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# DIPLOMARBEIT

Titel der Diplomarbeit

## Molecular, immunological and structural characterization of mite allergens

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Magistra der Naturwissenschaften (Mag. rer.nat.)


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The studies presented in this diploma thesis were conducted at the Department of Pathophysiology at the Medical University of Vienna under the supervision of Univ. Doz. Dr. Susanne Vrtala and Univ. Prof. Dr. Rudolf Valenta from March 2008 to March 2010.

An dieser Stelle möchte ich mich bei allen bedanken, die durch ihre fachliche und persönliche Unterstützung zum Gelingen der vorliegenden Diplomarbeit beigetragen haben.

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# Table of Contents

# I. Introduction

# I. The origin of the term "allergy"

**Fig. 1** Clemens von Pirquet (1)

It was in 1906, when the term "allergy" (from the Greek *allos* – "other" and *ergon* – "work") was first used by the Austrian paediatrician Clemens von Pirquet (Figure 1, (1)) in an article in the *Munchener Medizinische Wochenschrift* (2). The reason for this neologism was his attempt to have a general phrase for describing any immunological phenomenon in an organism where an external agent causes a state of "changed or altered reactivity" after one or more applications. His definition did not discriminate between agents that lead to harmful reactions in the organism (e.g. serum sickness or anaphylaxis) from those which result in immunity against them (1). Pirquet's very broad definition of "allergy" soon changed its meaning and is nowadays used in a restricted manner, namely as a “disease following a response by the immune system to an otherwise innocuous antigen” (3).

# 2. Hypersensitivity

Hypersensitivity reactions, defined as reactions that are mediated by immunologic mechanisms leading to tissue damage, are grouped into four types by a classification system designed by Robin Coombs and Philip Gell in 1963 (4) (Figure 2, (3)).

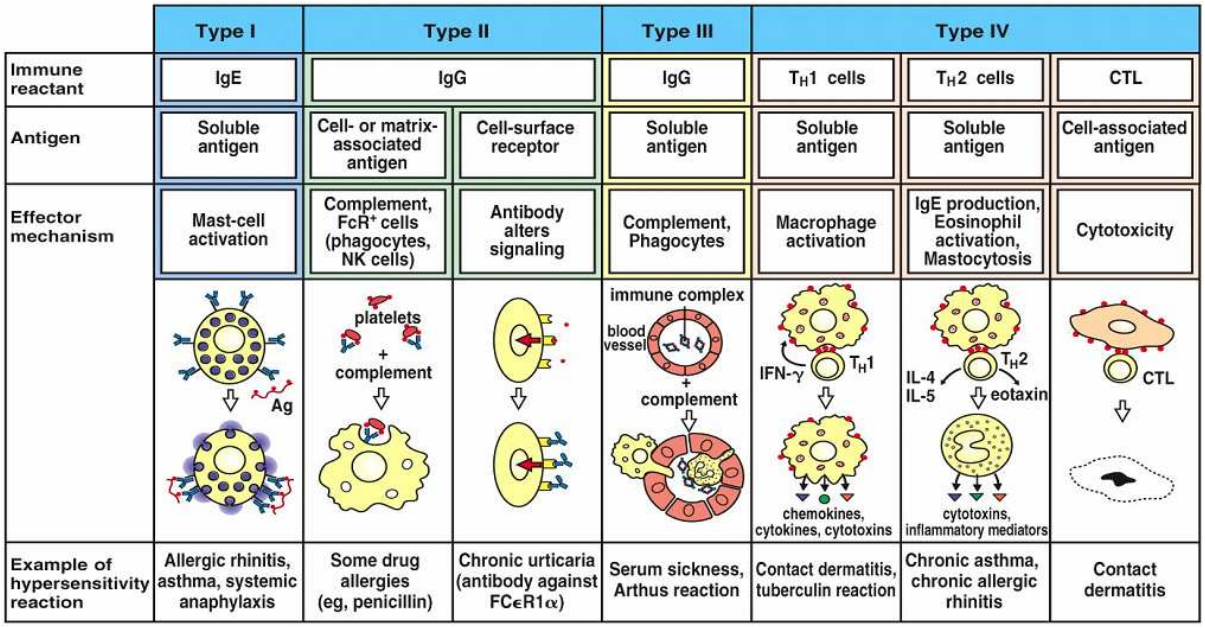


**Fig. 2** Summary of the characteristics of the four types of hypersensitivity (3)

## 2.I. Type I Hypersensitivity

Type I hypersensitivity is also known as immediate-type hypersensitivity due to the fact that reactions usually occur 15 to 30 minutes after exposure to the antigen. The mechanism of reaction involves IgE antibodies, which are produced in response to certain antigens called allergens. IgE binds to the surface of mast cells and basophils, via the high-affinity receptor FcεRI. Upon a subsequent exposure to the same allergen, the sensitized cells become activated when allergens induce cross-

linking of the FcεRI bound IgE antibodies. As a result of this activation, the cells degranulate and release various inflammatory chemical mediators that cause symptoms such as allergic rhinitis, acute urticaria, asthma or systemic anaphylaxis.

## 2.2. Type II Hypersensitivity

Hypersensitivity reactions of this type are caused by IgG antibodies that are directed against cell-associated antigens, and consequently mediate cell destruction via complement activation or ADCC (antibody dependent cellular cytotoxicity). Antigens recognized in this way, can either be part of the patient's cells, causing autoimmune diseases (e.g. autoimmune haemolytic anemia) or they are foreign, as it is the case in incompatible blood transfusion reactions. In special cases, the antibodies recognize and bind to cell surface receptors involved in cell signaling, where they either act as an agonist (e.g. in chronic urticaria) or antagonist (e.g. in myasthenia gravis, a muscle-weakness disease).

## 2.3. Type III Hypersensitivity

Type III hypersensitivity reactions are mediated by soluble antigen-antibody (IgG) immune complexes that are formed in large amounts. These complexes are normally removed by macrophages in the spleen or liver, but if they are produced in excess amounts this clearing mechanism is inadequate, resulting in aggregation of especially small complexes in capillaries between the endothelial cells. The immune complexes then activate the classical complement system leading to vasodilatation. Proteins of the complement system and the immune complexes themselves attract

neutrophiles that discharge proteolytic enzymes, thereby promoting massive inflammation which leads to tissue damage.

## 2.4. Type IV Hypersensitivity

In contrast to the other three types of hypersensitivity (I – III) described before which are antibody mediated, the type IV hypersensitivity is triggered by antigen-specific effector T-cells. This type of hypersensitivity is also called delayed-type hypersensitivity due to the fact that the reaction occurs usually not until 12-48 hours after exposure to the antigen. Similar to a response to an infectious pathogen, effector T-cells, that were primed by a previous exposure to the antigen migrate to the site where the antigen is present. There they become activated and produce $T_H1$ or $T_H2$ specific chemokines and cytokines, which cause the activation of macrophages or eosinophils. The following inflammatory reaction causes skin reactions (swellings, erythema, induration and dermatitis) if $T_H1$ cells are involved, or chronic asthma and chronic allergic rhinitis in a $T_H2$-dominated response.

# 3. Pathophysiology of Type I allergy

As already mentioned in chapter I.2.1, type I allergy is characterized by the formation of immunoglobulin E (IgE) antibodies against normally harmless environmental antigens, so-called allergens. Its time course can be sub-divided into different phases, which will be described in detail in the following chapters.

## 3.I. Sensitization and memory

The primary immune response to an allergen, which leads to the production of IgE, is called sensitization. Many factors are described in the literature, which can play a part in the triggering of this process. They can be classified as host and environmental factors. Host factors include the time of allergic sensitization, which is known to occur in the first few years after birth (5, 6). In addition to the age, genetic factors also contribute to the development of type I allergies (7-9). In atopic individuals, who have a higher disposition to develop a type I allergy, structural and promoter variants of some genes were found, which might be important in determining atopy. Examples are the genes coding for the β-chain of FcεRI or a genetic variant of the α-subunit of the IL-4 receptor. In addition to host factors, several environmental factors have so far been discussed to play a role in the formation of an allergic reaction. The fact, for example, that the prevalence of allergic disorders has increased over the last hundred years especially in industrialized countries, is often explained by the “hygiene hypothesis” (10, 11). This states that the reduced bacterial and parasitic exposure in developed countries due to a higher hygiene status and the vaccination of the population, results in an imbalance of the

immune system. The inadequate stimulation of the $T_H1$ fraction of the immune system give rise to an overactive $T_H2$ part, which then causes IgE-reactivity to normally harmless antigens. Nevertheless, proof of the contrary can be given by the observation that allergies are a common problem in developing countries too (12). Other factors, such as diesel exhaust particles, can act as a carrier for the allergens and thereby have an adjuvant effect in boosting the sensitization (13). One of the most important environmental factors is the allergen itself. Allergens have some characteristic properties, but it cannot be predicted whether an antigen acts as an allergen. It is known that almost all allergens are proteins or glycoproteins, that they are small in size (5 – 80 kDa) (14), are highly soluble, stable and are typically presented to the immune system at very low doses.

After describing the factors that may influence the development of an allergen-specific IgE response, a schematic representation of the course of sensitization and the formation of memory is shown in Figure 3 (15).

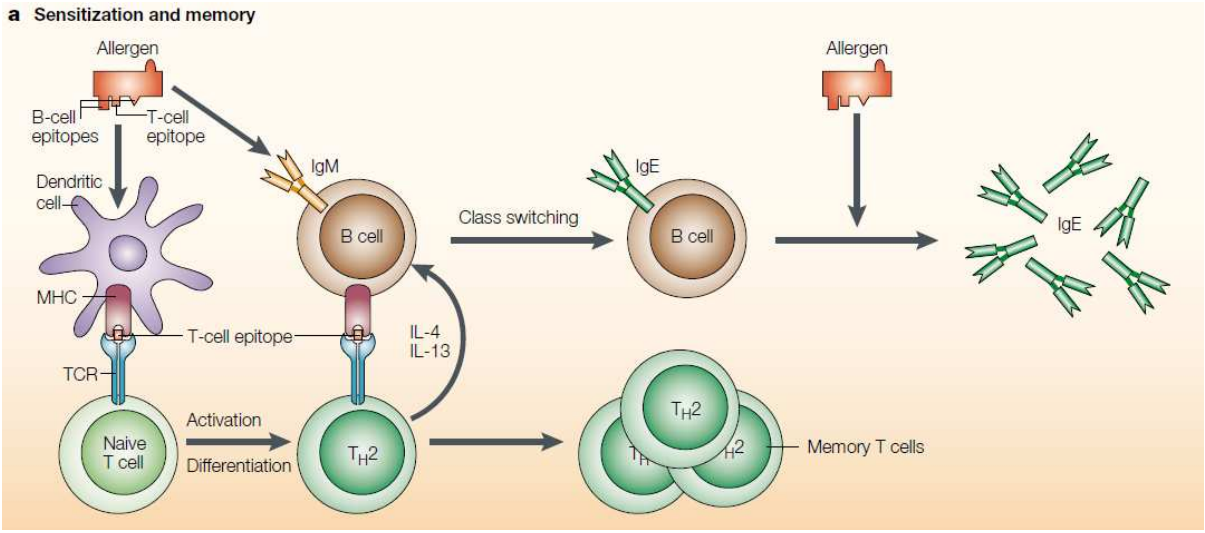


**Fig. 3** Overview of the allergen-specific immune response during sensitization and memory (15)

Production of IgE antibodies requires the differentiation of naïve T-cells into $T_H2$ cells, which then stimulate B-cells to switch the antibody isotype from IgM to IgE. The intrinsic properties of the allergen, the dose, the route of allergen presentation as well as certain cytokines are important factors that determine the fate of naïve $CD4^+$ cells to differentiate into $T_H2$ cells. Allergens are often delivered at low doses via the mucosa of the respiratory and gastrointestinal tract or through the epidermis of the skin. These sites of entry are also used by parasites to enter the body. As it is often claimed that the physiological function of IgE is to fight helminthic parasitic infections, cells of the immune system at these sites are specialized to produce cytokines that result in $T_H2$ responses (3). In summary, the microenvironment of the allergen entry sites favours the development of $T_H2$ cells (16). Allergens that enter the body are phagocytosed by antigen presenting cells, mainly myeloid dendritic cells, which then migrate to regional lymph nodes, where they present epitopes of the allergens to naïve T-cells using MHC. Once activated, the T-cells start to proliferate and to mature into effector $T_H2$ cells. As such, they produce interleukin-4 (IL-4) and IL-13, which favour the immunoglobulin-class switching of specific B-cells to IgE. Alternatively, B-cells may uptake allergens via their membrane-bound immunoglobulins and directly present them to T-cells.

Sensitization causes the production of allergen-specific IgE antibodies in allergic patients. Some of the allergen-specific lymphocytes form memory T- and B-cells that survive in an inactive state for a long period of time until they face the same allergen again and become reactivated (6, 17).

## 3.2. The immediate reaction

IgE is captured by the high-affinity Fcε receptor (FcεRI) (18), which is found on mast cells and basophils that reside in tissues. They become activated when allergens cross-link the receptors via binding to the IgE molecules. It is therefore necessary for allergens to have at least two epitopes for IgE binding. This cross-linking event leads to the induction of a signal cascade which leads to in the release of inflammatory mediators, such as enzymes (e.g. tryptase, chymase) and toxic mediators (e.g. histamine, heparin) by degranulation (Figure 4, (15)). The mediators cause an immediate increase in local blood flow and vessel permeability as well as tissue destruction. The clinical effects can be seen within minutes and are localized at the site at which mast cell degranulation occurs. Inhaled allergens cause allergic rhinitis, conjunctivitis and asthma, whereas food allergens provoke symptoms such as diarrhea and vomiting.

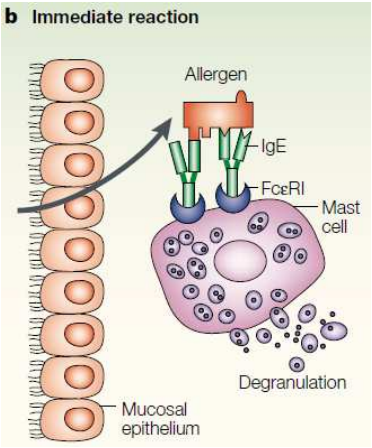


**Fig. 4** Events of the immediate reaction (15)

IgE not only cross-links FcεRI receptors, but also upregulates the expression of FcεRI (19-22), and promotes the survival of mast cells by preventing apoptosis (23, 24).

