Use of antisperm antibodies in differential display Western blotting to identify sperm proteins important in fertility

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BACKGROUND: Antisperm antibodies (ASA) may be an important cause of infertility but current tests for the detection of ASA have poor prognostic value. The inadequacy of current tests may reflect the inability of these tests to define the antigenic specificity of the sperm proteins with which the ASA react. Identification of the sperm proteins that ASA bind to is a necessary preliminary step to the development of more useful diagnostic tests for ASA. METHODS: A sensitive Western blotting technique was used to compare the antigenic specificities of ASA from men who were infertile (n = 6) with those who were fertile following vasectomy reversal (n = 3). Normal fertile men (n = 3) and infertile men with known ASA (n = 4) were also included in the analysis. RESULTS: All men, including the normal fertile controls, had ASA detectable in our system. Several sperm proteins were identified that react with ASA from infertile but not fertile men. Quantitative differences in the binding of ASA to some proteins were also demonstrated. Additionally, we demonstrated that normal motile sperm are coated with an antibody that appears to be bound to sperm by a non-antigenic mechanism. CONCLUSION: Sera from all men contained ASA, but clearly some of these did not cause infertility. Characterization of the proteins that are antigens for ASA from infertile but not fertile men may allow the development of more accurate tests for infertility-inducing ASA. The significance of immunoglobulin G coated on normal sperm remains to be determined.

Key words: antisperm antibodies/IgG/infertility/spermatozoa/vasectomy

Introduction

Infertility is estimated to affect up to one in six couples and poses a significant health problem (Templeton et al., 1990; Mosher and Pratt, 1991). Antisperm antibodies (ASA) are found in 26–55% of infertile couples (Ayvaliotis et al., 1985; Collins et al., 1993; Bronson, 1999). However, these antibodies are also present in up to 19% of fertile men (Sinisi et al., 1993; Heidenreich et al., 1994; Bronson, 1999) and as many as 43% of fertile women (Bronson, 1999). This high incidence of ASA in the fertile population indicates that not all ASA cause infertility. The confusion over the role of ASA in infertility to some extent reflects the inadequacies of the current diagnostic techniques such as the mixed antiglobulin reaction (MAR) test and the immunobead test. These tests do not examine the antigenic specificities of ASA, but rather measure gross binding of antibodies to sperm. The development of tests that distinguish the antigenic specificities of ASA may help to clarify the role of ASA in fertility.

A mainstay of treatment for ASA has previously been immunosuppression of antibodies (Hendry, 1992). However, immunosuppression has had limited success in controlled clinical trials and patients frequently suffered from treatment-related complications (Grigoriou et al., 1996). Part of the reason for the poor performance of immunosuppression for ASA may be that subjects were recruited into trials based on the presence of ASA as detected by diagnostic tests that were unable to distinguish between those antibodies that cause infertility and those that do not. Thus, developing a diagnostic test that can distinguish between ASA would be particularly valuable. In order to develop such a test, we examined the specificities of ASA by Western blot from men following vasectomy reversal. When vasectomies are reversed, ~50% of men with sperm in their ejaculates will be infertile, while the remaining 50% will regain fertility. Both groups are known to have antisperm antibodies (Royle et al., 1981; Parslow et al., 1983). Therefore, by comparing and contrasting the antigenic specificities of ASA from men following vasectomy reversal, we hoped to identify the sperm antigens that could be used to develop new and more specific diagnostic tests for ASA.

Materials and methods

Participants

This investigation was approved by the regional Ethics Committee and all samples were obtained after informed consent. Participants were grouped as follows: group 1, three men who had undergone vasectomy reversal and were fertile; group 2, three fertile men with normal sperm parameters serving as controls (World Health Organization, 1999); group 3, six men who were undergoing
investigations for infertility after 1 year of inability to impregnate their partners; group 4, four infertile men who had a positive (>80%) SpermMAR® sperm surface antibody test (Ferti-PRO, Belgium.) All individuals had a sperm concentration >10^6/ml and motility >75% (Meinertz et al., 1990). Blood was collected by venipuncture from all participants and their sera separated by centrifugation and stored at −80°C until use.

