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Short communication

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

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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 α -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 α -2HS-glycoprotein as control, were produced in different insect cell lines. In stark contrast to production in *Trichoplusia ni* (HighFive) cells, α -1,3-core fucosylation was absent or immunologically negligible after production in *Spodoptera frugiperda* (Sf9) cells. Consistently, co-expression of honeybee α -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 vespidae 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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1. Introduction

Hymenoptera stings may cause life-threatening and sometimes fatal IgE-mediated anaphylactic reactions in humans. Although venom immunotherapy is highly effective, systemic allergic side effects to injections have been observed in 20–40% of patients. According to sting challenge tests, 10–20% of patients were not protected by honeybee venom immunotherapy and continued to develop generalised allergic symptoms (Muller et al., 1992; Rueff

et al., 1996). Thus, there is considerable interest in improving diagnosis as well as design, safety and efficacy of therapy.

Although so far only a limited number of hymenoptera venom allergens are available as recombinant proteins (King and Spangfort, 2000; Muller, 2003) their use may improve existing strategies (Muller, 2002) by offering the potential for analyses on a molecular level beyond component resolution. The most prominent honeybee venom allergens include phospholipase A₂ (Api m 1), hyaluronidase (Api m 2), acid phosphatase (Api m 3) (King et al., 1976) and the basic 26 amino acid peptide Melittin (Api m 4) (Arbesman et al., 1976). Major yellow jacket allergens include phospholipase A₁ (Ves v 1), hyaluronidase (Ves v 2), and antigen 5 (Ves v 5) (King and Spangfort, 2000; Muller, 2002). Api m 1 and Api m 2 as well as Ves v 1, Ves v 2, and Ves v 5 could be expressed in bacteria, yeast or baculovirus-infected insect cells (Dudler et al., 1992; Gmachl and Kreil, 1993; Henriksen et al., 2001; Kuchler et al., 1989; Skov et al., 2006; Soldatova et al., 1998) and selected struc-

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tures were elucidated by X-ray crystallography (Markovic-Housley et al., 2000; Scott et al., 1990). Very recently, the acid phosphatase Api m 3 and the DPPIV enzymes Api m 5 and Ves v 3 were cloned and recombinantly produced (Blank et al., 2008, submitted for publication; Grunwald et al., 2006).

Although an increased availability of recombinant allergens will improve the dissection of individual IgE reactivities on a molecular level, allergenic cross-reactivity, a major handicap for accurate diagnosis in hymenoptera venom allergy, remains to be solved. Apart from true double sensitisation 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 (Aalberse et al., 2001). In general the N-glycans found on most hymenoptera venom 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. IgE with specificity for such glycotopes represents the underlying principle reactive with all proteins possessing CCDs (Aalberse et al., 1981). This has even led to the estimation that IgE binding to venom proteins with pronounced glycosylation may primarily or exclusively reflect CCD reactivities (Hemmer et al., 2004), 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 (Malandain, 2005), their diagnostic relevance is beyond any controversy. Identification of the culprit hymenoptera species that a patient is sensitised to remains key for proper diagnosis and for the selection of an appropriate therapeutic strategy. 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 sensitisation.

In this study, we report a molecular approach to assess the IgE reactivity to hyaluronidases Api m 2 and Ves v 2, currently recognised as the most relevant hymenoptera venom allergens displaying cross-reactivity. By exploiting both *Trichoplusia ni* and *Spodoptera frugiperda* insect cell lines, recombinant hyaluronidases and a suitable human reference protein, the high abundance serum component α -2HS-glycoprotein, could be obtained with authentic glycosylation while circumventing α -1,3-core fucose addition, which is the hallmark of CCDs. Evaluation of these proteins allowed for a detailed study of CCD-derived allergenic cross-reactivity and emphasised the potential of custom-tailored recombinant allergens for improvement of diagnostic and therapeutic strategies.

2. Methods

2.1. Materials

Whole honeybee venom was purchased from Latoxan (Valence, France) and venom of *Vespula species* (Euromix) was purchased from Vespa Laboratories (Spring Mills, Pennsylvania). 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 from Sigma (Taufkirchen, Germany). AlABLOTs as well as MUXF conjugated to bovine serum albumin were obtained from Siemens Healthcare Diagnostics (Bad Nauheim, Germany).

Two groups of sera were selected at random from the institutional serum bank: (i) Sera with a negative sIgE test to honeybee venom ($i1 < 0.35$ kU/L) but a positive test to vespid venom ($i3 \geq 0.35$ kU/L) ($n = 19$); (ii) Sera with a positive sIgE test to honeybee venom and to vespid venom ($i1$ and $i3 \geq 0.35$ kU/L) ($n = 22$).

All patients had given their informed written consent to draw an additional serum sample.

Specific antisera for Ves v 2a and b were raised by immunisation of rabbits with peptides according to amino acid position 222–235 for Ves v 2a and 224–237 for Ves v 2b using a C-terminal cysteine residue and LPH as a carrier. Immunisation was performed over a period of 35 days with four boosts according to standard protocols.

2.2. Cloning of cDNA

Total RNA was isolated from the separated stinger with attached venom-sack and additional glands of honeybee (*Apis mellifera*) and yellow jacket (*Vespula vulgaris*) using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to synthesise cDNA from the isolated total RNA. RNaseOut™ recombinant ribonuclease inhibitor (1 μ l) (Invitrogen, Karlsruhe, Germany) was added to the standard 20 μ l reaction mix containing 5 μ l venom-gland RNA. Reverse transcription was performed at 50 °C for 60 min. First strand cDNA was used as template for PCR amplification of DNA sequences.

Full-length Api m 2 was amplified from *Apis mellifera* venom-gland cDNA with *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) using the primers 5'-CCCACAACAACAAAACCG-3' and 5'-CACTTGGTCCACGCTCAG-3'. Full-length Ves v 2a and Ves v 2b were amplified from *Vespula vulgaris* venom-gland cDNA using the primers 5'-TCCGAGAGACCGAAAAAGAGTC-3' and 5'-GTTGACGGCT-TCTGTACAGTT-3', and 5'-GACAGAACAATTTGGCTAAG-3' and 5'-CTGTCAAAGAAAACACACCGTTAACTTT-3', respectively. Utilising the oligonucleotides 5'-GCCCCACATGGCCAGGGCTG-3' and 5'-GACCTTGAAGTGTCTGATCCT-3', full-length human α -2HS-glycoprotein was amplified from cDNA of human PBMCs.

