Molecular Human Reproduction Vol.7, No.2 pp. 113–118, 2001

Isolation and identification of sperm membrane antigens recognized by antisperm antibodies, and their possible role in immunological infertility disease

Claudia Bohring[1,3], Eberhard Krause[2], Barbara Habermann[1] and Walter Krause[1]

[1]Department of Andrology, Clinical Training Center of the European Academy of Andrology, University Hospital Marburg, 35033, and [2]Institute of Molecular Pharmacology, 10315 Berlin, Germany

[3]To whom correspondence should be addressed at: Deutschhausstraβe 9, D-35033 Marburg, Germany.
E-mail: bohring@mailer.uni-marburg.de

Antisperm antibodies (ASA) are the main cause of immunological infertility, as they impair sperm function by binding to the sperm membrane. In this study, we isolated highly enriched sperm membrane proteins by two-dimensional (2D) gel electrophoresis. Isoelectric focusing, as a first dimension, was performed on precast DryStrip IPG 4–7. The second dimension was carried out on 12% sodium dodecyl sulphate–polyacrylamide gels. A total of 18 antigens were identified by the subsequent 2D Western blotting using ASA from seminal plasma samples of infertile patients. Six of the recognized proteins were isolated and analysed by means of mass spectrometry and peptide matching. They were identified as heat shock proteins HSP70 and HSP70-2, the disulphide isomerase ER60, the inactive form of caspase-3 and two subunits of the proteasome (component 2 and ζ chain). The biochemical identification of these proteins will be helpful in understanding the mechanisms by which ASA impair both sperm function and fertilization. Thus, these proteins may also be useful in the development of reliable methods for ASA detection.

Key words: antisperm antibodies/2-D electrophoresis/caspase-3/heat shock protein/isomerase ER-60

Introduction

The occurrence of spontaneous antisperm antibodies (ASA) to human spermatozoa from infertile men was first described by Rümke and independently by Wilson in 1954 (Rümke, 1954; Wilson, 1954). Antibodies directed to sperm antigens can be detected in seminal fluid, where they may be bound to the sperm surface (Anderson and Hill, 1988) or solubilized in the seminal plasma (Clarke *et al.*, 1985a), cervical mucus (Clarke, 1984), oviductal fluid or follicular fluid (Kay *et al.*, 1985) of women. They also occur in blood serum of men and women (Clarke *et al.*, 1985b), but these appear to be iso-antisperm antibodies which are not important for fertilization. In the literature, the range of incidence of ASA in infertile couples (both men and women), has been given as 9–36% (Collins *et al.*, 1993; Menge and Naz, 1993; Lahteenmaki *et al.*, 1995; Nagy *et al.*, 1995). Of infertile male partners, 10% show ASA in the seminal plasma or attached to the surface of spermatozoa, while in ~5% of infertile female partners, ASA occur in cervical mucus or oviductal fluid (Lenzi *et al.*, 1997; Crosignani and Rubin, 1998). The occurrence of ASA in one of these conditions gives rise to the disease of 'immunological infertility'.

Surgical intervention, inflammation and trauma to the epididymis and the vas deferens (but not to the testis), are the main causes of the induction of ASA in man (Gubin *et al.*, 1998). It is likely that, in vital spermatozoa, only those ASA which bind to the sperm membrane will be of functional relevance. They will have heterogeneous effects, obviously depending on their binding sites (Ohl and Naz, 1995). ASA have been found to affect sperm motility (Barratt *et al.*, 1989; Zouari *et al.*, 1993), the acrosome reaction (Tasdemir *et al.*, 1996; Grundy *et al.*, 1998), penetration of the cervical mucus (Eggert-Kruse *et al.*, 1993), binding to the zona pellucida (Francavilla *et al.*, 1997) and sperm–oocyte fusion (Bronson *et al.*, 1989; Rajah *et al.*, 1993). However, at the present time, it is not possible to predict whether the ASA in individual men are of relevance to a particular function, because the antigens are mostly unknown.

Studies with the aim of isolation and characterization of sperm membrane antigens have to focus on human fluids which can interfere directly with the sperm membrane. For investigation in men the seminal plasma is the most suitable source. Using seminal fluids from men, for whom the result of a positive mixed antiglobulin reaction test indicated the presence of ASA we have, in previous work, discriminated

different sperm membrane antigens by their molecular weight and isoelectric point (pI), and identified them by immunoblotting with ASA from seminal plasma samples of infertile men or from men following vasectomy (Bohring and Krause, 1999). The identification of the nature of these antigens is the key to understanding the mechanism of immunological infertility.

