Diversity of the inhibitory effects on fertilization by anti-sperm antibodies bound to the surface of ejaculated human sperm

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BACKGROUND: The presence of anti-sperm antibodies (ASA) in males can reduce fecundity. However, it has been shown that there is a diversity of ASA bound to the sperm surface. This study was performed to investigate the inhibitory effects on fertilization by ASA in males. METHODS: ASA were detected using the direct-immunobead test (D-IBT) in 509 semen samples. In some cases, the direct-sperm immobilization test (D-SIT) was carried out. The fertilizing ability of infertile males with ASA was determined as follows; (i) an IVF fertilization rate of ≥50%, (ii) a hemizona index (HZI) of ≥50%, and (iii) pregnancy established without the use of ART. RESULTS: In total, 18 (3.54%) infertile males had ASA on the sperm surface. Except for one male with an absolute indication for ICSI because of severe asthenozoospermia and two males who dropped out of this study, fertilizing ability in 15 males could be determined. Four (26.7%) men did not satisfy the criteria. The existence of sperm immobilizing antibodies on the surface of ejaculated sperm had no impact on fertilization. In four (57.1%) of seven patients who had IB-bound sperm of >80%, fertilizing ability was inhibited, while none of the eight patients who had <80% IB-bound sperm had an inhibitory effect on fertilization. There was a significant difference between the two groups (P = 0.01). CONCLUSIONS: Some sperm-bound antibodies are related to the inhibitory effects on fertilization, indicating that a diversity of sperm-bound antibodies exists in males. This result might be one of the reasons for the controversy of the relationship between ASA and male immunological infertility. Based on the present study, a sperm–zona pellucida binding assay should be performed for appropriate decision making in infertile males with ASA.

Key words: anti-sperm antibody/fertilization/hemizona assay/immunobead test/sperm immobilization test

Introduction

An adverse immune response to certain tissues of the reproductive system can cause infertility. It has been shown that both males and females can make antibodies that react with human sperm. In males, for example, anti-sperm antibodies (ASA) can be detected in seminal plasma and serum, and are also located on the surface of sperm. ASA have usually been found in homosexual males (Witkin and Sonnabend, 1983) and in cases of testicular trauma (Hjort et al., 1974), varicocele (Golomb et al., 1986), mumps orchitis (Shulman et al., 1992), spinal cord injury (Siosteen et al., 1993), congenital absence of the vas (Patrizio et al., 1992), and vasectomy (Shulman et al., 1972).

The presence of ASA in males can reduce fertility. However, the relationship between ASA and immunological infertility in males has been disputed. We recently clarified that there is a diversity of ASA bound to sperm surface in males (Shibahara et al., 2002a). Moreover, a relatively high incidence of asthenozoospermia could be demonstrated in immunologically infertile males, and the significant effect of sperm immobilizing antibodies —discovered by Isojima et al. (1968)—bound to the surface of ejaculated sperm on sperm motility was confirmed in immunologically infertile males (Shibahara et al., 2003).

We have also demonstrated that ASA, especially sperm immobilizing antibodies, can inhibit fertilization in infertile women (Shibahara et al., 1991, 1993, 1996; Taneichi et al., 2002). It has also been shown that ASA in males have an inhibitory effect on fertilization (Mandelbaum et al., 1987; De Almeida et al., 1989; Rajah et al., 1993; Yeh et al., 1995). However, others have arrived at the opposite conclusion (Sukcharoen and Keith, 1995). These results might indicate that there is also a diversity of the blocking effects on fertilization by ASA bound to sperm surface.
This study was carried out to investigate the inhibitory effects on fertilization by ASA bound to the surface of ejaculated human sperm.

Materials and methods

Routine semen examinations

Between October 1999 and July 2002, a total of 509 fresh semen samples from 509 males was obtained for an initial semen examination. After liquefaction, sperm concentration and sperm motility were assessed using a computer-aided sperm analysis (CASA) system (Hamilton Thorne; Hamilton Thorne Research, Beverly, MA, USA) as we previously described (Hirano et al., 2001). Sperm morphology was evaluated following the method of Kruger et al. (1988). The ethics committee at our institution approved this study, and written informed consent was obtained from the patients.

Detection for ASA bound to sperm

The direct-immunobead test (D-IBT) was simultaneously performed on 509 semen samples following the method of Bronson et al. (1982) as we previously described (Shibahara et al., 2002a, 2003). In brief, 5 μl drops of each type of immunobead (IB; IgG, IgA, and IgM; Irvine Scientific, Santa Ana, CA, USA) were placed on glass slides and 5 μl of sperm suspension (10 × 10⁶/ml) was added and incubated for 10 min. The percentage of motile sperm that had one or more attached immunobeads (IBs) was recorded. A cut-off level of 20% was adopted in this study.