Preparation of normal sperm and leukocyte extracts for Western blotting
Normal semen (World Health Organization, 1999) was collected from donors by masturbation. The semen was allowed to liquefy at room temperature for 30 min and divided into aliquots containing 50×10^6 sperm in 1.5 ml microfuge tubes. Sperm were then separated from the seminal plasma by centrifugation (1500 g) for 15 min at 4°C. Each aliquot of sperm was washed by suspension in 1 ml of phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 1500 g at 4°C. The supernatant was determined by Bradford microprotein assay and stored at −80°C until use.

For experiments where proteins from motile sperm were needed, they were prepared from semen samples using a discontinuous 40–90% PureSperm® (Arctech, Australia) gradient centrifugation at 300 g. The sperm pellet was resuspended and adjusted to 50×10^6 then subjected to washing as above.

The sperm aliquots were subsequently suspended in 250 µl of protease inhibitor (PI) buffer (benzamidine hydrochloride hydrate 1 mmol/l; pepstatin A 1 µmol/l; N-Tosyl-L-Phenylalanine chloromethyl ketone 100 µmol/l in Tris 25 mmol/l/EDTA 10 mmol/l pH 8.0 buffer) and stored at −80°C until use. All materials, unless otherwise stated, were obtained from Sigma (Sydney, Australia).

Sperm protein extracts (SPE) were prepared freshly before use as follows. Cryopreserved sperm aliquots were thawed and the sperm were solubilized by mixing with Triton X114 (1% v/v) (Boehringer Mannheim, Auckland, New Zealand) by repeated pipetting with a micropipette. The samples were then incubated for 4–16 h at 4°C on a rotating platform. Debris was removed by centrifugation at 13 000 g for 15 min at 4°C. The concentration of the extracted protein in the supernatant was determined by Bradford microprotein assay (Bradford, 1976) using bovine serum albumin dissolved in PI buffer as standard protein.

Leukocytes were separated from 5 ml of freshly drawn heparinized blood by Ficoll gradient centrifugation (Lymphoprep-Nycomed®, Life Technologies, Auckland, New Zealand) according to the manufacturer’s instructions. The cells were washed twice with PBS, pH 7.4, and the leukocytic proteins were extracted using the method described above for sperm.

Isolation of immunoglobulin G from normal sperm extracts
Normal sperm extracts, prepared from 200×10^6 sperm, pooled from six donors as above, were passed through a 5 ml protein A-AffiGel® column (Bio-Rad, Auckland, New Zealand) that had been equilibrated with PBS, pH 7.4. Passage of proteins was monitored at optical density 280 nm with a UVcord II® flow cell (Pharmacia, Auckland, New Zealand). When protein had ceased to wash through the column, bound proteins were eluted with 0.1 mol/l glycine, pH 2.5. Fractions containing eluted proteins were pooled, neutralized by drop addition of a 1 mol/l Tris solution, concentrated in a centrifugal concentrator (Vivaspin 500®; Sartorius, Auckland, New Zealand), and equilibrated with PBS, pH 7.4, by dialysis. The proteins were then analysed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis.

