

Increased Level of Antibodies Cross-Reacting with Ves v 5 and CRISP-2 in MAR-Positive Patients

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Key Words

Anti-sperm antibodies · CRISP-2 · Cross-reactivity · Immunological infertility · Ves v 5

Abstract

Background: Anti-sperm antibodies (ASA) have been described to be involved in immunological infertility. A possible antigen for ASA is the human cysteine-rich secretory protein 2 (CRISP-2), a sperm surface protein important in sperm-oocyte interaction. Furthermore, anti-CRISP-2 antibodies were shown to decrease fertility rates in vitro. Recently, we have reported cross-reacting antibodies recognizing CRISP-2 and antigen 5 from yellow jacket venom (Ves v 5) in human serum. **Methods:** Here, we investigated anti-Ves v 5 and CRISP-2 antibodies in sera from two groups of donors: MAR+ and MAR– patients. **Results:** A higher incidence of allergy against hymenoptera venom was found in MAR+ patients. Interestingly, affinity-purified ASA from MAR+ patients' sera reacted against both Ves v 5 and CRISP-2, leading to sperm immobilization. Immunofluorescence analysis showed that ASA bound to the sperm surface, including the head part where CRISP-2 is localized. **Conclusion:** Taken together, these results showed a higher incidence of antibodies cross-

reacting with Ves v 5 and CRISP-2 in MAR+ patients. This leads to the hypothesis that MAR+ patients may have a higher risk to develop wasp allergy.

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Introduction

Anti-sperm antibodies (ASA) have been considered as a possible reason for immunological infertility. They can be detected in the seminal fluid where they bind to the sperm surface. The effects of ASA on sperm functions are very heterogeneous, as they may impair different steps of the fertilization process. ASA are described to reduce cervical mucus penetration [1, 2], reduce or increase capacitation and acrosome reaction [3, 4], inhibit the binding of the zona pellucida [5, 6] and oolema binding, and affect the sperm-egg fusion [7, 8] and the embryonic development, implantation and miscarriages [9, 10].

The incidence of ASA in infertile couples has been estimated between 6.2 and 30.3% [1, 11–14]. However ASA may also be present in both fertile men and women [14, 15]. This indicates that not all ASA cause infertility and their role in reproduction remains questionable.

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The controversial role of ASA may be linked to methodological problems used for their detection. Nowadays, several ways of detecting ASA in either semen or serum are available: tube slide agglutination test, gelatin agglutination test, sperm immobilization test, immunobead test and mixed antiglobulin reaction (MAR) test. However, just two of them are recommended by the WHO and thus performed in a routine laboratory: the immunobead test and the MAR test [16]. Both tests can be applied either as 'direct' test for the detection of antibodies bound to the sperm or as 'indirect' test for the detection of ASA in sperm-free fluids such as serum, or in our case in ASA-enriched serum. Both tests only show the binding of ASA on the sperm surface, but they do not give any information about the antigenic specificity.

Several membrane proteins that are specifically recognized by ASA in the seminal plasma have been characterized. Bohring and Krause [17] described 18 antigens that react with ASA from seminal plasma of infertile patients. Among them, 6 were identified as membrane proteins that may occur on the sperm surface. More recently, the group of de Mateo et al. [18] extracted spermatozoa from infertile patients' and healthy donors' ejaculates, analyzed them by 2D gel electrophoresis and quantified the expression of 101 proteins by MALDI-TOF analysis. Nevertheless, they largely remain uncharacterized, especially regarding their role on male infertility.

One antigen that has been described to be recognized by ASA is CRISP-2 (cysteine-rich secretory protein), also known as TPX-1. CRISP-2 was first described as an auto-antigen in the acrosome of guinea pig sperm [19]. Today, it is known that CRISP-2 is also expressed on the equatorial segment of the acrosome of human sperm [20]. Further, CRISP-2 was reported to remain attached to the sperm acrosomal region even after capacitation and acrosomal reaction. The additional observation that anti-CRISP-2 antibodies produce a significant reduction in the fertilization rates of zona-free hamster oocytes indicates that CRISP-2 participates in the sperm-egg fusion process [20, 21].

In a previous study, we have shown structural similarities between the major wasp venom allergen Ves v 5 from *Vespula vulgaris*, also designated antigen 5, and CRISP-2 [22]. Ves v 5 is a 23-kDa single-chain polypeptide and belongs to a superfamily of extracellular proteins present in different species such as mammals, plants, reptiles, fungi and insects. So far, its biological function remains unknown [23, 24]. In human sera, antibodies cross-reacting with Ves v 5 and CRISP-2 were found. However, a correlation between these antibodies and

wasp allergy was not observed, as wasp-allergic patients did not have more specific antibodies to Ves v 5 and CRISP-2. But the question remains open whether such antibodies may be important in other types of sera such as MAR+ sera containing ASA.