In some cases, direct-sperm immobilization test (D-SIT) was carried out as previously described (Shibahara et al., 2002a, 2003). In brief, 12 μl of the patient’s sperm suspension (40 × 10⁶ sperm/ml) and 1 μl of activated or heat-inactivated guinea-pig serum (Sigma, Tokyo, Japan) were mixed in a Terasaki plate (Greiner, Frickenhausen, Germany) and incubated at 32°C for 60 min. The sperm immobilization value (SIV) was calculated by dividing the sperm motility in the control containing the heat-inactivated complement by that containing the active complement. An SIV of ≥2 was regarded as positive for the sperm-immobilizing antibody.

IVF procedure

IVF–embryo transfer treatment was initiated with pituitary down-regulation using a gonadotrophin-releasing hormone (GnRH) agonist (Nafarelin acetate; Yamanouchi Pharmaceutical Co., Ltd. Tokyo, Japan) in the mid-luteal phase of the previous cycle as previously described (Shibahara et al., 1998; Obara et al., 2001). Semen samples were collected, mixed with Sydney IVF Sperm Buffer (Cook IVF, Australia) containing human sperm albumin (HSA) and centrifuged for 5 min at 569 g.

Swim-up procedure was performed using a combined migration-sedimentation method (Lucena et al., 1989) in a BIO-LABO tube (Jyuji Field Co., Tokyo, Japan) as previously described (Obara et al., 2001). Following the gonadotrophin administrations, oocyte retrieval was performed through transvaginal ultrasonography-guided aspiration.

The morning following insemination with swim-up sperm, oocytes were examined for the presence of pronuclei (PN), and cultured for another 24 h to allow for cleavage. On the second or third day following oocyte retrieval, morphological embryo assessment was performed under an inverted microscope. To avoid high-order multiple pregnancies, the elective transfer of two high-quality embryos was performed (Shibahara et al., 2002b). Clinical pregnancy was diagnosed 21 days after embryo transfer when the gestational sac was revealed under transvaginal ultrasonography.

Hemizona assay

Hemizona assay (HZA) was carried out following the method of Burkman et al. (1988). In brief, human oocytes were obtained from oocytes unsuccessfully fertilized after the treatment by ICSI. Some of the unfertilized oocytes were stored at 4°C in a highly concentrated salt solution containing 0.5 mol/l ammonium sulphate (Wakojunyaku, Osaka, Japan), 1 mol/l magnesium chloride (Wakojunyaku) and 0.1% dextran (Wakojunyaku) until use (Yanagimachi et al., 1979). The oocytes were cut roughly in half using Narishige micromanipulators (Narishige, Tokyo, Japan), and mounted on a phase-contrast microscope (Nikon, Garden City, NY, USA). After discarding the degenerated ooplasm, the two matched hemizona were placed overnight at 4°C in a droplet of medium under mineral oil. One hemizona was placed in a 100 μl drop of swim-up sperm suspension this test sample while the matched hemizona was placed in a drop of control sample with normal zona pellucida binding activity. After 4 h of co-incubation, each hemizona was removed and rinsed vigorously to detach loosely associated sperm. The number of sperm tightly bound to the outer hemizona surface was counted. As suggested for the quality control of the HZA, only donors with appropriate sperm–zona pellucida binding activities were selected (Burkman et al., 1990). The hemizona index (HZI) was the number of sperm bound to the hemizona in the test sample divided by that in the control, all multiplied by 100. When the HZI was ≥50%, the sperm binding to zona pellucida was considered to be inhibited in the test sample as compared with the control sample, according to the criterion of Mahony et al. (1991).

Determination of fertilizing ability of infertile men having ASA

The fertilizing ability of infertile men with ASA, detected by the D-IBT, was determined as follows; (i) an IVF fertilization rate of ≥50%, (ii) an HZI of ≥50%, and (iii) pregnancy established without the use of ART.

Statistical analysis

Statistical analysis of the data was performed by χ²-test using Statview 5.0 (Abacuss Concepts, Berkeley, CA, USA) for Macintosh, and P < 0.05 was defined as representing a significant difference.

Results

Incidence of infertile males having ASA detected by D-IBT

The characteristics associated with ASA were similar to those we reported previously (Shibahara et al., 2002a, 2003). In total, 18 (3.54%) of 509 infertile males had at least one Ig class of ASA detected by D-IBT. The 18 patients were aged between 27–43 years, with a mean age of 32.2 ± 4.7 years (mean ± SD). The mean infertile periods were 3.1 ± 2.6 years (mean ± SD). The causes of the patients’ infertility were as follows: a male factor in 11 cases, a male plus female infertility (one anovulation, one tubal occlusion, one endometrial polyp) in three cases, and unexplained in four cases.