PAGE and Western blotting
Normal sperm protein extracts and lymphocyte extract (LE) were boiled for 4 min with sample buffer (0.0625 mol/l Tris, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue dye). A total of 400 µg of proteins were then separated by electrophoresis on a preparatory, precast 4–15% gradient polyacrylamide gel with an 11 cm wide well using a Criterion® system (BioRad). The separated proteins were transferred to nitrocellulose membranes (Hybond-C extra; Amersham, Auckland, New Zealand) using a semi-dry blotting apparatus according to the manufacturer’s instructions (Gelman Science, Life Technologies). The membranes were then stained with a Ponceau-S solution [0.1% (w/v) Ponceau-S in 5% (v/v) acetic acid] to confirm the transfer and destained with PBS, pH 7.4, containing 0.05% (v/v) Tween 20 (PBS–T). The membranes were then blocked overnight at 4°C in 5% (w/v) non-fat milk powder (Pams, Auckland, New Zealand) dissolved in PBS–T (blocking buffer). They were cut into strips and incubated with patient sera, mouse anti-CD46 (Sorotec, Oxford, UK), mouse anti-CD 45 (Dako, Auckland, New Zealand) or rabbit anti-CD55 (gift from Dr A.R.A. Blanco, University of Liverpool, UK), all diluted 1/500 in blocking buffer under gentle rocking at room temperature for 1 h. The membranes were then washed three times with PBS–T and incubated for an additional 1 h, at room temperature, with horseradish peroxidase (HRP)-conjugated goat anti-human γ chain, anti-mouse immunoglobulin (IgG), or anti-rabbit IgG serum (Caltag, Auckland, New Zealand), as appropriate, diluted 1/5000 in blocking buffer. The membranes were again washed three times with PBS–T and incubated with enhanced chemiluminescence reagent (New England Nuclear, Life Science Technologies, Auckland, New Zealand) according to the manufacturer’s instructions. Finally, the membranes were placed in a heat-sealed polythene envelope and exposed to Hyperfilm® ECL (Amersham) for varying time periods ranging from 30 s to 30 min and developed in an automated film processor (Agfa Curix 60, Germany).

Molecular weights of detected sperm proteins were deduced by comparison with prestained recombinant molecular weight standards (BioRad).

Western blot of normal sperm extract with protein A-purified IgG from normal sperm extract
Normal sperm extract was prepared as above and resolved on a 10% SDS–PAGE using a BioRad MiniProtein II system (BioRad). The proteins were then transferred to a nitrocellulose membrane (Hybond-C®, Amersham) as above. The membrane was then blocked overnight at 4°C in blocking buffer and followed by probing either HRP-conjugated goat anti-human γ chain or with 100 µg of the IgG purified from sperm in blocking buffer, followed by goat anti-human γ chain HRP conjugate. The binding of the proteins on the blot was visualized by adding ECL reagent as above.

Results
Purity of sperm protein extracts
That sperm protein extract (SPE) was not significantly contaminated with proteins from non-sperm cells or seminal plasma was shown by examining the molecular weights of two proteins, CD46 and CD55, that have sperm-specific isoforms (Figure 1). Probing SPE with a monoclonal antibody to CD46 resulted in the detection of a single band of molecular weight of ~43 kDa, whereas the same antibody also reacted with a band of ~55 kDa in lymphocyte extract and seminal plasma. An antiserum to CD55 reacted with a single band of
Western blot analysis of ASA

The antigenic specificities of ASA from the fertile and infertile men were examined by Western blot analysis against normal sperm proteins. In order to confirm our results were repeatable, each blot was conducted on at least two separate occasions and each serum was used to probe SPE from at least two different normal donors. The antibody binding patterns for different individuals were complex and varied, but there were several striking features (Figure 2). Firstly, all men, including those who did not have ASA by MAR, had ASA detected by Western blot that reacted with proteins of molecular weights 144, 78, 51, 42, 41, 39–31 kDa (Figure 2). Secondly, sera from three infertile men (groups 3 and 4) reacted with sperm proteins of low molecular weights (9–19 kDa). None of the fertile men had ASA reactive with these proteins (Figure 2). Thirdly, ASA from all infertile men reacted with protein(s) of molecular weight 54 kDa, whereas none of the fertile men had ASA that bound this protein (Figure 2). The finding that none of the sera from fertile subjects reacted with these proteins make these proteins candidates for further study. Fourthly, the normal sperm extracts all contained sperm-bound IgG as shown by reactivity of the antihuman IgG conjugate directly with protein bands of 180, 110, 62 and 27 kDa (Figure 2). Fifthly, this sperm-associated IgG was also present on highly motile sperm prepared by density gradient centrifugation. This indicates that IgG was bound to normal sperm and was not removed by the washing procedures employed in this study.

Finally, although we did not conduct a densitometric analysis, some sera contained ASA that produced particularly intense reactivity against individual bands; e.g. serum from patient 1 (fertile) produced an intense reaction against a sperm protein of molecular weight 26 kDa and sera from patients 9, 12 and 14 (infertile) demonstrated consistently intense reactions against various proteins of low molecular weights from 9–19 kDa (Figure 2). This indicates that there are quantitative differences in the amount of some antibodies among the individuals that may be important in determining whether a specific antibody can disrupt fertility.

