Components and Mechanisms in Diagnosis and Therapy of Hymenoptera Venom Allergy

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

der Fakultät für Mathematik, Informatik und Naturwissenschaften der Universität Hamburg

vorgelegt von

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Hamburg 2009

The experimental part of this work was performed from November 2005 until November 2009 in the group of Prof. Dr. Reinhard Bredehorst at the Department of Chemistry, Institute for Biochemistry and Molecular Biology, University of Hamburg.

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Datum der Disputation:	04.12.2009

Abstract

Anaphylaxis due to Hymenoptera stings is one of the most severe clinical outcomes of IgE-mediated hypersensitivity reactions. Although allergic reactions to Hymenoptera stings are often considered as a general model for the underlying principles of allergic disease, diagnostic tests are still hampered by a lack of specificity and venom immunotherapies by severe systemic side-effects and incomplete protection.

Thus, aim of this work was the improvement of both diagnostic and therapeutic approaches by recombinant technologies. Since a recombinant availability of venom allergens might offer several promising possibilities for an improvement, novel allergenic components from *Apis mellifera* and *Vespula vulgaris* venom were identified as well as established allergens recombinantly produced and characterized in detail.

With the 100 kDa allergens Api m 5 and Ves v 3 a novel cross-reactive allergen family, the dipeptidylpeptidases IV, was identified. Both enzymes were generated in recombinant form, enzymatically characterized and their relevance as major allergens in Hymenoptera venom was demonstrated by different immunological and cellular methods assessing sIgE of venom-allergic patients.

For the recently identified Carbohydrate-rich protein from honeybee venom importance as sensitizing venom component in approximately 50% of venom-sensitized patients and applicability as novel surrogate marker candidate for honeybee venom sensitization were demonstrated by comparative assessment of differentially glycosylated recombinant molecules.

By the recombinant production of these new allergens, as well as established allergens like Api m 2, Api m 3, Ves v 1, Ves v 2a, Ves v 2b, and Ves v 5 in insect cells it was possible to provide a broad panel of properly folded and posttranslationally modified molecules for component-resolved approaches to Hymenoptera venom allergy.

Moreover, a novel diagnostic concept was introduced by the production of properly glycosylated allergens allowing reliable differentiation of protein versus cross-reactive carbohydrate determinant (CCD) reactivity and enabling identifycation of true sensitization with clinical impact.

Additionally, the generation of monoclonal recombinant allergen-specific IgE, IgG and IgY antibodies could be established for application in standardization and for avoidance of assay interference in immunoassays as well as for assessment of the complex molecular interactions of allergens, specific antibodies and their receptors. By the use of monoclonal IgE antibodies we were for the first time able to detect a putatively essential allergen in *A. mellifera* venom and, moreover, to demonstrate its absence in various therapeutic preparations, a finding with major implications for specific immunotherapy of allergy.

The results of this work demonstrate that a variety of recombinant technologies can provide novel, component-resolved concepts for the identification of clinically relevant allergens, proper allergy diagnosis and the design of adequate intervention strategies. Moreover, the newly identified and established recombinant allergens might contribute to a more detailed understanding of the molecular and allergological mechanisms of insect venoms.

Zusammenfassung

Anaphylaktische Reaktionen nach Hymenopterenstichen sind eine der schwersten klinischen Erscheinungsformen IgE-vermittelter Überempfindlichkeitsreaktionen. Obgleich allergische Reaktionen auf Hymenopterenstiche häufig als Modell der zugrunde liegenden Prinzipien allergischer Erkrankungen angesehen werden, sind diagnostische Tests noch immer durch einen Mangel an Spezifität und Insektengift-Immuntherapien durch schwere Nebenwirkungen und einen unvollständigen Schutz beeinträchtigt.

Das Ziel dieser Arbeit war somit die Verbesserung diagnostischer und therapeutischer Ansätze mit Hilfe rekombinanter Technologien. Da die rekombinante Verfügbarkeit der Insektengiftallergene eine Reihe viel versprechender Möglichkeiten für eine derartige Verbesserung bieten könnte, wurden neuartige Komponenten der Gifte von *Apis mellifera* und *Vespula vulgaris* identifiziert sowie etablierte Allergene rekombinant hergestellt und im Detail charakterisiert.

Mit den 100 kDa Allergenen Api m 5 und Ves v 3 haben wir eine neuartige kreuzreaktive Allergenfamilie, die Dipeptidylpeptidasen IV, identifiziert. Beide Enzyme wurden in rekombinanter Form erzeugt, enzymatisch charakterisiert und ihre Relevanz als Hauptallergene in Hymenopterengiften mittels unterschiedlicher, auf spezifischen IgE-Antikörpern allergischer Patienten basierender immunologischer und zellulärer Methoden, demonstriert.

Für das kürzlich identifizierte *Carbohydrate-rich protein* des Bienengiftes konnten wir anhand des Vergleichs differentiell glykosylierter, rekombinanter Moleküle die Wichtigkeit als sensibilisierende Giftkomponente in ungefähr 50% der Bienengift-sensibilisierten Patienten sowie die Anwendbarkeit als neuartigen Surrogatmarker Kandidaten für Bienengiftsensibilisierung demonstrieren.

Durch die rekombinante Herstellung dieser neuen, als auch etablierter Allergene wie Api m 2, Api m 3, Ves v 1, Ves v 2a, Ves v 2b und Ves v 5 in Insektenzellen, war es möglich, ein breites Panel korrekt gefalteter und posttranslational modifizierter Moleküle für einen komponentenaufgelösten Ansatz im Bereich der Hymenopterengiftallergie bereitzustellen.

Zusätzlich konnte die Generierung monoklonaler Allergen-spezifischer IgE-, IgG- und IgY-Antikörper für die Anwendung im Bereich der Standardisierung und zur Vermeidung von Interferenzen in immunologischen Tests sowie die Analyse der komplexen molekularen Interaktionen von Allergenen, spezifischen Antikörpern und deren entsprechenden Rezeptoren, etabliert werden. Durch die Nutzung eines solchen monoklonalen IgE-Antikörpers konnten wir erstmalig ein potentiell essentielles Allergen im Gift von *A. mellifera* detektieren und zusätzlich dessen Fehlen in verschiedenen therapeutischen Präparaten nachweisen; eine Entdeckung mit wichtigen Implikationen für die spezifische Immuntherapie der Allergie.

Die Resultate dieser Arbeit zeigen, dass verschiedenste rekombinante Technologien neue komponentenaufgelöste Konzepte für die Identifizierung klinisch relevanter Allergene, exakte Allergiediagnose und das Design adäquater Interventionsstrategien bereitstellen können. Zudem könnten die neu identifizierten und etablierten rekombinanten Allergene zu einem detaillierterem Verständnis der molekularen und allergologischen Mechanismen von Insektengiften beitragen.

Contents

1. Introduction

1.1 Allergy

The term “allergy” was coined by Clemens von Pirquet in 1906 to discriminate beneficial and harmful immune responses[1]. Today the term allergy is associated with an abnormal, pathogenic, adaptive immune response directed against non-infectious environmental substances (allergens). Allergic disorders, such as anaphylaxis, hay fever, eczema and asthma, now afflict roughly 25% of people in the developed world[2]. In allergic subjects, persistent or repetitive exposure to allergens, which typically are intrinsically innocuous substances common in the environment, results in chronic inflammation.

To unify the concepts of allergy, including the cutaneous immediate and delayed hypersensitivity reactions, Coombs and Gell proposed a classification of the immunopathologic mechanism[3]. They separated the reactions by which a specific antigen can induce cellular and tissue injury into four groups: Type I (immediate or anaphylactic), type II (cytotoxic or cytolytic), type III (antigen-antibody complex) and type IV (delayed or cell-mediated). In the situation of a type II response circulating IgG or IgM antibodies react with antigens that may actually be portions of cells such as erythrocytes and their membranes or with an unrelated antigen such as a drug that has become associated with these cells which may lead to complement-mediated lysis or cytotoxic action by killer cells. The type III reaction is referred to as immune-complex injury or tissue damage. In this immunopathologic reaction serum IgG antibodies interact with an antigen, antigen-antibody-complexes are formed and deposited in the tissue, complement is activated and immune cells are attracted to the site of antigen deposition, causing local damage[4]. The type IV reaction is the T cell-mediated immune response or delayed hypersensitivity reaction where antigen-sensitized T cells release cytokines following a secondary contact with the same antigen, thereby inducing inflammatory reactions.

The type I reaction of Coombs and Gell is referred to as the immediate, anaphylactic reaction. This reaction might also be called an atopic phenomenon, and is responsible for many of the common allergic diseases. Clinical examples include asthma, hay fever, urticaria, angiooedema, and anaphylaxis. In the majority of cases the term allergy is equalized with the type I hypersensitivity reaction.

1.1.1 Type I hypersensitivity

An established hypersensitivity type I reaction can occur in individuals, with increased susceptibility to mount IgE responses, termed atopy, after the primary response to an allergen, called sensitization. Although almost half of the urban population worldwide is atopic and most allergy suffers from atopy, it is possible to develop allergies in the absence of atopy; a common example is the allergy against Hymenoptera venoms[5]. Sensitization to an allergen reflects its ability to elicit a T helper type 2 (T_H2) cell response, in which Interleukin-4 (IL-4) and IL-13 drive IgE production by promoting immunoglobulin class-switch recombination in B cells[6-9]. Many factors affect the probability of developing clinically significant sensitization[10,11]: host genotype, type of allergen, allergen concentration in the environment and whether exposure occurs together with agents that can enhance the sensitization process like certain ligands of Toll-like receptors[12]. In the case of respiratory allergies, minute amounts of soluble antigen are released from allergen-bearing particles on mucosal surfaces, and in the case of allergies to stinging insects allergens are directly injected into the skin. Antigen-presenting cells (APCs), particularly dendritic cells (DCs), take up allergens and process them. Subsequently, activated DCs mature and migrate to regional lymph nodes or to sites in the local mucosa, where they present peptides derived from processed allergens in the context of major histocompatibility complex (MHC) class II molecules to naïve T cells (Fig. 2). In the presence of “early IL-4” -potentially derived from a range of cells, including basophils, mast cells, eosinophils, natural killer T cells and T cells- naïve T cells acquire the characteristics of T_H2 cells[2], in which the transcription factor GATA3 (GATA-binding protein 3) mediates cytokine secretion[13] (Fig.2). Different $CD4^+$ helper T cell effector lineages control host defenses against distinct classes of pathogens (Fig. 1). T helper type 1 cells (T_H1 cells), controlled by the transcription factor T-bet (T box expressed in T cells)[14], provide protective immunity to intracellular bacterial, viral and protozoan pathogens. IL-17-producing T helper cells (T_H-17 cells), characterized by the transcription factor RORγt (RAR-related orphan receptor γt)[15], regulate host defense against extracellular bacterial and fungal pathogens and T_H2 cells orchestrate immunity to multicellular parasites, including helminthes, which are mostly extracellular pathogens[16]. Inappropriate activation of these three arms of adaptive immunity can lead to different types of immunopathologies, including autoimmunity

in the case of T_H1 and T_H-17 responses and allergies in the case of T_H2 responses[16]. Although the basic aspects of the activation of T_H1 and T_H-17 immune responses are well characterized[17,18], the mechanisms of the induction of T_H2 responses remain obscure. Recent work now revealed an outstanding role for basophils as APCs for T_H2 differentiation in response to protease allergens[19].

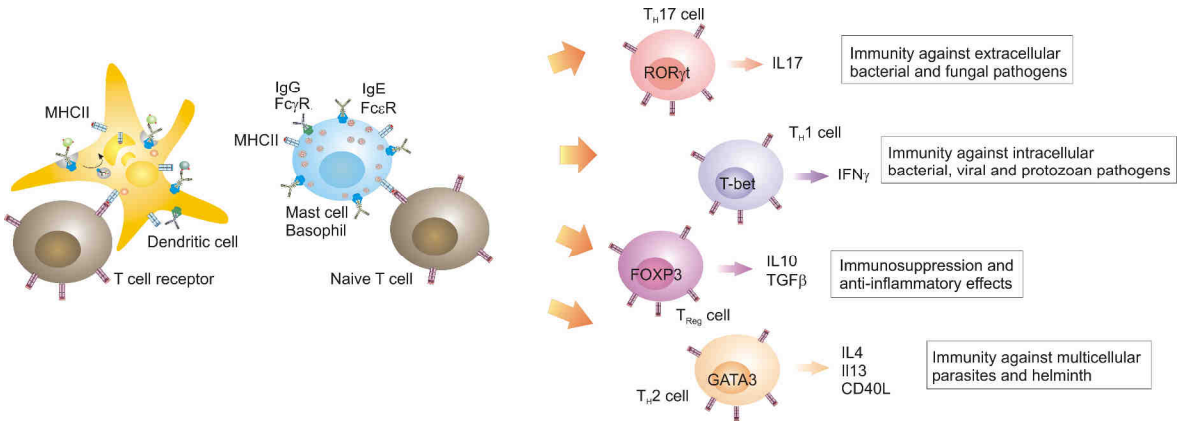

Fig. 1: T helper (T_H) cell differentiation. Schematic representation of different ways of T_H cell differentiation and overview of immunological functions of T_H cell subsets. MHC = major histocompatibility complex; T_{Reg} cell = T regulatory cell; FcεR = Fc recptor for IgE; FcγR = Fc receptor for IgG; IL = Interleukin; TGF = transforming growth factor; IFN = Interferon; CD = cluster of differentiation; CD40L = CD40 ligand; RORγt = RAR-related orphan receptor γt; T-bet = T box expressed in T cells; FOXP3 = forkhead box P3; GATA3 = GATA binding protein 3.

In the presence of IL-4 and IL-13, produced by T_H2 cells, together with the ligation of suitable co-stimulatory molecules (CD40 with CD40 ligand, and CD80 or CD86 with CD28), B cells undergo immunoglobulin class-switch recombination (Fig. 2), in which the gene segments that encode the immunoglobulin heavy chain are rearranged resulting in the production of antibodies of the IgE class[9,20]. IgE diffuses locally, enters the lymphatic vessels, subsequently the blood, and is then distributed systematically. After gaining access to the interstitial fluid allergen-specific IgE binds tightly to the high-affinity receptor for IgE (FcεRI) on tissue-resident mast cells, thereby sensitizing them to respond when the host is re-exposed to the allergen. Sensitization does not produce any symptoms but results in the establishment of an allergen-specific IgE antibody memory as well as of a pool of long-lived memory T cells that respond to repeated allergen contact[21,22].

Re-exposition to the allergen the individual is sensitized for leads to an early-phase reaction (type I hypersensitivity reaction) which occurs within minutes after exposure and mainly reflects the secretion of mediators by mast cells at the affected site [23]. In sensitized individuals, these mast cells already have allergen-

specific IgE bound to their surface Fcε receptors I. The cross-linking of adjacent IgE molecules by bivalent or multivalent allergens and subsequent aggregation of FcεRI (Fig. 2) triggers an intracellular signaling process, initialized by phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) at their cytoplasmic tail, that results in the secretion of three classes of biologically active products: mediators stored in the cytoplasmic granules, lipid-derived mediators, and newly synthesized cytokines, chemokines, growth factors and other products[24-27]. The secretion of preformed mediators occurs when the membrane of the mast cells cytoplasmic granules fuses with the plasma membrane in a process called degranulation, thereby releasing the granules content to the environment[28]. The released mediators include biogenic amines, particularly histamine[24,25], proteoglycans and serine proteases like tryptases and chymases[29-31] as well as prostaglandins and leukotrienes[32], and various other mediators. The release of these preformed and lipid-derived mediators contributes to the acute symptoms associated with early-phase reactions[33]. These symptoms vary according to the site of the reaction but can include vasodilation, marked increased vascular permeability, contraction of smooth muscles, increased mucus secretion, conjunctivitis and asthma. When

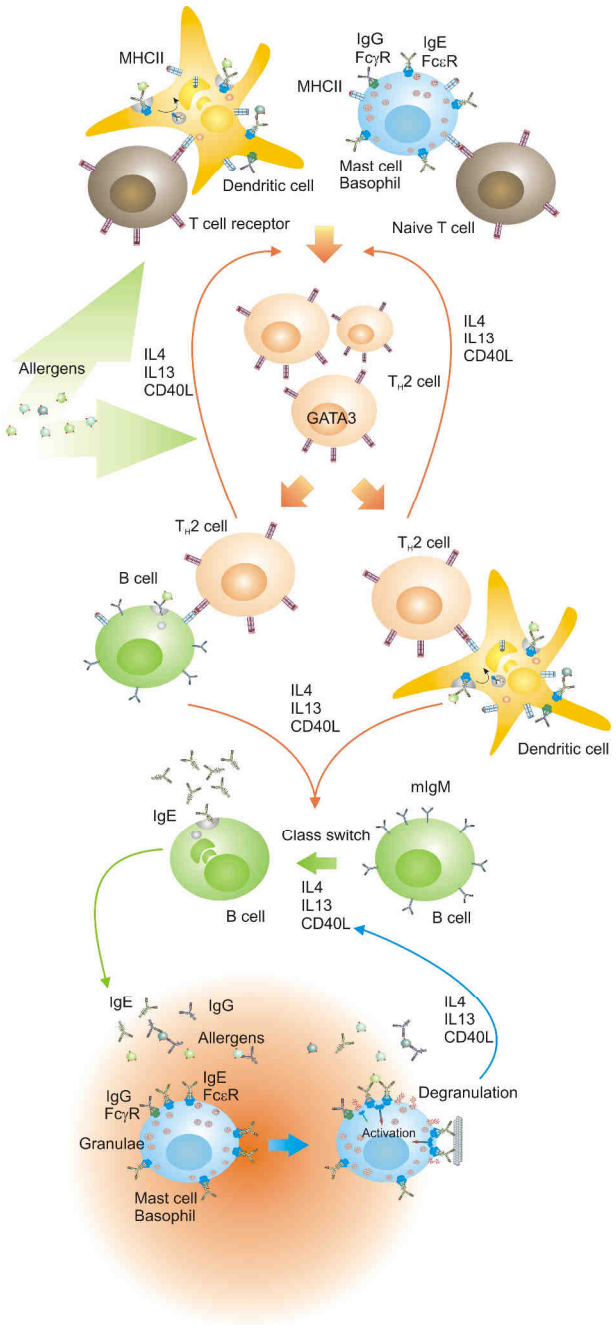

Fig. 2: Mechanism of allergic inflammation. Schematic representation of the way how allergens induce and maintain allergic inflammation. MHC = major histocompatibility complex; T_H cell = T helper cell; FcεR = Fc receptor for IgE; FcγR = Fc receptor for IgG; IL = Interleukin; CD = cluster of differentiation; CD40L = CD40 ligand; GATA3 = GATA binding protein 3.

such mediators are released locally, an early-phase reaction emerges. By contrast, the rapid and systemic release of those mediators from mast cells and basophils, which also express FcεRI[34], is responsible for the majority of symptoms associated with anaphylaxis[35]. The proinflammatory cytokines that are synthesized delayed by activated mast cells, like TNF-α, IL-5, IL-8, IL-10 and IL-13[26,27,36], can induce late-phase reactions, which develop typically 2-6 h after allergen-exposure, but not in all sensitized individuals[37]. Late-phase reactions are thought to reflect the action of innate and adaptive immune cells (neutrophils, monocytes, eosinophils, basophils, mast cells, T cells) which are recruited by the released mediators[38]. This leads for instance to degradation of type III collagen initiated by neutrophils or to tissue damage through basic peptides released by eosinophils which is reflected in symptoms like bronchoconstriction, oedema, erythema and pain. With persistent or repetitive allergen exposure a chronic inflammation develops, associated with tissue alterations as observed in asthma bronchiale[2,39].

Many features of allergic inflammation resemble those of the inflammation that results from immune responses to infection with enteric helminthes or from cutaneous responses to the bites of ectoparasites such as ticks[40], notably that both involve T_H2 cells and are associated with antigen-specific IgE. These similarities have led to the idea that in allergic disorders the immune system is “tricked” into reacting to otherwise innocuous allergens in the same way as it does to signals derived from enteric helminthes or ectoparasites. In addition to the enhancement of effector mechanisms that contribute to parasite clearance by T_H2 responses, chronic infections with certain parasites often also turns on immunological mechanisms that downregulate the inflammation and tissue damage that is associated with that infection[41]. Such mechanisms include the development of T regulatory cells (T_{Reg} cells), characterized by expression of the transcription factor FOXP3 (Forkhead box P3)[42], that secrete IL-10 which has various immunosuppressive and anti-inflammatory effects[43] (Fig. 1). In allergic disorders, it is thought that such downregulatory mechanisms do not fully develop, are lost or might be overcome by inflammatory factors. There is emerging evidence, that T_{Reg} cells control T_H2 responses in humans through IL-10 and TGF-ß secretion, with atopy resulting from an imbalance between T_H2 and T_{Reg} cells[44]. Observations of this type support the “hygiene hypothesis”[45] which is based on the observation

that, as living standards advance, there is reduced exposure to parasitic infections, in which the normal development of immune responses, with a bias towards T_H1 cells rather than T_H2 cells usually is promoted and which favor the development of appropriate control mechanisms of potentially harmful immune responses by various populations of regulatory T cells[46]. As exposure to infections is reduced, and exposure to certain otherwise harmless environmental allergens is increased, there is a propensity for genetically predisposed individuals to develop T_H2 cell type responses to a variety of common environmental allergens. However, the molecular mechanisms underlying the hygiene hypothesis continue to be explored[47].

