Presence of antibodies to sperm YLP\textsubscript{12} synthetic peptide in sera and seminal plasma of immunoinfertile men

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Using an enzyme-linked immunosorbent assay (ELISA), sera (n = 15) and seminal plasma (n = 30) from antisperm antibody-positive immunoinfertile men (n = 45) and from fertile men (n = 45), were tested for the immunoreactivity with the synthetic YLP\textsubscript{12} sperm peptide. Of the 15 immunoinfertile sera tested, 46% were positive for immunoglobulin (Ig)M, 73% for IgG, and 40% for IgA. Of the 30 samples of immunoinfertile seminal plasma tested, 10% were positive for IgM, 20% for IgG, and 43% for IgA. None of the sera or seminal plasma from fertile men showed a positive reaction. There was no significant correlation between the sperm immobilization technique (SIT) or tray agglutination technique (TAT) titres or percentage binding in immunobead binding technique (IBT) and the antibody reactivity for any class in the ELISA. The YLP\textsubscript{12} peptide conjugated to bovine serum albumin–Sepharose 4B beads pulled out IgG antibodies from the serum of the immunoinfertile, but not the fertile, men. The beads pulled out IgA antibodies from the immunoinfertile, but not the fertile, seminal plasma. The immuno-affinity purified antipeptide antibodies reacted with a specific band of 72 ± 5 kDa in the human testis and with a specific band of ~50 ± 5 kDa in the human sperm extracts. The YLP\textsubscript{12} peptide may have applications in the specific diagnosis and treatment of male infertility and in contraceptive vaccine development.

Key words: antisperm antibodies/fertilization/infertility/semen/sperm antigens

Introduction

Antisperm antibodies (ASA) have been implicated in involuntary infertility in humans (Mathur, 1986; Schulman, 1986; Naz and Menge, 1994; Ohl and Naz, 1995; Helmerhorst et al., 1999). Depending upon the centre, 2–30% of cases of infertility have been reported to be associated with the presence of ASA in the male and/or female partner of an infertile couple (Naz and Menge, 1994; Ohl and Naz, 1995). These ASA are not merely associated with, but are rather causative factors of infertility. These antibodies inhibit human sperm penetration of zona-free hamster oocytes and also reduce fertilization rates in IVF and embryo transfer. The inhibition of fertilization may be due to interference in sperm capacitation and acrosome reaction, in sperm–oocyte plasma membrane recognition and fusion, or in zona binding and penetration (Bronson et al., 1984).

Many ASA present in men and women, although reactive with spermatozoa in various techniques, may be irrelevant to infertility. These ASA should be called ‘sperm reactive’ rather than ‘antisperm’ antibodies, unless they are directed against sperm antigen(s) that are involved in fertility. Recently, using hybridoma and Western blotting techniques, attempts have been made by various laboratories to define antigens that are relevant to fertility, and the corresponding antibodies which are involved in immunoinfertility (Auer et al., 1995; Pillai et al., 1996; Clayton et al., 1998). A sperm-specific glycoprotein, designated as fertilization antigen-1 (FA-1), has been shown to be involved in human immunoinfertility (Naz, 1987; Bronson et al., 1989; Naz et al., 1990, 1993; Menge et al., 1999).

Recently, we described a novel human sperm dodecamer peptide sequence, designated YLP\textsubscript{12}, that is involved in binding to the zona pellucida of human oocyte (Naz et al., 2000). This sequence is specifically expressed only in the human testis and not in other human tissues tested. The peptide sequence is present on the acrosomal region of the human sperm cell. The present study was conducted to examine whether or not the sera and seminal plasma of immunoinfertile men have antibodies reactive with YLP\textsubscript{12} peptide. The overall aim of the study was to examine the involvement of this sperm-specific peptide in male infertility in order to find any potential clinical applications in the diagnosis and treatment of infertility, or in the development of a contraceptive vaccine.

Materials and methods

Patient sera

Sera (n = 15) and semen samples (n = 30) were collected from infertile men (n = 45; aged 24–37 years), who showed ASA in their
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Figure 1. Reaction of the immunoglobulin (Ig)M, IgG and IgA antibodies in immunoinfertile \((n = 15)\) and fertile \((n = 15)\) sera with the synthetic human sperm YLP\(_{12}\) peptide in enzyme-linked immunosorbent assay (ELISA). Sera from the immunoinfertile men strongly reacted with the peptide, seven (46\%) showing a positive reaction for IgM, 11 (73\%) showing a positive reaction for IgG and six (40\%) showing a positive reaction for IgA class of antibodies. None of the sera from the fertile men showed a positive reaction with the peptide for any class of antibody. The values above the dotted line are \(>2\) SD units.