Isolation and characterization of IgG bound to normal sperm

Protein A affinity chromatography was used to isolate the antibody from normal (MAR negative) sperm extracts. The isolated antibody, which proved to be IgG, was then used to probe normal sperm extracts by Western blot. This analysis demonstrated that the IgG isolated from normal sperm did not react with specific sperm proteins, suggesting the antibody is not bound to sperm via an antigen/antibody reaction (Figure 3).

Discussion

We investigated the antigenic specificity of ASA from fertile and infertile men using extracts of normal sperm as an antigen. Although we are not the first to use Western blotting to examine this question, there are several unique features of this study. Normal human ejaculates contain several cellular components other than sperm (Alexander and Anderson, 1987). Particularly, up to 10% of the cellular content of a normal ejaculate may be leukocytes (Wolff, 1995). The presence of proteins from these non-sperm cells in the antigen preparation used for Western blotting could lead to false positive results. Therefore, in this study we took great care to ensure that our sperm protein extracts were not contaminated with proteins from other cell types or seminal plasma proteins. The complement control proteins CD46 and CD55 are expressed by many cell types including leukocytes, epithelial cells and fibroblasts and are also found in seminal plasma (Taylor and Johnson,
Our analysis demonstrated a complex pattern of antibody reactivity with sperm proteins regardless of whether we used serum derived from fertile or infertile patients. However, there were several striking features.

Firstly, sperm protein(s) of molecular weights 9–19 kDa were reactive with antibodies from three of the infertile men but not with antibodies from their fertile counterparts. Sperm protein(s) of similar molecular weights have been found by others in males with unexplained infertility (Lehmann et al., 1985; Auer et al., 1997) and we are currently investigating the identities of these proteins.

Secondly, IgG was found to be present in the normal sperm extracts. These extracts were prepared from MAR-negative sperm; therefore, this antibody was not detected by a conventional ASA test. The washing procedure used in the preparation of the sperm protein extracts provides us with reasonable certainty that this IgG is not simply a carry-over from seminal plasma. Dead sperm have been known to be non-specifically coated with IgG (Rasanen et al., 1992). Our findings indicated that protein extracts prepared from highly motile sperm by density gradient centrifugation also contained IgG. That IgG is present on sperm is not a new finding (Allen and Bourne, 1978; Hjort, 1996). It is believed that the binding of this antibody is not via a specific antigen/antibody interaction but rather by some form of non-antigenic, possibly Ig Fc segment-mediated, interaction. In order to test this question, we purified the IgG from normal sperm and used the purified antibodies to probe a Western blot of sperm proteins. Our results confirm that the antibody present on normal sperm does not react with sperm antigens. However, the mechanism by which these antibodies bind to sperm remains unresolved. It has been suggested that direct disulphide bonding of the antibody to sperm may occur (Richards and Witkin, 1984). Alternatively, it has been suggested that there is an Fc receptor on sperm. A 16/20 kDa antibody-binding protein, suggested to be related to FcyRIII on the basis of monoclonal antibody reactivity, was reported (Kamada et al., 1991). However, we (unpublished data) and others have not found FcyRIII immunoreactivity on sperm (Bronson et al., 1992). If the IgG we have shown is associated with sperm by an Fc-mediated mechanism, this may explain why the sperm-associated antibody is not detected by the MAR test, as steric hindrance may prevent the MAR beads reacting with the sperm-bound antibodies.

The biological significance of having IgG attached to sperm is not clear. One could speculate that the presence of IgG on sperm could facilitate binding to macrophage or neutrophil Fc receptors to mediate disposal of excess sperm from the female reproductive tract (Overstreet and Mahi-Brown, 1993). On the other hand, the presence of IgG on the spermatozoal surface may be of benefit to the process of fertilization. For example, hamster oocyte plasma membranes are reported to express Fcy receptors and immunoglobulins on the surface of sperm, which may therefore act as a ligand to facilitate their binding or penetration of oocytes (Bronson et al., 1990).