Thus, in the present study, we analyzed purified ASA from sera for binding to CRISP-2 and Ves v 5 and sperm immobilization. From MAR+ and MAR- patients' serum, ASA were enriched on healthy donor sperm. Such enriched antibody reacts against Ves v 5 and CRISP-2 and, more importantly, leads to sperm immobilization. Furthermore, immunofluorescence analysis provides evidence that these antibodies recognize CRISP-2 and other epitopes on the sperm surface.

Materials and Methods

Study Population

This study was approved by the Cantonal Ethics Committee of Aarau, Switzerland, and all participating patients had to sign an informed consent. All patients undergoing a fertility checkup in our laboratory were evaluated for study participation. Therefore, semen was analyzed according to the 5th edition of the WHO laboratory manual [16]. The presence of ASA was investigated using MAR test. If, for any reason, the MAR test could not be performed, patients were excluded from our study. Based on MAR test results, the patients were separated into two groups (table 1): MAR- patients (n = 20) and MAR+ patients (n = 18). In addition to the ejaculates, 15 ml of sera were collected from all the participants. Furthermore, the patients filled out a questionnaire regarding medication, allergies and offspring.

ASA-free healthy donor sperm with sperm motility of at least 25% was used for ASA isolation, indirect MAR test and immunofluorescence. Semen specimens were collected by masturbation after a period of 24–72 h of sexual abstinence. The samples were either used directly or stored for maximally 1 h at 37°C.

Direct and Indirect MAR Test

For the detection of anti-sperm IgG and IgA, the spermMAR-IgG test (FertiPro NV, Beernem, Belgium) was used. This test can be used either as 'direct MAR test' for the detection of surface-bound ASA or as 'indirect MAR test' for the detection of ASA in sperm-free fluid, such as sera.

The tests were performed according to the manufacturer's instructions. Briefly, for the direct MAR test, 10 µl of patients' ejaculate were incubated with 10 µl of human IgG-coated latex beads and cross-linked with 10 µl of anti-human Fab IgG antiserum. The mixture was covered with a cover glass and incubated for 3 min at room temperature before 100 motile sperm cells were counted under the light microscope at ×40 magnification.

The indirect MAR test was performed on ASA-free healthy donor ejaculates, which were preincubated for 1 h at 37°C with sera of MAR+ and MAR- patients, IgG preparations from a large pool of healthy donors (IVIg), affinity-purified IgG on Ves v 5, CRISP-2 and control proteins, as well as ASA extracted from in-

fertile patients' sera. Sera used for the indirect MAR test were inactivated for 45 min at 57°C in order to get rid of interfering components.

Results for both tests were given as percent of agglutinated sperms. If more than 10% of the motile sperm were immobilized, the patient was considered as MAR+.

ASA Isolation from Serum

In order to enrich the concentration of ASA in the sera of MAR+ and MAR- patients, ASA were isolated as described by Shohat et al. [25] with some improvements. Briefly, healthy donor sperm was collected and washed 3 times with PBS at room temperature at 3,000 g. Then 20–40 × 10⁶ sperm cells were incubated with 2 ml of MAR+ patient sera for 2 h at 37°C. Afterwards sperm was washed once with PBS at room temperature at 3,000 g. ASA were then eluted from the pellet using 0.4 ml of 0.2 M glycine-HCl buffer (pH 2.7) at 37°C for 10 min and separated by centrifugation. The supernatant was neutralized with 1 M Tris buffer to pH 7.4 and then used immediately or stored at –20°C.

Determination of Specific IgE and IgG in MAR+ and MAR- Sera

To investigate the amount of total and specific IgE and specific IgG in the sera of MAR+ and MAR- patients, the ImmunoCAP system (Phadia, Uppsala, Sweden) was used. Specific antibodies directed against sperm extract (ImmunoCAP®, human sperm extract, o70), Ves v 5 (ImmunoCAP®, recombinant Ves v 5, i209) and wasp venom extract (ImmunoCAP®, common wasp, i3; all Phadia) were assessed (expressed in mg/l for IgG and in kU/l for IgE; 1 kU/l = 2.4 ng IgE/ml).