Enzyme-linked immunosorbent assay (ELISA)

The antibodies (IgM, IgG, and IgA) in the serum and seminal plasma samples were detected using ELISA as described elsewhere (Naz et al., 1989). Each well was incubated overnight (at 4°C) with peptide or bovine serum albumin (BSA; 1 \(\mu\)g/well in 200 \(\mu\)l) diluted in carbonate buffer (0.1 mol/l, pH 9.6). The wells were washed three times (for 5 min each) with phosphate-buffered saline (PBS) containing 0.05\% Tween-20 (PBS-T). To block non-specific binding sites, the wells were incubated with PBS-T containing 1\% BSA at 37°C for 45 min and washed three times (for 5 min each) with PBS-T. The wells were incubated (at 37°C, for 2 h) with serum (1:50 dilution) and seminal plasma (1:20 dilution) samples (200 \(\mu\)l/well), diluted in PBS-T containing 0.5\% BSA. The wells were washed (five times) with PBS-T and then incubated (37°C, 1.5 h) with alkaline phosphatase conjugated goat anti-human IgM, IgG, and IgA (\(\mu\)-, \(\gamma\)-, or \(\alpha\)-chain specific) immunoglobulins (Sigma Chemical Co, St Louis, MO, USA) diluted (1:1000) in PBS-T containing 0.5\% BSA (200 \(\mu\)l/well). The wells were washed as before, and then incubated (37°C, 30 min) with the substrate solution (1 mg/ml disodium p-nitrophenyl phosphate diluted in 0.05 mol/l carbonate buffer, pH 9.8). The reaction product was read at 405 nm. Each serum and seminal plasma sample was run in duplicate and the BSA-coated wells, treated identically, served as controls. The absorbance reading of the control wells was subtracted from the absorbance reading of the antigen-coated wells and the mean of the subtracted values was recorded. The absorbance readings were converted to SD units by using the following formula:

\[
SD\ units = \frac{\text{mean (test)} - \text{mean (control)}}{\text{SD of control group}}.
\]

Peptide synthesis

The YLP\(_{12}\) peptide was synthesized by solid-phase synthesis using Fmoc chemistry (Biosynthesis Inc, Lewisville, TX, USA). Deprotection was achieved by 20\% piperidine in dimethylformamide, and the peptide was cleaved from the resin by 85\% trifluoroacetic acid (TFA). The peptide was then precipitated in methyl tert-butyl ether and purified by using reverse-phase HPLC. The fractions eluted with 0.5\% TFA in acetonitrile were dried in a speed vacuum, redissolved in water, and lyophilized. The peptide was water soluble and had >95\% purity level.

sera as revealed by the sperm immobilization technique (SIT) (Isojima et al., 1968), tray agglutination technique (TAT) (Friberg, 1974) and immunobead binding technique (IBT) (Menge et al., 1999). All these sera and semen samples demonstrated \(>20\%\) sperm binding in IBT. These sera and the semen samples will be referred to as immunoinfertile samples. Sera and semen samples belonged to two different groups of men. The control sera \((n = 15)\) and semen samples \((n = 30)\) were collected from healthy fertile men \((n = 45;\) aged 24–35 years) of proven fertility record and who have fathered a child within the last year. Control sera and semen samples did not demonstrate presence of ASA when tested by SIT, TAT, or IBT. These sera and semen samples will be referred to as fertile samples. Semen was liquefied and centrifuged (1000 g, 15 min), and seminal plasma was separated, divided into aliquots, and frozen at \(-20°C\) until used. Serum samples separated from the blood were similarly divided and frozen at \(-20°C\) until used.
Involvement of YLP_{12} peptide in immunoinfertility

Figure 2. Reaction of the immunoglobulin (Ig)M, IgG and IgA antibodies in immunoinfertile (n = 30) and fertile (n = 30) seminal plasma samples with the synthetic human sperm YLP_{12} peptide in enzyme-linked immunosorbent assay (ELISA). Seminal plasma from the immunoinfertile men strongly reacted with the peptide, three (10%) showing a positive reaction for IgM, six (20%) showing a positive reaction for IgG and 13 (43%) showing a positive reaction for IgA class of antibodies. None of the seminal plasma samples from the fertile men showed a positive reaction with the peptide for any class of antibody. The values above the dotted line are >2 SD units.