1.1.2 Hymenoptera venom allergy and anaphylaxis

The potentially life-threatening clinical syndrome, anaphylaxis, was initially defined in 1902 by Portier and Richet as a fatal reaction to injection of a previously tolerated foreign protein in dogs[48,49]. Approximately 4500 years earlier, the Egyptians described fatal reactions to Hymenoptera insect stings, which probably represented anaphylaxis. Over the years, anaphylaxis has gradually come to mean the acute life-threatening syndrome that results from the rapid and systemic release of large amounts of inflammatory mediators from mast cells and basophils in response to a specific allergen in a previously sensitized host[50]. The clinical features of anaphylaxis are produced typically by immunologic mechanisms, but non-immune-mediated reactions also occur. Immune provoked anaphylaxis is mediated by IgE or immune antigen-antibody complexes, whereas, non-immune anaphylaxis occurs in relation to direct mast cell degranulating agents such as opiates or radio contrast media[51]. Perhaps the most common causes of anaphylaxis today are adverse drug reactions, especially to penicillin, reactions to food, most commonly occurring with eggs, shellfish, and nuts as well as to Hymenoptera venoms and saliva, latex, and to immunotherapy injections[35,51]. The mast cell mediator identified most commonly in anaphylaxis is histamine, and tissues rich in mast cells are the primary target tissues in anaphylaxis. Systemic anaphylaxis frequently involves multiple organ systems including the skin and the respiratory, gastrointestinal, genital, cardiac, and neural system[52]. The estimated frequency of anaphylaxis is 50–2000 episodes per 100.000 persons or a lifetime prevalence of 0.05–2.0%[53]. Anaphylaxis may develop rapidly, reaching peak severity within 5 to

30 minutes, but also late-phase reactions after 6 to 12 hours occur. The most life-threatening features are those involving the cardiovascular system and the respiratory tract. Increased vascular permeability can result in a transfer of 50% of the intravascular fluid into the extravascular space within 10 minutes[54]. The typical patient develops generalized itching, followed by cutaneous flushing, urticaria, a fullness in the throat, a feeling of “anxiety”, then tightness in the chest, faintness, and, finally, loss of consciousness[55].

Generalized systemic reactions to stinging Hymenoptera (Fig. 3) have been recognized as potentially life-threatening phenomenon related to the IgE antibodies to the various components of venom from the honeybee, bumblebee, yellow jacket, hornet, wasp and fire ant. The family *Apidae* consists of the honeybees (genus *Apis*) and bumblebees (genus *Bombus*). *Vespidae* are divided into the subfamilies *Vespinae* and *Polistinae*[56]. Three genera of the *Vespinae* exist: *Vespula* (called wasps in Europe, yellow jackets in the USA); *Vespa* (hornets) and *Dolichovespula*. *Polistinae* (called wasps in Europe and USA) are widespread in the Mediterranean areas. In Europe allergic reactions after insect stings are mainly caused by Hymenoptera of the families *Apidae* and *Vespidae*, particularly by the honeybee (*Apis mellifera*) and yellow jacket (*Vespula vulgaris*; *V. germanica*). Hornets (*Vespa crabro*, *V. orientalis* in Mediterranean areas) and *Polistinae* are much less aggressive than species of the genus *Vespula* and in this respect play an inferior role as elicitors of allergic reactions, but are locally from importance, especially the *Polistinae* in the whole Mediterranean area[57]. The bumblebee (*Bombus ssp.*) has gained significantly in importance since it is increasingly used for pollination in greenhouses[58]. In Europe allergic reactions to the family *Formicidae* (ants) seem to be rare[59] but are of great importance in America, especially the species *Pogonomyrex* and *Solenopsis*[60], and Australia, especially the species *Myrmecia*[61]. For appropriate diagnosis and therapy, it is important to define the specific insect venom responsible for the reaction. Most fatal reactions to insect venom occur in adults[62] and the diagnosis of IgE hypersensitivity to insect venom is best determined by skin testing[63]. The most frequent clinical patterns are large local reactions exceeding 10 cm in diameter and 24 h in duration, and generalized, immediate-type allergic reactions such as urticaria, angiooedema, asthma and anaphylactic shock[64].

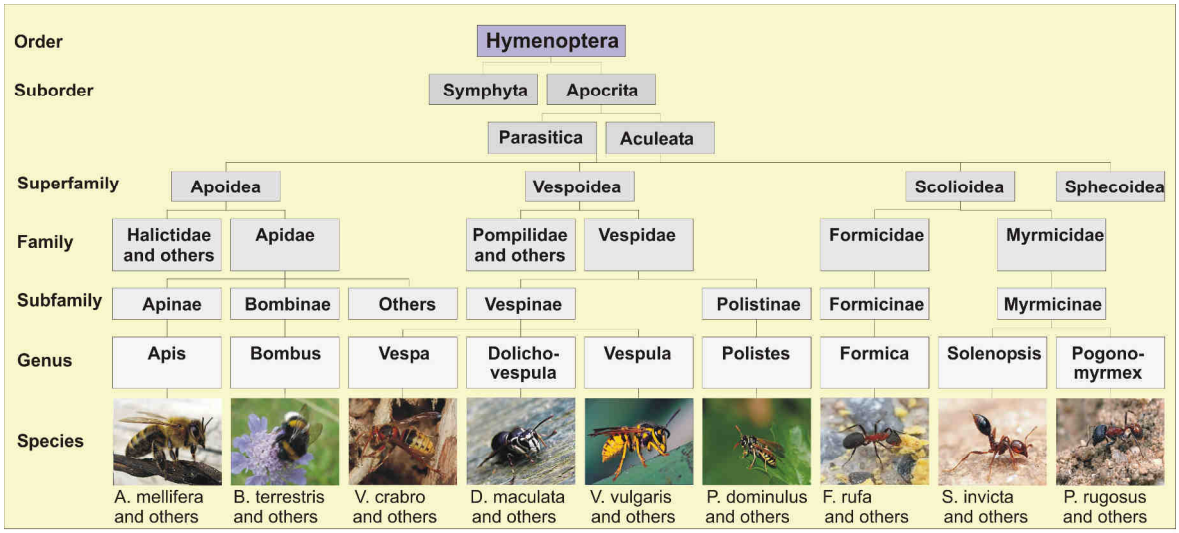

Fig. 3: Taxonomy of Hymenoptera. Classification according to Chinery[285]

The prevalence of sensitization to Hymenoptera venom is estimated at between 9.3 and 28.7% in adults[65]. Large local reactions occur in 2.4-26.4% of the general population and epidemiological studies report a prevalence of self-reported systemic anaphylactic sting reactions between 0.3% and 7.5% in adults[66-70] and of only 0.15-0.3% in children[71]. The prevalence of systemic reactions among beekeepers is high and falls between 14 and 43%[72,73]. The incidence of insect sting mortality ranges from 0.03 to 0.48 fatalities per 1.000.000 inhabitants per year[70,74-76]. However, the true number may be underestimated: a study reports the presence of venom-specific IgE in 23% of post-mortem serum samples taken from subjects, who had died outdoors suddenly and inexplicably between the end of May and the beginning of November[77]. Around 40-85% of the subjects with fatal reactions after Hymenoptera stings had no documented history of previous anaphylactic reactions[76,78]. Most often, symptoms appear within a few minutes to one hour after the sting[79], but rarely they can occur hours or even days later[80]. Normally, the patient recovers from anaphylactic reactions within a few hours, but rarely, a biphasic course is observed with an early onset, an apparent recovery and a subsequent relapse after 4-24 hours. Severe reactions after Hymenoptera stings are classified according to the severity of the reaction. The most frequently used classifications are those by Müller and by Ring with four grades of severity[81,82]. Symptoms of the grade I include generalized skin reactions like urticaria and of the grade II angiooedema and mild to moderate pulmonary, cardiovascular and gastrointestinal manifestations. The grade III is characterized by anaphylactic shock and loss of consciousness and the grade IV by cardiac arrest and apnea.

1.1.3 Hymenoptera venom allergens

Allergens are those antigens responsible for clinical allergic diseases. They are usually proteins or glycoproteins capable of inducing synthesis of IgE antibodies, thereby sensitizing the potentially allergic person[83]. Upon re-exposure to the same allergen, the previously sensitized patient manifests the signs and symptoms of allergy, as the allergen reacts with cell-related IgE tissue antibodies, and the cells generate the mediators of inflammation. Therefore, it is imperative that the circumstances of allergic disease are linked to allergen exposure, as allergens represent important etiologic factors in the pathogenesis of allergy[84].

The Allergen Nomenclature Committee of the International Union of Immunological Societies (IUIS) has devised a unified nomenclature system for purified allergens[85]. They are phenotypically designated by the first three letters of the genus followed by a space, the first letter of the species, another space, and finally an Arabic number; occasionally an additional letter must be added to either the genus or the species designation. Allergens can be further classified on the basis of nature or manner in which the patient is exposed into inhalants, ingestants, contactants and injectants. Those allergens responsible for allergic respiratory diseases including allergic asthma and allergic rhinitis are principally inhalants. These aeroallergens, which can be present outdoors (pollen, animal products like sheddings from skin and fur, algae) or indoors (molds, animal products, dusts) are responsible for the majority of allergic diseases. Foods and other ingestants, including drugs, are also important, especially for allergic gastrointestinal and skin diseases. The contactants are principally responsible for allergic contact dermatitis. In addition to drugs, the injectant group includes the venom and saliva of insects.

Hymenoptera venoms are a complex cocktail of biogenic amines, basic peptides, toxins and proteins, mostly enzymes and a variety of other compounds all of which may contribute to sensitization, allergic symptoms and success of venom immunotherapy[79]. The amount of venom released during a sting varies from species to species: bee stings release an average of 50 µg[86] up to 140 µg[87] of venom protein per sting, *Vespula* stings 1.7 to 3.1 µg, *Dolichovespula* stings 2.4 to 5 µg and *Polistes* stings from 4.2 to 17 µg of venom protein[88]. The insects of the order Hymenoptera have unique as well as common venom allergens. The until now identified allergenic molecules in bees, vespids and ants are listed in Table 1.

Venom	Allergen	Common name	MW (kDa)
Apis mellifera (honeybee) Accordingly in *A. cerana* (eastern hive bee), *A. dorsata* (giant honeybee) etc.	Api m 1 Api m 2 Api m 3 Api m 4 Api m 5 Api m 6 Api m 7 Api m 8 Api m 9 Api m 10	Phospholipase A2 Hyaluronidase Acid phosphatase Melittin Dipeptidylpeptidase IV putative protease inhibitor CUB-serine protease Carboxylesterase Carboxypeptidase Carbohydrate-rich protein	17 45 49 3 100 8 39 70 60 55
Bombus pennsylvanicus (bumble bee) Accordingly in *B. terrestris* etc.	Bom p 1 Bom p 4	Phospholipase A2 Protease	16 27
Vespula vulgaris (yellow jacket) Accordingly in *V. flavopilosa*, *V. germanica*, *V. maculifrons*, *V. pensylvanica*, *V. squamosa*, *V. vidua* etc.	Ves v 1 Ves v 2a Ves v 2b Ves v 3 Ves v 4 Ves v 5	Phospholipase A1B Hyaluronidase Hyaluronidase Dipeptidylpeptidase IV CUB-serine protease Antigen 5	34 45 47 100 35 23
Vespa crabro (European hornet) Accordingly in *V. mandarinia* (giant Asian hornet) etc.	Vesp c 1 Vesp c 5	Phospholipase A1B Antigen 5	34 23
Dolichovespula maculata (white face hornet) Accordingly in *D. arenaria* (yellow hornet), *D. media* etc.	Dol m 1 Dol m 2 Dol m 5	Phospholipase A1B Hyaluronidase Antigen 5	34 42 23
Polistes annularis (wasp) Accordingly in *P. dominulus* (Mediterranean paper wasp), *P. exclamans*, *P. fuscatus*, *P. gallicus*, *P. metricus* etc.	Pol a 1 Pol a 2 Pol a 5	Phospholipase A1B Hyaluronidase Antigen 5	34 38 23
Polybia paulista (wasp) Accordingly in *P. scutellaris* etc.	Poly p 1 Poly s 5	Phospholipase A1 Antigen 5	34 23
Solenopsis invicta (red imported fire ant) Accordingly in *S. geminata* (tropical fire ant), *S. richteri* (black fire ant), *S. saevissima* (Brazilian fire ant) etc.	Sol i 1 Sol i 2 Sol i 3 Sol i 4	Phospholipase A1B Antigen 5	18 14 26 12
Myrmecia pilosula (Australian jumper ant)	Myr p 1 Myr p 2 Myr p 3	 Pilosulin-3 Pilosulin-4.1	7,5 / 5,5 8,5 / 2-4

Tab. 1: Allergens of Hymenoptera venoms

Honeybee venom (HBV) contains various active substances. Melittin, the most prevalent substance, is one of the most potent anti-inflammatory agents known and shows strong hemolytic activity due to its strong membrane toxicity[89]. Adolapin is another strong anti-inflammatory substance, and inhibits cyclooxygenase; it thus has analgesic activity as well[90]. Apamin, a basic peptide, inhibits complement C3 activity, and blocks calcium-dependent potassium channels, thus enhancing

nerve transmission[91]. Other substances, such as Tertiapin, hyaluronidase, phospholipase A2, histamine, and mast cell degranulating peptide (MSD-peptide)[92], are involved in the inflammatory response to venom, with the softening of tissue and the facilitation of flow of the other substances. Additionally, measurable amounts of the neurotransmitters dopamine, norepinephrine and serotonin are present. The water content varies between 55-70% and the pH range is between 4.5-5.5. Many of the proteins and polypeptides in honeybee venom have been identified as sensitizing agents including phospholipase A2 (Api m 1), hyaluronidase (Api m 2), acid phosphatase (Api m 3)[93], melittin (Api m 4)[94], dipeptidylpeptidase IV (Api m 5; allergen C)[95,96], Api m 6 polypeptides (4 isoforms)[97], a 39 kDa CUB-serine protease (Api m 7)[98], a 70 kDa carboxylesterase which has been named Api m 8, Api m 9, a 60 kDa serine carboxypeptidase[99], and the Carbohydrate-rich protein (Api m 10), a component of unknown function[100]. Phospholipase A2 (Api m 1) and hyaluronidase (Api m 2) seem to be the mayor sensitizing allergens in honeybee venom[93,101] and the peptidic components Melittin (Api m 4) and Api m 6 are considered minor allergens[99,102]. In recent years, the designated genes could be assigned to the acid phosphatase Api m 3[103], the DPPIV enzyme Api m 5[96] as well as the Carbohydrate-rich protein (Api m 10)[100], the allergens recombinantly produced, and characterized for their allergenic properties. Other proteins including peptidases, esterases, and proteases, some of which are listed in the databases, are currently assessed for their allergenic potential (unpublished data). Considering the complex composition of insect venoms, however, additional allergens are likely present in honeybee venom.

Bumblebee venom has two allergens of known sequences: phospholipase A2 and a protease. The two bee venom phospholipases A2 have extensive sequence identity with each other and no sequence identity with vespid phospholipase A1[104]. Phospholipase A1 and A2 differ in their specificity of catalyzing the hydrolysis of fatty acid residues at positions 1 and 2 of phospholipids, respectively.

Among the vespids the venom of the yellow jacket *Vespula vulgaris* is best investigated. The three most prominent venom allergens include phospholipase A1 (Ves v 1), hyaluronidase (Ves v 2), and antigen 5 (Ves v 5)[105]. Recently, a second, inactive hyaluronidase (Ves v 2b) has been identified in *V. vulgaris* venom[106]. Additionally, the recently identified dipeptidylpeptidase IV (Ves v 3)[96] as well as the CUB-serine protease Ves v 4 exhibit IgE reactivity. In contrast to the phospho-

lipases, vespid hyaluronidases have about 50% sequence identity with HBV hyaluronidase and they have the same enzymatic specificity of endo-N-acetyl-hexosaminidase[104]. With the dipeptidylpeptidases Api m 5 and Ves v 3 a novel class of enzymes and homologous structures in Hymenoptera venom was described. For antigen 5 (Ves v 5) there exists no homologous protein in HBV. The biologic function of antigen 5 is not known, although it has been reported for antigen 5 from *Vespa mandarinia* to be a neurotoxin active at muscular junctions[107]. The homologous venom allergens from hornets, wasps and yellow jackets have varying degrees of sequence identity ranging from 60% for phospholipases and antigen 5 to about 80% for hyaluronidases[108-111]. Allergens of different species within a species group of each genus generally have a higher degree of sequence identity than those of a different species group.

To date, fire ant venoms are the only ant venoms showing high similarity with vespid venoms and contain four known allergens: Sol i 1 to 4. Sol i 3 has about 50% sequence identity with vespid antigen 5 and Sol i 1 is a homologue of vespid phospholipase A1[112]. Varying from all other known Hymenoptera venoms, the major allergens of the *Myrmecia* venoms are small peptides (pilosulins) which partially form homo- or heterodimers[113], but also phospholipase A2, acid phosphatase and, hyaluronidase activity was reported.

The further identification and characterization of all relevant allergens in Hymenoptera venoms is imperative for both improvement of diagnostic tests and design of effective immunotherapeutic approaches.

1.2 Allergy diagnosis

The diagnosis of allergic diseases should always begin with the procurement of a careful patient history and an appropriate physical examination. When an allergic disorder is suspected on the basis of clinical grounds, a variety of procedures can be used to confirm the diagnosis. Diagnostic tests can also be helpful in ruling out allergic disorders and clarifying the specific responsible antigens or allergens.

Skin testing is the tool used most widely to diagnose clinical allergies[114]. The basic procedure involves delivering an aqueous solution of antigen beneath the stratum corneum and barrier zone of the epidermis. As the antigen combines with IgE fixed to mast cells, mediator substances are released and cause local vasodilation and increased capillary permeability which results in wheal-and-flare reactions within

15 to 20 minutes[115] (Fig. 4A). There are two types of skin tests, the epicutaneous, also referred to as scratch, puncture, and prick technique, and the intracutaneous, or intradermal, test. The epicutaneous method has many advantages as it is easy and safe to perform, causes little discomfort and positive tests correlate well with clinical symptoms. One possible disadvantage to this method is that it can result in false-negative reactions due to a lack of sensitivity. In contrast, intracutaneous skin tests are more reproducible and 100 to 1000 times more sensitive[116,117]. Thus, they are associated with fewer false-negative reactions but the drawbacks to intradermal tests are that they are more time consuming and tedious to perform and are often associated with discomfort and an increased risk of systemic reactions. Even more important, they are more likely to produce false-positive results because of their increased sensitivity. Mildly positive intradermal reactions are not considered clinically relevant, thus, that the value of skin tests, like that of any diagnostic procedure, depends on the knowledge of their interpreter[118].

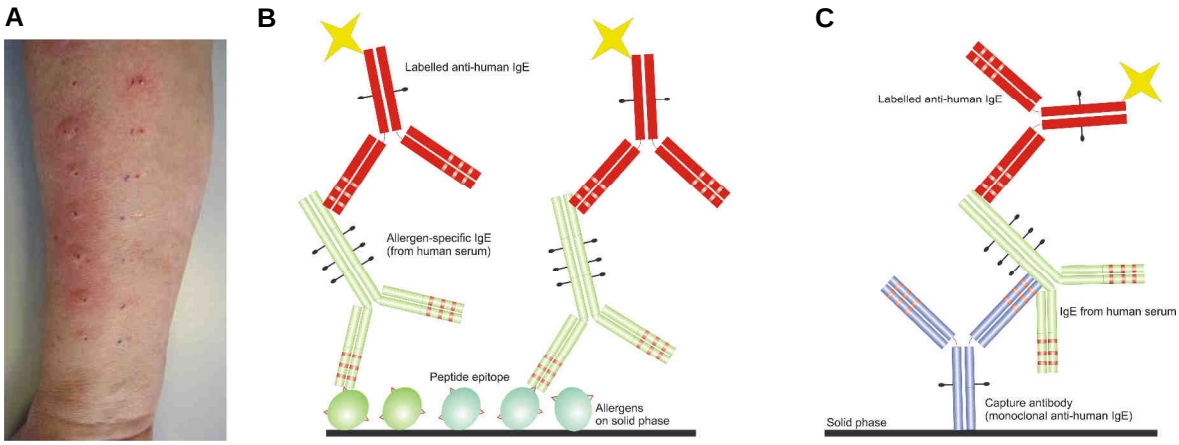

Fig. 4: Methods for allergy diagnosis. A: Result of skin prick test in an allergic patient. **B:** Principle of enzyme-linked immunoassay (EIA) for measurement of allergen-specific IgE. **C:** Principle of enzyme-linked immunoassay for detection of total IgE.

The discovery of IgE as the antibody responsible for allergic reactions in humans led to the development of sophisticated techniques for IgE measurement[119]. The two most commonly employed techniques for the measurement of IgE are radioimmunosorbent assay (RIA) and enzyme-linked immunosorbent assay (EIA) (Fig. 4B and 4C). EIAs are routinely used to quantify the amount of IgE antibody that is directed to a specific allergen[115,120,121] and have replaced the RAST (radioallergosorbent test), the original allergen-specific IgE test[122]. IgE levels are often elevated in cases of allergic disease, but these levels cannot be considered pathognomonic signs of allergy since IgE levels vary widely, both in allergic and non-allergic

individuals. A normal IgE level does not exclude allergy, while definitely elevated levels may be seen in non-atopic people[123].

The skin test and measurement of total IgE as well as specific IgE antibody levels in the serum are indirect assays of an allergic state (Fig. 4). Direct challenge, either inhaling or ingesting antigens, may be of greater diagnostic use[124]. In bronchial challenges the specific airway reactivity can be assessed by measuring the patients bronchial response to the inhalation of certain allergen solutions. Inhaled allergens can also be used to challenge the nasal mucosa to diagnose allergic rhinitis. In instances when a suspected allergen is ingested, an oral challenge can be performed. The challenge can be open, in which case the physician and the patient know the content of the substance ingested; single blind, with only the physician knowing the content; or double blind, with neither the physician nor the patient knowing the content of the challenge. Oral challenges serve several purposes. First, double-blind, placebo-controlled food challenges have proven useful in discerning IgE-mediated food sensitivities and second, oral challenges can also help diagnose sensitivity to ingested substances, such as aspirin or sulfites, in which the sensitivity is not on an IgE basis[118]. Other forms of challenge tests are the injection of drugs like penicillin or in the case of Hymenoptera venom allergy the sting challenge test.

