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Presence of PSA antibodies in seminal plasma of infertile men

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1. ABSTRACT

Immunoinfertility due to antisperm antibodies and semen hyperviscosity are among major causes of male infertility. Although the modulation of prostate-specific antigen (PSA) has been investigated in prostate abnormalities, its role and the effect of its dysfunction in male fertility/infertility have not been extensively examined. The present study was conducted to examine the presence of PSA antibodies locally in the seminal plasma of men having immunoinfertility and semen hyperviscosity. Seminal plasma samples from immunoinfertile men (*n*=25), men with hyperviscous semen (*n*=25), and normal men (*n*=24) were collected and analyzed for immunoreactivity with PSA in ELISA and Western blot. In the immunoinfertile group, seminal plasma from 20% of men reacted positively with PSA. In the hyperviscous group, seminal plasma from 28% of men reacted positively with PSA. None (0%) of the seminal plasma from the normal group showed immunoreactivity to PSA. This is the first study ever to indicate the presence of PSA antibodies in semen of men having immunoinfertility or hyperviscosity. These findings may have clinical significance in the specific diagnosis and treatment of infertility in men and contraceptive vaccine development.

2. INTRODUCTION

The presence of antisperm antibodies (ASA) has been implicated in infertility in both men and women (1). ASA can cause infertility by affecting various parameters of sperm function, including motility, penetration through cervical mucus, capacitation/acrosomal exocytosis, binding/penetration of oocyte zona pellucida, binding/fusion with oocyte plasma membrane, and inhibition of oocyte cleavage and embryonic development (1-6). ASA which are directed against a sperm antigen that is relevant to fertilization/fertility are especially pertinent to immunoinfertility. Several labs are searching for sperm antigens relevant to human immunoinfertility (1).

Prostate-specific antigen (PSA), also called human kallikrein 3 (hk3) or gamma-seminoprotein, is a serine protease produced primarily by the prostate gland (7-10). It is synthesized as a 261 aa pre-propolypeptide, including a 17-aa signal and a 7-aa activation peptide, which are cleaved by human glandular kallikrein 2 (hk2) during processing and secretion (8). Mature PSA is a glycoprotein of ~28 kDa comprised of 237 amino acids (aa) and a carbohydrate chain (11). PSA is secreted at concentrations of 0.5.-2 g/l into seminal fluid (12). Its

main function is to dissolve the coagulum formed after ejaculation by semenogelin, allowing sperm to swim freely in the female genital tract (13). PSA in seminal fluid is also important for the breakdown of cervical mucus, allowing the entry of sperm into the uterus (14). PSA is present in small quantities in serum of men with a normal prostate but is elevated during several prostate abnormalities, including prostate cancer (15-16). Although the modulation of PSA has been investigated in prostate abnormalities, its role and the effect of its dysfunction on human fertility/infertlity have not been examined. Most of the studies on immunoinfertility have focused on delineating/characterizing antibodies reactive with sperm and/or sperm-specific antigens. The role of seminal plasma components in normal fertility and its dysfunction leading to infertility has not been extensively investigated (17-18). Recently, we showed the presence of PSA antibodies in the sera of immunoinfertile men and women (19); however, the presence of PSA antibodies has not been investigated locally in the seminal plasma in relation to immunoinfertility.

Male infertility has been shown to contribute ~50% of cases of infertile couples, and of these, ~26% have been attributed to semen hyperviscosity (SHV) (20-21). Semen samples with SHV generally have a normal coagulum but impaired liquefaction (22). SHV can be caused by impairment of several physiological and biochemical factors involved in liquefaction. The presence and relevance of PSA antibodies in hyperviscous semen have not been investigated.

The present study was conducted to examine the presence of PSA antibodies locally in the seminal plasma of men having immunoinfertility and semen hyperviscosity. The long term objective of this study is to investigate the role of PSA autoantibodies in the specific diagnosis and treatment of male infertility, especially mediated through immune reactions and seminal dysfunction, and find novel targets for immunocontraception.

3. MATERIALS AND METHODS

3.1. Materials

The human PSA (catalog #MBS173180) purified from human seminal plasma was obtained from MyBioSource Inc. (San Diego, CA.) The rabbit polyclonal antibody (#PA1-38514) against purified human PSA was purchased from Pierce Biotechnology (Rockford, IL). All other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific Inc. (Pittsburg, PA).