## 3.3. The late-phase reaction

Activated mast cells also synthesize and release cytokines (e.g. IL-4, IL-13, TNF-α), chemokines and lipid mediators (e.g. leukotrienes, platelet-activating factor). These recruit other leukocytes, including eosinophils and $T_H2$ lymphocytes, to the site

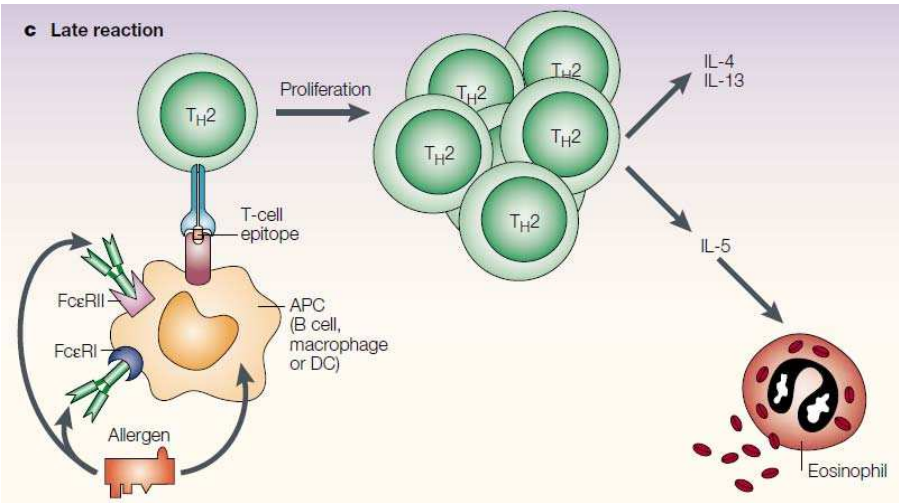


**Fig. 5** The late phase of an allergic reaction (15)

of inflammation. After T-cells become activated by IgE-facilitated and non-IgE facilitated presentation of allergen by antigen-presenting cells, they proliferate and release pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 (Figure 5, (15)). IL-5 is a key mediator in eosinophil activation, which causes the release of highly toxic granule proteins and free radicals from these cells, resulting in significant tissue damage. In addition, activated eosinophils synthesize chemical mediators, which activate epithelial cells. This leads to a recruitment and activation of more eosinophils and leukocytes. Clinical manifestations, such as oedema, pain, warmth and erythema can be seen two or more hours after allergen contact. The late-phase reaction can develop into chronic inflammation, which is characterized by the permanent presence of effector T-cells and eosinophils in the injured tissue (e.g. chronic allergic asthma).

# 4. Diagnosis of Type I allergy

Symptoms typical for an allergic disease such as rhinitis, redness and itching of the conjunctiva, sneezing and eczema often is a first evidence that a person is suffering from allergies. Nevertheless, further investigations, such as the medical history of the person, a physical examination as well as *in vivo* and *in vitro* tests are needed to confirm the diagnosis of an allergic disease.

Allergy can be diagnosed in relation to the different aspects of the allergic reaction, which is indicated by the various arrows shown in Figure 6 (25). Those framed with red boxes will be described in further detail.

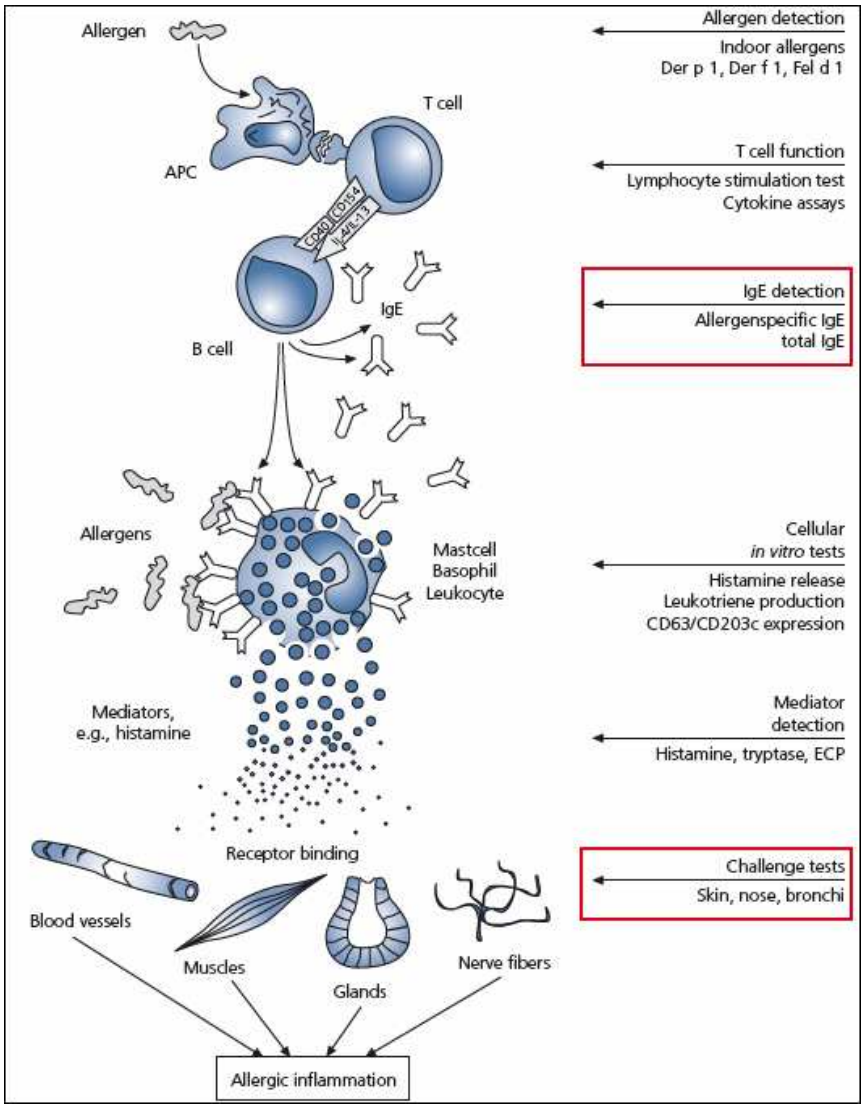


**Fig. 6** Tools for diagnosing an IgE-mediated allergy (25)

## 4.1. Extract-based diagnosis

After suggesting an allergy according to the medical history, sensitization to one or more allergen sources is usually confirmed by skin tests, which measure the allergic reaction in the skin. This technique is used since 1880 (26). The skin prick test is commonly performed on the forearm, where one drop of each allergen extract is placed on the skin and then a small prick is made with a lancet through the drop. A positive reaction, seen as a wheal and flare response, can be detected 10-15 min after exposure. Skin prick testing is the most often used technique for diagnosing allergy in patients as it is easy, quick to perform and cheap. Nevertheless, sometimes alternative diagnostic techniques must be used instead of the skin testing, for example if the patient is taking anti-histamines or if the skin is in such a bad condition that an interpretation of the skin test is very difficult.

While skin tests determine the skin reaction to different allergen sources, *in vitro* assays such as the radioallergosorbent (RAST) test measure the amount of IgE in the patient's serum directed against an allergen extract. The extract, immobilized to solid phase, is incubated with the serum and allergen specific IgE antibodies that bind to the components of the extract, are then detected with a labelled anti human IgE antibody. The amount of the allergen extract specific IgE is expressed as kilounits antigen per liter (kUA/L) with one unit representing 2.4 ng of IgE, and scored on a scale from 0 (< 0.35 kUA/L) to 6 (> 100.00 kUA/L ) (Phadia).

## 4.2. Component-resolved diagnosis

Both, the skin prick tests and the RAST assays are today performed with allergen extracts that are prepared from natural allergen sources using aqueous extraction (27). Although various methods have been developed in order to standardize these extracts, they show a great variability in the quantity of the different allergens (28, 29). In addition, contamination of the extracts with endotoxin or allergens from other allergen sources have been reported (30, 31).

These problems can be overcome if pure and defined allergens, in the form of recombinant allergens, were used which are produced since the late 1980s. While allergen extracts only allow to determine the allergen source against which a patient is sensitized, diagnosis with recombinant allergens reveals the patient's exact IgE reactivity profile against the disease-eliciting allergens (32). Valenta *et al.* started to test allergic patients with recombinant allergens in the early 1990s (33, 34) and in 1999 created the term "component-resolved diagnosis" (CRD) for the concept of using recombinant allergens for the diagnosis of type I allergy (32). Various studies have confirmed that recombinant allergens are a good alternative to allergen extracts for *in vitro* and *in vivo* diagnosis (reviewed in (35)). It has also been shown, that for most allergen sources a small number of recombinant allergens is sufficient for diagnosis of an allergy. Only four timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5 and profilin) are necessary to diagnose grass pollen allergic patients (34, 36, 37). For house dust mite allergy, it has been shown that Der p 1 and Der p 2, the two major allergens, are sufficient to diagnose 97% of *D. pteronyssinus* allergic patients. Addition of Der p 5 and Der p 7 increased the percentage to almost 100% (38).

Diagnosis with recombinant allergens also allows differentiation between co-and cross-sensitization. Several "species-specific" and cross-reactive marker allergens

have so far been identified (reviewed in (39), (40)). Examples for the former markers are the group 1, 2, 5 and 6 allergens of grass pollen, which is their exclusive source (41). Cross-reactive allergens are for example the tropomyosin Der p 10 from house dust mites, or profilin. The IgE reactivity of the patients against these marker allergens can give useful information about the sensitizing allergen source and whether an extract-based form of immunotherapy is advisable or not.

Since the end of the 1980s, when the first allergen was produced in recombinant form (42), a large collection of recombinant allergens from the most important sources became available that display the immunological properties of their natural counterparts. To test IgE-reactivity to the large panel of allergens, a new type of *in vitro* test, the so-called “Immuno Solid-phase Allergen Chip” (ISAC), was developed by Hiller *et al.* (43). In this multi-allergen test system, recombinant allergens are printed as microarrays in triplets on glass slides. It currently enables the determination of a patient’s IgE reactivity profile against 103 individual allergens in a single step using only 20 µl of serum. Further diagnostic *in vivo* tests such as provocation tests, are necessary to confirm the clinical relevance of the IgE reactivities.

# 5. Mite allergy

## 5.1. The history of the discovery of mite allergy

It was in the early 1920s, when house dust was discovered as a cause of allergic disease in the context of asthma by Kern (44) and Cooke (45). In 1928, Dekker suggested that asthma is caused by allergens produced by mites in the house, but this study received only little attention (46). In 1967, Voorhorst *et al.* showed that there is a strong relationship between the allergenic potency of dust samples and the number of mites in dust samples and suggested mites of the genus *Dermatophagoides* to be the main source of allergens in house dust (47). Many subsequent investigations confirmed these findings.

## 5.2. Description and life cycle of mites

**Fig. 7** *D. pteronyssinus*

House dust mites of the species *Dermatophagoides* are globular in shape and have a creamy white colour. Due to their small size they can only be seen under the microscope (Figure 7). The typical house dust mite is 420 µm long, while the width depends on the sex (245 µm for males, 320 µm for females). The body of the house dust mite also contains a striated cuticle.

The life cycle of *D. pteronyssinus* and *D. farinae* consists of five stages: egg, larva, protonymph, tritonymph and adult. The completion of their life cycle takes

about a month with the adult living an additional one to three months. During this time, the female mite lays about 60 to 100 eggs and produces about 2000 faecal particles, which are of great importance, as it is known that mite allergens are mainly present in the faeces.

The house dust mite is named after its habitat, the household dust. One of the main components of dust are shed skin flakes, which are their preferred food source. One of the major limiting factors for mite survival is the relatively high required humidity (60-90%) (48). This is the reason why the mite content in the house dust is higher in spring than in winter, where the heating dries out the houses and reduces the air moisture.

## 5.3. Allergenic mites species and their distribution

Among the over 50 000 known species of mites, allergens, which lead to mite allergy have been described from the families *Acaridae*, *Glycophagidae* and *Pyroglyphidae* (49, 50), all belonging to the order *Acariformes* (Figure 8), subclass *Acari*, class *Arachnida* and phylum *Arthropoda*.

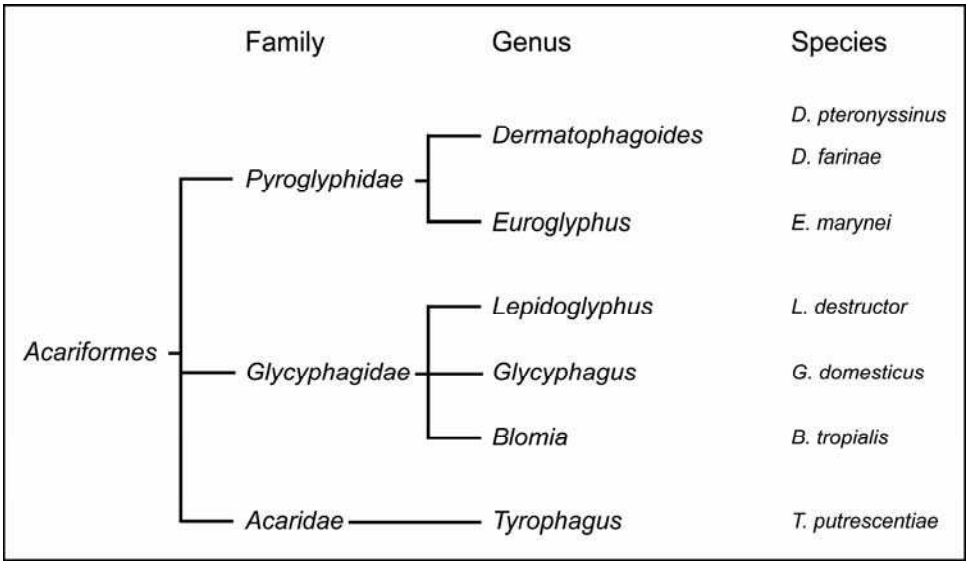


**Fig. 8** Phylogenetic relationships of the order *Acariformes*

Species of the family *Acaridae* (*T. putrescentiae*) are a minor source of mite allergens. The main species of the family *Glycyphagidae* are *L. destructor*, *G. domesticus* and *B. tropicalis*, also known as storage mites. Except for *B. tropicalis*, which is the prominent mite in tropical and subtropical regions (51), they play a less important role in mite allergy.

