Recombinant strategies in hymenoptera venom allergy and beyond

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

an der Fakultät für Mathematik, Informatik und Naturwissenschaften

der Universität Hamburg

vorgelegt von

Henning Seismann

Der experimentelle Teil der vorliegenden Doktorarbeit wurde in der Zeit von Februar 2006 bis Oktober 2009 unter der Leitung von Prof. Dr. R. Bredehorst im Institut für Biochemie und Molekularbiologie der Universität Hamburg durchgeführt.

Gutachter der Dissertation:	Prof. Dr. Reinhard Bredehorst
	Prof. Dr. Ulrich Hahn
Gutachter der Disputation:	Prof. Dr. Reinhard Bredehorst
	Prof. Dr. Markus Fischer
	Dr. Nicolas Piganeau
Datum der Disputation:	04.12.2009

Abstract

Hymenoptera venom allergies affect around one fourth of the population and 1-5% show systemic and sometimes life threatening symptoms. Even if the incidence for fatal events is low, it remains one of the main causes for fatal allergic reactions and the quality of live of affected individuals is significantly reduced. In Europe the main threat is emanating from the western honeybee *Apis mellifera* and the common wasp *Vespula vulgaris*. Actually, the only causative therapeutic intervention in hymenoptera venom allergy is the specific immunotherapy (SIT), whereby 80-95% of the treated patients are protected from severe reactions. A prerequisite for a successful therapy is a proper diagnosis, which mainly depends on the correct determination of the culprit venom. However, a considerable number of patients show reactivity to honeybee as well as wasp venom and are therefore diagnosed as double-positive, whereas in up to 75% of these the diagnosis is affected by molecular cross-reactivities. Reasons for this phenomenon are either specific IgE moieties directed against homologous proteins in the venoms, or antibody binding to similar carbohydrate structures on glycoproteins of both venoms, called cross-reactive carbohydrate determinants (CCDs), which cannot be differentiated by the current diagnostic tools. An improvement mainly depends on a thorough knowledge on the allergens involved in hymenoptera venom allergy. Therefore, in the present PhD thesis a new allergen from wasp venom was identified and characterised. Furthermore, already established components were re-evaluated in terms of their allergenic potential and with special regard to CCDs. As a basis for thorough characterisation, all proteins were recombinantly expressed in the baculovirus expression systems in functional form and analysed by various *in vitro* immunological and cellular assays. Moreover, recombinant antibody technologies were utilised for the generation of IgE, IgG and IgY antibodies as diagnostic tools in allergological and immunological assays in general and with special regard to molecular cross-reactivities.

In summary, the presented data allow a revision of the current view on hymenoptera venom allergens and their particular significance, especially concerning the role of CCDs. Moreover, together with the recombinant antibodies, a broad panel of new valuable diagnostic tools for the improvement of allergy diagnosis and beyond were generated which will contribute to a molecular understanding of pathophysiological and allergological mechanisms in hymenoptera venom allergy.

Zusammenfassung

Rund ein Viertel der Bevölkerung leidet an Insektengiftallergien, wobei 1-5% systemische bis lebensbedrohliche Reaktionen zeigen können. Auch wenn die Häufigkeit solch fataler Fälle gering ist, stellen sie doch eine der häufigsten Ursachen für fatale allergische Reaktionen dar und die Lebensqualität der Betroffenen ist signifikant reduziert.

In Europa geht die Hauptgefährdung von der westlichen Honigbiene *Apis mellifera* und der gewöhnlichen Wespe *Vespula vulgaris* aus. Zurzeit stellt die spezifische Immuntherapie (SIT) die einzige kausale therapeutische Intervention dar, wobei 80-95% der behandelten Patienten vor schweren allergischen Reaktionen geschützt sind. Eine Grundvoraussetzung für eine erfolgreiche Therapie ist eine entsprechende Diagnose, die hauptsächlich auf der Bestimmung des verantwortlichen Giftes beruht. Eine Vielzahl von Patienten zeigt jedoch Reaktionen auf Bienen- und Wespengift und wird daher als doppelt positiv eingeschätzt, wobei 75% dieser Diagnosen von molekularen Kreuzreaktionen beeinflusst sind. Gründe für dieses Phänomen sind zum einen IgE Antikörper mit Spezifität für homologe Proteine beider Gifte, oder Antikörper die sich gegen Carbohydratstrukturen auf Glykoproteinen beider Gifte, die als kreuzreaktive Carbohydrat Determinanten (cross-reactive carbohydrate determinants: CCD) bezeichnet werden und nicht in den aktuellen diagnostischen Verfahren differenziert werden können. Um diese diagnostischen Methoden verbessern zu können, bedarf es jedoch eingehender Kenntnis über die beteiligten Allergene in der Insektengiftallergie. Dazu wurde in der vorliegenden Doktorarbeit ein neues Allergen des Wespengifts identifiziert und charakterisiert und zudem bereits bekannte Komponenten im Hinblick auf ihre allergenes Potenzial und die Rolle von CCDs neu bewertet. Dafür wurden alle Proteine rekombinant im Baculovirus-System exprimiert und in verschiedenen immunologischen und zellulären *in vitro* Untersuchungen analysiert. Weiterführend wurden unterschiedliche rekombinante Antikörperformate für den Einsatz in allergologischen und immunologischen Diagnostikanwendungen hergestellt, wobei auch hier ein Focus auf molekularen Kreuzreaktionen lag.

Zusammenfassend erlauben die präsentierten Daten eine Neubewertung der Signifikanz einzelner Insektengiftallergene, besonders im Hinblick auf die Bedeutung von CCDs. Darüber hinaus, wurden zusammen mit den rekombinanten Antikörpern eine Reihe wertvoller diagnostischer Werkzeuge für den Einsatz in der Allergiediagnostik

und darüber hinaus zur Verfügung gestellt, die wesentlich zu weiteren Erkenntnissen über die pathophysiologischen und allergologischen Mechanismen der Insektengiftallergie beitragen können.

Index

1 Introduction

Symptoms related to allergic disease have already been described in antiquity by Hippocrates and a medical description of hay fever has been made in the 16th century. Therefore, allergy is not a modern disease, but prevalence has risen dramatically since the 1960s and nowadays it is considered a major public health concern [1]. The incidence for allergic disease in the general population of industrialised countries is about 25-30%, whereby most of the affected individuals suffer from atopic disease like atopic dermatitis (~ 10%), allergic asthma (5-10%), or allergic dermatitis (~ 10%) [2-4].

The term allergy was first introduced by Clemens von Pirquet in 1906 to distinguish immune responses that are harmful to the host from a physiological state of protective, beneficial immunity [5]. This description was increasingly confined to the actual definition of allergic disorders or atopic disease, from the Greek *atopos* meaning out of place, as an abnormal adaptive immune response directed against non-infectious environmental substances, including non-infectious components of certain infectious organisms. The responses to these substances, the allergens, are characterised by the involvement of allergen-specific IgE antibodies and T helper 2 cells (T_H2) that recognise allergen derived antigens. Nevertheless, in some forms of allergy, such as allergic contact dermatitis, IgE is thought to be irrelevant. Accordingly, two kinds of allergens can be distinguished, those representing non-infectious environmental substances that are capable of inducing IgE production and, thereby, sensitising a subject, and those capable of inducing an adaptive immune response associated with local inflammation independently of IgE.

On the basis of the underlying mechanisms a classification has been proposed describing four different types of reactions [6]. The type I reaction is the classical IgE dependent form of allergy, in which IgE-mediated cross-linking of cellular receptors by non-infectious environmental substances leads to the release of certain mediators that are causative for the clinical symptoms. Since this release occurs within minutes after allergen exposure, it has also been designated as immediate type. In the type II or cytotoxic reaction, which is independent from IgE, symptoms are related to an antibody dependent cellular cytotoxicity (ADCC) initiated by IgG antibodies directed against autoantigens or drugs bound to cell surfaces. The causative agents in type III reactions are immune complexes deposited along small vessels or the basal membrane which can mediate either complement activation or effector cell infiltration to the affected

tissue. Type IV reactions, also designated as delayed type reactions, are distinct from the other forms since the reaction is not antibody dependent and not directed against soluble, free antigens, but triggered by antigen specific T cells.

The type I or immediate type reaction accounts for the most common forms of allergy including asthma, hay fever, urticaria, angiooedema and anaphylaxis and, thus, is often exclusively associated with the term allergy. Therefore, and as it also represents an underlying principle of hymenoptera venom allergy the following will focus on this type of reaction.

Sources of allergens involved in type I reactions include pollen from grasses, trees and some other plants, animal dander, house dust mite faecal particles, certain foods like peanuts, fruits, fish, milk and eggs, latex as well as some drugs, moulds and insect venoms. In this context it is still an open question what establishes a protein as an allergen, since structural similarities have to date not been revealed. Although the use of bioinformatic tools could assign allergens from foods to a limited number of protein superfamilies, the consequences thereof are still unclear [7].

Allergic diseases are as diverse as the allergenic sources and include asthma, rhino conjunctivitis, sinusitis, food allergy, atopic dermatitis, angiooedema and urticaria, anaphylaxis, insect venom and drug allergy. Again a phenomenon of unclear etiology is the increasing prevalence of allergic diseases in the industrialised countries. The two explanations favoured at the moment are the hygiene hypothesis [8] and the environmental pollution hypothesis [9]. The hygiene hypothesis is based on the idea that the lack of exposure to infectious agents, symbiotic microorganisms and parasitic infections increases susceptibility to allergic diseases by modulating the immune homeostasis, mainly by preventing a T helper 1 cell (T_H1) dominated T cell milieu. The impact of environmental pollution is based on the observation that some potent allergens from plants are pathogenesis-related and hence, expressed in higher levels due to environmental stress and pollution [10]. Furthermore, soot particles seem to have an adjuvant effect if in complex with airborne allergens [11]. Additionally, environmental factors also exhibit detrimental effects on the organism by synergistically biasing the immune milieu towards T_H2. Since both hypotheses have been corroborated by several studies but still leave some aspects elusive and clear strategies for prevention are still missing, the main issue is how to fight existing allergies in terms of diagnosis and treatment.

The key components implicated in most types of allergic diseases are the allergens, the allergen specific antibodies of the IgE isotype and the corresponding cellular arm of the adaptive immune response and, therefore, will be highlighted in the following section.

1.1 IgE and the IgE network

After von Pirquet has coined the term allergy in 1906 it lasted another 15 years till Prausnitz and Küstner discovered the existence of a transferable serum factor [12] which was later described as “reagin” and finally identified as a new antibody subclass, the IgE [13]. IgE shares the same basic structural features with antibodies of other classes, with two identical heavy chains and two identical light chains, but in contrast to IgG the heavy ε chain contains one more domain. While the Cε3 and Cε4 domains are homologous in sequence and similar in quaternary structure to the Cγ2 and Cγ3 domains of IgG, the Cε2 domains corresponds to the hinge region of IgG and is the most variant feature of IgE. Although the Fc part of IgE seems to be extended, the three dimensional structure is more rigid compared to IgG, as the Cε3 domain folds back and gets in contact with the Cε2 domain.

The biological function of IgE is supposed to be associated with parasitic infections as elevated IgE titers are found during helminth infections. In this context, IgE was reported to bind the glycoprotein IPSE (*IL-4-inducing principle of S. mansonii* eggs) induced by *S. mansonii* infections and that cross-linking of FcεRI bound IgE upon binding of IPSE led to basophil activation [14]. Moreover, eosinophils release the major basic protein which is cytotoxic to helminths after binding of a parasite/IgE complex to the FcεRI. A more indirect finding is that the mean IgE level in industrialised countries is at least ten fold lower than in developing countries with parasitic infections being more common.

Two major cellular receptors have been described for IgE, a high affinity and a low affinity receptor. The high affinity FcεRI is expressed as a $\alpha\beta\gamma_2$ tetramer on basophils and mast cells and as a $\alpha\gamma_2$ trimer on human antigen-presenting cells, eosinophils, monocytes, platelets and smooth muscle cells [15]. The extracellular α-chain exhibits the IgE binding function, while the signalling motifs (immunoreceptor tyrosine-based activation motifs (ITAM) are located in the intracellular ß- and γ-domains. The affinity of the receptor is in the range of $10^{10}M^{-1}$ and the receptor binds

with a 1:1 stoichiometrie. Signalling via this receptor is initiated by cross-linking of receptor bound IgE molecules upon binding of an antigen. A prerequisite is the presence of multiple or at least two epitopes per antigen and a sufficient affinity of one or both IgE molecules involved in antigen binding [16].

CD23, the low affinity receptor, belongs to the C-type (calcium-dependent) lectins and differs from almost all other immunoglobulin receptors. It is found on mature B cells, activated macrophages, eosinophils, follicular dendritic cells and platelets with two different forms CD23a and CD23b being expressed, the latter one on normal B cells only. The affinities of CD23 to IgE differ between IgE-antigen complexes ($10^{-8}M^{-1}$) and free IgE ($10^{-7}M^{-1}$) [17].

1.2 IgE in allergy

Besides its putative role in parasitic infections, IgE antibodies are involved in most forms of allergy as one of the key molecules mediating binding of the allergenic proteins to the above mentioned Fc receptors. For the high affinity receptor this event can lead to a cross-linking of the IgE-FcεRI complexes on the cell surface of mast cells and basophils and their activation results in release of certain mediators responsible for the classical allergic syndromes.

Prior to this event an IgE based antibody response needs to be established. In this process, designated sensibilisation, (Fig.1 A) first contact with the allergen takes place, in case of food, some drugs and airborne allergens occurring via epithelial tissues, whereas insect venoms are directly injected into the skin. Antigen presenting cells (APC) within these tissues, like basophils, Langerhans or dendritic cells are involved in allergen uptake, processing and presentation via MHC (major-histo-compatibility complex) molecules. Moreover, immunoglobulin-mediated capturing of allergen by specific B cells occurs. As a result, naive T cells recognise the respective T cell epitopes presented on the MHC effecting activation and differentiation. Although T cell fate is mainly dependent on the local cytokine milieu, a predominant differentiation into T_H2 cells by IL4 is driven during sensitization. This T_H2 dominated immune response is a prerequisite for the triggering of allergic disease [18] and the principle underlying this imbalance in allergic individuals is one of the main topics actually discussed. One argument, based on the hygiene hypothesis, is a missing immune deviation caused by the westernised live style, whereby a reduced microbial burden

seems to lead to a reduction of T_H1 promoting cytokines like interferon-γ and IL12 as a consequence of missing stimulation with bacterial products of cells of the innate immune system via toll-like receptors (TLR) [19]. Somewhat contradictory to this is the epidemiological observation that the incidence for clearly T_H1 dominated diseases like insulin dependent diabetes mellitus (IDDM) or some autoimmune diseases has risen as well in industrialised countries. Another thesis favours a decreased activity of regulatory T cells (Treg) and, thereby, reduced immunosupression rather than missing immune deviation [8]. Furthermore, genetic factors have been identified [20], including gene products involved in T cell differentiation, antigen presentation or chemokine signalling, even though this finding can not constitute the sole explanation for the dramatic increase of allergic disease.

T_H2 cells can then induce a class switch of antigen specific B cells towards IgE producing plasma cells [21, 22] and allergen specific memory B cells [23] by secretion of IL4 and IL13. Furthermore, from these cells emerge T_H2 memory cells which are involved in allergic late phase reactions. Repeated contact with the allergen can further boost IgE B cells and thereby lead to establishment of high IgE levels.

After the individual is sensitised against a certain allergen, the next contact can lead to an immediate allergic reaction mainly driven by release of mediators from mast cells upon cross-linking of receptor bound IgE [24] (Fig. 1 B).

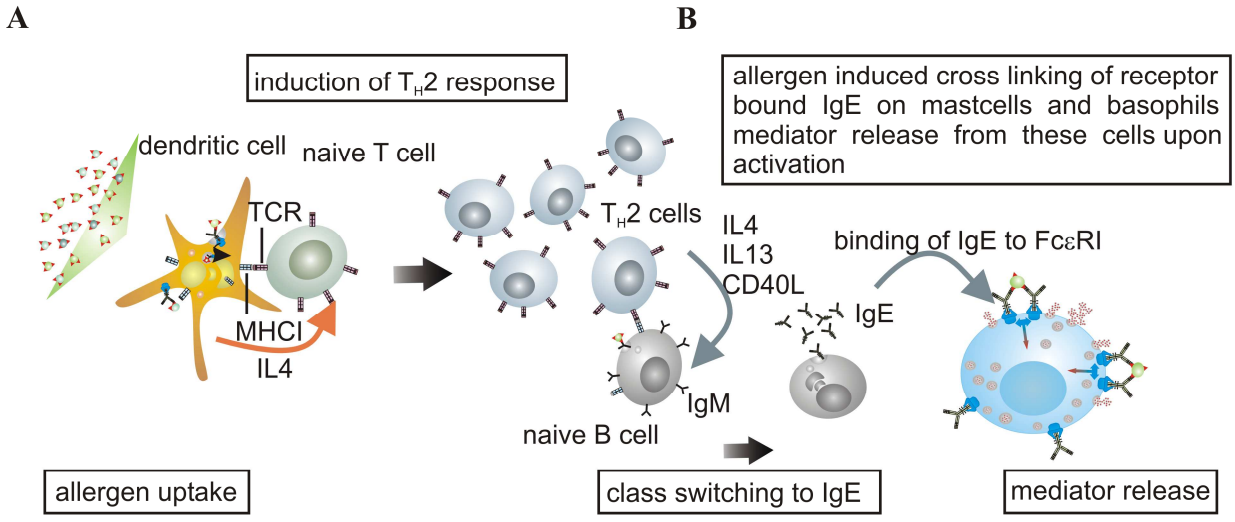

Figure 1: Sensitisation and immediate phase of type I reactions

TCR: T cell receptor; MHCII: major histocompatibility complex II; T_H2: T helper 2 cells; FcεRI: high affinity IgE receptor; IL4: interleukin 4; IL13: interleukin 13; CD40L: cluster of differentiation 40 ligand

Typical mediators released from these cells are preformed granule-associated mediators, newly generated lipid mediators as well as cytokines and chemokines. Most relevant for the acute symptoms of allergy like rhinitis, conjunctivitis, asthma and urticaria are the vasoactive histamine and leukotrienes. Further symptoms include increase of vascular permeability, smooth muscle contraction or increased mucus secretion. Since these mediators are readymade in preformed granules, the immediate reaction can occur within minutes, in contrast, chemokines and lipid mediators are newly synthesised and primarily account for the long term effects within 2-6 hours after allergen exposure by recruitment of effector cells. Hence, the allergic late phase reaction is characterised by a strong T cell infiltration, activation of eosinophils and release of inflammatory mediators from these cells which finally can lead to tissue damage upon repetitive allergen exposure [25].

1.3 Hymenoptera venom allergy

The prevalence for hymenoptera venom allergy in the general population is around 25 % [26] with most individuals showing mild to moderate symptoms upon stinging like generalised skin symptoms (e.g. flush, generalised urticaria, angiooedema) or mild to moderate pulmonary, cardiovascular, and/or gastrointestinal symptoms. Nevertheless, 3-5 % [27] show systemic reactions after a stinging event ranging from anaphylactic shock and loss of consciousness to cardiac arrest and apnoea which can have fatal consequences. Although mortality is relatively low, ranging from 0.03 to 0.48 fatalities per 1 000 000 inhabitants per year [28] hymenoptera stings belong to the most common causes for anaphylactic reactions beside drugs and foods.

The term anaphylaxis nowadays relates to the acute life threatening syndrome upon rapid and systemic release of inflammatory mediators from effector cells in response to allergen contact [29]. With the exception of radio contrast media and some opiates which show non immunologically mediated reactions as they act directly as mast cell degranulating agents [30], the clinical symptoms of anaphylaxis are mainly associated with immunological events initiated by allergen induced IgE cross-linking.

The potential severity of anaphylactic reactions renders the availability of an emergency kit including H1 receptor blocking antihistamine and corticosteroids for oral use, as well as an epinephrine inhaler and, in particular situations, an epinephrine auto-injector highly recommendable for patients which are known to show systemic reactions

upon stinging. Additional risk-factors aggravating severe systemic reactions have been identified, namely cardiovascular diseases or use of beta blockers [31], as well as elevated levels of serum tryptase, indicating mastocytosis [32]. Another observation relates to the causative insect, since systemic reactions after bee stings are more frequent than after vespid stings [33], whereas an even higher incidence was seen for hornet stings [34]. However, since specific immunotherapy is highly effective in hymenoptera venom allergy, patients with a history of severe systemic reactions are strongly encouraged to undergo this therapy.

In Europe and the US the main threat is emanating from the honeybee *Apis mellifera* as well as different wasp species including the three genera *Vespula, Dolichovespula* and *Vespa* all belonging to the family Vespinae (Table 1).

Family/Subfamily and species	**Common name**	**Main geographical distribution**
Apidae/		
Apinae		
Apis mellifera	honeybee	worldwide
Bombinae		
Bombus ssp	bumblebee	worldwide
Vespidae/		
Vespinae		
Vespa crabro	European hornet	Europe / NE/SE United states
Vespula germanica	Yellow jacket/ German wasp	worldwide
Vespula vulgaris	Yellow jacket/ Common wasp	worldwide
Vespula maculifrons	Yellow jacket	NE United States
Vespula squamosa	Yellow jacket	NE/SE United states
Vespula pensylvanica	Yellow jacket	NW/SW United states
Dolichovespula media	Median wasp	Central Europe
Dolichovespula saxonica	Saxon wasp	Central Europe
Dolichovespula maculata	Whitefaced hornet	Entire United States
Dolichovespula arenaria	Yellow hornet	NE/NW/SW United states
Polistinae		
Polistes gallicus	Paper wasp	Mediterranean
Polistes dominulus	Paper wasp	Mediterranean
Polistes annularis	Paper wasp	Entire United States
Polistes exclamans	Paper wasp	Entire United States

Table 1: Most common species among Apidae and Vespidae family accounting for allergic reactions in Europe and the US

Most common and with a worldwide distribution is the so called common wasp *Vespula vulgaris* designated yellow jacket in the US. Furthermore, in the Mediterranean as well as in the US different species of the family Polistinae such as *Polistes dominulus* are widely distributed. Less common are stings from bumblebees *Bombus ssp*. and ants like the fire ant *Solenopsis invicta* whereas sensitizations to bumblebees get more common due to their role in the pollination industry in green houses [35]. Stings from the European hornet are as well rare, since numbers of these insects have decreased significantly.