In the last years also cell-based *in vitro* tests are gaining ground in which a patients immune cells like basophils, are isolated, challenged with allergens and their activation assayed by colorimetric measurement of mediator release or activation markers are accessed flow-cytometrically[125-128].

1.2.1 Diagnosis of Hymenoptera venom allergy

The diagnosis of Hymenoptera venom allergy is based on a history of adverse sting reaction and on positive skin test to venom or the presence of venom-specific serum IgE[129], whereas the severity of sting reaction has no significant correlation with the serum levels of venom-specific IgE or skin tests[130,131]. Moreover, approximately 10% of patients experiencing an anaphylactic reaction to an insect sting have no detectable specific IgE or positive skin test result against the insect concerned[132]. When venom skin test and specific IgE measurement yield negative results in patients with a history of a systemic anaphylactic sting reaction, additionnal *in vitro* tests may be used to demonstrate immunologic sensitization.

In the basophil histamine release test peripheral blood leukocytes are incubated with venom allergens, reacting with cell-bound IgE antibodies and thus stimulating cells, mainly basophils, to release histamine, which can be detected calorimetrically[127,128]. In the leukotriene release test (cellular antigen stimulation test, CAST) blood leukocytes pre-stimulated with IL-3 are exposed to venom allergens and the release of sulfidoleukotrienes is determined by ELISA[126]. The basophil activation test is a novel method based on the flow cytometric demonstration of an altered membrane phenotype of basophils activated by allergen exposure. The currently most commonly used marker to demonstrate basophil activation is CD63[125]. Another diagnostic marker of insect hypersensitivity is an elevated level of the mast cell-specific enzyme tryptase, which is found in up to 30% of patients with a history of severe shock reaction[133]. The current finding indicates that basal tryptase levels, indicating an increased mast cell load, are a risk factor for severe or even fatal sting reactions.

Sting challenge tests are not recommended for routine diagnostic purposes[134,135] due to the high risk of fatal systemic reactions[136] and of boosting the sensitization[64], but are recommended in patients on maintenance venom immunotherapy (VIT) to identify those who are not yet protected and need an increase of the maintenance dose[137]. Nevertheless, the specificity of diagnostic tests is far from perfect[138], demonstrated by the fact that on the one hand up to 20% of individuals with no history of systemic sting reactions have positive tests and on the other hand, only 30-50% of those with positive tests will react to a subsequent sting by the respective insect[134]. Nowadays, preliminary trials indicate, that recombinant venom allergens may gain an improvement of Hymenoptera venom allergy diagnosis by increased specificity in both skin testing and in determining venom-specific IgE antibodies compared to natural venom allergen extracts[139].

1.2.2 Interference in diagnostic tests

Problems in allergy diagnosis are associated with false-positive, false-negative or even positive test results without clinical relevance. Several circumstances may account for negative results, e.g. in skin tests the stability of the antigen solution, the patients age (in general the skin of infants and elderly persons is less reactive), or a number of drugs, particularly antihistamines, interfering with the allergic response. The refractory period of a test may also contribute to a false-negative

result. Soon after a systemic reaction to an allergen, such as insect venom, penicillin, or food, the patient enters a refractory period during which a skin test reaction may be negative[79]. The reason is that specific IgE is consumed by the severe allergic reaction, so a 3-4 week period is needed for the allergic antibody to build back up to its pre-reaction levels.

Employing *in vitro* tests a lack of sensitivity may be the reason for false-negative test results, e.g. if the allergens the patient is sensitized to are underrepresented in natural allergenic extracts used for testing. False-positive results in skin tests may result from low-molecular-weight irritants which may be contained in natural extracts or from high histamine content present in some food extracts, particularly those from cheese[118].

Another problem in the diagnosis of allergies are positive test results without or with low clinical significance. Such results are mostly associated with allergenic cross-reactivity, and constitute a major handicap for accurate allergy diagnosis that remains to be solved. Apart from true double-sensitization and mimicry based on the primary structure, IgE may be directed against cross-reactive carbohydrate determinants (CCDs) provided by a broad panel of proteins in food, pollen and Hymenoptera venom[140]. In general the N-glycans found on most Hymenoptera venom proteins and many plant proteins possess a number of non-mammalian features rendering them potentially immunogenic. However, the supposed hallmark of CCDs on insect venom allergens comprises carbohydrates carrying α-1,3-linked core fucose residues. In plants ß-1,2-linked xylose builds a second immunogenic moiety (Fig. 5A). IgE with specificity for such glycotopes represent the underlying principle reactive with all proteins possessing CCDs[141], subsequently producing multiple positive test results of unknown clinical significance[142] (Fig. 5B). This has even led to the estimation that IgE binding to venom proteins with pronounced glycosylation may primarily or exclusively reflect CCD reactivities[143], rendering their postulated allergenic character questionable. Thus, the immunoreactivity of all glycosylated allergens demands thorough re-evaluation to verify their classification as allergens in *stricto sensu*. Although the clinical relevance of CCDs is still discussed[144], their diagnostic relevance is beyond any controversy. Identification of the culprit Hymenoptera species that a patient is sensitized to remains key for proper diagnosis and for the selection of an appropriate therapeutic strategy in Hymenoptera venom allergy[64]. Therefore, *in vitro* diagnosis

might be markedly improved when using strategies that eliminate CCD reactivities without affecting clinically relevant IgE reactivity and allow mere cross-reactivity to be distinguished from true multiple sensitization.

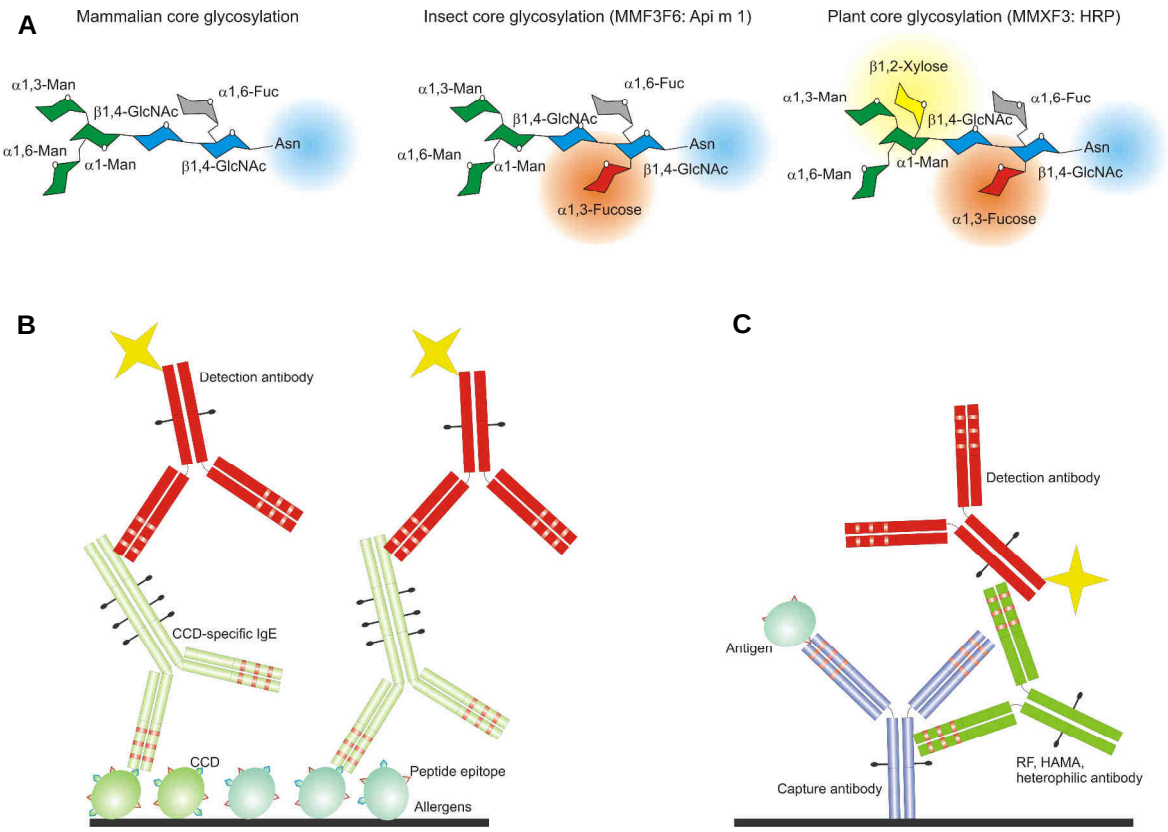

Fig. 5: Interference in diagnostic assays. A: Schematic representation of carbohydrate structures responsible for carbohydrate-based cross-reactivity in comparison to mammalian core glycosylation. **B:** Principle of assay interference phenomena caused by IgE directed against cross-reactive carbohydrate determinants (CCDs). **C:** Principle of assay interference phenomena caused by rheumatoid factor (RF), human anti-mouse antibodies (HAMA) and heterophilic antibodies.

Apart from IgE antibodies interference in immunoassays is increasingly recognized as a major diagnostic problem. Immunoglobulins are highly conserved among mammalian species, and their nature as specific binding moieties as well as their implication in many of immune-mediated pathologies renders them susceptible to interaction with a plethora of mammalian and bacterial proteins, thereby hampering their use in immunodiagnostic approaches[145]. In general, immunoassays are affected by cross-reactivity and non-specific binding. Furthermore, antigen-independent binding via specific immunoglobulin receptors and serum immunoglobulins is causative for false-positive and false-negative results in different diagnostic approaches. Heterophilic antibodies as well as rheumatoid factor (RF) and

human anti-mouse antibodies (HAMA) are the most prominent examples for interference in immunological assays[146] (Fig 5C). Heterophilic antibodies are recognized as mostly lower affinity, often IgM isotype immunoglobulins with unknown antigen, generating non-specific signals by binding to detection antibodies. In contrast, the appearance of HAMA is mainly a result of therapeutic approaches comprising administration of murine monoclonal antibodies, but may also be found in serum of untreated individuals. RF defines an auto-antibody that reacts with the Fc part of mammalian IgG, and is most often associated with rheumatoid arthritis, but can also be found in serum of patients with other diseases and also in 3-5% of healthy donors[147].

Exemplary it was recently demonstrated that tryptase immunoassays, indicating increased risk of anaphylaxis in patients with suspected mastocytosis, are sensitive to interference by heterophilic antibodies in over 15% of cases[148].

1.3 Therapy of allergic diseases

In the last decade, enormous improvements in the medical procedures used to treat allergic conditions were achieved. In atopic individuals, allergen sensitization is fundamental to the development of any allergic disease. Therefore, avoidance of allergens before or after sensitization should be beneficial as primary or secondary prophylaxis. In the case of house dust mites birth-cohort studies have shown that the level of allergen exposure early in live correlates with the extent of sensitization[149]. For domestic pets, the situation is more complex, with early-life exposure decreasing rather than increasing allergen sensitization, possibly as a result of simultaneous exposure to inhibitory products from non-pathogenic microorganisms, whereas exposure later in childhood leads to sensitization[38]. A similar situation applies to peanut allergy, in which avoidance during pregnancy and early infancy can increase rather than protect against sensitization. Therefore, exposure to a high dose of peanut, rather than avoidance in infancy, might be the way to induce protective tolerance[150,151]. These mixed results of primary prophylaxis can be explained by the fact that extremely low allergen exposures can lead to sensitization[152] and, as a result, anything than complete allergen avoidance is unlikely to be successful. Greater success has been obtained by using multiple early-life interventions in addition to the avoidance of house dust mites and pets, such as breast-feeding with the mother on a low-allergen diet[153]. However, avoidance as

secondary prophylaxis may help to reduce symptoms and avoid life-threatening anaphylaxis. In children which are already sensitized, single or combination interventions to decrease exposure to both dietary and aeroallergens result in a meaningful and sustained improvement in the control of asthma and rhinitis. However, in adults, the data are far less convincing, probably because of the many allergic factors that contribute to ongoing disease[154,155]. But strict avoidance still has a role in the treatment of allergies, and is often used in managing food allergies, but it is difficult to achieve for patients with pollen or similar air-borne allergies.

1.3.1 Pharmacotherapy of Allergy

In established pharmacotherapeutic treatment several antagonistic drugs are used to block the action of allergic mediators, or to prevent the activation of cells and in this way the degranulation processes. Corticosteroids and ß2-adrenoreceptor agonists are now the gold standard for asthma treatment. In the case of allergic rhinitis α-adrenoreceptor agonists are used to relieve nasal congestion, and non-sedating H1-antihistamines and corticosteroids are well-established control therapies. So, for most allergic disorders, a combination of symptom-relieving and control therapies forms the basis of therapy[38].

Corticosteroids suppress T_H2 cell-mediated inflammation through the inhibition of expression of cytokines, chemokines and adhesion molecules[156] by the interaction with cytoplasmic glucocorticoid receptors that modulate NF-κB- and activator protein 1-regulated gene expression [157,158]. Corticosteroids are highly effective at suppressing airway inflammation, but they do not influence the natural history of the disease[159]. ß2-adrenoreceptor agonists bind to the ß2-adrenoreceptor and subsequently activate cAMP production and protein kinase A activation which mediates smooth-muscle relaxation through phosphorylation of myosin light-chain kinase and by opening Ca^{2+}-dependent K^+ channels, which rapidly relieves bronchoconstriction and asthma symptoms. H1-antihistamines such as chlorpheniramine were the first specific agonists used to treat allergic reactions. The sedative and anti-cholinergic side-effects of the early products were overcome by a second generation of drugs (cetirizine, levocetirizine, loratadine, desloratadine, etc.) with decreased capacity to cross the blood-brain barrier and decreased cardiac toxicity[160]. Promising new agonists for asthma and rhinitis treatment are leukotriene modifiers like the CysLTs interacting with the CysLT receptor 1[161].

Mast cell stabilizing drugs are used since 1968 (sodium cromoglicate; SCG) to inhibit mast cells and subsequently allergen-induced early- and late-phase responses[162]. SCG inhibits the flux of chloride ions in mast cells in this way increasing their threshold for activation[163].

Because of the sentinel role that T_H2 cytokines have in orchestrating allergic inflammation, they and their receptors are key therapeutic targets. With almost no exceptions, this approach has required the application of biological agents in the form of blocking monoclonal antibodies, fusion proteins, soluble receptor constructs and most recently, inhibitors of the T_H2 cell transcription factors STAT6 and GATA3[38]. Recent studies dealing with the alteration of the T_H1-T_H2 cell balance concern the inhibition of T_H2 cell responses by administration of blocking agents for IL-4, IL-13 and IL-5[164-166], as well as the promotion of T_H1 cell responses by administration of INFγ, IL-12 or Il-10[167-169]. Inhibition of the allergic component of atopic asthma can also be achieved using IgE-specific monoclonal antibodies, like the humanized IgE-specific, non-anaphylactic IgG1 antibody Omalizumab, which is also effective for the treatment of allergic rhinoconjunctivitis. The binding to the C3 domain of IgE blocks its binding to FcεRI and subsequently decreases the level of circulating free IgE[170].

1.3.2 Specific immunotherapy of allergy

To date, the allergen-specific immunotherapy (SIT) is the only curative approach of allergy treatment. This immune modifying therapy has been recommended for the treatment of allergic rhinitis, venom allergy, some drug allergies and mild bronchial asthma, although the exact mechanism of therapy success is not clear. SIT modifies cellular and humoral responses to an allergen through repeated allergen-exposure (Fig. 5). The ratio of T_H1 cell cytokines to T_H2 cell cytokines is increased after SIT, and functional $CD4^+CD25^+$FOXP3 regulatory T cells (T_{Reg} cells) are induced raising immunological tolerance and the induction of blocking IgG4 antibodies[38,83]. Increased production of IL-10 by monocytes, macrophages, B cells and T cells might contribute together with TGF-ß to T_{Reg} cell function and immunoglobulin class-switching to IgA, IgG1 and IgG4[171], which compete, as so called blocking antibodies, with IgE for allergen binding, thereby decreasing the allergen capture and presentation that is facilitated by FcεRI or the low affinity-IgE receptor FcεRII (CD23)[172,173]. T_{Reg} cells in turn produce high levels of IL-10 and TGF-ß, two

cytokines that are known to attenuate allergen-specific T_H2 cell responses. IL-10 suppresses mast cell, eosinophil and T cell responses[174], and the pleiotropic functions of TGF-ß maintain a diverse and self-tolerant T cell repertoire[175].

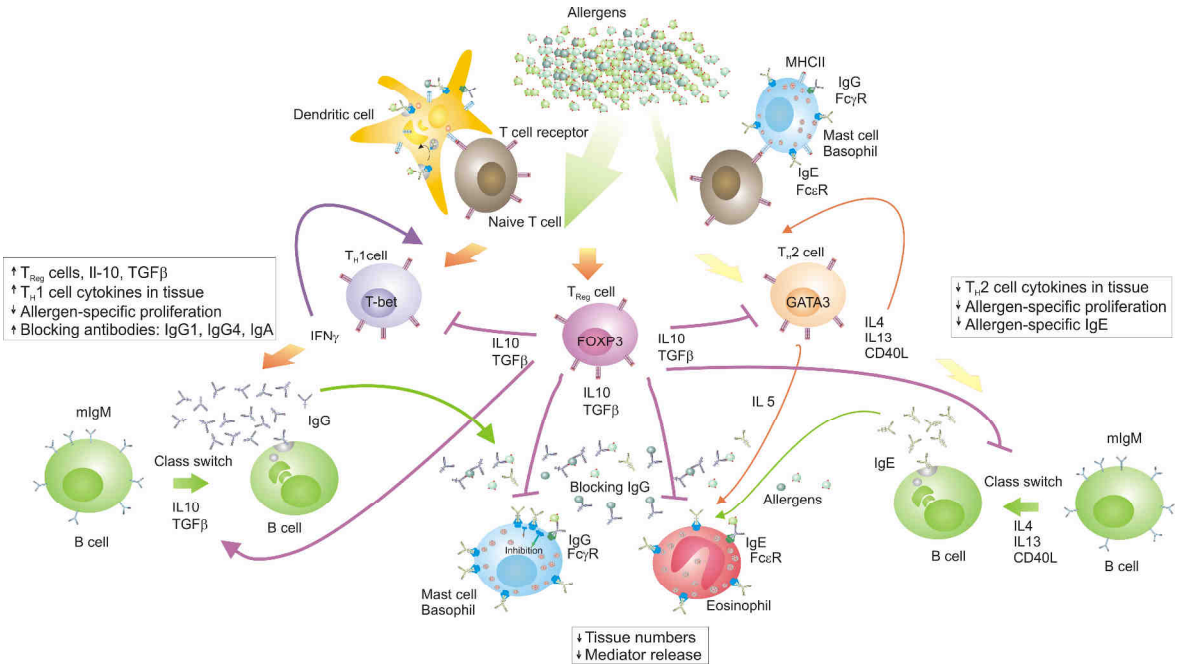

Fig.5: Mechanisms of allergen-specific immunotherapy. Schematic representation of immunological mechanisms leading to tolerance induction by specific immunotherapy. MHC = major histocompatibility complex; T_H cell = T helper cell; T_{Reg} cell = T regulatory cell; FcεR = Fc receptor for IgE; FcγR = Fc receptor for IgG; IL = Interleukin; TGF = transforming growth factor; IFN = Interferon; CD = cluster of differentiation; CD40L = CD40 ligand; T-bet = T box expressed in T cells; FOXP3 = forkhead box P3; GATA3 = GATA binding protein 3.

Subcutaneous immunotherapy (SCIT) involves the regular subcutaneous injection of allergen extracts or recombinant allergens using incremental regimens, with the induction of tolerance taking from several days to several month depending on the regimen used. The usual approach is a build-up phase (consisting of weekly injections) followed by a maintenance phase (consisting of monthly injections). Once tolerance is induced it can last for several years without further treatment[176]. The limiting factor in SCIT are anaphylactic side-effects, which vary in incidence from 0.1-5%[177]. Improved efficacy with decreased side-effects is the aim of new approaches to SCIT, including T cell-reactive peptides[178], hypoallergenic recombinant allergens[179] or chemically modified allergens (allergoids)[180]. Attaching CpG oligonucleotides, which induce innate immune responses through interaction with TLR9, in this way shifting the balance from a T_H2 towards a T_H1 phenotype, and other ligands of pathogen-recognition receptors, to allergens, seems to increase efficacy and decrease side-effects of SCIT[181].

The administration of allergens to the oral mucosa as a route for immunotherapy has only recently gained acceptance (sublingual immunotherapy; SLIT). Although much higher doses of allergen are required than are used for SCIT, the side-effect profile is impressively mild[182,183]. Clinical trials have shown, that SLIT is effective for the treatment of pollinosis caused by grass, olive, ragweed and birch, as well as rhinitis that is associated with house dust mite and cat allergies. Both SCIT and SLIT also decrease the development of sensitization to new allergens and the risk of asthma in patients with rhinitis.

To improve the safety and attractiveness of SIT for patients, alternative routes of allergen administration are being explored. A recent study evaluated direct intra-lymphatic allergen administration for SIT with bee venom allergen phospholipase A2 and cat allergen Fel d 1. Since injection into the lymph node delivers antigen more efficiently to subcutaneous lymph nodes than subcutaneous injection this therapy induced more than 10-fold higher IgG responses with 100-fold lower antigen doses than subcutaneous immunization in mice[184]. In the future such approaches may allow reducing both the number of allergen injections as well as the allergen dose, and improving efficacy and safety of SIT.

1.3.3 Therapy of Hymenoptera venom allergy

Systemic allergic reactions to Hymenoptera stings are treated according to the guidelines of anaphylactic shock. Medications used are intramuscular adrenaline, corticosteroids and antihistamines. Volume substitution and pressor substances are given if needed. Subjects with a history of potentially fatal insect sting hypersensitivity should have available an emergency kit containing aqueous adrenaline in a preloaded automatic syringe as well as tablets of corticosteroids and antihistamines[129].