Determination of Specific IgG by ELISA

ELISA was performed to evaluate the specificity of purified ASA against Ves v 5 and human CRISP-2. Recombinant proteins at a concentration of 5 µg/ml were coated on half-well flat-bottom Costar EIA/RIA plates (Costar, Cambridge, Mass., USA) overnight at 4°C. Plates were washed with PBS and blocked with PBS containing 0.15% casein for 2 h at 4°C. Sera as well as ASA-containing eluates from MAR+ patients' sera were serially diluted in blocking solution (1:2 to 1:100) and incubated for 2 h at 37°C by shaking at 320 rpm. After washing with PBS containing 0.01% Tween 20 and PBS, binding antibodies were detected with HRP-conjugated polyclonal anti-human IgG antibodies (Binding Site Group Ltd., Birmingham, UK) at a dilution of 1:1,000 in blocking solution for 1 h at 4°C. Antibody reaction was visualized with 3,3',5,5'-tetramethylbenzidine and the reaction was stopped after 5 min with 1 M H₂SO₄. The plates were then read at an optical density (OD) of 450 nm (ELISA reader EL808; Bio-Tek, Bad Friedrichshall, Germany). p values and means were calculated by GraphPad Prism (version 5.0c). Significant differences were defined as p < 0.05.

Detection of ASA Binding on Sperm by Immunofluorescence

Fresh, ASA-free sperm cells of healthy donors were used for these experiments. By 30 min after ejaculation, viable sperm cells were isolated by the swim-up method: 700 µl of Ringer lactate buffer (pH 7.4; Sintetica-Bioren SA, Couvet, Switzerland) were added to 5-ml round-bottom plastic tubes. Thereafter, 300 µl of ejaculate were gently placed onto the bottom of the tube. After a 1-hour incubation at 37°C at a 45° angle, 700 µl from the upper

Table 1. Basic data of the study population

| | MAR+ patients | MAR- patients |
|--|---------------|---------------|
| Patients, n | 18 | 20 |
| Mean age, years | 44.9 | 38.5 |
| Range | 38–56 | 27–59 |
| Mean agglutinated sperm by anti-sperm, % | | |
| IgG | 41.2 | 0 |
| IgA | 7.1 | 0 |
| Mean total IgE, kU/l | 81.4 | 71.3 |
| Questionnaire-based anamnesis | | |
| Allergy | 7/18 | 4/20 |
| Hymenoptera allergy | 2/18 | 0/20 |

part of each tube were pooled together. The sperm cell amount was adjusted to 6 × 10⁶ sperm cells/ml; 10 µl of the sperm preparation were then smeared onto a poly-L-lysine-coated glass slide and dried at 37°C. Next, 100 µl of ASA (diluted 1:2 in PBS containing 20% BSA) were added onto the immobilized sperm cells and incubated in a humid atmosphere for 1 h at 37°C. The slides were washed with 0.1 M PBS containing 5% BSA. Sperm cells were then incubated with a secondary fluorescein isothiocyanate (FITC)-conjugated primate-absorbed anti-human IgG (Inova Diagnostics Inc., San Diego, Calif., USA). After a 30-min incubation at room temperature in the dark, the slides were washed with 0.1 M PBS containing 5% BSA. As controls, slides incubated with the secondary antibody only without ASA as well as slides incubated with ASA-positive and -negative control sera were used. Propidium iodide staining (95–98%; TCL; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used at a 1:10⁶ dilution for counterstaining in order to localize the sperm head. Ficoll was used as mounting medium and anti-fading substance and cover glasses were added before samples were investigated under the fluorescence microscope.

Results

ASA in Human Ejaculates

Previous studies have shown that the wasp venom allergen Ves v 5 and CRISP-2 have a high structural similarity [22, 26]. Recently, we showed that cross-reactive antibodies reacting with Ves v 5 and CRISP-2 are present in human sera [22]. As CRISP-2 antibodies have been associated with a decrease in fertility rate [21] we investigated this cross-reactivity in two donor groups: MAR+ and MAR- patients. As listed in table 1, MAR+ patients have elevated IgG and/or IgA ASA in their ejaculates. According to the WHO guidelines, patients with sperm immobilization >10% are considered MAR+ [16].

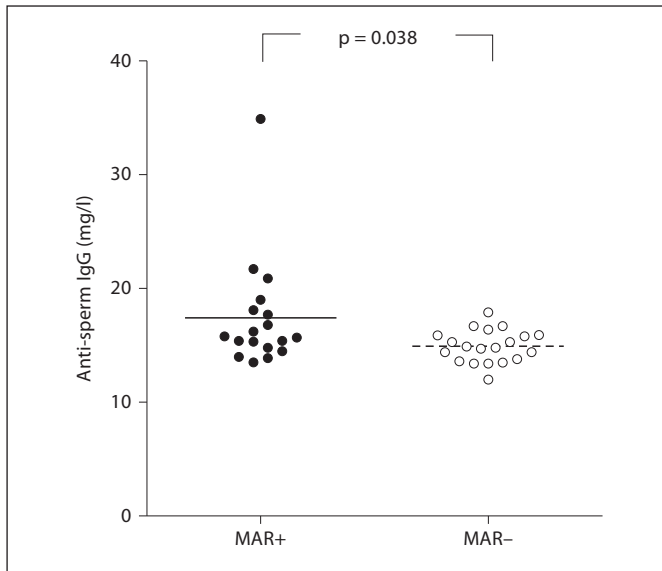


Fig. 1. Measurement of anti-sperm IgG in sera using the ImmunoCAP assay. Sera of MAR+ (●) and MAR- (○) patients were incubated on sperm extract proteins immobilized on the solid phase of the ImmunoCAP system. Bound antibodies were detected with fluorescence-labeled anti-human IgG and fluorescence was assessed with a laser scanner. A statistically significant difference was noted between MAR+ and MAR- patients for anti-sperm extract specific IgG.