Table I. Antipeptide antibody reactivity and sperm immobilization technique (SIT) and tray agglutination technique (TAT) titres in enzyme-linked immunosorbent assays (ELISA) of sera from immunoinfertile men

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>SIT (titre)</th>
<th>TAT (titre)</th>
<th>IgM</th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>256</td>
<td>1.6</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>256</td>
<td>2.8</td>
<td>4.6</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>512</td>
<td>4.3</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>128</td>
<td>2.3</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>256</td>
<td>3.2</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>1024</td>
<td>6.4</td>
<td>12.2</td>
<td>3.0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>128</td>
<td>3.8</td>
<td>2.9</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>641</td>
<td>1.4</td>
<td>5.7</td>
<td>7.4</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>64</td>
<td>2.8</td>
<td>0.8</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>0</td>
<td>1.2</td>
<td>4.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

There were no significant correlations between the SIT and TAT titres and any of the immunoglobulins (Ig).

test samples with ≥2 SD units were considered as having a positive reaction with the YLP_{12} peptide.

Immunoprecipitation procedure
To elute the antibodies reactive with the YLP_{12} peptide, the immunoprecipitation procedure was performed using the positive immunoinfertile sera and seminal plasma and the peptide conjugated to BSA beads. The peptide was conjugated to BSA using 1-ethyl-3-(3-dimethylaminopropyl) as described earlier (Tsong et al., 1985; Naz et al., 2000). The peptide–BSA conjugate or BSA alone was coupled to the cyanogen bromide (CNBr)-activated Sepharose-4B beads (Sigma) (Naz and Ahmad, 1994). The immunoinfertile sera (nos. 6 and 8) and seminal plasma (nos. 3, 16, 18 and 19) showing a strong positive reaction with the peptide, and the fertile sera (n = 3) and seminal plasma (n = 3), showing no reaction with the peptide, were selected and divided into two aliquots of 20 µl each. One aliquot was incubated (2 h, 4°C) with the peptide–BSA–Sepharose-4B beads and the second with the BSA–Sepharose-4B beads, suspended in radioimmunoprecipitation assay buffer (RIPA buffer) [50 mmol/l NaCl, 10 mmol/l Tris, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l phenylmethylsulphonylfluoride (PMSF)] (Naz and Ahmad, 1994). The unadsorbed proteins were washed five times with RIPA buffer (for 5 min each) and the YLP_{12}-reacted antibodies were eluted from the beads by treatment of glycine–HCl (0.1 mol/l, pH 2.8). The eluate was neutralized with solid K_{2}HPO_{4} to pH 7.4. A portion of the neutralized eluate was boiled with non-reduced sodium dodecyl sulphate (SDS) sample buffer, and was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (5–15% gradient) (Laemmli, 1970). The gel was stained with ultrasensitive silver nitrate to visualize the protein bands.

Western blot analysis
Western blotting was performed to examine the molecular identity of the cognate antigen recognized by the anti-YLP_{12} peptide antibodies in human testis and spermatozoa. The human testis extract was
obtained from Clonetech (Palo Alto, CA, USA). Human sperm extract was prepared as described elsewhere (Naz et al., 2000). Briefly, human spermatozoa were isolated by the swim-up procedure from liquefied (37°C, 30 min) semen collected from the healthy fertile donors. The swim-up spermatozoa were washed twice in PBS containing 1 mmol/l PMSF, and were solubilized overnight at 4°C in lysis buffer (20 mmol/l HEPES, pH 7.5, 1.5 mmol/l MgCl2, 10% glycerol, 1% Triton X-100, 10 mmol/l PMSF, and 10 µg/ml leupeptine). The lysate was centrifuged and the supernatant (sperm extract) was collected, divided into aliquots and stored at −20°C until used.