For patients being hypersensitive with having Grade III or IV reactions as well as a positive diagnostic test, either skin test or serum specific IgE, a venom immunotherapy is recommended[185]. The first attempts at specific immunotherapy involved extracts of venom sacs[186] but then only whole-body extracts were used for almost 50 years. In the late 70s, prospective studies showed clearly that venom preparations are largely superior to whole-body extracts for immunotherapy and that whole-body extracts give patients no better protection than placebo[187]. Various regimens are available for VIT. Conventional immunotherapy entails an initial

course of weekly injections over three month, starting with low doses of venom and reaching the highest dose of 100 µg (equivalent to two honeybee stings and probably many more Vespula stings). Thereafter, maintenance injecttions of the same dose are given at monthly or longer intervals for at least three to five years[64]. Rush protocols, with an up-dosing to maintenance dose in 4 days, provide more rapid protection than slow schedules, with sessions every 1-2 weeks and a dose increase to maintenance over several month[188]. However, slow protocols are usually better tolerated[189]. Newly described ultrarush protocols over 3.5 and 6 hours[190] seem to be very well tolerated in *Vespula* venom-allergic patients, but results in bee venom-allergic patients are controversial [191]. Another convenient alternative are cluster protocols which comprise 2–3 injections per day of treatment that are given once a week to rapidly reach the maintenance dose[192]. The standard for effective VIT is a well tolerated re-exposure, by either a field sting or a hospital provocation test (sting challenge). Although, venom immunotherapy is effective in the majority of Hymenoptera venom-allergic patients, systemic side-effects to VIT injections have been observed in 20-40% of patients and occur mainly during the initial phase of dose increase[193]. According to sting challenge tests during VIT, 10% of vespid-allergic patients and 20-25% of bee venom-allergic patients were not protected by venom immunotherapy and continued to develop generalized allergic symptoms[134,193]. In this respect, there is considerable interest in improving safety and efficacy of Hymenoptera venom immunotherapy.

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

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***Mol Immunol*, in press. [2009 Nov 5. Epub]**

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.

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doi:10.1016/j.molimm.2009.10.005

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

Braren, I., Blank, S., Seismann, H., Deckers, S., Ollert, M., Grunwald, T. and Spillner, E.

***Clin Chem* 53(5): 837-844 (2007). [2007 Mar 29. Epub]**

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.

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doi: 10.1373/clinchem.2006.078360

Recombinant IgY for improvement of immunoglobulin-based analytical applications

Greunke, K., Braren, I., Alpers, I., Blank, S., Sodenkamp, J., Bredehorst, R. and Spillner, E.

***Clin Biochem* 41:1237-1244 (2008). [2008 Jul 26. Epub]**

OBJECTIVES: In order to provide superior tools for diagnostic approaches and to prevent assay interference and background binding, the objective of this study was the establishment and evaluation of monoclonal IgY which are phylogenetically distant from mammalian immunoglobulins but have been unavailable so far.
DESIGN AND METHODS: Human, murine and avian monoclonal model antibodies were established and produced in mammalian cells. Their interaction with human serum components and Fc gamma receptors was compared by ELISA and fluorescence activated cell sorting (FACS).
RESULTS: The use of monoclonal IgY in contrast to mammalian antibodies prevented interference phenomena in absorbance measurements generated by human sera containing rheumatoid factor (RF) or heterophilic antibodies. Additionally, monoclonal IgY exhibited no interaction with the human and murine high-affinity receptor FCGR1 (CD64) and human low affinity receptor FCGR3a (CD16A).
CONCLUSIONS: The data obtained demonstrate the advantageous behaviour of monoclonal IgY as detection or capture antibodies compared to conventional mammalian immunoglobulins and provide a strategy for improvement of assay performance and accuracy.

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doi: 10.1016/j.clinbiochem.2008.07.004

3. Additional Results

3.1 Materials and Methods

3.1.1 Materials

Crude honeybee venom (HBV) collected by electrostimulation was purchased from Latoxan (Valence, France). Therapeutical grade HBV preparations were obtained from three different manufacturers. Yellow jacket venom (*Vespula spp.*) of immunotherapeutic grade (Venomil), which is obtained by venom sac extraction, was purchased from Bencard (Munich, Germany). Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate and anti-mouse IgG AP conjugate was obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). AlaBLOTs were obtained from Siemens Healthcare Diagnostics (Los Angeles, USA) . Patient sera were provided by 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 from hymenoptera venom-sensitized patients were selected: (i) sera with a negative sIgE test to vespid venom (i3 <0.35 kU/L) but a positive test to HBV (i1 $\geq$0.35 kU/L); (ii) sera with a negative sIgE test to HBV (i1 <0.35 kU/L) but a positive test to vespid venom (i3 $\geq$0.35 kU/L); (iii) sera with a positive sIgE test to HBV and to vespid venom (i1 and i3 $\geq$0.35 kU/L). All sera were derived from patients with a history of a systemic allergic reaction after a stinging event. Specific IgE tests for honeybee or yellow jacket venom were performed in all patients on the automated immunoassay systems UniCAP250 (Phadia, Upsala, Schweden) or Immulite2000 (Siemens Healthcare Diagnostics). All patients had given their informed written consent to draw an additional serum sample. Recombinant Ves v 3 was provided by Henning Seismann (University of Hamburg).

3.1.2 Protein biochemistry

Api m 5 was enriched from venom via chromatographic methods and subjected to sequencing by tandem mass spectrometry. Therefore, 200 mg of lyophilized honeybee venom were dissolved in 10 ml of 30 mM sodium citrate buffer (pH 4.5). Following removal of insoluble components by centrifugation at 4000 x g for 30

minutes the supernatant was incubated overnight with 5 ml of Sephadex C-25 ion exchange resin (GE Healthcare) pre-swollen in the same buffer. After settling of the resin by centrifugation, the supernatant was recovered and reduced to 800 µl by lyophilization, dialyzed against 3 mM Tris-HCl buffer (pH 7.0) and further reduced to 300 µl. This step enriches the approx. 100 kDa Api m 5 in relation to the abundant lower molecular weight protein fraction containing melittin and phospholipase A2. The enriched protein sample or 400 µg of whole venom dissolved in 30 µl 5x PAGE loading dye were subjected to fractionation by SDS-PAGE. Bands were excized, the proteins digested in-gel by trypsin (Roche Diagnostics, Penzberg, Germany) and resulting peptide fragments were sequenced on a Waters Micromass QToF2 mass spectrometer (Waters, Milford, MA, USA) by tandem mass spectrometry according to the manufacturers instructions.

3.1.3 cDNA cloning

Total RNA was isolated from the separated stinger with attached venom sac and additional glands of honeybee (*Apis mellifera*) using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen) and gene-specific primers were used to synthesize cDNA from the isolated total RNA. RNaseOut™ recombinant ribonuclease inhibitor (1 µl) (Invitrogen) was added to the standard 20 µl reaction mix containing 5 µg venom gland RNA. Reverse transcription was performed at 50 ºC for 60 minutes. First strand cDNA was used as a template for PCR amplification of Api m 5 and Carbohydrate-rich protein (CRP) DNA sequences. Full length Api m 5 was amplified from *Apis mellifera* venom gland cDNA with *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) using the primers 5'-ATGGAGGTACTGGTG-CAGCTGGCGCTGCTGCTG-3' and 5'-TCAGTGGGAGTA TCCCAGACA-3'. CRP was amplified from venom gland cDNA using the primers 5'-TTCCCTGGTGC-ACACGATGAGG-3' and 5'-TCAAGCAGTTAATACATCTCCTTGG-3'. DNA from the PCR reaction was isolated from 1% agarose gels (peqGOLD universal agarose, Peqlab Biotechnologie) using the peqGOLD Gel Extraction Kit (Peqlab Biotechnologie). Subcloning for sequencing was done using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with pCR-Blunt II-TOPO vector. The ligated DNA was used to transform *E. coli* of the strain XL1 Blue by electroporation (2 mm cuvettes) and selected on ampicillin agar plates.

3.1.4 Cloning and expression of venom allergens in insect cells and *E. coli*

After sequencing of selected subcloned cDNAs and verification of the sequence the clones were used for secondary amplification. The coding region of subcloned CRP was amplified in two consecutive PCR reactions adding a N-terminal 10-fold His-tag and V5 epitope using the primers 5'-AAGCCTATCCCTAACCCTCTCCT-CGGTCTCGATTCTACGTGGCTCGAGTCTAGATTCCCTGGTGCACACGATGA-GG-3', 5'-GATCGGATCCCATCACCACCACCATCATCACCACCACCATTCTTCT-GGTGGTAAGCCTATCCCTAACCCTCTCCTCGG-3' and 5'GATCGCGGCCGCT-CAAGCAGTTAATACATCTCCTTGG-3'. The mature chain coding region of subcloned Api m 5 was also amplified in two consecutive PCR reactions adding an N-terminal 10-fold His-tag and V5 epitope using the primers 5'-AAGCCTATCCCT AACCCTCTCCTCGGTCTCGATTCTACGTGGCTCGAGTCTAGAAAATCCGTTC CACGAGTGATCG-3', 5'-GATCGGATCCCATCACCACCACCATCATCACCACCA CCATTCTTCTGGTGGTAAGCCTATCCCTAACCCTCTCCTCGG-3'and 5'-GATC-GCGGCCGCTCAGTGGGAGTATCCCAGACAATTGGC-3'. The PCR products were subcloned into the BamHI and NotI digested baculovirus transfer vector pAcGP67-B (BD Pharmingen) after restriction with BamHI and NotI.

For expression of Api m 5 in *E. coli*, the coding region was cloned into the prokaryotic expression vector pMAL-c2X (New England Biolabs, Bad Schwalbach, Germany). Expression in *E. coli* XL1 Blue cells and purification of the fusion protein was performed according to the recommendations of the manufacturer.

For expression in *E. coli* the Carbohydrate-rich protein coding region was amplified using the primers 5'-GATCCATATGTTCCCTGGTGCACACGATG-3' and 5'-GGT-GGTTGCTCTTCCGCAAGCAGTTAATACATCTCCTTGG-3' and inserted into the digested vector pTXB1 (New England Biolabs) via NdeI and SapI. The vector was further modified by addition of a second chitin binding domain (CBD). Expression and purification of the fusion protein was performed according to the recommendations of the manufacturer.

3.1.5 Recombinant baculovirus production

Spodoptera frugiperda cells (Sf9) (Invitrogen) were grown at 27 °C in serum-free medium (Express Five SFM; Lonza, Verviers, Belgium) containing 10 µg/ml gentamycin; Invitrogen, Karlsruhe, Germany). 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 pAC-GP67-B containing Carbohydrate-rich protein or Api m 5. High titer stocks were produced by three rounds of virus amplification and optimal MOI for protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flasks (1.5-2 x 10^6 cells/ml in 20 ml suspension culture) with serial dilutions of high titer virus stock. High titer stock of recombinant baculovirus was used to infect 400 ml suspension culture of Sf9 or HighFive cells (Invitrogen) (1.5-2 x 10^6 cells per ml) in 2000 ml flasks. For protein production the cells were incubated at 27 ℃ and 110 rpm for 72 h.

3.1.6 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, Hilden, Germany). 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.

3.1.7 Enzymatic activity of the recombinant Api m 5

The DPPIV activity of the native and recombinant enzyme was assessed as follows. Recombinant Api m 5 from baculovirus expression at a concentration of 200 ng/ml in NTA-binding buffer containing 300 mM imidazole and honeybee 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).

3.1.8 IgE immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE immunoreactivity of sera, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with 20 µl of recombinant proteins (20 µg/ml) at 4 °C overnight and blocked with 4 0 mg/ml milkpowder in PBS at room temperature (RT). Thereafter, human sera were diluted 1:2 with PBS and incubated in a final volume of 20 µl for 4 hours at RT. Wells were washed 4 times with PBS before IgE was detected with a monoclonal alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD) 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) were added. The plates were read at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. For reasons of precision, reactivities only slightly higher than the cut off value were excluded.

For immunoblot procedures human sera were diluted 1:10 with 5 mg/ml BSA in PBS and applied to the corresponding AlaBLOTs (i1 *A. mellifera* venom; i3 *V. vulgaris* venom; Siemens Healthcare Diagnostics) or to the purified recombinant allergens, separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Visualization 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.

3.1.9 Basophil activation test

The basophil activation test was performed as described previously[194] with modifications as recommended by the manufacturer of the assay (Flow-CAST; Bühlmann Laboratories). 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. Subs equently, basophils were activiated for 40 min at 37 °C in a water bath with variou s concentrations of either native or recombinant allergens at a volume of 100 µl. Thereby, honeybee or yellow jacket 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 lyzed and white blood cells were fixed (FACS Lysing solution, BD Biosciences) for 5 min at room temperature. After centrifugation (5 min, 1200 x g) 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.

3.1.10 Other methods

SDS-PAGE, immunoblotting and molecular biology standard procedures were performed according to established protocols[195].

3.2 Results

The most abundant honeybee venom (HBV) allergens include phospholipase A2 (Api m 1), hyaluronidase (Api m 2), and the basic 26 amino acid peptide Melittin (Api m 4)[94], all constituting high abundance proteins with amounts of 12%, 2% and 50%, respectively, of dry weight in the venom[196]. Nevertheless, hymenoptera venoms comprise a more complex cocktail of a variety of different compounds all of which may contribute to allergic sensitization, allergic symptoms and success of VIT. Although venom allergens in recombinant form may provide significant improvements[197], only the most prominent ones are available so far[105,198]. Api m 1 and Api m 2 could be expressed in hosts like bacteria, yeast or baculovirus-infected insect cells[199-202] and selected structures have been elucidated by X-ray crystallography[203,204].

In the recent years significant progress has been made to identify additional HBV compounds of lower abundance, primarily by proteomic approaches. In general, recombinant approaches facilitate the assessment of the allergenicity and the clinical relevance of such venom compounds, whereby expression should meet the requirements of proper folding - if possible enzymatic activity - and correct posttranslational modifications, all of which are potentially important for the establishment of conformational epitopes[202]. In particular glycan structures can contribute significantly to biochemical and structural characteristics of venom proteins. Additionally, they constitute the underlying principle of cross-reactive carbohydrate determinants (CCD), a peculiarity of hymenoptera and plant allergens interfering with diagnosis and design of therapeutic strategies in hymenoptera venom allergy[140,143,205].

Since a recombinant availability of venom allergens might offer several promising possibilities for an improvement of diagnosis and therapy of Hymenoptera venom allergy, novel allergenic components from *Apis mellifera* venom were identified as well as established allergens recombinantly produced and characterized in detail.

3.2.1 Characterization of Carbohydrate-rich protein from *Apis mellifera* venom

A venom protein of considerable interest is the Carbohydrate-rich protein (CRP), also termed Icarapin or venom protein 2 (VP2). Peptides of this protein were identified by two independent groups in 2005[206,207]. Insoluble, non-glycosylated protein

obtained by recombinant production in *E. coli* exhibited an IgE reactivity with 4 out of 5 sera of beekeepers with HBV allergy[100] accompanied by an inherent molecular lability. However, without established recombinant expression of soluble Carbohydrate-rich protein, its relative abundance in whole HBV as well as its relevance in the context of sensitization and VIT remained elusive.

Thus, aim of this work was the recombinant production of Carbohydrate-rich protein in soluble, non-glycosylated form in *E. coli* and as a fully glycosylated protein in two variant baculovirus-infected insect cell lines and the comparative analysis of the differentially glycosylated proteins for IgE reactivity using sera from hymenoptera venom-allergic patients. Furthermore, a monoclonal Carbohydrate-rich protein-specific antibody should be generated for quantification of the native protein in *A. mellifera* venom and therapeutical preparations.