The most important elicitors of mite allergy are the house dust mites (HDM) *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and to much lesser extent *Euroglyphus manyei*, which all belong to the family *Pyroglyphidae*. The prevalence of these three major species of mites is dependent on the geographical region with *D. pteronyssinus* being the prevalent species in Europe, North and South America, Africa, Asia and Australasia (52). *D. farinae* is often misleadingly described as the American house dust mite, although it is dominant only in the north eastern regions of North America (53). *D. farinae* is predominately found in Sweden (54) and Poland (55), while the species distribution in most other countries is mixed (56).

## 5.4. Allergens from mites

Mite allergens are found in mite bodies, secreta and excreta with the faeces being the major source of house dust mite allergens (57). Der p 1, one of the two major house dust mite allergens, for example, becomes airborne almost exclusively on faecal particles (58).

Mite allergens are arranged in groups according to their homologies and order of description. So far, 24 groups of mite allergens have been identified. Table 1 (adapted from (59)) gives an overview of the biochemical function, the molecular weight as well as the IgE binding frequency of these allergens. Newly isolated allergens are given their official names by the World Health Organization and

International Union of Immunological Societies (WHO/IUIS) Subcommittee on Allergen Nomenclature and are listed at www.allergen.org. Nevertheless, several mite allergens have been described in the scientific literature that are not part of this list. These allergens can be found for example in the Allergome database (www.allergome.com).

**Table 1.** Characteristics of the 24 groups of mite allergens

| Group | Biochemical function | MW (kDa) SDS-PAGE | IgE binding* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Der p | Der f | Eur m | Lep d | Gly d | Blo t | Tyr p |
| 1 | Cysteine protease | 25 | 64-100 | 78-90 | ? | | | 60-93 | ? |
| 2 | ML domain lipid binding protein | 15 | 63-100 | 92-100 | ? | 59-78 | 94 | 7 | 56-72 |
| 3 | Trypsin | 31 | 9-97 | 16 | ? | | ? | 5-57 | ? |
| 4 | alpha-amylase | 57 | 28-74 | ? | 0 | | | 6-62 | ? |
| 5 | Unknown | 15 | 6-74 | ? | | 9 | ? | 20-73 | ? |
| 6 | Chymotrypsin | 25 | 41-65 | 41 | | | | 11 | |
| 7 | Unknown | 26, 29, 31 | 13-57 | 46 | | 62 | ? | ? | ? |
| 8 | Glutathione-S-transferase | 26 | 9-96 | ? | | ? | ? | ? | |
| 9 | Serine protease | 30 | 92 | ? | | | | ? | |
| 10 | Tropomyosin | 37 | 6-55 | 25, 81 | ? | 13 | ? | 10-29 | 13 |
| 11 | Paramyosin | 96 | 50, 75 | 50-82 | | | | 52, 67 | |
| 12 | Unknown | 14 | | | | | | 50 | |
| 13 | Fatty acid binding protein | 15 | ? | ? | | 13 | | 11 | 6 |
| 14 | Large lipid transfer protein | 177 | ? | 66, 84 | ? | | | ? | |
| 15 | Chitinase | 98, 105 | 70 | 70 | | | | ? | |
| 16 | Gelsolin | 55 | | 47 | | | | | |
| 17 | EF hand binding protein | 30 | | 35 | | | | | |
| 18 | Chitinase-like protein | 60 | 63 | 54 | | | | ? | |
| 19 | Anti-microbial peptide | 7 | | | | | | 10 | |
| 20 | Arginine kinase | 40 | 15-44 | ? | | | ? | ? | |
| 21 | Unknown | 15 | 56 | ? | | | | 57, 93 | |
| 22 | Unknown | 17 | | ? | | | | | |
| 23 | Chitin-binding protein | 14 | 61, 85** | | | | | | |
| 24 | Troponin C | 18 | | | | | | | ? |

allergens listed in the IUIS database

allergens additionally found in the Allergome database

* binding frequency (% patients, variation due to different studies)

** unpublished data

? no IgE frequency data available

A HDM protein is defined as an allergen when it binds IgE from more than 5% of sera from patients showing symptoms of a house dust mite allergy. The IgE binding frequencies that were described in different studies occasionally varied dramatically, not only between the species, but also within one species, as can be seen for groups 3, 8 or 10 in Table 1. These varieties can be explained by the different number of tested patients, their selection as well as the geographic area, in which the IgE-binding frequency was studied. Some of the allergens, initially designated as major allergens due to reports of IgE-binding frequencies of more than 50% (e.g. group 3 and 10) (60, 61) are nowadays described as low-potency allergens (62). A classification of house dust mite allergens according to their IgE-binding activity was recently given by Thomas WR (unpublished data) and is summarized in Table 2. The latest discovered mite allergens are missing in this classification by Thomas and are therefore listed in a separate column.

**Table 2.** IgE binding activity of HDM allergens

| **IgE binding activity** | **allergens** | |
|---|---|---|
| | ***Thomas WR*** | ***new*** |
| major | 1, 2 | 23 |
| mid-potency | 4, 5, 7, 21 | |
| low | 3, 6, 8, 9, 10, 13, 16, 17, 20 | 19 |
| uncertain | 11, 14, 15, 18 | 22, 24 |

Due to the high IgE-binding frequency, the high amount of specific IgE directed to this allergen and their high content in mite extracts, group 1 allergens (together with group 2) are considered as the most important mite allergens. Therefore group 1 allergens will be described in more detail in chapter I.5.6. Further information is also given about tropomyosin, the group 10 mite allergen, in chapter I.5.7., as it is subject of this diploma thesis.

## 5.5. Cross-reactivity of mite allergens

Cross-reactivity in allergy is a phenomenon, where IgE antibodies originally produced against one specific allergen react with other allergens, which are sequentially or structurally similar and therefore share IgE-epitopes.

Cross-reactivity among mite allergens is known with degrees varying from very low to very high dependent on the evolutionary relationship between the different mites. It was shown for example for group 2 allergens, that the cross-reactivity among the storage mites *L. destructor*, *T. putrescentiae* and *G. domesticus* was high, but only low between group 2 allergens from storage mites and Der p 2 from *D. pteronyssinus* (63). There is no IgE cross-reactivity of group 1 and 5 allergens between glycyphagid and pyrophagid mites (64, 65), which is consistent with their high sequence disparity of 65% (52). Tropomyosins, on the other hand, show a sequence identity of 95% among the order of *Acariformes*, which leads to extensive cross-reactions, also with tropomyosins from other arthropod species such as crustacean. They will be discussed in further detail in chapter I.5.7. and II. In summary one can state that mite allergens can be species-specific or cross-reactive.

## 5.6. Group I allergens - cysteine proteases

Der p 1 and Der f 1 are the most important allergens in this group. As indicated by the name, the group 1 allergens, or more precisely, Der p 1 was the first allergen described for house dust mites (1980) (66). It was also the first allergen of which the cDNA was isolated, cloned and sequenced (42). The group 1 allergens from *D. farinae*, *E. marynei*, *B. tropicalis* and *T. putrescentiae* have also been characterized some years later (1986, 1992, 2003 and 2006 respectively (67-69)).

Der p 1, Der f 1 and Eur m 1 show 85% sequence identity (52), whereas Blo t 1 is only 35% identical to the pyroglyphid group 1 allergens (69).

Group 1 HDM allergens are cysteine proteases, belonging to the family of papain (CA1) which is widely distributed in plants and animals. They are produced in the gut of mites, where they act as digestive enzymes and get later incorporated into dung balls, each containing about 0.1 ng of the allergen (57). Because Der p 1 is found in such high concentrations in the house dust, its content in the dust is used as an index of environmental exposure to house dust mites (defined at the Third International Workshop on mite allergens and asthma 1997).

Group 1 allergens are synthesized as enzymatically inactive pro-enzymes. Der p 1, being representative for this group, consists of an 18aa long signal peptide, a 80aa long pro-peptide and the mature region comprises 222aa. The pro-peptide inhibits the activity of the enzyme as it occludes the active site. Activation of the protein occurs under acidic conditions, when the solvent accessibility of the pro-peptide residues increases and therefore exposes the sequence to Der p 1, which then is able to cleave its own pro-sequence (70).

The allergenic activity of Der p 1 somehow seems to be to be related to its protease activity, as Der p 1 negatively influences functions of the innate and adaptive immune system. Der p 1 increases the permeability of bronchial mucosa by cleaving components of the intercellular tight junctions such as occludin, and consequently facilitates access to antigen-presenting cells (71). Der p 1 cleaves CD23 which may increase the production of IgE (72, 73) whereas digestion of CD25, the IL-2 receptor, by Der p 1, may result in a bias toward a $T_H2$ immune response (74).

### 5.6.I.I. Expression of recombinant group I allergens

Attempts to produce Der p 1 as a recombinant active allergen have been made since its cDNA was cloned in 1988, when it was tried to express the mature Der p 1 as a GST-fusion protein in *E.coli.* The protein, however, showed only a weak IgE-binding activity (42). The probable reason for this weak IgE reactivity was that Der p 1 was not expressed together with its pro-sequence. It is known for a lot of proteases, that the pro-sequence plays an important role in the correct folding of the molecules (75, 76). Therefore, Der p 1 was later expressed together with its pro-peptide, but this raised the problem of how to remove the additional amino acids after expression, to obtain the mature protein. It is published for papain, that the pro-sequence can be cleaved when incubated at 60°C for 15-30min at pH values between 3.7 and 4.2 (75). The literature shows that these conditions did not always lead to the activation of proDer p 1 (77, 78). Nevertheless, autocleavage of the propeptide worked when the protein was expressed in the yeast *Pichia pastoris*, but the mature protein showed a reduced IgE binding frequency (79). Because proteins expressed in yeast tend to be hyperglycosylated, the N-glycosylation motif was removed by the substitution of an asparagine residue to a glutamine residue. The resulting protein showed full enzymatic activity and IgE binding comparable to natural Der p 1 (80).

Der f 1, which shows 80% homology to Der p 1, was expressed as proDer f 1 in *E. coli* and was then activated under acidic conditions. The mature protein had equal protease and IgE binding activities as nDer f 1 (81). Attempts to produce recombinant Der p 1 in the same way, however failed (unpublished data).

## 5.7. Group I0 allergens - tropomyosins

Group 10 allergens belong to the family of tropomyosins, which together with troponin, actin and myosin play an important role in the Ca-dependent regulation of muscle contraction (82). The cDNA of tropomyosin encodes a 284 aa long poly-peptide, which is folded in an α-helical manner. Two of these proteins are wound around each other forming a coiled-coil structure (83). Being found in the skeletal muscle of the mites, it is not surprising that Der p 10 is the 23$^{rd}$ most abundant protein produced by the mite (84).

The first allergen described for the group 10 allergens was Der f 10 from *D. farinae* in 1995 (61). In this initial report an IgE-binding frequency of 80.6% was described, which led to the assumption that Der f 10 was a major mite allergen. Der p 10, however, which was cloned and expressed as recombinant protein three years later, bound IgE from only 5.6% of mite allergic patients (85). Low IgE-binding frequencies were also reported from the later identified group 10 allergens of glycyphagid mites, Lep d 10 (13%) and Blo t 10 (20-29%) as well as from Tyr p 10 (12.5%) (86-88).

The sequences of tropomyosin proteins are highly conserved among invertebrates. Der p 10 shares 91 – 96% identical amino acids with tropomyosins from storage mites (Tyr p 10, Gly d 10 and Lep d 10) and 80% with those of crustacean, while the sequence identity is only 50% to human tropomyosins (52). Being consistent with the conserved sequence of tropomyosins among invertebrates, IgE cross-reactivities of mite tropomyosins with tropomyosins from other arthropods such as cockroach and shrimp are reported (89, 90). Comparing the IgE-binding epitopes of the shrimp tropomyosin Pen a 1 with the homologous sequences from Der p 10 and Der f 10, revealed that four of the eight identified shrimp epitopes had

an identical amino acid sequence with the mite tropomyosins and two differed only in one amino acid (91).

The high IgE binding frequencies of group 10 allergens obtained in Japan (80.6%) (61) and Africa (55%) (12) are probably the result of a regional cross-reactivity, as studies in Europe (38), Korea (88) and Australia (62) showed low IgE binding frequencies for group 10 allergens.

The IgE binding frequencies for group 10 allergens were all obtained using recombinant tropomyosin proteins except for the study of Aki *et al.* (61), where the native form of the protein was also used resulting in the very high IgE binding frequency of 80.6%. Nevertheless, almost the same frequency was also obtained with the recombinant protein in this study excluding the possibility that the recombinant proteins show reduced IgE binding compared to their natural counterparts.

The recombinant tropomyosins were all expressed in *E. coli*, either as a Glutathione-S-transferase (GST) fusion protein as it is the case for Der p 10 (38), Der f 10 (61) and Blo t 10 (87), as a His tagged protein (Der p 10 (62), Tyr p 10 (88)) or in a non-fusion form (Der p 10 (85), Lep d 10 (86)).

A major interest in group 10 allergens is attributed to the fact that tropomyosin proteins are the major allergens in species belonging to the subphylum crustacean (e.g. shrimp, lobster and crab) and phylum mollusca (e.g. squid, snail and mussel), all known to cause adverse food reactions in allergic individuals. The sequences of a lot of these allergenic tropomyosins have so far been identified, most of them were cloned and expressed as recombinant proteins such as Pen a 1 (shrimp) (92), Cha f 1 (crab) (93) and Hom a 1 (lobster) (94).