1.4 Hymenoptera venoms

The amount of venom released during a sting is around 50-100 µg for honeybees in contrast to 2-10 µg for vespids with their capability of repeated stinging [36]. Additionally, for honeybees the amount of venom released by a sting depends of the duration till removal of the stinger since it continues to pump venom.

The venom is a complex mixture of active amines, lipids, amino acids, peptides and proteins, the latter ones responsible for the binding of IgE and, therefore, for the allergic reactions. The protein composition of hymenoptera venoms is considered elucidated in rough form (table 2), with the most prominent compounds being identified. This relates primarily to the phospholipases as well as the hyaluronidases, found throughout all venoms in significant amounts [37-39]. Further components that have been identified are the antigen 5 in different wasp venoms [40] and an acid phosphatase in honeybee [41] and bumblebee venom [42]. In honeybee venom some additional allergens have been described [43-46], however, the IgE prevalence for most of them is either initially or uncharacterised. Another allergen found in both honeybee and wasp venom is a dipeptidylpeptidase IV like enzyme which was designated Api m 5 and Ves v 3, respectively, and showed a high IgE prevalence [47].

Apis mellifera	**enzymatic function/ common name**	**molecular weight in kDa**
Api m 1	phospholipase A1	17
Api m 2	hyaluronidase	45
Api m 3	acid phosphatise	49
Api m 4	mellitin	3
Api m 5	dipeptidylpetidase	100
Api m 6	protease inhibitor	8
Api m 7	CUB-protease	39
Api m 8	carboxyesterase	70
Api m 9	carboxypeptidase	60
“Api m 10”	icarapin/venom protein 2	45
Vespula vulgaris		
Ves v 1	phospholipase A1	35
Ves v 2a	hyaluronidase	45
Ves v 2b	hyaluronidase (inactive)	47
Ves v 3	dipeptidylpetidase	100
“Ves v 4”	CUB-protease	39
Ves v 5	antigen5	25

Table 2: Venom allergens from *Apis mellifera* and *Vespula vulgaris*

Allergen nomenclature according to the I.U.I.S except those in quotation marks which have not yet been submitted

Apart from their significance as allergens most of the proteins fulfil enzymatic functions. The hyaluronidases specifically degrade hyaluronic acid in the extracellular matrix of the skin and, thereby, facilitate penetration of venom components into the body. In addition to the active enzyme a second hyaluronidase isoform without enzymatic activity was currently described in yellow jacket venom [48].

Dipeptidylpeptidases release amino-terminal dipeptides from polypeptides with either proline or alanine at the penultimate position [49] whereby substrates for the human enzyme are various chemokines [50] and a proposed function in the venom is the processing of the mellitin or mastoparan propeptide [47].

Phospholipases, which hydrolyse the ester linkage of phospholipids and fatty acids act on biological membranes and, therewith, are in concert with the membranolytic function of mellitin, haemolytic. Enzymatically, the phospholipases of honeybee and wasp venom are not related since they catalyse the release of fatty acids

from different positions of the phospholipids, a fact that is reflected in the reduced sequence identity.

The concentration of the different proteinic compounds range from 10% for the phospholipases and the antigen 5 to less than 1% for most of the other proteins [51]. In addition, a panel of lower molecular weight compounds can be found in the venoms. The predominant peptidic compound in honeybee venom is the mellitin, Api m 4, a membranolytic peptide of 26 amino acids in length, which comprises 50 % of dry mass of the *A. mellifera* venom. In vespid venoms no homologue can be found and the most relevant peptidic compound is the immune modulating peptide mastoparan, present in the range of 2 % of dry mass. In contrast to mellitin [52], IgE binding to mastoparan has not been reported.

Since the different hymenoptera venoms share certain protein functions and most of these proteins are structurally related, cross-reactivity of these proteins is observed especially among the vespid species but also vespids and honeybees [47, 53] show considerable cross-reactivity. Thereby, different Vespinae species show higher cross-reactivity among each other than between Vespinae and Polistinae species [54, 55]. Cross-reactivity between bee and wasp venom is primarily attributed to the hyaluronidases and dipeptidylpeptidases, the sequence identity of which is 52% (Api m 2/Ves v 2a) and 53% (Api m 5/ Ves v 3) respectively. Nevertheless, the hyaluronidases of honeybee and yellow jacket venom are believed to share no surface epitopes [56]. Beyond protein based cross-reactivity the presence of multiple glycoproteins in the venoms is responsible for another kind of cross-reactivity on the basis of so called cross-reactive carbohydrate determinants (CCD) which will be addressed below.

1.5 Allergy diagnosis

Diagnosis in the field of allergy addresses mainly three questions which are crucial either for successful treatment or for prevention of future allergic events and deterioration in health. First is to define the sensitisation pattern, next the severity of sensitisation and last the significance of sensitisation.

Initially the primary sensitization is assessed by skin prick testing or intradermal skin testing in which tiny amounts of allergens are applied to the skin by scratching or pricking. In case of a sensitisation degranulation of effector cells leads to local wheals the size of which is discussed to correlate with the severity of symptoms [57].

Moreover, the assessment of the sIgE level is of importance, since it may predict the severity of future allergic reactions. Allergy diagnosis by the assessment of sIgE in patient sera is a highly automated and standardised process with minimum amounts of patient serum needed for multiple tests. In general, three different methods are common in allergy diagnosis with automated systems for standard diagnosis being quantitative in terms of specific and total IgE measurement and lateral flow assays reflecting a certain sensitisation pattern only. Both systems are based on the binding of IgE to an immobilised allergen or allergen extract and the subsequent detection of IgE by a dye or enzyme labelled anti-IgE antibody. Immobilisation is facilitated by either biotinylation of allergen and binding to a streptavidin coated solid support, or by direct spotting to nitrocellulose membranes or activated glass surfaces. The latter system allows for screening of thousands of different allergens with a minimum amount of serum, since the area for each spot is in the micrometer range [58].

Even if in particular the routine systems are highly automated and allow for quantitative assessment of IgE levels, all systems essentially depend on allergens and allergen extracts. The phenomenon that these extracts, even if standardised by a certain manufacturer, can show batch to batch variations in terms of concentration and composition of particular components is the major limitation in allergy diagnosis. Other components can at least be missing from the extract and, therefore, reactivity can be lost [59]. Even if standardisation of these extracts or the respective assays is performed by most of the manufacturers, this will rarely be the case for an inter assay standardisation. Furthermore, standardisation is nowadays performed by use of polyclonal sera from allergic patients with varying specificities and total IgE amounts. Therefore, allergen extracts in these systems can only be analysed in terms of total IgE binding capacity without regard to single specificities. Moreover, even if different quantitative assays use the same unit definitions the results will not be interchangeable since they quantify different IgE moieties. In this context it was demonstrated by use of monoclonal chimeric IgE antibodies that even for a single specificity, different quantitative assays showed varying results [60]. In general, the use of monoclonal IgE antibodies would be a more sensitive method for standardisation, but the availability of such antibodies yet is very limited and the few reported ones are mainly chimeric ones with mouse origin, rendering a relevance of the detected epitopes unlikely.

Another concern about allergen extracts arises from homologous proteins in different extracts resulting in cross-reactivity of these extracts, an observation also

evident for bee and wasp venom. Moreover, such cross-reactivities can originate from cross-reactive carbohydrates (CCD) which will be highlighted in the next chapter. In general, the significance of diagnostic procedures is limited in those cases where sensitisation patterns indicate interference by such cross-reactivity. This might be the case in food allergy, where sensitisation to multiple foods with known cross-reactivity is a common feature, or in hymenoptera venom allergy, where sensitisation to both, bee and wasp venom in the majority is based on cross-reactivity to either homologous proteins or CCDs.

Since the essential requirement in hymenoptera venom allergy diagnosis remains the differentiation between sensitisation to either of the venoms for correct treatment or in general the identification of the relevant allergens, different diagnostic approaches aim to specifically address these problems. One approach relies on the assessment of sensitisation in a cellular activation test, by direct stimulation of patient derived basophils with the respective allergen *in vitro*, mimicking the *in vivo* situation after a stinging event [61]. As this is a laborious and expensive procedure it is not applicable to standard diagnosis but might be utilised in exceptional situations or for research purposes. Another approach is the use of inhibition assays by mutual inhibition of IgE binding to an allergen with the respective cross-reactive allergen or extract [62]. This again is primarily an experimental approach and the data obtained sometimes are difficult to interpret. However, for an appropriate therapeutic decision, clinical history complemented by diagnostic results should provide the clinician with essential information. Hence, reliable key messages should result from the diagnostic effort.

Taken together, increasing recognition of the molecular pitfalls in allergy diagnosis, even if highly automated and standardised, demands improvement in terms of specificity and differentiation between relevant sensitisations and irrelevant reactivities solely based on cross-reactivity. One strategy to address these needs could be the use of recombinant single allergens in combination with automated quantitative diagnosis, an approach which has already been introduced for particular applications [63-65]. By use of such molecular approaches, problems due to protein and CCD based cross-reactivity as well as those arising from allergenic extracts might be pursued.

1.6 Molecular cross-reactivity

A considerable number of allergens are glycoproteins with glycostructures characteristic for their origin from plants or insects. Identical structures in different glycans are causative for carbohydrate based cross-reactivities and, therefore, are called CCDs. Cross-reactive glycoproteins are known from different foods (nuts, fruits and vegetables) as well as from insect venoms, latex and some fungi [66]. Although not necessarily found on every N-linked carbohydrate the causative structures in plants is constituted by alpha-1,3-fucose and beta-1,2-xylose [67], both attached to the core structure of N-linked carbohydrates (Fig. 2).

In insects, only the alpha-1,3-fucose residue is present and, similar to the xylose modification in plants, represents a highly immunogenic structure in men since it is absent in mammalian glycosylation. Due to their immunogenic nature these carbohydrate structures will induce the generation of specific IgG and in allergic individuals also of IgE antibodies. An IgE reactivity directed against such carbohydrate structures will therefore occur in every plant or insect derived extract containing glycoproteins even if an underlying protein related sensitisation does not exist.

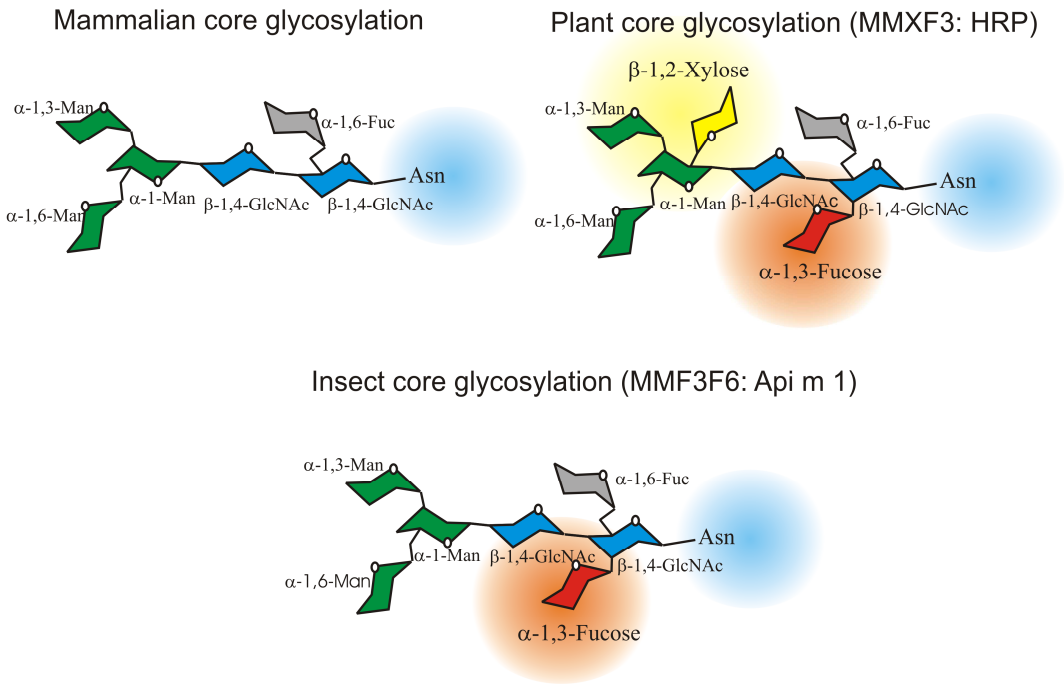

Figure 2: Schematic representation of mammalian, plant and insect N-linked core glycosylation
alpha-1,3-fucose residues are highlighted in red, beta-1,2-xylose residues in yellow; Man: mannose; GlcNac: N-acetylglucosamine; Fuc: fucose.

In hymenoptera venom allergy the rate of double-positive patients which have cross-reactive IgE for both honeybee and wasp venom is up to 30-40%, 70-80% of which being exclusively reactive on a carbohydrate level [68], and only a minority shows a true double-sensitisation or cross-reactivity on protein level. The hypothesis, that this exclusive CCD related reactivity can be dismissed as irrelevant is due to the low or not existing clinical significance of such a sensitisation as reports on clinical symptoms upon stimulation with non allergenic glycoproteins are scarce. Different studies have addressed this item, either by skin prick testing with non allergenic glycoproteins or deglycosylated allergens [69] or by direct stimulation of effector cells with such proteins [70, 71]. In summary, all used glycoproteins were no or only poor elicitors of allergic symptoms or cellular activation. As reason for this lack of clinical relevance, the valence of the used glycoproteins, affinity of anti-CCD IgE or existence of blocking IgG antibodies were discussed [72], however, the role of anti-CCD IgE antibodies in combination with protein specific IgE still remains elusive since a direct comparison of allergenic glycoproteins and defucosylated or dexylosylated ones yet has not been performed.

Even if the clinical relevance of CCDs is of minor relevance, their presence at least is cumbersome in allergy diagnosis since the determination of the relevant sensitisation is a prerequisite for proper treatment. Besides the already mentioned cross-inhibition approach a molecular tool for indicating potential cross-reactivities in standard diagnosis has been introduced. This marker for CCD specific IgE is a glycopeptide derived from the protein bromelain providing both alpha-1,3-fucose and beta-1,2-xylose residues and, therefore, enables detection of both kinds of specificities. Nevertheless, this marker only is suitable to indicate the presence of CCD specific IgE without improving the measurement of protein specific IgE.

In general, such ambiguity based on CCD dependent cross-reactivity is not limited to allergy diagnosis. All types of serological tests suffer from such cross-reactivities upon presence of the above mentioned glycostructures in a certain assay. Since glycoproteins of parasitic origin also provide alpha-1,3-fucose residues, serological assays for determination of parasite specific antibodies and sera of patients with helminth manifestations in all types of measurements may suffer from such cross-reactivity [73].

Another field of interaction potentially interfering with assay outcome beyond CCD based reactivity relates to binding of proteins within the sample either among each

other or to components utilised in the serological assay. Most prominent examples for such interfering molecules are heterophilic antibodies, human anti mouse antibodies (HAMA), or the rheumatoid factor (RF) [74]. While RF, an autoantibody directed against the Fc part of mammalian IgG, can interact with antibodies of diverse mammalian species, HAMA are directed against murine antibodies and found primarily in patients undergoing therapy with monoclonal antibodies of murine origin. Nevertheless, it can also be found in non treated individuals, and the presence of RF also is not limited to patients suffering from rheumatoid arthritis [75]. Heterophilic antibodies are defined as mostly lower affinity antibodies with specificity to the Fc part of diverse mammalian immunoglobulins [76]. A common assay that is often used in the context of allergy and which is affected by such interferences is the tryptase assay [77]. Elevated tryptase levels can indicate mastocytosis, which is a risk factor for severe allergic reactions e.g. in hymenoptera venom allergy.

Since all these molecular interferences are due to the high degree of conservation of the antibodies used in such assays a phylogenetically more distant immunoglobulin might fulfil the needs to reduce such interferences. An immunoglobulin which is not reacting with the aforesaid molecules due to its relatively large distance to those from mammals is the avian IgY antibody. By use of such antibodies assay interferences can markedly be reduced [78] and further advantages arise from the phylogenetic distance such as the broad spectrum of potential targets for immunisation, including conserved mammalian proteins.

1.7 Therapy of allergic disease

The avoidance of a certain allergen or allergenic source one is sensitised to obviously is the most intriguing way to prevent allergic syndromes but remains limited to those allergens which are not omnipresent in daily life such as some foods or pets. A symptomatic therapy by use of corticosteroids and antihistamines for repression of expression of certain chemokines and cytokines and smooth muscle relaxation particularly can be sufficient for seasonal allergens like pollen and in case of only weak syndromes. For severe allergic asthma the use of anti IgE antibodies (Omalizumab, Xolair®) is approved to reduce free IgE levels by formation of IgE/anti-IgE complexes [79].

In general, there is a plethora of different approaches targeting a variety of molecules in the complex network of allergic immune regulation and dysregulation. However, the only causative therapy for allergic disease is the specific immunotherapy (SIT) which was initially reported a century ago [80]. SIT relies on the repeated administration of the sensitising allergen by subcutaneous injection and modifies the responses of antigen presenting cells (APCs), T cells and B cells, as well as the number and the function of effector cells that mediate the allergic response. Furthermore, it induces the production of blocking antibodies of the IgG subclass [81].

Even if the mechanisms of SIT are still not known in detail one of the major events during SIT is a shift from a T_H2 to a T_H1 dominated T cell response and the induction of T regulatory cells (T_{reg}) [82] (Figure 4). In the T_H1 dominated milieu, class switch of allergen specific B cells to IgE is prevented and rather the production of IgG antibodies is promoted which can compete with IgE for binding to an allergen and, therefore, exert a blocking function [83]. Furthermore, T_{reg} cells fulfil diverse regulatory functions on T cells, B cells and effector cells [84].

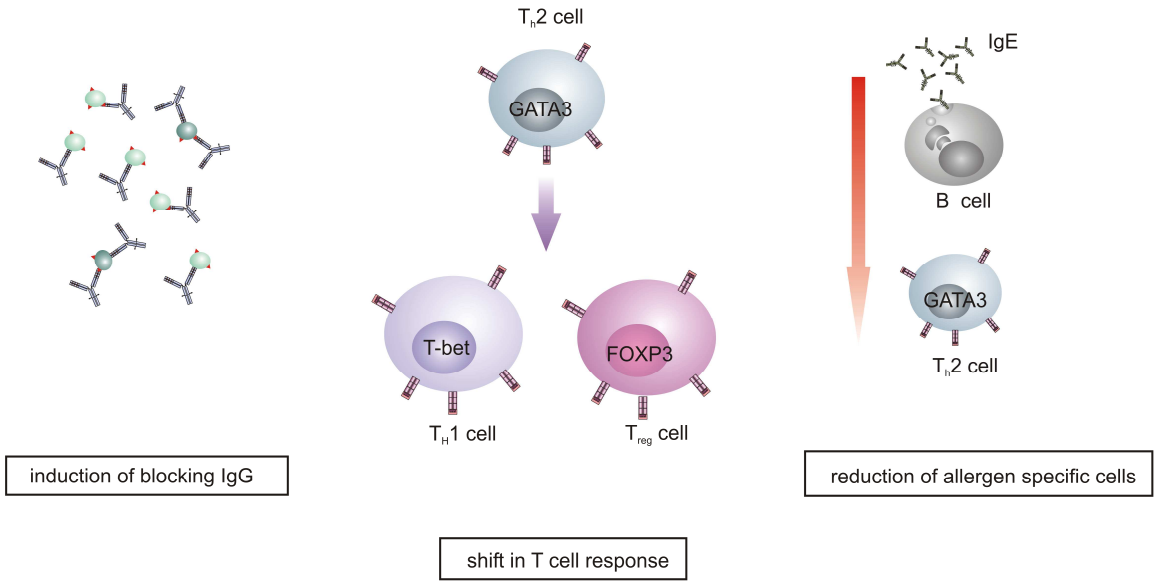

Figure 4: Schematic representation of immunological changes during specific immunotherapy
GATA3: essential transcription factor for T_H2 maintenance; T-bet: essential transcription factor for T_H1 maintenance; FOXP3: essential transcription factor for T_{reg} maintenance

The general efficacy and safety of SIT has been shown in numerous studies but strongly depends on the particular allergy and the therapeutic used. While in hymenoptera venom allergy efficacy is up to 90%, in house dust mite or cat dander allergy only low efficiencies were reported.

The original protocol for a specific immunotherapy involves regular subcutaneous injections of allergen extracts with an incremental dose increase reaching a maximal dose after 3-4 months. This build up phase with weekly injections is followed by a maintenance phase consisting of monthly injections with a maintenance dose which might be stopped after tolerance induction. Nevertheless, in some cases like hymenoptera venom allergy, the maintenance phase has to be extended to up to 3-5 years to achieve complete protection [85]. A limiting factor in SCIT is the possibility of severe reactions, mainly during build up phase, ranging from large local to anaphylactic reactions. These side effects and the long treatment schedule led to a limited acceptance of SCIT which, thereby, resulted in improved protocols, new treatment forms and modified therapeutics. Allergen extracts were chemically modified to allergoids having reduced side effects [86]. Furthermore, allergen extracts with sustained release were generated by coupling to aluminium hydroxide to reduce the number of injections. These developments resulted in modified treatment protocols designated rush and ultra rush SCIT which reach the maximal dose in three or even 1 day [87]. A further improvement is the introduction of the sublingual immunotherapy (SLIT) which is available for some pollen associated allergies and entirely avoids injection of the therapeutic agent [88].