3.2.1.1 Recombinant expression and characterization of the Carbohydrate-rich protein

Systematic MS-based proteomic analyses of *A. mellifera* venom proteins within the molecular mass range of 30-35 kDa yielded tryptic fragments that could be assigned to Carbohydrate-rich protein. Carbohydrate-rich protein is a putative venom allergen with a theoretical mass of 22 kDa. For recombinant expression the Carbohydrate-rich protein coding region was amplified from honeybee venom gland whole cDNA. Sequence analysis revealed the selective amplification of the splicing variant 2 of Carbohydrate-rich protein, 4 amino acids shorter than variant 1 (Fig. 6).

```
CRP V1  MKTLGVLFIAAWFIACTHSFPGAHDEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS 60
CRP V2  MKTLGVLFIAAWFIACTHSFPGAHDEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS 60
        ************************************************************

CRP V1  FEDSDEGSNWNWNTLLRPNFLDGWYQTLQSAISAHMKKVREQMAGILSRIPEQGVVNWNK 120
CRP V2  FEDSDEGSNWNWNTLLRPNFLDGWYQTLQT----HMKKVREQMAGILSRIPEQGVVNWNK 116
        ****************************:    ************************

CRP V1  IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTTVSSEAD 180
CRP V2  IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTTVSSEAD 176
        ************************************************************

CRP V1  SDVTTLPTLIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 223
CRP V2  SDVTTLPTLIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 219
        *******************************************
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Fig. 6: Alignment of Carbohydrate-rich protein variants. Shown are Carbohydrate-rich protein variant 1 and 2. Peptides identified by mass spectrometry are highlighted in light grey. Signal sequences are italicised and putative glycosylation sites are represented in dark grey.

For expression in *E. coli* as an agylcosylated protein the Intein-CBD system was employed. Release from the affinity resin by autocatalytic cleavage yielded soluble non-tagged Carbohydrate-rich protein with an apparent molecular weight of 35 kDa (Fig. 7A, B) suggesting a modified migration behaviour due to its low pI.
Glycosylated Carbohydrate-rich protein with or devoid of CCDs was produced by baculovirus-infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. Purification yielded recombinant Carbohydrate-rich protein (yield of approx. 1 µg per ml supernatant) from Sf9 and HighFive cells with an apparent molecular mass of approx. 50 to 55 kDa (Fig. 7A, B). Additionally, a very minor band in the range of 35 kDa could be detected. The migration behaviour of the insect cell derived proteins underlines the contribution of the carbohydrates to the overall characteristics, particularly when compared to the aglycosylated *E. coli*-derived protein. To verify their identity and immunoreactivity, all three protein variants were evaluated by means of different antibodies (Fig. 7C-E).

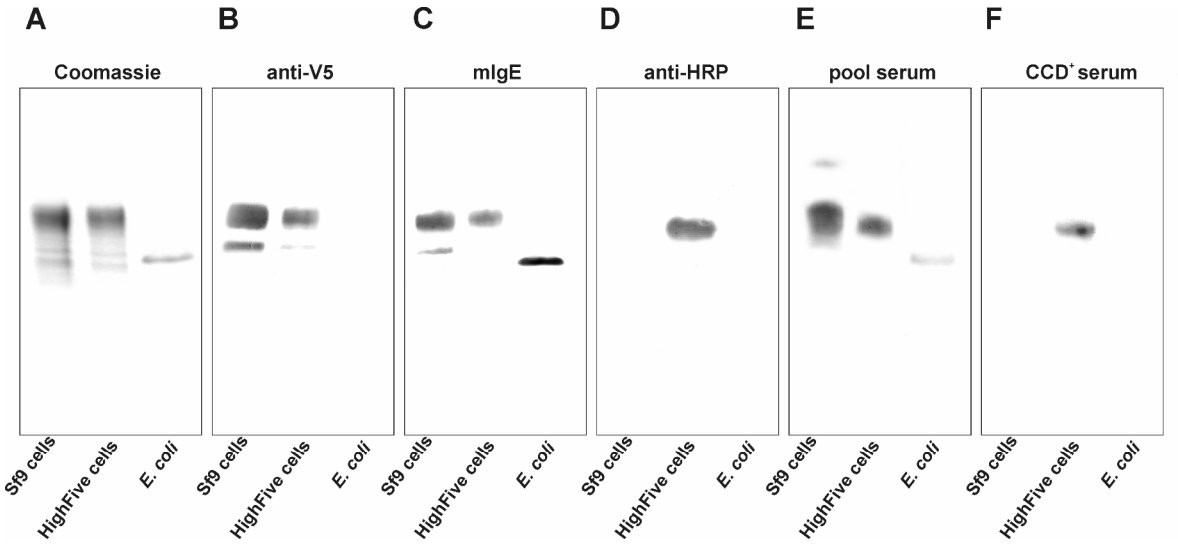

Fig. 7: Analysis of recombinant Carbohydrate-rich protein in immunoblot. SDS-PAGE and Immunoblot analysis of Carbohydrate-rich protein recombinantly produced in Sf9 and HighFive insect cells as well as in *E. coli* visualized by either coomassie staining or anti-V5 epitope antibody, monoclonal human anti-Carbohydrate-rich protein IgE antibody, anti-HRP antiserum, pooled HBV allergic patients sera and a CCD-positive serum.

In immunoblot, all three proteins were found reactive with a monoclonal anti-Carbohydrate-rich protein IgE and, additionally, with a serum pool of HBV-sensitized patients (Fig. 7C, E). The use of an anti-HRP rabbit serum specific for plant-derived glycostructures including α-1,3-core fucosyl and β-1,2 xylosyl residues verified pronounced α-1,3-core fucosylation, the causative structure for CCD-based cross-reactivity for Carbohydrate-rich protein produced in HighFive cells. In

contrast, glycosylated, Sf9-produced as well as *E. coli*-derived Carbohydrate-rich protein did not exhibit any CCD-reactivity (Fig. 7D). Comparable results were obtained with serum of a CCD-reactive but not HBV-allergic patient (Fig. 7F). Use of the analogous antibodies in ELISA (Fig. 8) corroborated the obtained data.

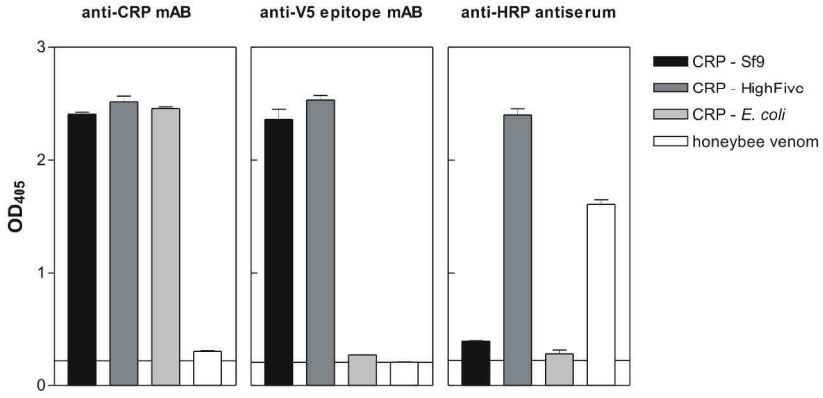

Fig. 8: Analysis of recombinant Carbohydrate-rich protein in ELISA. ELISA analysis of Carbohydrate-rich protein and HBV using the monoclonal anti-Carbohydrate-rich protein IgE, the anti-V5 epitope antibody, and the anti-HRP antiserum. Results are presented as triplicates.

Together these data demonstrate that choice of the expression host defines the state of glycosylation as shown previously[208] and, thereby, strongly influences the characteristics of the resulting proteins.

3.2.1.2 Screening of patient sera for IgE reactivity with Carbohydrate-rich protein variants

To assess the sensitization of allergic patients to Carbohydrate-rich protein, the IgE reactivity of individual sera was analyzed in ELISA and immunoblot. The impact of glycosylation was addressed by use of differentially glycosylated proteins produced in *E. coli* or Sf9 insect cells, thereby circumventing interference by CCD-reactivity.

Overall, sera of 85 randomly selected patients with a clinical history of insect venom allergy were assayed by ELISA for specific IgE antibodies to Carbohydrate-rich protein produced in Sf9 insect cells. These sera were separated into three groups, a group of 17 sera with negative sIgE to yellow jacket venom (YJV) implying sensitization to HBV only without CCD reactivity (Fig. 9A), a group of 17 sera with negative sIgE to HBV but positive sIgE to YJV (Fig. 9B), as well as a group of 51 sera double positive for HBV and YJV, thus, predominantly CCD-reactive (Fig. 9C). In the group with negative sIgE to HBV, none of the sera recognized re-

combinant Carbohydrate-rich protein, suggesting the absence of a cross-reactive, homologous molecule in YJV (Fig. 9B). From the HBV monosensitized group 8 sera (47%) reacted with Carbohydrate-rich protein (Fig. 9A). In the group of double positive sera including both HBV- and YJV-sensitized patients 27 sera (52%) exhibited pronounced reactivity with Sf9-derived Carbohydrate-rich protein (Fig. 9C).

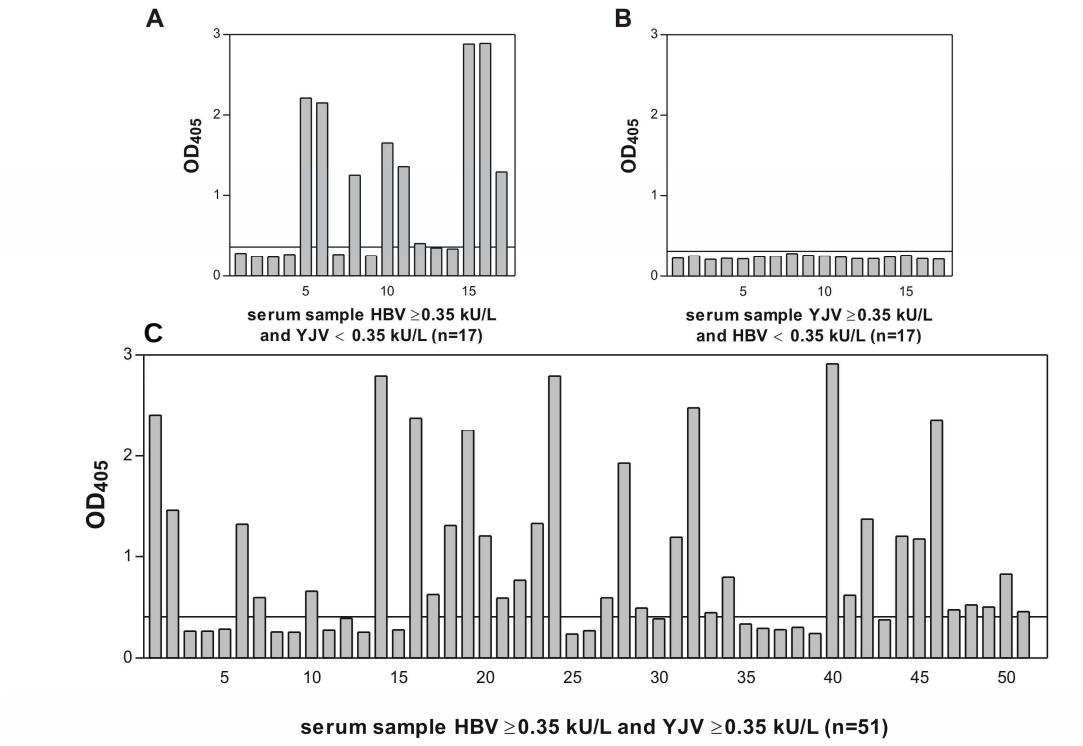

Fig. 9: IgE reactivity of individual patient sera with recombinant Carbohydrate-rich protein. Immunoreactivity of individual patient sera with recombinant Carbohydrate-rich protein produced in Sf9 insect cells. The IgE reactivity was assessed by ELISA with 17 sera of venom-sensitized patients with negative IgE to vespid venom (A), 17 sera of venom-sensitised patients with negative IgE to *A. mellifera* venom (B) and 51 double positive sera (C).

In comparative assessment of sIgE binding to Sf9- and *E. coli*-derived Carbohydrate-rich protein, 18/38 in the group of double positive sera (47%) and 17/38 (44%), respectively, exhibited reactivity with the respective protein variants (Fig. 10A). In the group of the patients with negative sIgE to vespid venom 5/11 sera (45%) showed pronounced reactivity with the Sf9- and prokaryotically derived Carbohydrate-rich protein (Fig. 10B). Although most Carbohydrate-rich protein reactive sera showed comparable reactivity to Sf9- and *E. coli*-derived Carbohydrate-rich protein, one serum interestingly exhibited a dramatically reduced reactivity to the *E. coli*-produced Carbohydrate-rich protein. The group with negative sIgE to HBV (Fig. 10C) exhibited no reactivity at all.

Reactivity of selected sera found positive in ELISA (patient 17, 19 and 24 in Fig. 9C) was further analyzed in immunoblot (Fig. 10D). All sera recognized Sf9- and *E. coli*-produced Carbohydrate-rich protein in an equivalent manner. In the case of the Sf9-produced protein all sera recognized the major band at 55 kDa as well as the minor band at 35 kDa, a pattern also observed for the pool serum.

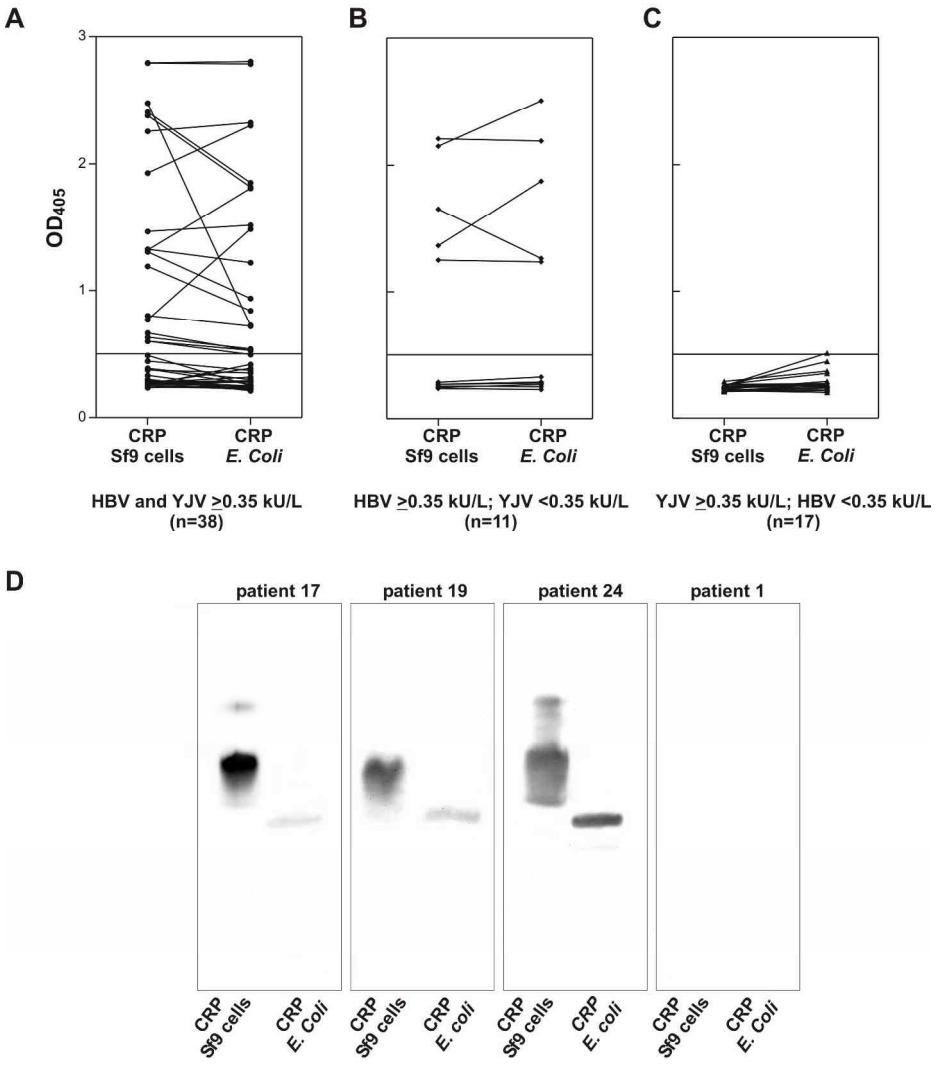

Fig. 10: IgE reactivity of individual patient sera with recombinant Carbohydrate-rich protein produced in insect cells and *E. coli*. Immunoreactivity of individual patient sera with Carbohydrate-rich protein produced in Sf9 insect cells and *E.coli*. The IgE reactivity was assessed with 38 double positive sera (A), 11 sera with negative IgE to vespid venom (B) and 17 sera with negative IgE to *A. mellifera* venom (C). In parallel, the reactivity of 4 particular sera with recombinant Carbohydrate-rich protein was assessed in immunoblot (D).

Together these results suggest that Carbohydrate-rich protein has to be considered as an important and genuine allergen in *Apis mellifera* venom. Moreover, carbohydrates beyond the CCDs appear to contribute to the overall IgE reactivity to a minor extent only.

3.2.1.3 Evaluation of native Carbohydrate-rich protein in *Apis mellifera* venom

The finding of Carbohydrate-rich protein derived peptides in the range of 30-35 kDa and a previously reported tendency to degradation[100,207] may suggest an inherent molecular lability. To clarify the molecular integrity and concentration of Carbohydrate-rich protein in the native HBV and in therapeutic preparations used for VIT a monoclonal human IgE antibody was generated (Fig. 11), the reactivity of which with the Carbohydrate-rich protein variants was verified in ELISA and immunoblot (Fig. 7C, 8A).

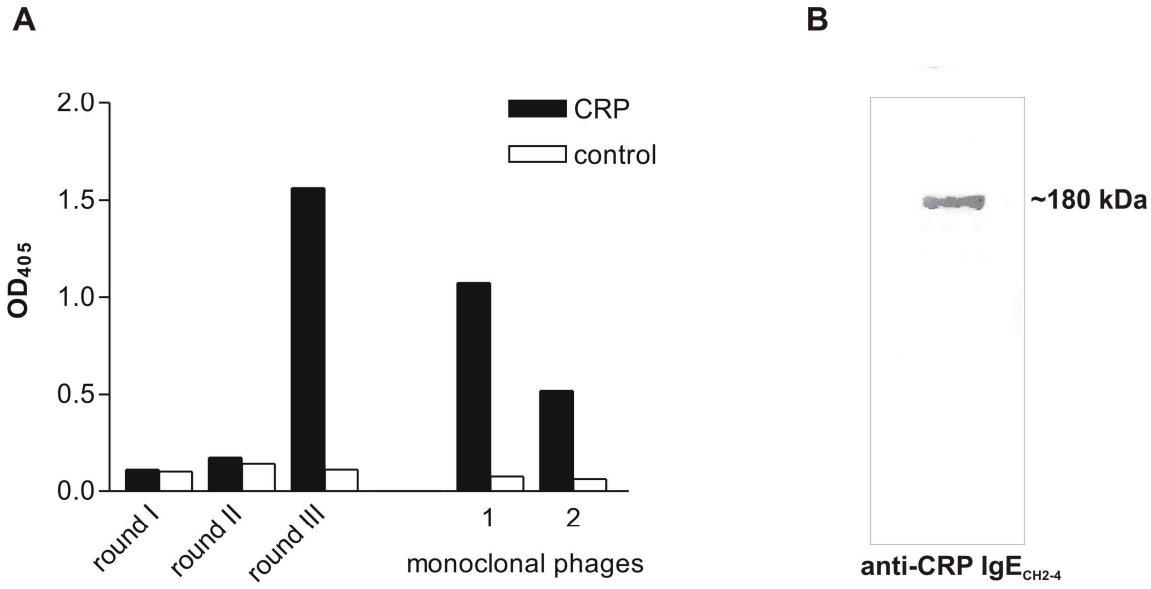

Fig. 11: Generation and expression of a monoclonal human IgE by selection of a combinatorial library. The immunoreactivity of phage-displayed polyclonal phages from three panning rounds and selected monoclonal phages against carbohydrate-rich protein was analysed by ELISA. Controls (white bars) were performed by omission of antigen (A). After conversion of the antibody gene into the format of a monoclonal human IgE antibody, clone 1 was produced in HEK293 cells and detected in cellular supernatant by using monoclonal anti-human IgE-AP conjugate.

Applying this monoclonal antibody in immunoblots of crude HBV a major band at 55 kDa as well as a minor product of 35 kDa were detected (Fig. 12A) corresponding to the pattern recognized for the insect cell-derived proteins (Fig. 7A,B). To address the quantity of Carbohydrate-rich protein in HBV, the monoclonal IgE was applied to immunoblots providing serial dilutions of recombinant Sf9-derived Carbohydrate-rich protein and HBV (Fig. 12A, upper panel). In parallel, a human monoclonal IgE antibody with specificity for acid phosphatase (Api m 3), another low abundance allergen in HBV, was applied analogously using insect cell-pro-

duced Api m 3[103] (Fig. 12A, lower panel). Densitometric quantification suggested a Carbohydrate-rich protein concentration in the range of 8 ± 1 µg per g of crude HBV, corresponding to 0.8% ± 0.1% of dry weight compared with 1.7% ± 0.4% for Api m 3. Together these data suggest that Carbohydrate-rich protein is present in the venom as intact component in amounts roughly comparable to that of other allergens of already established relevance such as Api m 3.

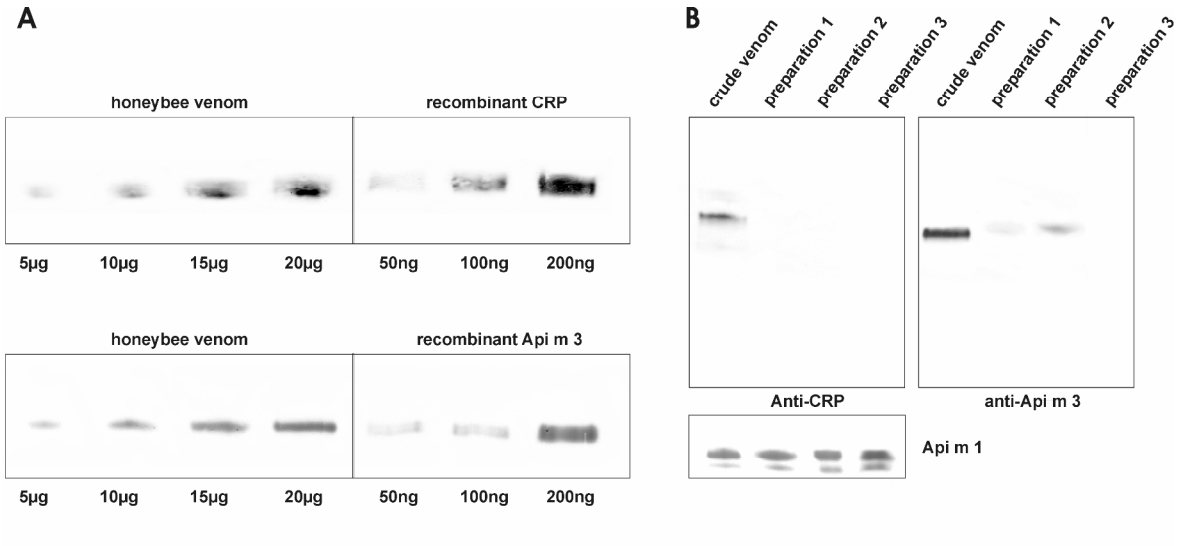

Fig. 12: Determination of the presence of CRP in venom and venom preparations. Concentration of Carbohydrate-rich protein in HBV was assessed by densitometric analyses of serial dilutions of recombinant Carbohydrate-rich protein and Api m 3 and in crude venom by immunoblotting using an anti-Api m 3 and an anti-Carbohydrate-rich protein IgE antibody (A). The presence of Carbohydrate-rich protein and Api m 3 in crude venom and venom preparations (each 25 µg per lane, coomassie staining of Api m 1 was employed as additional loading control) was addressed analogously (B).

To assess the integrity and presence of Carbohydrate-rich protein in HBV preparations 25 µg of crude venom and three different HBV preparations for VIT from three different allergen extract producers were separated on SDS-PAGE and analyzed with the monoclonal anti-Carbohydrate-rich protein IgE (Fig. 12B, left panel). Staining for Api m 1 was used as a control for loading equal amounts. In stark contrast to the crude venom, no reactivities for none of the preparations were obtained at all. Api m 3 could be readily detected in whole HBV as well as in two of the preparations, although in significantly lesser amounts (Fig. 12B, right panel). These data demonstrate that the concentrations of the putatively labile HBV allergen Carbohydrate-rich protein and the already established major allergen Api m 3, although present in HBV as intact proteins in substantial amounts, are dramatically reduced in some or all therapeutic venom preparations investigated in this study.

3.2.1.4 Carbohydrate-rich protein as a relevant *Apis mellifera* venom component

In the last decades much effort has been spent to characterize a plethora of allergens in a variety of sources including pollen, food, moulds, animals, etc., however, the allergenic source causing the highest incidence of anaphylaxis and sometimes even fatal consequences – the venoms of hymenoptera - remain inadequately characterized with regard to their molecular composition. Notably, this holds also true for HBV, despite the fact that the higher abundance allergens (Api m 1, Api m 2, and Api m 4) have already been characterized in detail years ago. With a few exceptions (e.g., Api m 3), comparatively little is still known about the identity, the sensitizing potential, the allergenicity, and the clinical relevance of the lower abundance allergens in HBV. One of these putative lower abundance allergens is Carbohydrate-rich protein, a protein of unknown function, peptides of which were recently identified in HBV independently by two groups[206,207]. In support of their finding, in this work it have also been identified CRP-derived peptides in two different batches of HBV obtained by electrostimulation in the range of 30 to 35 kDa. Its nature as a true venom compound is further supported by the presence of a signal peptide typical for secreted proteins, an immunodetection at the cuticular lining of the venom duct[100] and the presence in fractionated HBV[206], as additionally confirmed in this study by the use of a monoclonal Carbohydrate-rich protein-specific antibody.

Carbohydrate-rich protein shares a consensus sequence with a number of mostly unknown insect proteins; one of them is the *Ae. aegypti* putative salivary secreted mucin 3, however, Carbohydrate-rich protein lacks common features of mucins or mucin-like proteins rendering a mucin function at least questionable.

In general, it could be demonstrated that production of insect venom allergens in insect cells as nearly autologous system is superior in terms of functionality and folding[96,202,209]. This strategy also favours the establishment of discontinuous IgE epitopes as well as linear epitopes requiring a rigid and properly folded framework. However, contrary to a previous report in this work it was possible to obtain soluble recombinant Carbohydrate-rich protein using both, the eukaryotic baculovirus expression system or the strategy of CBD fusion followed by autocatalytic intein-mediated cleavage in *E. coli*. The insect cell produced Carbohydrate-rich protein showed a molecular weight of approx. 50-55 kDa. In contrast, *E. coli* produced soluble Carbohydrate-rich protein exhibited a molecular weight of 35 kDa

without indications for inherent lability as reported previously for the insoluble protein obtained also from *E. coli*[100].

The peculiar discrepance between the predicted molecular weight of 22 kDa of the aglycosylated protein variant and the actual migration behaviour at 35 kDa in SDS-PAGE might be explained by the acidic pI of 4.4. The additional difference between the aglycosylated and the insect cell-derived, glycosylated proteins obviously stems from extensive posttranslational modifications, such as glycosylation which is anticipated due the presence of 4 potential N- and 6 potential O-glycosylation sites. Although the capacity for the latter type of modification was demonstrated for different lepidopteran insect cell lines recently, including *T. ni* and *S. frugiperda* cells[210] the N-glycosylation most likely will be of major importance.

Since a few years it is increasingly been recognized that carbohydrate-based cross reactivities, namely by α-1,3-core fucose residues, represent a major concern for diagnostic approaches in hymenoptera venom allergy. However, the use of Sf9 and HighFive insect cell lines constitutes a strategy recently reported by us to define the establishment of CCDs and their detrimental role[208] under the aegis of an autologous eukaryotic expression.

Using the differentially glycosylated protein variants, up to approx. 50% of HBV-allergic patients showed reactivity with recombinant Carbohydrate-rich protein, thus rendering it an important sensitizing component of HBV beyond the presence of CCDs. Interestingly, the findings obtained with the aglycosylated protein expressed in *E. coli* matched those with the glycosylated proteins, although reactivity of prokaryotically produced protein appeared slightly reduced. Only singular sera exhibited drastically reduced or loss of sIgE reactivity with the aglycosylated Carbohydrate-rich protein. This might hint for a predominance of IgE epitopes that are not affected by structural rearrangements due to glycosylation. Future studies will have to address the allergen status of CRP as a major or minor allergen of HBV and its clinical relevance in venom allergy.

Notably, the complete lack of reactivity in the group of YJV-sensitized patients without sensitization to HBV clearly suggests the absence of a homologous structure in vespid venom. This would render Carbohydrate-rich protein a novel genuine marker for HBV allergy.

As evident from the data of this work Carbohydrate-rich protein appears to be a crucial but delicate component of HBV preparations. By the use of the monoclonal

anti-Carbohydrate-rich protein IgE it was for the first time possible to detect native Carbohydrate-rich protein in unprocessed *A. mellifera* venom and to provide evidence for its molecular integrity. Present as intact protein with concentrations only slightly lower than those of Api m 3 (1.7%) and Api m 2 (2%) the Carbohydrate-rich protein appears as an allergen analogous to since long established proteins which are easier to prove, inter alia for their enzymatic activity. Delineated from these observations, it can be calculated that during a bee sting, given an injection of 50-140 µg of total protein per sting[86,87], approx. 400-1120 ng of intact Carbohydrate-rich protein are introduced into the individual. Thus, it was surprising to find that Carbohydrate-rich protein is apparently absent or at least vastly underrepresented in three therapeutical preparations of HBV from independent vendors as assessed by use of the monoclonal antibody. Obviously, downstream processing of venoms for VIT affects the distribution of venom proteins, resulting in the potential loss of particular low abundance components, as evident for Carbohydrate-rich protein and Api m 3, although the latter not to the extent of Carbohydrate-rich protein.

Although VIT is relatively efficient, the high effort of therapy regarding safety and time, the difficulties to achieve full protection, and the reasons for the 10-20% treatment failures remain to be addressed. The findings of this work might hint for an implication of lower abundance components of hymenoptera venoms in the success and, thereby, also the failure of venom immunotherapies.

Although these data need further validation and the exact role of lower abundance components for therapeutic efficacy remains to be analyzed, the presence of lower abundance or inherently labile venom components should be imperative and might serve as proof of quality. With regard to such standardization purposes the use of monoclonal antibodies as shown here for Carbohydrate-rich protein as well as Api m 3 may open interesting prospects to improvement and standardization of therapeutics.

3.2.2 Identification, recombinant expression and characterization of the 100 kDa allergen Api m 5

