



Sensitization to wasp venom does not induce autoantibodies leading to infertility

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ABSTRACT

Antigenic cross-reactivity has been described between the venom allergen (antigen 5) and mammalian testis proteins. Based on an allergen database we have previously shown that allergens can be represented by allergen motifs. A motif group was found containing venom antigen 5 sequences from different vespids. Using an optimized amino acid profile based on antigen 5 sequences for searching cross-reactive proteins, three human semen proteins belonging to the family of cysteine-rich secretory proteins (hCRISP) were found in the Swiss Protein database. To analyze antigenic cross-reactivity between antigen 5 and hCRISPs, antigen 5 from yellow jacket venom (Ves v 5) and two hCRISPs (CRISP-2 and -3) were chosen and produced as recombinant proteins in *E. coli*. A correlation was found between antibodies reacting with rVes v 5 and rhCRISP-2, -3 in a small human sera population indicating the presence of cross-reactive antibodies in human serum. Using intravenous immunoglobulin (IVIg), a therapeutic multidonor IgG preparation, cross-reactive antibodies were isolated that recognize rVes v 5, hCRISP-2 and -3 suggesting the presence of common epitopes between Ves v 5 and hCRISPs. However this cross-reactivity seems not to be linked to allergy to wasp venom as we could show no correlation between increasing CAP-class IgE level to wasp venom and IgG to sperm extract and hCRISPs. These data suggest that higher sensitization to wasp venom does not induce more antibodies against autoantigens and might not represent a higher risk to develop autoantibodies leading to infertility.

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1. Introduction

The identification of structural similarities between allergen and autoantigen has led to the concept that there is a link between allergy and autoimmunity (Valenta et al., 1997). Former works have shown that patients with severe and chronic forms of atopy have IgE autoantibodies against a variety of autoantigens suggesting that allergens with similarities to human proteins may be responsible for cross-reactivities. Thus it was reported that patients who were allergic to manganese superoxide dismutase (MnSOD) and ribosome P-2 protein from *Aspergillus fumigatus* exhibited autoimmune reactions to the human homologous (Fluckiger et al., 2002; Mayer et al., 1999).

We have recently developed a bioinformatic approach to define shared motifs in the amino acid sequences of allergenic proteins

that can be used to predict cross-reactivity (Stadler and Stadler, 2003). In a first study we used an allergen database for the motif analysis and identified two motifs that contain two allergens, tropomyosin and manganese superoxide dismutase (Marti et al., 2007). Both allergens share structural similarities with human proteins and have been shown to play a role in IgE autoimmunity linked to severe forms of atopy (Valenta et al., 2000). A motif consensus sequence was defined for each allergen and expressed as recombinant peptides representing allergenic structures comparable to the full length protein and suggesting that the motif generated algorithm can be used to define IgE binding structures.

In this study using the same allergen database as before we found a motif that contains all the sequences from the antigen 5 of different vespids including the one from *Vespula vulgaris* (Ves v 5). Ves v 5 is a single polypeptide chain consisting of 204 amino acids (23 kDa) and belongs to a super family containing extracellular proteins within different species such as plants, fungi, reptile, mammals and insects. Ves v 5 is one of the major wasp venom allergen but its biological function remains unknown (Hoffman, 1993; Lu et al., 1993; Wood et al., 1983). From the sequences found in

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the motif an optimized amino acid profile sequence was defined that allowed us to find cross-reactive proteins in the Swiss Protein database (SwissProt) belonging to the family of cysteine-rich secretory proteins (hCRISPs). This finding corresponds to former studies based on amino acid sequence similarity of antigen 5, which revealed cysteine-rich secretory proteins (CRISPs) as an additional homologue protein family (Henriksen et al., 2001). Thus experiments carried out in mice using Dol m 5 (antigen 5 from white-face hornet venom) and mouse testis-specific protein 1 (TPX-1, murine CRISP-2) have revealed cross-reactivity at both T- and B-cell levels (King and Lu, 1997).

The proteins of the CRISP family are single chain polypeptides that consist of two distinct domains: an amino terminal PR-1-like domain and a cysteine-rich domain (CRD) on the carboxyl terminus (Guo et al., 2005). The CRISPs are widely distributed in a variety of organisms and have been shown to share 40–80% sequence identities among each other (Guo et al., 2005). In previous studies mammalian CRISP proteins have been isolated and their biological functions have been characterized. CRISP-1 is expressed exclusively in the epididymis of mammals and is reported to bind loosely to spermatozoa and participate in gamete fusion (Eberspaecher et al., 1995; Hayashi et al., 1996; Klemme et al., 1999; Kratzschmar et al., 1996; Sivashanmugam et al., 1999). CRISP-2, also known as testis-specific protein 1 (TPX-1), is mainly expressed in testis and is associated with sperm-egg gamete fusion as well as sperm development and maturation, respectively (Kasahara et al., 1989; Kratzschmar et al., 1996; Maeda et al., 1999). Compared to CRISP-1 and CRISP-2, CRISP-3 is more widely distributed. It is expressed in salivary gland, human neutrophils, plasma, prostate and exocrine secretions (Kjeldsen et al., 1996; Kratzschmar et al., 1996; Schambony et al., 1998; Udbj et al., 2002a,b).

Antibodies against human CRISP-1 (hCRISP-1) and CRISP-2 (hCRISP-2), have shown to inhibit dose-dependently the percentage of penetrated zona-free hamster oocyte, when co-incubated with human sperms (Busso et al., 2005; Cohen et al., 2001), indicating that antibodies against CRISP proteins could lead to reduced fertility. Since hCRISPs and Ves v 5 share sequence similarity, sensitization with Ves v 5 may lead to the production of cross-reactive autoantibodies that could be involved in the development of autoimmune infertility.

In our study we analyzed the cross-reactivity existing between Ves v 5 and the two human CRISP-2 and -3. As CRISPs 1 and 2 show high sequence similarity we chose CRISP-2 as representative of CRISP proteins found mainly in the reproductive tract. For control purpose CRISP-3 protein was chosen because of its wider tissue distribution in the human body. Ves v 5 was produced as full length recombinant protein and hCRISP-2 and hCRISP-3 as truncated (PR-1 domain only) forms in *E. coli*. Using intravenous immunoglobulin (IVIg) preparation as a source of IgG we found antibody reactivity against both rVes v 5 and trunc-hCRISP-2, -3 indicating the presence of cross-reactive antibodies in human serum. However IgG against rVes v 5 and trunc-hCRISPs did not correlate with CAP-class IgE level to wasp venom extract indicating that high sensitization to wasp does not represent a risk for the development of an immune response against hCRISPs which may lead to infertility.

2. Experimental

2.1. Materials and methods

2.1.1. Determination of Ves v 5 cross-reactivity in silico

Construction of protein sequence motifs and searching for matching sequences were performed using generalized sequence profiles (Gribskov et al., 1987) as implemented in the Pftools soft-

ware package (Bucher et al., 1996). The sequences of wasp venom proteins reported to be cross-reactive, were collected (accession numbers: Q05108, Q05109, Q05110, P10736, P10737, P35759, P35760, P35786). The Ves v 5 profile was calculated based on a multiple sequence alignment of these proteins using ClustalW (Thompson et al., 1994). The profile was calibrated empirically on a randomized version of the SwissProt database (Pagni and Jongeneel, 2001). Potential Ves v 5 cross-reactive proteins were searched using the calibrated profile and SwissProt release 40 as a source of sequences.

2.1.2. Cloning, expression and purification of rVes v 5

Ves v 5 DNA sequence (Kasahara et al., 1989) was produced by using 10 overlapping oligonucleotides which were synthesized at Microsynth (Balgach, Switzerland) (Table 1). Pairs of oligos (#1–2, #3–4, #5–6, #7–8, #9–10) were assembled by PCR filling reactions, using Pfx Platinum™ Polymerase (Invitrogen, CA, USA). These pairs were consecutively linked together by PCR and the whole Ves v 5 DNA sequence was amplified using the primers f-Ves v 5 and r-Ves v 5 (Table 2).

Ves v 5 PCR product encoding DNA was isolated and cloned into the restriction sites EcoRI/XbaI (Roche, Basel, Switzerland) of the pMal-p2x vector (New England Biolabs GmbH, Frankfurt am Main, Germany). This vector contains a periplasm leader sequence and an N-terminal maltose-binding protein (MBP)-tag followed by a protease restriction site for factor Xa (New England Biolabs) upstream of the multiple cloning sites. *E. coli* XL-1 Blue (Stratagene, San Diego, USA) cells were used for transformation.