Further developments were designed to substitute allergen extracts by recombinant allergens to circumvent side effects of particular extracts due to additional, non allergenic components. By use of extracts de novo sensitisation during SIT can not be avoided [89] and extracts poses serious problems from a regulatory point of view. The use of recombinant proteins further should allow for modifications yielding hypoallergenic proteins providing reduced IgE binding capacity. Amino acid substitutions in the IgE epitopes or other approaches of modification might be suitable to reduce adverse reactions during SIT, if the induction of blocking IgG antibodies is not affected [90].

However, all of these novel approaches depend on a precise knowledge about the allergens on a molecular level. The most advanced state to date is found in some pollen associated allergies, where such recombinant therapeutics have passed first clinical phases [91]. Nevertheless, in other fields, e.g. hymenoptera venom allergy, this knowledge needs to be extended to allow for improvement of our molecular understanding of the diagnostic performance and the therapeutic approaches.

Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody fragments

Kerstin Greunke, Edzard Spillner, Ingke Braren, Henning Seismann, Sabine Kainz, Ulrich Hahn, Thomas Grunwald, Reinhard Bredehorst

J Biotechnol. 2006 Jul 13;124(2):446-56. Epub 2006 Feb 21

Abstract: Monoclonal IgY have the potential to become unique tools for diagnostic research and therapeutic purposes since avian antibodies provide several advantages due to their phylogenetic difference when compared to mammalian antibodies. The mechanism of avian immunoglobulin gene diversification renders chicken an excellent source for the generation of recombinant scFv as well as Fab antibody libraries of high diversity. One major limitation of these antibody fragments, however, is their monovalent format, impairing the functional affinity of the molecules and, thereby, their applicability in prevalent laboratory methods. In this study, we generated vectors for conversion of avian recombinant antibody fragments into different types of bivalent IgY antibody formats. To combine the properties of established mammalian monoclonal antibodies with those of IgY constant domains, we additionally generated bivalent murine/avian chimeric antibody constructs. When expressed in HEK-293 cells, all constructs yielded bivalent disulfide-linked antibodies, which exhibit a glycosylation pattern similar to that of native IgY as assessed by lectin blot analysis. After purification by one step procedures, the chimeric and the entire avian bivalent antibody formats were analyzed for antigen binding and interaction with secondary reagents. The data demonstrate that all antibody formats provide comparable antigen binding characteristics and the well established properties of avian constant domains

 PMID: 16490273; doi:10.1016/j.jbiotec.2005.12.032

Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries

Ingke Braren, Simon Blank, Henning Seismann, Susanne Deckers, Markus Ollert, Thomas Grunwald, Edzard Spillner

Clin Chem. 2007 May;53(5):837-44. Epub 2007 Mar 29

Abstract: BACKGROUND: Allergen-specific IgE and IgG antibodies play pivotal roles in the induction and progression of allergic hypersensitivity reactions. Consequently, monoclonal human IgE and IgG4 antibodies with defined specificity for allergens should be useful in allergy research and diagnostic tests. We used combinatorial antibody libraries and subsequent recombinant production to make and assess IgE, IgG1, and IgG4 allergen-specific antibodies. METHODS: We used phage display to select a synthetic single-chain antibody fragment (scFv) library against 3 different allergens, from bee venom, bovine milk, and apple. The scFv obtained were converted into IgG1, IgG4, and IgE antibody formats and assessed for their biochemical properties by ELISA, immunoblotting, and fluorescence-activated cell sorting. RESULTS: Two different antibody formats for each IgG1, IgG4, and IgE antibody were produced in mammalian cells as disulfide-linked and glycosylated Ig, which were usable in allergen-specific ELISA assays and immunoblots. In addition, the recombinant IgE antibodies mediated the binding of allergens to HEK-293 cells transfected with the high-affinity IgE receptor, and this binding was blocked by corresponding IgG antibodies. CONCLUSIONS: The use of synthetic libraries for the generation of allergen-specific recombinant IgE and IgG antibodies should have broad applications in allergological research and diagnosis.

Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of α-1,3-core fucosylation

Henning Seismann, Simon Blank, Ingke Braren, Kerstin Greunke, Liliana Cifuentes, Thomas Grunwald, Reinhard Bredehorst, Markus Ollert and Edzard Spillner

Mol Immunol. 2009 Nov 5. [Epub ahead of print]

Abstract: Hymenoptera venom allergy is known to cause life-threatening and sometimes fatal IgE-mediated anaphylactic reactions in allergic individuals. About 30-50% of patients with insect venom allergy have IgE antibodies that react with both honeybee and yellow jacket venom. Apart from true double sensitisation, IgE against cross-reactive carbohydrate determinants (CCD) are the most frequent cause of multiple reactivities severely hampering the diagnosis and design of therapeutic strategies by clinically irrelevant test results. In this study we addressed allergenic cross-reactivity using a recombinant approach by employing cell lines with variant capacities of alpha-1,3-core fucosylation. The venom hyaluronidases, supposed major allergens implicated in cross-reactivity phenomena, from honeybee (Api m 2) and yellow jacket (Ves v 2a and its putative isoform Ves v 2b) as well as the human alpha-2HS-glycoprotein as control, were produced in different insect cell lines. In stark contrast to production in Trichoplusia ni (HighFive) cells, alpha-1,3-core fucosylation was absent or immunologically negligible after production in Spodoptera frugiperda (Sf9) cells. Consistently, co-expression of honeybee alpha-1,3-fucosyltransferase in Sf9 cells resulted in the reconstitution of CCD reactivity. Re-evaluation of differentially fucosylated hyaluronidases by screening of individual venom-sensitised sera emphasised the allergenic relevance of Api m 2 beyond its carbohydrate epitopes. In contrast, the vespid hyaluronidases, for which a predominance of Ves v 2b could be shown, exhibited pronounced and primary carbohydrate reactivity rendering their relevance in the context of allergy questionable. These findings show that the use of recombinant molecules devoid of CCDs represents a novel strategy with major implications for diagnostic and therapeutic approaches.

 PMID: 19896717; doi:10.1016/j.molimm.2009.10.005

3 Further Results

3.1 Materials and Methods

3.1.1 Materials

Wasp venom (*Vespula spp.*) of immunotherapeutic grade (Venomil), which is obtained by venom sac extraction, was purchased from Bencard Allergy (Munich, Germany). Purified native Api m 1 was purchased from Latoxan (Valence, France). Anti-V5 antibody was purchased from Invitrogen. Polyclonal rabbit anti-HRP serum was obtained from Sigma (Taufkirchen, Germany). AlaBLOTs and the CCD marker MUXF-BSA were obtained from Siemens Healthcare Diagnostics (Los Angeles, USA). The monoclonal alkaline phosphatase conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). Recombinant Api m 5 expressed in insect cells and as a prokaryotic maltose binding protein (MBP) fusion protein was present in the workgroup and was provided by Simon Blank (University of Hamburg).

Sera from insect-venom allergic patients were provided from Prof. Dr. Markus Ollert (Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy, Biederstein, Technische Universität München, Germany), three groups of sera were obtained without further preselection from the institutional serum bank: (i) Sera with a positive sIgE test to wasp venom extract (i3: sIgE $\geq$0.35 kU/L); (ii) Sera with a positive sIgE test to wasp venom venom extract (i3: sIgE $\geq$0.35 kU/L) and honeybee venom (i1: sIgE $\geq$0.35 kU/L); (iii) Control sera with a negative sIgE test to either honeybee or wasp venom extract (i1 and i3: sIgE <0.35 kU/L). All sera of the first two groups were derived from patients with a history of a systemic allergic reaction after a stinging event. Specific IgE tests for honeybee or wasp venom were performed in all patients on the automated immunoassay systems UniCAP250 (Phadia, Uppsala, Sweden)) or Immulite2000 (Siemens Healthcare Diagnostics) as described in detail elsewhere [92]. All patients had given their informed written consent for additional blood samples to be drawn.

3.1.2 Cloning of the Ves v 3 cDNA

Total RNA was isolated from the separated stinger with attached venom sack and additional glands from *Vespula vulgaris* using peqGold TriFast™ (Peqlab

Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to synthesise cDNA from the isolated total RNA. RNaseOut™ recombinant ribonuclease inhibitor (1 µl) (Invitrogen) was added to the standard 20 µl reaction mix containing 5 µl venom gland RNA. Reverse transcription was performed at 50 º C for 60 minutes. First strand cDNA was used as a template for PCR amplification of Ves v 3 DNA sequences.

Due to the lack of genomic data for *V. vulgaris* a C-terminal fragment of Ves v 3 was amplified from venom gland cDNA using the oligonucleotides 5´-CCN ATG CTN GTN AAY GTN TAY GCN-3´ as deduced from peptides obtained by MS/MS sequencing and oligo-dT back. After sequence determination of subcloned cDNA fragments the oligonucleotide 5´-GTG AGC GTG AGA CAG ACT G-3´ was deduced from the sequence and used for reverse transcription as described before. The cDNA was used for 5´RACE employing the 5´/3´RACE Kit, Second Generation (Roche, Mannheim, Germany) according to the recommendations of the manufacturer. Subsequent nested PCR was performed using the primers 5´-CAC GCT ACG ATT CGT TGT C-3´ and 5´-CAA CGT ATT TGG TCC AGC G-3´. The obtained cDNA fragments were used as a basis for further sequence determination. Full length cDNA was then amplified using the forward primer 5´-ATG GTT CCA CTA CGA AGT TTC G-3´ and the reverse primer 5´-GTG AGC GTG AGA CAG ACT G-3´. DNA from the PCR reaction was isolated from 1 % agarose gels (peqGOLD universal agarose, Peqlab) using the peqGOLD Gel Extraction Kit (Peqlab). Subcloning for sequencing was done using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with pCR-Blunt II-TOPO vector. *Escherichia coli* of the strain XL1Blue were transformed with the ligated DNA by electroporation (2 mm cuvettes, EasyJect+; Eurogentec, Seraing, Belgium) and selected on ampicillin agar plates. After sequencing of selected full length cDNA clones and verification of the sequence, the clone was used for secondary amplification of the mature chain coding region with *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) using primers incorporating 5´ *BamH*I and 3´ *Not*I restriction sites as well as a 3´ V5 epitope and a 10 fold His-tag. The PCR product was subcloned into the *BamH*I and *Not*I digested baculovirus transfer vector pACGP67-B after digest with the respective enzymes. For cloning into the prokaryotic expression vector pMAL-c2X (NEB, Frankfurt, Germany) a 3´ *EcoR*I and a 5´ *Sac*II site were added by PCR.

3.1.3 Cloning of the Ves v 1 and Ves v 5 cDNA

Full length Ves v 1 was amplified from *Vespula vulgaris* venom gland cDNA with *Pfu* DNA polymerase (Fermentas) using the primers 5'-GGA CCC AAA TGT CCT TTT AAT TC -3' and 5´-AAC CGC GGT TAA ATT ATC TTC CCC TTG TTA-3´. DNA from the PCR reaction was isolated from 1 % agarose gels (peqGOLD universal agarose, Peqlab) using the peqGOLD Gel Extraction Kit (Peqlab). Subcloning for sequencing was done using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with pCR-Blunt II-TOPO vector. Full length Ves v 5 was amplified and cloned in the same way employing the primers 5´-AAC AAT TAT TGT AAA ATA AAA TGT TTG AAA-3´ and 5´-CTT TGT TTG ATA AAG TTC CTC-3´ for amplification from venom gland cDNA.

After sequencing of selected subcloned cDNA clones and verification of the sequence, the clone was used for secondary amplification of the mature chain coding region with *Pfu* DNA polymerase (Fermentas) in two consecutive PCR reactions adding an N-terminal 10-fold His-tag and V5 epitope as well as a 5´ *BamH*I and 3´ *Not*I restriction sites. The PCR product was subcloned into the *BamH*I and *Not*I digested baculovirus transfer vector pACGP67-B (BD Pharmingen, Heidelberg, Germany) after restriction digest with *BamH*I and *Not*I.

3.1.4 Site directed mutagenesis

For generation of an inactive Ves v 1 form two amino acid residues in the potential active site were altered by site directed mutagenesis, using the QuikChange Site directed mutagenesis Kit (Stratagen, La Jolla, USA) according to the recommendation of the manufacturer employing the primers 5´-CGA TTA ATT GGA CAT GGC TTA GGA GCA CAT G-3´ and 5´-CAT GTG CTC CTA AGC CAT GTC CAA TTA ATC G-3´ for serine 137 to glycin exchange and 5´-GAAA TTATT GGG CTT GCT CCT GCT AGGCCT T3´ and 5´- AAG GCC TAG CAG GAG CAA GCC CAA TAA TTT C-3´ for the aspartic acid 165 to alanine exchange.

3.1.5 Recombinant bacterial expression and purification of allergens

For expression of Ves v 3 in *E. coli*, the cDNA was cloned into the prokaryotic expression vectors pMAL-c2X (NEB). Expression in *E. coli* XL1 Blue cells and purification of the fusion protein via amylose resin (NEB) were performed according to the recommendations of the manufacturers.

3.1.6 Recombinant baculovirus production

Spodoptera frugiperda Sf9 cells (Invitrogen) were grown at 27 °C in ser um-free medium (Express Five SFM, containing 16.5 mM glutamine and 10 µg/ml gentamycin; Invitrogen). Cell density was determined by haemocytometer counts, cell viability was evaluated by staining with Trypan Blue. Recombinant baculovirus was generated by cotransfection of Sf9 cells with BaculoGold bright DNA (BD Pharmingen) and the baculovirus transfer vector pACGP67-B Ves v 1, Ves v 3 or Ves v 5 respectively according to the recommendations of the manufacturer. High titer stocks were produced by three rounds of virus amplification and optimal MOI for recombinant protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flask (1.5-2x10^6 cells/ml in 20 ml suspension culture) with serial dilutions of high titer virus stock.

3.1.7 Expression in baculovirus-infected insect cells

High titer stocks of recombinant baculovirus containing the Ves v 3, Ves v 1 or Ves v 5-coding DNA respectively were used to infect Sf9 or HighFive cells (Invitrogen) (1.5-2.0x10^6 cells per ml) in a 2000 ml suspension flask (400 ml suspension culture). For protein production the cells were incubated at 27 °C and 110 rpm for 72 h.

3.1.8 Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8 and centrifuged at 4000 x g for 5 minutes. Supernatants were applied to a nickel-chelating affinity matrix (NTA-agarose, Qiagen). The column was washed with NTA-binding buffer (50 mM sodium phosphate, pH 7.6, 500 mM NaCl) and pre-eluted with NTA-binding buffer containing 20 mM imidazole. The recombinant protein was eluted from the matrix

with NTA-binding buffer containing 300 mM imidazole. Purification was confirmed by SDS-PAGE.

3.1.9 Biophysical analysis of rVes v 5

Dynamic light scattering was performed using a Spectroscatterer 201 (RiNA GmbH, Berlin, Germany) at a concentration of 0.12 mg/ml in 50 mM sodium phosphate, 150 mM NaCl , pH 7.6. CD-spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Groß-Umstadt, Germany) at a protein concentration of 0.015 mg/ml in 50 mM sodium phosphate, 150 mM NaCl , pH 7.6.

3.1.10 Measurement of DPPIV activity of rVes v 3

The DPPIV activity of the native and recombinant enzyme was assessed as follows. Recombinant Ves v 3 from baculovirus expression at a concentration of 200 ng/ml in NTA-binding buffer containing 300 mM imidazole and wasp venom at a concentration of 100 µg/ml in 50 mM sodium phosphate, pH 7.6, 100 mM NaCl were used for activity testing. The synthetic DPPIV substrate glycine-proline p-nitroanilide hydrochloride (Sigma) was applied at a final concentration of 0.5 mM and DPPIV activity was assayed at 405 nm using a spectrophotometer. For determination of the IC50 values, activity assays were repeated using serial dilutions of the specific DPPIV inhibitor Diprotin A (Sigma) ranging from 2.5 mM to 1.25 µM final concentration. All values were measured in triplicates. Non-linear regression curves and inhibition values were calculated using Prism 3.0 (Graphpad Software).

3.1.11 Measurement of phospholipase activity of rVes v 1

The enzymatic activity of the recombinant Ves v 1 was assessed by use of the EnzChek Phospholipase A1 Assay Kit (Invitrogen) according to the recommendation of the manufacturer. In brief rVes v 1 was diluted in 1x PLA1 reaction buffer to a final concentration of µg/ml. To 50 µl of the diluted rVes v 1 50 µl of a substrate-liposome mix containing dioleoylphosphatidylcholine, dioleoylphosphatidylglycerol and PLA1 substrate were added. After a 30 minute incubation in the dark fluorescence emission was measured at 535 nm.

3.1.12 IgE immunoreactivity of patient sera with rVes v 1, rVes v 3 and rVes v 5

For assessment of specific IgE immunoreactivity of human sera with purified recombinant Ves v 1, Ves v 3 or Ves v 5 in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with 20 µl of purified recombinant protein (20 µg/ml) at 4 °C overnight and blocked with 40 mg/ml MPBS at room temperature. Afterwards human sera were diluted 1:1 with 5 mg/ml BSA in PBS and incubated in a final volume of 20 µl for 4 hours at room temperature. Wells were washed 4 times with PBS before bound IgE was detected with a monoclonal alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD Pharmingen) diluted 1:1000. Wells were again washed 4 times with PBS and 50 µl of substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) per well were added. After 30 minutes the plates were read at 405 nm. The cut-off was calculated as the mean of the negative controls plus 2 SDs. This resulted in a working range of $OD_{405nm} \geq 0.32–3$. For reasons of precision, reactivities only slightly higher than the cut-off value were excluded for both allergens ($OD_{405nm} \geq 0.32<0.40$). Thus, an effective working range of $OD_{405nm} \geq 0.40–3$ was applied in all IgE ELISAs used for allergen identification. For immunoblot procedures human sera were diluted 1:10 with 5 mg/ml BSA in PBS and applied to the corresponding AlaBLOTs (i3 *V. vulgaris* venom; Siemens Healthcare Diagnostics) or to the purified recombinant allergen, separated by SDS-PAGE and immobilised onto nitrocellulose membranes. Visualisation of bound IgE was then performed with monoclonal anti-human IgE conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate according to recommendations of the manufacturer.

3.1.13 Basophil activation with rVes v 3

The basophil activation test was performed as described previously [61] with modifications as recommended by the manufacturer of the assay (Flow-CAST; Bühlmann Laboratories, Schönenbuch, Switzerland). In brief, within 3 h after sampling of patient blood in endotoxin free EDTA tubes, aliquots of 50 µl whole blood were pre-incubated for 10 min at 37 °C with stimulation buffer containing IL-3. Subsequently, basophils were activiated for 40 min at 37 °C in a water bath with various concentrations of either native or recombinant allergens at a volume of 100 µl. Thereby, honey bee or wasp venom (in a concentration range of 0.25 ng/ml to 25 ng/ml) as well

as the recombinant allergens (Ves v 3 and Api m 5, for comparison Ves v 1, Ves v 2, and Ves v 5, all in a concentration range of 0.001 ng/ml to $2x10^3$ ng/ml) were applied in concentrations according to those reported in literature. As positive stimulation control served a murine monoclonal antibody against the human high affinity Fcε receptor (FcεRI) (Bühlmann Laboratories). Plain stimulation buffer was used as negative stimulation control. The optimal stimulation time and temperature were determined in preliminary experiments. To quantify activated basophils, cells were stained with 20 µl reagent containing a mixture of monoclonal antibodies to human CD63 labelled with phycoerythrin (anti-CD63-PE) and to human IgE labelled with fluoresceine isothiocyanate (anti-IgE-FITC) for 30 min on ice. Red blood cells were lysed and white blood cells were fixed (FACS Lysing solution, BD Biosciences) for 5 min at room temperature. After centrifugation (5 min, 1200xg) cells were resuspended in 500 µl of stop solution. Flow cytometric analysis of basophil activation was performed on a FACScan flow cytometer (BD Immunocytometry Systems). IgE-staining and side scatter were employed to gate on at least 500 basophils that expressed high density of surface IgE. Subsequently, within this gate the percentage of activated basophils, i.e. coexpressing CD63, was measured.

3.1.14 Basophil activation with rVes v 1 and rVes v 5

The basophil activation test was essentially performed as described above. Stimulation with recombinant Ves v 1 and Ves v 5 was performed at protein concentrations of 0.1, 200 and 2000 ng/ml and 50ng/ml for control stimulation with wasp venom.

3.1.15 Other methods

SDS-PAGE, Western blotting and ELISA as well as standard procedures in molecular biology were performed according to established protocols [93]. The recombinant venom allergens Ves v 1, Ves v 2, and Ves v 5 were cloned, expressed and purified according to established procedures [41]. The chimeric human IgE antibody against Api m 5 was generated essentially as described recently [94].

3.2 Results

3.2.1 Recombinant Ves v 1 for improved diagnosis of hymenoptera venom hypersensitivity

Since diagnosis in hymenoptera venom allergy is severely hampered by molecular cross-reactivities, especially emanating from cross-reactive carbohydrates, a possible approach for improvement could rely on the use of non glycosylated major allergens with high IgE prevalence which are unique for the particular venom. In wasp venom the major allergens Ves v 1 and Ves v 5 fit to those criteria and a high IgE prevalence has been demonstrated with either recombinant [95] or native proteins. For the phospholipase recombinant approaches were not successful so far [37]. Therefore, the aim was the recombinant expression and a thorough analysis of Ves v 1 in combination with the well characterised allergen Ves v 5.

