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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 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.

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 A_2 (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 A_1 (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 ≥ 0.35 kU/L) ($n = 19$); (ii) Sera with a positive sIgE test to honeybee venom and to vespid venom (i1 and i3 ≥ 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 cystein 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 μl) (Invitrogen, Karlsruhe, Germany) was added to the standard 20 μl reaction mix containing 5 μl 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′-CCCGACAACAACAAAACCG-3′ and 5′-CACTTGGTCCACGCTCACG-3′. Full-length Ves v 2a and Ves v 2b were amplified from *Vespula vulgaris* venom-gland cDNA using the primers 5′-TCCGAGAGACCGAAAAGAGTC-3′ and 5′-GTTGACGGCTTCTGTCACGTT-3′, and 5′-GACAGAACAATTTGGCCTAAG-3′ and 5′-CTGTCAAAGAAAACACACCGTTAAACTTT-3′, respectively. Utilising the oligonucleotides 5′-GCCCCACATGGCCCAGGGCTG-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′-GATCAAGCTTATGGGTCTGCCGCGTCTCTCCCTG-3′ and 5′-GATCCCGCGGAGTATTTTTTAAATTTCCCGCGCGAACCG-3′. The DNA was isolated from a 1% agarose gel using the peqGOLD Gel Extraxtion 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 μg/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