Fifteen (2.95%), nine (1.77%), and two (0.39%) of 509 infertile males were tested positive (>20%) in IgG, IgA, and IgM of the D-IBT respectively (Table I). Two males had positive D-IBT in the three Ig classes, while four males had positive D-IBT in the two Ig classes. The range of percentage ASA-positive sperm in each IgG, IgA, and IgM positive case
was 20±98, 20±99 and 21±33% respectively (Table I). In 18 immunologically infertile males, the sites on the sperm bound by the IBs were five (27.8%) in head plus tail, four (22.2%) in only the head, and nine (50.0%) in only the tail (Table I).

Thirteen infertile males, with ASA bound to sperm surface, diagnosed using D-IBT, were also tested for D-SIT bound to sperm surface. Nine (69.2%) of 13 infertile males tested positive in D-SIT (Table I).

For semen characteristics, four (22.2%) of 18 males were diagnosed with normozoospermia. The number of males with oligozoospermia, asthenozoospermia and terato-asthenozoospermia was 0, 12 (66.7%), and two (11.1%) respectively (data not shown).

Fertilizing ability of infertile males with ASA

Except for one male with an absolute indication for ICSI because of severe asthenozoospermia (motility <2% with progressive motility of 0%) and two males who dropped out of this study, the fertilizing ability of 15 patients with ASA bound to the sperm surface could be determined. As shown in Table I, IVF–embryo transfer treatments were carried out in five males with ASA detected using D-IBT. Four (80.0%) of the five patients had fertilization rates of ≥50%. One patient had a fertilization rate of 0% by IVF, and was subsequently treated by ICSI, resulting in a fertilization rate of 62.5%. The patient conceived by ICSI–embryo transfer treatment. Three (75.0%) of the four patients with the higher fertilization rates by IVF–embryo transfer also established pregnancies.

One (25.0%) of the four patients was assessed and his fertilizing ability was confirmed by HZA before he proceeded to the infertility treatments. The other three males had HZI of ≤50%, indicating they did not seem to have satisfactory sperm–zona pellucida binding activities.

Thus, four patients had a fertilization rate of ≥50% in IVF, one patient had HZI of ≥50%, and six patients established pregnancies without the use of ART (Table II). Therefore, the remaining four (26.7%) of 15 infertile males having ASA did not satisfy the criteria.

Association of the incidence of the inhibitory effects on fertilization with the number of 1B bound

In four (57.1%) of seven patients who had IB-bound sperm of ≥80%, the fertilizing ability was inhibited, while none of the eight patients who had <80% IB-bound sperm had an inhibitory effect on fertilization (Table III). There was a significant difference between the two groups (P = 0.01). However, there was no relationship between the inhibitory effects on fertilization and the Ig classes or localization of ASA (Table I).

Association of the incidence of the inhibitory effects on fertilization with the results of D-SIT

In 12 infertile males (except for patient code No. 3 with severe asthenozoospermia, Table I) tested for D-SIT bound to sperm surface, the incidence of the inhibitory effects on fertilization was compared between eight males with sperm immobilizing antibodies and four males without the antibodies bound to sperm surface. However, the existence of sperm immobilizing antibodies on the surface of ejaculated sperm had no impact on fertilization (Table IV).
are strongly associated with asthenozoospermia (Shibahara et al., 2002a, 2003). In some studies, tail-directed ASA tended to impair sperm motility, resulting in the reduction of fertilization rates in vitro (Witkin et al., 1992). In the present study, an association of the localization of ASA on the sperm surface with the impairment of fertilization could not be demonstrated (Table I). For the Ig class as well, no specific relationship could be identified with fertilizing ability. It has been reported (Yeh et al., 1995) that IgA of ASA bound on the sperm surface induced a statistically significant reduction of fertilization only when it was present on the head. They also concluded that IgM was the Ig isotype that most significantly affected fertilization rates when localized at both the head and at the tail tip. One of the reasons for the different conclusions drawn by each investigator might be due to the heterogeneity of ASA themselves.

We previously reported that sperm immobilizing antibodies impair sperm–zona pellucida binding in female immunological infertility (Shibahara et al., 1991, 1993, 1996; Taneichi et al., 2002). As shown in Table IV, however, there was no relationship between the incidence of the inhibitory effects on fertilization and the results of D-SIT. Once sperm are incubated with serum samples with sperm immobilizing antibodies or monoclonal sperm immobilizing antibodies, almost all sperm are immediately bound by the antibodies, which leads to the blocking of the fertilizing ability of sperm. However, in males with sperm immobilizing antibodies on the surface of sperm, the rate of ASA positive sperm was 28–98% (Table I), indicating that there are fertilizable sperm without having the antibodies.