In the present investigation, we isolated and separated highly enriched sperm membrane proteins from pooled donor samples by a two-dimensional (2D) electrophoresis technique. By immunoblotting with ASA from seminal plasma samples of infertile men, several membrane antigens were recognized. The proteins detected were isolated and analysed using matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS) and peptide matching.

Materials and methods

Seminal plasma samples

Semen samples were obtained by masturbation after 3–6 days abstinence from 20 infertile patients who had been referred to the Department of Andrology of the University Hospital, Marburg, Germany, for infertility investigation. Using the MAR test, these patients were shown to have ASA in their seminal plasma. The ejaculate was centrifuged for 15 min at 3000 *g* at room temperature. The seminal plasma was then stored at –20°C until assayed. In addition, 10 seminal plasma samples from infertile patients without ASA and 10 samples from healthy donors with normal sperm parameters were collected as controls.

ASA testing with indirect mixed antiglobulin reaction

For the detection of ASA, the SpermMAR immunoglobulin G (IgG) test (FertiPro NV, Sint Martens Latem, Belgium) was used. This test used IgG-coated latex particles, which bind to the sperm-bound ASA, with an antihuman Fab' fragment added as a link. The ASA solubilized in the patients seminal plasma sample were tested using the indirect MAR test method (Hinting *et al.*, 1988). The soluble ASA in patients seminal plasma bound first to healthy donor spermatozoa as the antigen; after this donor spermatozoa will react positively in a subsequent MAR test. From the evaluation of 100 motile spermatozoa, the percentage of spermatozoa with attached latex particles represents the test result. According to the World Health Organization (WHO, 1999) manual, the test is considered to be positive if latex particles are attached to >50% of the motile spermatozoa. Therefore, we used only seminal plasma samples with an indirect MAR test result of >50%.

Membrane preparation

Semen samples from 50 donors with normal sperm parameters (WHO, 1999) were obtained by masturbation after 3–6 days abstinence. Spermatozoa were enriched and purified from other cells by means of a swim-up preparation. 1 ml of semen was diluted 1:5 with IVF buffer (Medi Cult a/s, Copenhagen, Denmark) followed by a centrifugation at 300 *g* for 10 min at room temperature. The pellet containing spermatozoa was resuspended in 5 ml IVF buffer and centrifuged again. IVF medium (2 ml) was then carefully placed above the final pellet, after which the tube was inclined to an angle of 45° and incubated for 1 h at 37°C. The supernatant containing the enriched fraction of motile spermatozoa was aspirated and centrifuged for 10 min at 500 *g* at room temperature. The membrane preparation was performed as previously described (Hinsch *et al.*, 1992), with some modifications. Briefly, the final pellet was diluted at 1:9 with a modified hypo-osmotic medium (Jeyendran *et al.*, 1984). The spermatozoa were swollen in this hypo-osmotic medium for 2 h in a water bath (37°C), after which the solution was transferred to ice. The membranes were then stripped off with a homogenizer (Ultra-turrax; 1200 IU/min; 2 min; 4°C). The suspension was sonicated in a sonication bath for 2 min (10 kHz) and centrifuged at 4000 *g* (4°C for 15 min) to remove the cell debris and unbroken spermatozoa. The supernatant was centrifuged at 10 000 *g* (4°C for 10 min) and the resultant supernatant underwent a further ultracentrifugation at 100 000 *g* (4°C for 2 h). The final pellet containing the membrane proteins (plasmalemma, inner and outer acrosomal membrane) was resuspended, pooled and dissolved in isoelectric focussing (IEF) buffer, consisting of 2 mol/l thiourea (Sigma-Aldrich GmbH, Steinheim, Germany) 9 mol/l urea, 20 mmol/l dithiothreitol (DTT), 2% CHAPS [3-(cyclohexylamino)-1-propane-sulphonic acid], 0.4% Pharmalyte and 0.4% Ampholine (pH 3, 5–10) (all from Pharmacia Fine Chemicals, Uppsala, Sweden) for the 2D electrophoresis.