ASA in Human Sera

In order to investigate whether ASA are restricted to ejaculates only, we tested the sera of the MAR+ and MAR- patients for the presence of specific sperm extract IgG and IgE by ImmunoCAP. Figure 1 shows that in both patient groups anti-sperm IgG was detectable. However, sperm anti-IgG levels were higher in MAR+ patients ($p = 0.038$). These results indicate that anti-sperm reactivity is not limited to the ejaculates but also detectable in the sera of both groups. In contrast, no IgE levels against sperm extract could be reported in both groups (data not shown).

Questionnaire-Based Allergy Profiling

As we intended to investigate a potential link between autoimmunity and wasp venom allergy, participants were requested to fill out a questionnaire concerning their allergies (table 1). In the group of MAR- patients, only 4 of 20 patients reported any allergy. In contrast, 7 of 18 MAR+ patients suffered from allergies and 2 of them were allergic to hymenoptera including wasp and bee venom. These results suggest a higher prevalence of allergy i.e. hymenoptera allergy in MAR+ than in MAR- patients.

Determination of Total and Specific IgE

The same sera were tested for the presence of specific IgE against wasp venom extract (i3) and Ves v 5 by ImmunoCAP. Variable specific IgE levels were found in MAR+ and MAR- patients, but significant differences were neither observed for total IgE ($p = 0.691$) nor for wasp venom extract ($p = 0.458$) or Ves v 5 ($p = 0.465$) specific IgE (fig. 2).

Specific IgG against Ves v 5 and CRISP-2

To investigate the presence of specific IgG in the sera of MAR+ and MAR- patients, sera were tested against Ves v 5 and CRISP-2 by ELISA. No significant difference could be found between the sera of MAR+ and MAR- patients for both specific IgG (data not shown). However, as the signal in this test was only barely above the detection limit, further analysis for the detection of specific IgG to Ves v 5 and wasp venom extract in MAR+ and MAR- patients' sera was performed by ImmunoCAP, a quantitative standardized method routinely used for the determination of specific IgE [27]. Figure 3 shows that again no significant difference in wasp venom extract-specific IgG was detected in MAR+ and MAR- patients ($p = 0.3$). However, the level of Ves v 5 specific IgG was significantly higher in MAR+ than in MAR- patients ($p = 0.0042$), suggesting that anti-Ves v 5 antibodies might be of importance in MAR+ patients.

Sperm Agglutination by Affinity-Purified Antibodies

In a former study using an IgG preparation of a large pool of healthy donors (IVIg), we isolated IgG that recognized Ves v 5 and CRISP-2 [22]. As CRISP-2 is a sperm surface antigen, we analyzed here whether such antibodies may induce sperm agglutination in indirect MAR tests by incubating such Ves v 5 or CRISP-2 affinity-purified IgG on ASA-free ejaculates. Both anti-Ves v 5 and anti-CRISP-2 IgG induced sperm agglutination in a concentration-dependent manner indicating that both types of antibodies bind to the sperm surface (fig. 4). In contrast, when affinity-purified IgG on factor VIII, a control protein and a monoclonal IgG, were used, no sperm agglutination was observed.

Sperm Agglutination by ASA Extracted from Human Sera

Further, we investigated whether MAR+ sera containing specific antibodies against Ves v 5 are also able to induce sperm immobilization. However, incubating ASA-free ejaculates with MAR+ sera did not lead to sperm immobilization suggesting that the serum con-

Fig. 2. Determination of serum IgE. Sera of MAR+ (●) and MAR- (○) patients were incubated with wasp venom extract (i3) and recombinant Ves v 5. Binding of total and specific IgE was detected using the ImmunoCAP assay (1 kU/l = 2.4 ng IgE/ml). No statistically significant differences were found.

