SAFETY OF ALLERGY VACCINES

Side effects are related to the production of allergen-specific IgE antibodies but can also be mediated by the allergen-specific T cells. Several studies during the last years have used an allergen-unrelated carrier molecule, which provides T cell help for the

production of IgG antibodies but avoid the activation of allergen-specific T cells. This approach would reduce T cell mediated side effects. Different strategies to reduce both IgE as well as allergen-specific T cell epitopes have been reported. These approaches will lead to less IgE and T cell-mediated side effects (11, 13).

- Strategies targeting allergen-specific T cells by using T cell epitope-containing peptides (synthetic molecules produced by chemical synthesis), which give no IgE-mediated activation of effector cells.
- Strategies reducing allergen-specific IgE reactivity, but conserving T cell reactivity.
- Strategies based on carrier-bound fusion proteins using peptides. These allergen derived peptides are obtained from the IgE-binding sites of the allergen but contain no or reduced allergen-specific T cell epitopes and exhibit no or strongly reduced IgE reactivity. The peptides are fused to an allergen-unrelated viral carrier molecule and expressed as recombinant proteins. This technology was promoted by the Vienna group and a grass-pollen allergy vaccine based on carrier-bound peptides of the four major grass-pollen allergens is now under evaluation in a clinical phase II trial (11, 13–15).

Several other strategies for improving SIT have been described in the studies published within the last years: e.g., targeting the allergen to Fcγ receptors (16), targeting the MHC class II pathway (17), linking allergens to CpG-containing nucleotides (18, 19) or to carbohydrate-based particles (20), and co-administrating allergens with immunomodulatory substances such as vitamin D3 (21, 22). DNA vaccines favoring specific Th1 responses may also be used with different adjuvants such as CpG. mRNAs encoding allergens are another possible strategy, which would improve the safety of therapeutic nucleic acid-based vaccines. Moreover, different routes for SIT have been investigated using recombinant allergens especially sublingual, epicutaneous, and intra-lymphatic delivery of the allergen (23).

Table 1 provides a summary of these approaches that have been applied for furry animal allergen SIT. It is interesting to note that the majority of studies have focused on the major cat allergen Fel d 1 due to its dominant role in cat allergy and to its extensively characterization in terms of IgE and T cell epitopes.

T CELL PEPTIDES

T cell peptides were originally designed to modulate allergen-specific T cell responses without IgE-mediated activation of effector cells (35). Following preclinical studies (36), a series of clinical trials were performed to evaluate the safety and efficacy of two polypeptides from the major cat allergen Fel d 1. Initially, patients received a subcutaneous injection weekly for 4 weeks with an equimolecular mixture of the peptides (ALLERVAX CAT; Immunologic Pharmaceutical Corporation, Waltham, MA, USA, an equimolar combination of two 27-aminoacid synthetic peptides), 7.5, 75, and 750 μg, or placebo. Significant improvements in lung and nasal symptoms were observed in the high dose group but many adverse events occurred several hours after peptide injection (37). A subsequent multicenter study in which patients received eight injections of 750 μg of vaccine reported only modest clinical improvement and immediate and late side effects were observed

Table 1 | Concepts and strategies for improving SIT for mammalian allergy.

	Technologies	Specific approaches	Reference
Synthetic molecules	Peptides		
		Mixture of 12 short peptides	Oldfield et al. (24)[a]
		Mixture of 7 short peptides	Worm et al. (25)[a], Patel et al. (26)[a]
Recombinant molecules	Hypoallergens		
	Mutants	Mus m 1	Ferrari et al. (27)
	Hybrids	Fel d 1 duplicated T cell epitopes and disruption of disulfide bounds	Saarne et al. (28)
	Random mutation	Fel d 1	Nilsson et al. (29)
	Peptide carrier fusion proteins		
	Derived virus particles	Qß-Fel d 1	Schmitz et al. (30)
	Pre-S domain of hepatitis B	Fel d 1 peptides fused to Pre-S	Niespodziana et al. (31)
Conjugation with immunomodulatory molecules	Fcγ receptor	Truncated IgG Fcγ Fel d 1	Zhu et al. (16)
	Truncated invariant chain peptide + transactivator of transcription	MAT-Fel d 1	Martinez-Gomez et al. (17), Senti et al. (32)[a]
	Vitamin D3	Fel d 1-Vitamin D3	Jeffery et al. (33), Grundström et al. (22)
	Carbohydrate-based particles	CBP-Fel d 1	Neimert-Andersson et al. (20), Thunberg et al. (34)

MAT, modular antigen transporter; CBP, carbohydrate-based particle.