2D gel electrophoresis

The 2D polyacrylamide gel electrophoresis (PAGE) of pooled sperm membrane proteins was carried out as recently described (Bohring and Krause, 1999), with some modifications in the urea compound to improve the hydrophilic bilayer membrane isolation. Briefly, isoelectric focusing, as a first dimension, was performed on a precast DryStrip IPG 4–7 L gel (linear immobilized pH 4–7 gradient gel), using Multiphor II (Pharmacia). For the rehydration of the gels, the urea compound in the solution was modified, we used only 7 mol/l urea (instead of 9 mol/l urea) with an additional 2 mol/l thiourea. The gels were loaded with 75 µg membrane proteins for immunoblotting and 500 µg proteins for MALDI–MS analysis (see below). The second dimension was performed on 12% sodium dodecyl sulphate (SDS) polyacrylamide gels (10×13×0.1 cm).

For the determination of molecular mass, prestained SDS–PAGE standards and the SDS–PAGE standards low range (BioRad, Krefeld, Germany) were used. In addition one IPG gel with 2D SDS–PAGE Standards (BioRad) and Carbamylyte™ Calibration Kit (Pharmacia) run parallel to probing gels. After electrophoresis the membrane protein pattern was visualized by silver staining (Rabilloud *et al.*, 1994) or Coomassie Brilliant Blue G-Colloidal (Sigma-Aldrich GmbH). Stained gels were scanned in a wet state using a ScanJet 6100CIT and the HP DeskScan II software (Hewlett Packard, Greeley, Colorado, USA).

Immunoblotting

Separated pooled donor membrane proteins were electrotransferred from the gels in a semi-dry NovaBlot device (Pharmacia) on Immobilon-P polyvinylidene difluoride (PVDF) sheets (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. The blots were exposed to seminal plasma samples from patients and controls diluted 1:20–1:50 for 1 h, according to a previously described procedure (Lee *et al.*, 1994) for specific probing. The secondary antibody, conjugated with peroxidase [Affini Pure Goat Anti-Human IgG, IgM, IgA (H + L) Code: 196–035–064; Dianova, Hamburg, Germany] was used at a 1:10 000 dilution and incubated for 30 min. The enhanced chemoluminescence (ECL) kit (Amersham, Buckinghamshire, UK) was used to identify the immunoreactive antigenic proteins. The ECL procedure was performed according to the instructions of the manufacturer. The immunodetection of the sperm membrane antigens by ASA were compared with the 2D separated membrane pattern visualized with staining and selected Coomassie Brilliant Blue G-Colloidal stained spots were excised from the 2D gel for MALDI–MS identification.

