Diversity of the blocking effects of antisperm antibodies on fertilization in human and mouse

Hiroaki Shibahara1, Minoru Shigeta1, Miyuki Inoue1, Akiko Hasegawa1, Koji Koyama1,4, Nancy J.Alexander2 and Sinzo Isojima3

1Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo, 663, Nishinomiya, Japan, 2National Institute of Child Health and Human Development, Center of Population Research, Bethesda, Maryland, USA and 3Advanced Fertility Center, Fuchu Hospital, Izumi, Osaka, Japan

4To whom correspondence should be addressed

The blocking effects of complement-dependent sperm immobilizing antibodies in the sera of infertile women and monoclonal antisperm antibodies against humans and mice on fertilization were investigated. The hemizona assay (HZA) and sperm penetration assay (SPA) were used to study the inhibitory effects of sera from 22 infertile patients positive for sperm immobilizing antibodies. Use of these tests allowed us to differentiate whether the antibody blocked sperm–zona pellucida tight binding and/or sperm penetration into the ooplasm. The zona pellucida penetration assay (ZPA) was also used to study the effects of four monoclonal antibodies (mAbs) on human sperm penetration into the zona pellucida. Seven mAbs against murine spermatozoa were tested for their inhibitory effects on in-vitro fertilization (IVF) and HZA in mice. Of 22 patient sera with sperm immobilizing antibodies, 21 (95.5%) inhibited HZA attachment and penetration, whereas this did not occur in any of 13 patient sera without these antibodies. However, 19 of 22 (86.4%) patient sera with sperm immobilizing antibodies and eight of 13 (61.5%) patient sera without these antibodies inhibited the SPA. Two (2C6, 1G12) of four mAbs against human spermatozoa showed strong inhibitory effects in all the assays (HZA, ZPA and SPA). One mAb (3B10) did not inhibit HZA but blocked ZPA and SPA. Another mAb (H6-3C4) seemed to have no inhibitory effects on fertilization. Two (Vx 5 and Vx 8) of seven mAbs against murine spermatozoa inhibited IVF in mice but did not block mouse HZA. These findings suggest that antisperm antibodies block fertilization at specific stages. Some of them may inhibit sperm capacitation and thus prevent all processes of fertilization that follow. Some other antibodies may not affect capacitation and sperm binding to zona pellucida but inhibit the acrosome reaction, followed by the blocking of sperm penetration through zona pellucida and ooplasm.

Key words: antisperm antibody/hemizona assay/monoclonal antibody/sperm penetration assay/zona penetration assay

Introduction
Infertile patients with antisperm antibodies are less likely to conceive because some of the antibodies secreted into their genital tracts not only interfere with sperm migration (Koyama et al., 1979; Shibahara et al., 1995) but also inhibit the fertilization process (Bronson et al., 1982; Alexander, 1984; Kamada et al., 1985; Tsukui et al., 1988; Liu et al., 1991; Mahony et al., 1991; Shibahara et al., 1991, 1993, Bandoh et al., 1992). We previously reported (Shibahara et al., 1993) that various sperm immobilizing antibodies are heterogeneous in the manner in which they block sperm–zona pellucida tight binding as assessed by the hemizona assay (HZA; Burkman et al., 1988). There are at least two kinds of sperm immobilizing antibodies, one that causes sperm immobilization in the presence of complement as well as directly blocking sperm–zona pellucida tight binding and another which only causes sperm immobilization.

As there are various stages of the normal fertilization process including sperm capacitation (Benoff et al., 1993), sperm–zona pellucida binding (Coddington et al., 1992, 1993), acrosome reaction (Zouari et al., 1992), sperm penetration through zona pellucida, sperm–egg fusion and post-fusion events, the use of the HZA alone may not be sufficient to detect the blocking effects of antisperm antibodies on fertilization.

In the present study, several diagnostic tests for predicting the fertilization potential of spermatozoa, including the HZA (Burkman et al., 1988), zona-free hamster egg penetration assay (SPA; Yanagimachi et al., 1976) and zona pellucida penetration assay (ZPA; Yanagimachi et al., 1979), were used to investigate the blocking effects of antisperm antibodies on fertilization in human and mouse.