It was revealed that infertile males with high levels (>80%) of ASA bound sperm were less likely to fertilize (Table III). It was also demonstrated by a few investigators that the IVF rate significantly decreased when the inseminated sperm were coated with higher numbers of ASA (De Almeida et al., 1989; Lahteenmaki, 1993). These findings also suggest that in infertile males having positive but lower levels of ASA on the ejaculated sperm, ASA-free sperm might contribute to higher fertilization rates in IVF. In contrast, those with higher levels of ASA have fewer ASA-free sperm, which leads to a significant reduction in fertilization. If so, a higher sperm concentration for insemination might contribute to acceptable fertilization rates in IVF for such immunologically infertile males.

In conclusion, some sperm-bound antibodies are related to the inhibitory effects on fertilization, indicating that a diversity of ASA exists in males. This result might be one of the reasons for the controversy regarding the relationship between ASA and male immunological infertility. Based on the present study, the sperm–zona pellucida binding assay, HZA, should be performed for appropriate decision-making in infertile males with ASA.

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**Table III.** Association of the incidence of the inhibitory effects on fertilization with the number of immunobeads (IB) bound

<table>
<thead>
<tr>
<th>No. of IB bound</th>
<th>No. of patients Tested</th>
<th>No. of patients With inhibitory effects on fertilization</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;80%</td>
<td>8</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>≥80%</td>
<td>7</td>
<td>4</td>
<td>57.1*</td>
</tr>
</tbody>
</table>

*P = 0.01.

**Table IV.** Association of the incidence of the inhibitory effects on fertilization with the results of direct-sperm immobilization test (D-SIT) in 12 infertile males

<table>
<thead>
<tr>
<th>D-SIT</th>
<th>No. of patients Tested</th>
<th>No. of patients With inhibitory effects on fertilization</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>4</td>
<td>1</td>
<td>25.0*</td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>3</td>
<td>37.5*</td>
</tr>
</tbody>
</table>

*P > 0.05.

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**Discussion**

Understanding the heterogeneity of the ASA themselves as well as their biological effects on sperm motility and fertilizing ability is one of the important issues in immunological infertility in males. Because of the heterogeneity of sperm antigens, it has been shown that some of the sperm surface proteins were recognized by the sera from infertile women having sperm immobilizing antibodies, or by the sera from infertile males and females with unexplained infertility having ASA detected by indirect-IBT, using 2-dimensional gel electrophoresis (Shetty et al., 1999; Shibahara et al., 2002c). However, other sperm surface proteins did not react with those sera. Therefore, it was suggested that ASA are directed against different types of sperm antigens.

As for ASA themselves, there is also a diversity such as Ig classes of ASA, the localization of the corresponding antigens against ASA, and the biological activity of ASA bound to sperm surface in males (Shibahara et al., 2002a, 2003). In some cases, it has been shown that ASA bound to the sperm surface are sperm immobilizing antibodies. However, others were not sperm immobilizing antibodies (Shibahara et al., 2002a). Sperm immobilizing antibodies on the surface of human sperm are strongly associated with asthenozoospermia (Shibahara et al., 2003).

In the present study, another reason for the controversy of the relationship between ASA and male immunological infertility was investigated. As shown in Table II, 11 (73.3%) of 15 infertile males having ASA had no inhibitory effects on fertilization. From among them, six conceived without the use of ART. HZA, which is believed to be one of the ideal tests to examine human sperm fertilizing abilities, was utilized to determine the inhibitory effects of ASA-bound sperm on fertilization. When counselling infertile couples with male ASA-associated infertility before they proceed to ART, the HZA assessed by the HZA seems to be useful.

When the vast majority of sperm are coated with ASA over their heads, fertilization is often impaired by a reduction of the number of sperm binding to the oocyte (Bronson et al., 1982; Mandelbaum et al., 1987; Yeh et al., 1995). However in other studies, tail-directed ASA tended to impair sperm motility, resulting in the reduction of fertilization rate in vitro (Withkin et al., 1992). In the present study, an association of the localization of ASA on the sperm surface with the impairment of fertilization could not be demonstrated (Table I). For the Ig class as well, no specific relationship could be identified with fertilizing ability. It has been reported (Yeh et al., 1995) that IgA of ASA bound on the sperm surface induced a statistically significant reduction of fertilization only when it was present on the head. They also concluded that IgM was the Ig isotype that most significantly affected fertilization rates when localized at both the head and at the tail tip. One of the reasons for the different conclusions drawn by each investigator might be due to the heterogeneity of ASA themselves.

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In conclusion, some sperm-bound antibodies are related to the inhibitory effects on fertilization, indicating that a diversity of ASA exists in males. This result might be one of the reasons for the controversy regarding the relationship between ASA and male immunological infertility. Based on the present study, the sperm–zona pellucida binding assay, HZA, should be performed for appropriate decision-making in infertile males with ASA.


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