3.2.1.1 cDNA cloning and recombinant expression in eukaryotic insect cells

For recombinant production of the wasp venom allergens Ves v 1 and Ves v 5 the particular cDNA was amplified from wasp venom gland cDNA. Subsequently, full-length Ves v 1 and Ves v 5 were produced in insect cells as secretory proteins. Wild type Ves v 1 was expressed and served as an indicator of proper folding, however, to avoid potential detrimental effects on expression yields a mutant of phospholipase A1, Ves v 1 S137G/N165A, lacking phospholipase activity was generated by site directed mutagenesis. Thereby, both variants of Ves v 1 were yielded in comparable amounts suggesting that the phospholipase activity exerts no adverse effect on expression. The epitope-tagged rVes v 1 and rVes v 5 obtained with yields of approx. 0.2 µg and 1.5 µg, respectively, per ml of culture supernatant exhibited an apparent molecular mass of approx. 37 kDa and 27 kDa, corresponding to the native proteins in venom (Fig. 3).

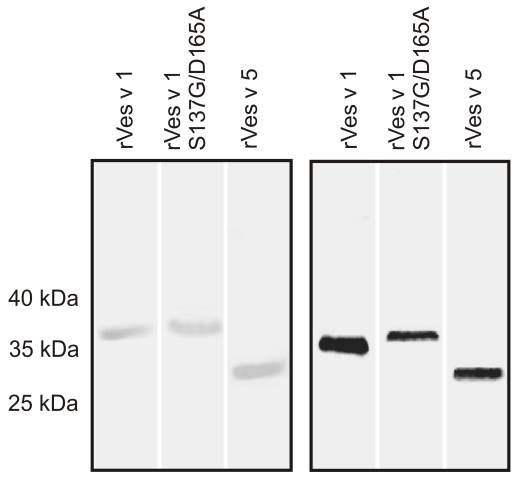

Figure 3: SDS-PAGE and Immunoblot of rVes v 1 and rVes v 5

Left panel: SDS-PAGE analysis of recombinant allergens (lane 1: rVes v 1; lane 2: rVes v 1 S137G/D165A; lane 3: rVes v 5). Right panel: Immunoblot analysis of recombinant allergens with anti-V5 epitope antibody (Invitrogen) (lane 1: rVes v 1; lane 2: rVes v 1 S137G/D165A; lane 3: rVes v 5).

3.2.1.2 Biochemical characterisation of rVes v 1 and rVes v 5

Due to the lack of inherent enzymatic activity, the physicochemical nature of rVes v 5 was addressed by biophysical methods. Oligomerisation of *E. coli* derived rVes v 5 has been postulated [96], however, insect cell derived rVes v 5 exhibited clear monodispersity with a hydrodynamic radius of 2.6 +/- 0.41 nm as assessed by DLS measurements (Fig. 4A). Furthermore, the structural features of rVes v 5 as assessed by CD spectroscopy (Fig. 4B) were identical to those reported for nVes v 5 [97].

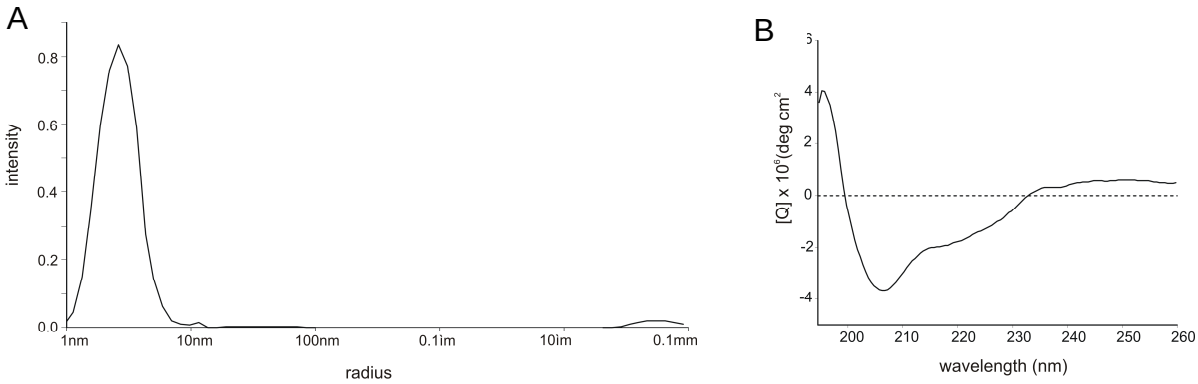

Figure 4: DLS measurement and circular dichroism spectroscopy of rVes v 5

(A) Dynamic light scattering measurements were carried out using the Spectroscatterer 201 (RiNA GmbH) with a He–Ne laser at 20 °C. Protein concentration of rVes v 5 was 0.12 mg/ml in 50 mM sodium phosphate, pH 7.6. CD spectra were recorded with a Jasco J-715 spectropolarimeter (Jasco) at 20 °C. (B) For CD-spectroscopy protein concentration was 0.015 mg/ml in 50 mM sodium phosphate, pH 7.6. The CD spectrum for rVes v 5 with a minimum at 208 nm and a shoulder at 225 nm was superimposable to data reported for native Ves v 5.

For the rVes v 1 protein, functionality could be addressed by determining the inherent phospholipase activity using an enzymatic assay (Fig. 5). The specific activity of the wild type protein was determined to be approx. 2.5 U/ml at a concentration of 10 µg per ml. As anticipated, the mutant rVes v 1 S137G/N165A did not exhibit enzymatic activity. The biophysical data and the enzymatic activity clearly suggest proper folding of both insect cell produced proteins and, furthermore, corroborate the idea that insect cells are an ideal host for expression of hymenoptera venom proteins.

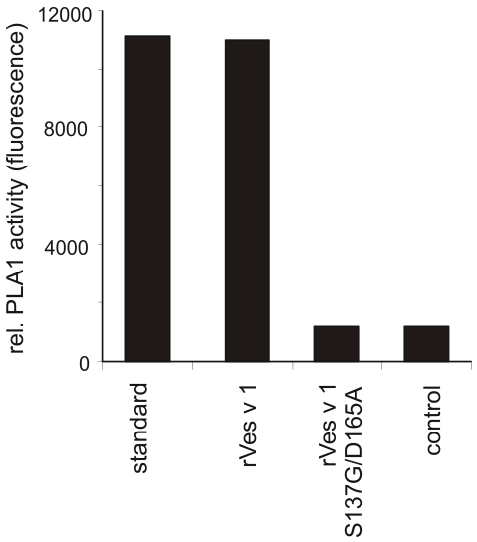

Figure 5: Enzymatic activity of rVes v 1

Phospholipase activity assay of rVes v 1 and rVes v 1 S137G/D165A. The recombinant proteins were used at a concentration of 10 µg/ml, the standard (Lecithase® ultra) at 2.5 U/ml.

Furthermore, human basophils isolated from whole blood of venom allergic patients were stimulated with rVes v 1 and rVes v 5, whereby the inactive rVes v 1 S137G/N165A mutant was employed to avoid unspecific basophil degranulation through phospholipase activity. Stimulation with wasp venom at a concentration of 50 ng/ml served as control. Clear cellular activation as quantified by CD63 upon stimulation with the recombinant allergens could be observed over a concentration range from 0.1 ng/ml to 2 µg/ml (Fig. 6). These data corroborate proper folding and correct molecular characteristics of the recombinant allergens but also underline their applicability for cellular approaches in hymenoptera venom diagnosis.

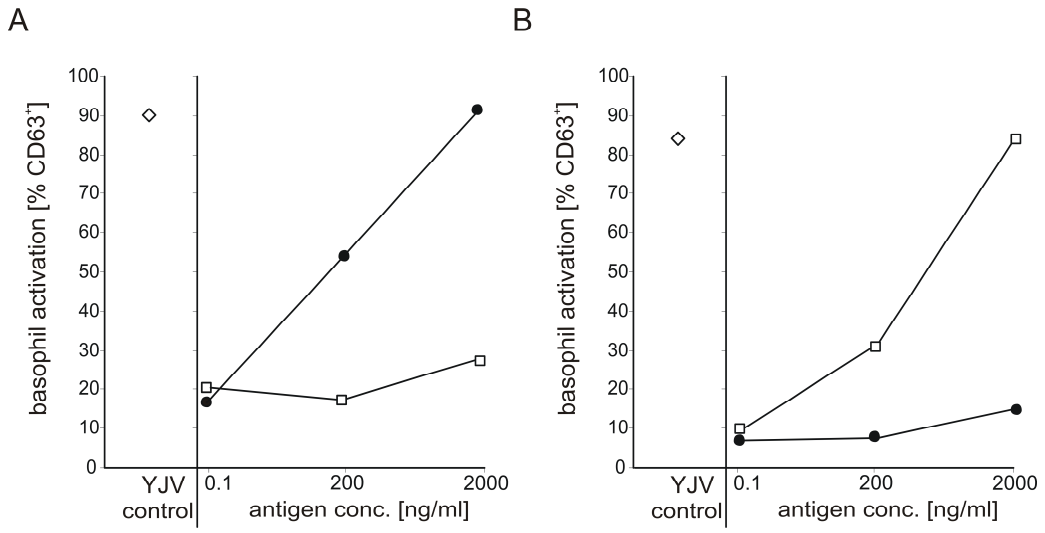

Figure 6: Basophil activation

Basophils from two wasp venom sensitised patients (A and B) were stimulated with 0.1–2000 ng/ml of rVes v 1 (open squares) and rVes v 5 (filled circles). Activation was determined by CD63 upregulation in FACS. Control stimulation was performed with 50 ng/ml wasp venom (diamonds).

3.2.1.3 Immunoreactivity of rVes v 1 and rVes v 5

To study the immunoreactivity and diagnostic relevance of rVes v 1 and rVes v 5, individual patient sera of 34 patients with a positive sIgE test to either honeybee venom and wasp venom or wasp venom only were assayed by ELISA for specific IgE antibodies. To provide a broad reactivity profile and allow for assignment of sensitisation, nApi m 1, considered a surrogate marker for sensitisation to *A. mellifera* venom, and the CCD marker MUXF-BSA were included.

Of the 20 double-positive sera (Fig. 7A), 15 showed reactivity to rVes v 1, 9 of which additionally had specific IgE to rVes v 5. Interestingly, only 1 out of these 20 sera had sIgE to rVes v 5 exclusively, while 2 sera exhibited reactivity to Api m 1. In this group an overall diagnostic coverage of 80% could be achieved by use of two wasp venom allergens, compared to 40% when using rVes v 5 solely.

Of the remaining 4 patients 2 had sIgE for nApi m 1 and 1 was reactive to the CCD marker MUXF-BSA only. Thus, for 16/20 patients a particular culprit venom could convincingly be assigned (Fig. 7C) whereas 2 patients showed a true double-sensitisation. Only 1 patient showed no reactivity to any of the proteins.

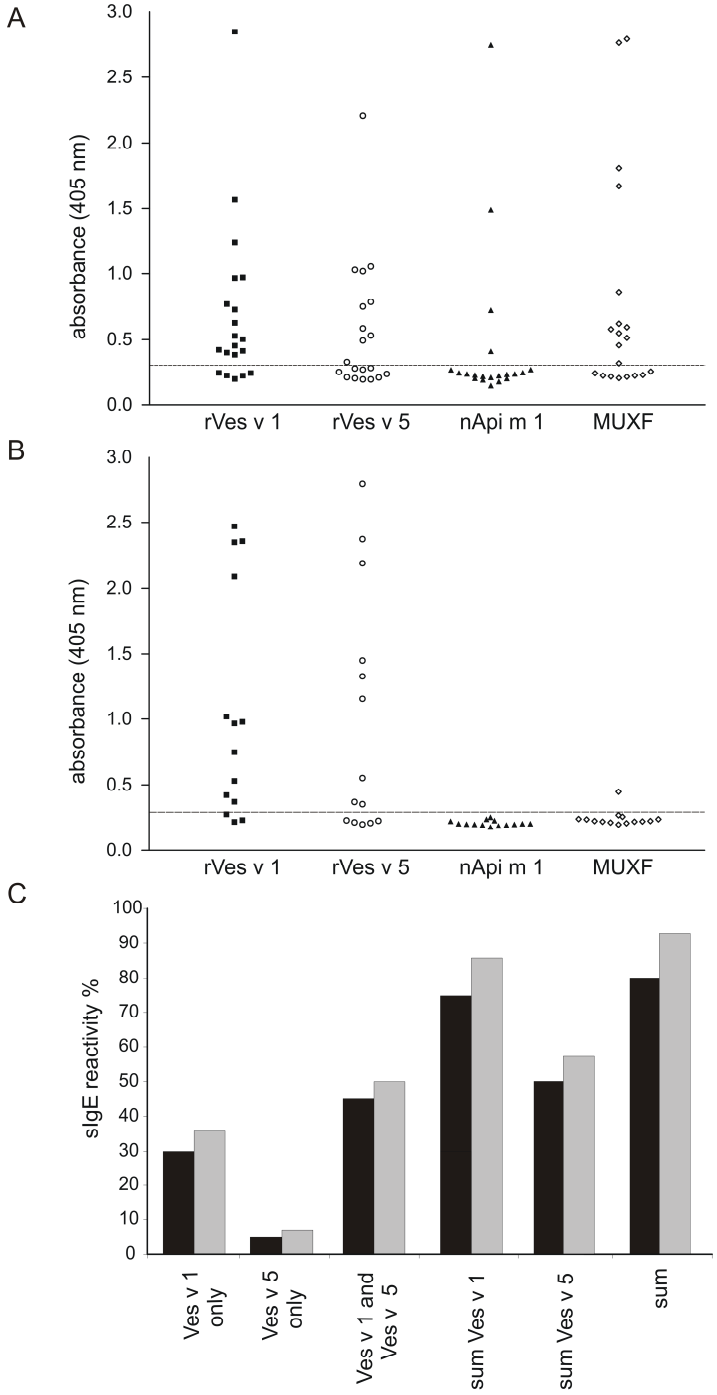

Figure 7: IgE reactivity of patient sera

IgE reactivity of individual sera from double-positive (A) or wasp venom-positive (B) patients to rVes v 1, rVes v 5, nApi m 1, and MUXF-BSA. The cut-off of the ELISA is indicated by a dashed line (mean background plus two fold the SD). The percentage of sIgE reactivity to either each allergen or both allergens is represented in C for double-positive (black bars) or wasp venom-positive (grey bars) patients.

In the wasp venom mono-sensitised group (Fig. 7B) 12 of 14 sera were reactive to rVes v 1, 8 of which exhibited additional sIgE reactivity to rVes v 5. One further patient showed sIgE reactivity exclusively to rVes v 5. Thus, 13/14 had detectable sIgE either to rVes v 1, rVes v 5 or both (Fig. 7C) while 1 patient with low total wasp venom sIgE showed no reactivity. In accordance with the sensitisation of this group, no reactivities to nApi m 1 were observed, however, 1 patient exhibited low sIgE reactivity to the CCD marker.

These data demonstrate that recombinant Ves v 1 is an essential component to assess the sensitisation of individuals to wasp venom and its recombinant availability complemented with Ves v 5 and Api m 1 allows for clear assignment of sensitisation patterns.

3.2.1.5 Implications of the presented data

Standard diagnostic approaches in allergy but particularly in hymenoptera venom allergy are often characterised by multiple IgE reactivities affecting the interpretation of ambiguous results and the correct choice of the proper venom for immunotherapy, a prerequisite for efficient therapy [98].

Causative for this phenomenon are IgE binding either to peptide epitopes of closely related or homologous proteins or to conserved carbohydrate structures of otherwise unrelated glycoproteins. Obviously, the more common incidence in hymenoptera venom allergy is the latter one, the molecular basis of which could be attributed to α-1,3-core-fucose and, exclusively in plants [67], ß-1,2-xylose. These residues are absent in mammalian glycosylation and, therefore, constitute a highly immunogenic epitope in men. In contrast to protein-directed cross-reactivity, the carbohydrate-directed reactivity in food and hymenoptera venom allergy is mainly believed to be clinically irrelevant, but diagnostically cumbersome [69, 99].

A sophisticated method to identify and circumvent such reactivities are inhibition tests based on mutual inhibition of IgE by venom of the particular species [100]. However, these tests are not applicable in standard diagnosis and the obtained data difficult to interpret. Hence, the more advanced and promising option relies on the use of unique recombinant major allergens which are representative for the respective venom and fulfil all criteria regarding high prevalence and low cross-reactivity of both types.

For honeybee venom, phospholipase A2 (Api m 1) is considered an ideal surrogate marker as it shows a high prevalence of sIgE recognition [95]. Other proteins in honeybee venom had to be excluded due to presence of multiple sites and confirmed CCD reactivities. Wasp venom contains two non-glycosylated major allergens without cross-reactive homologues in other species, Ves v 1 and Ves v 5, both showing high IgE prevalence, as shown for proteins purified from venom [101]. Allergens like Ves v 2 a and b and Ves v 3 do not meet those criteria [102, 103].

The phospholipase A2 from honeybee venom can be purified from the easy to obtain venom, nevertheless, recombinant production has been shown in bacteria and enzymatic and biological activity in terms of effector cell activation was demonstrated to be comparable to the native protein [104] In contrast to Api m 1 and Ves v 5 [97, 105] and to the best of our knowledge, no expression of functional phospholipase A1 from wasp venom has been reported so far [37]. However, the approach used in this attempt, production of hymenoptera venom allergens in a nearly autologous system, yielded Ves v 1 for the first time as a soluble and enzymatically active molecule. Interestingly, expression of the active enzyme appears not to be detrimental for the host cells, as shown with the inactive variant of Ves v 1. Biophysical and biochemical measurements of both recombinant Ves v 1 and Ves v 5 were entirely in accordance with the activity and native folding. Under the aegis of insect cell-mediated folding and expression the allergenic characteristics were compatible even with activation of human basophils, fitting best to the pathophysiological situation.

By use of rVes v 1 and rVes v 5 a true sensitisation to wasp venom for 80% of the patients could be confirmed, while a true double-sensitisation was verified in only 6% of these patients as indicated by additional sIgE reactivity to nApi m 1. Thereby, the prevalence of CCD reactive patients was approx. 60% in the double-positive cohort which is in accordance with the literature [98] and can be assumed the only reason for cross-reactivity.

In summary, the use of defined recombinant major allergens like Ves v 1 and Ves v 5 provides a significant improvement for the identification of the culprit venom which is indispensable for the choice of the appropriate immunotherapeutic strategy. For the first time implementation of rVes v 1 to routine diagnosis may allow to assess its IgE prevalence beyond estimations from immunoblot studies. Furthermore, recombinant allergens may provide new insights into the role and relevance of particular venom compounds during sensitisation and hyposensibilisation.

3.2.2 Identification, recombinant expression and characterisation of the high molecular weight wasp venom allergen Ves v 3

Even if a considerable number of allergens have already been investigated in hymenoptera venoms, some components with unknown relevance still remain elusive. One such component is the 100 kDa honeybee venom allergen Api m 5, currently described in our workgroup, for which a dipeptidylpeptidase activity could be allocated. In wasp venom as well a 100 kDa protein with known IgE reactivity is present and dipeptidylpeptidase activity could be demonstrated for this venom, which renders the existence of a corresponding protein in wasp venom possible. Therefore the aim was to verify the presence of such a protein in wasp venom and to elucidate its sequence. Furthermore, recombinant expression and characterisation in terms of IgE reactivity should verify the allergenic nature of this protein.

3.2.2.1 Identification and molecular cloning of Ves v 3

Since venoms of both *A. mellifera* and *V. vulgaris* contain a prominent 100 kDa band detected by sIgE in individual and pooled sera of sensitised patients (Fig. 9) we aimed for identification of the Api m 5 homologue in *Vespula spp*. venom. MS/MS approaches yielded peptide sequences with a high degree of identity to Api m 5. Based on this information oligonucleotides were employed to amplify fragments from cDNA of *V. vulgaris* venom glands. Subsequent 5´RACE approaches finally led to the cloning of the full length ORF and contiguity was verified by amplification of the entire cDNA. The nucleotide sequence (Genbank accession: EU420987) encodes a 776-amino acid polypeptide with a calculated molecular mass of 88.9 kDa which is in accordance with the apparent molecular mass (approx. 105 kDa) and that of Api m 5 considering the presence of six predicted sites for N-glycosylation (Fig. 10).

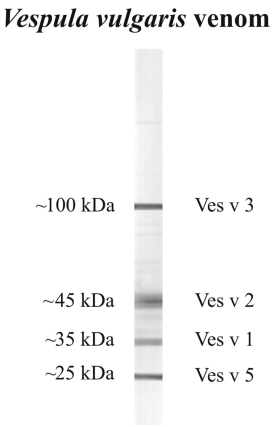

Figure 9: Venom immunoreactivity of Ves v 3 from wasp venom

Specific IgE immunoreactivity of pooled sera from wasp venom-sensitised patients with wasp venom.