3.2. Patient population

This study was approved by the Institutional Review Board (IRB) for human studies. Semen was collected by masturbation from men who came to the clinic for infertility diagnosis and treatment. Semen was analyzed for volume, viscosity, sperm concentration, motility, and sperm morphology following WHO procedures and criteria (23). The patients' sperm were also analyzed for the presence of antisperm antibodies using the direct immunobead binding technique (IBT) (24). The viscosity was estimated by performing gentile aspiration of the semen after liquefaction (>30 min, 37°C) into a wide-bore 5 ml pipette and then observing the length of the thread as the semen was allowed to drop by gravity. The sample was defined as hyperviscous if, instead of discrete drops which is normally made, it formed a thread of >2 cm long (23). Semen was centrifuged, and seminal plasma was collected and stored at -80° C until used. Samples were divided into three groups: immunoinfertile group (Group A, *n*=25), hyperviscous group (Group B, *n*=25), or normal group (Group C, *n*=23). The immunoinfertile group included men (age, mean ± SD; 32 ± 6 yr old) whose sperm were positive for ASA by the direct IBT (>20% binding) for IgG and/or IgA and had normal viscosity, as defined above (drops and not a thread) (23). The majority of these men (>80%) also tested positive for ASA in the seminal plasma by the indirect IBT using the donor sperm. The hyperviscous group included men (age, mean ± SD; 36 ± 7 yr old) whose sperm were negative for ASA by the direct IBT. These men also tested negative for ASA in the seminal plasma by the indirect IBT using the donor sperm. The seminal plasma from these men showed hyperviscosity as defined above (23). The normal group included men (age, mean ± SD; 31 ± 8 yr old) who were negative for ASA by direct and indirect IBT, had normal viscosity, and had normal semen analysis.

3.2. Enzyme-linked immunosorbent assay (ELISA)

The immunoreactivity of human seminal plasma with PSA was examined using ELISA, as described previously (16,19). Wells were coated overnight with purified human PSA (0.1.-0.4. µg/200 µL), washed with PBS (pH 7.4.) containing 0.0.5% Tween-20 (PBS-T) to remove unbound proteins, and wells were incubated (37°C, 45 min) with PBS-T containing 0.2.5% bovine serum albumin (BSA) to block uncoated sites. Wells were then washed (3x) and incubated (37°C, 2 h) with seminal plasma samples in PBS-BSA (1:20 dilution). After washing with PBS-T (3x), wells were incubated (37°C, 1.5. h) with goat anti-human IgG conjugated to alkaline phosphatase (Fisher Scientific Inc, Pierce Biotechnology, Rockford, IL, USA) in PBS-BSA (1:1500). Wells were again washed(3x) with PBS-T, incubated with substrate (1.0. mg/mL *p*-nitrophenyl phosphate in 0.0.5 M Na_2CO_3 buffer containing 1.0. mM $MgCl_2$, pH 9.6.), and read at 405 nm. The PSA antibody was used as a positive control in every ELISA plate. Each sample was run in triplicate in each plate and tested 3-5 times on different days. The mean absorbance values of the

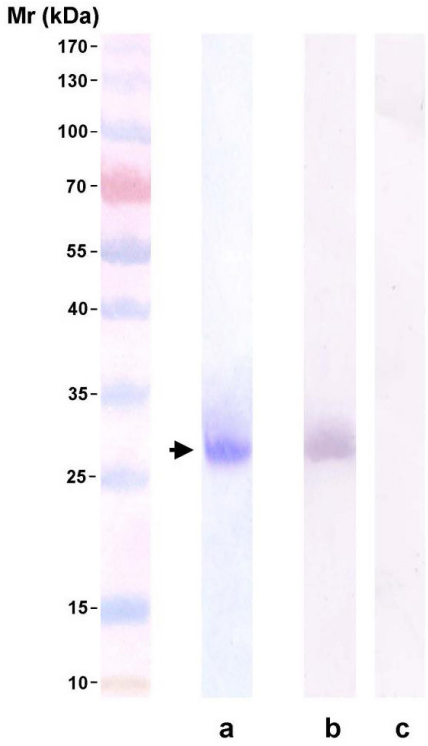

Figure 1. Immunoreactivity of PSA rabbit antibody (lane b) and control rabbit immunoglobulins (lane c) with PSA in the Western blot procedure. The PSA protein band of ~28 kDa is shown by the arrow in SDS-PAGE (lane a). The molecular weight markers have been included for comparison.