A notable feature of our analysis is the demonstration that serum from all men studied contained sperm-reactive antibodies. In most instances antibodies from fertile men

1996). Humans express sperm specific isoforms of both CD46 and CD55 with reported molecular weights of 43 and 50 kDa respectively (Seya et al., 1993; Simpson and Holmes, 1994; Taylor and Johnson, 1996). By examining the molecular weight of CD46 and CD55 contained in our sperm protein extracts, we have been able to confirm that only the sperm-specific isoforms of CD46 and CD55 were present. Thus, our sperm preparation and washing procedures were effective in removing seminal plasma proteins and non-sperm cells. The absence of CD45 immunoreactivity confirmed the absence of contaminating leukocyte proteins from our sperm protein extracts.

The presence of the sperm-specific isoforms of proteins CD46 and CD55 in our sperm protein extract confirm that these extracts contain membrane proteins but it is also likely that the extracts contain cytoplasmic proteins. We plan to determine the sub-cellular localization of these sperm protein(s) once they are further characterized.

Some previous Western blotting studies have employed denaturing conditions (Lehmann et al., 1985; Naaby-Hansen and Bjerrum, 1985; Mathur et al., 1988; Auer et al., 1995) which may have caused the loss of conformation-dependent epitopes on sperm proteins (Shai and Naot, 1992). We have tried to minimize antigen denaturation by employing a non-ionic detergent, Triton X114 (Helenius and Simons, 1975), for sperm protein extraction and non-reducing electrophoresis. We also used a very sensitive detection system, enhanced chemilluminescence (Bronstein et al., 1990), as opposed to the more traditional colorimetric methods used by others (Lehmann et al., 1985; Naaby-Hansen and Bjerrum, 1985; Parslow et al., 1987).

Figure 3. Antibody (IgG) bound to normal [mixed antiglobulin reaction (MAR)-negative] sperm was isolated by protein A chromatography of solubilized normal sperm protein extract. This protein A eluant was shown to contain only whole IgG and fragments of IgG by SDS–PAGE (lane 1) and Western blot using a horseradish peroxidase-conjugated goat anti-human IgG antibody (lane 2). The purified IgG was then used to probe a normal sperm protein extract by Western blot (lane 3). This analysis demonstrated that no additional reactivities were present other than those produced by the direct binding of the conjugate to the normal sperm proteins (lane 4).
reacted with apparently the same sperm proteins as antibodies from fertile men. At face value one might then draw the conclusion that there is no difference in the antigenic specificity of sperm antibodies from fertile and infertile men. However, it is quite possible that antibodies from infertile men are reacting with a protein of given molecular weight while antibodies from fertile men are reacting with a different protein of the same molecular weight. Furthermore, even if antibodies from fertile and infertile men are reacting with the same protein(s), it is possible that the antibodies from these two groups react with different epitopes within the protein(s). It is well known that antibodies that react with biologically active proteins can be either neutralizing or non-neutralizing depending upon the epitope with the protein with which the antibody reacts. For example, antibodies reactive with the first short consensus repeat (SCR) domain of CD46 inhibit the ability of sperm to interact with oocytes, whereas antibodies reacting with other domains of CD46 do not inhibit sperm-oocyte interaction (Taylor et al., 1994; Taylor and Johnson, 1996). Thus, where antibodies from fertile and infertile men react with the same protein, it is possible that fertile men produce non-neutralizing antibodies while infertile men produce neutralizing antibodies. Furthermore, quantitative differences in the amount of antibody may also be important in determining whether a given antibody disrupts fertility, as is believed to be the case with MAR tests. Our results show that some individuals have substantially more antibodies reactive with particular proteins than other individuals who have antibodies reactive with the same proteins. These quantitative differences in antibody levels may be very important in determining the effects of ASA on fertility.

The complications discussed above make the interpretation of Western blots for ASA difficult, but our use of differential Western blot analysis of fertile and infertile men has enabled identification of candidate proteins that may be involved in fertility/infertility and we are continuing our investigations to identify these proteins.

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References


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**Antigenic specificities of antisperm antibodies**