The DNA was isolated from 1% agarose gels using the peqGOLD Gel Extraction Kit (Peqlab). Subcloning for sequencing was done using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector. The ligated DNA was transformed into the *E. coli* XL1Blue strain by electroporation (2 mm cuvettes, Easy-Ject+; Eurogentec, Seraing, Belgium) and selected on ampicillin agar plates.

After sequencing of selected cDNA sub-clones and verification of the sequence, the clones were used for secondary amplification of the coding region using *Pfu* DNA polymerase in two consecutive PCR reactions adding a 10-fold His-tag and V5 epitope. The PCR product was subcloned via BamHI and NotI restriction enzyme sites into the baculovirus transfer vector pAC-GP67-B (BD Pharmingen, Heidelberg, Germany).

Full-length fucosyltransferase X was amplified from *A. mellifera* venom-gland cDNA using *Pfu* DNA polymerase and the primers 5'-GATCAAGCTTATGGGTCTGCCGCTCTCTCCCTG-3' and 5'-GATCCCGCGGAGTATTTTAAATTTCCCGCGCAACCG-3'. The DNA was isolated from a 1% agarose gel using the peqGOLD Gel Extraction Kit. After verification of the sequence, the PCR product was cloned into the insect cell expression vector pIB/V5-His (Invitrogen, Karlsruhe, Germany) via HindIII and SacII restriction enzyme sites and was fused with a C-terminal V5 epitope and a 6-fold His-tag. The ligated DNA was transformed into *E. coli* XL1Blue by electroporation and selected on ampicillin agar plates.

2.3. Recombinant baculovirus production

Sf9 cells (Invitrogen, Karlsruhe, Germany) were grown at 27 °C in serum-free medium (Express Five SFM, containing 16.5 mM glutamine and 10 μ g/ml gentamycin; Invitrogen, Karlsruhe, Germany). Cell density was determined by haemocytometer counts and cell viability was evaluated by staining with Trypan Blue. Recombinant baculovirus was generated by co-transfection of Sf9

cells with BaculoGold bright DNA (BD Pharmingen, Heidelberg, Germany) and the baculovirus transfer vector pAC-GP67-B containing either Api m 2, Ves v 2a, Ves v 2b, or AHSG according to recommendations of the manufacturer. High titer stocks were produced after three rounds of virus amplification and the optimal MOI for recombinant protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flasks ($1.5\text{--}2 \times 10^6$ cells/ml in 20 ml suspension culture) with serial dilutions of high titer virus stock.

2.4. Expression in baculovirus-infected insect cells

High titer stocks of recombinant baculovirus containing the particular coding DNA were used to infect Sf9, HighFive (Invitrogen, Karlsruhe, Germany), or Sf9-FucTX cells ($1.5\text{--}2.0 \times 10^6$ cells/ml) in a 2000 ml suspension flask (400 ml suspension culture) according to recommendations of the manufacturer. For protein production the cells were incubated at 27 °C with agitation at 110 rpm for 72 h.

2.5. Stable transfection of insect cells

For transfection of purified DNA into cells, the reagent Cellfectin (Invitrogen, Karlsruhe, Germany) was used. Sf9 cells were grown in serum-free medium and selected for stable integration of the recombinant product by addition of 80 mg/ml Blasticidin S (Invivogen) antibiotic to the medium.

2.6. Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8 and centrifuged at $4000 \times g$ for 5 min. 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 thereafter eluted with NTA-binding buffer containing 300 mM imidazole. Purity was confirmed by SDS-PAGE.

2.7. Activity tests

Quantitation of hyaluronidase activity was determined by a method based on the colorimetric determination of *N*-acetyl-D-glucosamine at the reducing end of sugars liberated from hyaluronic acid (Reissig et al., 1955), as described elsewhere in detail (Muckenschnabel et al., 1998). Briefly, a standard solution of 5 mg/ml of hyaluronic acid was prepared in water and stored at 4 °C. To 250 µl of water, 50 µl of the hyaluronic acid stock solution and 100 µl of buffer were added. The buffer contained 0.2 M sodium formate, 0.1 M NaCl and 0.2 mg/ml of bovine serum albumin (BSA) and was adjusted to pH 3.5 with formic acid. Incubation mixtures were equilibrated at 37 °C for 10 min and the reaction was started by the addition of 50 µl of venom or recombinant protein.

The reaction was stopped by the addition of 110 µl of alkaline borate solution (2.8 M H_3BO_3 and 1.4 M KOH in H_2O) and heating for 4.5 min at 100 °C. To 10 ml of the alkaline solution, 1 ml of potassium carbonate solution (5.8 M K_2CO_3 in H_2O) was added before use. The tubes were placed on ice and 1.5 ml of *p*-dimethylaminobenzaldehyde solution (1.3 M *p*-dimethylaminobenzaldehyde dissolved in 25 ml of concentrated HCl and 75 ml glacial acetic acid was diluted with 4 volumes of glacial acetic acid immediately before use) was added. For color development, the tubes were incubated at 37 °C for 20 min. After centrifugation at $6000 \times g$ for 10 min, absorbance was measured at 595 nm.

2.8. IgE immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE immunoreactivity of human sera in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with 20 µl of purified recombinant proteins (20 µg/ml) at 4 °C overnight and blocked with 40 mg/ml milk powder in PBS at room temperature. Thereafter, human sera were diluted 1:2 with 5 mg/ml BSA in PBS and incubated in a final volume of 20 µl for 4 h at room temperature. Wells were washed 4 times with PBS before bound IgE was detected with a monoclonal alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD Pharmingen, Heidelberg, Germany) diluted 1:1000. Wells were again washed 4 times with PBS and 50 µl of substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) per well was added. The plates were read at 405 nm. The cut-off was calculated as the mean of the negative controls plus 2 SDs. This resulted in a working range of $\text{OD}_{405 \text{ nm}} \geq 0.24\text{--}3$. In the interest of precision, reactivities only slightly higher than the cut-off value were excluded for all allergens ($\text{OD}_{405 \text{ nm}} \geq 0.24 < 0.40$). Thus, an effective working range of $\text{OD}_{405 \text{ nm}} \geq 0.40\text{--}3$ was applied in all IgE ELISAs used for allergen identification.