[a] *Clinical studies.*

(38). Further developments using a mixture of 12 short peptides comprising the majority of the T cell epitopes from Fel d 1 demonstrated that changes in the quality of life of the active treated group versus the placebo group, but isolated late asthmatic reactions were still observed (24). In a recent study, a new vaccine was developed consisting of a mixture of seven immunodominant peptides (ToleroMune cat®, also known as cat PAD, Circassia Ltd, Oxford, UK), which were selected on the basis of MHC-binding characteristics. This approach allowed reducing the number of peptides without substantially compromising population coverage. The safety and tolerability of the vaccine was evaluated in a phase IIa study (25) where cat-allergic individuals were given a single dose of the vaccine either intradermally or subcutaneously. Inhibition of the late cutaneous reaction was considered as a surrogate of clinical efficacy and 3 nmol was determined as the best concentration for intradermally desensitization. Treatment was performed in patients with cat-induced allergic rhinoconjunctivitis and in an environmental exposure chamber they were exposed to cat allergen before and after therapy. The clinical efficacy was observed after 18–22 weeks and 50–54 weeks after the start of the treatment and the highest dose of cat PAD (6 nmol 4 weeks apart, compared to 3 nmol 2 weeks apart) gave the greatest efficacy. The treatment effect was apparent on nasal and ocular symptoms and persisted 9 months later without any further retreatment (26). Other clinical studies should confirm these encouraging results.

T cell peptides containing major allergen epitopes have been generated from the primary structures of other mammalian allergens such as Can f 1 (39), Bos d 2 (40), and Equ c 1 (41) but the potential application of these molecules in allergic patients have not been investigated.

RECOMBINANT HYPOALLERGENIC DERIVATIVES

Hypoallergenic derivatives are characterized by a strongly reduced IgE reactivity. The IgE-binding epitopes of Fel d 1 have been modified through disruption of disulfide bonds and duplication of T cell epitopes. Three of the modified Fel d 1 derivatives displayed a strong reduction in allergenicity with 400–900 times lower IgE-binding capacity (hypoallergens) compared to wild-type Fel d 1 (28). The therapeutic capacity of the hypoallergen with the most reduced IgE reactivity was evaluated in a mouse model for cat allergy and by skin tests on cat-allergic patients. An induction of Fel d 1-specific IgG antibodies with capacity of blocking patients' IgE to rFel d 1 and a reduction in airway responsiveness was noted. Furthermore, the hypoallergen showed a tendency of reduced SPT reactivity compared to rFel d in seven cat-allergic patients (42).

Other hypoallergenic mutants of rFel d 1 have been generated by the introduction of random mutated allergen sequences and the selection of derivatives with a maintained tertiary structure by phage display using IgE antibodies from cat-allergic patients. The mutants had a lower IgE-binding and T cell activation capacity and could induce strong IgG-anti Fel d 1 protective responses by mouse immunization experiments. Thus they should be good candidates for safe alternatives for SIT (29).

Furthermore, in search of a vaccine for mouse allergy a structure-guided single point mutation has been performed for Mus m 1, the major mouse allergen which is an urinary protein. This mutation induced a spatial rearrangement of aromatic side chains and a lower allergenic activity although the T cell reactivity was preserved (27).

PEPTIDE CARRIER FUSION PROTEINS

One strategy to improve SIT has been to couple allergens to the bacteriophage Qß-derived virus-like particles. In mice, one injection of Qß coupled to Fel d 1 induced a Fel d 1-specific IgG response, and reduced anaphylactic reactions after rFel d 1 challenge (30). However, the chemical coupling process might be difficult to standardize for vaccine production.