Sequence identity to Api m 5 is in the range of 53% (59% on DNA level, data not shown), to human DPPIV in the range of 31% (11% on DNA level), and to the *Gloydius blomhoffi brevicaudus* DPPIV in the range of 32% (17% on DNA level). According to its presence in the venom and the IUIS allergen nomenclature, we termed the protein Ves v 3.

```
Bee    Api m 5   MEVLVQLALLLVVHGSLVVLVAGKSVPRVIDQDLERYEPLEEEDHRGARVPFNLEETYDOSFRANSFNGTWKTDREILYSDNYVGDIRLFDVTTGSGTVLLDSSVTADFDKAS--VMFSF 118
Vespid Ves v 3   MVPLRSFVLLNSLF--LVLLAARTVVTRVIDKDNSDRIVKTQNDQNLSKVPFNLEETYTADFLAYVFNGTWTSDTTIVYTDRRTGDILQFDVIKQRSTLIVDSSVMDAYIVSN--YVLSP 116
Human  DPP IV    ---------MKTPWKVLLGLLGAAALVTIITVPVVLLNKGTDDATADSRKTYTLTDYLKNTYRLKLYSLRWISDHEYLYKQ--ENNILVFNAEYGNSSVFLENSTFDEFGHSINDYSISP 109
Snake  Gbb-IVa   ---------MKTVVKCLLGLLALGVIITAIVVPVVLLTR--DDS--DIRRKFSLEDYLSDEFQYKSYNLRWMSGHEYVYTN--QNNVLLYNIDDERESIVLSNDTLDSFNSSQ--AILSP 103
                           :     *: * .   :   *           ::     :  :.* :     :    :.  * :.   :*.:   .::  ::      ::.:....   :  :     :*

Bee    Api m 5   DNSHVAIGHDYVNGFRYSIHQKCTVYNIKSR-TFTDIANGDRIPLFKWSPTRNALIYVHKNDIYYOVFFEGGS-DTRRITNTGVPDIVFNGIPDWVYEEEVLGSPVAFWISPDGRHLAFA 236
Vespid Ves v 3   KGRYLLIGYDLKKGYRYSTFMRYVIYDIEHR-AYHKIGNDMHIALAKWAPLTDDLIYILDNDIYYMRFSNNGFNDVQRVTYDGISGIVYNGVPDWVYEEEVLQDSSAIWFSPDGNHLAYA 235
Human  DPP IV    DGQFILLEYNYVKQWRHSYTASYDIYDLNKRQLITEERIPNNTQWVTWSPVGHKLAYVWNNDIYVKIEPNLPS---YRITWTGKEDIIYNGITDWVYEEEVFSAYSALWWSPNGTFLAYA 226
Snake  Gbb-IVa   DRKFALLQYSYEKVWRHSYTASYHIYDLNNRTKITENPLPTNIQYISWSPVGHKLAYVYRNNVYVKATPNASP---VQITENGAENKILNGLADWVYEEEMFGTHSALWWSPNGRFLAFA 220
                  .  .  : :.  : :*:*      :*::: *    .     .    .*:*  . * *:  *:::*     :       ::*  *  . : **:.********::    *:* **:* .**:*

Bee    Api m 5   TFNDTNVRDIVISKYGSPGNSRDQYPNEIRIKYPKAGTTNPFVSLSVIDLHD----PSSKLIDLPPPVDVVGADNVLYTANWRRDGEIVATWTNRVQNKAQLVLYDTKGNANNIYYEEET 352
Vespid Ves v 3   SFDDRNVQEILYLHYGEPGNLDDQYPTEVKIKYPKVGTLNPVVSLTLVDLHD----PTLNKIDLKAPHYAVGTDNLLYNVQWKDFDHVVVTWSNRVQNKTEIVWYNMYGEIVKTLHVVEH 351
Human  DPP IV    QFNDTEVPLIEYSFYSDE---SLQYPKTVRVPYPKAGAVNPTVKFFVVNTDSLSSVTNATSIQITAPASMLIGDHYLCDVTWATQERISLQWLRRIQNYSVMDICDYDESSGRWNCLVAR 343
Snake  Gbb-IVa   EINDTEVFVMEYSFYSED---TLQYPKTIKIPYPKAGAINFTIRLFVLDIS----LSFKNISEIVAPSSIISGDHYLSAVTWVTDERICVQWLRRIQNFSVLTICDY---SGAWHCPKER 330
                   ::* :*  :    *..      ***. ::: ***.*: ** : : :::       .  . :: .*   :  *: *  . *    .:   * .*:** : :   :

Bee    Api m 5   EGWLRIQPPLYHDRYVIVAKLQDSGTKAGRFLHATR----LEYRNGALVDETDLTPGTCEVISLLLVDHARARLYYLGTELG-KPSHKNLYSVQLSGNEPPVCLSCDVLTPEGNRCTYAY 467
Vespid Ves v 3   KGWLDIKHLFFYKGSVYIRKLQPSGTKAGRFHHVTR----YDETFKQSPTQMDLTPDAIEVQNICTIDQSNGRIYYLASGLG-KPSQKNLYSVPADGSEKPTCISCNVLTPEGNVCTYAD 466
Human  DPP IV    QHIEMSTTGWVGRFRPSEPHFTLDGNSFYKIISNEEGYRHICYFQIDKKDCTFITKGTWEVIGIEALTS--DYLYYISNEYKGMPGGRNLYKIQLSDYTKVTCLSCELNP---ERCQYYS 458
Snake  Gbb-IVa   EHLEESKTGWVGRFQPSEPYFTSDKISYYRIISDSEGYKHIHYTDSAGK-VKPITSGKWEVISISAVTN--NSLYFISNEFEGRPGGRHLYKVDLKNDLKKICITCNSKE---EACQYFS 444
                  :                    :  .  .  ::    .                  :* .  ** .:  :      :*::..     *. ::**.:  ..     *::*:      : *

Bee    Api m 5   AYFSTNGSHYALYCAGPDPVFIAIVN-ANHRQISIWEENRSLRRKLAARTQPIVKNFNVNANGYTNKVKLYLPPDFDETKKYPLLITVYAGPNTIRITEEATYGFESYIVTNRSVIYGRI 586
Vespid Ves v 3   AIFSPLGQYYVLVCHGPDPAFVSIFN NAHQKVYSWENNLSLRKKLAKRHLLPLVKDLDVRANGYESKVRLLFLPHNFDESKSYPMLVNVYAGPNTLKIIDAASYGHQVYMTTNRSVIYAYI 585
Human  DPP IV    VSFSKEAKYYQLRCSGPGLPLYTLHSSVNDKGLRVLEDNSALDKMLQNVQMPSKKLDFIILNETKFWYQMILPFHFDKSKKYPLLLDVYAGPCSQKADTVFRLNWATYLASTENIIVASF 578
Snake  Gbb-IVa   VSFSTDSRYYKLNCYGPDLPYFTLQNSITDKAIKTLEDNNNLKNVLKEIQMPCKRLSNITLHGQTYWYQMILPPNFDESKKYPLLIDVYAGPCSQKADAAFRINWSTYLASSEGIIVASF 564
                  . **  . :* * * **.    :: .  .: :   *:*  * . *     *  :   :  :      :: ** .**::*.**:*: ***** : :       .   *:.:...:* . :

Bee    Api m 5   DGRGSAYKGSKMLFEIYRRLGTVEIEDOIIITRTLOEKYSWIDSNRTGIWGWSYGGFSAAMVLATDAESVFKCGISVAPVTSWIYYDSLYTERFMGLPTPEDNQSGYNDTDVSRRVEGMR 706
Vespid Ves v 3   DGRGSSNKGSKMLFSIYRKLGTVEVEDQITVTRQLQEMFPWIDSKRTGVWGWSYGGFSTAMILAKDTSFVFKCGIAIAPVSSWIYYDSIYTERFMGFPTPEDNLSGYNETDVSRRVEDIR 705
Human  DPP IV    DGRGSGYQGDKIMHAINRRLGTFEVEDQIEAARQFSKMG-FVDNKRIAIWGWSYGGYVTSMVLGSGSG-VFKCGIAVAPVSRWEYYDSVYTERYMGLPTPEDNLDHYRNSTVMSRAENFK 696
Snake  Gbb-IVa   DGRGSGFQGDKILHAIYRRLGTYEVEDQISAAKLFSEMS-FVDKDRIAIWGWSYGGYVTSMVLGAGSD-VFKCGIAVAPVSRWQYYDSIYTERYMGLPEKNDNLNFYENSTVMARAKNFR 682
                  *****. :*.*::. * *:*** *:****  :: :.:   ::*..* .:******: ::*:*. .:  ******::***: * ****:****:**:*  :** . *.:: *  *.:.::

Bee    Api m 5   GKKYMLIHGTADDNVHYQQTMMLNKALVNSDIMFQQQTYTDEARALGN--VFPHLYHTTDRFWANCLGYSH-- 775
Vespid Ves v 3   GKKFMLIHGSGDDNVHYQQSLALAKALEKADVMFEQITYTDEAHALFG--VLPHLYHTMDRFWSDCFSLSHAH 776
Human  DPP IV    QVEYLLIHGTADDNVHFQQSAQISKALVDVGVDFQAMWYTDEDHGIASSTAHQHIYTHMSHFIKQCFSLP--- 766
Snake  Gbb-IVa   TVDYLLIHGTADDNVHFQQAAQISKALVDAEVDFQAMWYTDKDHGIGG-HAHSHIYQHMSHFMKQCFKLP--- 751
                    .::****:.*****:**:  : *** .  : *:   ***: *.: .  .  *:*   . :*  :*:  .
```

Figure 10: Alignment of DPPIV amino acid sequences

Alignment of Ves v 3 with Api m 5 and other related proteins. Shown are Ves v 3, Api m 5, dipeptidylpeptidase IV of the snake *Gloydius blomhoffi brevicaudus* (e.g. Genbank accession AB158224) and human dipeptidylpeptidase IV (e.g. Genbank accession BC65265). Peptides identified by mass spectrometry are underlined. Signal sequences are italicised, the residues of the conserved active centre of the enzymes are represented boxed and putative glycosylation sites in grey.

3.2.2.2 Recombinant expression of Ves v 3 in insect cells

In order to provide recombinant protein for subsequent functional and immunological studies and to verify the presence of the identified protein in the venom we produced full length Ves v 3 as secreted protein in insect cells. The cDNA of Ves v 3 was cloned into the particular expression vector for baculovirus based infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. Culture supernatants of HighFive cells were subjected to Ni-NTA-agarose chromatography, and resulting proteins analysed by SDS-PAGE and IgE immunoblotting. The epitope-tagged recombinant protein (yield of approx. 0.2 µg per ml supernatant) exhibited an apparent molecular mass of approx. 105 kDa (Fig. 11C, D) corresponding to the natural allergen in wasp venom and, additionally, was reactive with sIgE from pooled sera of venom-sensitised patients (Fig. 11E).

Employing a recombinant human IgE antibody specific for the homologues Api m 5, insect cell-derived recombinant Ves v 3 could be detected indicating a shared epitope in both proteins (Fig. 11F). Moreover, it detected the corresponding natural form of Ves v 3 in the venom of *V. vulgaris* (Fig. 11A). Together, these data verified the identity of the recombinantly produced allergen with the IgE immunoreactive 100 kDa allergen in the native venom.

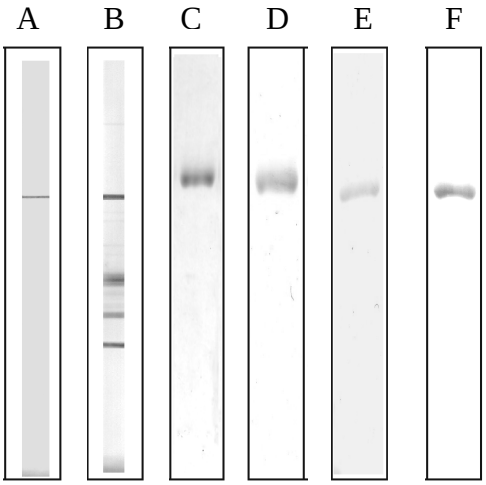

Figure 11: Immunoreactivity of native and recombinant Api m 5 and Ves v 3

Immunoblot of yellow jacket venom with a monoclonal human IgE antibody specific for Api m 5 (A) and with pooled wasp venom allergic patient sera (B). SDS-PAGE (C) and immunoblot analysis of purified Ves v 3 expressed in Sf9 insect cells, visualised by either Coomassie Blue staining (C) or anti-V5 epitope antibody (D), pooled wasp venom allergic patient sera (E) and a monoclonal human anti-Api m 5 IgE antibody (F).

3.2.2.3 Enzymatic activity of rVes v 3

Its sequence renders Ves v 3 a putative homologue of DPPIV from humans and other species. Using glycine-proline nitroanilide, a synthetic substrate of human DPPIV, specific DPPIV activity could be detected in the venom of both *Vespula. spp.* and *A. mellifera* (Fig. 12A, B). Moreover, this activity could be abolished by Diprotin A, a highly specific inhibitor of human DPPIV. Purified recombinant Ves v 3 proved to exhibit significant DPPIV activity, demonstrating its DPPIV nature and, thereby, correct folding of the insect cell produced protein (Fig 12C). The inhibition constants of Diprotin A for the DPPIV activitiy in the venom and of the recombinant allergen Ves v 3 matched the reported activity of the human enzyme very closely, suggesting mechanistical conservation. Together these data suggest that the DPPIV-like activity contributes to the diverse panel of enzymatic activities exhibited by hymenoptera venoms and that this activity relies on Ves v 3 and its homologues.

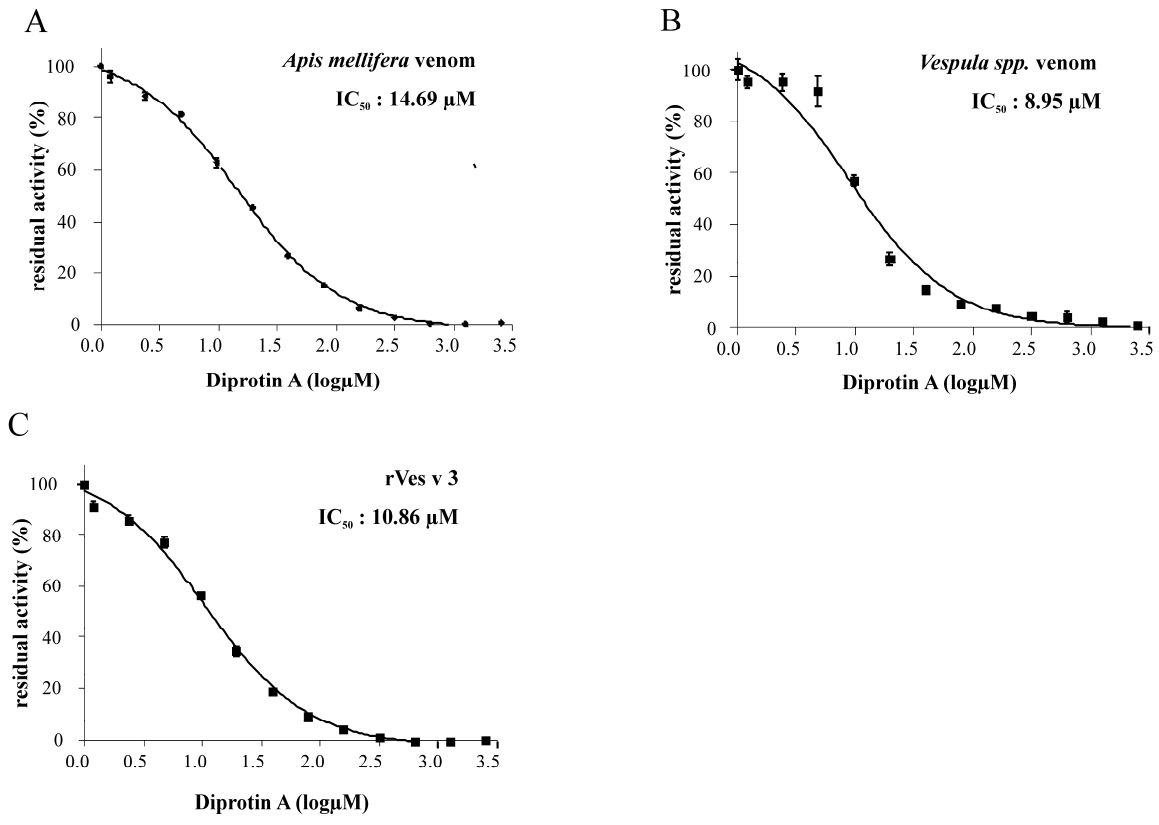

Figure 12: Kinetic analyses of DPPIV activity

Inhibition of DPPIV activity by the inhibitor Diprotin A (Ile-Pro-Ile) of *A. mellifera* venom (A), *Vespula spp.* venom (B) and purified rVes v 3 (C) was analysed. The Y axis shows the residual activity (%) of cleavage of the chromogenic substrate Gly-Pro p-nitroanilide hydrochloride for serial dilutions of the specific DPPIV inhibitor Diprotin A (log µM). IC_{50} values are depicted on each plot.

3.2.2.4 IgE immunoreactivity of rVes v 3

To evaluate the IgE immunoreactivity of Ves v 3 produced in Sf9 insect cells, individual sera of patients with a clinical history of an allergic reaction after a stinging event were assayed by ELISA for specific IgE antibodies. Fig. 13 shows the reactivity of patients who were characterised by a positive sIgE test to honeybee or wasp venom. Of the 35 wasp venom-positive sera, 16 showed high sIgE reactivity (OD_{450nm} $\geq$1.0), whereas additional 4 sera showed a positive sIgE reactivity to a medium to lower degree (OD_{450nm} $\geq$0.4<1.0; cut-off value of 0.35). Overall, 20/35 (57%) of the patient sera had detectable sIgE to recombinant Ves v 3 (Fig. 13).

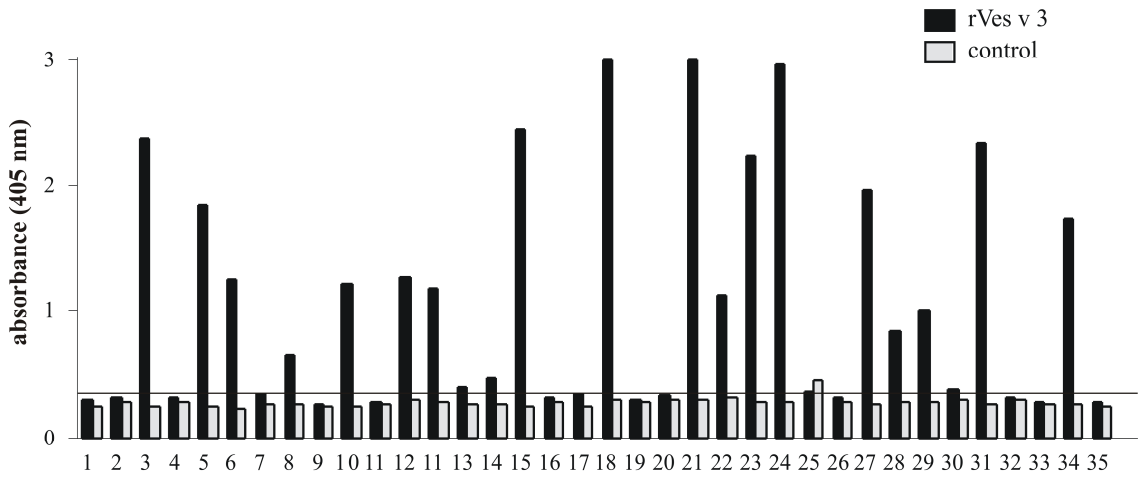

Figure 13: IgE immunoreactivity of rVes v 3

Immunoreactivity of individual patient sera with recombinant Ves v 3. The IgE reactivity was assessed by ELISA with sera of wasp venom-sensitised patients (n=35). Controls were performed by omission of antigen. The lower end functional cut-off of the ELISA was OD_{405nm} $\geq$0.40 as represented by a dashed line.

In order to verify the obtained reactivities we further compared eukaryotically-produced, posttranslationally modified, and enzymatically active protein with prokaryotically produced Ves v 3 maltose binding protein (MBP) fusion protein (Fig 14 A, B) in ELISA (Fig. 14 C). The fact that most reactive sera were also reactive with the prokaryotically produced counterparts corroborates the IgE reactivity of the insect cell produced proteins and further supports the conclusion, that the recombinant high molecular weight proteins exhibit IgE reactivity beyond CCD reactivity.

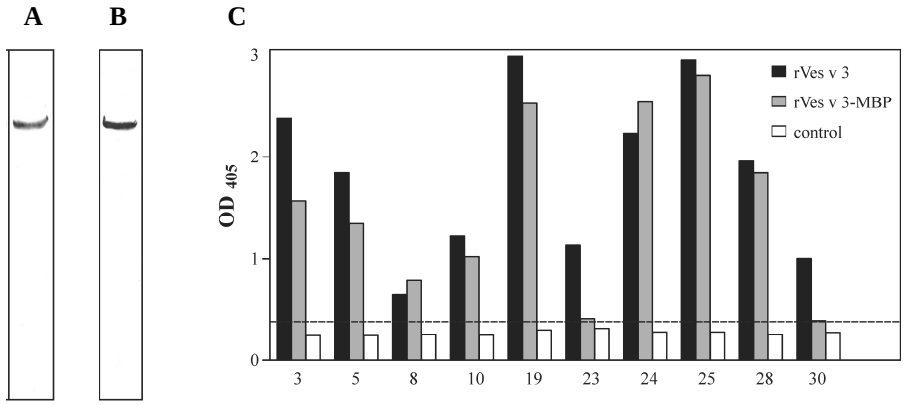

Figure 14: Comparative analyses of the immunoreactivity of prokaryotically versus eukaryotically produced rVes v 3

For immunoblot analysis rVes v 3 expressed as maltose binding protein fusion protein in *E. coli* was visualised by either anti-V5 epitope antibody (A) or a monoclonal human anti-Api m 5 specific IgE antibody (B). Immunoreactivity of individual patient sera with recombinant Ves v 3 was assessed by ELISA using sera of wasp venom sensitised patients for rVes v 3 and rVes v 3-MBP (C) (exemplary n=10 each) found reactive in Fig. 13. Controls were performed by omission of antigen. The lower end functional cut-off of the ELISA was OD405nm >0.40 as represented by a dashed line.

An initial analysis of cross-reactivity between Ves v 3 and Api m 5 using honeybee venom-positive sera from sera, 8/21 (38%) of the Api m 5-reactive sera were cross-reactive with Ves v 3, and 11/20 (55%) of the Ves v 3-reactive sera with Api m 5 (data not shown). To further substantiate the finding of serologic cross-reactivity of the DPPIV allergens in honeybee and vespid venom, we selected another group of patients with clinically relevant allergy to wasp venom (n=14) and a positive sIgE reactivity to Ves v 3 (n=11). The remainder of the patients had a negative sIgE reactivity to Ves v 3 (n=3). All sera were subsequently tested in parallel for serologic sIgE reactivity to Ves v 3 and Api m 5 (Fig. 15). Of the 11 Ves v 3-positive patient sera, 6/11 (54.5%) showed a medium to high sIgE reactivity with Api m 5 (OD$\geq$0.15), whereas 2/11 sera were Api m 5-negative and 3/11 sera showed a very low degree of positivity slightly above the lower end cut-off of the assay (cut-off value of 0.1). None of the 3 Ves v 3-negative sera showed sIgE reactivity with Api m 5.