2.9. Other methods

SDS-PAGE, Western blotting and ELISA as well as standard procedures in molecular biology were performed according to established protocols (Ausubel, 1996).

3. Results

3.1. Cloning of Api m 2, Ves v 2a, Ves v 2b, and α -2HS-glycoprotein

For production of the recombinant venom hyaluronidases, Api m 2 and Ves v 2a, the specific cDNA was amplified from bee and yellow jacket venom-gland cDNA. Additionally, the corresponding cDNA of Ves v 2b, a putative hyaluronidase isoform recently suggested as another component of the hyaluronidase band in *V. vulgaris* venom, was amplified. As a control protein to provide a non-reactive, non-allergenic protein backbone for different glycan structures we employed human fetuin, termed also α -2HS-glycoprotein (AHSG), a plasma protein highly abundant in most mammalian species. AHSG consists of 2 polypeptide chains and provides two sites each for N- and O-glycosylation. The AHSG cDNA was amplified from human PBMC cDNA.

3.2. Expression in insect cells and analyses of CCD reactivities

Full-length Api m 2, Ves v 2a, Ves v 2b, and AHSG were produced by baculovirus-infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. Following expression, culture supernatants were subjected to Ni-affinity chromatograph, and the resulting proteins were analysed by SDS-PAGE and immunoblotting (Fig. 1A and B). Purification yielded recombinant Api m 2, Ves v 2a, Ves v 2b, and AHSG (yield of approx. 0.2–1 µg/ml supernatant) from Sf9 and HighFive cells with apparent molecular masses of approx. 42–45 kDa.

In order to definitely verify the presence of two distinct hyaluronidase isoforms in the venom of *V. vulgaris*, peptide-specific rabbit sera were raised against both proteins. After verification of reactivity with and specificity for their particular target protein the sera were employed for detection of their cognate antigens in yellow jacket venom. Antibody dilutions which were found capable at detecting equal amounts of the recombinant proteins with identical intensity were applied to AlaBLOTs of *V. vulgaris* venom. Not

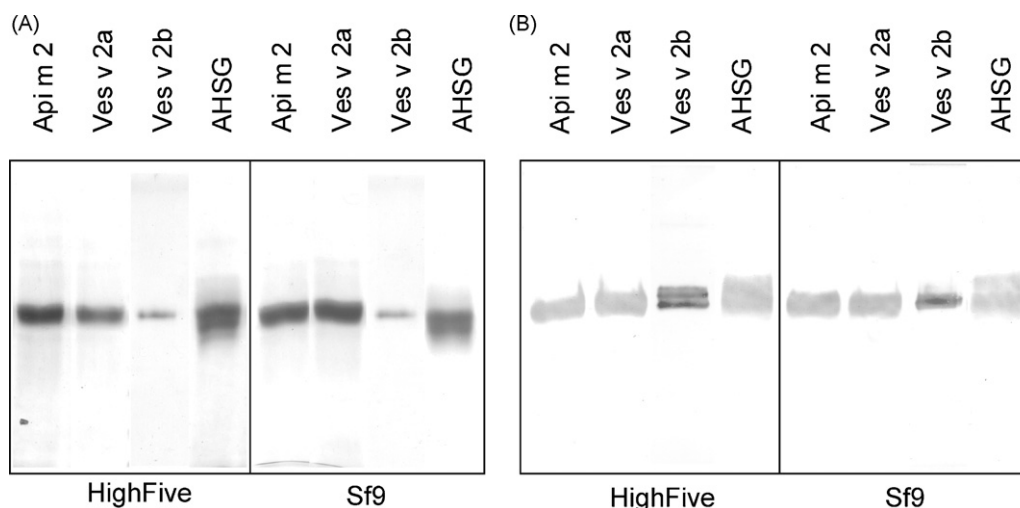


Fig. 1. SDS-PAGE and Immunoblot analysis of Api m 2, Ves v 2a, Ves v 2b, and α -2HS-glycoprotein recombinantly produced in Sf9 and HighFive insect cells. Purified Api m 2, Ves v 2a, Ves v 2b, and AHSG expressed in Sf9 or HighFive insect cells were subjected to SDS-PAGE and visualised by either Coomassie Blue staining (A) or detected in immunoblotting (B) using an anti-V5 epitope antibody and anti-mouse IgG-AP conjugate.

only was presence of both isoforms of the vespid hyaluronidases demonstrated, but the predominance of the isoform b in the venom of *V. vulgaris* was also clearly shown (Fig. 2A). Densitometric analyses of the AlABLOTs suggested a ratio of the Ves v 2a and b isoforms to be 1:4.

Specific enzymatic tests determined hyaluronidase activity for Api m 2 and Ves v 2a (Fig. 2B). In contrast, Ves v 2b showed no hyaluronidase activity, a finding that is consistent with primary structure analyses which suggest a mutation within the active site

(Kolarich et al., 2005). Thus, the hyaluronidase activity of the venom seems to be entirely a result of the isoform Ves v 2a. Additionally, the activity of the protein suggests proper folding.