In addition to the major components of *Apis mellifera* venom with known enzymatic function a panel of high molecular weight proteins exhibits IgE reactivity, the most prominent of which in the range of 100 kDa is termed Api m 5 or allergen C and is supposed to be another major allergen recognized by specific IgE in a majority of honeybee venom-allergic patients. Although present in substantial concentrations, identity and function of this allergen defied elucidation. As determined by gel-electrophoretic analysis, Api m 5 has an apparent molecular weight ranging between 102 kDa[211] and 105 kDa[95]. In immunodiffusion, it has been demonstrated to be non-cross-reactive with other major bee venom allergens including Api m 1, Api m 2, Api m 3, and Api m 4 as well as with other minor components[95].

Thus, aim of this work was the identification and molecular cloning of the cDNA of the high molecular weight allergen Api m 5, the expression of the gene in insect cells, and the biochemical and immunological characterization of the purified recombinant molecule.

3.2.2.1 Identification of Api m 5

Major sIgE reactivities with proteins of higher molecular weight were evident in immunoblot of *A. mellifera* venom employing pool sera of venom-sensitized patients as shown in Fig. 13A. Thereby, the most remarkable reactivity was detected with a high molecular weight allergen at approx. 100 kDa, putatively corresponding to Api m 5 or allergen C in honeybee venom. After enrichment by chromatographic procedures the IgE-reactive putative Api m 5 with an apparent molecular weight of 105 kDa (Fig. 13B) was subjected to sequencing by tandem mass spectrometry. Four peptide sequences (shown in Fig. 14) could be identified, three of which yielded hits in a database search of the *Apis mellifera* genome with bioinformatic tools[212,213]. According to the automated gene prediction program GNOMON, a putative gene (XP_393818) codes for the isolated Api m 5. A Blast search for short, nearly exact matches yielded a corresponding result with the fourth peptide sequence. Although with low probability scanning for a potential signal peptide cleavage site[214] indicated a putative N-terminus for the Api m 5 protein.

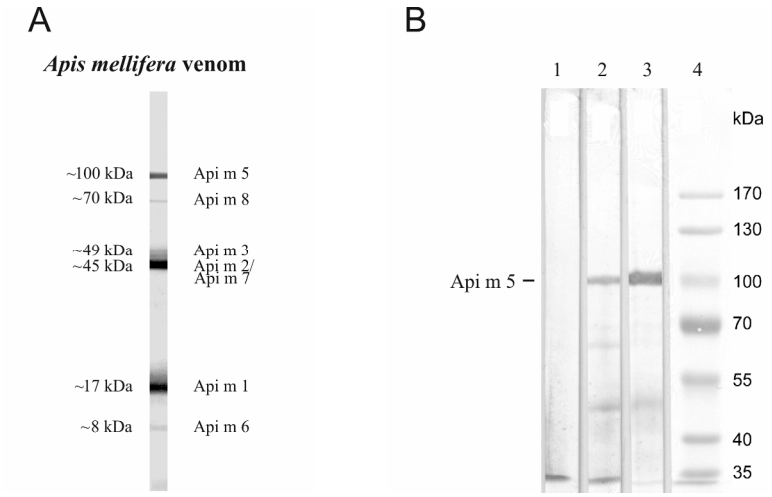

Fig. 13: Venom immunoreactivity and enrichment of the high molecular weight allergen Api m 5 from honeybee venom. Specific IgE immunoreactivity of pooled sera from honeybee venom-sensitized patients with venom of *A. mellifera* (A). Immunoblot analysis of Api m 5 enriched from venom of *A. mellifera*. Detection was performed using pooled serum of honeybee venom-sensitized patients and anti-IgE alkaline phosphatase conjugate (lane 1: bovine serum albumin as negative control; lane 2: honey bee venom; lane 3: enriched Api m 5 fraction; lane 4: protein marker) (B).

3.2.2.2 cDNA cloning and sequence analysis

First attempts to amplify the gene from bee venom gland cDNA failed. However, reevaluation of the genomic sequence using the alternative automated gene prediction program GenMark suggested a variant N-terminal splicing un-ambiguously providing a signal peptide. Based on this information a DNA fragment of 2328 bp in length could be amplified.

The Api m 5 nucleotide sequence (Genbank accession: EU564832) encodes a 775-amino acid polypeptide with a calculated molecular mass of 87.9 kDa which is compatible with the apparent molecular weight (approx. 105 kDa) of native Api m 5 (Fig. 13, Fig. 14). The discrepancy between the deduced molecular weight of Api m 5 and its apparent molecular weight of 105 kDa in SDS-PAGE is most likely due to posttranslational modification by glycosylation as suggested by the presence of six predicted sites for N-glycosylation. The amino acid sequence shows significant homology to dipeptidylpeptidase IV (DPPIV) proteins known to cleave dipeptide units from the N-terminus of growth factors and other peptidic compounds. The enzyme is composed of an N-terminal dipeptidyl peptidase domain and a C-terminal prolyl peptidase domain. Identity to human DPPIV (CD26) is in the range of 32% (10% on DNA level) and to a DPPIV from the venom of the snake *Gloydius blomhoffi brevicaudus* in the range of 32% (11% on DNA level).

Fig. 14: Alignment of Api m 5 with other related proteins. Alignment with database-derived sequences revealed homologies to peptidases from other species. Shown are Api m 5, Ves v 3 from YJV, 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 italicized, the residues involved in the conserved active centre of the enzymes are represented boxed and putative glycosylation sites in grey.

3.2.2.3 Recombinant expression of Api m 5 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 full length Api m 5 was produced as secreted protein in insect cells. The cDNA of Api m 5 was cloned into the particular expression vector for baculovirus based infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. The culture supernatant was subjected to Ni-NTA-agarose chromatography, and the resulting protein analyzed by SDS-PAGE and IgE immunoblotting (Fig. 15). The epitope-tagged recombinant protein (yield of approx. 0.2 µg per ml supernatant) exhibited an apparent molecular mass of approx. 105 kDa corresponding to the natural allergen in honeybee venom and, additionally, was reactive with sIgE from pooled sera of venom-sensitized patients (Fig. 15B). Recombinant Api m 5 was analyzed in comparison to its homologue from Yellow jacket venom, Ves v 3, which was present in the laboratory.

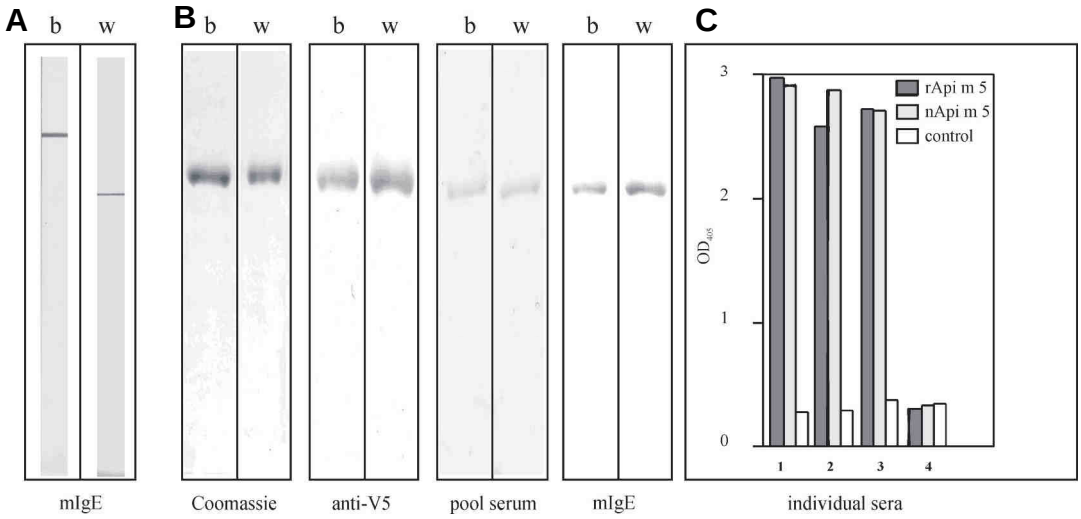

Fig. 15: Immunoreactivity of native and recombinant Api m 5. Immunoblot of honeybee (b) and yellow jacket (w) venom with a monoclonal human IgE antibody specific for Api m 5 (mIgE) (A). SDS-PAGE and immunoblot analysis of purified Api m 5 (b) and Ves v 3 (w) expressed in Sf9 insect cells, visualized by either Coomassie Blue staining or anti-V5 epitope antibody, pooled HBV or YJV allergic patient sera and monoclonal human anti-Api m 5 IgE antibody (B). Immunoreactivity of individual sera HBV allergic patients (1-3) and a control serum of a nonallergic individual (4) with recombinant and native Api m 5 in ELISA. Api m 5 was expressed in HighFive insect cells (C).

Employing prokaryotically expressed Api m 5 fusion protein a recombinant human IgE antibody was generated and produced in mammalian cells. This Api m 5-specific monoclonal IgE showed reactivity with insect cell-derived recombinant proteins Api m 5 and Ves v 3 (Fig. 15B). Moreover, the corresponding natural form of Api m 5 and Ves v 3 was detected in the venoms of *A. mellifera* and *V. vulgaris* (Fig. 15A). Furthermore, as shown in Fig. 15C, comparable IgE binding to purified native Api m 5 and recombinant Api m 5 produced in insect cells was verified via ELISA employing three exemplary sera of honey bee venom-sensitized patients that were selected by sIgE immunoreactivity with Api m 5 in immunoblots. Together, these data verified the identity of the recombinantly produced allergen with the IgE immunoreactive 100 kDa allergens in the native venom.

3.2.2.4 Enzymatic activity of recombinant Api m 5

Its sequence renders Api m 5 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 *A. mellifera* (Fig. 16A). Moreover, this activity could be abolished by Diprotin A, a highly specific inhibitor of human DPPIV. Purified recombinant Api m 5 proved to exhibit significant DPPIV activity, demonstrating its DPPIV nature and, thereby, correct folding of the insect cell produced protein (Fig. 16B).

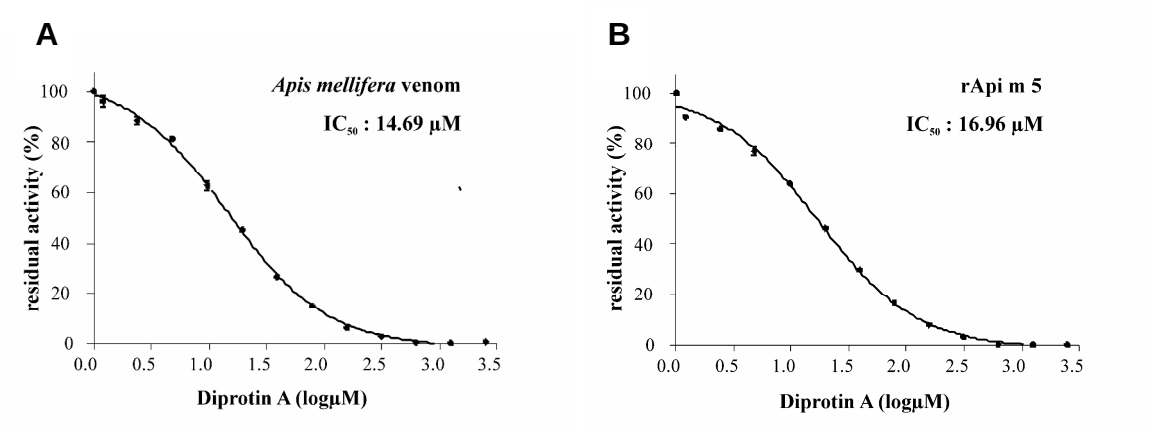

Fig. 16: Kinetic analyses of DPPIV activity. Inhibition of DPPIV activity by the inhibitor Diprotin A (Ile-Pro-Ile) of *A. mellifera* venom and purified rApi m 5 (B) and was analyzed as described in *Materials and Methods*. The Y axis shows the residual activity (%) of cleavage of the chromogenic substrate 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.

The inhibition constants of Diprotin A for the DPPIV activity in the venom and of the recombinant Api m 5 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 Api m 5 and its homologues.

3.2.2.5 IgE immunoreactivity of recombinant Api m 5

To evaluate the IgE immunoreactivity of Api m 5 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. 17 shows the reactivity of patients who were characterized by a positive sIgE test to honeybee venom. Of the 35 honeybee venom-positive sera, 9 showed high sIgE reactivity (OD_{450nm} $\geq$1.0), whereas additional 12 sera showed a positive sIgE reactivity to a medium to lower degree (OD_{450nm} $\geq$0.4<1.0). Overall, 21/35 (60%) patient sera had detectable sIgE to recombinant Api m 5 (Fig. 17).

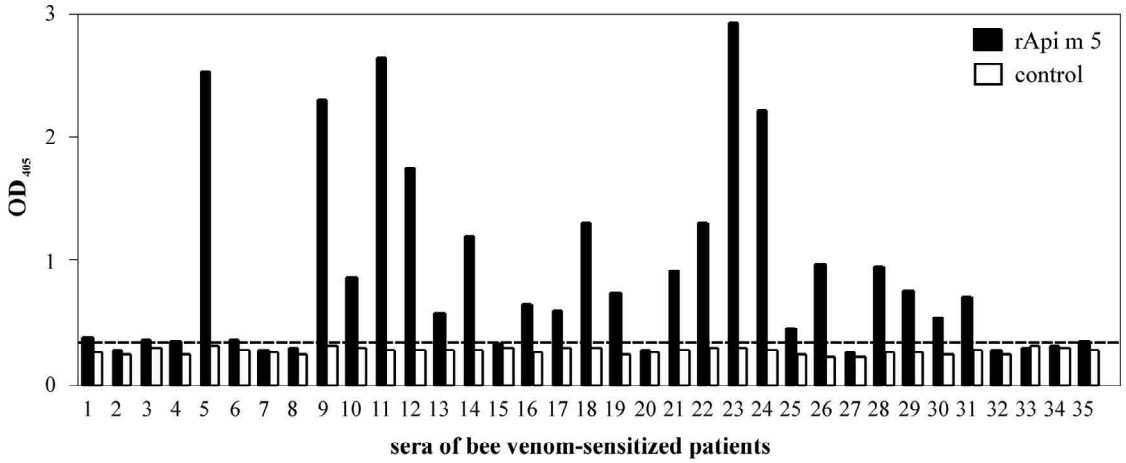

Fig. 17: Immunoreactivity of individual patient sera with recombinant Api m 5. The IgE reactivity was assessed by ELISA as described in *Materials and Methods* with sera of honeybee venom-sensitized patients (n=35). Controls were performed by omission of antigen. The lower end functional cut-off of the ELISA is represented by a dashed line.

In order to verify the obtained reactivity eukaryotically produced, posttranslationally modified, and enzymatically active Api m 5 was further compared with prokaryotically produced Api m 5 maltose binding protein (MBP) fusion protein in ELISA (Fig. 18). The fact that most reactive sera were also reactive with the prokaryotically produced counterpart corroborates the IgE reactivity of the insect cell produced protein and further supports the conclusion, that the recombinant high molecular weight protein exhibits IgE reactivity beyond CCD reactivity.

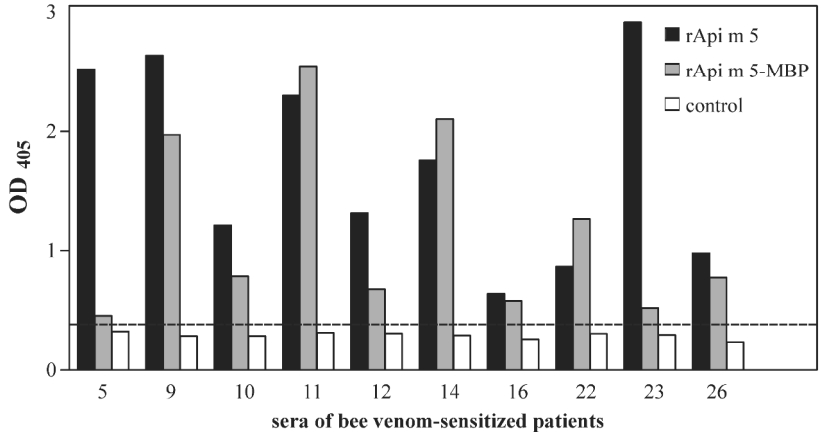

Fig. 18: Comparative analyses of the immunoreactivity of prokaryotically versus eukaryotically produced Api m 5. Immunoreactivity of individual patient sera with recombinant Api m 5 and recombinant Api m 5-MBP was assessed by ELISA as described in *Materials and Methods* using sera of honeybee venom-sensitized patients (exemplary n=10) found reactive in Fig. 17. Controls were performed by omission of antigen. The lower end functional cut-off of the ELISA is represented by a dashed line.

An initial analysis of cross-reactivity between Api m 5 and the homologous Ves v 3 from YJV using the sera from Fig. 17 showed 8/21 (38%) of the Api m 5-reactive sera to be cross-reactive with Ves v 3 (data not shown). To further substantiate the finding of serologic cross-reactivity of the DPPIV allergens in honeybee and vespid venom, another group of patients with clinically relevant allergy to yellow jacket venom (n=14) and a positive sIgE reactivity to Ves v 3 (n=11) was selected. 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. 19). 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≥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.

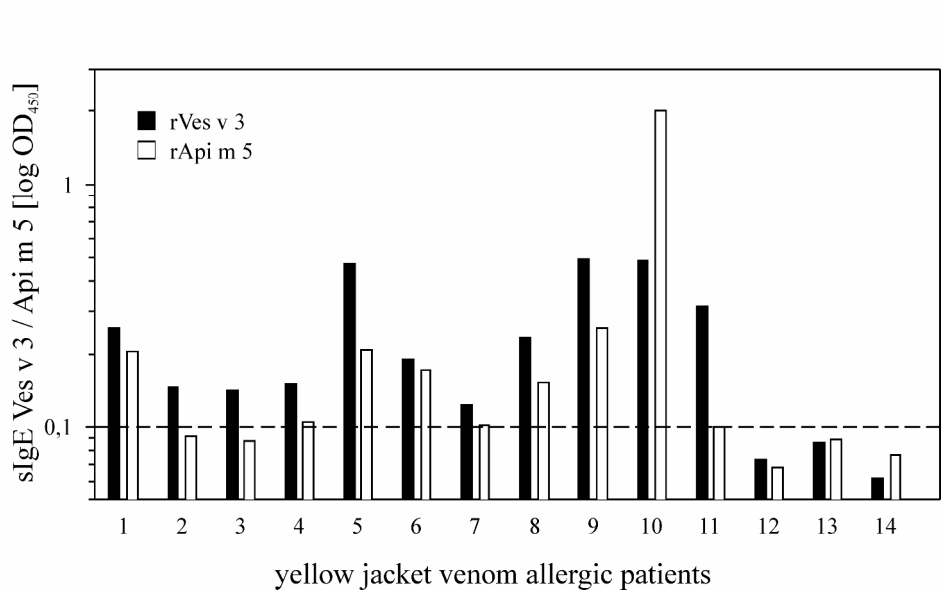

Fig. 19: IgE-based cross-immunoreactivity of Api m 5 and Ves v 3. IgE immunoreactivity of individual sera from patients with clinically relevant allergy to yellow jacket venom (n=14) and a positive (n=11; patient ID *1-11*) or negative (n=3; patient ID *12-14*) sIgE reactivity to Ves v 3 with recombinant 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 IgE reactivity was assessed by ELISA as described. The lower end cut-off of the ELISA is represented by a dashed line.

3.2.2.6 Activation of basophils from venom-allergic patients by Api m 5

The capability of Api m 5 as well as Ves v 3 produced in Sf9 cells for activation of human basophils, derived from a yellow jacket venom-allergic patient with a clinical history of an anaphylactic reaction after a stinging event (≥ grade 2), was assessed by a flow cytometry-based basophil activation test employing CD63 as activation marker. This patient also had a positive intradermal skin test and sIgE

test for YJV and HBV extract. In order to ensure a maximum of validity of the basophil activation test a broad concentration range was covered and the extent of basophil activation was related to that of known major allergens.

Basophil activation was analyzed 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. 20A, both Ves v 3 and Api m 5 lead to a robust basophil activation of up to 42% and 65%, respectively, 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. 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 sensitization to high molecular weight DPPIV allergens through yellow jacket venom Ves v 3 in this patient. The use of whole *A. mellifera* and *V. vulgaris* venom revealed clear-cut basophil activation only with vespid venom (Fig. 20B). Together, these data suggest that the high molecular weight venom allergens Api m 5 and Ves v 3 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.

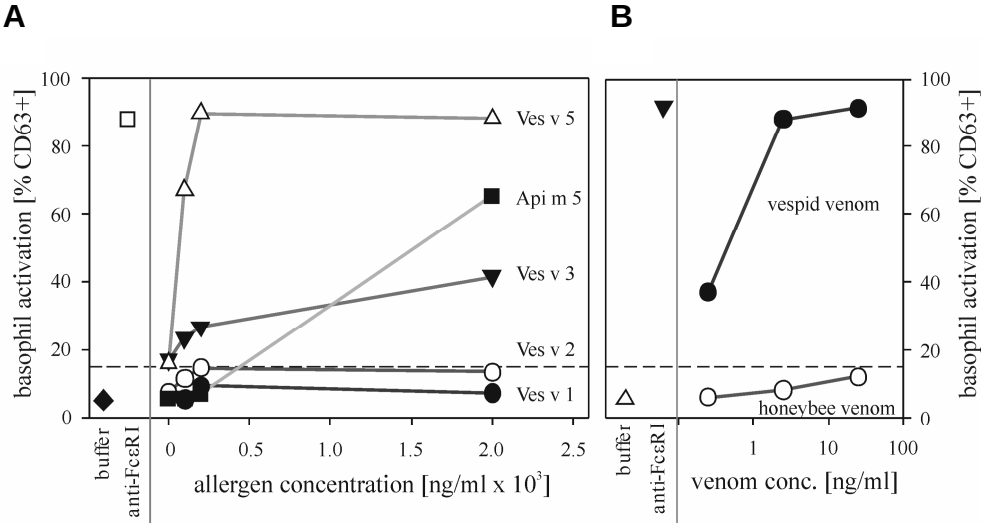

Fig. 20: Basophil activation tests with recombinant Api m 5 and Ves v 3. Human basophils from a YJV-sensitized patient were exposed to serial dilutions of rApi m 5, rVes v 3 (A), other *V. vulgaris* allergens (rVes v 1, rVes v 2, rVes v 5) (A) or whole venom (B) of *A. mellifera* or *V. vulgaris*. Incubations with a monoclonal anti-FcεRI or with plain stimulation buffer were used as positive or negative stimulation controls (A, B). Activation was assessed by flow cytometric analysis using anti-CD63 and anti-human IgE antibodies as described in the *Methods* section and is shown as percentage of CD63-positive cells. The dashed line represents the lower end functional cut-off of the assay (15% CD63+ cells).

3.2.2.7 DPPIV enzymes as important allergens of Hymenoptera venoms

In this work, the 100 kDa high molecular weight allergen in the venom of *A. mellifera* was identified. Using advanced sequencing strategies to overcome quantity limitations, detrimental abundance of the major component in honeybee venom, the cytolytic peptide melittin (55% of dry venom mass), and potential N-terminal modifications sequence information of enriched Api m 5 was obtained, allowing an assignment to a predicted open reading frame on the basis of available genomic sequence information. Finally, the full length cDNA providing an alternative N-terminal exon could successfully be amplified from venom gland cDNA. The cDNA of another honeybee venom protein of 94 kDa recently proposed to correspond to allergen C[215] could not be amplified from venom gland cDNA. Moreover, Api m 5 is reported to exhibit an apparent molecular weight ranging between 102 kDa[211] and 105 kDa[95]. The dipeptidylpeptidase IV (Ves v 3) from YJV corresponds to Api m 5 regarding molecular weight, amino acid sequence, enzymatic function, IgE immunoreactivity, and functional allergenic capability using basophils from venom-allergic patients.

Insect cells appeared to be the most appropriate system for expression of the putative Api m 5. In contrast to mammalian systems insect cells most likely will provide a similar glycosylation as found in the natural isoforms, a fact which is supported by the apparent molecular mass of the expressed recombinant allergen[216]. 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[217]. This monoclonal anti-Api m 5 IgE antibody reacted to a similar extent with the natural venom isoforms and the insect cell-expressed isoforms of Api m 5 and Ves v 3, suggesting the presence of a conserved protein epitope in Ves v 3 and Api m 5. 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 in immunoblot and ELISA analyses with IgE from >50% of honeybee venom-sensitized patients demonstrated the general presence of human IgE epitopes on Api m 5.

Furthermore this work supports relevance of Api m 5 in venom allergy beyond CCD reactivity by the fact that Sf9 insect cells were employed for production of the enzymatically active recombinant protein. Sf9 cells are considered to exhibit significantly reduced α-1,3-core fucosyltransferase activity[218,219] resulting in proteins

without or with only minute amounts of CCDs. Using the Sf9 produced allergen, more than 50% of not preselected honeybee venom sIgE-positive sera were reactive with recombinant Api m 5, thus rendering it an allergen containing proteinic IgE epitopes with clinical relevance. Additional evidence in this direction is derived from the fact that even prokaryotically expressed Api m 5 devoid of both glycosylation and, most likely, proper folding exhibited significant IgE reactivity with most patient sera found reactive with the eukaryotically produced Api m 5. 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.

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 Api m 5 and Ves v 3. The basophil activation pattern closely matched the serologic sIgE reactivity of the patient. 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[220-222].

Moreover, these data provide for the first time evidence for both serologic and cellular sIgE cross-reactivity between Api m 5 and Ves v 3 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 CCDs[143,223]. With the identification, characterization, and recombinant expression of Api m 5 and Ves v 3, a new pair of cross-reactive homologous allergens becomes available for future clinical applications in diagnosis and therapy.

Sequence analysis and characterization of the enzymatic activities provide clear evidence that both enzymes 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. Human DPPIV is a membrane-anchored 110-kDa serine protease expressed on various cell types[224-226]. 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[227,228]. Therefore, DPPIV shows a variety of functions including regulation of inflammatory and immunological responses, signal transduction, and apoptosis by degrading physiological substances such as chorionic gonadotropin and substance P, TNF-α, IL-2, and various chemokines including CCL5 (RANTES)[226,229,230]. Originally characterized as a T-cell differentiation antigen (CD26) human DPPIV plays a role in tumor progression such as cell adhesion, invasion, and cell cycle arrest[231-234]. Interestingly, soluble DPPIV activity is found in human serum and supposed to exert similar effects[235].

DPPIV activity has also been reported in snake venoms of different species[236] 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[237], nevertheless, neither in *A. mellifera* nor *V. vulgaris* 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*[237]. Given a relative protein content of 1% Api m 5 in native honeybee venom and an amount of 50-140 µg protein delivered per honeybee sting[63], 0.5-1.5 µg of DPPIV are injected into the skin per stinging event. However, further studies will be necessary to prove whether the amounts of enzyme injected are actually sufficient to modulate local immune responses in humans.

The characterization of DPPIV homologues may provide further insights into potential immunomodulatory functions of different hymenoptera venoms. Additionally, the results of this work demonstrate for the first time the clinical relevance of Api m 5 as high molecular weight allergen in IgE-mediated hymenoptera venom allergy. The recombinant Api m 5 will represent a valuable tool for the improvement of current diagnostic tests and immunotherapy of insect venom allergy.

4. Summary and discussion

Allergic reactions to Hymenoptera stings are one of the major causes of IgE-mediated anaphylaxis. According to epidemiologic studies in Europe, the United States and Australia, between 1 and 4% of an unselected adult population report systemic reactions to stings by honeybees or vespids[70,238]. Since the introduction of Hymenoptera venoms instead of the previously used whole body extracts in the diagnosis and treatment of this allergy in the late 1970s[187,239,240], allergy to stinging insects is often considered as a model for allergic disease and for immunotherapy of IgE-mediated allergy[241]. However, the specificity of the main diagnostic tests, skin tests and venom-specific IgE antibodies, is far from perfect and both efficacy and tolerance, especially in patients receiving honeybee venom (HBV) immunotherapy, are still suboptimal[138]. Up to 20% of individuals with no history of systemic sting reactions have positive tests. On the other hand, only 30-50% of those with positive tests will react to a subsequent sting by the respective insect[134]. According to a sting provocation test during venom immunotherapy the complete protection rate is around 95% for patients allergic to vespid stings but only 80-90% for those allergic to honeybee venom[134,193]. Systemic allergic side-effects to immunotherapy injections may occur in 20-40% of patients during immunotherapy with honeybee venom and in 5-10% during immunotherapy with vespid venoms[193].