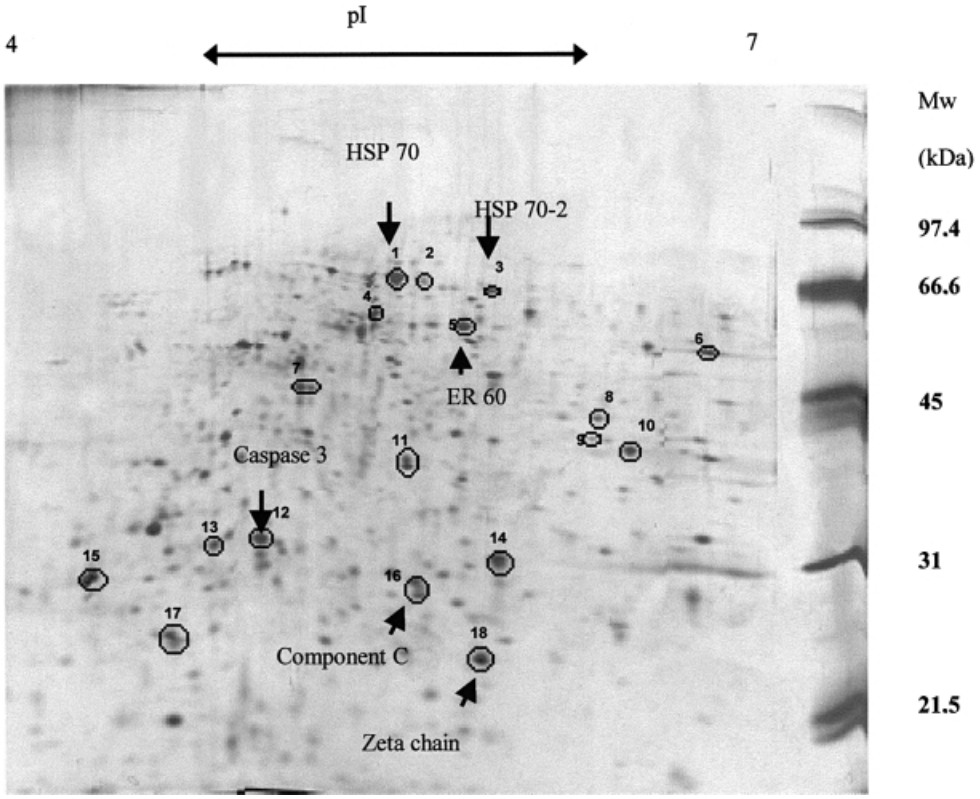

Figure 1. Two-dimensional (2D) electrophoresis of spermatozoa membrane proteins performed with 75 µg protein sample. The first dimension was carried out on IPG (linear 4–7 pH gradient). The second dimension was run on a homogeneous running sodium dodecyl sulphate (SDS) gel (12%) and 4% stacking gel. The proteins were detected by silver staining. The numbered and encircled spots indicate proteins which could be matched with antigens detected by immunoblots using the patients' antisperm antibodies (ASA) from seminal plasma (see Table I for isoelectric points and molecular weights). The six proteins indicated by an arrow were identified using matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS) and peptide matching.

Table I. Biochemical data of 18 sperm antigens (spag) recognized by sperm antibodies

Antigen	Molecular weight (kDa)	Isoelectric point	Infertile patients (n = 20) No. of positive samples (frequency in %)
spag1	71.0	5.3	15 (75)
spag2	70.5	5.4	7 (35)
spag3	70.0	5.6	15 (75)
spag4	60.0	5.2	16 (80)
spag5	58.0	5.5	19 (95)
spag6	57.2	6.5	9 (45)
spag7	48.0	5.1	15 (75)
spag8	44.8	5.8	8 (40)
spag9	42.6	5.75	8 (40)
spag10	41.4	5.9	9 (45)
spag11	40.0	5.3	14 (70)
spag12	33.0	4.9	18 (90)
spag13	32.5	4.8	16 (80)
spag14	31.0	5.6	15 (75)
spag15	30.0	4.5	3 (15)
spag16	29.0	5.35	8 (40)
spag17	26.0	4.6	9 (45)
spag18	24.0	5.5	6 (30)

Identification of proteins by MALDI–MS fingerprinting and peptide matching

A slightly modified in-gel digestion method (Shevchenko *et al.*, 1996), was performed. The protein spots were excised, washed with 50% acetonitrile (Baker, S Planfild, USA) in 25 mmol/l ammonium bicarbonate (Merck, Darmstadt, Germany), shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The gel pieces were reswollen in 10 µl of 5 mmol/l ammonium bicarbonate, containing 300 ng trypsin (sequencing grade, Boehringer Mannheim, Germany). After 60 min, 5 µl of 5 mmol/l ammonium bicarbonate was added to keep the gel pieces wet during tryptic cleavage (37°C, overnight). To extract the peptides, 15 µl of 0.5% trifluoroacetic acid (TFA; Fluka, Buchs, Switzerland) in acetonitrile was added, and the samples were sonicated for 5 min. Peptides were analysed directly from the separated liquid by MALDI–MS.

The mass spectrometry measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI–TOF mass spectrometer (Perseptive Biosystems Inc). The analyte solution (2 µl) was mixed with 2 µl of α-cyano-4-hydroxy cinnamic acid (4-HCCA; Sigma, St Louis, MO, USA) matrix solution consisting of 10 mg of matrix dissolved in 1 ml of 0.3% TFA in acetonitrile–water (1:1, v/v). From the resulting mixture, 1 µl was applied to the sample plate. Samples were air-dried at ambient temperature (24°C). All measurements were performed in the reflection mode at an acceleration voltage of 20 kV, 70% grid voltage and a delay of 200 ns. Each spectrum obtained was the mean of 256 laser shots. Mass spectra were calibrated using known trypsin fragments as internal standards. The peptide masses determined were matched with theoretical peptide masses of proteins from the SWISS-PROT database using the search program MS-FIT (http://falcon.ludwig.ucl.ac.uk/msfit.htm). Searching was performed using a mass uncertainly of ± 0.05 Da and a molecular weight range of 20% of the relative molecular weight (M_R) and ± 1 pI unit, as determined by 2D gel electrophoresis.