After sequencing (Microsynth), protein expression and purification was performed using the pMAL™ Protein Fusion and Purification System (New England Biolabs) according to the manufacturer's instructions with small modifications. Briefly, a single colony was used to grow an overnight (o/n) culture. After 1:100 dilution, the culture was grown in 1 l Luria-Bertani (LB)-Medium containing 5 g glucose and 50 µg/ml carbenicillin until optical density (OD₆₀₀) reached 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 20 h while shaking at 220 rpm. Bacterial cells were harvested by centrifugation at 4000 × g for 20 min and resuspended in 400 ml 30 mM Tris(hydroxymethyl)-aminomethan (Tris) containing HCl, 20% sucrose, pH 8.0. While stirring, ethylene diamine tetraacetic acid (EDTA) to a final concentration of 1 mM was added and incubated for 10 min at room temperature (RT). After centrifugation (8000 × g, 20 min, 4 °C), the pellet was resuspended in 400 ml of ice-cold 5 mM MgSO₄ and stirred for 10 min in an ice-water bath. Subsequent to an additional centrifugation step (8000 × g, 20 min, 4 °C), the supernatant (osmotic shock fluid) was used for affinity purification of MBP-Ves v 5 on an amylose column via the MBP-tag and was competitively eluted with 10 mM maltose. Fractions were analyzed by SDS-PAGE (10% polyacrylamide) and Western blot analysis under non-reducing conditions as described elsewhere (Scheppeler et al., 2005). After blocking with phosphate buffer saline pH 7.4 (PBS) including 0.15% casein (PBS/0.15% casein), the Western blots were incubated o/n at 4 °C with rabbit anti-MBP serum (New England Biolabs) (1:10 000 diluted in PBS/0.15% casein). After washing with PBS, bound antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Nordic Immunology Laboratories, Tilberg, Netherlands), 1:1000 diluted in PBS/0.15% casein.

MBP-Ves v 5 fusion protein was separated using 3% (w/w) factor Xa and size exclusion chromatography (HiLoad 26/60 Superdex-75 prep grade, Amersham Pharmacia Biotech, USA). Size and purity were analyzed by SDS-PAGE and Western blot analysis as described above. Human anti-wasp Serum (1:80) was used to detect rVes v 5, followed by a HRP-conjugated anti-human IgG (Fc) as a sec-

Table 1

Oligonucleotides used for the synthesis of the Ves v 5 DNA sequence

Oligo 1	5'-AAC AAT TAT TGT AAA ATA AAA TGT TTG AAA GGA GGT GTC CAT ACT GCC TGC AAA TAT GGA AGT CTT AAA CCG AAT-3'
Oligo 2	5'-TAA GAT GTC TTG TTT CTC TTG TTT CGT TAG ACC ATA GGA TAC CAC TAC CTT ATT ACC GCA ATT CGG TTT AAG ACT T-3'
Oligo 3	5'-GAA ACA AGA CAT CTT AAA GGA GCA CAA TGA CTT TAG ACA AAA AAT TGC ACG AGG ATT GGA GAC TAG AGG TAA TCC T-3'
Oligo 4	5'-TAA GCT AAC TCG TCG TTC CAT ACC AAA TTT TTC ATA TTC TTC GCT GGA GGC TGT GGT CCA GGA TTA CCT CTA GTC-3'
Oligo 5	5'-ACG ACG AGT TAG CTT ATG TCG CCC AAG TGT GGG CTA ATC AAT GTC AAT ATG GTC ACG ATA CTT GCA GGG ATG TAG-3'
Oligo 6	5'-ATC GTA TTT AGC AGC CGT GCT ACC TGT TAA GGC TAC GTT TTG TCC AAC CTG ATA TTT TGC TAC ATC CCT GCA AGT-3'
Oligo 7	5'-GGC TGC TAA ATA CGA TGA TCC AGT TAA ACT AGT TAA AAT GTG GGA AGA TGA AGT GAA AGA TTA TAA TCC TAA GAA A-3'
Oligo 8	5'-GTT AGC CCA AAC CAT TTG AGT GTA ATG GCC GGT TTT CAG AAA GTC GTT TCC CGA AAA CTT TTT CTT AGG ATT ATA A-3'
Oligo 9	5'-AAT GGT TTG GGC TAA CAC CAA GGA AGT TGG TTG TGG AAG TAT AAA ATA CAT TCA AGA GAA ATG GCA CAA ACA TTA C-3'
Oligo 10	5'-CTT TGT TTG ATA AAG TTC CTC ATT CAT AAA GTT CCC GCT GGG TCC ATA ATT ACA TAC AAG GTA ATG TTT GTG CCA T-3'

ondary antibody (Nordic Immunology Laboratories), both diluted in PBS/0.15% casein.

2.1.3. Cloning, expression and purification of trunc-hCRISP-2 and trunc-hCRISP-3

The coding DNA sequence of hCRISP-2 was PCR amplified out of the MegaManTM human transcriptoma cDNA library (Stratagene, Edinburgh, Scotland) by PCR using f-hCRISP-2 and r-hCRISP-2 as primers (Table 2). Isolated full length hCRISP-2 DNA was re-amplified with the primer pair f-trunc-hCRISP-2/r-trunc-hCRISP-2 (Table 2) in order to obtain the truncated version of hCRISP-2. DNA encoding trunc-hCRISP-2 was cloned into the BamHI/HindIII (Roche) restriction sites of pQE30 (Qiagen, Hilden, Germany) and transformed into *E. coli* XL-1 Blue.

After sequencing (Microsynth), an overnight culture of one single clone was used to inoculate 1 l LB-Medium containing 50 µg/ml carbenicilline. At an OD_{600nm} of 0.6, IPTG was added to a final concentration of 1 mM and the culture was shaken (220 rpm) for 15 h at 30 °C. Bacterial cells were pelleted at 4000 × g for 30 min at 4 °C, resuspended in 20 ml ice-cold PBS and frozen. After thawing on ice, sonication and centrifugation (4000 × g for 30 min at 4 °C), the obtained inclusion bodies were washed 3 times with 20 ml wash buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM 1,4-dimercapto-2,3-butandiol (DTT), 1 mM EDTA, 0.5% Triton X-100, 0.1% NaN₃, pH 8.0) using a glass homogenizer. Triton X-100 was removed by two washes with resuspension buffer (50 mM Tris/HCl, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 8.0) as described above. Purified inclusion bodies were opened with 32 ml of 6 M guanidine hydrochloride buffered in 50 mM Tris/HCl pH 8.0 by rotating o/n at 4 °C.

8 ml of solubilized inclusion bodies were fully denatured by adding DTT to a final concentration of 20 mM for 1 h at 37 °C. Proteins were refolded in 1 l of refolding buffer (5 M Urea, 400 mM L-Arg, 3.7 mM cystamine, 6.6 mM cysteamine) by dialysis against 20 mM Tris/HCl pH 6.7 buffer. Charged proteins (pI of 8.2 for trunc-hCRISP-2) were bound o/n at 4 °C to a cation-exchange HiLoad 16/10 SP Sepharose HP column (Amersham Pharmacia Biotech). After washing the column, concentrated protein was eluted using a 100 ml linear gradient of 20 mM Tris/HCl pH 6.7 containing 1 M NaCl. Refolded proteins were purified by size exclusion (HiLoad 26/60 Superdex-75 prep grade,

Amersham Pharmacia Biotech) and protein containing fractions were analyzed by SDS-PAGE and Western blot analysis, respectively.

Cloning, expression and purification of trunc-hCRISP-3 was done as described for trunc-hCRISP-2, with small modifications: f-hCRISP-3/r-hCRISP-3 primers (Table 2) were used to get full length cDNA out of the MegaManTM human transcriptoma cDNA library (Stratagene). F-trunc-hCRISP-3 and r-trunc-hCRISP-3 (Table 2) were used to obtain the coding sequence for the truncated version of hCRISP-3. Protein concentration was performed as described for trunc-hCRISP-2, but this time 20 mM Tris/HCl pH 7.5 buffer (pI of 8.9 for trunc-hCRISP-2) was used for dialysis and subsequent cation-exchange chromatography.

2.1.4. Patients' sera

Intravenous immunoglobulin (Sandoglobulin SAGL, CSL Behring AG, Bern, Switzerland) was used as an IgG source representing the total IgG of more than 60 000 apparently healthy individuals.

For the studies on rVes v 5, trunc-hCRISP-2, trunc-hCRISP-3 and human sperms, three groups of donors were used: patients with high levels of anti-sperm antibodies, patients tested on wasp venom extract and control subjects. Individuals with high levels of anti-sperm antibodies were diagnosed by positive direct mixed-agglutination reaction (MAR) as described in Jager et al. (1978) and were designated as MAR positive. Specific IgE to wasp venom extract (common wasp, i3) was determined by ImmunoCAP® (Pharmacia, Uppsala, Sweden). Sera with CAP-class ≥ 1 were designated as wasp positive, while patients with CAP-class = 0 were named as wasp negative. 272 sera were selected to achieve two gender and age matched groups. Sera of patients with age ≥ 14 were termed as adults and sera of patients age < 14 as children. Finally, 146 wasp positive (67 male, thereof 16 male children; 79 female, thereof 7 female children) and 126 wasp negative (56 male, thereof 12 male children; 70 female, thereof 5 female children) were used in enzyme linked immunosorbent assays (ELISA). Individuals without allergy were used as controls.

As part of ongoing validations at our in house diagnostic laboratory, this study was approved by the ethical committee of the University of Berne.