Materials and methods

Patient sera
Blood samples were collected from 22 infertile women with sperm immobilizing antibodies, 13 women with unexplained infertility without the antibodies, and 10 normal postpartum women at the hospital of Hyogo College of Medicine, Japan. All sera were heat-treated at 56°C for 30 min to inactivate complement and kept frozen at -20°C until use.

Human spermatozoa
Semen samples were obtained from fertile healthy donors. After liquefaction, semen was centrifuged and washed twice in Ham’s F-10 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 3.5% human serum albumin (HSA fraction V, Sigma, St Louis, MO, USA). The mouse spermatozoa, collected by a standard
swim-up technique, were suspended in the same medium at a concentration of $2 \times 10^6$ motile spermatozoa/ml for each assay.

**Monoclonal antibodies against human spermatozoa**

One human and three mouse monoclonal antibodies (mAbs) against human spermatozoa were used in this study. Three of the mAbs (2C6, 1G12 and H6-3C4) had sperm immobilizing activities. The human mAb H6-3C4 (immunoglobulin (Ig) M) possessing sperm immobilizing activity was produced by cell fusion between peripheral blood lymphocytes from an uninfertile woman with sperm immobilizing antibodies in her serum and mouse myeloma cells (NS-1) as described previously (Isojima et al., 1987). The three mouse mAbs against human spermatozoa were produced by cell fusions between the spleen cells of immunized mice and mouse myeloma cells as follows. The mAb 2C6 (IgM) was derived from a Balb/c mouse immunized with human seminal plasma (Kameda et al., 1991). The mAb 1G12 (IgM) was derived from a Balb/c mouse immunized with human sperm membrane fraction (S Komori, unpublished data).

Both mAbs 2C6 and 1G12 showed sperm immobilizing activities. The third mouse mAb 3B10 (IgM) was derived from a Balb/c mouse immunized with human sperm membrane fraction but did not show sperm immobilizing activity (Toji, 1991).

**Purification of monoclonal antibodies**

The IgM fraction of mAb H6-3C4 was purified from hybridoma supernatant cultured in serum-free culture medium (Hybrynt-1, Sankojunyaku, Japan) by gel filtration through Sephacryl S-300 (Pharmacia, Uppsala, Sweden). The IgM fractions of mAbs 2C6, 1G12 and 3B10 were prepared from hybridoma supernatant cultured in RPMI 1640 medium containing 10% fetal bovine serum by salt precipitation with 50% saturated ammonium sulphate followed by gel filtration through Sephacryl S-300 (Pharmacia). All purified mAbs were tested to determine quantitative sperm immobilizing antibody titres (SI50) and sperm agglutination test (SAT) was carried out as described below.

**Sperm immobilization test**

The sperm immobilization test (SIT) was performed as described by Isojima et al. (1968). Briefly, 0.25 ml of inactivated patient’s serum or purified mAb, 0.025 ml of human sperm suspension ($4 \times 10^6$ spermatozoa/ml) and 0.05 ml of guinea pig serum (10 C'HSO (50% hemolytic) unit as complement) were mixed in a small test tube and incubated at 32°C for 60 min. The sperm immobilization value (SIV) was calculated by dividing the sperm mobility of the control serum by that of test serum. All sera and mAbs with sperm immobilizing antibodies were tested to determine the antibody titres by a quantitative SIT (SI50 = 50% sperm immobilization units) as described by Isojima and Koyama (1974). The SAT was performed as described by Fnberg (1974), and a titre above 20 was considered as positive. For the application of the serum samples or mAbs with both sperm immobilizing and sperm agglutinating activities to HZA, SPA and ZPA, appropriate dilution in order to avoid the effects of sperm agglutination on fertilization was performed according to the value of SAT, so that no more than 10% of motile spermatozoa were agglutinated.