Table II. Human sperm membrane proteins as identified by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS)

Spag no.	Protein description	Experimental pI/MW	Theoretical pI/MW	Database accession code[a]	Peptides matched
1	heat shock 70 kDa protein 1-HOM (HSP70-HOM)	71/5.3	70.4/5.1–6.2	P34931	15
3	heat shock related 70 KD protein 2	70/5.6	70.0/5–6	P54652	15
5	probable protein disulfide isomerase ER-60 precursor (ER-60) (58 kDa microsomal protein) (P58) (GRP58) (ER-57)	58/5.5	56.8/5.4–5.8	P30101	18
12	apopain precursor (cysteine protease CPP32) (yama protein) (caspase-3) (CASP-3) (SREBP cleavage activity 1) (SCA-1)	33/4.9	31.6/5.0–6.8	P42574	6
16	proteasome component C2. macropain subunit C2 proteasome NU chain. multicatalytic endopeptidase complex subunit C2	29/5.35	29.6/5.4–6.6	P25786	5
18	proteasome ζ chain. macropain ζ chain multicatalytic endopeptidase complex ζ chain	24/5.5	26.4/4.2–5.4	P28066	5

spag = sperm membrane antigen; pI = isoelectric point; MW = molecular weight.
[a]The accession code refers to the SWISS-PROT database; the theoretical MW and pI were obtained by SWISS-PROT 2D database determined for other human tissue.

Results

Reactivity of seminal plasma samples

The seminal plasma samples of 20 infertile patients (IP) and control samples ($n = 20$) were tested using 2D Western blotting. ASA from immunologically infertile patients bound to a wide variety of sperm membrane proteins. The antigens detected are iso-antigens in this sense. The sperm membrane antigens as detected by ASA from seminal plasma samples of infertile patients were matched with 2D separated membrane protein pattern, visualized using silver staining.

The experimental molecular weight and pI of these proteins were determined by matching them with standard proteins, with precisely defined pI and molecular weight, thus allowing a repeatable and accurate calibration of the 2D separation. The results of immunoblotting assays are summarized in Table I. Altogether 18 membrane antigens were detected by 2D immunoblotting with ASA-positive seminal plasma samples. The positions of all the 18 sperm antigens (spag) recognized, numbered from 1 to 18, are indicated in a standard silver stained 2D gel (Figure 1) and marked by circles.

The absolute number and the percentage of samples of infertile patients containing ASA positive for a distinct antigen (spag 1–18) are shown in Table I. Some of the sperm antigens (spag) were recognized by a large number of ASA samples, while others were identified only in few cases. No sample detected all 18 antigens. The control samples, the ASA-negative seminal plasma samples from infertile patients ($n = 10$), and the ASA-negative samples from healthy donors ($n = 10$), showed no immunoreaction with any of the human sperm membrane proteins. The highest rates of binding were found for the sperm antigens spag 5 (95%) and spag 12 (90%) in all samples. At least three quarters of the ASA-positive seminal plasma samples also recognized the sperm antigens spag 1 (75%), spag 3 (75%), spag 4 (80%), spag 7 (75%), spag 13 (80%), and spag 14 (75%).

Protein identification by MALDI–MS

All the spots recognized as antigens were processed for protein identification. Proteins were distinguished by peptide mass fingerprinting using the MS-FIT search algorithms in the SWISS-PROT database. Six proteins could be identified using peptide mass profiling matched to the database. In Figure 1, the matched proteins are indicated by an arrow.

A minimal number of five matching peptides were used for protein search. In Table II, the sperm proteins identified are listed according to their experimental molecular weight and pI, in comparison with the theoretical molecular weight and theoretical pI ranges, as obtained by the SWISS-PROT database. The proteins spag 1, spag 3, spag 5 and spag 12 could be identified with a significant number of matching proteins as heat shock 70 protein, heat shock 70–2 protein, disulphide isomerase ER-60 and caspase-3. The proteins spag 16 (proteasome component 2) and spag 18 (proteasome ζ chain) could be identified based on five matching peptides. These results just reach the level of significance of five findings. Since the two proteins are subunits of one enzyme, we could consider the findings as probable.

Discussion

The characterization of sperm membrane antigens which are recognized by ASA from seminal plasma of infertile men is a first step to understanding immunological infertility and the interaction between ASA and spermatozoa. In the present study 18 proteins associated with sperm membranes were detected as antigens and six proteins were biochemically

identified. Using MALDI–MS, the heat shock 70 kDa protein (HSP70), the heat shock-related protein HSP70-2, disulphide isomerase ER60, caspase-3 and two subunits of the multicatalytic endopeptidase proteasome, the component C2 and proteasome ζ chain were found (Table II). No peptide matching was possible for the residual proteins. No amino acid sequencing could be performed due to the very small amount of protein obtained.

The heat shock proteins are a family of molecular chaperones involved in protein folding and transport and in the development of tolerance to various physico–chemical stresses. The HSP70 related protein (HSP70-2) is stress-induced by damage or heat shock; HSP70 expression is not thermo-inducible.