triplicate wells of each sample was calculated after subtracting the absorbance value of the blank wells. The values of each ELISA plate were normalized using the absorbance value obtained using the control rabbit antibody which was run as a positive control in each plate. After normalization, the mean absorbance value of each sample obtained in 3-5 plates was calculated. Thus, the mean value of each sample is comprised of a total of 9-15 wells run in 3-5 plates after normalization. Absorbance values were converted to standard deviation (SD) units with the equation: SD units = mean (test) – mean (control)/SD of control (16,19). In this formula, the mean(control) and SD values are of the whole normal group (Group C). The mean (test) indicates the mean value of a sample which needs to be examined for SD units. Samples of seminal plasma with an absorbance value equating to ≥ 2 SD units were considered to have a positive reaction with PSA.

3.3. Western blot procedure

The immunoreactivity of human seminal plasma with PSA was also confirmed by Western blot, as described previously (16,19). PSA (5-20 µg per lane) was loaded and run in SDS-PAGE. After gel electrophoresis, the proteins were transferred onto nitrocellulose membrane and analyzed for PSA immunoreactivity using seminal plasma/PSA antibodies. Seminal plasma samples (primary antibody) were tested at a 1:2500 dilution. PSA antibody was used as a positive control. The secondary antibody (goat anti-human conjugated to alkaline phosphatase) was tested at a 1:5000 dilution. The reactive protein bands were visualized by chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT)).

3.4. Statistical analysis

The significance of difference among the mean age of various groups and the mean values of SD units of immunoinfertile, hyperviscous, and normal groups was statistically analyzed by the analysis of variance (ANOVA) (GraphPad Software Inc., La Jolla, CA, USA). A p-value ≤ 0.0.5 was considered significant. Depending upon whether the distribution was parametric or non-parametric, the correlation between SD units and various seminal parameters was analyzed either by the Pearson or Spearman test, respectively.

4. RESULTS

4.1. Assessment of purity and authenticity of PSA and PSA antibody

In SDS-PAGE, the purified PSA showed a single protein band of ~28 kDa (Fig 1. lane a). In the

Table 1. Antibodies to PSA in seminal plasma of men

Group	Samples (*n*)	Absorbance (mean ± SD) (range)	SD Units (mean ± SD) (range)	% Positive
Immunoinfertile	25	0.9.31 ± 0.2.29[1] (0.5.71-1.3.51)	0.7.59 ± 1.3.58[1] (-1.3.79-3.2.55)	20% (5/25)
Hyperviscous	25	0.9.90 ± 0.3.31[2] (0.5.61-1.9.97)	1.1.09 ± 1.9.67[2] (-1.4.38-7.0.93)	28% (7/25)
Normal	24	0.8.13 ± 0.1.72[3] (0.5.92-1.1.66)	-0.0.15 ± 0.9.70[3] (-1.2.54-1.8.22)	0% (0/24)

[1] and [2] versus [3] are significantly different ($p < 0.0.4$ to $< 0.0.1$); [1] versus [2] are non-significant ($p > 0.0.5$)

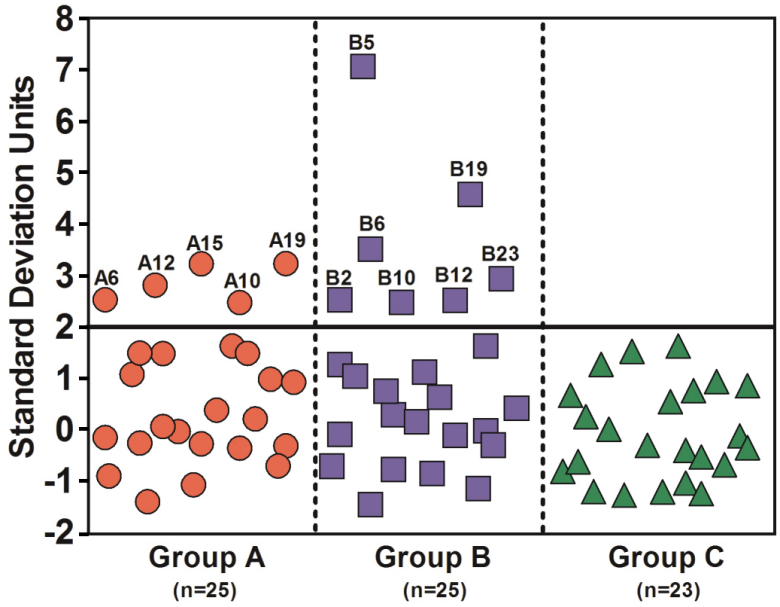

Figure 2. Immunoreactivity of seminal plasma from the immunoinfertile, hyperviscous, and normal control groups with PSA in ELISA. The solid horizontal line indicates 2 SD units, and values above the line were considered to have a positive immunoreactivity.