Table 2

Primers used for PCR amplification of Ves v 5 and CRISPs DNA sequences

f-Ves v 5	5'-TCC ACA GGC GCG CAC TCC AAC AAT TAT TGT AAA ATA-3'
r-Ves v 5	5'-ATA GAT CTA GAT TAG TGA TGG TGA TGG TGA TGC TTT GTTT GAT AAA GTT C-3'
f-hCRISP-2	5'-AAG GAT CCC GCT TTT ACT G-3'
r-hCRISP-2	5'-TTA GTA AAT TTT GTT CTC ACA TAG GC-3'
f-trunc-hCRISP-2	5'-GTA TGG ATC CAA AGA TCC CGC TTT TAC T-3'
r-trunc-hCRISP-2	5'-CAG TAA GCT TTT AGT ACG GGG TAT TCT TTC TAT TC-3'
f-hCRISP-3	5'-ATG ACA TTA TTC CCA GTG CTG TTG TT-3'
r-hCRISP-3	5'-TTAATAAATGCTGTTTGAACAATTGCTG-3'
f-trunc-hCRISP-3	5'-GTA TGG ATC CAA TGA AGA TAA AGA TCC C-3'
r-trunc-hCRISP-3	5'-GCT TAA GCT TTT AAT AAG GCA CAT ATA GTC TAT-3'

2.1.5. Preparation of human sperm extracts

Ejaculates of four donors (25–35 years old) were received after 48 h of sexual abstinence. After liquefaction, the four semen samples were pooled and centrifuged at ($1000 \times g$) to separate sperm cells from the seminal plasma. After resuspension in 10 ml ice-cold PBS, sperm cells were frozen. Thawing on ice and subsequent sonication was followed by centrifugation $8000 \times g$ at 4°C for 20 min. The supernatant was then designated as sperm extract.

2.1.6. Isolation and characterization of specific IgG to rVes v 5 and trunc-hCRISP-2

To purify rVes v 5 and trunc-hCRISP-2-specific IgG, rVes v 5 and trunc-hCRISP-2 were coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacture's instructions. Briefly, the proteins were dissolved in coupling buffer (0.1 M NaHCO_3 pH 8.3 containing 0.5 M NaCl) using polyethersulfone VivaSpin 20 concentration tubes (Milian, Geneva, Switzerland) for buffer exchange. 6.6 mg of an irrelevant protein control containing a His tag (DARPin D40) (Vogel et al., 2007) were mixed with 1.5 g of CNBr-activated Sepharose 4B and incubated o/n at 4°C by rotating. For rVes v 5 and trunc-hCRISP-2, respectively, 1 mg protein was coupled to 0.5 g CNBr-activated Sepharose 4B as described above. After washing, remaining active groups were blocked with 0.1 M Tris/HCl buffer pH 8.0 for 2 h at RT.

12 g of lyophilized IVIg (CSL) was dissolved in 100 ml PBS and then dialyzed against PBS. The obtained 180 ml [65 mg/ml] of IVIg were loaded on the D40-column by circulating at a flow rate of 0.4 ml/min at 4°C for 16 h. For the following experiments, the resulting flow through was splitted in four equal volumes. Therefore 45 ml [65 mg/ml] D40-preabsorbed IVIg was loaded on either rVes v 5- or trunc-hCRISP-2-coupled CNBr-columns. Bound IVIg was eluted by Tris/HCl pH 4, neutralized, pooled and dialyzed against PBS.

Affinity purified IVIg was then tested in different concentrations [100, 50, 10, 5, 1, 0.5, 0.1, 0.05 $\mu\text{g/ml}$] on rVes v 5, trunc-hCRISP-2, trunc-hCRISP-3 and two control proteins serving as His-tag controls (DARPin D40 and D7 (Vogel et al., 2007)).

For each protein 5 $\mu\text{g/ml}$ were coated on Costar EIA/RIA half-well plates (Costar, Cambridge, MA) o/n at 4°C . Plates were washed with PBS and blocked with PBS containing 0.5% bovine serum albumin (BSA) by shaking 2 h at 4°C . IVIg was diluted in blocking solution and incubated 2 h at 37°C while shaking at 320 rpm. After washing with PBS followed by PBS containing 0.01% Tween 20, HRP-conjugated anti-human IgG-(Fc) (The Binding Site; For all experiments except Fig. 2: Nordic), 1:1000 diluted in blocking solution, was added and incubated for 1 h at 4°C . To develop the ELISA, 3,3',5,5'-tetramethylbenzidine (TMB) was used as a substrate. The reaction was stopped after 5 min by addition of 1 M sulphuric acid. $\text{OD}_{450\text{nm}}$ was measured using a standard ELISA reader (BIO-TEK EL808, BioTek, Bad Friedrichshall, Germany).

2.1.7. Inhibition experiment

Half-well ELISA plates were coated with rVes v 5, trunc-hCRISP-2 or trunc-hCRISP-3 [5 $\mu\text{g/ml}$] and blocked as described above. rVes v 5- or trunc-hCRISP-2-specific IgG was pre-incubated 2 h at 37°C and 320 rpm with either 0, 2, 10, 50 and 250 $\mu\text{g/ml}$ of rVes v 5 or trunc-hCRISP-2, respectively. His-tagged DARPin D40 was used as a control. Pre-incubated sera were added on coated plates and incubated during 2 h at 37°C , 320 rpm. Residual IgG was analyzed as forgoing described.

2.1.8. Determination of specific IgG levels

Specific IgG levels to rVes v 5, trunc-hCRISP-2, trunc-hCRISP-3 and sperm extract were evaluated by ELISA. Proteins were coated with 5 $\mu\text{g/ml}$ and sperm extract was diluted 1:4 in PBS. ELISA was

performed as described above for direct ELISA. Correlation coefficients (R -values) were calculated with Microsoft Excel 2007.

2.1.9. Accession numbers

Protein accession numbers from ExPasy: Q05108, Q05109, Q05110, P10736, P10737, P35759, P35760, P35786, P54108, P16562
DNA accession numbers from EMBL: Ves v 5 (M98858), CRISP-2 (M25532), CRISP-3 (X95240)
PDB ID: 1QNX, 1RC9, 1WVR, 1XTA, 1XX5

2.2. Results

2.2.1. Identification of CRISP proteins that cross-react with the venom allergen Ves v 5

From the *in silico* analysis of an allergen database (Marti et al., 2007), a motif group was identified that contains 8 cross-reactive allergens including the venom antigen Ves v 5. An amino acid profile, based on the sequences, was defined and used to search in the Swiss protein sequence database for other cross-reactive proteins. Interestingly we found three human semen proteins belonging to the family of cysteine-rich secretory proteins (CRISPs). As shown in Fig. 1, Ves v 5 showed around 30% amino-acid sequence identity with the PR-1 domain of the three human CRISP proteins (hCRISP-1, -2, -3). Moreover, structure alignment of Ves v 5 and hCRISP-3 demonstrated a high structural similarity apart from the cysteine-rich domain which is missing in Ves v 5. Thus to verify cross-reactivity between Ves v 5 and hCRISPs, full length rVes v 5 and truncated forms of hCRISP-2 and -3, missing CRD, were produced and compared for immunogenicity *in vitro*.

2.2.2. Cloning and expression of Ves v 5, trunc-hCRISP-2 and trunc-hCRISP-3

To clone recombinant Ves v 5 (rVes v 5), cDNA was generated by PCR and then expressed in the periplasm of *E. coli* as a fusion protein with maltose-binding protein. MBP was chosen as fusion partner in order to enhance solubility of Ves v 5 in *E. coli*. MBP-Ves v 5 fusion protein was isolated from the periplasmic fraction and affinity purified by an amylose column. Purified MBP-Ves v 5 was then digested by factor Xa and separated from MBP using size exclusion chromatography. Analysis by SDS-PAGE and Western blot resulted in a protein band of 23 kDa corresponding to the molecular weight of Ves v 5, indicating that MBP was successfully removed from rVes v 5 (data not shown). rVes v 5 was produced with a final yield of 0.6 mg/l bacterial culture.

To generate the cDNA of CRISP-2 and CRISP-3, MegaManTM Human Transcriptome Library was screened and PCR amplified using suitable primers hybridizing at both 5' and 3' ends of the hCRISPs. Truncated forms of hCRISP-2 and hCRISP-3 were then generated by re-amplifying full length cDNAs with primers exclusively complementary to both ends of PR-1 domains.

Sequencing of trunc-hCRISP-2 and trunc-hCRISP-3 revealed correct sequences as reported previously (Kasahara et al., 1989; Kratzschmar et al., 1996). Trunc-hCRISP-3 was produced as a variant with a known single nucleotide polymorphism (SNP) at position 120 (S $\rightarrow$ P, SwissProt ID VAR.011718 in P54108).

Trunc-hCRISP-2 and trunc-hCRISP-3 were produced in *E. coli* using the pQE-30 expression vector. Both proteins were mainly expressed as inclusion bodies in the cytoplasm of *E. coli*. Inclusion bodies were opened using guanidiniumchloride and mainly contained either trunc-hCRISP-2 or trunc-hCRISP-3, respectively, as analyzed by SDS-PAGE (data not shown).