It has been demonstrated that heat shock proteins are abundant components of the sperm surface (Miller *et al.*, 1992). HSP70 was localized by immunhistochemistry on the total sperm surface and by gel electrophoresis and immunoblotting. The function of HSP70 on the sperm surface is discussed; it is unlikely that it is involved in a stress response, because spermatozoa have highly condensed chromatin and so, unlike somatic cells, they are unable to develop a stress response. The involvement of HSP70 and HSP70-2 in mediating protein folding and the process of translocation across the membrane seems more likely. The impairment of the chaperone functions of HSP70 and HSP70-2 during capacitation and the acrosome reaction by ASA may inhibit the fertilization process.

The catalytic activity of ER60 comprises the rearrangement of both intra- and inter-chain disulphide bonds in proteins to form the native structures. The expression of ER60 in the developing acrosome of spermatids during rat spermiogenesis has been demonstrated (Ohtani *et al.*, 1993). Recent studies have shown that the protein disulphide isomerase assists protein folding by expressing both an isomerase and chaperone-like activity (Wang, 1998; Chen *et al.*, 1999). The chaperone activity is independent of its isomerase activity. ER60 has recently been found in the plasma membrane of several cell types (Kroning *et al.*, 1994) and several functions have been attributed to ER60 on the cell surface (Zai *et al.*, 1999). The existence of ER60 on the sperm membranes, however, has not been described before and its function is still unclear. Although we are not sure whether it is associated with the surface membrane, we would suggest that the chaperone activity of this protein is important for capacitation and the acrosome reaction. Future experiments are needed to clarify whether ER is a surface protein and whether it has chaperone activity. The binding of ASA to this protein may also impair the process of fertilization.

We have also identified caspase-3 in the inactive 32 kDa conformation as an antigen for ASA. Active caspase-3 is one of the key executioners of the early stage of apoptosis and consists of a heterodimer of 17 kDa (p17) and 12 kDa (p12) subunits. The role of caspase-3 in ejaculated spermatozoa and the association of the inactive form with the sperm membrane is unclear. Previous findings (Weil *et al.*, 1998) raised the possibility that mouse spermatozoa carry an apoptosis programme independent of caspases. We suggest that caspase-3 is a rudiment from the apoptotic pathway during spermatogenesis.

We also recognized two subunits of the proteasome, a multicatalytic proteinase complex which is characterized by ATP-dependent proteolytic activity, as antigens for ASA. Two subunits were isolated, the component C2 (30 kDa) and the ζ chain (26.5 kDa). The function of the proteasome has been characterized for salmon spermatozoa (Inaba *et al.*, 1998). In these cells, the proteasomes are involved in ATP-dependent regulation of sperm motility by modulating the activity of outer arm dynein by regulation of cAMP. An association between proteasomes and sperm motility was first described in mammals (de Lamirande and Gagnon, 1986). In other species, e.g. the ascidian, the proteasomes are involved in sperm binding and sperm penetration, by functioning as lysins (Sawada *et al.*, 1998). Other authors (Tipler *et al.*, 1997) were able to purify 26S proteasomes from human spermatozoa by gradient centrifugation; these authors showed that there are differences in the isoforms of proteasomes in human spermatozoa compared with proteasomes of other tissues. During spermatogenesis, proteasomes (together with ubiquitin) are probably involved in protein degradation and cellular remodelling. However, it is unlikely that the proteasomes in mature spermatozoa are involved in the ubiquitin system because other important components of this system are not present.

In the present study, we isolated and separated highly enriched sperm membrane proteins. By mapping, we summarized 18 proteins to which ASA from infertile patients bind. Six of these proteins were identified by using MALDI–MS and peptide matching. Additional experiments (using specific antibodies) are required to demonstrate that the protein spots characterized are indeed related to the proteins identified by MALDI–MS. The interaction of ASA with these proteins may influence the function of spermatozoa and may play a role in infertility. Since proteins which are an integral part of inner cell membranes may also occur on the sperm surface, these proteins may also play a role in immunological infertility. Hence, the next step in our research is to determine whether the proteins identified are associated with the membrane as integral compounds or are loosely bound to the surface, and then to clarify the role of these proteins in sperm function.

The characterization and identification of human sperm antigens are important for understanding the mechanism by which ASA may impair sperm fertilization capacity. The binding of ASA to sperm-specific antigens involved in fertilization offers an attractive approach to immunocontraception. In the mouse model, a systemic immunization can be induced with the sperm antigen FA-1, resulting in reduced sperm fertilization capacity (Naz and Zhu, 1998). In addition the knowledge of the immunological relevant proteins on sperm membranes offers the possibility of developing methods for determining ASA with minimal intra- and inter-assay variations.