# 6. Aims of the Diploma thesis

The major aim of this thesis was to study the relevance of the house dust mite allergen Der p 10 in mite allergic patients. The allergen should be produced as a recombinant protein that equals its natural counterpart regarding biochemical and immunological properties. A cDNA encoding Der p 10 was obtained from *D. pteronyssinus* RNA by reverse transcription with specific primers and should be expressed as a non-fusion protein in *E. coli*. After purification to homogeneity, mass spectroscopy and circular dichroism (CD) analysis should confirm the correct size and folding of recombinant Der p 10. The IgE-binding frequency of the mite tropomyosin in an Austrian population should be tested with sera from over 1000 *D. pteronyssinus* allergic patients. The allergenic activity of rDer p 10 should be studied in an *in vitro* mediator release assay using rat basophil leukaemia (RBL) cells transfected with the human FcεRI receptor, which are loaded with mite allergic patients' serum IgE before.

In the second part of the Diploma thesis the existence of mite specific IgE antibodies in the serum of a patient who showed an allergic anaphylactic reaction to a traditional Chinese medicine (TCM) tea should be tested.

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# II. Manuscript I

Der p 10, a marker for broad sensitizations to house dust mite allergens

# Der p 10, a marker for broad sensitizations to house dust mite allergens

Short title: Characterization of Der p 10

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## Abstract

***Background:*** The mite allergen Der p 10 cross-reacts with tropomyosins in seafood but has not been studied in detail in house dust mite (HDM) allergic patients.

*Objective:* To express and purify a recombinant Der p 10 equivalent to natural Der p 10 and to evaluate it in HDM allergic patients.

***Methods:*** rDer p 10 was expressed in *E. coli*, purified and characterized by mass spectrometry and circular dichroism. It was tested for IgE reactivity in 1322 HDM allergic patients. Detailed IgE-reactivity profiles to six HDM allergens (Der p 1, 2, 5, 7, 10, 21) were established for a subgroup of Der p 10-positive and negative sera. The allergenic activity was evaluated in basophil degranulation experiments.

***Results:*** rDer p 10 is an α-helical protein sharing IgE epitopes with nDer p 10. It is recognized by 15.2% of HDM-allergic patients. The majority of Der p 10-positive patients showed IgE reactivity to several other HDM allergens besides the major allergens (Der p 1, Der p 2) while some showed a rather selective Der p 10 reactivity. Der p 10-negative patients were primarily sensitized to Der p 1 and/or Der p 2. The allergenic activity of Der p 10 was high only in few patients with strong and selective IgE reactivity to Der p 10.

***Conclusion:*** Der p 10 may be a diagnostic marker for HDM allergic patients with additional sensitization to allergens other than Der p 1 and Der p 2 who may require attention when allergen-specific immunotherapy is considered.

**Clinical implications:**

Diagnostic testing with Der p 10 may be used to identify patients who may be not suitable for immunotherapy with most of the currently available HDM-extracts.

**Capsule summary:**

We produced rDer p 10 equivalent to its natural counterpart that may be used as a diagnostic marker allergen to prescribe HDM-specific immunotherapy.

**Key words:**

House dust mite allergy, Der p 10, tropomyosin, recombinant allergens, serological diagnosis, allergenic activity, RBL assay, immunotherapy

**Abbreviations:**

HDM, house dust mite

cDNA, complementary deoxyribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

MALDI-Tof, matrix-assisted laser desorption and ionization-time of flight mass spectrometry

UV CD, ultraviolet circular dichroism

BSA, bovine serum albumin

RBL, rat basophil leukaemia

## Introduction

House dust mites, especially the species *Dermatophagoides pteronyssinus* and *D. farinae*, are a frequent cause of allergy worldwide with around 20% of the population and more than 50% of allergic patients being sensitized to them.[1, 2] House dust mite allergens are among the most common triggers of asthma and allergic rhinitis.[3, 4] The frequency of IgE recognition, allergenic activity and clinical relevance of the major HDM allergens, Der p 1 and Der p 2 has been well investigated.[5-8] Both allergens are recognized by more than 90% of HDM allergic patients and are considered as the major HDM allergens.[5] More than 20 other mite allergens have been identified but their importance for mite allergy has only partly been studied.[9] Among these allergens, Der p 10, a tropomyosin, has received attention because it occurs as cross-reactive allergen in invertebrates[10] and in particular in sea food where it has been described as an important allergen responsible for severe systemic anaphylaxis.[11]

Although the first cDNA coding for a HDM tropomyosin Der f 10 was isolated already in 1995,[12] information regarding the prevalence of recognition and importance of HDM tropomyosins are rare or highly contradictory. In the initial study describing Der f 10 a prevalence of IgE recognition of 80.6% was reported whereas for Der p 10 much lower IgE-binding frequencies (5 – 18%) were described.[5, 13, 14] However, in an African population 55% of the tested mite allergic patients showed IgE reactivity to Der p 10.[15] Since Der p 10 has not been studied well in a large population of HDM allergic patients we first expressed recombinant *D. pteronyssinus* tropomyosin as a biologically active recombinant non-fusion protein in *E. coli* which equals its natural counterpart. We then screened sera from more than 1300 HDM allergic patients for IgE reactivity to rDer p 10 and performed a detailed analysis of

the IgE reactivity profiles to various HDM allergens in subgroups of Der p 10-positive and Der p 10-negative patients. The allergenic activity of rDer p 10 was compared with that of clinically important and highly allergenic HDM allergens, Der p 1 and Der p 2 in basophil degranulation assays. Our results indicate that Der p 10-positive patients differ substantially from Der p 10-negative patients regarding their sensitization profiles to HDM allergens which has implications for specific immunotherapy treatment.

## Materials and methods

### Patients' sera

One thousand three hundred and twenty two sera were collected from persons living in or in the surrounding area of Vienna, Austria who had attended an allergy outdoor clinic. For these persons the diagnosis of house dust mite allergy could be established based on respiratory symptoms (i.e. rhinitis and/or asthma) of allergy at home, positive skin prick test-reactivity to *D. pteronyssinus* and/or presence of mite-specific IgE antibodies as determined by ImmunoCAP (Phadia AB, Uppsala, Sweden) or IgE reactivity to nitrocellulose blotted *D. pteronyssinus* extract.[14] IgE-reactivity to dot-blotted Der p 10 was performed for sera from all 1322 mite allergic patients. An extensive assessment of IgE reactivities to purified HDM allergens nDer p 1, rDer p 2, rDer p 5, rDer p 10 and rDer p 21 was performed for those patients where serum volumes ≥ 200 µl were available (i.e. 39 rDer p 10-positive and 31 Der p 10-negative patients).

### Purified house dust mite allergens

Natural Der p 1 was isolated by affinity chromatography.[16] Recombinant Der p 2 was expressed in *E. coli* with a C-terminal hexa-histidine tag using the pET17b expression system (Novagen, Madison, WI, USA) and purified by Ni-NTA agarose (Qiagen, Hilden, Germany).[17] Recombinant Der p 5, Der p 7 and Der p 21 were expressed as non-fusion proteins in *E. coli* and purified to homogeneity by ion exchange and hydrophobic interaction chromatography.[18, 19]

**Expression in E. coli and purification of recombinant Der p 10**

A cDNA coding for Der p 10 (data base #AF016278) was obtained by reverse transcription from *D. pteronyssinus* RNA by RT-PCR with a Platinum Tag Kit (Invitrogen, Carlsbad, California) and the following primers: forward 5'-CG**GGATCC**CATATGGAGGCCATCAAAAAGAAAATGC-3' and reverse 5'-CG**GGATCC**TTAATAACCAGTAAGTTCGGC-3', containing *BamHI* (bold letters) and *NdeI* (underlined) restriction sites (MWG, Ebersberg, Germany). The PCR product was cut with *BamHI* and *NdeI*, gel-purified and subcloned into the *NdeI* and *BamHI* sites of pET17b vector (Novagen, EMD, Madison, WI, USA). Both DNA strands were sequenced (MWG).

Recombinant Der p 10 was expressed in *E. coli* BL21 (DE3) (Stratagene) as described.[18] After lysis of cells, the protein was found in the supernatant, which was dialyzed against 1 M $(NH_4)_2SO_4$, 50 mM $Na_2HPO_4$ pH 7, 1 mM DTT and loaded onto a Phenyl-Sepharose fast-flow column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Bound proteins were eluted by a 0% to 100% linear gradient to 50 mM $Na_2HPO_4$ pH 7, 1 mM DTT. Fractions containing rDer p 10 were pooled, dialyzed against 10 mM $Na_2HPO_4$ pH 7, 0.25 mM DTT, loaded onto a column prepacked with Bio-Gel HT Hydroxyapatite (BioRad, Hercules, Calif., USA) and eluted by continuously increasing the concentration of $Na_2HPO_4$ to 300 mM. Fractions containing more than 90% pure rDer p 10 were pooled, dialyzed against 5 mM $Na_2HPO_4$ pH 7 and stored at –20°C. The purity of the protein was determined by SDS-PAGE and Coomassie blue staining[20], and its concentration was measured using the Micro BCA Assay Kit (Pierce, Rockford, Ill., USA).

**Matrix-assisted laser desorption and ionization–time of flight (MALDI-Tof) mass spectrometry and CD (Circular dichroism) analysis of rDer p 10**

rDer p 10 was desalted and further purified via reversed phase-HPLC (HP 1050 binary gradient; column, Nucleosil-100 RP-18 150 x 4 mm, 5 µm; solvent A, water, 0.1% FTA; solvent B, acetonitrile, 0.1% FTA; gradient, 10% B to 90% B in 15 min; flow, 1 ml/min; detection, UV at 215 nm; injected volume, 10 µl). Laser desorption mass spectra were acquired in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.; piCHEM, Research and Development, Graz, Austria). rDer p 10 was dissolved in 10% acetonitrile, 0.1% trifluoroacetic acid and α-cyano-4-hydroxycinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Far UV CD spectra of rDer p 10 were collected on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 1mm path length quartz cuvette at a protein concentration of 0.26 mg/ml. Spectra were measured from 260 to 180 nm, with 0.5 nm resolution at a scanning speed of 50 nm/min and resulted from averaging of three scans. All measurements were performed in 10 mM $Na_2HPO_4$ pH 7. For the temperature scan, spectra of rDer p 10 were recorded by gradually increasing the temperature (5°C steps) from 25°C to 95°C with a heat rate of 1°C/min, and after cooling down to 25°C again. The final spectra were baseline corrected by subtracting the corresponding buffer spectrum. Results were expressed as the mean residue ellipticity [θ] at a given wavelength. The secondary structure content of rDer p 10 was calculated using the secondary structure estimation program CDSSTR.[21]

**Preparation of an aqueous whole body mite extract**

An aliquot of 0.3 g of purified *Dermatophagoides pteronyssinus* whole bodies (Allergon, Vällinge, Sweden) was homogenized in 5 ml $H_2O$ containing 1 mM benzamidine and 1 mM PMSF using an Ultra-Turrax T25 Basic disperser (IKA, Staufen, Germany). The homogenate was extracted for 2 h at 4°C and the unsoluble fraction was removed by centrifugation (20 min, 3220 g, 4°C). The protein concentration was determined in the extract with the Micro BCA Assay Kit (Pierce). Aliquots were stored at -20°C.

**IgE immunoblot inhibition assays**

For IgE inhibition experiments, 0.4 mg/cm of the aqueous *D. pteronyssinus* extract or 1.5 µg/cm of rDer p 10 were separated by 14% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Sera from mite allergic patients with IgE reactivity to Der p 10 were diluted 1:10 in gold buffer [50 mM sodium phosphate pH 7.4 0.5% (v/v) Tween-20, 0.5% (w/v) BSA and 0.05% (w/v) sodium azide] and pre-incubated with 350 µg/ml of the aqueous *D. pteronyssinus* extract or 20 µg/ml rDer p 10 o/n at 4°C. The pre-incubated serum samples or, for control purposes, the untreated serum (diluted 1:10) were then exposed to the nitrocellulose-strips o/n at 4°C and bound IgE antibodies were detected with $^{125}$I-labeled anti-human IgE antibodies (Demeditec Diagnostics, Kiel, Germany) and visualized by autoradiography (Kodak XOMAT film).[22]

**IgE reactivity to purified allergens in a non-denaturating RAST-based dot blot assay**

Two µl aliquots of natural and recombinant house dust mite allergens (nDer p 1, rDer p 2, rDer p 5, rDer p 7, rDer p 10 and rDer p 21)[16-19] as well as BSA as control

protein (each 0.25 µg/µl) were dotted onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with gold buffer, twice for 5 min and once for 30 min, and incubated with mite allergic patients' sera diluted 1:10 dilution in gold buffer o/n at 4°C. Bound IgE antibodies were detected as described for the inhibition experiments and visualized by autoradiography. A quantification of dot blot signals was achieved with the NIH ImageJ software (version 1.42; http://rsb.info.nih.gov/ij/). The scanned film was analysed after conversion into an 8-bit greyscale image, background correction and inversion of the image. Thus, pixel values ranged from 0 (black) to 255 (white). After outlining the individual dots using the elliptical tool, the integrated density was calculated as a product of “area” value (area of selection in square pixels) and "mean grey value" (sum of the grey values of all the pixels in the selection divided by the number of pixels). An example of the dot’s integrated density is shown for patient A39 (see Fig. E1 in the Online Repository at www.jacionline.org). Three independent measurements were done and averaged for each dot. The individual dots were grouped into three categories (weak, middle and strong) according to their “integrated density” values and marked white, grey and black in Table E1 (see Online Repository at www.jacionline.org) for better visualization.

**Rat basophil leukemia cell mediator release assay**

The RBL (rat basophil leukaemia) cell-release assay was performed as described previously by Schulmeister *et al.*[23] Briefly, human FcεRI-transfected RBL cells (clone RBL-703/21)[24] were sensitized with mite allergic patients' serum IgE in a final dilution of 1:30 o/n at 37°C. Degranulation of the cells was induced by adding nDer p 1, rDer p 2, rDer p 10 (100 ng/ml, 10 ng/ml, 1 ng/ml and 0.1 ng/ml) or buffer as control and assessed by measuring the β-hexosaminidase release. Results are

reported as percentage of total β-hexosaminidase release, where values obtained with buffer instead of allergen were subtracted.