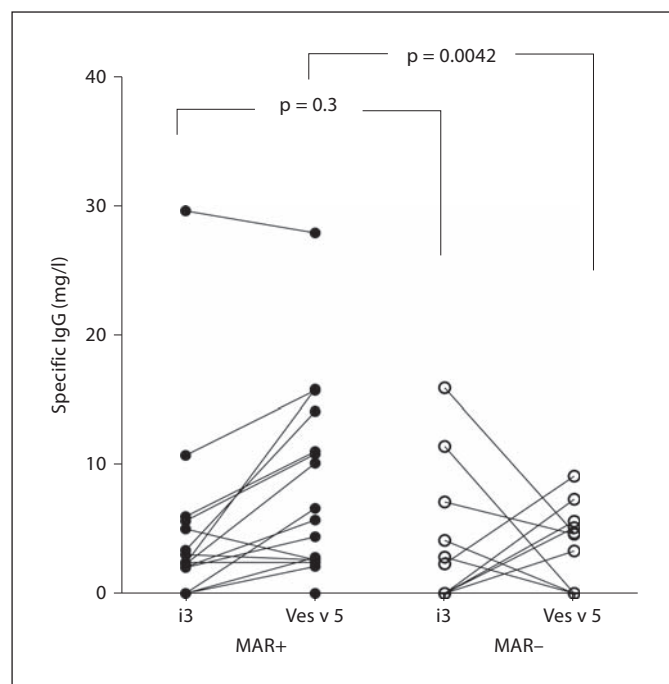
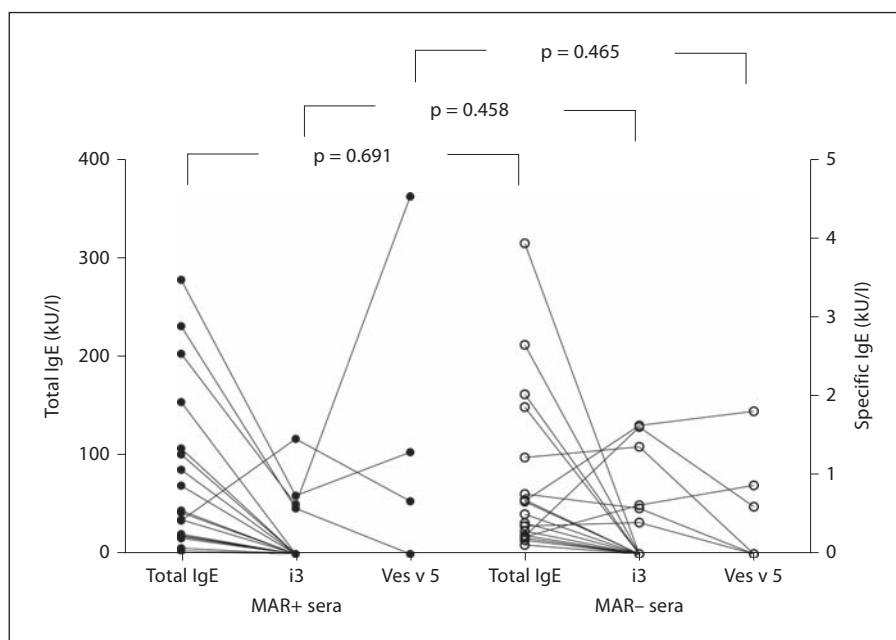


Fig. 3. Measurement of specific IgG in the serum. Specific IgG reactivity to wasp venom extract (i3) and recombinant Ves v 5 was measured in sera of MAR+ (●) and MAR- (○) patients by the ImmunoCAP assay. There was no significant difference in the wasp venom extract specific IgG, but Ves v 5 specific IgG levels were significantly different between MAR+ and MAR- patients' sera ($p = 0.0042$).

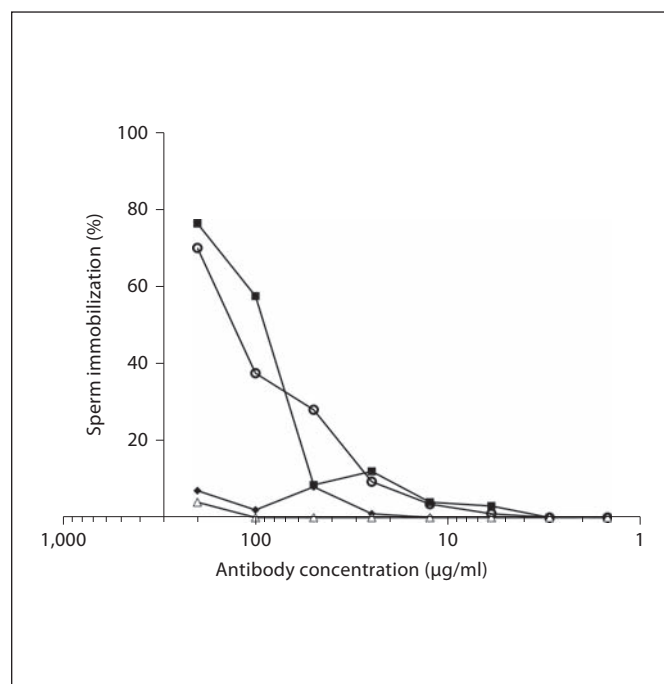


Fig. 4. Affinity-purified IVIg on Ves v 5 (○) or CRISP-2 (■) were incubated with ASA-free healthy donor sperm in the indirect MAR test. Affinity-purified IVIg on factor VIII (◆) as well as a monoclonal IgG4 (Δ) were used as unspecific controls. Sperm-bound antibodies leading to sperm immobilization were detected by incubating sperm with human IgG-coated latex beads together with cross-linking anti-human IgG. The amount of immobilized sperm was investigated under the light microscope.