To perform efficient folding of denatured solubilized hCRISP, refolding was performed using a redox refolding buffer. Properly refolded proteins were first concentrated by cation-exchange

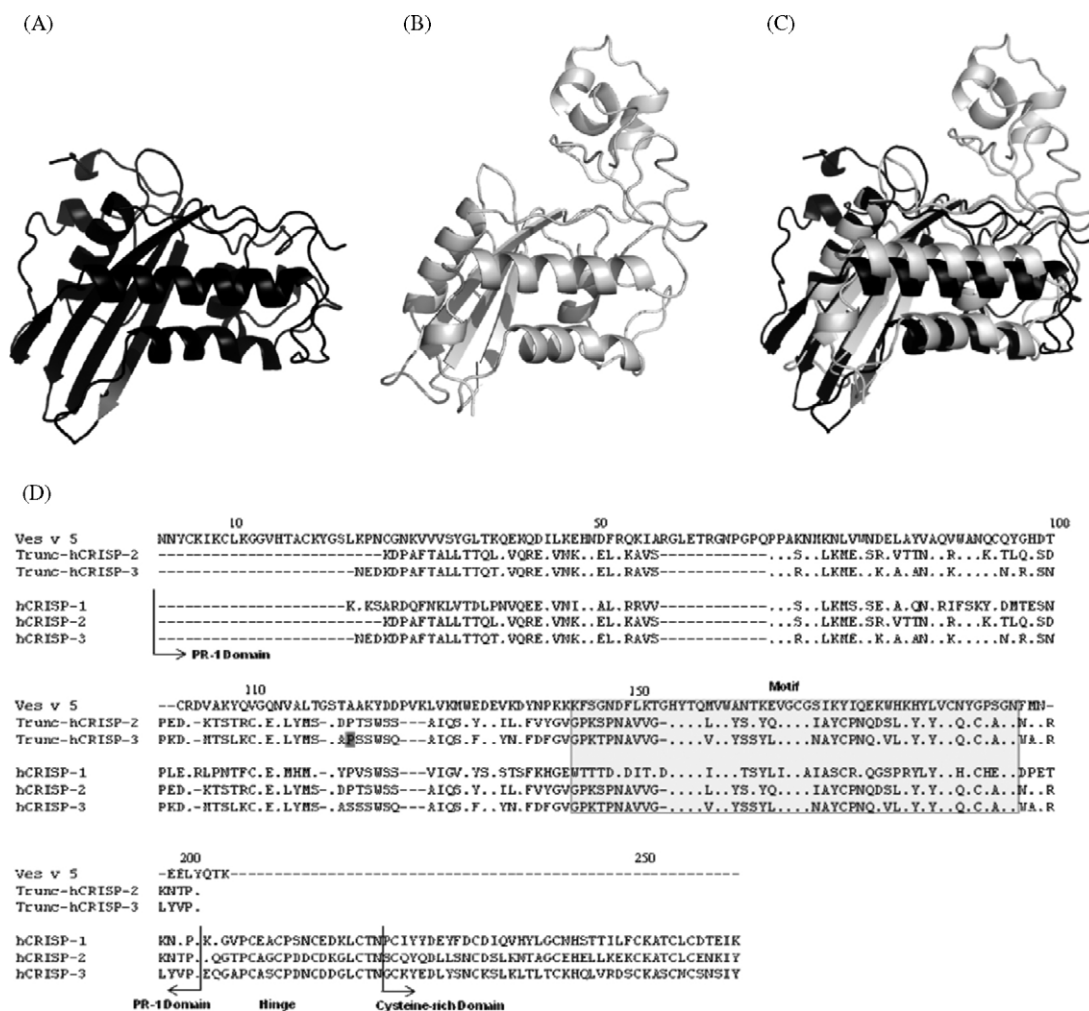


Fig. 1. (A) Protein structure of Ves v 5 (PDB ID: 1QNX¹⁰). (B) Protein structure of hCRISP-3 was modeled by Swiss-Model (Schwede et al., 2003) against stecrisp, a member of the CRISP family from *Trimeresurus stejnegeri* (PDB ID: 1RC9 (Guo et al., 2005)), triflin, a CRISP isolated from the venom of the habu snake *Trimeresurus flavoviridis* (1WVR (Shikamoto et al., 2005)), and natriin toxin from *Naja atra* (1XTA (Wang et al., 2004), 1XX5 (Wang et al., 2005)). (C) Superimposition of Ves v 5 and modeled hCRISP-3 protein. The figure was generated using PyMOL (DeLano, 2002) and Swiss PDB Viewer (Guex and Peitsch, 1997). (D) Protein sequence alignment of Ves v 5 (Lu et al., 1993) from *V. vulgaris*, our produced truncated human CRISP-2/CRISP-3 and the three published full length CRISP-1 (Hayashi et al., 1996), CRISP-2 (Kasahara et al., 1989) and CRISP-3¹⁵. Amino acid identities are indicated by dots. Gaps are indicated by dashes. Numbers above correspond to the protein sequence of Ves v 5. SNP of trunc-hCRISP-3 at pos. 120 is marked in grey. The PR-1, hinge and cysteine-rich domain corresponding to the three CRISPs designated by arrows.

chromatography and then separated from aggregates by size exclusion chromatography. The purity of proteins was checked by SDS-PAGE which showed for both CRISPs a band at 19 kDa corresponding to the truncated forms of hCRISP-2 and -3 (data not shown). A final yield of 5.8 mg for trunc-hCRISP-2 and 2.4 mg for trunc-hCRISP-3, respectively, was achieved per liter bacterial culture.

2.2.3. Antibody recognition of rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3

To determine the specific recognition of to rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 by IgG, ELISA experiments were performed. Wasp positive, MAR positive and control sera were tested on all three recombinant antigens. All wasp positive sera recognized rVes v 5 although these sera were selected according to their IgE reactivity to whole wasp venom extract. Interestingly, these sera also recognized both human trunc-CRISPs to the highest extent. The same results were obtained with MAR positive sera and control sera which showed a similar recognition pattern, although with a lower signal level. Thus, all three types of sera contained IgG specific for rVes v 5 as well as for the truncated forms of the

two autoantigens hCRISP-2 and -3. For each graph *R*-values >0.7 were obtained demonstrating that IgG reactivity against rVes v 5 correlated well with the IgG binding to trunc-hCRISP-2 and trunc-hCRISP-3 (Fig. 2). These data suggest the presence of cross-reactive antibodies in these tested sera.

2.2.4. Isolation and characterization of specific IgG to rVes v 5 and trunc-hCRISP-2

Our previous experiments showed a comparable IgG reactivity to rVes v 5 and trunc-hCRISPs in healthy, MAR- and wasp positive sera. Therefore, in order to investigate whether these cross-reactive antibodies detected in an immune assay may be physically isolated, we used IVIg, representing IgG from a large pool of healthy donors, as an IgG source.

Recombinant Ves v 5 and trunc-hCRISP-2 were coupled to affinity columns and IVIg, previously pre-absorbed on a His-tagged protein to eliminate anti-His-specific IgG, was affinity purified in order to isolated specific IgG to Ves v 5 and hCRISP-2.

ELISA data suggested antibody titers corresponding to specific immune responses, but not to titer as generated by a strong immunogens. Indeed, we isolated 3.3 mg specific anti-Ves v 5

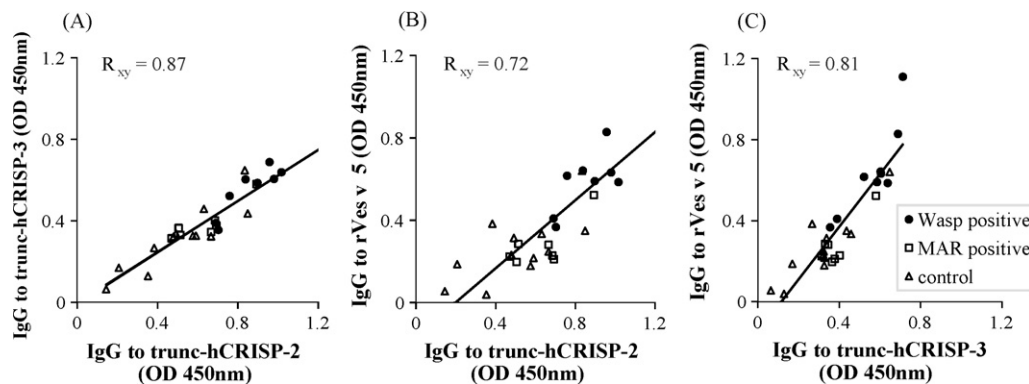


Fig. 2. Correlation of values of serum IgG to (A) trunc-hCRISP-2 and trunc-hCRISP-3. (B) Trunc-hCRISP-2 and rVes v 5. (C) Trunc-hCRISP-3 and rVes v 5. Binding of specific serum IgG to 5 μ g/ml of solid phase coated rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 proteins was analyzed by ELISA. Sera of patients allergic to wasp venom (9 donors), sera containing anti-sperm antibody (7 donors) and sera of healthy individuals (12 donors) were diluted 1:250. Bound IgG was detected using HRP-conjugated anti-IgG (Fc) antibody.

IgG and 3.9 mg specific anti-trunc-hCRISP-2 IgG, respectively, both derived from 3 g of IVIg indicating a higher titer compared to ELISA data. The total yield of specific antibodies might be even higher, since some remaining reactivity to rVes v 5 and trunc-hCRISP-2 could be measured in the corresponding flow through.

These purified specific IgG pools were tested for reactivity against rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 by ELISA (Fig. 3). His-tagged proteins (DARPin D7, D40) were used as controls. As shown in Fig. 3A, purified rVes v 5-specific IgG recognized rVes v 5 best. Interestingly, the same antibodies recognized trunc-hCRISP-3 to a lesser extent but not trunc-hCRISP-2 (Fig. 3A) suggesting these isolated IgG being only weakly cross-reactive.

IVIg purified on trunc-hCRISP-2 was also analyzed for its IgG reactivity to rVes v 5, trunc-hCRISP-2 and -3. The same binding to trunc-hCRISP-2 as well as to trunc-hCRISP-3 and rVes v 5 was found (Fig. 3B). For comparison, only background reactivity was observed to the control proteins. These data indicate that cross-reactive IgG antibodies are present in most human sera and that these antibodies are recognizing epitopes shared by Ves v 5 and hCRISPs.