Western blot procedure, the PSA antibody recognized the PSA band of ~28 kDa (Fig 1. lane b), which was not recognized by the control immunoglobulins (Fig 1. lane c).

4.2. Analysis of seminal plasma for PSA antibodies by ELISA

In ELISA, the absorbance values were converted to SD units. The mean value of SD units of the immunoinfertile group for PSA immunoreactivity was significantly ($p < 0.0.4$) higher than the mean value of SD units of the normal control group (Table I). The mean value of SD units of the hyperviscous group for PSA immunoreactivity was also significantly ($p < 0.0.1$) higher than the mean value of SD units of the normal control group (Table I). The mean value of SD units between the immunoinfertile and hyperviscous groups was non-significant ($p > 0.0.5$), though approaching significance ($p = 0.0.6$). Using ≥ 2 SD units as a cut-off for positivity, 20% (5/25) of the seminal plasma of the immunoinfertile group and 28% (7/25) of the seminal plasma of the hypervisous group showed a positive reaction with PSA (Figure 2). None (0%) of the seminal plasma of the normal control group reacted positively with PSA (Table I, Figure 2).

4.3. Analysis of seminal plasma for PSA antibody by Western blot

The seminal plasma samples, two each from the immunoinfertile (#A15 and A19) and hyperviscous (#B5 and B19) groups that showed the highest immunoreactivity in ELISA were also analyzed for immunoreactivity with PSA in the Western blot. All of these samples showed a specific reaction with PSA in the Western blot. The results of two of these samples (#A15 and B5) are shown in Figure 3. PSA was not recognized by the seminal plasma from the normal control group (Figure 3).

4.4. Correlation of PSA antibodies with seminal parameters

In the immunoinfertile and normal control groups, there was no significant ($p > 0.0.5$) linear correlation of SD units for PSA immunoreactivity with any of the seminal parameters (volume/concentration/

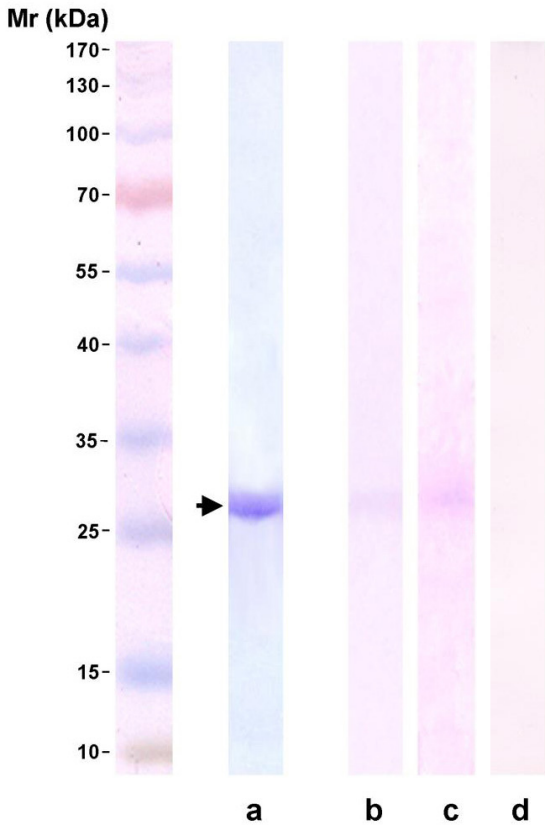

Figure 3. Immunoreactivity of seminal plasma (# A15) from immunoinfertile group (lane b), hyperviscous group (#B5) (lane c), and normal control group (#C5) (lane d) with PSA in Western blot. The PSA showed a protein band of ~28 kDa in SDS-PAGE (shown by the arrow) (lane a), which was transferred to nitrocellulose membrane for examining the immunoreactivity of various seminal plasma samples in the Western blot. The molecular weight markers have been included for comparison.

motility/percent abnormal sperm/IBT IgG and IgA bead binding values). In the hyperviscous group, a significant (p < 0.0.5) linear correlation was found only with sperm motility (r = 0.4.) and not with any other seminal parameters (p > 0.0.5). There was no significant difference (p > 0.0.5) among the mean age of men in the three groups.