Another carrier protein, the Pre-S domain of hepatitis B virus has been evaluated more recently (31). The cat hypoallergen vaccine was produced by fusion of Pre-S of hepatitis B to two non-allergenic Fel d 1 derived peptides. This approach has shown to eliminate both IgE-mediated and T cell mediated side effects. The T cell help comes from a Fel d 1-unrelated carrier protein, the Pre-S domain of hepatitis B virus, which contains antigenic sites for both B and T cells (43). The recombinant fusion protein exhibited more than 1000-fold reduction in allergenic activity compared with rFel d 1 (31). After immunization of mice and rabbits the fusion protein induced Fel d 1-specific IgG antibodies, which inhibited IgE-binding of cat-allergic patients to Fel d 1. In addition, the T cell responses in immunized mice were specific for Pre-S and very low for Fel d 1. This vaccine has to be further evaluated in clinical studies to confirm its promising qualities.

CONJUGATION TO MOLECULES WITH IMMUNOMODULATORY FUNCTIONS

A chimeric human–cat fusion protein composed of a truncated human IgG Fc gamma 1 and Fel d 1 has been proposed for a new approach of SIT (16). This conjugate induced a dose dependent inhibition of Fel d 1 driven IgE histamine release from cat-allergic donors' basophiles and from sensitized human cord-blood derived mast cells. The vaccine potential was also demonstrated in a mouse model for cat allergy (16, 44).

A new technology called modular antigen translocation (MAT) has been applied to Fel d 1 to enhance immunogenicity and safety of SIT. By attaching a truncated invariant chain peptide, and a transactivator of transcription peptide to allergens, they are converted into cytoplasmic proteins targeting the MHC class II pathway (17). MAT fusion of rFel d 1 has shown to enhance protective antibody and Th1 responses in mice, while reducing human basophil degranulation (17). A recent paper has described a phase I/IIa, randomized, placebo-controlled, and double-blind trial, using this construct by intra-lymphatic injections in cat dander allergic patients (20 patients were randomized) (32). The intra-lymphatic route has shown to reduce both the number of allergen injections as well as the allergen dose, improving both efficacy and safety of SIT (45). Three monthly injections with increasing doses of MAT-Fel d 1 elicited no adverse events and there was significant increase in allergen tolerance after nasal provocation. Regulatory T cell responses were stimulated and IL10 cytokine secretion and increased cat dander specific IgG4 production were observed. This clinical study represents the first immunotherapy trial with a recombinant cat allergen. It is also a major improvement over classical immunotherapy due to improved safety, low allergen doses, few injections, and a short treatment period. This

promising vaccination approach has to be confirmed in larger patient studies and also by assessing efficacy on reduction of symptoms and medication use and long term follow-up.

1,25-Dihydrovitamin D3 has been shown to induce dendritic cells with tolerogenic properties, thus, increasing regulatory T cell responses (33). In an attempt to use this effect, VD3 was covalently coupled to rFel d 1 and tested in a mouse model for cat allergy (22). rFel d 1 VD3 was superior to rFel d 1 in reducing airway inflammation, and airway hyperresponsiveness, and in producing allergen-specific IgG blocking antibodies.

rFel d 1 has also been covalently coupled to carbohydrate-based particles (CBP) for targeting of dendritic cells and enhanced adjuvanticity in SIT (34). This approach was evaluated in a mouse model for cat allergy (20). CBP-rFel d 1 treated mice showed reduced features of allergic inflammation and increased allergen-specific IgM and IgG responses. In a prophylactic protocol it was also shown that CPB rFel d 1 prevents the induction of airway inflammation possibly through the induction of allergen-specific IgG and IgM and by a prolonged tissue exposure to rFel d 1 (34).

CONCLUSION

Better characterization of recombinant allergens from mammalian species has contributed to formulate new allergy vaccines to improve SIT for patients allergic to furry animals. The identification of the major allergens in allergen sources is essential for generating vaccines, which may replace the natural allergen extract (11, 46). This explains in part why a majority of the new furry animal allergy vaccines have been restricted to the major cat allergen Fel d 1. Further studies are needed to point out which allergen component/s are needed for treatment of furry animal allergy, taking in account the great variability of commercial extracts regarding their allergen contents. This is especially true for dog allergens (4) where the panel of allergens so far is incomplete. Several studies presented here explore new concepts for improving the safety of SIT, by using different approaches and various technologies. However, only a few of them have been investigated in clinical trials, which will be the gold standard for evaluation of safety and efficacy in allergic patients.

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EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org