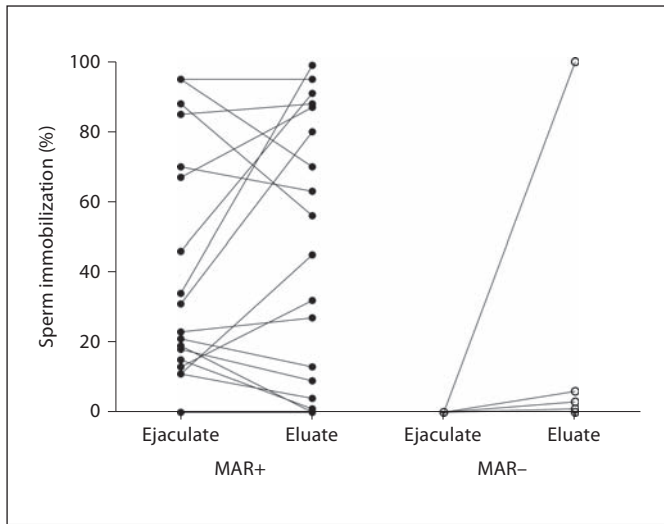


Fig. 5. Indirect MAR test with affinity-purified ASA. Affinity-purified ASA from sera of MAR+ (●) and MAR- (○) patients was incubated with ASA-free healthy donor sperm. Sperm immobilization was visualized under the light microscope using human IgG-coated latex beads and anti-human IgG for cross-linking and compared to the ejaculates of the corresponding MAR+ and MAR- patients in the direct MAR assay.

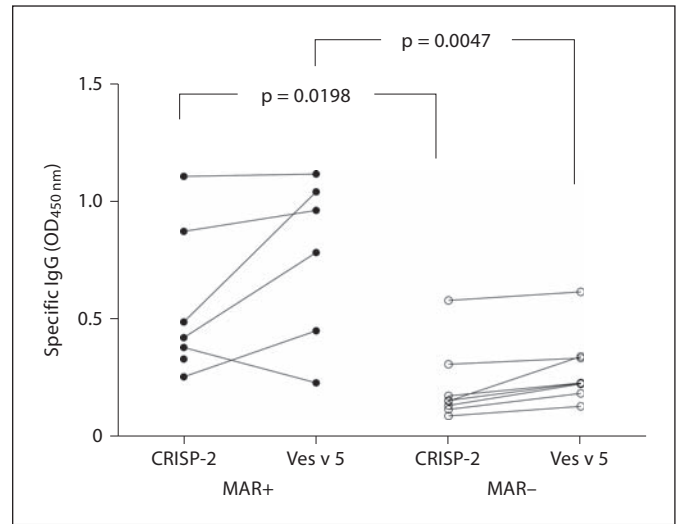


Fig. 6. Specific IgG reactivity to Ves v 5 and CRISP-2 of affinity purified ASA of MAR+ (●) and MAR- (○) patients was determined by ELISA. Significant differences ($p = 0.0047$ and $p = 0.0198$) were obtained between MAR+ and MAR- patients' sera.

centration of ASA is too low to induce such an effect (data not shown).

Thus, to enrich antibodies from MAR+ and MAR- patient sera, they were absorbed on healthy donor sperm. Eluted IgG were measured by a semiquantitative ELISA and showed no significant difference between the two groups indicating that the same amounts of antibodies were eluted from MAR+ and MAR- patient sera (data not shown). To investigate the agglutination capacity of these affinity-purified ASA, an indirect MAR test was performed using ASA-free healthy donor sperm. The results showed that affinity-purified ASA from 83.4% of MAR+ patients induced sperm immobilization >10%, whereas no effect was observed with ASA extracted from 95% of MAR- patients' sera (fig. 5). These results indicate that mainly ASA from MAR+ patients' sera are functional and induce sperm agglutination.