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 in ELISA, however, revealed striking differences in the differentially expressed Api m 2, Ves v 2a, and Ves v 2b (Fig. 3A–C). In contrast to proteins produced in Sf9 cells, all of the proteins produced in HighFive cells exhibited strong reactivity with the

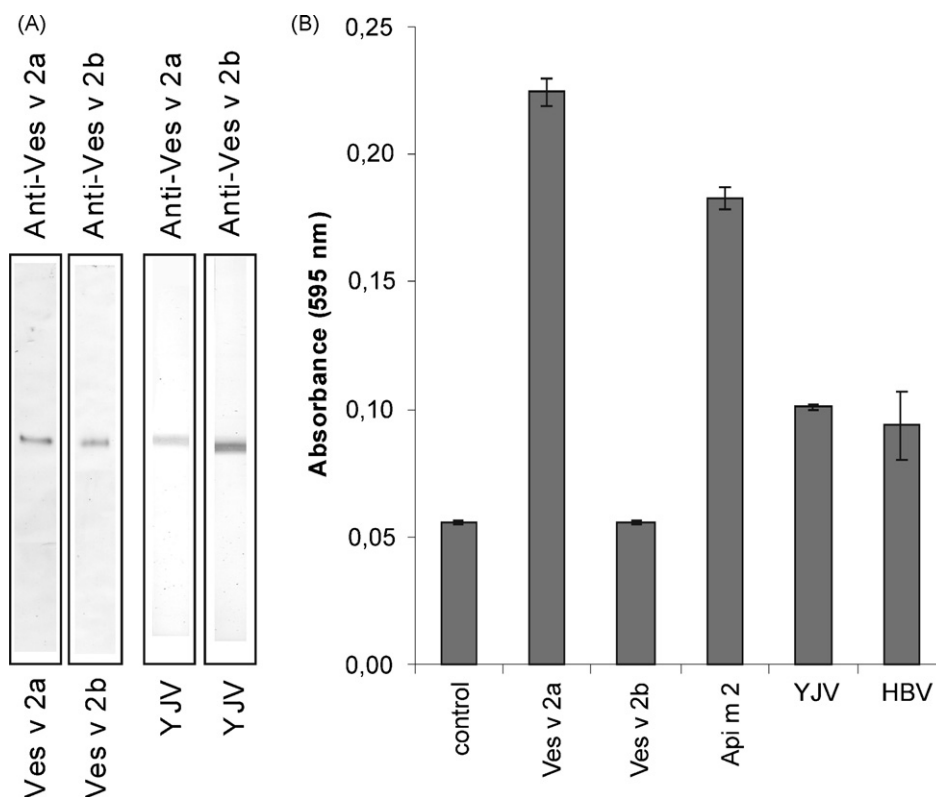


Fig. 2. Analyses of the vespid hyaluronidases. (A) After SDS-PAGE of purified recombinant Ves v 2a and Ves v 2b, equal amounts were detected in immunoblotting with anti-Ves v 2a (diluted 1:2000 in DMEM) or anti-Ves v 2b (1:5000 in DMEM) monospecific antibodies (right panel). Employing the anti-Ves v 2a or anti-Ves v 2b monospecific antibodies and anti-rabbit IgG-AP conjugate in identical dilutions the presence of the particular hyaluronidases in venom of *V. vulgaris* were verified in AlABLOTs (right panel). (B) Hyaluronidase activity of purified Api m 2, Ves v 2a, and Ves v 2b, and *A. mellifera* venom (HBV), *Vespula spp.* venom (YJV) was analysed as described in Section 2. Shown is the absorbance as produced by colorimetric determination of *N*-acetyl-D-glucosamine at the reducing end of sugars liberated from hyaluronic acid.

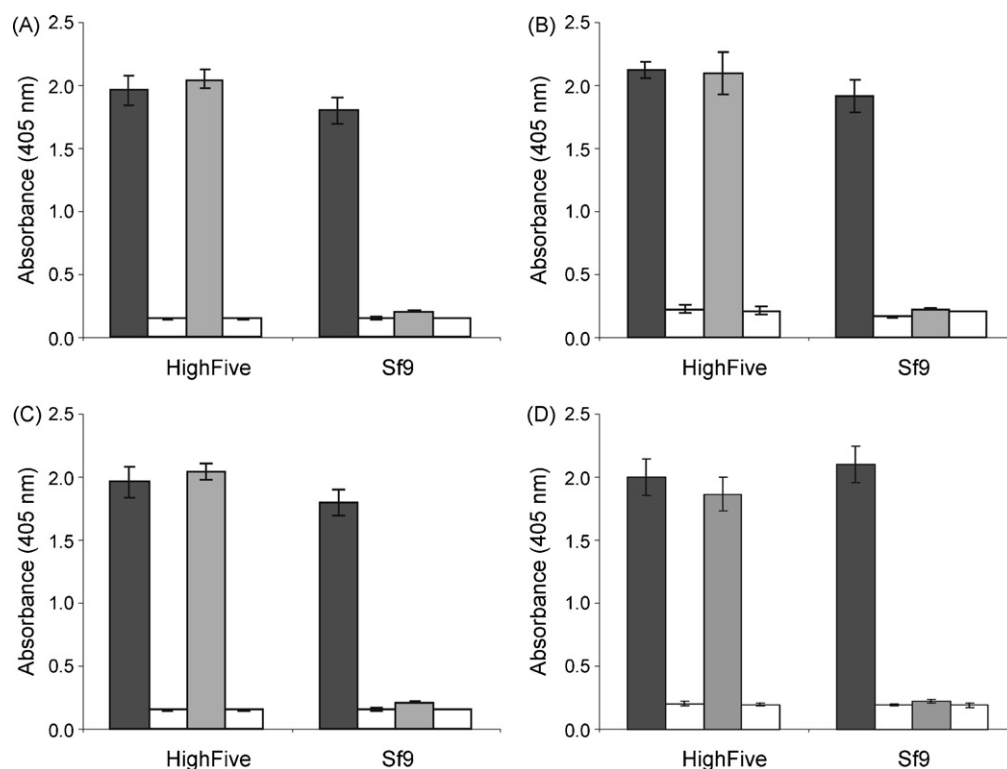


Fig. 3. ELISA analyses of the immunoreactivity of recombinant proteins. Assessment of recombinant Api m 2 (A), Ves v 2a (B), Ves v 2b (C), and AHSG (D) for presence of α -1,3-core fucosylation was performed using the anti-HRP-antibody and anti-rabbit-IgG-AP conjugate (grey bars) as described in Section 2. Normalisation of the amounts of immobilised proteins was performed employing an anti-V5 epitope antibody and anti-mouse IgG-AP conjugate (dark bars), for controls antigen was omitted (white bars).

anti-HRP serum, indicating α -1,3-core fucosylation. Analogously, whereas pronounced interaction was observed upon expression in HighFive cells, no reactivities were obtained for the Sf9-derived control protein AHSG (Fig. 3D). The immunologically inert nature of the control protein verifies the specific recognition of α -1,3-core fucose without any contributions from the protein backbone. Thereby, immobilisation of equal amounts of proteins was performed using a V5 epitope directed antibody.