**Statistical analysis**

Statistical significant differences of the allergen-specific IgE-reactivity frequencies in the Der p 10-positive and negative patients were analyzed using the multiple chi-square testing. The Mann-Whitney *U* test was applied to determine statistically significant differences regarding the induction of basophil degranulation with the different allergens. The p-values were adjusted using the Bonferroni-Holm correction. Differences were considered statistically significant if the p-value was lower than 0.05.

## Results

### Expression and purification of folded rDer p 10

The cDNA coding for Der p 10, which was obtained by RT-PCR from *D. pteronyssinus* RNA, was completely identical to the earlier described nucleotide sequence of Der p 10 (AF016278). The cDNA coding for the Der p 10 protein was subcloned into expression plasmid pET17b and was expressed as a soluble non-fusion protein in *E. coli* BL21 (DE3). Between 3 - 7 mg of recombinant protein/liter of bacterial culture was purified by hydrophobic interaction and ion exchange chromatography to homogeneity (Fig. 1, *A*). In SDS-PAGE (Fig. 1, *A*) rDer p 10 migrated at a molecular mass (i.e. 36 kDa) which was greater than that calculated from the amino acid sequence (32.972 kDa). However, MALDI-Tof mass spectrometry confirmed the calculated mass (32.939 kDa) (Fig. 1, *B*). The far UV-CD spectrum of purified recombinant Der p 10 recorded at 25°C showed two minima at 209 and 221.5 nm and a large maximum at 191 nm, which is characteristic for a protein with predominantly α-helical structure (Fig. 2, *A*). Calculation of the secondary structure using the program CDSSTR with the reference data set 7 resulted in 92% α-helix, 2% β-sheets, 4% turns and 2% random-coils with an excellent fit between the calculated and measured spectrum (normalized root mean square difference value = 0.004). A melting temperature, defined as midpoint of transition, of 44°C was determined at 222 nm (data not shown). Total unfolding of rDer p 10 occurred at 50°C. However, even after heating to 95°C, the protein regained almost completely its fold after cooling to 25°C (Fig. 2, *B*).

**rDer p 10 is immunologically equivalent to nDer p 10 and reacts with IgE from 15% of house dust mite allergic patients**

To investigate whether rDer p 10 contains the IgE-epitopes of the natural Der p 10, IgE inhibition experiments were performed. As exemplified in Fig. 3, preincubation of HDM allergic patients' sera with rDer p 10 completely inhibited IgE binding to nDer p 10, as well as to rDer p 10 but not to other HDM allergens (Fig. 3).

Der p 10 was then used to search for specific IgE antibodies in 1322 sera from *D. pteronyssinus* allergic patients. These patients were typical HDM-allergic suffering from respiratory symptoms to HDM. We found that 201 (15.2%) of the patients had specific IgE antibodies to rDer p 10 (data not shown).

**rDer p 10-reactive patients are more often sensitized to HDM allergens other than Der p 1 and Der p 2**

A detailed analysis of the IgE reactivity profiles to other HDM allergens was performed in 39 of the 201 Der p 10-positive sera where sufficient serum was available. For comparison, 31 sera from the Der p 10-negative patients were randomly picked and analysed. Fig. 4 shows the frequency of IgE reactivity to nDer p 1, rDer p 2, rDer p 5, rDer p 7 and rDer p 21 in the two groups. Table E1 displays a comparison of the intensities of IgE reactivity to the individual HDM allergens obtained by screening of the autoradiographies of the dot blots.

Comparing the frequencies of IgE reactivity to the indicated HDM allergens in the Der p 10-positive and negative group, we observed striking differences regarding the reactivity profiles. More than 50% of the Der p 10-positive sera reacted with at least 5 of the tested HDM allergens (Fig. 4, Table E1). Twelve patients showed IgE reactivity to all 6 tested HDM allergens (30.8%) and nine reacted with 5 allergens (23.1%). The frequencies of IgE reactivities of rDer p 5 ($p = 0.002$), rDer p 7 ($p =$

0.024) and rDer p 21 (p = 0.005) were approximately 4 fold higher in the Der p 10 positive compared to the Der p 10 negative patients (Fig. 4). By contrast, the majority of the Der p 10 negative sera (77.5 %) were characterized by a sensitization to 1 - 2 HDM allergens (Table E1). Interestingly, five patients showed IgE reactivity only to Der p 10 but to none of the other HDM allergens (Table 1).

**Der p 10 has low allergenic activity in the majority of sensitized patients but can induce strong basophil degranulation in certain patients**

In order to study the allergenic activity of recombinant Der p 10, mediator release assays with an RBL cell line that had been transfected with the human FcεRI receptor, were performed. RBL cells, loaded with serum IgE from the 39 Der p 10 positive patients, were exposed to different concentrations of nDer p 1, rDer p 2 and rDer p 10. The mediator release at the allergen concentration (i.e. 100 ng/ml) giving the strongest basophil degranulation, is displayed in Fig. 5 and suppl. Table 1. In the majority of the patients, rDer p 10 induced weak or no basophil degranulation. Relevant degranulation upon exposure with rDer p 10 was however obtained with 4 sera which were derived from patients who had also strong IgE reactivity to Der p 10 (Fig. 5, suppl. Table 1). A relevant mediator release was observed for 22 (73.3%) and 20 (64.5%) sera with nDer p 1 and rDer p 2, respectively, indicating that these allergens have much higher allergenic activity than Der p 10.

## Discussion

Tropomyosins are important food allergens which can cause severe anaphylactic reactions in sensitized patients.[11] They show extensive cross-reactivity with mite tropomyosins but their relevance in house dust mite allergy has not been studied in detail. In order to investigate the importance of mite tropomyosin in HDM allergy, we expressed and purified rDer p 10, the tropomyosin from the mite *D. pteronyssinus* as authentic and folded protein which equals natural Der p 10 in terms of IgE reactivity. The reported prevalences of IgE recognition of mite tropomyosins in HDM patients showed a large variability from 5-80%.[12, 13] In a population of more than 1300 HDM allergic patients we found that Der p 10 is recognized by 15.2% patients.

When we performed an in depth analysis of sera from Der p 10-positive and negative patients regarding IgE reactivity to 5 other HDM allergens (nDer p 1, rDer p 2, rDer p 5, rDer p 7 and rDer p 21) striking differences of IgE reactivity profiles between these two groups were noted. The majority of the Der p 10-positive patients was polysensitized to most of the tested allergens and certain patients showed strong and exclusive IgE reactivity to Der p 10. By contrast, the majority of Der p 10-negative patients reacted only with Der p 1 and/or Der p 2 but not with the other HDM allergens. Our finding may have important clinical implications for HDM-specific immunotherapy. In fact, at present HDM extracts are only tested for the presence of Der p 1 and Der p 2 so that no information can be given by allergen extract manufacturers regarding the contents of allergens other than Der p 1 and Der p 2 in their extracts.[25] Furthermore, it has been demonstrated that allergens other than Der p 1 and Der p 2 are missing in most of the commercially available allergen extracts.[26] (Casset & Vrtala, unpublished data). Based on our results it is very likely that patients

from the Der p 10-positive group who are also sensitized against clinically highly relevant allergens such as Der p 5, Der p 7, Der p 21 may not benefit from HDM-specific immunotherapy as well as patients who are primarily sensitized to Der p 1 and Der p 2.

The clinical relevance of Der p 10 among HDM allergic patients seems to be rather low compared to patients with food allergy to tropomyosin because only relatively few HDM allergic patients were sensitized to Der p 10, their IgE reactivities were rather weak and also the allergenic activity of Der p 10 was very low compared to that of Der p 1 and Der p 2. However, for a few patients Der p 10 appeared to be the only and most important HDM allergen which also exhibited high allergenic activity in the basophil degranulation assay.

One possible explanation for the low frequency of sensitization, the low IgE reactivity and allergenic activity of Der p 10 may be that the allergen is not well represented in the respirable mite feces compared to other HDM allergens (e.g., Der p 1, Der p 2, Der p 5, Der p 7 and Der p 21)[27, 28] but only occurs in the possibly less immunogenic dead mite body particles.[29]

In conclusion, Der p 10 seems to be relevant only for a few HDM allergic patients. However, it allows identification of a subset of HDM allergic patients with a broad sensitization to HDM allergens other than Der p 1 and Der p 2 which may have important implications for the prescription of HDM-specific immunotherapy.

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## Figure legends

**Figure 1.** Biochemical characterization of rDer p 10. (A) Coomassie Blue-stained SDS-PAGE. Molecular weight marker (lane M), 3 µg of purified rDer p 10 (lane rDer p 10). (B) MALDI-Tof analysis of rDer p 10. The *x*-axis shows the mass/charge ratio. Signal intensities as percentage of the most abundant signal (*y*-axis).

**Figure 2.** CD analysis of rDer p 10. The molecular ellipticities [θ] (*y*-axes) at different wavelengths (*x*-axes) are displayed for different temperatures (A), (B) CD spectra of the protein at 25°C and refolding after heating to 95°C and cooling down to 25°C.

**Figure 3.** Inhibition of IgE reactivity to blotted nDer p 10 by rDer p 10. Nitrocellulose-blotted *D. pteronyssinus* extract (left panels) or rDer p 10 (right panels) were incubated with sera from Der p 10-sensitized patients (A12 and A30), which had been pre-incubated with (+) or without (-) rDer p 10. Molecular weights (kDa) were shown at the margins.

**Figure 4.** Frequencies (% of reactive sera) of IgE recognition (*y*-axis) of HDM allergens (*x*-axis) are shown for HDM allergic patients with (grey bars) or without (white bars) IgE antibodies to rDer p 10. Statistically significant differences between the two groups are indicated (* $p < 0.05$).

**Figure 5.** Allergenic activity of nDer p 1, rDer p 2 and rDer p 10. RBL cells were loaded with IgE from Der p 10-sensitized patients and degranulation was induced with nDer p 1, rDer p 2 or rDer p 10 (x-axis). The intensities of basophil degranulation are shown as percentages of total β-hexosaminidase release (y-axis) as a scatter

plot. Median mediator release values and the cutoff (5% mediator release) are indicated by a horizontal black and dashed line, respectively. Statistically significant differences are indicated (* $p < 0.05$).

**Supplemental Figure E1.** Quantification of the IgE reactivity intensities to the individual HDM allergens using the image analysis software Image J exemplified on patient A39. (A) A grey-scaled, background-subtracted and inverted image of the autoradiography film was prepared before the individual dots were outlined (see yellow circles). (B) “Area” value (area of selection in square pixels) and “mean grey value” (sum of the grey values of all the pixels in the selection divided by the number of pixels) were measured by the program and multiplied resulting in the “integrated density” value (IntDen).

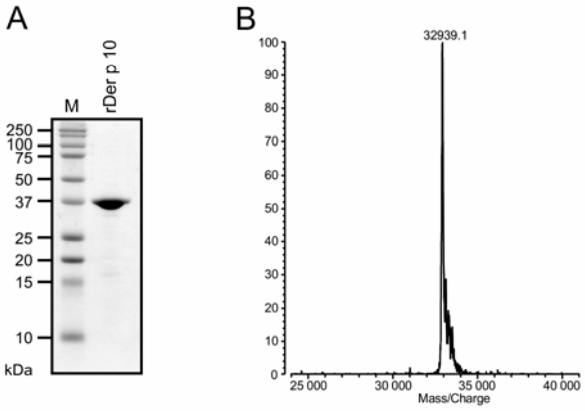

A
M
rDer p 10
250
100
75
50
37
25
20
15
10
kDa
B
32939.1
100
90
80
70
60
50
40
30
20
10
0
25 000
30 000
35 000
40 000
Mass/Charge

Figure 1

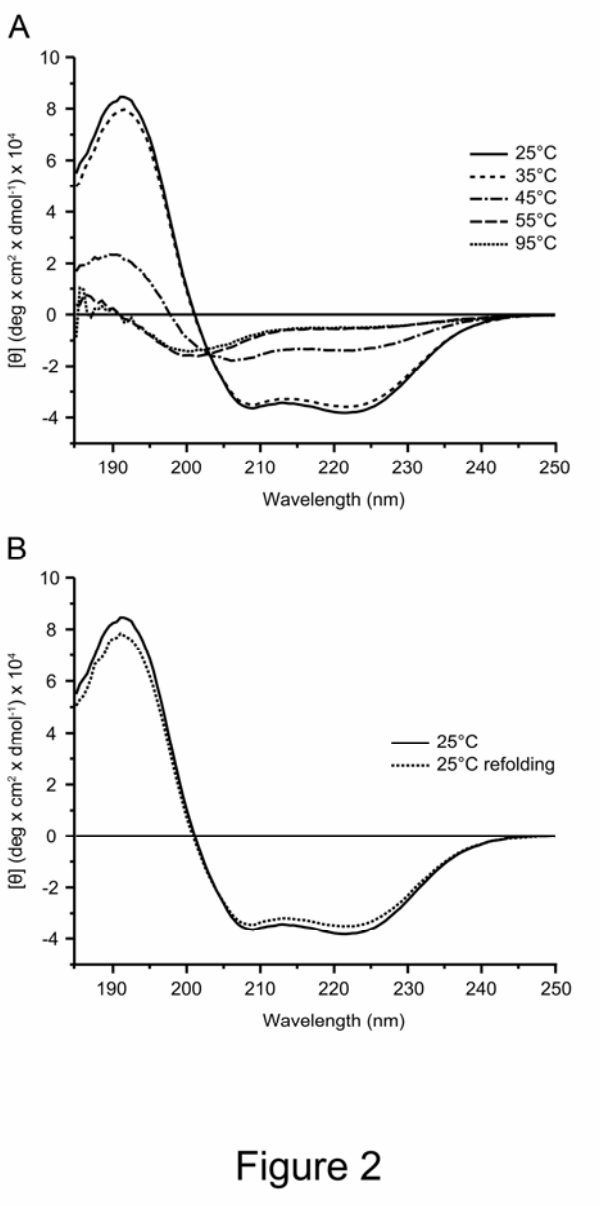

A
[θ] (deg x cm² x dmol⁻¹) x 10⁴
Wavelength (nm)
25°C
35°C
45°C
55°C
95°C
B
[θ] (deg x cm² x dmol⁻¹) x 10⁴
Wavelength (nm)
25°C
25°C refolding