The affinity-purified ASAs were then tested by ELISA for specificity against Ves v 5 and CRISP-2 (fig. 6). A significant difference between the affinity-purified ASA from MAR+ and MAR- patients could be observed for both specific CRISP-2 IgG ($p = 0.0047$) and specific Ves v 5 IgG ($p = 0.0198$). These data indicate a higher prevalence of antibodies directed against Ves v 5 and CRISP-2 in MAR+ patients.

Sperm Surface Binding Site of ASA Extracted from Human Sera

To further confirm the specificity of ASA for CRISP-2, eluted antibodies were analyzed for binding on sperm by immunofluorescence. Affinity-purified ASA from both MAR+ and MAR- patients were incubated together with immobilized ASA-free healthy donor sperm and then allowed to develop with anti-human IgG FITC. As shown in figure 7, ASA extracted from MAR+ patients' sera bind to the flagellum as well as to the sperm head where CRISP-2 is expressed. In comparison, commercial sera containing ASA show similar binding to the whole sperm surface. In contrast, no binding was observed with a commercial ASA-free serum as well as with ASA extracted from MAR- patients' sera. These results indicate that even if anti-sperm IgG was eluted from the sera of both groups only antibodies from MAR+ patients' sera could be visualized on the surface of the sperm. Furthermore, ASA extracted from sera of MAR+ patients recognized CRISP-2 antigen but also other sperm surface antigens, which remain to be further characterized.

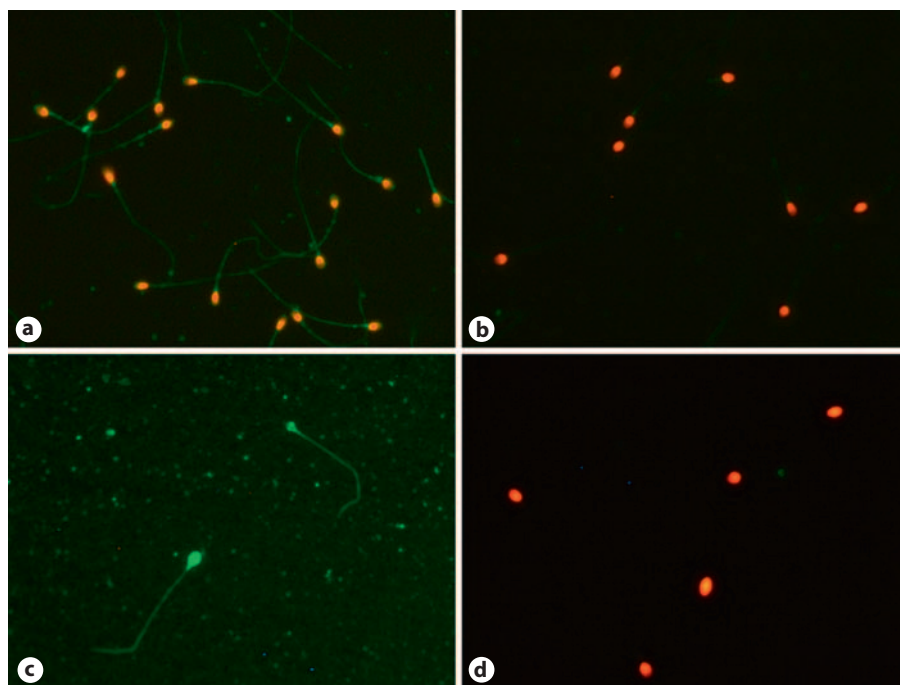


Fig. 7. Binding of affinity-purified ASA on human immobilized sperm. Affinity-purified ASA from sera of MAR+ (a) and MAR- sera (b) were incubated with immobilized healthy donor sperm on poly-L-lysine-coated slides. MAR+ (c) and MAR- control sera (d) were used as controls. ASA reactivity to sperm was detected using FITC-conjugated anti-human IgG and visualized under a fluorescence microscope.

Discussion

The characterization of sperm antigens recognized by ASA may be important to determine their role in male immunological infertility. Thus, recent proteomic studies have proposed potential ASA antigens, eventually being helpful for understanding the impairment in sperm function and fertilization [28]. Our study is based on the observation that Ves v 5 and CRISP-2 share sequence homology and cross-react in immunoassays. Our first hypothesis was that such cross-reactive antibodies may interfere with sperm function. However, wasp-allergic individuals did not have specific IgE and IgG reactivities to CRISP-2 [22]. Here we turned the question around and looked whether MAR+ individuals specifically recognize CRISP-2 and thereby the homologous wasp venom allergen. We demonstrated that ASA purified from suspected infertile men's sera indeed reacted against both Ves v 5 and CRISP-2. Further, we showed that these purified ASA are leading to sperm immobilization. Through visualization by immunofluorescence we demonstrated that the purified ASA from MAR+ patients' sera recognized CRISP-2 as well as different epitopes on the sperm surface.