5. DISCUSSION

Our findings indicate that the seminal plasma from men having immunoinfertility or hyperviscosity have antibodies reactive with PSA. Seminal plasma from normal men showed no immunoreactivity with PSA. The PSA used in the present study was highly pure and showed a single protein band of ~28 kDa in SDS-PAGE that was specifically recognized by the PSA rabbit antibody. Mature PSA has a molecular weight of ~28 kDa and is comprised of 237 amino acids (aa) and a carbohydrate chain (11). PSA antibodies can be directed against the peptide and/or carbohydrate moiety of PSA.

Using ≥ 2 SD units as a cutoff for positivity, seminal plasma from 20% of immunoinfertile men showed a positive reactivity with PSA in ELISA. The ELISA assay was repeated 3-5 times on different days providing similar results. The findings of ELISA were confirmed using Western blot. In our previous study, it was found that 16% of immunoinfertile men were ASA-positive by sperm immobilization test (SIT)/tray agglutination technique (TAT)/IBT (19). The seminal plasma in the present study was collected from men whose sperm were positive for ASA by IBT. There was no correlation between ASA positivity by IBT and PSA positivity in ELISA. It was expected since IBT measures antibodies which are directed against sperm proteins, and PSA antibodies are directed against PSA which is a seminal plasma component and not a sperm membrane protein.

Interestingly, seminal plasma from 28% of men having hyperviscosity also showed a positive reactivity with PSA in ELISA which was confirmed by Western blot. The PSA immunoreactivity (SD units) of the hyperviscous group and not the immunoinfertile/normal control groups significantly (p < 0.0.5) correlated with the sperm motility grade. Hyperviscosity is expected to reduce seminal motility. No other seminal parameters correlated with this and

any of the other groups tested. Defective liquefaction leading to hyperviscosity can be attributed to the concentration and/or defective PSA molecule or other factors involved in coagulation and liquefaction. A decrease in PSA concentration has been correlated with hyperviscosity in semen (25-26). An association between decreased/abnormal hK2, an activator of PSA, has been correlated with abnormal liquefaction (27-28). The present study reports, for the first time ever, that antibodies to PSA are present in the hyperviscous semen that can contribute to defective liquefaction. These antibodies can bind to PSA and inactivate its function. Our findings further indicate that these antibodies cannot be detected by IBT, a routine assay used in the clinics for detection of ASA.

The mechanism for production of antibodies to PSA in the immunoinfertile and hyperviscous groups is unclear. PSA is a "self" molecule in men, thus should not be autoantigenic. The immune response to PSA-like molecules has been implicated in some cases of allergy to coitus (29-30). No association has been found between chronic inflammatory and infectious prostatitis/epididymitis/urethritis with the development of antisperm antibodies (31). Some prostate abnormalities induce PSA antibodies (16,32). However, prostate abnormalities (chronic prostatitis, benign prostate hyperplasia, and prostate cancer) do not induce immunity to spermatozoa or seminal plasma (32).

In conclusion, the presence of PSA antibodies was found in the seminal plasma of men having immunoinfertility or hyperviscosity. Whether PSA antibodies are associated with or are a causative factor of infertility needs further investigation. The main function of PSA is to liquefy the seminal coagulum and allow sperm to swim freely for traveling to the oocyte for fertilization. It is also believed that PSA participates in dissolving cervical mucus for sperm transit into the uterus. Antibodies to PSA can interfere in these processes. The findings may have clinical significance in the specific diagnosis and treatment of infertility in men and contraceptive vaccine development. PSA can provide an interesting target for immunocontraception. The antibodies to PSA developed after vaccination can block liquefaction causing contraception. This is presently being investigated in our laboratory.

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Abbreviations: PSA, prostate specific antigen; ASA, antisperm antibodies; ELISA, enzyme-linked immunosorbent assay; hk3, human kallikrein 3; hk2, human glandular kallikrein 2; kDa, kilodaltons; IBT, immunobead binding technique; SHV, semen hyperviscosity; SIT, sperm immobilization technique; TAT, tray agglutination technique

Key Words: Antibodies, hyperviscous semen, IBT, Immune Infertility, Male Infertility, Psa, Semen

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