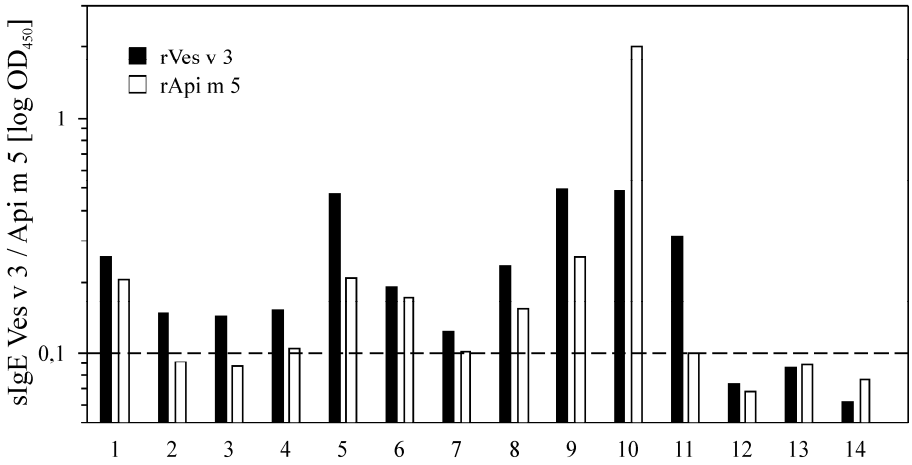

Figure 15: IgE-based cross-immunoreactivity of Ves v 3 and Api m 5

IgE immunoreactivity of individual sera from patients with clinically relevant allergy to yellow jacket venom (n=14) and a positive (n=11; patient ID *1-11*) or negative (n=3; patient ID *12-14*) sIgE reactivity to Ves v 3 with recombinant Api m 5. All sera were tested in parallel for serologic sIgE reactivity to rVes v 3 and rApi m 5 expressed in Sf9 cells. The lower end cut-off of the ELISA was $OD_{450nm} \geq 0.1$ as represented by a dashed line in the graph.

3.2.2.5 Activation of basophils from venom-allergic patients with rVes v 3

The capability of Ves v 3 produced in Sf9 cells for activation of human basophils derived from wasp venom-allergic patients was assessed by a flow cytometry-based basophil activation test employing CD63 as activation marker. In order to ensure a maximum of validity of the basophil activation tests a broad concentration range was covered and the extent of basophil activation was related to that of known major allergens. All blood samples were obtained from consecutively selected patients (n=7) with a clinical history of an anaphylactic reaction after a stinging event (≥ grade 2). The 7 patients represented a subgroup of the 14 patients depicted in Fig. 15. All selected patients had a positive intradermal skin test and sIgE test for wasp venom extract, whereas 4/7 patients had a positive result in either skin testing and/or sIgE testing - although to a lesser degree - with honeybee venom extract. All patients were tested for serological IgE reactivity against Ves v 3 and Api m 5. Four of 7 patients had positive sIgE titers for Ves v 3 (patients 1-4). With Api m 5, 2/7 showed either a strongly positive (patient 1) or a borderline positive sIgE titer (patient 4). Patients 5-7 had no detectable sIgE against either Ves v 3 or Api m 5. Basophil activation results using recombinant

Ves v 3 for all 7 patients are shown in Fig. 16 A. Each patient with a positive sIgE against Ves v 3 in serum also demonstrated positive basophil activation through this allergen (patients 1-4). In contrast, basophils from patients lacking sIgE reactivity to Ves v 3 could not be activated by Ves v 3 (patients 5-7). Both stimulation controls, whole vespid venom and a monoclonal anti-FcεRI, gave positive results for all patients whereas plain stimulation buffer used as a negative control did not induce reactivity.

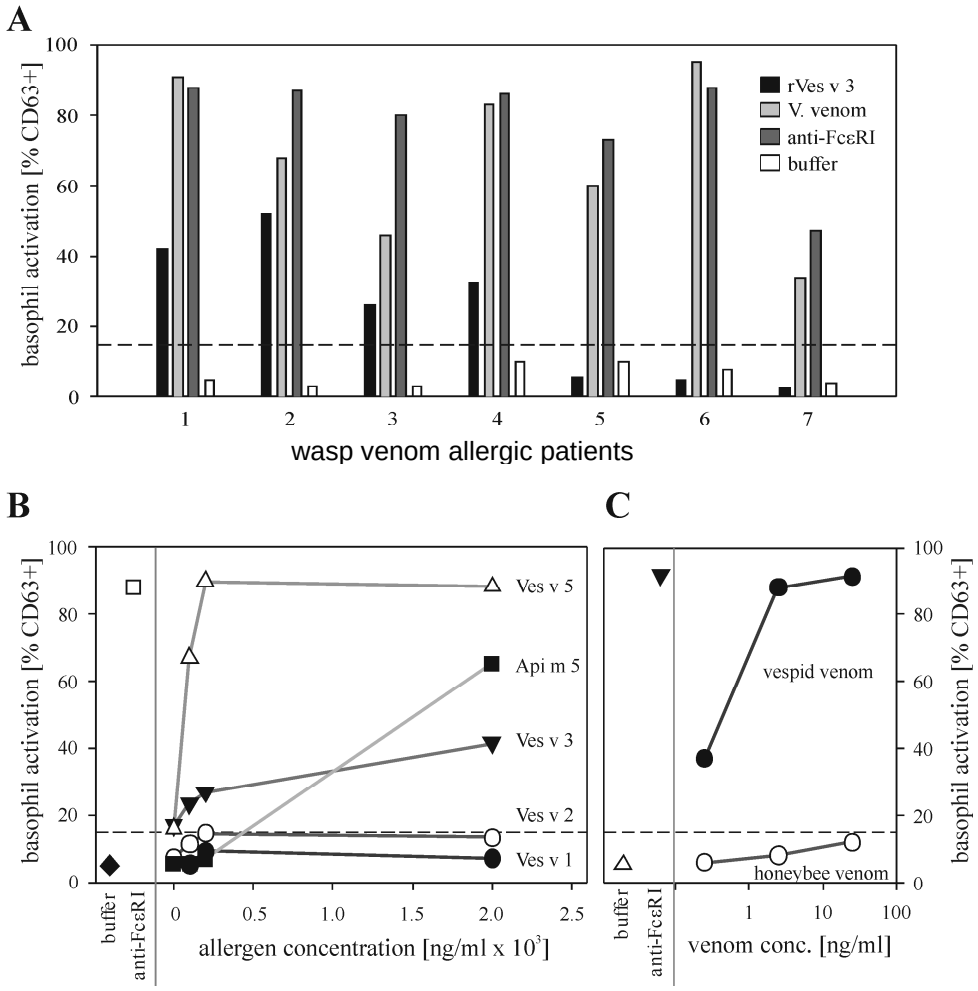

Figure 16: Basophil activation tests with recombinant rVes v 3

Human basophils from wasp venom-sensitised patients (n=7) were exposed to serial dilutions of rVes v 3 (A, B), other *V. vulgaris* allergens (rVes v 1, rVes v 2, rVes v 5) and rApi m 5 (B) or whole venom (C) of *A. mellifera* or *V. vulgaris*. Incubations with a monoclonal anti-Fc□RI or with plain stimulation buffer were used as positive or negative stimulation controls (A-C). (A) Shown is the percentage of activated basophils on incubation with the maximally tested concentration of rVes v 3 (2 µg/ml) or whole *V. vulgaris* venom (25 ng/ml) in 4 patients with positive sIgE against Ves v 3 (patient ID *1-4*) and 3 patients with negative sIgE against Ves v 3 (patient ID *5-7*). (B, C) Detailed analysis of patient *1* using a concentration range of recombinant hymenoptera venom allergens (B) or of whole honeybee or vespid venom (C) as indicated. The dashed line in A-C represents the lower end functional cut-off of the assay (15% CD63+ cells).

Basophil activation was analysed in more detail in patient 1 - the patient exhibiting strong Api m 5-directed sIgE cross-reactivity - using a panel of recombinant *V. vulgaris* allergens (Ves v 1, Ves v 2, Ves v 3, Ves v 5) together with the honeybee venom allergen Api m 5. As shown in Fig. 16B, both Ves v 3 and Api m 5 lead to a robust basophil activation of up to 42% and 65%, respectively, in patient 1 within the uniformly tested allergen concentration range (up to 2 µg/ml Ves v 3/Api m 5). This patient also had a strong basophil activation with Ves v 5. At higher allergen concentrations (up to 10 µg/ml Ves v 3/Api m 5), basophil activation of up to 87% (Ves v 3) and 72% (Api m 5) was recorded (data not shown). Such a concordant basophil activation by Api m 5 and Ves v 3 was only evident in the higher concentration range, whereas the capability for basophil activation by Api m 5 in the lower concentration range was clearly less pronounced as compared to Ves v 3. This most likely reflects primary sensitisation to high molecular weight DPPIV allergens through wasp venom Ves v 3 in patient 1. The use of whole *A. mellifera* and *V. vulgaris* venom revealed clear-cut basophil activation only with vespid venom (Fig. 16C). Together, these data suggest that the high molecular weight venom allergens Ves v 3 and Api m 5 are not only able to induce effector cell activation in venom allergic patients, but are also likely candidates for clinical cross-reactivity in hymenoptera venom allergy.

3.2.2.6 Implications of the presented data

Allergens in hymenoptera venoms have been investigated for more then 50 years and several components have been described in terms of sequence and even structure, but some proteins could not be identified so far, even if IgE binding to these proteins has been mentioned in different studies. One such component is a 100 kDa protein from honeybee venom, for which IgE reactivity was reported but the identity of the protein so far defied elucidation. After the protein, designated Api m 5, was recently identified in our workgroup this paved the way for the identification of a corresponding 100 kDa protein (Ves v 3) as a new allergen in the venom of *V. vulgaris*

This protein corresponds to Api m 5 regarding molecular weight, amino acid sequence, enzymatic function, IgE immunoreactivity using a broad range of wasp venom-sensitised individuals, and functional allergenic capability using basophils from venom-allergic patients. Although a protein of this size has been reported as putative

IgE reactive protein in another *Vespula* species, no attribution of a name or function in *V. vulgaris* has been made so far [36].

Insect cells appeared to be the most appropriate system for expression of the putative Ves v 3 as insect cells most likely will provide a similar glycosylation as found in the natural isoforms in contrast to mammalian systems, a fact which is supported by the apparent molecular masses of the expressed recombinant allergens [106]. The identity of the expressed open reading frame with the venom protein was further proven by a recombinant human monoclonal IgE antibody specific for Api m 5 selected by phage display [94]. This monoclonal anti-Api m 5 IgE antibody reacted to a similar extent with the natural venom isoform and the insect cell-expressed isoforms of Ves v 3, suggesting the presence of a conserved protein epitope in Ves v 3 and Api m 5 (see Fig. 11). Such an epitope hints to the possible occurrence of cross-reactive protein epitope-specific IgE also in venom-allergic patients. Indeed, the immunoreactivity of recombinant Api m 5 and Ves v 3 in immunoblot and ELISA analyses with IgE from >50% of honeybee or wasp venom-sensitised patients demonstrated the general presence of human IgE epitopes on the two high molecular weight allergens.

However, it is well known that Ves v 3 and the corresponding Api m 5 are glycoproteins [107, 108] containing multiple potential glycosylation sites. Moreover, most of the proteins in hymenoptera venom provide carbohydrates carrying α-1,3-linked core fucose residues that form a type of human IgE epitope also known as cross-reactive carbohydrate determinant (CCD) [109]. IgE with specificity for CCD are considered to play a key role in allergen cross-reactivity, representing a major concern for the specificity of diagnostic approaches in hymenoptera venom allergy [99]. In this context, it was postulated in a recent immunoblot study that the immunoreactivity of high molecular weight hymenoptera venom allergens including Ves v 3 and Api m 5 reflects almost exclusively CCD-specific sIgE reactivity in patient sera [53], thus rendering them allergens of potentially minor interest.

In contrast to these findings, the presented data point to a more important role of Ves v 3 and Api m 5 as allergens recognised by patient sIgE beyond CCD reactivity. We could further support their relevance in venom allergy by the fact that Sf9 insect cells were employed for production of the enzymatically active recombinant proteins. Sf9 cells are considered to exhibit significantly reduced α-1,3 core fucosyltransferase activity [110, 111] resulting in proteins without or with only minute amounts of CCDs. Using the Sf9 produced allergens, more than 50% of not preselected honeybee or wasp

venom sIgE-positive sera were reactive with recombinant Ves v 3, thus rendering the 100 kDa protein a major allergen containing proteinic IgE epitopes with clinical relevance. Additional evidence in this direction is derived from the fact that even prokaryotically expressed Ves v 3 devoid of both glycosylation and, most likely, proper folding exhibited significant IgE reactivity with most patient sera found reactive with the eukaryotically produced Ves v 3. This also fits into the context of the basophil activation, in which only clinically relevant IgE reactivies (not including IgE directed against CCDs) are documented. Both findings support the conclusion that the recombinant high molecular weight protein exhibits a clear IgE reactivity beyond CCD reactivity. Additionally, previous in-depth cross-reactivity studies demonstrated that Api m 5 is not cross-reactive with other honeybee venom allergens [112].

As mentioned, best evidence that high molecular weight hymenoptera venom allergens are of clinical relevance in venom allergy was provided by activation of human patient-derived basophils through recombinant Ves v 3. The basophil activation pattern closely matched the serologic sIgE reactivity in each of the patients. Thereby, upon consideration of the high molecular weight of Api m 5 and Ves v 3 the concentrations (and, thus, molarities) required for efficient activation of basophils are in the range employed throughout a plethora of studies regarding allergenic potential of proteins from different sources including venom, pollen, food or animal [113-115]. However, best evidence for comparable allergenic potential is given in an extensive evaluation of native and recombinant venom allergens employing 1 µg/ml as concentration of all allergens [101].

Moreover, the data provide for the first time evidence for both serologic and cellular sIgE cross-reactivity between Ves v 3 and Api m 5 in hymenoptera venom allergy. So far, double-positivity in venom allergic patients had been largely attributed to IgE directed against either hyaluronidases (Api m 2, Ves v 2) or against CCD [53, 116]. With the identification, characterisation, and recombinant expression of Ves v 3 and Api m 5, a new pair of cross-reactive homologous allergens becomes available for future clinical applications in diagnosis and therapy.

Sequence analysis and characterisation of the enzymatic activities provide clear evidence that Ves v 3 and Api m 5 belong to the class of DPPIV enzymes. In general, proline-specific dipeptidyl peptidases (DPPs) are emerging as a protease family with important implications for the regulation of signalling by peptide hormones. The extracellular domain of DPPIV encodes an ectopeptidase and is able to cleave amino-

terminal dipeptides from polypeptides with either proline or alanine at the penultimate position [117, 118]. Therefore, DPPIV shows a variety of functions including regulation of inflammatory and immunological responses, signal transduction, and apoptosis by degrading physiological substances such as substance P, TNF-α, IL-2, and various chemokines including CCL5 (RANTES) [49, 50, 119].

DPPIV activity has also been reported in snake venoms of different species [120] and a corresponding cDNA of a snake DPPIV has been cloned. However, the presence of DPPIV in hymenoptera venoms has remained obscure. A single report of DPPIV activity suggested the presence of the enzyme in venom gland extracts of queen bees [121], nevertheless, neither in *V. vulgaris* nor *A. mellifera* venom such an activity has been reported. Insect venom DPPIV may function through the conversion of venom components into their active forms in the venom gland on the one hand and the enhancement or decrease of the chemotactic activity of immune cells after the insect sting on the other hand. The former may be confirmed by the hypothesis that promelittin is processed into its active form in a stepwise manner by enzymes of the DPPIV type in vitro [121].

In summary, with Ves v 3 a DPPIV homologue to Api m 5 in the venom of *Vespula vulgaris* as major allergen in insect venom allergy was identified. The characterisation of DPPIV homologues may provide further insights into potential immunomodulatory functions of different hymenoptera venoms. Additionally, the results demonstrate for the first time the clinical relevance of Ves v 3 as high molecular weight allergen in IgE-mediated hymenoptera venom allergy. The recombinant allergens Ves v 3 and Api m 5 will represent valuable tools for the improvement of current diagnostic tests and immunotherapy of insect venom allergy. These findings provide a novel view on the molecular patterns of allergic IgE sensitisation in venom allergy which requires reconsideration of current and future concepts for component-resolved diagnosis and specific immunotherapy of hymenoptera venom allergy.

3 Summary

Allergic diseases are an increasing problem in industrialised countries and incidence in the general population has risen throughout the last decades to 25-30% nowadays. Albeit of lower incidence, allergy to insect stings is considered a severe threat due to the more severe and sometimes fatal reactions after a stinging event. In Europe and the US the major threat is emanating from the honeybee *Apis mellifera* and from different yellow jacket species, mainly the common wasp *Vespula vulgaris* also designated as yellow jacket in the US with a worldwide distribution. Allergens from the venoms of these two species have been investigated throughout the last 50 years, but the list is far away from completeness. Even if specific immunotherapy for hymenoptera venom allergy is effective, there are at least 5-15% treatment failures. Moreover diagnosis of hymenoptera venom allergy is severely affected by molecular cross-reactivities on the basis of cross-reactive epitopes of homologous proteins and cross-reactive carbohydrate epitopes from unrelated sources.

Both, therapy and diagnostic additionally are affected by the use of allergen extracts, which in the case of the hymenoptera venoms are complex mixtures of proteins, only some of which are allergens, peptides and non-proteinic compounds. Beside the presence of further components other than the allergens, which renders a therapeutic agent difficult to handle and is cumbersome for a diagnostic tool, the even more severe obstacles arise from the natural origin of these extracts, since protein concentrations and compositions can differ significantly between various source materials.

To address both therapeutic and diagnostic problems, further knowledge about the allergenic venom compounds, their role during sensitisation and hyposensibilisation, as well as the molecular mechanisms of carbohydrate related cross-reactivities is indispensable. Even if a considerable number of allergens has been described from the venoms of *A. mellifera* and *V. vulgaris* a thorough characterisation of most of these components is hampered by the availability of pure and functional protein, either from venom or from recombinant expression. Furthermore, allergen extracts need to be standardised in terms of protein content, concentration, and composition and remain to be characterised in terms of additional allergenic compounds.

Therefore, in the present thesis three scientific publications are presented describing different aspects in the field of molecular allergology with a focus on

recombinant antibody technologies and hymenoptera venom allergy. Beside these published data further results about the identification of new venom components and the characterisation of these and already known components are presented. Indispensable prerequisite for this were to gain access to the venom allergens in quantities which allow for a thorough characterisation and a quality equal to that of the native proteins.

Since the purification of particular proteins from venom in most cases is high in effort due to the amount of venom needed and the scarcity of source material, we aimed for a recombinant approach to guarantee sufficient quantity and constant quality of the proteins. In general prokaryotic expression is limited due to the lack of correct protein folding and post-translational modification. Due to the protein origin from insects, the most suitable system appeared to be the baculovirus based expression in eukaryotic insect cells. This system is based on the introduction of the respective gene of interest into the virus genome with a strong viral promotor allowing high expression yields. Protein folding and posttranslational modification can be assumed to be similar to those of the native proteins and expression as secreted proteins simplifies purification from the cellular supernatants via affinity chromatography.

Even if a correct folding and, thereby, functionality of theses recombinant proteins appears to be likely, the assessment of functionality and comparison to the native protein is the first step in further characterisation. Enzymatic activity is the ideal way to examine structural integrity of the proteins by indirect means. Direct structural comparison can be performed by circular dichroism (CD)-spectroscopy if data of the native proteins are available. Another key feature of a functional allergen is the IgE binding which might be compared to the native protein. Moreover, prevalence of IgE reactivity may be used as indicator for the importance of a certain allergen. Since sole binding of IgE to an allergen is not a clear proof for its allergenicity, another *in vitro* assay, the basophil activation test (BAT), can clarify its importance in the allergic response. As mediator release from effector cells like basophils is causative for the allergic symptoms, cellular activation gives best evidence for the relevance of a certain allergen. Furthermore, since IgE binding to different epitopes in the correct structural arrangement is needed for cross-linking and, thereby, for cellular activation, again the structural integrity can be verified.