Figure 2

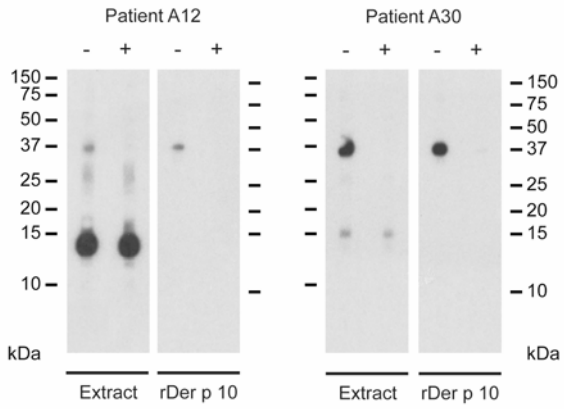

Patient A12
Patient A30
- + - +
- + - +
150
75
50
37
25
20
15
10
kDa
Extract
rDer p 10
Extract
rDer p 10
kDa

Figure 3

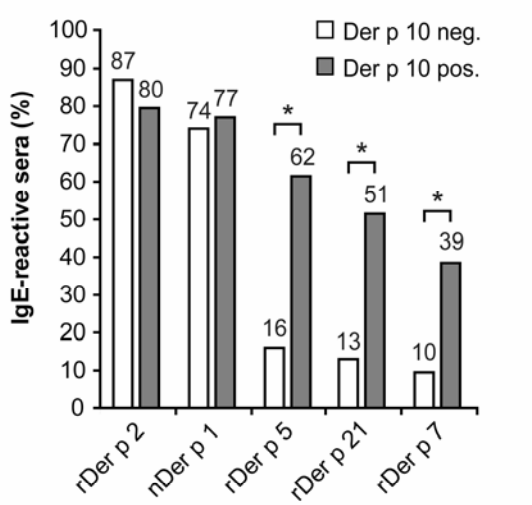

Der p 10 neg.
Der p 10 pos.
IgE-reactive sera (%)
100
90
80
70
60
50
40
30
20
10
0
87
80
74
77
16
62
13
51
10
39
*
*
*
rDer p 2
nDer p 1
rDer p 5
rDer p 21
rDer p 7

Figure 4

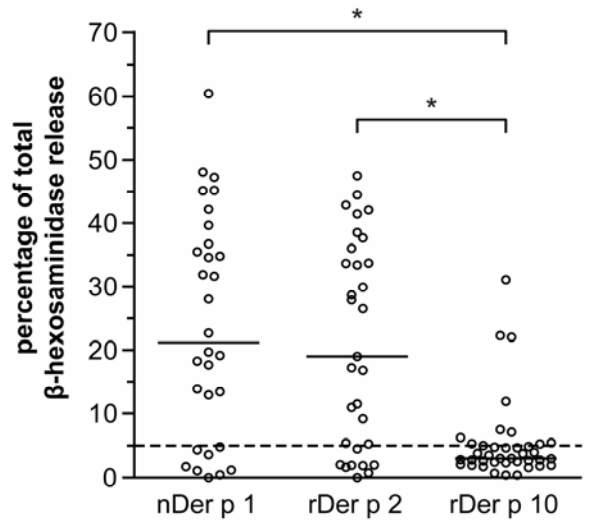
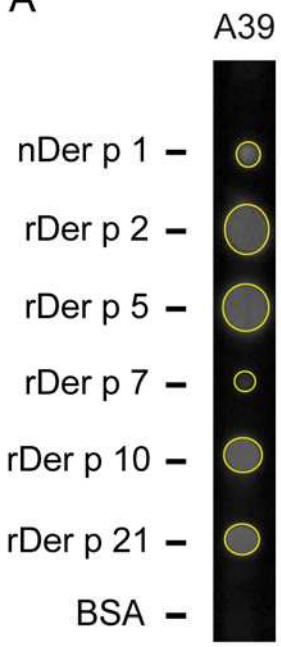

percentage of total
β-hexosaminidase release
70
60
50
40
30
20
10
0
*
*
nDer p 1
rDer p 2
rDer p 10

Figure 5

A




B

| | Area | Mean | IntDen |
|---|---|---|---|
| **Der p 1** | 4779 | 74.834 | 357633 |
| **Der p 2** | 15552 | 81.648 | 1269782 |
| **Der p 5** | 14924 | 85.977 | 1283124 |
| **Der p 7** | 1792 | 44.349 | 79473 |
| **Der p 10** | 9152 | 88.017 | 805534 |
| **Der p 21** | 8641 | 82.887 | 716223 |

Figure E1

**Table E1.** Allergen specific IgE reactivity and basophil degranulation measured for sera from Der p 10-positive and negative HDM allergic patients

**Der p 10-positive sera**

| | IgE reactivity | | | | | | Basophil degranulation | | |
|---|---|---|---|---|---|---|---|---|---|
| | Integrated density (x $10^4$) | | | | | | Percentage of total β-hexosaminidase release | | |
| Patient | nDer p 1 | rDer p 2 | rDer p 5 | rDer p 7 | rDer p 10 | rDer p 21 | nDer p 1 | rDer p 2 | rDer p 10 |
| A1 | 26.88 | 38.12 | 32.74 | - | 0.93 | 38.37 | 0.46 | 5.27 | 2.12 |
| A2 | 20.49 | 87.13 | - | - | 43.52 | - | 12.94 | 5.42 | 2.46 |
| A3 | 3.53 | 55.61 | 85.44 | 29.21 | 13.75 | - | 4.83 | 9.17 | 2.67 |
| A4 | 2.02 | 49.94 | - | - | 75.58 | - | 0.00 | 4.50 | 5.31 |
| A5 | 53.32 | 106.55 | 121.13 | 50.44 | 1.16 | 89.29 | 19.13 | 17.21 | 0.71 |
| A6 | 24.61 | 118.27 | 147.50 | 77.63 | 1.52 | 137.09 | 36.73 | 28.67 | 4.66 |
| A7 | 6.44 | 95.26 | 98.91 | - | 2.10 | - | 17.68 | 19.00 | 2.55 |
| A8 | - | 1.61 | - | - | 14.37 | - | - | 0.78 | 2.81 |
| A9 | 2.05 | 82.39 | 147.08 | - | 1.03 | 1.09 | 31.88 | 33.66 | 3.00 |
| A10 | - | - | 111.59 | 13.37 | 1.27 | 59.33 | - | - | 1.73 |
| A11 | 59.02 | 139.14 | 3.47 | - | 0.82 | 70.80 | 45.13 | 42.86 | 2.78 |
| A12 | 1.29 | 37.64 | 24.81 | 22.64 | 5.41 | 1.67 | 1.74 | 10.93 | 3.85 |
| A13 | - | - | - | - | 98.74 | - | - | - | 22.03 |
| A14 | 15.48 | 39.95 | 86.25 | 20.30 | 57.43 | 2.13 | 13.41 | 16.84 | 7.47 |
| A15 | 48.43 | 124.23 | - | - | 1.00 | - | 45.19 | 47.49 | 3.54 |
| A16 | 43.06 | 102.55 | 69.23 | 27.53 | 34.90 | 1.15 | 60.44 | 42.05 | 3.80 |
| A17 | 35.40 | 109.79 | 72.35 | 39.38 | 1.34 | 1.15 | 35.50 | 38.52 | 4.85 |
| A18 | 12.02 | 79.89 | 68.91 | 61.67 | 1.33 | 56.68 | 4.39 | 26.51 | 4.68 |
| A19 | 2.14 | 86.38 | 114.72 | - | 1.24 | 2.09 | 28.04 | 44.49 | 4.75 |
| A20 | 18.24 | 97.29 | 84.69 | 105.90 | 6.65 | - | 47.25 | 41.42 | 6.25 |
| A21 | 30.62 | 83.58 | 21.13 | - | 1.55 | 20.84 | 31.66 | 33.41 | 5.27 |
| A22 | 23.66 | - | - | - | 22.05 | - | 34.82 | - | 5.02 |
| A23 | 25.34 | 77.88 | 107.98 | 35.52 | 1.34 | 105.71 | 19.68 | 27.88 | 5.48 |
| A24 | 34.25 | 12.33 | 4.73 | - | 185.50 | - | 34.62 | 1.88 | 31.10 |
| A25 | 44.06 | 93.34 | 62.71 | 42.89 | 1.26 | 12.37 | 48.10 | 37.67 | 2.86 |
| A26 | - | - | - | - | 1.20 | - | - | - | 3.05 |
| A27 | 23.73 | 101.69 | 86.08 | 41.17 | 1.94 | 119.62 | 22.66 | 33.71 | 1.60 |
| A28 | - | 1.06 | - | - | 38.31 | - | - | 0.00 | 2.65 |
| A29 | 1.98 | - | - | - | 56.64 | - | 1.23 | - | 7.10 |
| A30 | - | - | - | - | 41.67 | - | - | - | 2.36 |
| A31 | 24.18 | 137.17 | - | - | 27.42 | - | 39.62 | 36.04 | 3.99 |
| A32 | 10.22 | 92.03 | 70.37 | 1.06 | 37.88 | 6.14 | 13.87 | 1.67 | 1.95 |
| A33 | 2.99 | 72.09 | 90.96 | - | 34.02 | 49.48 | 3.61 | 1.95 | 1.95 |
| A34 | 0.92 | 29.48 | - | - | 1.08 | - | 1.13 | 2.00 | 3.03 |
| A35 | - | 36.32 | - | - | 4.25 | - | - | 2.03 | 0.43 |
| A36 | - | - | - | - | 15.45 | - | - | - | 1.85 |
| A37 | - | - | - | - | 172.09 | - | - | - | 22.25 |
| A38 | 33.43 | 99.57 | 60.15 | - | 1.15 | 66.85 | 18.24 | 11.50 | 0.44 |
| A39 | 32.28 | 127.55 | 132.97 | 10.78 | 84.01 | 73.55 | 42.18 | 29.92 | 11.87 |

**Der p 10-negative sera**

| Patient | nDer p 1 | rDer p 2 | rDer p 5 | rDer p 7 | rDer p 10 | rDer p 21 |
|---|---|---|---|---|---|---|
| B1 | 13.52 | 34.87 | - | - | - | - |
| B2 | 35.49 | 103.83 | - | - | - | - |
| B3 | - | 4.37 | - | - | - | - |
| B4 | 64.14 | 90.53 | - | 55.40 | - | - |
| B5 | 3.16 | 19.63 | - | - | - | - |
| B6 | - | 59.10 | - | - | - | - |
| B7 | 1.24 | 28.23 | | - | - | - |
| B8 | 10.53 | 36.84 | 0.90 | - | - | - |
| B9 | 17.14 | 45.25 | - | - | - | - |
| B10 | 5.90 | 17.78 | - | 1.73 | - | - |
| B11 | 2.95 | - | - | - | - | - |
| B12 | - | 29.06 | - | - | - | - |
| B13 | 1.86 | 19.01 | - | - | - | - |
| B14 | 4.29 | - | - | - | - | - |
| B15 | - | 54.86 | - | - | - | - |
| B16 | 1.06 | - | - | - | - | 16.35 |
| B17 | 6.20 | 74.17 | 55.33 | - | - | - |
| B18 | 7.90 | 47.55 | - | - | - | - |
| B19 | 21.81 | 52.59 | - | - | - | - |
| B20 | 37.48 | 46.58 | - | - | - | - |
| B21 | 21.72 | 35.13 | 85.03 | 7.80 | - | 44.97 |
| B22 | 28.90 | 38.51 | - | - | - | - |
| B23 | - | 37.34 | - | - | - | - |
| B24 | 42.95 | 76.78 | - | - | - | - |
| B25 | 1.39 | 33.17 | - | - | - | - |
| B26 | - | 13.78 | 0.75 | - | - | 15.52 |
| B27 | - | 3.28 | - | - | - | - |
| B28 | 2.32 | - | - | - | - | - |
| B29 | - | 19.55 | - | - | - | - |
| B30 | 3.43 | 3.86 | - | - | - | - |
| B31 | 41.59 | 53.93 | 106.56 | - | - | 57.90 |

| Integrated density x $10^4$ |
|---|
| 0 - 15 |
| 15.01 - 60 |
| 60.01-185.50 |

| percentage of total β-hexos-aminidase release |
|---|
| 0 - 5 |
| 5.01 - 10 |
| 10.01 - 60.44 |

# III. Manuscript 2

Anaphylaktische Reaktion auf einen TCM-Tee

(Allergologie 2009; 32:146-151)

**Originalarbeit**
**Original paper**

*Allergologie, Jahrgang 32, Nr. ■/2009, S. ■–■*

# Anaphylaktische Reaktion auf einen TCM-Tee

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**Schlüsselwörter**
■■■

**Key words**
■■■

**Anaphylaktische Reaktion auf einen TCM-Tee**

Hintergrund: Tee gilt als unbedenkliches Genuss- und Heilmittel. Tatsächlich wurden Unverträglichkeitsreaktionen bis hin zum anaphylaktischen Schock auf Kamillen- oder Grünen Tee beschrieben. Fallbericht: Wir berichten über eine 55-jährige Patientin, die eine anaphylaktische Reaktion auf einen TCM- (traditionelle chinesische Medizin) Tee entwickelte. Methodik: Es wurde ein Routine-Pricktest auf inhalative Allergene durchgeführt. Der Tee sowie seine Einzelkomponenten wurden im Pricktest getestet. Die Tryptase, das Gesamt-IgE und das spezifische IgE wurden bestimmt. Darüber hinaus wurde eine In-vitro-Inhibition und ein Immunblot durchgeführt. Ergebnisse: Der Pricktest zeigte mittelgradige Reaktionen auf diverse Vorratsmilben sowie eine stark positive Reaktion auf den TCM-Tee. Die Einzelkomponenten des Tees waren negativ. Das spezifische IgE auf Vorratsmilben, Beifuß- und Ragweedpollen war negativ, die Tryptase und das Gesamt-IgE waren unauffällig. Ein Immunblot mit dem TCM-Tee war positiv, aber negativ auf Vorratsmilben, so dass eine zuerst angenommene Kontamination des TCM-Präparats mit Vorratsmilben ausgeschlossen werden konnte. Eine neuerliche Anamnese ergab eine 20 Jahre zurückliegende Diagnose einer Seidenallergie, die mittels IgE-Bestimmung bestätigt werden konnte (Wildseide 5,1 kU/l). In der In-vitro-Inhibition konnte die IgE-Bindung an Wildseide mit dem TCM-Tee zu 87% inhibiert werden. Schlussfolgerungen: Es schien wahrscheinlich, dass eines der Ausgangsprodukte des TCM-Teegranulats von Lebensmittelmotten oder anderen Schmetterlingen befallen und dadurch mit Seidenproteinen aus deren Puppenkokons kontaminiert war. Die anaphylaktische Reaktion auf den TCM-Tee war daher auf die Verunreinigung des Produkts mit Seidenallergenen bei vorbestehender Seidensensibilisierung der Patientin zurückzuführen.