**Hemizona assay in human**

We previously described the procedure of the HZA in human to detect the inhibitory effects of sperm immobilizing antibodies on sperm–zona pellucida tight binding (Shibahara et al., 1991, 1993). Briefly, human oocytes obtained from excised ovarian tissues were stored at −70°C in a 2.0 M dimethylsulphoxide solution in phosphate-buffered saline. The frozen oocytes were thawed and cut almost in half using Narishige micromanipulators (Narishige, Tokyo, Japan) 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. Swim-up human sperm were incubated with patient’s serum or test solution containing mAb against human spermatozoa before exposure to hemizona at 37°C for 1 h. One hemizona was placed in a 100 µl drop of swim-up sperm suspension with test sample while the matched hemizona was placed in a drop of control serum or diluent of mAb confirmed to be non-inhibitory to sperm–zona binding. After 4 h of co-incubation, each hemizona was removed and rinsed vigorously to detach loosely associated spermatozoa. Then the number of spermatozoa tightly bound to the outer hemizona surface was counted. Each zona and each sample was tested twice. The hemizona index (HZI) was the number of spermatozoa bound to the hemizona in the test sample divided by that in the control, all multiplied by 100. When the HZI was 50% or less, 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).

**Sperm penetration assay**

The SPA was performed according Yanagimachi et al. (1976). Female golden hamsters at the age of 8 weeks were induced to ovulate by an i.p. injection of 30 IU of pregnant mare serum (PMS) on the morning of post-oestrus vaginal discharge. At 48 h later, each animal received a i.p. injection of 30 IU human chorionic gonadotrophin (HCG; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). Each hamster was killed 15–17 h later, its oviducts were removed, and the cumulus mass was freed; 0.1% hyaluronidase (Sigma) and 0.1% trypsin (Sigma) were used to free the ova from the cumulus and to remove the zona pellucida. From 10 to 20 ova were added to 0.1 ml of capacitated spermatozoa plus patient’s serum, covered with mineral oil, and allowed to incubate for 3 h at 37°C. The ova were then mounted on a slide, compressed with a coverslip, and examined at ×400 magnification with a phase-contrast microscope. Penetration was determined by the presence of a swollen sperm head and attached tail within the cytoplasm. Sperm samples with no serum added served as a control and an indicator of the integrity of the SPA on any given day. Each sample was tested twice in different experiments. The sperm penetration index (SPI) was calculated by dividing the number of eggs penetrated in the test sample by that in the control sample and multiplying by 100. A value <50% was considered to show inhibition of sperm penetration.

**Zona pellucida penetration assay**

The ZPA (Yanagimachi et al., 1979) was used to test the blocking effects of antisperm antibodies on sperm penetration through the human zona pellucida. Human immature oocytes obtained from excised ovarian tissue were incubated in Ménézo's B2 medium (France) containing 1% bovine serum albumin (BSA) for 48 h, then stored at 4°C in highly concentrated salt solution containing 0.5 M ammonium sulphate (Wako-junyaku, Osaka, Japan), 1 M magnesium chloride (Wako-junyaku) and 0.1% dextran (Wako-junyaku) until use. Swim-up spermatozoa were incubated with test solution containing mAb against human spermatozoa or control solution containing NS-1 (mouse myeloma cells) before exposure to salt-stored human zona pellucida at 37°C or 24 h. Three eggs were used in each assay and each mAb was tested three times. The zona pellucida penetration index (ZPI) was calculated by dividing the number of sperm penetrated into the perivitelline space in the test sample by that in the control sample and multiplying by 100. A value <50% was considered to show inhibition of zona pellucida penetration by spermatozoa.
Monoclonal antibodies against murine spermatozoa

Ben et al. (1988) developed and characterized seven mAbs against murine spermatozoa from spleen cells of vasectomized BDF-1 mice. Five mAbs (Vx 5, 8, 10, 16 and 18) possessed complement-dependent sperm immobilizing activities. In-vitro fertilization (IVF) was strongly impaired by two mAbs (Vx 5 and Vx 8) in mice. These mAbs were frozen and kept at -20°C.

Mouse epididymal spermatozoa

Mouse epididymal spermatozoa were collected from C57BL/6 mice by excision and flushing of the epididymus into modified Krebs-Ringer bicarbonate solution (mKRB) containing 3.0 mg/ml of BSA (Fraction V, Sigma, St Louis, MO, USA) with 100 IU/ml of penicillin-streptomycin. Spermatozoa were incubated for 15 min at 37°C and 5% CO₂ in air for dispersion, and adjusted to 2×10⁶ motile spermatozoa/ml.