Since diagnosis is severely hampered by molecular cross-reactivities, especially emanating from cross-reactive carbohydrates, one approach for improvement could rely on the use of major allergens with high IgE prevalence which are not glycosylated and are unique for the particular venom. In yellow jacket venom the major allergens Ves v 1 and Ves v 5 (antigen 5) fit to those criteria and for Ves v 5 a high IgE prevalence has been demonstrated with recombinant proteins [92]. For the phospholipase recombinant approaches were not successful so far [37] and scarce data for this allergen originate from studies with purified protein and from immunoblot studies [93], indicating as well a high IgE prevalence. Therefore, our aim was the recombinant expression and a thorough analysis of Ves v 1 in combination with the well characterised allergen Ves v 5, data presented in the section 3.2. Recombinant expression could be achieved by use of the baculovirus expression system (BVES) and proteins were purified from cellular supernatants by affinity chromatography. For Ves v 1, functionality was verified by an enzymatic activity test. As for Ves v 5 no enzymatic activity has been reported so far, we compared its structural features to those of the native protein by mean of CD-spectroscopy. Another functional test was the use of the recombinant proteins in a basophil activation test. Here, basophils derived from allergic patients were stimulated with either yellow jacket venom or the recombinant proteins and activation was followed by upregulation of the marker molecule CD63. Both proteins showed clear upregulation of the marker CD63 and, thus, activation gave best evidence for their equivalence to native proteins. After this characterisation of the recombinant proteins we assessed the prevalence of IgE binding, using two types of sera, characterised either by positive sIgE to honeybee and yellow jacket venom (double- positive), or to yellow jacket venom alone (single-positive). In addition to a high IgE prevalence for Ves v 1 that unanticipated was even higher than for Ves v 5, we observed a considerable number of sera with IgE reactivity exclusively to Ves v 1, a finding that was significantly less pronounced for Ves v 5. In total, over 90% of the single positive patients showed IgE reactivity to one or both of the recombinant proteins, while the diagnostic coverage using Ves v 5 alone was around 60%. The same tendency was observed for double-positive patients, whereas in this group 4 patients did not react with either of the proteins indicating a sensitisation to honeybee venom. This could be confirmed by additional evaluation of IgE reactivity to the major bee venom allergen Api m 1 as a marker in two sera. Furthermore, we investigated the presence of CCD specific IgE by use of the CCD marker MUXF which revealed a high rate of CCD specific reactivities in

the double-positive patients indicating a reason for their reactivity pattern. In contrast, the rate of true double-sensitisation classified by sIgE for both Api m 1 and one of the yellow jacket allergens was low, a findingin accordance with the clinical situation.

In summary, the recombinant Ves v 1, which was produced in functional form for the first time, allowed for a first immunological characterisation in terms of IgE binding and together with the recombinant Ves v 5 as another unique marker for sensitisation to yellow jacket venom, improved diagnostic by more than 30%. Therefore, recombinant Ves v 1 is an ideal candidate for improvement of standard diagnostic approaches, a prerequisite for proper diagnosis in hymenoptera venom allergy.

Even if the aforesaid strategy can improve diagnostic approaches, an improvement for therapeutic considerations relies on the knowledge on the protein components involved in hymenoptera venom allergy. Even if a considerable number of allergens have already been investigated, some components with unknown relevance still remain elusive. One such component is a 100 kDa protein from honeybee venom, for which IgE reactivity was reported more than 30 years ago [96] but the identity of the protein so far defied elucidation. After the protein, designated Api m 5, was recently identified in our workgroup this paved the way for the identification of a identical 100 kDa protein (Ves v 3) from wasp venom which is described in section 3.1.

The 100 KDa protein Api m 5 from *A. mellifera* venom [97] was allocated to a dipeptidylpeptidase IV (DPPIV) like enzyme and a corresponding dipeptidylpeptidase activity could also be detected in yellow jacket venom. Additional reports of an IgE reactive 100 kDa protein in wasp venom [98] rendered the existence of a corresponding protein likely. Accordingly, mass spectrometry based sequencing yielded several peptides that could be allocated to a dipeptidylpeptidase, but due to the lack of genomic data for *Vespula vulgaris* direct cloning was not possible and an alternative strategy using degenerated primers and 5´RACE approaches had to be conducted for cloning of the full length gene.

The derived gene from *V. vulgaris* showed a sequence identity of 53% to the homologous protein from *A. mellifera* venom. The gene was cloned in the respective vector for recombinant expression in eukaryotic insect cells and recombinant protein could be yielded in soluble form. Characterisation of the enzymatic function by use of a synthetic DPPIV substrate and the specific inhibitor Diprotin A revealed a comparable activity of the vespid DPPIV to the honeybee venom enzyme and, in addition, closely matched the reported activity of the human enzyme, suggesting mechanistical

conservation. If such an enzymatic activity may contribute to the immunological and toxicological function of venoms, as it is capable for processing of human cytokines like RANTES and other substances, or if this function is intrinsic for the venom, since mastoparan is also a proper substrate, needs to be further analysed and will contribute to a more detailed understanding of insect venoms in general.

Even more interesting, from the immunological point of view, is the IgE prevalence assessed by ELISA. In this analysis a sIgE prevalence to the recombinant protein of around 50% was observed for yellow jacket venom specific sera. Additionally, Ves v 3 was found to be cross-reactive to Api m 5 as indicated by recognition of both proteins by either bee or yellow jacket specific sera. Due to its clear allergenic nature the protein was added to the official list of allergens of the I.U.I.S [99] designated Ves v 3. To verify the significance of the new allergen in hymenoptera venom allergy cellular activation assays using basophils from bee or yellow jacket venom sensitised patients were conducted with the recombinant allergen. Clear cellular activation as determined by CD63 upregulation could be observed over a broad concentration range.

Since Ves v 3, as well as its homologue Api m 5 provides several potential N glycosylation sites, IgE reactivity based on CCDs had to be prevented, especially as the eukaryotic expression in insect cells should result in completely glycosylated and, thereby, also fucosylated proteins. The finding, that certain insect cell lines exhibit unique variant glycosylation patterns in terms of fucosylation and can therefore be used for production of CCD free proteins is presented in **“Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation”** focussing on the hyaluronidases in honeybee and yellow jacket venom, both being glycoproteins. Prior to the identification of the dipeptidylpeptidases, the hyaluronidases were believed to be the only molecules capable for protein related cross-reactivity between honeybee and yellow jacket venom due to a sequence identity of approximately 50%. Additionally, the proteins were for a long time considered major allergens with high sIgE prevalence whereas a re-evaluation of IgE reactivity taking into account the CCD based IgE reactivity indicated a strongly diminished importance of the yellow jacket hyaluronidases Ves v 2a and Ves v 2b [100]. To address this point we recombinantly expressed the hyaluronidases in different insect cell lines for which we had observed varying fucosylation patterns to further compare the differentially fucosylated proteins in terms of CCD specific IgE reactivity. Furthermore, the different fucosylation capacity of the HighFive (*Trichoplusia ni*) and Sf9 (*Spodoptera frugiperda*)

insect cells was applied for the hyaluronidases of honeybee and yellow jacket venom as well as for a human control glycoprotein by use of an alpha-1,3-fucose specific rabbit anti-serum and CCD positive patient sera. The proteins produced in HighFive cells exhibited clear reactivity with both agents, indicating the presence of alpha-1,3-fucosylation, whereas for the Sf9 derived proteins such fucosylation was not detectable. Employing the differentially fucosylated proteins for an ELISA based screening of double-positive sera we could confirm IgE binding to Api m 2 beyond CCD based reactivity even if less pronounced than assumed in literature, while for the yellow jacket hyaluronidases IgE binding to the unfucosylated proteins from Sf9 was negligible, indicating the predominant role of CCD based IgE reactivity for these proteins. Furthermore, protein based cross-reactivity between the honeybee and yellow jacket hyaluronidases could not be observed, which confirms structure based data that indicate a reduced presence of identical surface areas [56].

In addition we were able to quantify the hyaluronidase isoforms Ves v 2a and b in wasp venom by use of polyclonal, isoform-specific rabbit sera which led to the conclusion, that the currently identified Ves v 2b comprises the major hyaluronidase isoforms in wasp venom. In summary, these data emphasise the influence of CCD based cross-reactivity on hymenoptera venom allergy diagnosis. Furthermore, these findings point to the necessity for re-evaluation of numerous allergens with regard to glycosylation and depict the presented approach as a generally applicable strategy not limited to hymenoptera venom allergens only.

After the demonstration of the usefulness of a standardisation of allergen extracts applying monoclonal antibodies, the underlying technology, published in “**Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries**” is going to be presented. Quantitative sIgE measurements are a well established diagnostic tool in allergology and represent the basis for therapeutic interventions and their follow up. Most systems still rely on allergen extracts even if the number of single components, either as purified native or recombinant proteins, is extending. Standardisation of these systems is nowadays performed using polyclonal sera from allergic patients with varying specificities and total IgE amounts. Therefore, allergen extracts in these systems can only be analysed in terms of total IgE binding without regard to single specificities.

To improve these standardisation procedures, monoclonal agents are needed allowing for detection with the system immanent moieties to guarantee comparability. Therefore, we generated allergen-specific monoclonal IgE and IgG antibodies for the major allergen from honeybee venom Api m 1 and to allergens from apple and bovine milk. Basis of this approach was the selection of a human synthetic antibody library comprising a high diversity of scFv formatted variable regions of human origin against the native or recombinant allergens by use of the phage display technology. Allergen-specific scFv obtained from this procedure were converted to artificial scFv based antibody constructs or complete IgE and IgE antibodies by cloning into respective vectors for eukaryotic expression. The recombinant proteins were found to be reactive in immunoblot and ELISA employing both, the recombinant proteins and natural extracts. Furthermore, the antibodies were capable for binding to the high affinity FcεRI IgE receptor indicating the structural integrity of the Fc portion.

In summary, in this approach we gained access to monoclonal IgE and IgG antibodies of virtually any specificity for the improvement of quantitative sIgE measurements and, more generally, for a better standardisation of allergenic extracts particularly regarding therapeutic aspects. Furthermore, this technique might be extended with the generation of patient derived antibody libraries to yield monoclonal IgE directed to native epitopes which would allow for a further characterisation of allergens with regard to fundamental aspects of epitope structure.

Based on this general molecular approach for generation of recombinant antibodies we aimed for the generation of other antibody isotypes, presented in "**Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody fragments**".

Since in mammalian immunoglobulins a high grade of conservation is present in the Fc portion of the antibodies, cross-reactivity can occur in serological assays due to the presence of certain antibody species like RF, HAMA or heterophilic antibodies. This phenomenon can also be observed in the context of allergic disease, since the determination of tryptase levels, an indicator for mastocytosis as a risk factor e.g. in hymenoptera venom allergy, is strongly affected by heterophilic antibodies. Moreover, Fc receptors in human sera show some degree of variability in terms of species specificity and can also account for cross-reactivities in cellular assays. To circumvent these interactions, an immunoglobulin with large phylogenetic distance to mammalian

antibodies, the avian IgY antibody, was investigated, which is the major low molecular weight serum immunoglobulin in chicken and can be obtained easily from the egg yolk.

In the present publication we describe the generation of such IgY in monoclonal form, as well as IgY derived and chimeric IgY antibodies by molecular cloning and their recombinant expression, since classical hybridoma technology is insufficient in terms of IgY [94]. As a model system two different antibody specificities with avian and murine origin were used for the construction of either complete IgY antibodies directed against the IBDV virus major structural protein VP2 [95] or chimeric murine/avian IgY against chicken egg lysozyme. Beside the entire heterotetrameric IgY antibodies, further artificial constructs, based on the respective scFv and lacking the C_H1 region were generated for reasons of expression efficiency. All constructs could be expressed in mammalian cell lines in functional form and proteins could be applied in ELISA and immunoblot analyses. In further analyses, employing different secondary reagents for detection, we observed specific interaction of the generated antibodies with anti IgY antibodies, while protein A/G or anti human IgG antibodies failed to detect the avian IgY molecules, a finding that was further validated in a subsequent study [78].

By generation of these so far limited monoclonal IgY antibodies by recombinant means we made available defined agents for use in serological assays, circumventing all kinds of species related cross-reactivity.

The generation of allergen specific antibodies depends essentially on the availability of the respective antigen/allergen which requires the knowledge on all allergens within a certain extract.

Taken together, the presented data contribute to a broadened view on diagnostic and therapeutic approaches in hymenoptera venom allergy and, moreover, on fundamental aspects of allergy and allergy diagnosis in general. Beside the thorough analysis of already known components from honeybee and yellow jacket venom which for the first time was conducted with functional, high quality recombinant proteins, the identification of two new allergens from these venoms and their characterisation emphasises the complexity of hymenoptera venoms. In addition to the presented data further venom components of *Apis mellifera* (Api m 8 and Api m 9) and *Vespula vulgaris* (Ves v 4 and a further putative allergenic moiety) have been identified and initially characterised although a conclusive evaluation remains to be conducted to estimate their role in hymenoptera venom allergy. Nevertheless, the current work gained access

to a widely extended, if not complete, set of venom allergens and the sophisticated expression strategies allowing for defined fucosylation enabled a re-evaluation of so believed major allergens. Thereby, the relevance of so far underestimated components and the irrelevance of others could be demonstrated. Furthermore, some of these components currently are implemented into an automated diagnostic system in close collaboration with a leading supplier in allergy diagnosis. Evaluation of these allergens can be considered as a first step for a so called “component resolved diagnosis” (CRD) in hymenoptera venom allergy and will constitute a significant improvement of diagnosis. In addition, the generation of a comprehensive panel of hymenoptera venom allergen-specific antibodies allows for further evaluation of venom extracts as well as quality control in future CRD approaches.

4. Outlook

Beside mere diagnostic applications the availability of this comprehensive set of recombinant allergens allows for detailed analyses of the relevance of single components in hymenoptera venom allergy on a molecular level which may contribute to a more general understanding on the mechanisms of sensitisation to certain allergens. Such insights are a prerequisite for the development of strategies for therapeutic interventions which is also orchestrated by the establishment of CRD approaches. The use of defined recombinant components allows for a future component resolved and patient tailored intervention. First attempts in this direction have already been performed with either single components, fragments thereof [101], or hybrid molecules [102, 103], although the number of components and, therefore, the efficiency of such approaches remained limited. The comprehensive set of allergens closely resembles a synthetic venom and now should allow a more convincing set-up of such attempts.

In addition to a component resolved therapy a future direction of special interest would include the generation of hypoallergenic molecules with reduced IgE binding potential but retained characteristics in terms of induction of blocking IgG and T cell stimulation during SIT. Initial investigations in this direction also were limited by the reduced number of so far identified hymenoptera venom allergens [102, 104]. The sophisticated expression strategies established in the present work provide an excellent basis for comparative structural analysis, so far available for Api m 1, Api m 2 and Ves v 5 only.

Taken together all these analyses in hymenoptera venom allergy can be considered as a model for different types of allergies and, thus, provide elucidating insights in mechanisms of sensitisation, hyposensibilisation and diagnosis.

5 References

1. Sears, M.R., *Descriptive epidemiology of asthma.* Lancet, 1997. 350 Suppl 2: p. SII1-4.
2. Thyssen, J.P., et al., *The epidemiology of contact allergy in the general population--prevalence and main findings.* Contact Dermatitis, 2007. 57(5): p. 287-99.
3. Sicherer, S.H. and D.Y. Leung, *Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects in 2008.* J Allergy Clin Immunol, 2009. 123(2): p. 319-27.
4. Cookson, W.O. and M.F. Moffatt, *Asthma: an epidemic in the absence of infection?* Science, 1997. 275(5296): p. 41-2.
5. von Pirquet, C., *Allergie.* MünchMed Wochenschrift, 1906. 30: p. 1457-1458.
6. Gell, P.G.H. and R.R.A. Coombs, *The classification of allergic reactions underlying disease.* Clinical Aspects of Immunology, 1963.
7. Breiteneder, H. and C. Radauer, *A classification of plant food allergens.* J Allergy Clin Immunol, 2004. 113(5): p. 821-30; quiz 831.
8. Yazdanbakhsh, M., P.G. Kremsner, and R. van Ree, *Allergy, parasites, and the hygiene hypothesis.* Science, 2002. 296(5567): p. 490-4.
9. Ring, J., B. Eberlein-Koenig, and H. Behrendt, *Environmental pollution and allergy.* Ann Allergy Asthma Immunol, 2001. 87(6 Suppl 3): p. 2-6.
10. Bryce, M., et al., *Impact of Urbanization on the Proteome of Birch Pollen and Its Chemotactic Activity on Human Granulocytes.* Int Arch Allergy Immunol, 2009. 151(1): p. 46-55.
11. Chambellan, A., et al., *[Diesel particles and allergy: cellular mechanisms].* Allerg Immunol (Paris), 2000. 32(2): p. 43-8.
12. Prausnitz, C. and H. Küstner, *Studien über die Überempfindlichkeit.* ZBL Bakt, 1921. 86: p. 160.
13. Ishizaka, K. and T. Ishizaka, *Physicochemical properties of reaginic antibody. 1. Association of reaginic activity with an immunoglobulin other than gammaA- or gammaG-globulin.* J Allergy, 1966. 37(3): p. 169-85.
14. Schramm, G., et al., *Molecular characterization of an interleukin-4-inducing factor from Schistosoma mansoni eggs.* J Biol Chem, 2003. 278(20): p. 18384-92.
15. Kraft, S. and J.P. Kinet, *New developments in FcepsilonRI regulation, function and inhibition.* Nat Rev Immunol, 2007. 7(5): p. 365-78.
16. Christensen, L.H., et al., *Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge.* J Allergy Clin Immunol, 2008. 122(2): p. 298-304.
17. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nat Rev Immunol, 2008. 8(3): p. 205-17.
18. Romagnani, S., *The Th1/Th2 paradigm and allergic disorders.* Allergy, 1998. 53(46 Suppl): p. 12-5.
19. Romagnani, S., *Immunologic influences on allergy and the TH1/TH2 balance.* J Allergy Clin Immunol, 2004. 113(3): p. 395-400.
20. Vercelli, D., *Discovering susceptibility genes for asthma and allergy.* Nat Rev Immunol, 2008. 8(3): p. 169-82.
21. Shimoda, K., et al., *Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene.* Nature, 1996. 380(6575): p. 630-3.
22. Geha, R.S., H.H. Jabara, and S.R. Brodeur, *The regulation of immunoglobulin E class-switch recombination.* Nat Rev Immunol, 2003. 3(9): p. 721-32.

23. Niederberger, V., et al., *Evolution of IgM, IgE and IgG(1-4)antibody responses in early childhood monitored with recombinant allergen components: implications for class switch mechanisms.* Eur J Immunol, 2002. 32(2): p. 576-84.
24. Marshall, J.S., *Mast-cell responses to pathogens.* Nat Rev Immunol, 2004. 4(10): p. 787-99.
25. Galli, S.J., M. Tsai, and A.M. Piliponsky, *The development of allergic inflammation.* Nature, 2008. 454(7203): p. 445-54.
26. Golden, D.B., et al., *Natural history of Hymenoptera venom sensitivity in adults.* J Allergy Clin Immunol, 1997. 100(6 Pt 1): p. 760-6.
27. Charpin, D., et al., *Prevalence of allergy to hymenoptera stings in different samples of the general population.* J Allergy Clin Immunol, 1992. 90(3 Pt 1): p. 331-4.
28. Bilo, B.M. and F. Bonifazi, *Epidemiology of insect-venom anaphylaxis.* Curr Opin Allergy Clin Immunol, 2008. 8(4): p. 330-7.
29. Simons, F.E., et al., *Risk assessment in anaphylaxis: current and future approaches.* J Allergy Clin Immunol, 2007. 120(1 Suppl): p. S2-24.
30. Kemp, S.F. and R.F. Lockey, *Anaphylaxis: a review of causes and mechanisms.* J Allergy Clin Immunol, 2002. 110(3): p. 341-8.
31. Lantner, R. and R.E. Reisman, *Clinical and immunologic features and subsequent course of patients with severe insect-sting anaphylaxis.* J Allergy Clin Immunol, 1989. 84(6 Pt 1): p. 900-6.
32. Biedermann, T., et al., *Mastocytosis associated with severe wasp sting anaphylaxis detected by elevated serum mast cell tryptase levels.* Br J Dermatol, 1999. 141(6): p. 1110-2.
33. van der Linden, P.W., et al., *Insect-sting challenge in 324 subjects with a previous anaphylactic reaction: current criteria for insect-venom hypersensitivity do not predict the occurrence and the severity of anaphylaxis.* J Allergy Clin Immunol, 1994. 94(2 Pt 1): p. 151-9.
34. Antonicelli, L., et al., *European hornet (Vespa crabro) sting: a new risk factor for life-threatening reaction in hymenoptera allergic patients?* Eur Ann Allergy Clin Immunol, 2003. 35(6): p. 199-203.
35. Kochuyt, A.M., E. Van Hoeyveld, and E.A. Stevens, *Occupational allergy to bumble bee venom.* Clin Exp Allergy, 1993. 23(3): p. 190-5.
36. Hoffman, D.R. and R.S. Jacobson, *Allergens in hymenoptera venom XII: how much protein is in a sting?* Ann Allergy, 1984. 52(4): p. 276-8.
37. King, T.P., et al., *Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy.* J Allergy Clin Immunol, 1996. 98(3): p. 588-600.
38. Sobotka, A.K., et al., *Allergy to insect stings. II. Phospholipase A: the major allergen in honeybee venom.* J Allergy Clin Immunol, 1976. 57(1): p. 29-40.
39. King, T.P., et al., *Allergens of honey bee venom.* Arch Biochem Biophys, 1976. 172(2): p. 661-71.
40. Hoffman, D.R., *Allergens in Hymenoptera venom. XXV: The amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity.* J Allergy Clin Immunol, 1993. 92(5): p. 707-16.
41. Grunwald, T., et al., *Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3).* J Allergy Clin Immunol, 2006. 117(4): p. 848-54.