2.2.5. Affinity purified IgG cross-reacts with rVes v 5 and trunc-hCRISPs

In order to further characterize these cross-reactive IgG antibodies found by affinity purification of IVIg on either rVes v 5 or trunc-hCRISP-2, inhibition experiments were performed.

The first inhibition assay was performed with affinity purified IgG to rVes v 5 using as inhibitor either rVes v 5 or trunc-hCRISP-2. As shown in Fig. 4A, a 10-fold molar excess of rVes v 5 [50 μ g/ml] resulted in an 80% inhibition of IgG binding to solid phase rVes v 5. In contrast, 10-fold molar excess of trunc-hCRISP-2 could only inhibit 10% of IgG reactivity. Interestingly, reactivity to solid phase trunc-hCRISP-3 could be inhibited to more than 80% by a 10-fold molar excess of either rVes v 5 or trunc-hCRISP-2 (Fig. 4B). These data confirm our previous results showing that IgG purified on rVes v 5 best recognized rVes v 5. Furthermore it showed that IgG purified on rVes v 5 not only weakly cross-reacted with trunc-hCRISP-3 but also reacted to trunc-hCRISP-2 as their binding to solid phase trunc-hCRISP-3 could be inhibited by both rVes v 5 and trunc-hCRISP-2.

In a second set of inhibition assays, affinity purified IgG on trunc-hCRISP-2 was tested against rVes v 5 and trunc-hCRISPs. Fig. 5A shows that 10-fold molar excess of trunc-hCRISP-2 inhibited IgG reactivity to solid phase trunc-hCRISP-2 to more than 60%. 10-fold molar excess of rVes v 5 resulted in 30% inhibition

of binding to trunc-hCRISP-2 (Fig. 5A). Equal inhibition was observed when trunc-hCRISP-3 was inhibited with either rVes v 5 or trunc-hCRISP-2, although this time 10-fold molar excess resulted in an inhibition to around 50 and 20%, respectively (Fig. 5B). Finally, affinity purified trunc-hCRISP-2-specific IgG was inhibited with a 10-fold molar excess of either rVes v 5 or trunc-hCRISP-2

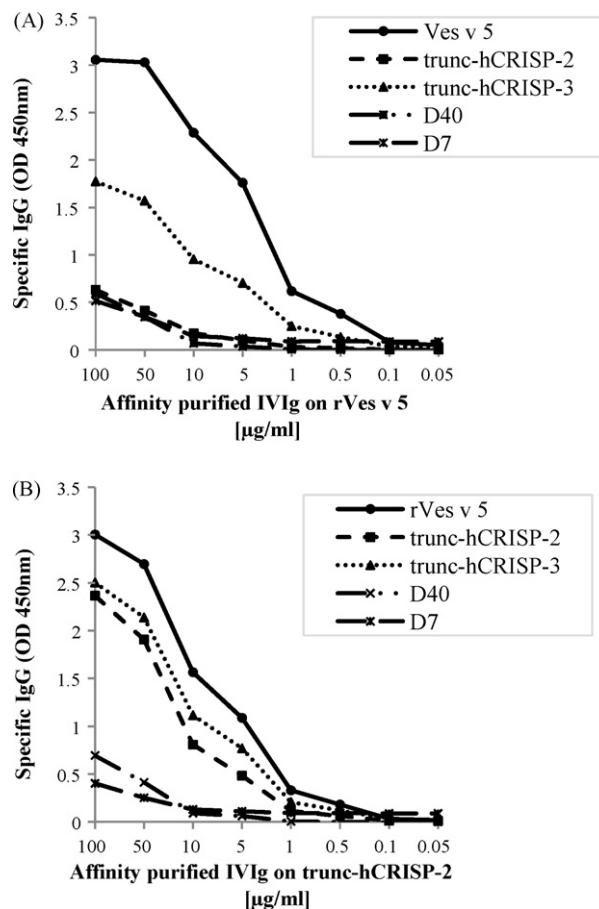


Fig. 3. Reactivity of affinity purified IVIg against rVes v 5 and trunc-hCRISPs. IVIg was purified either on (A) rVes v 5 or on (B) trunc-hCRISP-2. Five different solid phase antigens (rVes v 5, trunc-hCRISP-2, trunc-hCRISP-3, DARPin D40 and D7) were coated at 5 μ g/ml on ELISA plates and incubated with different concentrations of affinity purified IVIg. Values shown represent mean values of duplicates. HRP-conjugated anti-IgG (Fc) antibodies were used for detection.

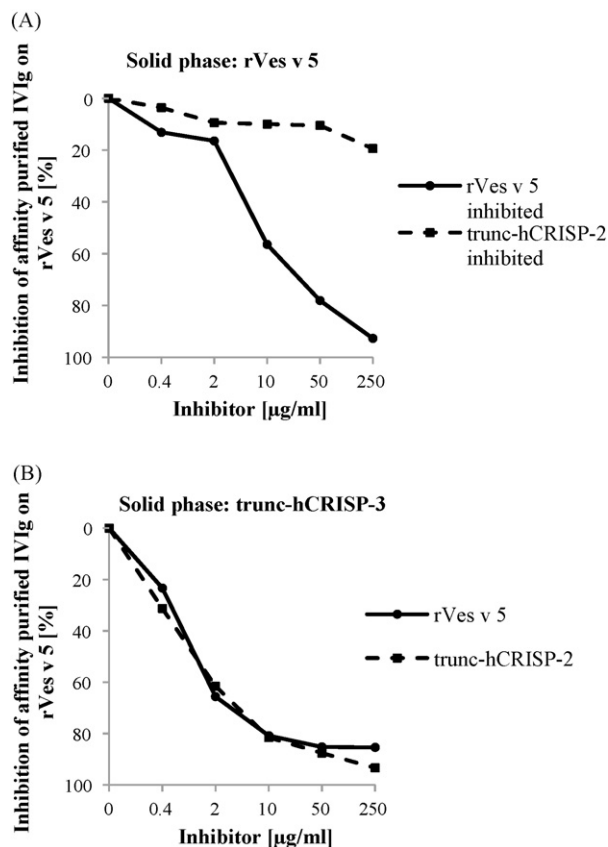


Fig. 4. Inhibition of binding of affinity purified IVIg to rVes v 5 and trunc-hCRISP-3. Affinity purified IVIg on rVes v 5 was pre-incubated with increasing concentration of either rVes v 5 or trunc-hCRISP-2 and were tested on 5 µg/ml coated: (A) rVes v 5 or (B) trunc-hCRISP-3. Mean values of duplicates are shown. D7 was used as an unspecific control and obtained values were subtracted from each data point obtained for rVes v 5 and trunc-hCRISP-2, respectively. HRP-conjugated anti-IgG (Fc) antibody was used for detection.

(Fig. 5C) resulting in an inhibition of around 60% in both cases. This second inhibition assay showed that IgG antibodies, when purified on the putative autoantigen trunc-hCRISP-2, are highly cross-reactive recognizing cross-reactive epitopes on Ves v 5 and trunc-hCRISPs.

Taken together, these data confirm the existence of cross-reactive antibodies in normal human sera and exclude an artifact linked to the use of a small population of different sera.

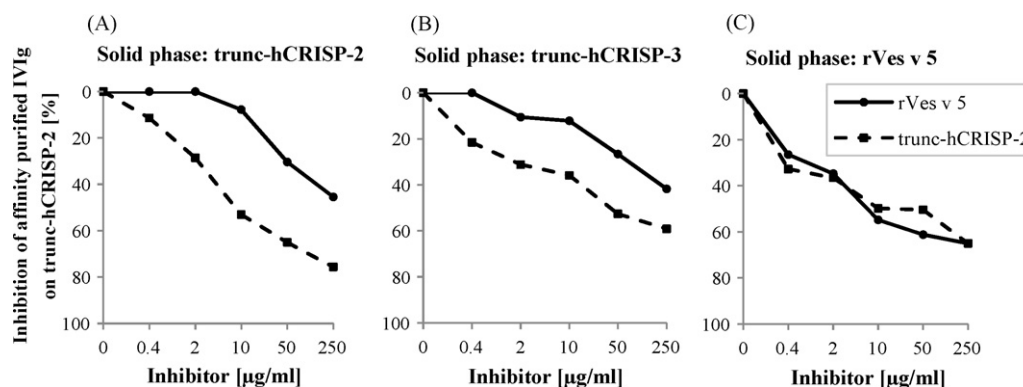


Fig. 5. Inhibition of binding of affinity purified IVIg to rVes v 5 and trunc-hCRISPs. Affinity purified IVIg on trunc-hCRISP-2 was pre-incubated with increasing concentration of either rVes v 5 or trunc-hCRISP-2 and were tested on 5 µg/ml coated: (A) trunc-hCRISP-2, (B) trunc-hCRISP-3 and (C) rVes v 5. Mean values of duplicates are shown. D7 was used as an unspecific control and obtained values were subtracted from each data point calculated for rVes v 5 and trunc-hCRISP-2, respectively. HRP-conjugated anti-IgG (Fc) antibody was used for detection.