Hemizona assay in mice

Superovulation was induced by i.p. injections of 5 IU of PMS and 5 IU of HCG administered 48 h apart to CD-1 mice. At 16–20 h after HCG administration, oocytes in cumulus masses were collected from the oviducts. Hyaluronidase was used to free the oocytes from cumulus. The oocytes were cut into small pieces using micromanipulators as described for HZA in humans. Mouse epididymal spermatozoa adjusted to 2×10⁶/ml were incubated with test solution containing mAb against murine spermatozoa before exposure to hemizona at 37°C for 1 h. One hemizona was placed in a 100 μl drop of sperm suspension with test sample while another matched hemizona was placed in a drop of control solution containing NS-1. The procedure was the same as described above for HZA in human except for the concubination time of 2 instead of 4 h for mAb treated spermatozoa with the hemizona because there was no perceptible increase in the number of spermatozoa tightly bound to the zona pellucida after 2 h (data not shown). After 2 h of concubination, each hemizona was removed and rinsed vigorously to detach loosely associated spermatozoa. Then the number of spermatozoa tightly bound to the outer hemizona surface was counted. Each mAb was tested twice. The HZI was calculated as shown in HZA in humans.

Results

Comparison of the hemizona index and sperm penetration index in infertile patients

Sera from 22 infertile women with sperm immobilizing antibodies and 13 women with unexplained infertility without the antibodies were tested for their effects on sperm–zona pellucida binding by using HZA, and on sperm penetration into the ooplasm by using SPA. To study the diversity of the blocking effects of antisperm antibodies on fertilization in human, the HZI and the SPI were compared in patients with sperm immobilizing antibodies in Figure 1A and those without the antibodies in Figure 1B. Of 22 patient sera with sperm immobilizing antibodies, and 13 patient sera without the antibodies, 21 (95.5%) and none showed inhibitory effects on HZA respectively. Of 22 patient sera with sperm immobilizing antibodies and 13 patient sera without the antibodies, 19 (86.4%) and eight (61.5%) inhibited SPA respectively. There was no correlation between the HZI and the SPI in either group (Figure 1A and 1B). No statistical correlations were found between SI₅₀ values and HZI, nor between SI₅₀ values and SPI (data not shown).

Effects of monoclonal antibodies against human spermatozoa on fertilization

Four mAbs against human spermatozoa were used to investigate the diversity of the antisperm antibodies on fertilization by using the sperm functional assays including HZA, SPA and ZPA. The effects of mAb against human spermatozoa on the three assays and SI₅₀ titres are summarized in Table I. Both of the two mAbs 2C6 and 1G12 had strong sperm immobilizing activities and showed significant inhibitory effects in all the three assays. One mAb 3B10, which did not have sperm immobilizing activity, never inhibited sperm binding to the zona pellucida, but showed blocking effects on sperm penetration through both the zona pellucida and the ooplasm. A human mAb, H6-3C4, had strong sperm immobilizing activities but did not show any inhibitory effects in any of the assays.

Effects of monoclonal antibodies against murine spermatozoa on fertilization

Seven mAbs against murine spermatozoa were used to investigate the diversity of the antisperm antibodies on fertilization by using previous IVF results (Ben et al., 1988) and HZA. The effects of seven mAbs on IVF and HZA in mice are summarized in Table II. Five of the mAbs (Vx 5, Vx 8, Vx 10, Vx 16 and Vx 18) had sperm immobilizing activities, but another two mAbs (Vx 4 and Vx 13) did not show sperm immobilizing activities (Ben et al., 1988). Two mAbs, Vx 5 and Vx 8, strongly impaired IVF in mice, while none of the mAbs, including mAbs Vx 5 and Vx 8, inhibited sperm–zona pellucida tight binding.