In the present work, we investigated whether this cross-reactivity might even be associated with male infertility. For that we used two groups of donors: patients with the presence of functional ASA in their ejaculates

(MAR+) and a control group of suspected infertile patients without ASA in the ejaculates (MAR-). By means of a questionnaire-based allergy anamnesis, we found a higher prevalence of hymenoptera allergy in MAR+ than MAR- patients. However, no significant difference was observed for specific IgE levels between the two patient groups. In contrast, a significant difference was found for Ves v 5 specific IgG, suggesting a higher prevalence of anti-Ves v 5 antibodies in MAR+ than in MAR- patients' sera. The presence of such antibodies in MAR+ patients led us to the hypothesis that they may also have a functional effect on the sperm. Because of the structural similarity between Ves v 5 and CRISP-2, anti-Ves v 5 antibodies should bind to the sperm surface. Indeed we could demonstrate that antibodies specific for Ves v 5 or CRISP-2 isolated from IVIg by affinity purification effectively lead to sperm immobilization in the indirect MAR test.

Surprisingly, in contrast to Ves v 5 specific IgG the measurement of anti-CRISP-2 IgG in the serum revealed no significant difference between MAR+ and MAR- patients. One possible explanation may be related to the different methods used for the detection of anti-Ves v 5 and anti-CRISP-2 IgG. Assessment of anti-Ves v 5 specific IgG was made by means of ImmunoCap. Such an assay is not available for anti-CRISP-2 IgG, and a homemade ELISA was used for the determination of these antibodies.

ImmunoCAP is a standardized quantitative method routinely used for the determination of specific IgE, which has been shown to have a significantly higher sensitivity than ELISA [27]. Another explanation may be connected to the fact that CRISP-2 is a protein which is mainly expressed in the male testis [20] and, under normal conditions, remains hidden from the immune system by the blood-testis barrier. Thus, disruption of this barrier is required for the immune system to encounter CRISP-2 [29]. Hence, because of the hidden character of the CRISP-2 antigen, only low amounts of anti-CRISP-2 antibodies might be present in the sera. Therefore, an enrichment process seemed to be necessary for detecting measurable anti-CRISP-2 antibody.

Previous studies have shown that ASA can be obtained from the sera of infertile couples by absorption on ejaculated human sperm [25]. In the present study, we used a similar enrichment procedure from MAR+ and MAR- patient sera. Both patient groups yielded IgG, but only affinity-purified ASA from MAR+ patients' sera induced sperm immobilization. Furthermore, a higher IgG reactivity to both CRISP-2 and Ves v 5 was obtained for ASA eluted from MAR+ patients than for ASA from MAR- patients. This is in accordance with our previous results showing significant differences in the amount of specific Ves v 5 IgG between MAR+ and MAR- patients' sera.

Our immunofluorescence data showed reactivity with whole sperm surface indicating that affinity-purified ASA recognized CRISP-2 antigen as well as several other antigens present on the sperm surface. Further experiments using 2D gel electrophoresis would be required to identify the other proteins recognized by the affinity-purified ASA. In contrast, even if antibodies from MAR- patients were purified on sperm cells no agglutination and no visualization of sperm binding was detected. As the test used to detect antibodies on sperm surface requires several washing steps, the interaction of MAR- antibodies might be too low to be visualized.

In the present study, there were no significant differences between MAR+ and MAR- patients' sera regarding total and specific IgE levels. These results are in line with our previous study showing no correlation between increasing wasp venom specific IgE levels and sperm extract or CRISP-2 specific IgG levels [22]. However, we were able to show that MAR+ patients have higher levels of cross-reactive antibodies directed against CRISP-2 and Ves v 5 than MAR- patients. Due to the structural similarity between CRISP-2 and Ves v 5 these autoantibodies bind the wasp venom allergen Ves v 5, suggesting that they may serve as parental antibodies to Ves v 5 antibodies. Higher amounts of such antibodies in MAR+ patients might suggest that MAR+ patients are more susceptible to be sensitized to the wasp venom than MAR- patients. Thus, upon sensitization with wasp venom allergen these cross-reactive antibodies would undergo class switch and affinity maturation. This hypothesis is in accordance with our previous study showing two groups of IgG, one that mostly reacts with Ves v 5 but only weakly with CRISP-2, and a second one that reacts equally with Ves v 5 and CRISP-2 [22].

In summary, we have been able to demonstrate the presence of higher levels of cross-reactive anti-Ves v 5 and anti-CRISP-2 antibodies in MAR+ than MAR- patients' sera. Affinity-purified antibodies from MAR+ sera were able to induce sperm immobilization and showed binding to the flagellum of the sperm as well as to its head, where CRISP-2 is expressed. A higher incidence of such antibodies in the sera of MAR+ patients might increase their sensitization to wasp venom.

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