2.2.6. IgG recognition of rVes v 5, trunc-hCRISP-2 and -3 is independent of sensitization to wasp venom

Since our original purpose was to investigate whether a link exists between allergic and autoimmune reactions, we analyzed sera, sent to our in-house routine allergy lab with the request to test IgE against wasp venom extract (ImmunoCAP®, common wasp, i3, Pharmacia). 272 sera were selected to achieve two gender and age matched groups. These sera were grouped according to their CAP-class: 146 wasp positive (CAP-class 1–6) and 126 wasp negative (CAP-class 0) and then tested for specific IgG to the three recombinant proteins. A majority of sera recognized all three proteins but most of them had low OD values (Fig. 6). In this wasp venom suspicious group we found between the recognition of hCRISP-2 and hCRISP-3 a correlation coefficients of $R_{xy} = 0.72$ for wasp negative (WN) (Fig. 6A) and $R_{xy} = 0.76$ for wasp positive (WP) (Fig. 6D). This is comparable with our previous finding of the first experiment (Fig. 2). In contrast, lower correlation coefficients were found when comparing rVes v 5 with trunc-hCRISP-2 ($R_{xy} = 0.20$, for WN and $R_{xy} = 0.22$, for WP, Fig. 6B and E) or trunc-hCRISP-3 ($R_{xy} = 0.20$, for WN and $R_{xy} = 0.32$, for WP, Fig. 6C and F), respectively, indicating that cross-reactive antibodies recognizing both Ves v 5 and CRISP proteins are not equally found in this sera population.

Additionally our results suggest that the presence of specific antibodies to Ves v 5 and hCRISPs does not depend on the sensitization state to wasp venom of the donors and that such antibodies can also be found in non-wasp allergic patients.

Analysis of the sera in respect to gender and age of the donor, showed no significant difference in IgG reactivity to rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 comparing female vs. male and children vs. adults, respectively (data not shown).

2.2.7. IgG reactivity does not correlate to CAP-class IgE level

To investigate whether the higher level of anti-sperm antibodies in wasp positive sera is connected to exposure to wasp venom, sera belonging to different CAP-classes were tested for their IgG reactivity towards rVes v 5, trunc-hCRISP-2, and sperm extract (Fig. 7). Fig. 7 shows a similar recognition pattern of high titer IgG to rVes v 5 and sperm extract compared to trunc-hCRISP-2 (and trunc-hCRISP-3, data not shown). However, high levels of IgG to rVes v 5, trunc-hCRISP-2 and sperm extract did not correlate with high IgE binding to wasp venom extract suggesting that allergenicity to wasp venom extract is not linked to an increased IgG reactivity to human autoantigens (hCRISPs).

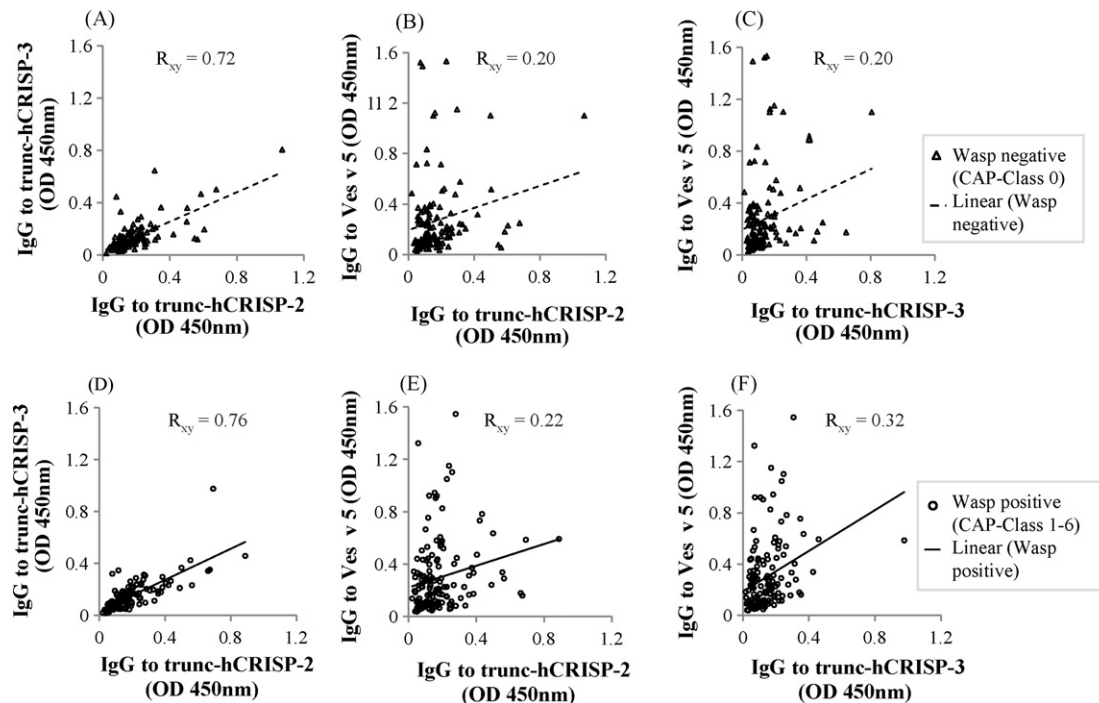


Fig. 6. Correlation of serum IgG values to (A and D) trunc-hCRISP-2 and trunc-hCRISP-3, (B and E) Trunc-hCRISP-2 and rVes v 5, (C and F) Trunc-hCRISP-3 and rVes v 5. Wasp negative sera are shown in (A–C). Wasp positive sera are shown in (D–F). 5 μ g/ml of rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 were coated on ELISA plates. Wasp positive (146) and wasp negative (126), age and sex matched sera, were diluted 1:256 and mean values of duplicates are shown in three correlation diagrams. Bound IgG was detected using HRP-conjugated anti-IgG (Fc) antibody.

2.2.8. Wasp positive sera recognize human sperm extract

As hCRISP-2 and hCRISP-3 represent two proteins present in human sperm, we investigated whether antibodies reacting to Ves v 5 and hCRISPs also recognize human sperms (Kratzschmar et al., 1996). We compared IgG specific for rVes v 5, trunc-hCRISP-2 and trunc-hCRISP-3 with IgG binding to sperm extract (Fig. 8). Interestingly, wasp positive sera better recognized sperm extract proteins compared to sera negative to wasp venom. A slight increase of the correlation coefficient of rVes v 5 and sperm extract recognition could be observed when comparing wasp negative ($R_{xy}=0.16$) and wasp positive ($R_{xy}=0.35$), sera indicating a tendency of higher anti-sperm extract antibody in such sera. This result can be explained by the presence of two additional homologue antigens (hyaluronidase and phospholipase A₁) in sperm which are also found in wasp venom extract.

3. Discussion

Previous studies have postulated that IgE autoreactivity is initiated by sensitization against allergens that share sequence similarity with human proteins (Orenge et al., 1994). Thus in atopic individuals exposure to an exogenous allergens might stimulate the production of cross-reactive autoantibodies. In this study we demonstrate cross-reactivity between Ves v 5 and trunc-hCRISP proteins by isolating cross-reactive antibodies from IVIg, which represents IgG from a large pool of healthy donors. A correlation between these antibodies and CAP-class IgE level to wasp venom extract was not found suggesting that sensitization to wasp does not induce such cross-reactive antibodies.

We carried out our studies because of sequence and structural similarities found between Ves v 5 and hCRISPs. Similarity was detected by using an optimized motif profile to search the

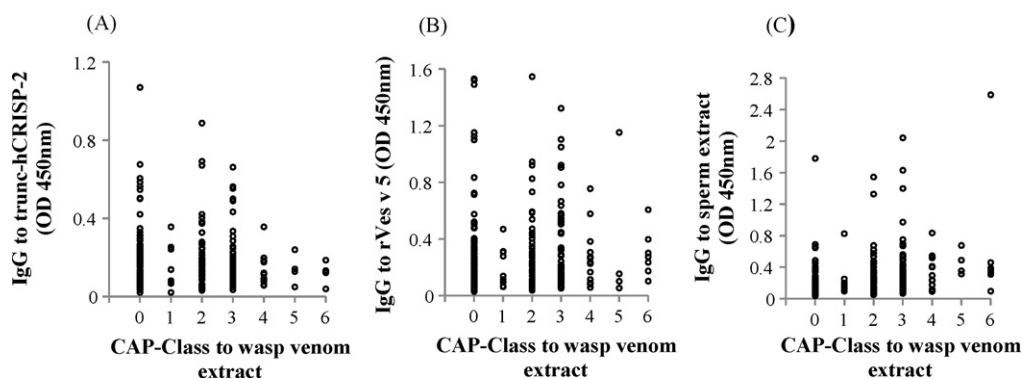


Fig. 7. Correlation between CAP classes and serum IgG specific to (A) trunc-hCRISP-2, (B) rVes v 5 and (C) sperm extract of patients allergic to wasp venom extract. IgG reactivity to trunc-hCRISP-2, rVes v 5 and sperm extract was measured by ELISA (values of Figs. 6 and 8).