References

Anderson, D.J. and Hill, J.A. (1988) Cell-mediated immunity in infertility. *Am. J. Reprod. Immunol. Microbiol.*, **17**, 22–30.

Barratt, C.L., Havelock, L.M., Harrison, P.E. and Cooke, I.D. (1989) Antisperm antibodies are more prevalent in men with low sperm motility. *Int. J. Androl.*, **12**, 110–116.

Bohring, C. and Krause, W. (1999) The characterization of human spermatozoa membrane proteins–surface antigens and immunological infertility. *Electrophoresis*, **20**, 971–976.

Bronson R.A., Cooper G.W. and Phillips, D.M. (1989) Effects of anti-sperm antibodies on human sperm ultrastructure and function. *Hum. Reprod.*, **4**, 653–657.

Chen, J., Song, J.L., Zhang, S. *et al.* (1999) Chaperone activity of DsbC. *J. Biol. Chem.*, **274**, 19601–19605.

Clarke, G.N. (1984) Detection of antispermatozoal antibodies of IgG, IgA, and IgM immunoglobulin classes in cervical mucus. *Am. J. Reprod. Immunol.*, **6**, 195–197.

Clarke, G.N., Elliott, P.J., and Smaila, C. (1985a) Detection of sperm antibodies in semen using the immunobead test, a survey of 813 consecutive patients. *Am. J. Reprod. Immunol. Microbiol.*, **7**, 118–123.

Clarke, G.N., Stojanoff, A., Cauchi, M.N. and Johnston, W.I. (1985b) The immunoglobulin class of antispermatozoal antibodies in serum. *Am. J. Reprod. Immunol. Microbiol.*, **7**, 143–147.

Collins, J.A., Burrows, E.A., Yeo, J. and Young Lai, E.V. (1993) Frequency and predictive value of antisperm antibodies among infertile couples. *Hum. Reprod.*, **8**, 592–598.

Crosignani, P.G. and Rubin, B.L. (1998) Male infertility update. The ESHRE Capri Workshop Group. *Hum. Reprod.*, **13**, 2025–2032.

de Lamirande, E. and Gagnon, C. (1986) Effects of protease inhibitors and substrates on motility of mammalian spermatozoa. *J. Cell. Biol.*, **102**, 1378–1383.

Eggert-Kruse, W., Bockem-Hellwig, S., Doll, A. *et al.* (1993) Antisperm antibodies in cervical mucus in an unselected subfertile population. *Hum. Reprod.*, **8**, 1025–1031.

Francavilla, F., Romano, R., Santucci, R. *et al.* (1997) Interference of antisperm antibodies with the induction of the acrosome reaction by zona pellucida (ZP) and its relationship with the inhibition of ZP binding. *Fertil. Steril.*, **67**, 1128–1133.

Grundy, C.E., Killick, S.R., Hay, D.M. *et al.* (1998) A prospective clinical trial investigating the efficacy of a method of preparing subpopulations of antibody-free spermatozoa from the ejaculates of antibody-positive patients. *Int. J. Androl.*, **21**, 261–270.

Gubin, D.A., Dmochowski, R. and Kutteh, W.H. (1998) Multivariant analysis of men from infertile couples with and without antisperm anibodies. *Am. J. Reprod. Immunol.*, **39**, 157–160.

Hinsch, K.D., Hinsch, E., Aumüller, G. *et al.* (1992) Immunological identification of G protein alpha- and beta-subunits in tail membranes of bovine spermatozoa. *Biol. Reprod.*, **47**, 337–346.

Hinting, A., Vermeulen, L. and Comhaire, F. (1988) The indirect mixed antiglobulin reaction test using a commercially available kit for the detection of antisperm antibodies in serum. *Fertil. Steril.*, **49**, 1039–1044.

Inaba, K., Morisawa, S. and Morisawa, M. (1998) Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. *J. Cell Sci.*, **111**, 1105–1115.

Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M. *et al.* (1984) Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, **70**, 219–228.

Kay, D.J., Boettcher, B., Yovich, J.L. and Stanger, J.D. (1985) Antispermatozoa antibodies in human follicular fluid. *Am. J. Reprod. Immunol. Microbiol.*, **7**, 113–117.