42. Hoffman, D.R. and R.S. Jacobson, *Allergens in Hymenoptera venom. XXVII: bumblebee venom allergy and allergens.* J Allergy Clin Immunol, 1996. 97(3): p. 812-21.
43. Hoffman, D.R., *Hymenoptera venom allergens.* Clin Rev Allergy Immunol, 2006. 30(2): p. 109-28.
44. Kettner, A., et al., *Api m 6: a new bee venom allergen.* J Allergy Clin Immunol, 2001. 107(5): p. 914-20.
45. Peiren, N., et al., *Molecular cloning and expression of icarapin, a novel IgE-binding bee venom protein.* FEBS Lett, 2006. 580(20): p. 4895-9.
46. Winningham, K.M., et al., *Hymenoptera venom protease allergens.* J Allergy Clin Immunol, 2004. 114(4): p. 928-33.
47. Blank, S., et al., *Identification, recombinant expression and characterization of the 100 kDa high molecular weight hymenoptera venom allergens Api m 5 and Ves v 3.* submitted, 2008.
48. Kolarich, D., et al., *The N-glycans of yellow jacket venom hyaluronidases and the protein sequence of its major isoform in Vespula vulgaris.* Febs J, 2005. 272(20): p. 5182-90.
49. Nemoto, E., et al., *Increase of CD26/dipeptidyl peptidase IV expression on human gingival fibroblasts upon stimulation with cytokines and bacterial components.* Infect Immun, 1999. 67(12): p. 6225-33.
50. Yaron, A. and F. Naider, *Proline-dependent structural and biological properties of peptides and proteins.* Crit Rev Biochem Mol Biol, 1993. 28(1): p. 31-81.
51. Edery, H., et al., *Venoms of Vespidae.* Arthropod venoms, handbook exp. pharmacol., 1978: p. 690-771.
52. Paull, B.R., J.W. Yunginger, and G.J. Gleich, *Melittin: an allergen of honeybee venom.* J Allergy Clin Immunol, 1977. 59(4): p. 334-8.
53. Hemmer, W., et al., *Identification by immunoblot of venom glycoproteins displaying immunoglobulin E-binding N-glycans as cross-reactive allergens in honeybee and yellow jacket venom.* Clin Exp Allergy, 2004. 34(3): p. 460-9.
54. Hoffman, D.R. and C.A. McDonald, *Allergens in hymenoptera venom. VIII. Immunologic comparison of venoms from six species of Vespula (yellow jackets).* Ann Allergy, 1982. 48(2): p. 78-81.
55. Hoffman, D.R., *Allergens in Hymenoptera venom XV: The immunologic basis of vespid venom cross-reactivity.* J Allergy Clin Immunol, 1985. 75(5): p. 611-3.
56. Skov, L.K., et al., *Structure of recombinant Ves v 2 at 2.0 Angstrom resolution: structural analysis of an allergenic hyaluronidase from wasp venom.* Acta Crystallogr D Biol Crystallogr, 2006. 62(Pt 6): p. 595-604.
57. Radcliffe, M.J., et al., *Do skin prick and conjunctival provocation tests predict symptom severity in seasonal allergic rhinoconjunctivitis?* Clin Exp Allergy, 2006. 36(12): p. 1488-93.
58. Ferrer, M., et al., *Molecular diagnosis in allergology: application of the microarray technique.* J Investig Allergol Clin Immunol, 2009. 19 Suppl 1: p. 19-24.
59. Rudeschko, O., et al., *Optimization of apple allergen preparation for in vivo and in vitro diagnostics.* Allergy, 1995. 50(3): p. 262-8.
60. Wood, R.A., et al., *Accuracy of IgE antibody laboratory results.* Ann Allergy Asthma Immunol, 2007. 99(1): p. 34-41.
61. Eberlein-Konig, B., et al., *In vitro basophil activation using CD63 expression in patients with bee and wasp venom allergy.* J Investig Allergol Clin Immunol, 2006. 16(1): p. 5-10.

62. Hemmer, W., et al., *Antibody binding to venom carbohydrates is a frequent cause for double positivity to honeybee and yellow jacket venom in patients with stinging-insect allergy.* J Allergy Clin Immunol, 2001. 108(6): p. 1045-52.
63. Ballmer-Weber, B.K., et al., *Component-resolved in vitro diagnosis in carrot allergy: does the use of recombinant carrot allergens improve the reliability of the diagnostic procedure?* Clin Exp Allergy, 2005. 35(7): p. 970-8.
64. Hansen, K.S., et al., *Component-resolved in vitro diagnosis of hazelnut allergy in Europe.* J Allergy Clin Immunol, 2009. 123(5): p. 1134-41, 1141 e1-3.
65. Constantin, C., et al., *Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis.* Allergy, 2009. 64(7): p. 1030-7.
66. Aalberse, R.C., J. Akkerdaas, and R. van Ree, *Cross-reactivity of IgE antibodies to allergens.* Allergy, 2001. 56(6): p. 478-90.
67. van Ree, R., et al., *Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens.* J Biol Chem, 2000. 275(15): p. 11451-8.
68. Hemmer, W., *[Cross-reactivity to honeybee and wasp venom].* Hautarzt, 2008. 59(3): p. 194-9.
69. Mari, A., *IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity.* Int Arch Allergy Immunol, 2002. 129(4): p. 286-95.
70. van Ree, R., *Carbohydrate epitopes and their relevance for the diagnosis and treatment of allergic diseases.* Int Arch Allergy Immunol, 2002. 129(3): p. 189-97.
71. Iacovacci, P., et al., *Comparison between the native glycosylated and the recombinant Cup a1 allergen: role of carbohydrates in the histamine release from basophils.* Clin Exp Allergy, 2002. 32(11): p. 1620-7.
72. Altmann, F., *The role of protein glycosylation in allergy.* Int Arch Allergy Immunol, 2007. 142(2): p. 99-115.
73. Hancock, K., et al., *False positive reactivity of recombinant, diagnostic, glycoproteins produced in High Five insect cells: effect of glycosylation.* J Immunol Methods, 2008. 330(1-2): p. 130-6.
74. Boscato, L.M. and M.C. Stuart, *Heterophilic antibodies: a problem for all immunoassays.* Clin Chem, 1988. 34(1): p. 27-33.
75. Hennig, C., L. Rink, and H. Kirchner, *Evidence for presence of IgG4 anti-immunoglobulin autoantibodies in all human beings.* Lancet, 2000. 355(9215): p. 1617-8.
76. Hennig, C., et al., *The influence of naturally occurring heterophilic anti-immunoglobulin antibodies on direct measurement of serum proteins using sandwich ELISAs.* J Immunol Methods, 2000. 235(1-2): p. 71-80.
77. van Toorenenbergen, A.W., et al., *Heterophilic antibody interference in a tryptase immunoassay.* Clin Biochem, 2008. 41(4-5): p. 331-4.
78. Greunke, K., et al., *Recombinant IgY for improvement of immunoglobulin-based analytical applications.* Clin Biochem, 2008. 41(14-15): p. 1237-44.
79. Strunk, R.C. and G.R. Bloomberg, *Omalizumab for asthma.* N Engl J Med, 2006. 354(25): p. 2689-95.
80. Cohen, S.G., A.W. Frankland, and M. Dworetzky, *Noon and Freeman on prophylactic inoculation against hay fever.* J Allergy Clin Immunol, 2003. 111(5): p. 1142-50.
81. Wachholz, P.A. and S.R. Durham, *Mechanisms of immunotherapy: IgG revisited.* Curr Opin Allergy Clin Immunol, 2004. 4(4): p. 313-8.

82. Akdis, C.A. and M. Akdis, *Mechanisms and treatment of allergic disease in the big picture of regulatory T cells.* J Allergy Clin Immunol, 2009. 123(4): p. 735-46; quiz 747-8.
83. Akdis, C.A., K. Blaser, and M. Akdis, *Mechanisms of allergen-specific immunotherapy.* Chem Immunol Allergy, 2006. 91: p. 195-203.
84. Verhagen, J., et al., *T regulatory cells in allergen-specific immunotherapy.* Int Rev Immunol, 2005. 24(5-6): p. 533-48.
85. Golden, D.B., *Fatal insect allergy after discontinuation of venom immunotherapy.* J Allergy Clin Immunol, 2001. 107(5): p. 925-6.
86. Alvarez-Cuesta, E., et al., *Immunotherapy with depigmented glutaraldehyde-polymerized extracts: changes in quality of life.* Clin Exp Allergy, 2005. 35(5): p. 572-8.
87. Roll, A., et al., *Safety of specific immunotherapy using a four-hour ultra-rush induction scheme in bee and wasp allergy.* J Investig Allergol Clin Immunol, 2006. 16(2): p. 79-85.
88. Akdis, C.A., et al., *Immunological mechanisms of sublingual immunotherapy.* Allergy, 2006. 61 Suppl 81: p. 11-4.
89. Moverare, R., et al., *Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP System.* Allergy, 2002. 57(5): p. 423-30.
90. Wald, M., et al., *Generation of a low immunoglobulin E-binding mutant of the timothy grass pollen major allergen Phl p 5a.* Clin Exp Allergy, 2007. 37(3): p. 441-50.
91. Jutel, M., et al., *Allergen-specific immunotherapy with recombinant grass pollen allergens.* J Allergy Clin Immunol, 2005. 116(3): p. 608-13.
92. Muller, U.R., et al., *Hymenoptera venom allergy: analysis of double positivity to honey bee and Vespula venom by estimation of IgE antibodies to species-specific major allergens Api m1 and Ves v5.* Allergy, 2009. 64(4): p. 543-8.
93. Binder, M., et al., *Individual hymenoptera venom compounds induce upregulation of the basophil activation marker ectonucleotide pyrophosphatase/phosphodiesterase 3 (CD203c) in sensitized patients.* Int Arch Allergy Immunol, 2002. 129(2): p. 160-8.
94. Matsuda, H., et al., *A chicken monoclonal antibody with specificity for the N-terminal of human prion protein.* FEMS Immunol Med Microbiol, 1999. 23(3): p. 189-94.
95. Sapats, S.I., et al., *Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV).* Arch Virol, 2003. 148(3): p. 497-515.
96. Hoffman, D.R., W.H. Shipman, and D. Babin, *Allergens in bee venom II. Two new high molecular weight allergenic specificities.* J Allergy Clin Immunol, 1977. 59(2): p. 147-53.
97. *Insights into social insects from the genome of the honeybee Apis mellifera.* Nature, 2006. 443(7114): p. 931-49.
98. Hoffman, D.R. and C.L. Wood, *Allergens in Hymenoptera venom XI. Isolation of protein allergens from Vespula maculifrons (yellow jacket) venom.* J Allergy Clin Immunol, 1984. 74(1): p. 93-103.
99. King, T.P., et al., *Allergen nomenclature.* Allergy, 1995. 50(9): p. 765-74.
100. Seppala, U., et al., *Structural and immunological characterization of the N-glycans from the major yellow jacket allergen Ves v 2: the N-glycan structures are needed for the human antibody recognition.* Mol Immunol, 2009. 46(10): p. 2014-21.

101. Muller, U., et al., *Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom.* J Allergy Clin Immunol, 1998. 101(6 Pt 1): p. 747-54.
102. Karamloo, F., et al., *Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes.* Eur J Immunol, 2005. 35(11): p. 3268-76.
103. Kussebi, F., et al., *A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy.* J Allergy Clin Immunol, 2005. 115(2): p. 323-9.
104. Suck, R., et al., *Purification and immunobiochemical characterization of folding variants of the recombinant major wasp allergen Ves v 5 (antigen 5).* Int Arch Allergy Immunol, 2000. 121(4): p. 284-91.

6.1 Gefahrstoffe und Sicherheitsdaten

Folgende verwendete Reagenzien und Lösungsmittel waren mit Gefahrenhinweisen und Sicherheitsratschlägen gemäß §6 der Gefahrstoffverordnung versehen. Krebserzeugende, erbgutverändernde oder fortpflanzungsgefährdende Stoffe (KMR) sind fett gedruckt:

Verbindung	Gefahrensymbol	R-Sätze	S-Sätze
ABTS	Xi	36/37/38	26-36
Acrylamid	**T**	**45-46-20/21-25-36/38-43-48/23/24/25-62**	**53-45**
Ammoniumpersulfat	Xn	8-22-36/37/38-42/43	22-24-26-37
Ampicillin	Xn	36/37/38-42/43	22-26-36/37
BCIP	Xn,T		22-24/25
Calciumchlorid Dihydrat	Xi		36 22-24
Chloroform	**Xn**	**22-38-40-48/20/22**	**36/37**
Diethylpyrocarbonat	Xn	20/22-36/37/38	26-36
Dimethylformamid	**T**	**61-20/21-36**	**53-45**
Dimethylsulfoxid	Xi		36/38 26
Dithiothreitol	Xi	36/37/38	36/37/39-22
EDTA-Dinatriumsalz-Dihydrat	Xn	22	-
Ethidiumbromid	**T+**	**22-26-36/37/38-40**	**26-28.2-36/37-45**
Ethanol	F	11	7-16
Glutardialdehyd 25%	T, N	22-23-34-42/43-50	26-26/37/39-45-61
Imidazol	C	22-34	22-26-36/37/39-45
Kanamycin Sulfat	T	61	26-36/37-39-45
Methanol	F, T	11-23/24/25-39/23/24/25	7-16-36/37-45
NaOH	C	35	26-37/39-45
Ni-NTA-Agarose	Xn	10-22-40-42/43	13-26-36-46
NBT	Xn	20/21-33	22-45
Phenol (Tris-gesättigt)	**T**	**24/25-34**	**28.6-45**
2-Propanol	F, Xi	11-36-67	7-16-24/25-26
Salzsäure, konz.	C	34-37	26-36/37/39-45
SDS	Xn	22-36/38	22-24/25
TEMED	C, F	11-20/22-34	16-26-36/37/39-45
Triethylamin	F, C	11-20/21/22-35	3-16-26-29-36/37/39-45
Tris	Xi	36/38	-
Wasserstoffperoxid 30%	C	34	3-28-36/39-45
Xylen Cyanol	Xi	36	24
Zitronensäure	Xi	36	24/25

6.2 Selbständigkeitserklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte fremde Hilfe angefertigt habe. Ich habe keine anderen als die im Literaturverzeichnis angeführten Quellen benutzt und sämtliche Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen wurden, und alle Angaben, die auf mündlichen Auskünften beruhen, als solche kenntlich gemacht.

Die Arbeit wurde zuvor keiner Prüfungsbehörde in gleicher oder ähnlicher Form vorgelegt.

Hamburg, den 05. November 2009

_____

Henning Seismann

6.3 Angaben zum individuellen Beitrag an den Publikationen

Greunke, K., Spillner, E., Braren, I**., Seismann, H**., Kainz, S., Hahn, U., Grunwald, T., Bredehorst, R. Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody fragments.
J Biotechnol **124(2)**:446-56 82006) (Impact Factor 2008: 2,7)
Eigener Anteil: ca. 30% (Klonierung unterschiedlicher monoklonaler IgY Antikörper mit Spezifität für VP2, Expression der Antikörper In HEK293, Reinigung der Antikörper und Reaktivitätsanalyse)

Braren, I., Blank, S., **Seismann, H.**, Deckers, S., Ollert, M., Grunwald, T., and Spillner, E. Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries.
Clinical Chemistry **53(5)**: 837-844 (2007) (Impact Factor 2008: 5,6)
Eigener Anteil: ca. 30% (Generierung monoklonaler Antikörper mit Spezifität für Gal d 4, Klonierung als IgE und IgG4 Vollantikörper und verkürzter Antikörperkonstrukte, Expression und Reinigung der Antikörper, Reaktivitätsanalyse der Antikörper, Stabilitätsanalyse der Antikörper)

Seismann, H., Blank, S., Braren, I., Greunke, K., Cifuentes, L., Grunwald, T., Bredehorst, R., Ollert, M., and Spillner, E. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3 core fucosylation.
Molecular Immunology , im Druck (2009). (Impact Factor 2008: 3,6)
Eigener Anteil: ca. 45% (Klonierung, Expression in verschiedenen Zelllinien und Reinigung von der Hyaluronidase Isoformen Ves v 2a und b, Charakterisierung von rekombinantem Ves v 2a und b, immunologische Charakterisierung der differentiell glykosylierten Proteine, Charakterisierung verschiedener Insektenzelllinien bezüglich der Fähigkeit zur alpha-1,3-Fucosylierung, Quantifizierung der Hyaluronidase Isoformen Ves v 2a und b im Gift von *Vespula vulgaris*, Bestimmung der Hyaluronidaseaktivität der rekombinanten Proteine, sowie des Bienen- und Wespengifts, Textbeiträge: Einleitung, Methoden, Ergebnisse und Diskussion)

Prof. Dr. Reinhard Bredehorst

6.4 Curriculum Vitae

Personal data	Henning Seismann Rögen 8 23863 Nienwohld
Date of birth	11.08.1978 in Bad Oldesloe
Education	08.85-06.89 primary school Bargfeld-Stegen 08.89-06.98 Kreisgymnasium I Bargteheide
Community service	08.98-08.99 at the Heinrich-Sengelmann Krankenhaus Bargfeld-Stegen
Internship	11.99 at the Forschungszentrum Borstel/ Leibniz-Zentrum für Medizin und Biowissenschaften
Studies	03.00-06.05 Graduated in biology at the University of Hamburg
Diploma thesis	09.04-06.05 at the Institute for Biochemistry and Molecular Biologie; Work group of Prof. Dr. R. Bredehorst Title: Generation and selection of avian antibody libraries
Phd thesis	02.06-12.09 at the University of Hamburg Institute for Biochemistry and Molecular Biologie; Work group of Prof. Dr. R. Bredehorst Title: Recombinant strategies in hymenoptera venom allergy and beyond

Publications

Greunke, K., Spillner, E., Braren, I., Seismann, H., Kainz, S., Hahn, U., Grunwald, T., Bredehorst, R. Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody fragments. J Biotechnol 124(2):446-56 2006

Braren, I., Blank, S., Seismann, H., Deckers, S., Ollert, M., Grunwald, T., Spillner, E. Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries. Clin Chem. 53(5):837-44 2007

Seismann, H., Blank, S., Braren, I., Cifuentes, L., Bredehorst, R., Grunwald, T., Ollert, M., Spillner, E. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. Mol Immunol 2009 in press

Abstracts

Blank, S., Seismann, H., Bockisch, B., Cifuentes, L., Ring, J., Grunwald, T., Ollert, M., Spillner, E. Recombinant production of a panel of hymenoptera venom allergens in insect cells. *Allergo J* **16(1)**: 33 (2007).

Cifuentes, L., Seismann, H., Blank, S., Bockisch, B., Spillner, E., Grunwald, T., Darsow, U., Ring, J., Ollert, M. Honey bee venom allergy with negative venom-specific IgE: The use of allergenic molecules provides an improved diagnostic solution. *Allergo J* **16(1)**: 46 (2007).

Blank, S., Seismann, H., Bockisch, B., Braren, I., Bredehorst, R., Ollert, M.W., Ring, J., Grunwald, T., Spillner, E. Identification, recombinant expression and characterization of high molecular weight hymenoptera venom allergens. *Allergo J* **17(1)**: 36 (2008).

Seismann, H., Blank, S., Braren, I., Grunwald, T., Bredehorst, R., Ollert, M., Spillner, E. Generation of a panel of monoclonal IgE antibodies with specificity for hymenoptera venom allergens. *Allergo J* **17(1)**: 43 (2008)

Blank, S., Seismann, H., Bockisch, B., Braren, I., Bredehorst, R., Ollert, M.W., Grunwald, T., Spillner, E. Identification, recombinant expression and characterization of high molecular weight hymenoptera venom allergens. *Allergy* **63 (Suppl. 88)**: 13-14 (2008)

Seismann, H., Blank, S., Braren, I., Grunwald, T., Bredehorst, R., Ollert, M., Spillner, E. Generation of a panel of monoclonal IgE antibodies with specificity for hymenoptera venom allergens. *Allergy* **63 (Suppl. 88)**: 13 (2008)

Braren, I., Hecker, J., Blank, S., Seismann, H., Rühl, D., Bredehorst, R., Grunwald, T., Ollert, M., Spillner, E. Addressing current challenges of allergy diagnostics using recombinant human antibody isotypes. *Allergy* **63 (Suppl. 88)**: 42-43 (2008)

Conference contributions

Cifuentes, L., Balzer, L., Seismann, H., Braren, I., Blank, S., Spillner, E., Ring, J., Mempel, M., Ollert, M. Up-regulation of CD63 in basophils from wasp venom-allergic patients by insect cell-expressed recombinant Antigen 5 (Ves v 5). *Allergy* **63 (Suppl. 88)**: 442 (2008)

Seismann, H., Blank, S., Braren, I., Greunke, K., Cifuentes, L., Grunwald, T., Bredehorst, R., Ollert, M., Spillner, E. Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. *Allergo J* **18(1)**: 31 (2009)

Seismann, H., Blank, S., Braren, I., Cifuentes, L., Bredehorst, R., Grunwald, T., Ollert, M., Spillner, E. Improvement of molecular diagnosis in hymenoptera venom hypersensitivity by the use of the recombinant major allergens Ves v 1 and Ves v 5. *Allergy* **64 (Suppl. 90)**: 39 (2009)

Blank, S., Seismann, H., Braren, I., Greunke, K., Cifuentes, L., Grunwald, T., Bredehorst, R., Ollert, M., Spillner, E. Dissecting CCD reactivity in hymenoptera venom allergy by diminution of alpha-1,3-core fucosylation. *Allergy* **64 (Suppl. 90)**: 39 (2009)

Braren, I., Blank, S., Greunke, K., Seismann, H., Ollert, M and Spillner, E. Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries. 2nd International Symposium on Molecular Allergology. Rome, Italy, 22.-24 April 2007.

Blank, S., Seismann, H., Bockisch, B., Braren, I., Bredehorst, R., Grunwald, T., Ollert, M.W., Spillner, E. Identification and recombinant expression of a novel IgE-reactive 70 kDa carboxylesterase from *Apis mellifera* venom. 3nd International Symposium on Molecular Allergology. Salzburg, Austria, 18.-20 April 2008.

Seismann, H., Blank S., Braren, I., Bredehorst, R., Ollert, M.W., Grunwald, T., Spillner, E. Identification, recombinant expression and characterization of high molecular weight hymenoptera venom allergens. XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona, Spain, 7.-11. June 2008.

Seismann H.; Blank S., Braren I., Cifuentes L., Bredehorst R., Grunwald T.,Ollert M., Spillner E. Improvement of molecular diagnosis in hymenoptera venom hypersensitivity by the use of the recombinant major allergens Ves v 1 and Ves v 5. XXVIII Congress of the European Academy of Allergology and Clinical Immunology. Warsaw, Poland, 6.-10. June 2009.