Thus, there is considerable interest of improving both diagnosis and treatment of Hymenoptera venom allergy. Aim of this work was the establishment and evaluation of recombinant technologies contributing to this objective. The first important topic that needs to be addressed for the rational design of recombinant allergen-based diagnostic tests and therapeutics is the selection of the important allergens. Selection criteria may include the frequency of sensitization, the clinical relevance and the extent of IgE response. Since decades the high abundance compounds of honeybee and yellow jacket venom (YJV) are considered as allergens, mostly based on their IgE reactivity only. In contrast, little is known about lower abundance compounds regarding their relevance for sensitization and allergic potential.

4.1 Recombinant expression of Hymenoptera venom allergens

Prior to the development of optimized diagnosis and therapy the true clinical relevance of an individual allergen has to be determined. Recombinant allergens are clearly superior to highly purified natural preparations since it was coherently shown by inhibition studies that even highly purified allergens contain trace amounts of other venom allergens[138], interfering in diagnostic assays[242,243].

The clinical studies performed with recombinant wild-type allergens (*Phleum pratense*; Bet v 1)[244,245] and genetically modified hypoallergenic allergen variants (Bet v 1)[246] to date indicate that these molecules can be used for immunotherapy for allergen sources containing one predominant allergen as well as for complex allergen sources, provided that all relevant allergens have been identified and included in the vaccine.

Our data show that protein expression still is a very empirical process. Interestingly, in the case of Carbohydrate-rich protein our findings for aglycosylated protein matched those of the glycosylated protein, but, in stark contrast, the high molecular weight proteins Api m 5 and Ves v 3 which could also be expressed as soluble fusion proteins in *E. coli* showed no enzymatic activity and decreased IgE reactivity when compared to insect cell produced protein.

Hence, for every new allergen it should be determined which expression system is the best choice with regard to the need for and character of posttranslational modifications. Since most of the IgE binding epitopes are considered conformational, recombinant allergens expressed in eukaryotic systems such as yeast or insect cells should preferably be used for diagnostic approaches[247].

However, to date even the recombinant availability of well established major allergens of honeybee and vespid venom is still limited. The mayor honeybee venom allergen phospholipase A2 (Api m 1) was expressed in *E. coli* and biologic properties found to be comparable to that of natural purified Api m 1[199,248]. The enzymatic activity of purified and refolded recombinant Api m 1 was similar to that of natural purified Api m 1. For antigen 5 (Ves v 5) from vespid venom it was shown that folding of the bacterial preparation was incorrect and IgE binding avoided[249]. After application of refolding procedures it was possible to obtain Ves v 5 from *E. coli* with an IgE reactivity similar to that of the natural purified allergen[250]. Identically, recombinant bee venom hyaluronidase (Api m 2), a 45 kDa enzyme, was first expressed in *E. coli*[202]. The enzymatic activity of this preparation was, however,

clearly inferior and accounted for only 30% of that of natural purified allergen. Likewise, the IgE binding capacity was strongly reduced compared to natural Api m 2.
In contrast, use of the Baculovirus-based expression system resulted in a preparation with an enzymatic activity and IgE binding capacity similar to that of natural Api m 2[202], as found by us in the case of Api m 5 and Ves v 3. These observations indicate that in individual allergens post-translational modifications may be essential for the correct three-dimensional conformation of the molecule, its biologic activity and the correct conformation of its B cell epitopes. The crystal structures of Api m 1, Api m 2 and synthetic Api m 4 and Ves v 5 have been elucidated[203,204,251,252]. Phospholipase A1 (Ves v 1) and hyaluronidase (Ves v 2a) from vespid venom have so far only been expressed in prokaryotic systems[104].
In this work we have generated a panel of the most prominent honeybee and yellow jacket venom allergens, produced in baculovirus-infected insect cells as well in *E. coli*, including Api m 2, Ves v 2a, Ves v 2b (Seismann, Blank *et al.*, 2009, in press), Api m 1, Api m 3, Api m 6 and Api m 7. Additionally, we were able to identify and recombinantly express carboxylesterase (Api m 8) and carboxypeptidase (Api m 9) as well as other novel proteins from honeybee and yellow jacket venom, the relevance of which has to be further investigated (see Table 2 for an overview of the actual state of recombinant allergens produced by us; unpublished data). The insect cell-based expression of this entire set of hymenoptera venom allergens will for the first time allow for development of comprehensive component-resolved diagnostic approaches as well as safer and more efficacious treatment modalities.
Especially for the preparation of defined hypoallergenic allergen derivatives, the recombinant availability of allergens is imperative and offers considerable advantage over the traditional chemical modifications. The concept of allergen modification postulates that allergens can be modified in a way to reduce or even destroy IgE binding B cell epitopes, which are responsible for allergic side-effects, while linear T cell epitopes mediating protective immunity are preserved. Different ways of allergen modification for venom immunotherapy have been proposed. While the results of chemical modifications due to a lack of reproducibility of difficult to control chemical modifications of allergen extracts were not entirely convincing[253], recent studies with T cell epitope peptides from the major bee venom allergen phospholipase A2 (Api m 1) look promising[254].

Allergen	Common name	Insect cell expression	*E. coli* expression	Antibody
Api m 1	Phospholipase A2	+	+	+
Api m 2	Hyaluronidase	+	+	+
Api m 3	Acid phosphatase	+	+	+
Api m 5	Dipeptidylpeptidase IV	+	+	+
Api m 6	putative protease inhibitor	+	+	
Api m 7	CUB-serine protease	+		
Api m 8	Carboxylesterase	+	+	
Api m 9	Carboxypeptidase	+	+	
Api m 10	Carbohydrate-rich protein	+	+	+
Ves v 1	Phospholipase A1B	+	+	
Ves v 2a	Hyaluronidase	+	+	+
Ves v 2b	Hyaluronidase	+		+
Ves v 3	Dipeptidylpeptidase IV	+	+	+
Ves v 4	CUB-serine protease	+	+	+

Table 2: Overview of recombinantly expressed Hymenoptera venom allergens and allergen-specific antibodies

The availability of recombinant allergens has to be considered an improvement in this field because genetic information is the basis for a rational design of hypoallergenic variants. Conformational epitopes have been shown to be strongly reduced in unfolded recombinant allergens[248,250] and can also be destroyed by point mutations in B cell epitopes[255]. Such preparations, in which all relevant T cell epitopes of the allergen are preserved since they are linear, will have a strongly reduced IgE reactivity, and will, therefore, induce far less mediator release, and will be better tolerated. T cell epitope peptides could also be expressed as recombinant fragments and used for immunotherapy[256]. Nevertheless, such studies also showed that T cell peptides from all allergens to which the patient is sensitized seems to be required in order to achieve complete protection by peptide immunotherapy.

Yet another experimental strategy for immunotherapy is DNA vaccination, consisting of the injection of DNA plasmids encoding the relevant allergens. In contrast to environmental allergen exposure and to classical immunotherapy, this kind of vaccination induces T_H1 responses[257]. Many Hymenoptera venom-allergic patients are sensitized to several different venom allergens, thus treatment with one major allergen in recombinant form may be insufficient. One elegant solution for this has recently been presented by the production of a chimeric protein consisting of two

fragments each of Api m 1, Api m 2 and Api m 4. The fragments were designed in a way to preserve all relevant T cell epitopes while conformational B cell epitopes were destroyed. The molecule induced strong proliferation in lymphocyte cultures from bee venom-allergic patients, but did not react with specific IgE, nor did it induce mediator release from blood basophils[258].

4.2 Identification of Hymenoptera venom allergens

In order to contribute to a more detailed knowledge of the composition of Hymenoptera venoms and of the allergic potential of each component in this work the identification and molecular cloning of the high molecular weight allergen Api m 5 is reported. The expression of the genes of Api m 5 and its homologue from yellow jacket venom in insect cells, and the biochemical and immunological characterization of the purified recombinant molecules is demonstrated. Api m 5 or allergen C is a 100 kDa protein of *Apis mellifera* venom with pronounced IgE reactivity. Since decades Api m 5 was supposed a major allergen[95], but, although present in substantial concentrations, identity and function of this allergen defied elucidation. We employed MS-MS-based strategies for the identification of Api m 5 and used the obtained sequence information to scan the published honeybee genome[259]. The cDNA of Api m 5 could be amplified from honeybee venom glands. Since venoms of both *A. mellifera* and *V. vulgaris* contain a prominent 100 kDa band detected by sIgE of sera of sensitized patients we aimed for identification of the Api m 5 homologue in *V. ssp.*. Based on sequence information of Api m 5 we were then able to identify and clone the homologous protein from *Vespula vulgaris* venom, Ves v 3, as new allergen applying homology- and RACE-based approaches. Subsequently, both proteins were expressed in insect cells and the purified proteins further biochemically and immunologically characterized.

Sequence analysis and characterization of the enzymatic activities provided clear evidence that Api m 5 and Ves v 3 belong to the class of dipeptidylpeptidase IV (DPPIV) enzymes, a protease family with important implications for the regulation of signaling by peptide hormones. Identity to human DPPIV (CD26) is in the range of 32%. The accordant enzymatic activity and molecular weight of recombinant Api m 5 and Ves v 3 expressed in insect cells further hinted for correct folding and proper posttranslational modifications. The presence of DPPIV enzymes in Hymenoptera venoms may shed light on molecular mechanisms of insect venom

allergy and potential physiological and pathophysiological implications. 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 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*[260]. Further studies will be necessary whether the amounts of enzyme injected are actually sufficient to modulate local immune responses in humans.

Moreover, analysis of recombinant allergens revealed a pronounced reactivity with a majority of sera of hymenoptera venom-sensitized patients. Using the allergens expressed in Sf9 insect cells more than 50% of not preselected honeybee or yellow jacket venom sIgE-positive sera were reactive with recombinant Api m 5 or Ves v 3, respectively, thus rendering both 100 kDa proteins major allergens containing proteinic IgE epitopes with clinical relevance. Moreover, an initial analysis revealed serologic cross-reactivity between the DPPIV allergens Api m 5 and Ves v 3, which show an identity of 53% on protein level. The capability of recombinant Ves v 3 and Api m 5 for activation of human basophils derived from yellow jacket venom allergic patients with history of an anaphylactic reaction after a stinging event was shown employing CD63 as activation marker in flow cytometry, underlining the clinical relevance of these two new allergens. Each patient with a positive sIgE against Ves v 3 in serum also demonstrated positive basophil activation through this allergen and also by Api m 5, although less pronounced, most likely reflecting primary sensitization to vespid venom.

With Api m 5 and Ves v 3, a new pair of cross-reactive homologous allergens has become available for future clinical applications in diagnosis and therapy which may also contribute to the understanding of the molecular mechanisms of insect venoms. Moreover, the pronounced patient IgE reactivity demonstrated for the first time the relevance of high molecular weight allergens in the context of hymenoptera venom allergy. These findings provide a novel view on the molecular patterns of allergic IgE sensitization in venom allergy which requires reconsideration of current and future concepts for component-resolved diagnosis and specific immunotherapy of hymenoptera venom allergy.

“Component-resolved diagnosis” (CRD) is the concept of using separate allergens to determine the patients sensitization profile. Originally aimed for providing the basis for patient-tailored forms of immunotherapy[261], this approach was found to have several other advantages related to the diagnostic test requirements (little amounts of serum in protein microarrays), performances (sensitivity, specifivity), standardization (concentration, structural integrity, batch-to-batch variation), and interpretation (risk likelihood and severity of allergic reactions)[262]. However, further progress leading to such an approach lies in the completion of the repertoire of recombinant allergens. The development of an artificial recombinant Hymenoptera venom preparation for clinical practice is essentially based on an in depth knowledge of the allergic components that exist in natural venom in order to refine the composition of the recombinant cocktail.

Nevertheless, Hymenoptera venoms comprise a more complex cocktail of a variety of components all of which may contribute to sensitization, allergic outcome and success of venom immunotherapy. Nowadays it is also remarkable that the biological function of some of the newly discovered low abundant venom constituents do not correspond necessarily with the principal function of venom as defense weapon, as for instance for DPPIV that has possibly no function once injected into the victim. It increasingly becomes clear, primarily by proteomic approaches, that there are various “venom trace elements” present, occurring in comparably low quantities and may have only a local function in the venom duct or reservoir or which are normal cell components, released by leakage from the gland tissue. Nevertheless, the case of Api m 5 and Ves v 3 demonstrated inter alia in cellular assays that such components might be immunologically highly relevant.

4.3 Characterization and Evaluation of Hymenoptera venom allergens

In this work Carbohydrate-rich protein, a recently identified *A. mellifera* venom component initially described as allergen, was comparatively assessed, with special focus on the relevance of glycosylation and cross-reactive carbohydrate determinants in order to identify new venom components with allergic potential. Evaluation of the allergological relevance of venom proteins is mainly based on the reactivity with IgE of venom-sensitized individuals rendering low abundance components difficult to address. A recombinant approach can facilitate the assessment of such proteins but should meet the requirements of proper folding, if

possible enzymatic activity, and correct posttranslational modifications all of which are potentially important for establishment of conformational epitopes[202]. In particular carbohydrates can contribute significantly to biochemical and structural characteristics of venom proteins.

Carbohydrate-rich protein was identified by two independent groups in 2005[206,207] and insoluble protein obtained by recombinant production in *E. coli* exhibited IgE reactivity in 4 out of 5 sera of beekeepers with HBV allergy[100]. We describe the comparative production of Carbohydrate-rich protein in soluble, non-glycosylated form in *E. coli* and as fully glycosylated protein in different baculovirus-infected insect cell lines providing a varying degree of α-1,3-core fucosylation, the hallmark of cross-reactive carbohydrate determinants (CCDs). Applying the differently glycosylated recombinant molecules we were able to show that the authentically and the non-glycosylated protein reacted with IgE antibodies of approximately 50% of honeybee-venom sensitized individuals. Beyond CCD reactivity glycosylation appears to have only a minor impact on the IgE reactivity. This might hint for at least the presence of epitopes that are not affected by structural rearrangements due to glycosylation. Moreover, we showed a lack of reactivity with sera of YJV-sensitized patients suggesting the absence of a highly homologous, cross-reactive structure in vespid venom. This would render Carbohydrate-rich protein a novel surrogate marker for HBV-sensitization. Together our results suggest an important role as sensitizing component in HBV beyond its carbohydrate-based cross-reactivity for the low abundance component Carbohydrate-rich protein.

4.4 Evaluation of diagnostic concepts for Hymenoptera venom allergy

Diagnosis of Hymenoptera venom allergy is not only hampered on the level of component-resolution. Although an increasing availability of recombinant allergens will improve the dissection of individual IgE reactivities on this level, allergenic cross-reactivity, a major handicap for accurate diagnosis in venom allergy, remains to be solved. Generally, physicians have to rely on quantification of specific IgE antibodies and skin tests to diagnose venom allergy. Unfortunately, these tests lack sensitivity and specificity, making the diagnosis of Hymenoptera venom allergy not always easy[263]. Indeed, up to 50% of diagnostic test results suggest double-positivity to both bee and vespid venoms. This can be explained by either true double sensitization if the patient was stung by both insects, or cross-

reactivity of allergens of the two venoms, particularly between the carbohydrate epitopes they share[142]. In contrast, the small portion of patients showing adverse reactions to both venoms implies cross-reactions due to cross-reactive carbohydrate determinants (CCDs), as reported for 75% of double-positive patients[205], and a limited clinical relevance of glycans epitopes. As the patient cannot always provide the entomologic identification of the culprit insect, it sometimes remains obscure which life-saving VIT should be initiated. Promising *in vitro* test methods based on the venom-specific stimulation of basophils are increasingly introduced, and pushed the sensitivity and specificity of the diagnostic tools[264,265]. However, serologic as well as effector cell-based diagnosis of venom allergy is currently performed with whole venom preparations, containing other non-allergenic components in addition to allergens. At the best, current diagnosis of bee or yellow jacket venom allergy only permits the identification of a given allergen source, but not the molecular entities involved in the adverse immunological reactions.

As general strategy to address allergenic cross-reactivity in the study **"Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation"** (Seismann, Blank *et al.*, 2009, Mol Immunol, in press) we used a recombinant approach by employing cell lines with variant capacities of α-1,3-core fucosylation, the hallmark of CCDs, in order to establish and evaluate novel strategies providing properly folded recombinant allergens for an improved diagnosis of Hymenoptera venom allergy. 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 isoforms Ves v 2b) venom as well as the human α-2HS-glycoprotein as control, all of them containing at least two glycosylation sites, were produced in different insect cell lines. We were able to show, that in stark contrast to production in *Trichoplusia ni* cells, α-1,3-core fucosylation was absent or immunologically negligible after production in *Spodoptera frugiperda* cells. Moreover, overexpression of the *A. mellifera* α-1,3-core fucosyltransferase in *S. frugiperda* cells led to restoration of the fucose-dependent epitope, demonstrating that CCD reactivity is exclusively dependent on α-1,3-core fucosylation and that the glycans added by *S. frugiperda* cells differ from those added by *T. ni* cells primarily with respect to α-1,3-core fucosylation. Proper folding of enzymes expressed in both cell lines could be demonstrated by measurement of hyaluronidase activity of Api m 2 and Ves v 2a. Re-evaluation of