Taken together, these data suggest that the α -1,3-core fucosylation of allergens produced in Sf9 cells may either be absent or below the immunological detection limit in the experimental setup followed here using highly specific antibodies.

3.3. Re-establishment of a CCD phenotype upon co-expression of α -1,3-core fucosyltransferase

The modified properties of the recombinant proteins upon expression in Sf9 vs. HighFive insect cells as shown above might not exclusively rely on the unique addition of α -1,3-linked core fucose, but rather on a potential modification of the entire glycan composition. To exclude this possibility, we aimed to overcome the putative α -1,3-core fucosyltransferase-deficient phenotype of the Sf9 cells by over-expressing the corresponding fucosyltransferase X gene from *A. mellifera*.

Therefore, we established stably transfected Sf9 insect cells (Sf9-FucTX) expressing the recently identified α -1,3-core fucosyltransferase from honeybee venom-gland (Rendic et al., 2007), thus allowing defined addition of 1,3-core fucose residues. After selection of transfectants, the recombinant enzyme could be visualised in cellular lysates by immunoblotting (Supplemental data Fig. 1). Detection with the anti-HRP antibody revealed pronounced α -1,3-core fucosyltransferase activity in the lysates of HighFive and Sf9-FucTX cells, in contrast to those of Sf9 cells (Fig. 4, middle panel).

To exclude the possibility that, in contrast to the anti-HRP antibody, serum IgE directed against core glycan structures exhibits reactivity with glycans even either lacking or reduced in α -1,3-core fucose residues due to their higher affinity, analyses of a strongly CCD-positive serum (class 4 against the CDD reagent MUXF as assessed by CAP; O214) were performed. However, as already observed employing the anti-HRP antibody, α -1,3-core fucosyl-

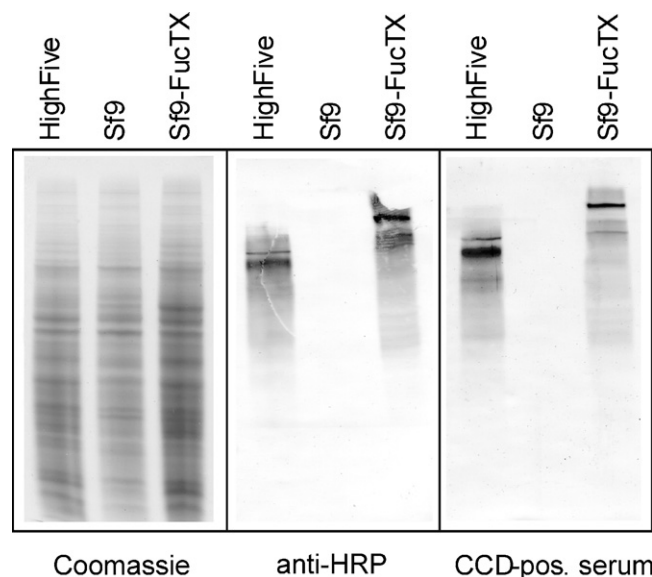


Fig. 4. Fucosyltransferase activity in cellular supernatants. After SDS-PAGE using equal amounts of lysates from different insect cell lines, α -1,3-core fucosylation was detected in immunoblotting with anti-HRP antibody (diluted 1:2000 in DMEM) and anti-rabbit IgG-AP conjugate (left panel) or a human CCD-positive serum (diluted 1:20 in DMEM) and anti-human IgE-AP conjugate.

transferase activity was obvious in HighFive and Sf9-FucTX cells, whereas such activity was absent in mock-transfected Sf9 cells (Fig. 4, right panel). These data also suggest that the interaction of human anti-CCD serum with mixed epitopes consisting of core glycan/protein backbone can be neglected.

Subsequently, these Sf9-FucTX cells were employed for production of Api m 2, Ves v 2a, Ves v 2b, and AHSG by means of baculovirus-based expression. In SDS-PAGE, the migration behaviour of all three resulting proteins did not differ significantly from that observed upon expression in mock-transfected Sf9 cells, as an example for the AHSG shows (Fig. 5A). Nevertheless, when employing the anti-HRP antibody in immunoblot analyses, the immunoreactivity of the Sf9-FucTX cell derived AHSG was comparable to that derived from HighFive cells, while Sf9-derived protein again exhibited no reactivity at all (Fig. 5A). These data were further corroborated by analogous reactivities of the three AHSG proteins as documented in ELISA analyses (Fig. 5B).

Together, these data suggest that the glycans added by Sf9 cells differ from those added by HighFive cells primarily with respect to the α -1,3-core fucosylation, a phenotype which can be entirely reverted by recombinant expression of the corresponding FucTX. This finding also implies that the molecular composition of the natural glycans beyond fucosylation does not contribute to the establishment of CCD reactivity.

3.4. Screening of patient sera for assessment of double sensitisation

After providing evidence that IgE reactivity to proteins produced in Sf9 cells is confined to the peptide backbone, we aimed for re-evaluation of the venom hyaluronidases from *A. mellifera* and *V. vulgaris* with respect to their IgE reactivity and the extent of interference by CCDs. Hence, the recombinant proteins were subjected to analysis of their IgE reactivities. Sera of 41 patients with clinical history of insect venom allergy were assayed by ELISA for specific IgE antibodies to the hyaluronidases and AHSG, with or without CCDs. These sera were separated into a group of 19 sera with negative sIgE to honeybee venom (Fig. 6A) and of 22 sera double positive for honeybee and yellow jacket venom (Fig. 6B).

None of the honeybee venom negative sera, implying sensitisation by vespid proteins accompanied by a lack of CCD reactivity, showed high sIgE reactivity to any of the proteins. However, a few sera did show medium to lower degree sIgE reactivity to Ves v 2b. Reactivity was not observed utilising proteins derived from HighFive cells or the MUXF conjugate, supporting a lack of CCD reactivity of these sera.