**Anaphylactic reaction to TCM tea**

Background: Tea is considered a healthy and harmless beverage. However, adverse reactions to several tea species, like chamomile or green tea, have been reported. Case report: We report a 55-year-old woman, who developed palmar and plantar itching, dyspnea, angioedema and dysphagia 10 min after drinking a tea prepared from a TCM granulate. Methods: Skin prick tests with common inhalant allergens, as well as with the tea and its individual components were performed. Total IgE, specific IgE and tryptase were measured. In addition, immunoblotting and CAP inhibition tests were done. Results: Skin prick tests showed moderately positive reactions to various storage mites and a strongly positive reaction to the TCM tea. Subsequent testing with all individual tea components was negative. Specific IgE to storage mites, mugwort and ragweed pollen was negative. Total IgE and tryptase were within normal limits. The immunoblot showed positive reactions to proteins between 15 and > 100 kDa in the TCM tea, but was negative to extracts from Tyrophagus, Lepidoglyphus, Acarus and Blomia, excluding contamination of the granulate with storage mite antigens. A recheck of the patients history revealed a former allergy to silk diagnosed 20 years ago. Subsequent assessment of specific IgE to wild silk by CAP was positive (5.1 kU/l). In a CAP inhibition assay, IgE binding to wild silk was inhibited by the TCM tea by 87%. Conclusion: In the present case, the anaphylactic reaction resulted from the presence of silk allergens in the TCM tea granulate, presumably due to infection of one of its raw materials by insect pests like Plodia interpunctella, Ephestia kuehniella or other related moth species and subsequent contamination of the plant material by silk proteins from pupal cocoons.

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## Einleitung

Tee stellt eines der beliebtesten und am häufigsten konsumierten Heil- und Genussmittel dar. Als reines Naturprodukt gilt Tee

Tab. 1. Zusammensetzung des TCM-Produkts, **gerundet.*

| Chinesischer Name | Lateinischer Name | Deutscher Name | Genützter Pflanzenteil | *Teile im Granulat (%)** |
|---|---|---|---|---|
| SUAN ZAO REN | Ziziphus spinosa, S. | Stacheljujube, Chin. Dattel | Samen | 9 |
| YE JIAO TENG | Polygonum multiflorum, Caulis | Vielblütiger Knöterich | Zweige | 18 |
| MAI MEN DONG | Ophiopogon, Radix | Schlangenbart | Wurzel | 9 |
| CHAI HU | Bupleurum, Radix | Hasenohr | Wurzel | 9 |
| BAI SHAO | Paeonia alba, Radix | Pfingstrose, weiß | Wurzel | 9 |
| HUANG QIN | Scutellaria, Radix | Sumpfhelmkraut (S. galariculata) | Wurzel | 9 |
| ZHI MU | Anemarrhena, Rhizoma | Muttergedenken | Rhizom | 6 |
| YU JIN | Curcuma, Tuber | Kurkuma | Wurzel | 9 |
| SHI CHANG PU | Acorus, Rhizoma | Kalmus | Wurzel | 9 |
| ZHI GAN CAO | Glycyrrhiza, Radix praep. | Süßholz | Wurzel | 6 |
| DA ZAO | Zizyphus jujuba, Fructus | Chin. Datteln | Früchte | 6 |

als harmlos und unbedenklich. In der traditionellen chinesischen Medizin (TCM) nimmt Tee einen besonderen Stellenwert ein. Er wird als wichtiges therapeutisches Mittel eingesetzt und ist Bestandteil der chinesischen Heilkräutertherapie, die auf der Verwendung zahlreicher Pflanzen, Mineralien und Tierprodukte basiert.

Es sind jedoch auch Überempfindlichkeitsreaktionen auf Tee bekannt, so beispielsweise auf Kamillen- oder Grünen Tee. Die Reaktionen reichen von Kontaktallergien über Asthma bronchiale bis hin zur Anaphylaxie [5, 10, 12]. Wir berichten über einen Fall einer anaphylaktischen Reaktion nach der Einnahme eines TCM-Tees.

## Kasuistik

Eine 55-jährige Patientin wurde in unserem Allergiezentrum vorstellig, nachdem sie eine anaphylaktische Reaktion auf ein ihr gegen Menopausebeschwerden empfohlenes chinesisches Kräutergranulat entwickelte. Anamnestisch bestanden bis zu diesem Zeitpunkt keine inhalativen Beschwerden oder Nahrungsmittelunverträglichkeiten.

Das Kräutergranulat "Schlaf" bestand aus 11 Einzelkomponenten (Tab. 1) und wurde in einer spezialisierten TCM-Apotheke frisch zusammengemischt. 2 Teelöffel des Granulats, gelöst in 250 ml heißen Wassers, sollten jeden Abend eingenommen werden. Die erste Packung wurde gut vertragen, die Patientin zeigte während der ersten 3 Wochen der Therapie keine Beschwerden.

Nach einer 2-wöchigen urlaubsbedingten Pause begann die Patientin mit der Zubereitung des TCM-Tees aus einer neuen Packung. 10 Minuten nach erstmaliger Einnahme entwickelte sie Juckreiz an Hand- und Fußflächen sowie am Abdomen und im Genitalbereich, Atemnot, oropharyngealen Juckreiz und ausgeprägte Schluckbeschwerden. Zusätzlich bestanden ein Angioödem der Lippen, Husten, Magenschmerzen und Angst. Die Patientin erhielt ein Antihistaminikum sowie Glukokortikoide und wurde vorübergehend stationär aufgenommen.

## Methodik

Im Routine-Pricktest wurden inhalative Allergene (Erle, Birke, Esche, Gräser, Wegerich, Beifuß, Ragweed, Alternaria, Cladosporium, Penicillinum, Dermatophagoides pteronyssinus, D. farinae, Lepidoglyphus, Tyrophagus, Acarus, Katze, Hund, Pferd und Latex) mittels kommerziell erhältlichen Testlösungen getestet (ALK-Abelló). Zusätzlich erfolgte ein Pricktest mit der Originalcharge des TCM-Tees in der empfohlenen Anwendungskonzentration (2 TL/250 ml) sowie mit Verdünnungen von 1 : 10 und 1 : 100. Außerdem wurden die von der zuständigen TCM-Apotheke zur Verfügung gestellten Einzelkomponenten, die jeweils aus neuen Produktchargen stammten, in den jeweils im fertigen Tee vorliegenden Konzentrationen sowie in 10fach höherer Konzentration getestet. Als positiv wurde ein Quaddeldurchmesser von mindestens 3 mm gewertet.

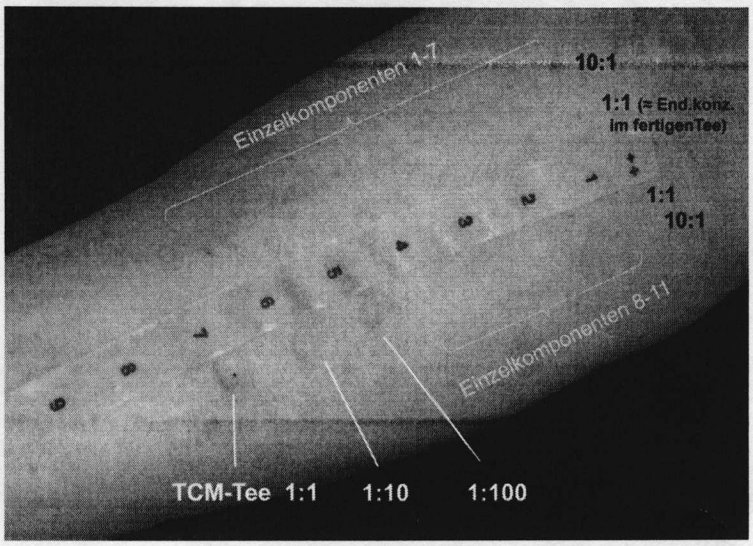


Abb. 1. Pricktest mit dem TCM-Tee und den Einzelsubstanzen.

Gesamt-IgE, spezifisches IgE und Tryptase wurden mittels UniCAP® (Phadia, Schweden) bestimmt. Darüber hinaus erfolgten ein Immunblot und ein CAP-Inhibitionstest.

## CAP-Inhibition

Für die CAP-Inhibition wurden 100 µl des Patientenserums mit 25 µl des TCM-Tees (as is) oder mit 25 µl PBS (Positivkontrolle) für 3 h bei 20 °C vorinkubiert und anschließend im CAP auf Wildseide (k73) getestet.

## Immunblot

Das Patientenserum wurde im Immunblot auf verschiedene Milbenextrakte (D. pteronyssinus, Blomia tropicalis, Lepidoglyphus destructor, Glycyphagus domesticus, Tyrophagus putrescentiae) und den TCM-Tee getestet. Zur Herstellung der Milbenextrakte wurden je 0,3 g Milben mit 5 ml SDS-Puffer (62 mM Tris, 200 mM SDS, 10% (v/v) Glyzerin, 5% (v/v) β-Mercaptoethanol, 2,5% (v/v) Bromphenolblau gelöst in 1% Methanol, pH 6,8) versetzt. Zum Aufbrechen der Milben wurden die Milbensuspensionen mit einem Ultra-Turrax T25 Basic disperser (IKA, Staufen, Deutschland) behandelt und 10 Minuten bei 95 °C inkubiert. Der TCM-Tee wurde as is ohne weitere Behandlung elektrophoretisch aufgetrennt. Eine Proteinbestimmung war, möglicherweise infolge von Interferenzen mit im Tee enthaltenen Farbstoffen, nicht erfolgreich.

Gleiche Mengen der Milbenextrakte und des TCM-Tees wurden auf ein 14% SDS-PAGE-Gel aufgetragen und auf Nitrozellulose geblottet (Schleicher und Schuell, Dassel, Deutschland). Die Nitrozellulose wurde mit Puffer A (50 mM Natriumphosphat, pH 7,4, 0,5% (v/v) Tween-20, 0,5% (w/v) BSA and 0,05% (w/v) Natriumazid) gewaschen und mit dem 1 : 5 in Puffer A verdünnten Serum der Patientin über Nacht bei 4 °C inkubiert. Gebundenes IgE wurde wie beschrieben detektiert [14].

## Ergebnisse

Der Routine-Pricktest zeigte mittelgradig positive Reaktionen auf alle getesteten Vorratsmilben (Lepidoglyphus, Tyrophagus, Acarus) sowie fragliche Reaktionen auf Hausstaubmilben. Das spezifische IgE auf Tyrophagus, Lepidoglyphus, Dermatophagoides pteronyssinus, Beifuß- und Ragweedpollen war negativ (< 0,35 kU/l). Das Gesamt-IgE (68 kU/l) sowie die Tryptase (4,6 µg/l) waren im Normbereich.

Der Pricktest mit dem TCM-Tee ergab deutlich positive Reaktionen bis zur Verdünnung 1 : 100 (Abb. 1). Die Einzelkomponenten des Tees, die allesamt neuen Produktchargen entstammten, waren hingegen negativ.

Angesichts der positiven Testergebnisse auf Vorratsmilben ergab sich zunächst die Vermutung einer Kontamination des TCM-Granulats mit Vorratsmilbenantigenen. Der Immunblot mit Vorratsmilbenextrakten war jedoch negativ, während sich mit dem TCM-Tee eine starke IgE-Reaktivität zeigte (Abb. 2).

In einer neuerlichen, vertiefenden Anamnese berichtete die Patientin über eine 20 Jahre zurückliegende Diagnose einer Seidenallergie, die sich als nächtliche Rhinokonjunktivitis äußerte. Nach dem Entsorgen der Seidendecke sei sie damals beschwerdefrei gewesen. Eine daraufhin von uns durchgeführte Bestimmung des spezifischen IgEs auf Rohseide k74 (1,7 kU/l) und Wildseide k73

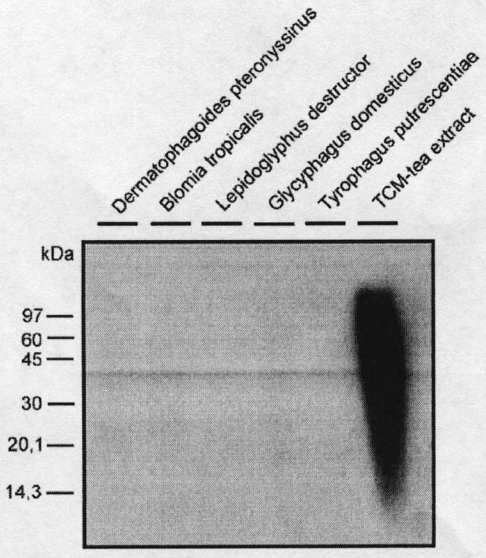


Abb. 2. Immunoblot mit dem Patientenserum auf Milbenextrakte und dem TCM-Tee.