Kroning, H., Kahne, T., Ittenson, A. and Ansorge, S. (1994) Thiol–proteindisulfide–oxireductase (proteindisulfide isomerase), a new plasma membrane constituent of mature human B lymphocytes. *Scand. J. Immunol.*, **39**, 346–350.

Lahteenmaki, A., Reima, I., and Hovatta, O. (1995) Treatment of several male immunoligical infertility by intracytoplasmatic sperm injection. *Hum. Reprod.*, **10**, 2824–2828.

Lee, S.L., Stevens, J., Wang, W.W. and Lanzillo, J.J. (1994) A liquid gelatin blocking reagent for western blotting with chemiluminescent detection. *Biotechniques*, **17**, 60–62.

Lenzi, A., Gandini, L., Lombardo, F. *et al.* (1997) Antisperm antibody detection, 2. Clinical, biological, and statistical correlation between methods. *Am. J. Reprod. Immunol.*, **38**, 224–230.

Menge, A.C. and Naz, R.K. (1993) Immunoglobulin (Ig) G, IgA, and IgA subclass antibodies against fertilization antigen-1 in cervical secretions and sera of women of infertile couples. *Fertil. Steril.*, **60**, 658–663.

Miller, D., Brough, S. and al-Harbi, O. (1992) Characterization and cellular distribution of human spermatozoal heat shock proteins. *Hum. Reprod.*, **7**, 637–645.

Nagy, Z.P., Verheyen, G., Liu, J. *et al.* (1995) Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. *Hum. Reprod.*, **10**, 1775–1780.

Naz, R.K. and Zhu, X. (1998) Recombinant fertilization antigen-1 causes a contraceptive effect in actively immunized mice. *Biol. Reprod.*, **59**, 1095–1100.

Ohl, D.A. and Naz, R.K. (1995) Infertility due to antisperm antibodies. *Urology*, **46**, 591–602.

Ohtani, H., Wakui, H., Ishino, T. *et al.* (1993) An isoform of protein disulfide isomerase is expressed in the developing acrosome of spermatids during rat spermiogenesis and is transported into the nucleus of mature spermatids and epididymal spermatozoa. *Histochemistry*, **100**, 423–429.

Rabilloud, T., Vuillard, L., Gilly, C. and Lawrence, J.J. (1994) Silver-staining of proteins in polyacrylamide gels, a general overview. *Cell. Mol. Biol.*, **40**, 57–75.

Rajah, S.V., Parslow J.M., Howell, R.J. and Hendry, W.F. (1993) The effects on in-vitro fertilization of autoantibodies to spermatozoa in subfertile men. *Hum. Reprod.*, **8**, 1079–1082.

Rümke, P. (1954) The presence of sperm antibodies in the serum of two patients with oligospermia. *Vox. Sang*, **4**, 135–140.

Sawada, H., Pinto, M.R. and De Santis, R. (1998) Participation of sperm proteasome in fertilization of the phlebobranch ascidian *Ciona intestinalis*. *Mol. Reprod. Dev.*, **50**, 493–498.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.*, **68**, 850–858.

Tasdemir, I., Tasdemir, M., Fukuda, J. *et al.* (1996) Sperm immobilization antibodies in infertile male sera decrease the acrosome reaction, a possible mechanism for immunologic infertility. *J. Assist. Reprod. Genet.*, **13**, 413–416.

Tipler, C.P., Hutchon, S.P., Hendil, K. *et al.* (1997) Purification and characterization of 26S proteasomes from human and mouse spermatozoa. *Mol. Hum. Reprod.*, **3**, 1053–1060.

Wang, C.C. (1998) Protein disulfide isomerase assists protein folding as both an isomerase and a chaperone. *Ann. N.Y. Acad. Sci.*, **864**, 9–13.

Weil, M., Jacobson, M.D. and Raff, M.C. (1998) Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. *J. Cell Sci.*, **111**, 2707–2715.

Wilson, L. (1954) Sperm agglutinins in human semen and blood. *Proc. Soc. Exp. Biol. Med.*, **85**, 652–654.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Semen Cervical–Mucus Interaction.* 4th edn. Cambridge University Press, Cambridge, UK.

Zai, A., Rudd, M.A., Scribner, A.W. and Loscalzo, J. (1999) Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *Clin. Invest.*, **103**, 393–399.

Zouari, R., De Almeida, M., Rodrigues, D. and Jouannet, P. (1993) Localization of antibodies on spermatozoa and sperm movement characteristics are good predictors of *in vitro* fertilization success in cases of male autoimmune infertility. *Fertil. Steril.*, **59**, 606–612.

Received on August 2, 2000; accepted on November 28, 2000