The protein concentration in the sperm extract was measured by the Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA) following the manufacturer’s protocol. Testis and sperm extracts were subjected to SDS–PAGE (5–15% gradient), and electrophoretically transferred to the nitrocellulose membranes (Towbin et al., 1979). The blots were incubated (2–3 h, room temperature) with blocking solution (3% BSA) and then incubated overnight with the anti-YLP12 antibodies (immunoprecipitated from the immunoinfertile sera or seminal plasma) or an equal amount of immunoglobulins from the fertile control sera and seminal plasma, diluted in incubation buffer (∼10–15 µg/ml). The blots were washed, incubated (2 h) with goat anti-human alkaline phosphatase-conjugated antibodies (α- or γ-chain specific, 1:1000 dilution), washed again and treated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate, to visualize the reacted bands.

Statistical analysis

Differences were calculated using paired and unpaired Student’s t-tests. P < 0.05 was considered to be statistically significant. Correlations were examined by analysing for linear regression.

Results

Of the 15 immunoinfertile sera tested against the YLP12 peptide, seven (46%) were positive for IgM, 11 (73%) were positive for IgG, and six (40%) for IgA, taking ≥2 SD units as having a positive reaction (Figure 1). There were more IgG antibody-positive sera than IgM or IgA antibody-positive sera. Of these, one (no. 6) (6.6%) was positive for all three classes, three (nos. 2, 5 and 7) (20%) were positive for both IgM and IgG, one (no. 9) (6.6%) for both IgM and IgA, and four (no. 8, 10, 11 and 14) (26.6%) for both IgG and IgA classes (Figure 1). There was no significant linear correlation between the SIT(~102) peptide–bovine serum albumin (BSA)–Sepharose beads. The peptide–BSA beads (lane b) and not the BSA beads (lane a) pulled out antibodies from the immunoinfertile serum that showed a band of ~160 kDa. Neither the peptide–BSA beads (lane d) nor the BSA beads (lane c) pulled out any protein from the fertile serum.

Table II. Antisperm antibody (ASA) binding in immunobead binding technique (IBT) and antipeptide antibody reactivity in enzyme-linked immunosorbent assays (ELISA) of seminal plasma from immunoinfertile men

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Percentage binding in IBT</th>
<th>SD units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>1</td>
<td>45 (H, T)</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>64 (H, T)</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>100 (H, T)</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>100 (H, T)</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>40 (H, T)</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>80 (H, T)</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>50 (H, T)</td>
<td>−1.2</td>
</tr>
<tr>
<td>8</td>
<td>97 (H, T)</td>
<td>0.0</td>
</tr>
<tr>
<td>9</td>
<td>50 (H)</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>28 (H)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

There was no significant correlation between IBT and any of the immunoglobulins (Ig). H = head binding; T = tail binding.

Figure 3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) pattern of the antibodies eluted from the immunoinfertile and fertile sera by the YLP12 peptide–bovine serum albumin (BSA)–Sepharose beads. The peptide–BSA beads (lane b) and not the BSA beads (lane a) pulled out antibodies from the immunoinfertile serum that showed a band of ~160 kDa. Neither the peptide–BSA beads (lane d) nor the BSA beads (lane c) pulled out any protein from the fertile serum.
antibodies from the immunoinfertile sera in the immunoprecipitation procedure, showing a distinct band of \(~160\) kDa in the SDS–PAGE (Figure 3, lane b). No antibodies were pulled out by the BSA–Sepharose beads that lacked the YLP\(_{12}\) peptide (Figure 3, lane a). No antibodies were eluted from the fertile sera by either the peptide–BSA (Figure 3, lane d) or BSA beads without peptide (Figure 3, lane c).

The YLP\(_{12}\)–BSA–Sepharose 4B beads (but not the BSA beads without peptide) also pulled out specific antibodies from each of the four immunoinfertile seminal plasma samples. No antibodies were eluted from the fertile seminal plasma. The immunoinfertile seminal plasma used for the immunoprecipitation was only positive for the IgA class of antibodies. Although the eluates from the immunoinfertile seminal plasma did not show any band in the non-reduced SDS–PAGE due to the high molecular weight of the eluted polymeric IgA that did not run in the gel, they showed the presence of specific IgA antibodies in ELISA examined using the \(\alpha\)-chain specific alkaline phosphatase-conjugated secondary antibodies (\(>9\) SD units).