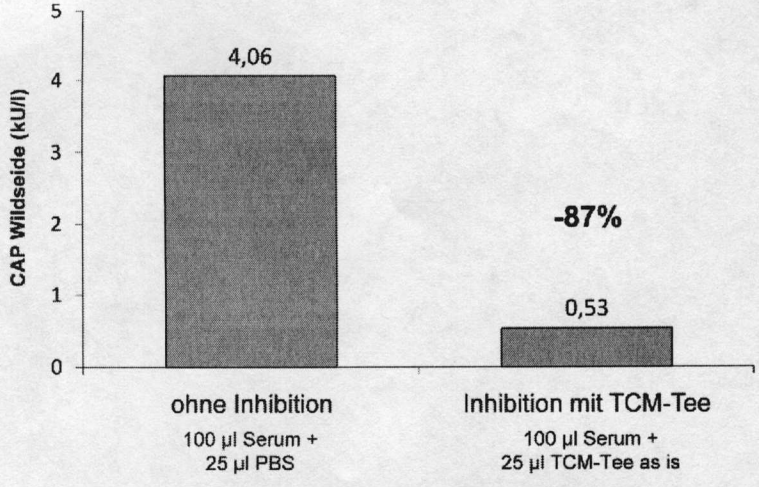


Abb. 3. CAP-Inhibition. Vorinkubation des Patientenserums mit dem TCM-Tee führte zu einer 87%igen Hemmung der IgE-Bindung an Wildseidenallergene (CAP k73).

(5,1 kU/l) bestätigte das Fortbestehen einer Seidensensibilisierung.

Aufgrund des Verdachts, das TCM-Präparat könnte mit Lebensmittelmotten bzw. seinen Puppengespinsten verunreinigt gewesen sein, wurde eine In-vitro-Inhibition durchgeführt. Es zeigte sich, dass die IgE-Bindung an Wildseide mit dem TCM-Tee zu 87% gehemmt werden konnte (Abb. 3). Die anaphylaktische Reaktion auf den TCM-Tee ist daher mit großer Wahrscheinlichkeit auf eine Kontamination des Produkts mit Seidenproteinen aus Puppenkokons zurückzuführen.

## Diskussion

Tee gilt als harmloses Genussmittel. Als häufig angewandtes Mittel in der traditionellen chinesischen Medizin weist jedoch auch Tee unerwünschte Wirkungen bis hin zu schweren Nebenwirkungen auf. In der TCM zählen allergische Reaktionen auf Inhaltsstoffe und direkte toxische Wirkungen der Substanzen, darunter Hepato- und Nephrotoxizität, zu den häufigsten unerwünschten Wirkungen [1]. Unsachgemäße Herstellung oder Verarbeitung sowie Kontaminationen der Produkte, unter anderen mit Schwermetallen oder Pestiziden, stellen weitere Ursachen dar [2]. Obwohl allergische Reaktionen als häufige Nebenwirkung traditioneller chinesischer Medizin genannt werden, fehlen genaue Angaben, welche Substanzen als Auslöser identifiziert wurden und wie das Vorliegen von Typ-1-Allergien verifiziert wurde. Lediglich eine rezente Kasuistik nennt Rhizome des asiatischen Glockenblumengewächses Codonopsis lanceolata als Auslöser einer anaphylaktoiden Reaktion [4].

Bekannt sind allergische Reaktionen hingegen bei einigen Teesorten. Kamille löst ein breites Spektrum an allergischen Reaktionen aus. Ihre Verwendung in Kosmetika und diversen Cremes kann zu Kontaktekzemen führen [7, 15], IgE-vermittelte Reaktionen reichen von Konjunktivitiden nach lokaler Applikation bis hin zu Anaphylaxien nach Genuss von Kamillentee oder nach Kamillentee-Einläufen [5, 11]. Großteils scheint es sich dabei um Kreuzreaktionen mit Beifuß zu handeln [8, 12].

Kreuzreaktionen zwischen Lippenblütlern können zu allergischen Reaktionen auf Melissentee führen. So wurde eine Patientin in unserem Zentrum vorstellig, die nach dem Genuss von Melissentee über Übelkeit, Meteorismus, Diarrhö und Erbrechen klagte. Mittels Prick- und Prick-Prick-Test konnten Sensibilisierungen gegenüber Melisse und anderen Lippenblütlern wie Oregano, Basilikum, Rosmarin und Lavendel bestätigt werden (unveröffentlichte Daten).

Erhöhte inhalative Exposition gegenüber Grünem Tee, wie es bei Plantagenarbeitern berichtet wurde, kann zu einer Sensibilisierung über die Atemwege und so zu Asthma bronchiale führen, das sich nach einer gewis-

sen Latenz auch durch oralen Genuss von grünem Tee auslösen lässt [9].

Da bei unserer Patientin jedoch eine allergische Reaktion auf Einzelbestandteile des Teegranulats mittels negativen Prick Tests ausgeschlossen werden konnte, mussten andere Gründe für die Anaphylaxie verantwortlich sein. Aufgrund des positiven Pricktests auf Lepidoglyphus, Acarus und Tyrophagus wurde bei unserer Patientin zunächst eine Kontamination des Tees mit Vorratsmilben angenommen, die aber aufgrund der negativen Testergebnisse auf Vorratsmilbenantigene in CAP und Immunblot unwahrscheinlich erschien. Erst durch die anamnestischen Hinweise auf eine zurückliegende Seidenallergie, die schließlich serologisch bestätigt werden konnte, wurde der Verdacht auf eine Kontamination des TCM-Präparats mit Seidenproteinen gelenkt, der mittels einer In-vitro-Inhibition untermauert werden konnte. Woher diese Seidenproteine letztlich stammen, bleibt spekulativ. Wir halten den Befall eines der TCM-Rohstoffe mit Schadschmetterlingen wie z.B. den kosmopolitischen Lebensmittelmotten Plodia interpunctella (Dörrobstmotte) oder Ephestia kuehniella (Mehlmotte) und eine daraus resultierende Kontamination mit den Puppengespinsten (Kokons) ihrer Larven als wahrscheinlichste Erklärung. Voraussetzung dafür ist eine Kreuzreaktivität dieser Seidenproteine mit denen der Wildseidenspinner (Antherea spp.) bzw. des Maulbeerspinners (Bombyx mori). Die relevanten Seidenallergene sind aber noch unzureichend charakterisiert, und zur Kreuzreaktivität zwischen Seidenproteinen verschiedener Schmetterlingsarten liegen nur wenige Untersuchungen vor.

Die Seidenallergie ist selten und kommt hauptsächlich in China und Japan vor, wo Seide ein weitverbreiteter Rohstoff ist, und erklärt sich durch die höhere berufliche Exposition bei Webern und Spinnern [13]. Über die genaue Prävalenz der Seidenallergie in der Gesamtbevölkerung gibt es wenige Angaben. In Mitteleuropa verursacht diese Form der Allergie vorwiegend Asthma und wird gelegentlich, wie bei unserer Patientin, als Auslöser nächtlicher Beschwerden in Verbindung mit dem Gebrauch von Seidendecken gefunden [3, 16]. Interessanterweise gibt es auch Berichte über anaphylaktische Reaktionen nach dem Genuss von Seidenraupen, denen allerdings andere Allergene zugrundeliegen dürften [6].

Es handelte sich bei unserer Patientin also um eine anaphylaktische Reaktion aufgrund einer Verunreinigung des TCM-Produkts mit Seidenproteinen bei einer bestehenden Seidenallergie. Trotz der weitverbreiteten Annahme, Naturprodukte wie Tees und TCM-Heilkräuter seien harmlos, kann es bei Vorliegen prädisponierender Faktoren wie Sensibilisierungen gegen Bestandteile durch intensiven beruflichen Kontakt oder Kontaminationen der Produkte mit potentiellen Allergenen zu schwerwiegenden Überempfindlichkeitsreaktionen kommen.

Bei Produkten der traditionellen chinesischen Medizin sollte an Kontaminationen gedacht werden. Klinische Relevanz resultiert jedoch erst, wenn zusätzliche Faktoren, wie in diesem Fall die Sensibilisierung, beim Patienten vorliegen. Aufgrund der geringen Prävalenz von Allergien auf Seide sowie aus Kosten- und Zeitgründen erschien es uns nicht sinnvoll, weitere Untersuchungen in TCM-Apotheken zu veranlassen.

Dieser Fall zeigt, dass in der Allergologie neben medizinischem Fachwissen und Kenntnissen der Biologie auch ein kriminalistischer Spürsinn gefragt ist.

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# IV. Summary - Zusammenfassung

Type I hypersensitivity, better known as allergy, represents a major health problem in industrialized countries, with more than 20% of the population being affected. House dust mites (HDM) are among the most important elicitors of Type I allergy, with a prevalence of sensitization of more than 50% among allergic patients.

The introduction of recombinant DNA technology into the field of allergy represents an important step towards the improvement of diagnosis and treatment of allergic patients. Allergen extracts that are nowadays in use, are difficult to standardize, certain important allergens are under-represented or the extracts may be contaminated with allergens from other sources or bacterial endotoxins. Thus, they may fail in diagnosis or induce unwanted side-effects in immunotherapy. The use of pure and defined recombinant allergens might overcome these problems. The successful diagnosis and immunotherapy of HDM-allergic patients with recombinant allergens requires a panel of all important allergens that induce relevant symptoms in patients. IgE-binding frequency, allergen-specific IgE level and in particular the allergenic activity have to be taken in consideration when deciding about the importance of an allergen.

The major aim of this thesis was to evaluate the relevance of the house dust mite tropomyosin, Der p 10, in HDM allergic patients. A recombinant protein was produced in a bacterial expression system that equals its natural counterpart regarding biochemical, structural and immunological properties. Der p 10 showed low IgE reactivity (15.2%) and low allergenic activity in the vast majority of the tested Der p 10-positive HDM-allergic patients, which indicates a rather low clinical relevance of the mite tropomyosin in HDM allergic patients. Nevertheless, our results revealed that Der p 10-positive and negative patients showed substantial differences regarding their sensitization profiles to HDM allergens which has implications for specific immunotherapy treatment.

Chapter III describes a case report of a 55-year-old woman who developed an anaphylactic shock after drinking a TCM (traditional Chinese medicine) tea. As the individual components of the tea failed to cause a positive reaction in skin prick tests, a contamination of the tea was assumed. Our results excluded house dust mites and storage mites from being the contaminating source and identified silk allergens probably derived from insect pests as being the reaction-causing source.

Typ I Hypersensitivität, besser bekannt als Allergie, stellt ein bedeutendes Gesundheitsproblem in industrialisierten Ländern dar, von dem mehr als 20% der Bevölkerung betroffen ist. Hausstaubmilben zählen zu den wichtigsten Auslösern der Typ I Allergie, mit einer Sensibilisierungsprävalenz von mehr als 50% unter Allergikern.

Die Einführung der rekombinanten DNA Technologie in den Bereich der Allergie stellt einen wichtigen Schritt in Richtung Verbesserung von Diagnose und Therapie von allergischen Patienten dar. Allergenextrakte, die heutzutage verwendet werden, sind schwierig zu standardisieren, einige wichtige Allergene sind unterrepräsentiert, oder die Extrakte sind mit Allergenen anderer Quellen oder bakteriellen Toxinen kontaminiert. Diese können daher ein falsch negatives Diagnoseergebnis und unerwünschte Nebenwirkungen in der Immuntherapie verursachen. Die Verwendung von reinen und definierten rekombinanten Allergenen könnte diese Probleme beseitigen. Eine erfolgreiche Diagnose und Immuntherapie von Hausstaubmilben-allergikern mit rekombinanten Allergenen benötigt die Summe aller wichtigen Allergene, die relevante Symptome in Patienten hervorrufen. Die IgE-Bindungs-frequenz, der allergenspezifische IgE-Spiegel und im Speziellen die allergene Aktivität müssen in Betracht gezogen werden wenn über die Wichtigkeit eines Allergens entschieden werden soll.

Das Hauptziel dieser Diplomarbeit war es, die Relevanz des Hausstaub-milbentropomyosins, Der p 10, in Milbenallergikern zu untersuchen. Es wurde ein rekombinantes Protein in einem bakteriellen Expressionssystem produziert, dass seinem natürlichen Gegenstück in biochemischer, struktureller und immunologischer Hinsicht glich. Der p 10 zeigte eine niedrige IgE-Reaktivität (15.2%), sowie eine schwache allergene Aktivität in der Mehrheit der getesteten Der p 10 positiven Hausstaubmilbenallergikern, was auf eine eher niedrige klinische Relevanz des

Milbentropomyosins hindeutet. Unsere Ergebnisse zeigten allerdings, dass sich Der p 10 positive und negative Patienten beachtlich in ihrem Hausstaubmilben-sensibilisierungsprofil unterscheiden. Dies könnte Auswirkungen auf eine spezifische Immuntherapiebehandlung haben.

Kapitel III beschreibt den Fallbericht einer 55-jährigen Frau, die nach Konsum eines TCM- (traditionelle chinesische Medizin) Tees einen anaphylaktischen Schock erlitt. Da die Einzelkomponenten des Tees keine positive Reaktion im Pricktest verursachten, wurde eine Kontamination des Tees vermutet. Unsere Ergebnisse schlossen eine Verunreinigung mit Hausstaub- und Vorratsmilben aus. Im Folgenden konnte eine Kontamination mit Seidenproteinen, die wahrscheinlich von Lebensmittelmotten stammen, gezeigt werden. Diese scheinen für die starke allergische Reaktion verantwortlich zu sein.

# V. Curriculum vitae

**Personal Details**

| | |
|---|---|
| Name: | Yvonne Resch |
| Date of Birth: | June 26th, 1984 |
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**Education:**

| | |
|---|---|
| Since March 2008: | Diploma Thesis at the Department of Pathophysiology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, under supervision of Dr. Susanne Vrtala<br>Title: Molecular, immunological and structural characterization of mite allergens |
| Since October 2003 | University of Vienna<br>Study of Genetics and Microbiology |
| Oct. 2002 - June 2003 | University of Vienna<br>Study of Medicine |
| Sept. 1994 – June 2002 | Grammar school, BG/BRG Pichelmayergasse, Vienna |
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**Memberships:**

Member of the Austrian Society of Allergology and Immunology (ÖGAI)

Member of the European Academy of Allergy and Clinical Immunology (EAACI)

**Poster presentations:**

"Recombinant Der p 10 as a diagnostic tool to identify patients with genuine shrimp sensitizations" – International Workshop "Current problems in allergen vaccine development and manufacturing", Varadero, Cuba, October 18-23, 2009

"Molecular and immunological characterization of the mite allergen Der p 10, a diagnostic marker allergen for tropomyosin sensitization" – Karl Landsteiner Meeting, Salzburg, Austria, November 6-7, 2009