Discussion

SIT is a reliable test to detect antisperm antibodies related to infertility as compared with other assay methods (Isojima et al., 1968; Isojima, 1989). Approximately 13–15% of women with unexplained infertility have circulating sperm immobilizing antibodies (Isojima, 1989). It was clearly established that the sperm immobilizing antibodies contribute to infertility by interfering with sperm migration in the female reproductive tract, e.g. in the cervical mucus (Koyama et al., 1979) or in the uterine cavity through the Fallopian tubes (Shibahara et al., 1995). It is also well known that the other mechanisms by which the antibodies lead to infertility include the blocking effects on fertilization (Bronson et al., 1982; Alexander, 1984; Kamada et al., 1985, Tsukii et al., 1988, Liu et al., 1991, Mahony et al., 1991; Shibahara et al., 1991, 1993; Bandoh et al., 1992) and the impairment of the early embryo development (Koyama et al., 1984; Shibahara et al., 1996). Previous reports concerning the blocking effects of antisperm antibodies on fertilization have involved only a single diagnostic test of sperm function even though there are several stages in the normal fertilization process. In this study, several diagnostic tests for predicting the fertilization potential of spermatozoa were used to study the diversity of the blocking effects of antisperm antibodies on fertilization in humans and mice. From the results of HZA and SPA in humans, no correlation was observed between the HZI and the SPI in the patient sera with and without sperm immobilizing antibodies (Figure 1A,
Figure 1. Comparison of the hemizona index (HZI) and the sperm penetration index (SPI) in the sera of 22 infertile women with sperm immobilizing antibodies (A) and those of 13 women with unexplained infertility without the antibodies (B). There was no correlation between the HZI and the SPI in either group.

Table I. Effects of monoclonal antibodies (mAb) against human spermatozoa on fertilization

<table>
<thead>
<tr>
<th>mAb</th>
<th>Ig class</th>
<th>SI50 units</th>
<th>HZI*</th>
<th>ZPI*</th>
<th>SPI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C6</td>
<td>mouse M</td>
<td>200</td>
<td>9</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>1G12</td>
<td>mouse M</td>
<td>100</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3B10</td>
<td>mouse M</td>
<td>&lt;1</td>
<td>114</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>H6-3C4</td>
<td>human M</td>
<td>100</td>
<td>76</td>
<td>106</td>
<td>78</td>
</tr>
</tbody>
</table>

IH = immunoglobulin; SI50 units = quantitative sperm-immobilizing antibody titres, HZI = hemizona index, ZPI = zona pellucida penetration index, SPI = sperm penetration index
*Statistical analysis was considered unnecessary since these are averaged data obtained from only two or three samples in each experiment

Table II. Effects of monoclonal antibodies (mAb) against murine spermatozoa on fertilization

<table>
<thead>
<tr>
<th>mAb</th>
<th>SIT*</th>
<th>IVFI*</th>
<th>HZI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vx5</td>
<td>Positive</td>
<td>6</td>
<td>68</td>
</tr>
<tr>
<td>Vx8</td>
<td>Positive</td>
<td>13</td>
<td>52</td>
</tr>
<tr>
<td>Vx10</td>
<td>Positive</td>
<td>67</td>
<td>81</td>
</tr>
<tr>
<td>Vx16</td>
<td>Positive</td>
<td>77</td>
<td>99</td>
</tr>
<tr>
<td>Vx18</td>
<td>Positive</td>
<td>89</td>
<td>68</td>
</tr>
<tr>
<td>Vx4</td>
<td>Negative</td>
<td>95</td>
<td>94</td>
</tr>
<tr>
<td>Vx13</td>
<td>Negative</td>
<td>100</td>
<td>112</td>
</tr>
</tbody>
</table>

mAb = monoclonal antibody, HZI = hemizona index, SIT = sperm immobilization test, IVFI = in-vitro fertilization index, values <50% were considered to be inhibitory.
*Adapted from Ben et al. (1988)

B). These findings indicate that there is diversity in fertilization blocking antibodies and the antibodies seem to block fertilization in a stage-specific manner. It was seen that 21 of 22 (95.5%) of the patient sera with sperm immobilizing antibodies inhibited HZA while none of 13 patient sera without the antibodies inhibited HZA. These results suggest two possibilities. One is that the sperm immobilizing antibodies in the sera of infertile patients have inhibitory effects on sperm–zona pellucida tight binding. Another is that the patients with sperm immobilizing antibodies in their sera are likely to have simultaneous blocking antibodies. Conversely, 19 of 22 (86.4%) patient sera with sperm immobilizing antibodies blocked SPA while eight of 13 (61.5%) patient sera without them blocked SPA. These results suggest that most of the patient sera with sperm immobilizing antibodies also have blocking effects on sperm penetration into the ooplasm. Furthermore, there were some women who had inhibitory factor on sperm penetration into the ooplasm in their sera, despite not having sperm immobilizing antibodies.