Antibodies eluted from the immunoinfertile sera and seminal plasma, reacted with a specific band of \(72 \pm 5\) kDa (Figure 4A, lane a) in the human testis extract and with a specific band of \(~50 \pm 5\) kDa in the human sperm extract (Figure 4A, lane b) in the Western blot analysis. Both the IgG antibodies eluted from the immunoinfertile sera (Figure 4) and the IgA antibodies (data not shown) eluted from the immunoinfertile seminal plasma reacted with the specific bands of similar molecular identities. The same amount of immunoglobulins from fertile sera and seminal plasma did not react with these specific bands (Figure 4B). There were some non-specific protein bands in the testis lane that were recognized by both the immunoinfertile antibodies and fertile immunoglobulins, but the specific band was recognized only by the former.

**Discussion**

The results of the present investigation indicate that the sera and seminal plasma from immunoinfertile men react strongly with the sperm-specific YLP\(_{12}\) synthetic peptide, while the sera and seminal plasma from fertile men showed minimal ‘immunological background’ reactions. Taking \(>2\) SD units as a cut-off for positive reactivity, at least 73% of the immunoinfertile sera, and 43% of the immunoinfertile seminal plasma were positive for at least one class of antibody. None of the fertile sera or the fertile seminal plasma demonstrated antibody reactivity of \(>2\) SD units. As expected, more serum samples showed positive reactions for the IgG class and more seminal plasma samples showed positive reactions for the IgA class of antibodies.

There was no significant linear correlation between the SIT or TAT titres in the serum and any class of antipeptide antibodies detected by ELISA. This was not surprising since the antibodies raised in rabbits against the synthetic peptide
do not immobilize or agglutinate human spermatozoa (Naz et al., 2000). There was also no significant linear correlation between the percentage binding in the IBT and any class of antipeptide antibodies detected by ELISA. These findings indicate that both these techniques are measuring different types of antibodies. ASA present in serum and seminal plasma are directed against various antigens including FA-1 antigen, cleavage signal-1 (CS-1) protein, protamine and several as yet unidentified antigens (Naz et al., 1989; Naz and Menge, 1994). Antibodies that are directed against the sperm surface antigens may be detected by IBT. One type of these antibodies (among several others) is the type directed against the YLP_{12} peptide epitope.

To elute the antibodies specifically reacting with the peptide, the immunoprecipitation procedure was performed. The peptide–BSA beads, but not the BSA beads without the peptide, pulled out the specific antibodies from the immunoinfertile sera, showing a single band of ~160 kDa, corresponding to IgG, in the non-reduced SDS–PAGE. The peptide–BSA beads did not react with the non-specific immunoglobulins present in the serum of fertile men. Similarly, the peptide–BSA beads (but not the BSA beads) pulled out the specific IgA antibodies from the immunoinfertile seminal plasma and not the fertile seminal plasma. The presence of the antibodies in the eluate was detected using the α-chain-specific secondary antibodies. These findings confirm the results obtained by ELISA.

The antibodies eluted from the immunoinfertile serum and seminal plasma by the peptide–BSA beads, recognized a specific band of ~72 ± 5 kDa in the human testis extract and ~50 ± 5 kDa band in the human sperm extract. Both the IgG class of antibodies eluted from the immunoinfertile sera and the IgA class of antibodies eluted from the seminal plasma showed binding to the protein bands of similar molecular identities. These results observed with the human antibodies are in agreement with those observed with the antipeptide antibodies raised in rabbits. The rabbit antipeptide antibodies reacted with the antigens of similar molecular identities as detected with the human antipeptide antibodies (Naz et al., 2000). These findings suggest that the YLP_{12} peptide epitope is part of a 72 ± 5 kDa protein that is synthesized during spermatogenesis in the testis and later modified/cleaved to form a 50 ± 5 kDa protein in the mature ejaculated sperm cell.

In conclusion, our data indicate that the immunoinfertile men have circulating and local antibodies against the sperm-specific YLP_{12} peptide epitope. Since these antibodies are present only in the immunoinfertile men and not in the fertile men, it indicates that they are associated with infertility. Antipeptide antibodies inhibit human sperm binding to human oocyte zona pellucida in vitro (Naz et al., 2000). Similar anti-fertility effects of the antibodies can be expected in vivo, making these antibodies possibly causative, rather than just associated, factors of infertility. Thus, the auto-antigenic sperm-YLP_{12} peptide may have clinical applications in the specific diagnosis and treatment of male infertility, and in contraceptive vaccine development.

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References
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