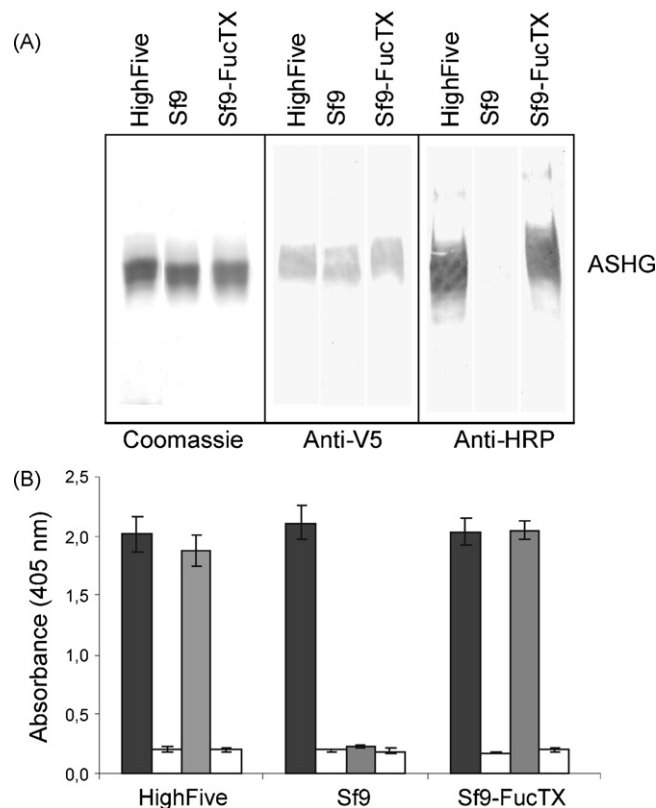


Fig. 5. Immunoblot and ELISA analysis of α -2HS-glyco-protein co-expressed with α -1,3-core fucosyltransferase from *A. mellifera*. Assessment of the recombinant AHSG for the presence of α -1,3-core fucosylation was performed in immunoblot after SDS-PAGE (A) and in ELISA (B) using the anti-HRP-antibody and anti-rabbit-IgG-AP conjugate as described in Section 2. Normalisation of the amounts of immobilised proteins was performed employing an anti-V5 epitope antibody and anti-mouse IgG-AP conjugate (dark bars), for controls in B antigen was omitted (white bars).

The group of double-positive sera included both honeybee venom-sensitised and yellow jacket venom-sensitised individuals, potentially accompanied by cross-reactivity based on structural similarity or CCD reactivity. Accordingly, a significant portion of the sera exhibited CCD reactivity to the HighFive derived proteins and MUXF conjugate (Fig. 6B, right panel), in clear contrast to the Sf9-derived proteins. For these CCD-devoid proteins, 3/20 sera exhibited pronounced reactivity with Api m 2, additional 2/20 to lower degree. However, no reactivities were found to Ves v 2a and

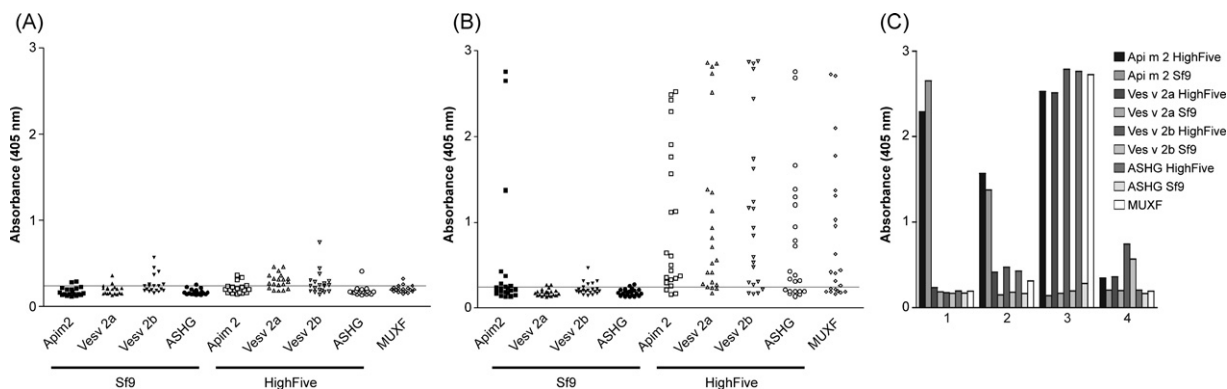


Fig. 6. IgE reactivity of individual patient sera with Api m 2 and Ves v 2 with and without CCD. Immunoreactivity of individual patient sera with recombinant Api m 2, Ves v 2a, Ves v 2b and AHSG from Sf9 and HighFive cells as well as MUXF conjugate. IgE reactivity was assessed in ELISA as described in Section 2 with 19 sera from venom-sensitised patients with negative IgE to *A. mellifera* (A) and with 22 double-positive sera (B). For controls antigen was omitted. The lower end functional cut-off of the ELISA was OD_{405 nm} > 0.24 as represented by the line. The reactivity of 4 particular sera is shown to illustrate distinct profiles (C).

while a few reactivities were observed to Ves v 2b, they were not as pronounced as those to Api m 2.

To illustrate this finding, reactivity profiles of individual sera are shown in Fig. 6C. Serum 1 specifically recognises Api m 2 from Sf9 and HighFive cells, without reaction to any of the other proteins, including MUXF. This picture corresponds to the specific recognition of Api m 2, without contribution of CCDs or cross-reactivity to other hyaluronidases. In contrast, serum 2 shows reactivity to Api m 2, but CCDs contribute to the profile as suggested by the reactivity observed for the other HighFive derived proteins. Serum 3 is reactive with all proteins from HighFive cells but lacks reactivity to Sf9-derived allergens, indicating mere CCD reactivity, also supported by the pronounced MUXF reactivity. Serum 4 exhibits reactivity with Ves v 2b without significant contributions of the CCDs.

These data provide evidence that Sf9-derived proteins lack detectable α -1,3-core fucosylation de facto. Furthermore, the data suggest that most IgE reactivities to venom hyaluronidases are essentially based on the recognition of CCDs, and not proteinic epitopes. Hence, these results raise questions regarding the relevance of vespid hyaluronidases, in particular the more common isoform Ves v 2a.