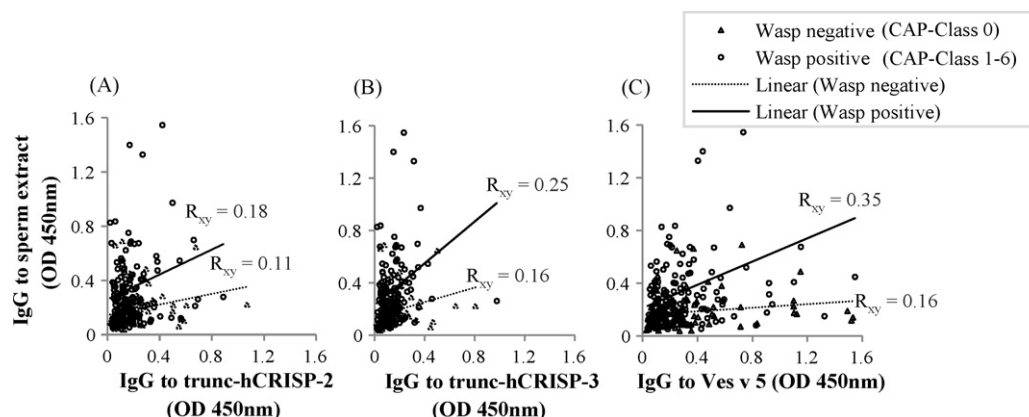


Fig. 8. Correlation values of serum IgG from wasp positive and wasp negative patients to (A) trunc-hCRISP-2 and sperm extract, (B) trunc-hCRISP-3 and sperm extract; (C) rVes v 5 and sperm extract. Sperm extract (1:4 diluted) and 5 μ g/ml of trunc-hCRISP-2, trunc-hCRISP-3 and rVes v 5 were coated on ELISA plates. Wasp positive (146) and wasp negative (126), age and sex matched sera, were diluted 1:256 and mean values of duplicates are shown in three correlation diagrams. Bound IgG was detected using HRP-conjugated anti-IgG (Fc) antibody.

SwissProt database. Comparison of the PDB structures of crystallized Ves v 5 and modeled hCRISP-3 was striking, especially when taken into account that the Ves v 5 PDB structure was excluded from the modeling (Fig. 1). Interestingly, in the work of King and Lu, cross-reactive antibodies were observed in mice immunized with a 61 amino acid stretch of the C-terminal part of the PR-1 domain of either Dol m 5 or TPX-1 that included our motif sequence (King and Lu, 1997).

To test the immunological cross-reactivity between Ves v 5 and trunc-hCRISPs, we used in a first set of experiments a small population of human sera from three different sources (wasp positive, MAR positive and healthy sera). We found a correlation of IgG reactivity between Ves v 5 and trunc-hCRISP-2, -3 and between the two trunc-hCRISP-2 and -3 in all types of sera suggesting cross-reactive antibodies present in human serum. Although hCRISP-2 and -3 are human sperm proteins, no difference in respect to sex and age could be observed, indicating that anti-hCRISPs IgG antibodies can be found independently of gender and stage of life. These results brought us to the hypothesis that these antibodies may be part of the human natural antibody repertoire. Accordingly they may cross-react with other human proteins but that was not the case as blast analysis could not find highly similar known protein sequences to CRISPs in human genome (data not shown).

To investigate whether such antibodies can be isolated from human serum we used IVIg. Remarkably anti-Ves v 5 and anti-trunc-hCRISP-2 IgGs were isolated by affinity purification. By means of inhibition assays we have shown the isolation from IVIg of two populations of IgG that recognize rVes v 5 and trunc-hCRISPs. A first one recognized mostly rVes v 5 and only weakly cross-reacted to the trunc-hCRISPs suggesting the binding of an immunodominant epitope on Ves v 5. In contrast the second one reacted with rVes v 5 and to the same extent with hCRISPs which might indicate that these antibodies recognize a highly cross-reactive epitope present in trunc-hCRISPs as well as in rVes v 5. This is in line with our previous observation that anti-Fc ϵ RI α antibodies with low affinity can be isolated from IVIg that cross-react with an external antigen, tetanus toxoid (TTd) (Horn et al., 1999). These autoantibodies were postulated to belong to the natural antibody repertoire and serve as the parental antibodies for anti-TTd antibodies. In our study the ubiquitous presence of anti-CRISPs antibodies in human sera that cross-react with Ves v 5 may indicate that they also belong to the natural antibody repertoire and may be at the origin of mature anti-Ves v 5 IgG antibodies which only weakly cross-react with trunc-hCRISPs. However this hypothesis needs to be further evalu-

ated by measuring the affinity of the two populations on rVes v 5 and trunc-hCRISPs.

Since for immunological infertility anti-sperm antibodies play a central role, hCRISPs are potential candidates and therefore of particular interest. Anti-sperm antibodies, recently reviewed by Chamley et al., were shown to be mainly of IgG and IgA isotypes and were also found in sera of infertile women and in low titers even in fertile couples (Chamley and Clarke, 2007). Supporting infertility thoughts, CRISPs were reported to be involved in egg/sperm interaction (Busso et al., 2003; Cohen et al., 2007, 2001). Antibodies against hCRISP-1 and -2 have been reported to inhibit sperm/egg penetration in an artificial fertilization test (Busso et al., 2005; Cohen et al., 2001). Hence, the role of cross-reactive antibodies between an allergen Ves v 5 and self-proteins such as CRISPs would be of interest in the field of immunological infertility. So far the presence of anti-sperm antibodies in men has been correlated to conditions that disrupt the blood testis barrier between the male reproductive tract and the immune system (Fuchs and Alexander, 1983; Haas and Beer, 1986). However through the 'hidden' character of antigens in the male human reproductive tract peripheral tolerance is limited and therefore an autoimmunity is conceivable.

In our study no correlation could be observed between anti-trunc-hCRISPs, anti-sperm IgG and CAP-class IgE level to wasp venom extract. This means that exposure to wasp does not result in a higher induction of autoantibodies. In contrast individuals who belong to a higher CAP-class seem to have less IgG antibodies against sperm extract and trunc-hCRISPs. These results are in line with our previous observation, namely that in IVIg there is a population of IgG that mostly react with Ves v 5 and only weakly react with trunc-hCRISPs. Thus one can assume that this population arises after high exposure to wasp venom which would be the case in individuals having a CAP-class of 5 and 6. Because IVIg contains a second population of IgG cross-reacting at the same extent with both Ves v 5 and trunc-hCRISPs, these antibodies may have undergone affinity maturation that increased their specificity to wasp venom extract and to Ves v 5. As our data showed an unexpected low anti-Ves v 5 IgG reactivity in individuals of CAP-class 5 and 6, it is possible that these cross-reactive antibodies may have, upon higher exposure, undergone class switch to give antibodies of other isotypes such as IgG4 or IgE. Thus determination of Ig isotypes using appropriate developing reagent would help to answer this question. It has been previously shown that IgG4 antibodies are the ones which show high specificity to the allergens. In contrast, IgG1 antibodies are ubiquitously found and are also present in non-atopic individuals (Olsson et al., 2001).

Another explanation might be given for the relative low anti-Ves v 5 IgG reactivity found in individuals with CAP-class 5 and 6. Using a larger population of sera of donors a tendency was observed for wasp positive sera to have a higher amount of IgG antibodies against sperm extract. This reactivity was independent of the IgG response to hCRISP-2, -3 and to Ves v 5 and might be the result of sensitization against other allergens in wasp venom that cross-react with human proteins in sperm extract. In fact it has been shown that wasp venom contains two other major venom allergens, hyaluronidase and phospholipase A₁ which have partial sequence similarity with their human homologous present in sperm (Lu et al., 1995). Further experiments using the two other major venom allergens as targets and other types of sera such as MAR positive sera would help to understand whether a correlation might exist between anti-sperm antibodies and wasp allergens.

In summary we have been able to demonstrate cross-reactivity between a wasp venom allergen, Ves v 5 and two human CRISP proteins (CRISP-2 and -3). However this cross-reactivity does not correlate with the level of sensitization to wasp venom and thus seems not to represent a risk for inducing autoimmunity that may lead to infertility.