the differently fucosylated, properly folded hyaluronidases by screening of individual venom-sensitized sera emphasized the allergenic relevance of Api m 2 beyond its carbohydrate epitopes. In contrast, the vespid hyaluronidases, for which a predominance of Ves v 2b in the venom could be demonstrated for the first time by the use of specific antisera, exhibited pronounced and primary carbohydrate reactivity rendering their relevance in the context of allergy questionable.
Together, these data clearly suggest that the recombinant production in *S. frugiperda* vs. *T. ni* cells provides a novel concept for the reliable analysis of protein vs. CCD reactivities. Recombinant allergens with tailor-made CCD reactivity may enable differentiation of true sensitization with clinical impact from mere CCD-based cross-reactivity. Such strategies should therefore have an impact on the identification of clinically relevant allergens, proper allergy diagnosis and design of adequate intervention strategies.

In summary, these data show that the use of defined recombinant major allergens provides a significant improvement for the identification of the culprit venom which is indispensable for the choice of the appropriate immunotherapeutic strategy.

The application of recombinant DNA technology to allergen characterization has revealed the molecular nature of many important allergens and has advanced the characterization of their immunological and structural features[266]. On the basis of this work, it will become possible to modify important allergens in ways simultaneously reducing allergenic activity and preserving relevant T cell epitopes and structures that are necessary for the induction of antibody responses[267]. Finally, recombinant allergens provide increased specificity for diagnostic testing[247] in both skin testing[248] and in determining venom-specific IgE antibodies[242] as well as a good performance in cellular assays[268] when compared to natural venom allergens. By the use of all relevant recombinant venom allergens, specificity of up to 100% should be possible enabling replacement of natural extracts for *in vitro* and *in vivo* diagnosis by such a recombinant cocktail in the future[247].

4.5 Generation of recombinant allergen-specific antibody formats

Imperative for an improvement of diagnostic and theraupeutic approaches as well as for the understanding of the molecular mechanisms of allergic reactions and immunotherapy are not only defined recombinant allergen molecules, the counterpart to the environmental allergen as elicitor of the allergic reaction are the individual's allergen specific antibodies.

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 will be useful in allergy research and diagnostic tests. As mentioned, reliable determination of allergen-specific serum IgE or IgG4 antibodies is limited by the fact that human IgE antibody pools are not standardized and cannot be reproducibly prepared. Standardization will likely become more important with the use of recombinant allergens for advanced diagnostic interventions. Due to the scarcity of IgE producing cells approaches of generating human allergen-specific IgE secreting hybridomas from immunized donors have not been successful[269]. The unavailability of monoclonal IgG and IgE antibodies has thus far prohibited detailed analyses of their characteristics in pathophysiology as well as their molecular interplay.

In the study **"Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries"** (Braren *et al*., Clinical Chemistry 53:837-844, 2007) we established the generation of reproducible allergen-specific antibodies for the most relevant isotypes for allergy diagnosis and research to overcome such limitations. As a model system we generated fully human IgE, IgG4, and IgG1 antibodies with defined specificity for 3 different allergens (Api m 1 from honeybee venom, Bos d 5 from bovine milk, and Mal d 1 from apple) and assessed their biochemical properties by different immunological assays. The combinatorial selection of a human synthetic antibody library yielded antibody fragments with specificity for these allergens, which were then converted by recombinant antibody technology into different formats of fully human monoclonal allergen-specific IgE, IgG1 and IgG4 antibodies. Expression of homodimeric or heterotetrameric recombinant antibodies was performed in HEK293 cells as a human system. The immunoreactivity of these antibodies in direct ELISA when added to nonallergic human serum and their reactivity in commercial immunoblots

demonstrated their potential use in different types of immunological and allergy diagnostic methods. The analyses of IgE binding to recombinant high-affinity receptor FcεRI also demonstrated compatibility of the recombinant proteins with cellular assay systems and effector functions. Moreover, allergen binding to FcεRI could be blocked by IgG antibodies of the same specificity, a situation thought to mimic the situation after affinity maturation of IgG antibodies in patients undergoing SIT. In summary, this study demonstrated that synthetic libraries of human origin can quickly provide reactive antibody fragments against broad panels of available allergens which can easily converted into different monoclonal antibody formats of various isotype, capable of forming functional allergen/IgE/IgE-receptor-complexes. Such reagents should have broad application in allergological research and diagnosis. The use of such reliable and precise reagents may help to aid in the standardization of allergen-specific diagnostic immunoassays and to minimize interassay variance. In addition, such defined antibodies are attractive tools for basic and applied research to evaluate the complex molecular interplay of allergens, different allergen-specific antibodies and Fc receptors to better understand modulation of the allergic reaction. But also therapeutic applications like passive or adjuvant immunotherapy are thinkable[270].

Such defined monoclonal regents were further refined for their application in a broad range of immunological assays in the study **“Recombinant IgY for improvement of immunoglobulin-based analytical applications”** (Greunke, Braren *et al*., Clinical Biochemistry 41:1237-1244, 2008). The aim of this study was to provide superior tools for diagnostic approaches preventing assay interference and background binding by the use of monoclonal IgY antibodies which have been unavailable so far. IgY are the major low molecular weight serum immunoglobulins in oviparous animals[271] and, thus, phylogenetically distant from mammalian immunoglobulins. Interference in immunoassays is increasingly recognized as a major diagnostic problem[272,273]. Mammalian antibodies can affect immunoassays by cross-reactivity and non-specific binding. Furthermore, antigen-independent binding via specific immunoglobulin receptors and serum immunoglobulins is causative for false-positive and false-negative results in different diagnostic approaches. The use of polyclonal IgY, especially for detection of molecules from sources like blood or serum, provides minor background due to the lack

of interaction with heterophilic antibodies, rheumatoid factor (RF), human anti-mouse antibodies (HAMA) and complement components[274,275], a major problem reported to affect for instance the tryptase immunoassay, recommended to perform in patients with suspected mastocytosis, a risk factor for anaphylaxis[148].
In this study we evaluated recombinant monoclenal IgY-based antibodies regarding their performance in diagnostic assay formats in comparison to human or murine IgG analogues. We found that monoclonal recombinant IgY entirely reflected the characteristics of their native counterparts and, therefore, provide the same advantageous properties. The use of monoclonal IgY in contrast to mammalian antibodies prevented interference phenomena in absorbance measurements generated by human sera containing RF or heterophilic antibodies. Additionally, they exhibited no interactions with the human and murine high-affinity receptor FcγRI (CD64) and human low-affinity receptor FcγRIIIa (CD16A). The data obtained demonstrate the advantageous behavior of monoclonal IgY as detection or capture antibodies compared to conventional mammalian immunoglobulins in avoidance of assay interference, thus rendering IgY-based constructs valuable tools for all types of immunoassays.

After establishment of such recombinant antibody technologies in the context of allergen-specific antibodies, we were able to show the advantages of such reagents in basic research on venom components and their potential for standardization of allergen extracts.
In this work a monoclonal IgE antibody directed against Api m 5, generated by using the same methods as described above was used, to confirm the identity of the newly identified open reading frame with the component in *Apis mellifera* venom. Moreover, the reactivity of this monoclonal IgE antibody with the homologous protein of yellow jacket venom, Ves v 3, suggested the presence of a conserved protein epitope in these two molecules. Such an epitope hints to the possible occurrence of cross-reactive protein epitope-specific IgE in venom-allergic patients, also demonstrated by specific IgE reactivity of allergic patients and basophil activation.
The quality of natural allergen extracts has improved over the years through increased standardization and characterization, however, products from natural sources are often heterogeneous and may contain many non-allergenic mole-

cules, which can also vary in composition and quantity[266,276], and certain of which have shown to prime T_H2 responses[277] may even contain contaminating allergens from other sources[278]. Another potentially important problem related to unpredictable complexity of allergen extracts is that therapy-induced new IgE reactivities towards extract components that were not recognized prior to therapy can arise[173,279]. On the other hand, in many cases important allergens are present in small amounts or lacking, und their biological potency is subject to broad variability[280]; e.g. the presence of bioactive molecules like proteolytic enzymes that degrade allergens would be a limiting factor for stability[281]. Due to these reasons, products from different companies and even batches from the same company are not necessarily comparable and strongly vary in their composition[179], likely hampering success of specific immunotherapy.

A particular finding emphasizing this problem is described in this work. We generated a monoclonal human IgE antibody with specificity for Carbohydrate-rich protein applying the above described methods and recombinant Carbohydrate-rich protein. Using this monoclonal IgE we were able for the first time to detect native Carbohydrate-rich protein in whole Apis mellifera venom. Additionally we could show that Carbohydrate-rich protein is present primarily as intact component in significant amounts comparable to other allergens of relevance such as Api m 3. Analyzing three different honeybee venom preparations routinely used for venom immunotherapy we interestingly obtained no reactivities for none of the preparations with the monoclonal anti-Carbohydrate-rich protein IgE, in stark contrast to the crude venom, demonstrating absence of this putatively essential component in therapeutical preparations. These data confirm that downstream processing of venom for therapeutic preparations at least affects the distribution of venom proteins, and underline the need for reagents for standardization of such preparations. Although these data need further and broader validation, the presence of inherently labile venom components should be imperative and might serve as proof of quality. With regard to such standardization purposes the use of monoclonal antibodies, as shown here, may open novel prospects to improvement and quality control of therapeutics. Hence, the establishment of a panel of recombinant monoclonal antibodies with specificity for various important Hymenoptera venom allergens will become valuable for further applications in both standardization and basic research (Table 2).

4.6 Outlook

Recombinant technologies have opened a wide range of possibilities to improve the diagnosis and treatment of Hymenoptera venom allergy. Once all relevant allergens of a venom will be available in recombinant form, the sensitization pattern of an individual patient could exactly be determined by estimating specific IgE antibodies to all of them. A patient-tailored cocktail containing the allergens the patient shows IgE reactivity to could then be tailored for immunotherapy[282]. Any of the problems associated with allergen extracts can be easily overcome with recombinant allergens, for instance, vaccines could be formulated containing molecules defined on the basis of mass units without irrelevant components.

Moreover, recombinant allergens can be produced as molecules that exactly mimic natural allergens, as modified variants with advantageous properties such as reduced allergenic activity or increased immunogenicity, or as hybrid molecules resembling the entity of epitopes of several different allergens to include the relevant epitopes of complex allergen sources[283,284].

With the use of defined recombinant molecules instead of crude allergen extract-based mixtures, it will become possible to decipher more precisely the mechanisms underlying immunotherapy, to develop new forms of immunotherapy and perhaps prophylactic strategies as well as to monitor its success, allowing adaptation of the treatment strategy.

Furthermore, recombinant allergens combined with the corresponding monoclonal antibodies will contribute to a more detailed understanding of the molecular and allergological mechanisms of allergic disease including but not limited to Hymenoptera venom allergy.

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Curriculum Vitae

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Education

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Working experience

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Publication as first author

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. *Mol Immunol*, in press.

Other publications

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. *Clin Chem* **53(5)**: 837-844 (2007).

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Abstracts

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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)

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Conference contributions - oral presentations

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. 19. Mainzer Allergie-Workshop, Mainz, Germany, 16./17. March 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. 20. Mainzer Allergie-Workshop. Mainz, Germany, 7./8. March 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. XXVII Congress of the European Academy of Allergy and Clinical Immunology. Barcelona, Spain, 7.-11. June 2008.

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. XXVIII Congress of the European Academy of Allergology and Clinical Immunology. Warsaw, Poland, 6.-10. June 2009.

Conference contributions - posters

Blank, S., Bockisch, B., Bredehorst, R., Ollert, M.W., Grunwald, T. and Spillner, E. Identification, recombinant production and characterization of honeybee venom major allergen C (Api m 5). 2nd International Symposium on Molecular Allergology. Rome, Italy, 22.-24 April 2007.

Cifuentes, L., Blank, S., Vosseler, S., Grunwald, T., Mempel, M., Darsow, U., Ring, J., Bredehorst, R., Spillner, E. and Ollert, M. Insect venom allergy with negative venom-specific IgE: The use of alergenic molecules provides an improved diagnostic solution. 2nd International Symposium on Molecular Allergology. Rome, Italy, 22.-24 April 2007.

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.

Braren, I., Plum, M., Blank, S., Spillner, E. Adressing erbB1 by human and murine IgE. 2nd International AllergoOncology Symposium. Wien, Österreich, 11./12. April 2008.

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.

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). XXVII Congress of the European Academy of Allergy and Clinical Immunology. Barcelona, Spain, 7.-11. June 2008.

Ollert, M., Cifuentes, L., Blank, S., Grunwald, T., Darsow, U.G., Ring, J., Bredehorst, R., Spillner, E. The use of recombinant allergens provides improved solutions for patients with insect venom allergy. 27th Symposium of the Collegium Internationale Allergologicum. Curacao, Netherlands Antilles, 1.-6. Mai 2008.

Blank, S., Seismann, H., Braren, I., Greunke, K., Cifuentes, L., Ring, J., Grunwald, T., Bredehorst, R., Ollert, M., Spillner, E. A comprehensive set of hymenoptera venom allergens as tool for diagnosis and therapy. 6th Symposium on environmental Allergy And Allergotoxicology, Munich, Germany, 29./30. January 2009.

Prizes

Best abstract in the oral abstract session 5: Hymenoptera Venom Allergy. XXVII Congress of the European Academy of Allergy and Clinical Immunology. Barcelona, Spain, 7.-11. Juni 2008

Best abstract in the oral abstract session 14: Advances in Hymenoptera Venom Allergy. XXVIII Congress of the European Academy of Allergy and Clinical Immunology. Warsaw, Poland, 6.-10. June 2009

Gefahrstoffe und Sicherheitshinweise

Folgende verwendete Reagenzien und Lösungsmittel waren mit Gefahrenhinweisen und Sicherheitsratschlägen gemäß §6 der Gefahrstoffverordnung versehen. Krebserzeugende, erbgutverändernde und fortpflanzungsgefährdende Stoffe (KMR-Stoffe) der Kategorie I und II sind fett dargestellt:

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	O, Xn	8-22-36/37/38-42/43	22-24-26-37
Ampicillin	Xn	36/37/38-42/43	22-26-36/37
BCIP	-	-	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	-
Ethanol	F	11	7-16
Ethidiumbromid	**T+**	**22-26-36/37/38-40**	**26-28.2-36/37-45**
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 FF	Xi	36	24
Zitronensäure	Xi	36	24/25

Angaben zum individuellen Beitrag an den Publikationen, die in dieser kumulativen Dissertation enthalten sind

Erstautorenschaft:

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.
(Impact Factor 2008: 3,6)
Eigener Anteil: ca. 45% (Klonierung, Expression in verschiedenen Zelllinien und Reinigung von Api m 2 und AHSG, Charakterisierung von rekombinantem Api m 2 und AHSG, immunologische Charakterisierung der differentiell glykosylierten Proteine, Charakterisierung verschiedener Insektenzelllinien bezüglich der Fähigkeit zur alpha-1,3-Fucosylierung, Klonierung der Fucosyltransferase A aus *A. mellifera*, Etablierung der Zelllinie Sf9 FucTA, immunologische Charakterisierung der Zelllinie Sf9 FucTA, Expression in der etablierten Zelllinie Sf9 FucTA and Charakterisierung der rekombinanten Allergene, Textbeiträge Methoden, Ergebnisse und Diskussion)

Weitere Publikationen:

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. 40% (Generierung monoklonaler Antikörper mit Spezifität für Bos d 5 und Mal d 1, Klonierung der Antikörper ins IgE und IgG4 Format, Expression und Reinigung der Antikörper, Reaktivitätsanalyse der Antikörper, Stabilitätsanalyse der Antikörper, Klonierung des IgE-Rezeptors FcεRI, Generierung der stabilen Zelllinie HEK393 FcεRI, durchflusszytometrische Analyse der Zelllinie HEK 293 FcεRI)

Greunke, K., Braren, I., Alpers, I., **Blank, S.**, Sodenkamp, J., Bredehorst, R., and Spillner, E. Recombinant IgY for improvement of immunoglobulin-based analytical applications.
Clinical Biochemistry **41**:1237-1244 (2008)
(Impact Factor 2008: 1,9)
Eigener Anteil: ca. 30% (Klonierung humaner IgG Antikörper, Etablierung der Zelllinie HEK293 FcγRIA)

Prof. Dr. Reinhard Bredehorst

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Arbeit selbstständig und ohne fremde Hilfe sowie nur mit den angegebenen Hilfsmitteln und Quellen erstellt zu haben. Ergebnisse aus Diplom- oder Schwerpunktarbeiten, die in diesem Arbeitskreis angefertigt wurden und die teilweise in diese Arbeit eingeflossen sind, wurden von mir initiiert und unter meiner Anleitung angefertigt.

Ebenfalls versichere ich, noch keinen weiteren Promotionsversuch an einer anderen Einrichtung unternommen zu haben.

Hamburg, den 05.11.2009

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Simon Blank