4. Discussion

The glycosylation of a variety of species including hymenoptera is increasingly being investigated, not only to understand the complexity of glycotypes in nature, but also to determine the clinical implications of immunogenic or allergenic CCDs in hypersensitivities. Hymenoptera venom glycoproteins display some features not found in mammals including a Lewis-type structure (Kubelka et al., 1995, 1993) that plays a detrimental role in inflammation and cancer in humans (Becker and Lowe, 2003; Kannagi et al., 2004; Sakamoto et al., 1986). However, the most prominent feature are α -1,3-linked core fucose residues which are considered essential for the establishment of cross-reactive glycan-based epitopes (Tretter et al., 1993). IgE with specificity for such CCDs are key players in allergen cross-reactivity and represent a major concern for diagnostic and, thus, therapeutic approaches. Today, almost 50% of patients with suspected hymenoptera allergy appear to be sensitised to both honeybee and yellow jacket venom according to in vitro assays (Egner et al., 1998). In contrast, only a small portion of patients show adverse reactions to both venoms. This implies cross-reactions due to CCDs, as reported for 75% of double-positive patients (Jappe et al., 2006), and a limited clinical relevance of glycan epitopes.

Although tempting CCD reactivity and clinical relevance of proteins are not mutually exclusive as hypothesised in particular for the high molecular weight allergens in the venom. It also has to be considered that a higher molecular weight to some extent might correlate with increased number of glycosylation sites. Recently, true sensitisation and clinical relevance could be shown for Api m 5 containing 5–7 potential glycosylation sites and often considered to reflect mere CCD reactivity, and its vespid homologue (Blank et al., 2008, submitted for publication).

In general, the role of CCDs for occurrence of allergic symptoms still is controversial (Malandain, 2005). CCD-specific IgE has recently been shown to effect IL-4 release of basophils (Fotisch et al., 1999; Wicklein et al., 2004). Moreover, it is reported that some hymenoptera venom allergic individuals with severe clinical manifestations possess IgE specific for CCDs only (Jappe et al., 2006). Interestingly, the recent finding of IgE-mediated carbohydrate-specific anaphylaxis via Gal- α -1,3-Gal structures of the humanised antibody cetuximab provides convincing evidence for the detrimental potential of glycan epitopes (Chung et al., 2008). On the other hand, artificially or recombinantly synthesised glycoproteins

that provide CCDs on an inert protein backbone are evidence of the specificity of anti-CCD IgE, although these appear to have no effect in mediator release assays or skin prick tests (Bencurova et al., 2004; Mari et al., 2008).

To further dissect these inconsistencies it is crucial to address the molecular basis of the observed CCD reactivity. For IgG raised in rabbits against insect and plant glycoproteins, such as bee venom or HRP, the α -1,3-core fucose on the glycans constitutes the most essential epitope (Kurosaka et al., 1991; Prenner et al., 1992) and an analogous molecular recognition is assumed to be implicated for the human immune response. Evidence for this is provided by the finding that the sera of approx. 25% of patients with bee venom allergy contain IgE that cross-reacts with N-glycans from plants, a finding which can adequately be explained by the presence of α -1,3-core fucose in both plants and insects (Tretter et al., 1993) including fire ant (Hoffman et al., 2005) and yellow jacket venoms (Hemmer et al., 2001). The nearly exclusive role of α -1,3-core fucose is further corroborated by the finding that glycan structures devoid of α -1,3-core fucose are not able to interfere with IgE binding (Bencurova et al., 2004), in contrast to peptides containing α -1,3-core fucose (Hemmer et al., 2004). Additionally, CCD-reactive sera can be separated into two different fractions recognising either the fucose-dependent or the xylose-dependent epitope (Faye et al., 1993). These steric arrangements in the overall assembly of glycans containing both xylose and fucose have been demonstrated by structural analyses, rendering simultaneous antibody binding unlikely (Elgavish and Shaanan, 1998; Shaanan et al., 1991). In summary, it appears convincing that insect core glycans devoid of α -1,3-core fucose resemble mammalian core structures. If this core would retain any immunogenicity, immune responses with severe consequences would be triggered by endogenous glycoproteins.

The analyses performed here focused on honeybee and yellow jacket venom hyaluronidases (E.C. 3.2.1.35). Both 42–45 kDa proteins are considered major allergens and are putatively responsible for the cross-reactivity of these two venoms with allergic patient's sera. Since Api m 2 and the Ves v 2a/b proteins are glycoproteins comprising up to 4 potential glycosylation sites of which at least 2 are occupied, CCDs will at least be partially responsible for the cross-reactivities also observed with hyaluronidases not only from hornet (*Dolichovespula* sp.) and paper wasp (*Polistes* sp.) (King et al., 1996), but also bumble bee and fire ant venom (Hoffman et al., 2005; Kochuyt et al., 2005). Therefore, it appears likely that yellow jacket and honeybee venom hyaluronidases (and others) carry α -1,3-core fucosylated N-glycans (Kubelka et al., 1995). The glycosylation pattern of Ves v 2 was very recently addressed in detail (Seppala et al., 2009). However, in these findings the distribution of CCD reactivity and true reactivity based on clinically relevant epitopes remained elusive.

In our study, the predominance of the recently identified hyaluronidase isoform Ves v 2b in the venom of *V. vulgaris* (Kolarich et al., 2005) and, hence, its significant contribution to overall IgE reactivity is shown for the first time. Additionally, the data point to equivalent reactivities of the two hyaluronidases including CCD directed IgE. Regarding the exact ratio of Ves v 2a and Ves v 2b, no definitive statement can be given, since venoms from particular *Vespula* species are difficult to obtain and the isoform ratio may also vary significantly between different species and sources. Nevertheless, AlaBLOTs provide a reliable source of *V. vulgaris* venom and at least allow for an estimation of the isoform ratio which is 1:4 (Ves v 2a/b). This evidence suggests that the majority of diagnostic and therapeutic studies focusing on Ves v 2a solely may need to be thoroughly revisited and, potentially, redesigned.