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References

- Bucher, P., Karplus, K., Moeri, N., Hofmann, K., 1996. A flexible motif search technique based on generalized profiles. *Comput. Chem.* 20, 3–23.
- Busso, D., Cohen, D.J., Da Ros, V., Fissore, R., Cuasnicu, P.S., 2003. Studies on the participation of epididymal sperm protein DE/CRISP-1 in egg activation. *Cell Mol. Biol. (Noisy-le-grand)* 49, 407–412.
- Busso, D., Cohen, D.J., Hayashi, M., Kasahara, M., Cuasnicu, P.S., 2005. Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. *Mol. Hum. Reprod.* 11, 299–305.
- Chamley, L.W., Clarke, G.N., 2007. Antisperm antibodies and conception. *Semin Immunopathol.* 29, 169–184.
- Cohen, D.J., Da Ros, V.G., Busso, D., Ellerman, D.A., Maldera, J.A., Goldweic, N., Cuasnicu, P.S., 2007. Participation of epididymal cysteine-rich secretory proteins in sperm-egg fusion and their potential use for male fertility regulation. *Asian J. Androl.* 9, 528–532.
- Cohen, D.J., Ellerman, D.A., Busso, D., Morgenfeld, M.M., Piazza, A.D., Hayashi, M., Young, E.T., Kasahara, M., Cuasnicu, P.S., 2001. Evidence that human epididymal protein ARP plays a role in gamete fusion through complementary sites on the surface of the human egg. *Biol. Reprod.* 65, 1000–1005.
- DeLano, W.L., 2002. The PyMOL Molecular Graphics System. World Wide Web, <http://www.pymol.org>.
- Eberspaecher, U., Roosterman, D., Kratzschmar, J., Haendler, B., Habenicht, U.F., Becker, A., Quensel, C., Petri, T., Schleuning, W.D., Donner, P., 1995. Mouse androgen-dependent epididymal glycoprotein CRISP-1 (DE/AEG): isolation, biochemical characterization, and expression in recombinant form. *Mol. Reprod. Dev.* 42, 157–172.
- Fluckiger, S., Scapozza, L., Mayer, C., Blaser, K., Folkers, G., Cramer, R., 2002. Immunological and structural analysis of IgE-mediated cross-reactivity between manganese superoxide dismutases. *Int. Arch. Allergy Immunol.* 128, 292–303.
- Fuchs, E.F., Alexander, N.J., 1983. Immunologic considerations before and after vasovasostomy. *Fertil. Steril.* 40, 497–499.
- Gribskov, M., McLachlan, A.D., Eisenberg, D., 1987. Profile analysis: detection of distantly related proteins. *Proc. Natl. Acad. Sci. U.S.A.* 84, 4355–4358.
- Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 18, 2714–2723.
- Guo, M., Teng, M., Niu, L., Liu, Q., Huang, Q., Hao, Q., 2005. Crystal structure of the cysteine-rich secretory protein stecrisp reveals that the cysteine-rich domain has a K⁺ channel inhibitor-like fold. *J. Biol. Chem.* 280, 12405–12412.
- Haas Jr., G.G., Beer, A.E., 1986. Immunologic influences on reproductive biology: sperm gametogenesis and maturation in the male and female genital tracts. *Fertil. Steril.* 46, 753–766.
- Hayashi, M., Fujimoto, S., Takano, H., Ushiki, T., Abe, K., Ishikura, H., Yoshida, M.C., Kirchhoff, C., Ishibashi, T., Kasahara, M., 1996. Characterization of a human glycoprotein with a potential role in sperm-egg fusion: cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEG1). *Genomics* 32, 367–374.
- Henriksen, A., King, T.P., Mirza, O., Monsalve, R.I., Meno, K., Ipsen, H., Larsen, J.N., Gajhede, M., Spangfort, M.D., 2001. Major venom allergen of yellow jackets. Ves v 5: structural characterization of a pathogenesis-related protein superfamily. *Proteins* 45, 438–448.
- Hoffman, D.R., 1993. Allergens in Hymenoptera venom. XXV: The amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity. *J. Allergy Clin. Immunol.* 92, 707–716.
- Horn, M.P., Gerster, T., Ochensberger, B., Derer, T., Kricke, F., Jouvin, M.H., Kinet, J.P., Tschernig, T., Vogel, M., Stadler, B.M., Miescher, S.M., 1999. Human anti-FcεR1α autoantibodies isolated from healthy donors cross-react with tetanus toxoid. *Eur. J. Immunol.* 29, 1139–1148.
- Jager, S., Kremer, J., van Slochteren-Draaisma, T., 1978. A simple method of screening for antisperm antibodies in the human male. Detection of spermatozoal surface IgG with the direct mixed antiglobulin reaction carried out on untreated fresh human semen. *Int. J. Fertil.* 23, 12–21.
- Kasahara, M., Gutknecht, J., Brew, K., Spurr, N., Goodfellow, P.N., 1989. Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics* 5, 527–534.
- King, T.P., Lu, G., 1997. Hornet venom allergen antigen 5. Dol m 5: its T-cell epitopes in mice and its antigenic cross-reactivity with a mammalian testis protein. *J. Allergy Clin. Immunol.* 99, 630–639.
- Kjeldsen, L., Cowland, J.B., Johnsen, A.H., Borregaard, N., 1996. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett.* 380, 246–250.
- Klemme, L.M., Roberts, K.P., Hoffman, L.B., Ensrud, K.M., Siiteri, J.E., Hamilton, D.W., 1999. Cloning and characterization of the rat Crisp-1 gene. *Gene* 240, 279–288.
- Kratzschmar, J., Haendler, B., Eberspaecher, U., Roosterman, D., Donner, P., Schleuning, W.D., 1996. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. *Eur. J. Biochem.* 236, 827–836.
- Lu, G., Kochoumian, L., King, T.P., 1995. Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. *J. Biol. Chem.* 270, 4457–4465.
- Lu, G., Villalba, M., Coscia, M.R., Hoffman, D.R., King, T.P., 1993. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. *J. Immunol.* 150, 2823–2830.
- Maeda, T., Nishida, J., Nakanishi, Y., 1999. Expression pattern, subcellular localization and structure–function relationship of rat Tpx-1, a spermatogenic cell adhesion molecule responsible for association with Sertoli cells. *Dev. Growth Differ.* 41, 715–722.
- Marti, P., Truffer, R., Stadler, M.B., Keller-Gautschi, E., Cramer, R., Mari, A., Schmid-Grendelmeier, P., Miescher, S.M., Stadler, B.M., Vogel, M., 2007. Allergen motifs and the prediction of allergenicity. *Immunol. Lett.* 109, 47–55.
- Mayer, C., Appenzeller, U., Seelbach, H., Achatz, G., Oberkofler, H., Breitenbach, M., Blaser, K., Cramer, R., 1999. Humoral and cell-mediated autoimmune reactions to human acidic ribosomal P2 protein in individuals sensitized to *Aspergillus fumigatus* P2 protein. *J. Exp. Med.* 189, 1507–1512.
- Olsson, S., van Hage-Hamsten, M., Magnusson, C.G., 2001. IgG1, IgG4 and IgE antibody reactivity to mutant forms of the major dust mite allergen Lep d 2 among atopic and nonatopic subjects naturally exposed to *Lepidoglyphus destructor*. *Int. Arch. Allergy Immunol.* 126, 50–58.
- Orengo, C.A., Jones, D.T., Thornton, J.M., 1994. Protein superfamilies and domain superfolds. *Nature* 372, 631–634.
- Pagni, M., Jongeneel, C.V., 2001. Making sense of score statistics for sequence alignments. *Brief Bioinform.* 2, 51–67.
- Schambony, A., Gentzel, M., Wolfes, H., Raida, M., Neumann, U., Topfer-Petersen, E., 1998. Equine CRISP-3: primary structure and expression in the male genital tract. *Biochim. Biophys. Acta* 1387, 206–216.
- Scheppler, L., Vogel, M., Marti, P., Müller, L., Miescher, S.M., Stadler, B.M., 2005. Intranasal immunisation using recombinant *Lactobacillus johnsonii* as a new strategy to prevent allergic disease. *Vaccine* 23, 1126–1134.
- Schwede, T., Kopp, J., Guex, N., Peitsch, M.C., 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31, 3381–3385.
- Shikamoto, Y., Suto, K., Yamazaki, Y., Morita, T., Mizuno, H., 2005. Crystal structure of a CRISP family Ca²⁺-channel blocker derived from snake venom. *J. Mol. Biol.* 350, 735–743.
- Sivashanmugam, P., Richardson, R.T., Hall, S., Hamil, K.G., French, F.S., O'Rand, M.G., 1999. Cloning and characterization of an androgen-dependent acidic epididymal glycoprotein/CRISP1-like protein from the monkey. *J. Androl.* 20, 384–393.
- Stadler, M.B., Stadler, B.M., 2003. Allergenicity prediction by protein sequence. *FASEB J.* 17, 1141–1143.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Udby, L., Calafat, J., Sorensen, O.E., Borregaard, N., Kjeldsen, L., 2002a. Identification of human cysteine-rich secretory protein 3 (CRISP-3) as a matrix protein in a subset of peroxidase-negative granules of neutrophils and in the granules of eosinophils. *J. Leukoc. Biol.* 72, 462–469.
- Udby, L., Cowland, J.B., Johnsen, A.H., Sorensen, O.E., Borregaard, N., Kjeldsen, L., 2002b. An ELISA for SGP28/CRISP-3, a cysteine-rich secretory protein in human. *J. Immunol. Methods* 263, 43–55.

- Valenta, R., Natter, S., Seiberler, S., Grote, M., 1997. Isolation of cDNAs coding for IgE autoantigens: a link between atopy and autoimmunity. *Int. Arch. Allergy Immunol.* 113, 209–212.
- Valenta, R., Seiberler, S., Natter, S., Mahler, V., Mossabeh, R., Ring, J., Stingl, G., 2000. Autoallergy: a pathogenetic factor in atopic dermatitis? *J. Allergy Clin. Immunol.* 105, 432–437.
- Vogel, M., Keller-Gautschi, E., Baumann, M.J., Amstutz, P., Ruf, C., Kricek, F., Stadler, B.M., 2007. Designed ankyrin repeat proteins as anti-idiotypic-binding molecules. *Ann. N. Y. Acad. Sci.* 1109, 9–18.
- Wang, J., Shen, B., Guo, M., Lou, X., Duan, Y., Cheng, X.P., Teng, M., Niu, L., Liu, Q., Huang, Q., Hao, Q., 2005. Bocking effect and crystal structure of natrin toxin, a cysteine-rich secretory protein from *Naja atra* venom that targets the BKCa channel. *Biochemistry* 44, 10145–10152.
- Wang, Y.L., Goh, K.X., Wu, W.G., Chen, C.J., 2004. Purification, crystallization and preliminary X-ray crystallographic analysis of cysteine-rich secretory protein (CRISP) from *Naja atra* venom. *Acta Crystallogr D Biol Crystallogr.* 60, 1912–1915.
- Wood, C.L., Timmons, B.E.T., Hoffman, D.R., 1983. Allergens in hymenoptera venoms. X. Vespidae venoms versus venom sac extracts: comparison by two-dimensional polyacrylamide gel electrophoresis. *Ann. Allergy* 51, 441–445.