As shown in Table I, among four mAbs against human spermatozoa tested, two (2C6, 1G12) showed strong inhibitory effects in all the assays (HZA, ZPA and SPA). One mAb 3B10 did not inhibit HZA but blocked ZPA and SPA. Another mAb, H6-3C4, seemed to have no inhibitory effects on fertilization. These results indicate that the two mAbs, 2C6 and 1G12, may block sperm capacitation because the corresponding antigens to these mAbs were found to be sperm-coating antigens derived from seminal plasma (Kameda et al., 1991; S.Komori, manuscript in preparation).

Several authors have demonstrated that there is greater binding of serum immunoglobulins to capacitated spermatozoa than to those isolated soon after ejaculation (Fusi and Bronson, 1990; Monroe et al., 1990; Margalioth et al., 1992). Benoff et al. (1993) suggested that head-directed antisperm immunoglobulins inhibited the changes in membrane sterol content associated with sperm capacitation. Our mAb, 2C6 and 1G12, seemed to react with non-capacitated spermatozoa and blocked capacitation so that all the following process of fertilization might be inhibited. The mAb 3B10 may block fertilization at the level of acrosome reaction because it was found to react with the inner acrosome membrane (Toji, 1991). Bandoh et al. (1992) found that sperm immobilizing antibodies blocked fertilization at least in part by inhibiting the acrosome reaction of spermatozoa. Zouari et al. (1992) also demonstrated that sperm capacitation and acrosome reaction could be altered by
antisperm antibodies present on human ejaculated spermatozoa. Our mAb 3B10 seemed to have blocking effect on acrosome reaction of spermatozoa so that it allowed sperm binding to the zona pellucida but inhibited sperm penetration through the zona pellucida and also sperm penetration into the ooplasm. These results show that there is a diversity of the blocking effects of antisperm antibodies on fertilization

In mice, two of seven mAbs (Vx5 and Vx8) against murine spermatozoa inhibited 1VF but never blocked HZA (Table II). Both of the two mAbs with sperm immobilizing activities reacted with the murine sperm tail (Ben et al., 1988). They allowed sperm binding to the zona pellucida but seemed to block sperm penetration through the zona pellucida or sperm penetration into the ooplasm. These findings also suggest that antisperm antibodies block fertilization in a stage-specific manner.

We found that some of the antisperm antibodies inhibited sperm capacitation and blocked all the subsequent stages of fertilization. Some other antibodies allowed the capacitation and sperm binding to zona pellucida but inhibited the acrosome reaction, followed by the blocking of sperm penetration through zona pellucida and ooplasm. This study is the first to have demonstrated the diversity of the blocking effects of antisperm antibodies on fertilization; the combined use of several diagnostic tests for predicting fertilization by spermatozoa might be useful in the evaluation of the effects of the patient sera or monoclonal antisperm antibodies on fertilization.

References


Ishijima, S and Koyama, K. (1974) Quantitative estimation of sperm immobilizing antibodies in the sera of women with sterility of unknown etiology the 50% sperm immobilization unit (S1\textsubscript{50}). Excerpta Med. Int. Congr Ser., 370, 10-15

Downloaded from https://academic.oup.com/humrep/article-abstract/11/12/2595/714409 by guest on 16 April 2019

Received on February 12, 1996, accepted on September 26, 1996


Margalioth, E.J., Cooper, G.W., Taney, F.H. et al. (1992) Capacitated sperm cells react with different types of antisperm antibodies than fresh ejaculated sperm. Fertil. Steril., 57, 393-398