We employed the 40 kDa protein fetuin as a reference protein in this study (Pedersen, 1944). In the human system it is also termed α -2HS-glycoprotein or α -2-Heremans-Schmid glyco-

protein (Anderson and Anderson, 1977) and represents a highly abundant plasma protein in a plethora of mammalian species. AHSG contains a long A-chain (Yoshioka et al., 1986) and short B-chain (Gejyo et al., 1983) providing 2 sites each for N- and O-glycosylation. Since the Sf9-produced AHSG exhibits no IgE reactivity at all, it appears to be an ideal non-allergenic candidate retrospectively fostering its use as carbohydrate carrier.

In this study, all proteins were produced using different insect cell lines. Due to their homologous nature these cells appear to be an ideal expression host for hymenoptera venom compounds. Another advantage is the added glycosylation, which is more authentic than that from other routine hosts (Altmann et al., 1999). To the best of our knowledge, our results are the first report of the comparative production and assessment of Api m 2 and Ves v 2a and b in insect cells. Since prokaryotically produced, non-glycosylated proteins suffer limitations regarding folding, activity, and IgE epitope conservation among others, and production in yeasts as performed for Api m 2 (Reitinger et al., 2008) is associated with aberrant glycosylation, the cell lines examined here provide expression hosts that are phylogenetically as close as possible to the parental organism, making them indispensable for recombinant insect venom protein production.

The strategy we followed here is based on observations of variant N-glycosylation in different lepidopteran cell lines (Tomiya et al., 2003, 2004). Although both derived from ovarian tissue Sf9 insect cells (Vaughn et al., 1977) are considered to exhibit no or drastically reduced α -1,3-core fucosyltransferase activity compared to HighFive cells (Davis et al., 1992) in the context of baculovirus-based expression (Staudacher et al., 1992; Voss et al., 1993). This behaviour should allow for the production of proteins with only minute amounts of CCDs, or lacking CCDs entirely. We could confirm the validity of this approach using an anti-HRP antibody that is established since a long time to detect α -1,3-core fucosylation (Kurosaka et al., 1991; Rendic et al., 2007; Tretter et al., 1993).

Overexpression of the *A. mellifera* α -1,3-core fucosyltransferase in Sf9 cells led to restoration of the fucose-dependent epitope, demonstrating that CCD reactivity is exclusively dependent on α -1,3-core fucosylation and absent in conventional Sf9 cells. Although the affinity of IgE antibodies to α -1,3-core fucosylated glycans is reported to be high (Jin et al., 2008), neither increased sensitivity of IgE compared to the anti-HRP antibodies, nor residual CCD reactivities due to IgE binding to non-fucosylated core glycans could be observed for the AHSG control protein from Sf9 cells.

In a recent report the establishment of carbohydrate-directed IgG reactivities and false-positive signals upon production of proteins utilized as diagnostics in parasitic infections was observed (Hancock et al., 2004, 2008). Thereby, the prevalence of up to 12% of individual sera exemplifies the future need of defined glycosylation of biologicals. To circumvent problems such as the lack of terminal sialylation (Butters et al., 1981; Fabini et al., 2001; Hooker et al., 1999; Lopez et al., 1999; North et al., 2006; Roth et al., 1992) and processing (Donaldson et al., 1999; Hollister et al., 2002; Wagner et al., 1996), mammalian glycosyltransferases have been integrated into SfSWT-1 cells (Jarvis et al., 1998), which are modified Sf9 cells (O'Reilly et al., 1992; Vaughn et al., 1977). However, the immunogenic α -1,3-core fucosylation remains the major problem (Paschinger et al., 2005; Tretter et al., 1993). In this regard, identification of the α -1,3-core FucTs from the most relevant insect cell lines might pave the way for specifically abolishing with the α -1,3-core fucose moiety.

Assessment of the differentially fucosylated proteins revealed that for the apid as well the vespid hyaluronidases the IgE reactivities are strongly dominated by CCD reactivities. In absence of CCDs, a significant portion of sera showed clear IgE reactivities with Api m 2. Since only a minor fraction of the double-positive sera was

allergic to honeybee venom, which is in accordance with the lower prevalence of honeybee venom allergy in Germany as compared to yellow jacket venom allergy with an estimated ratio of 1/3, the number of Api m 2 positive sera is roughly in accordance with other data. In contrast to this finding, for both Ves v 2a and Ves v 2b the scarce reactivities in the absence of CCDs suggest only minor contributions of the vespid hyaluronidases to yellow jacket venom hypersensitivity. The nearly absence of IgE reactivities for Ves v 2a might also reflect the scarcity of this isoform in vespid venom compared to Ves v 2b.

Another interesting finding is the lack of IgE reactivity of Api m 2 positive sera with the vespid hyaluronidases in absence of CCDs. This emphasises the hypothesis that cross-reactivity between the hyaluronidases at the mere protein level is limited. This is further attributed to the absence of surface areas that possess a significant degree of identity, rendering cross-reactivities unlikely (Skov et al., 2006).

When considering IgE reactivities in absence of CCDs only, the venom hyaluronidases of honeybee and yellow jacket may not be classified as major allergens. This estimation is also supported by a recent report on the predominance of carbohydrate-directed reactivity for Ves v 2a (Seppala et al., 2009). It was demonstrated that serum pooled from 14 donors did not recognise prokaryotically produced Ves v 2a, as it lacked both fucosylation and glycosylation.

Our data also demonstrate that a careful re-evaluation of IgE reactivities is essential towards understanding the allergenic characteristics of individual venom glycoproteins, a concept that also applies to other allergen sources including plants, mites, etc., and that should have implications for therapeutic approaches in hymenoptera venom allergy.

In summary, the obtained results clearly suggest that the recombinant production in Sf9 vs. HighFive cells provides a novel concept for the reliable analysis of protein vs. CCD reactivities. As shown for the hyaluronidases, including the as yet uncharacterised isoform Ves v 2b, recombinant allergens with tailor-made CCD reactivity may enable differentiation of true sensitisation 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 modalities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.10.005.

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Seismann et al., Supplemental data:

Supplementary Figure 1: Expression of α -1,3-core fucosyltransferase from *A. mellifera* in Sf9 insect cells

After SDS-PAGE (right side) of equal amounts of cellular lysate from different insect cell lines, expression of the recombinant protein was detected by immunoblotting employing anti-V5 epitope antibody (diluted 1:2000 in DMEM) and anti-mouse IgG-AP conjugate.

