Flow cytometry study on the effect of serum and peritoneal fluid of women on sperm-binding activity of immunoglobulin G antisperm antibodies*

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Objective: To investigate the influence of sera and peritoneal fluids (PFs) from fertile and infertile women on the binding of antisperm antibodies to the surface of spermatozoa.

Design: The immunoglobulin (Ig) G antisperm antibodies binding to the surface of live spermatozoa was evaluated after their incubation in antisperm antibodies-positive serum from an infertile male in the presence and absence of female sera or PFs.

Setting: Russian Scientific Center for Obstetrics, Gynaecology, and Perinatology.

Patient(s): Serum and PF samples from fertile and infertile women; antisperm antibodies-positive serum from infertile men; high-quality fresh semen from healthy donors.

Intervention(s): Serum samples were obtained from fertile and infertile women and from infertile men. Peritoneal fluids were collected during routine laparoscopy.

Main Outcome Measure(s): The proportion of spermatozoa positive for IgG antibodies and the quantity of antisperm antibodies on the sperm surface measured by flow cytometry (FCM).

Result(s): The addition of sera from fertile or infertile women with endometriosis or pelvic adhesion disease to an IgG antisperm antibodies-positive male serum resulted in significant inhibition of the antisperm antibodies binding to the sperm surface.

Conclusion(s): Sera of fertile as well as infertile woman contain factors that block IgG antisperm antibodies binding to the surface of live spermatozoa. (Fertil Steril® 1997;67:680–6. © 1997 by American Society for Reproductive Medicine.)

Key Words: Antisperm antibodies, flow cytometry, human spermatozoa, infertility

There are numerous detailed reports on human antisperm antibodies and interference of some of them with reproductive processes. Binding of antisperm antibodies to sperm surface inhibits sperm function and fertilization and the presence of circulating antisperm antibodies in serum of women has been implicated as a contributing factor to infertility. This is a recent occurrence, given that the sperm-binding and sperm-immobilizing activity of antisperm antibodies can depend on the factors present in blood serum (1, 2). Neutralization of antisperm antibodies binding to the sperm surface by female blood also has been described (3). Currently, flow cytometry (FCM) is used widely as an objective method of quantifying antisperm antibodies on the sperm surface (4–7). Not only the number of antisperm antibodies-positive spermatozoa, but also the quantity of antibodies bound to the cell surface, can be estimated using this method. In the present study, we attempted to use FCM for evaluating the effect of sera and peritoneal fluids (PFs) from fertile and infertile women on IgG antisperm antibodies binding to the surface of live spermatozoa.

MATERIALS AND METHODS

Subjects

The subjects were 10 fertile and 35 infertile women (infertility period ranging from 2 to 10 years)
undergoing laparoscopy for either tubal ligation or as part of infertility evaluation; 10 healthy women in the first trimester of pregnancy (7 to 13 weeks); and 5 infertile men whose serum contained IgG anti-sperm antibodies. High-quality fresh semen was collected from five healthy donors.

The study group consisted of infertile women with laparoscopic evidence of endometriosis (stage I-II according to the revised American Fertility Society Classification (8), n = 20; infertile women with pelvic adhesion disease, n = 15; fertile women without laparoscopic evidence of endometriosis, n = 10; and healthy pregnant women (primigravidas, with a normal course of pregnancy), n = 10. Informed consent was obtained from all patients.

Specimen Collection and Preparation

Five-milliliter blood samples without anticoagulant were taken from each patient participating in the study. The blood was allowed to clot and then was centrifuged. The serum was aliquoted in 100-PL portions and kept at -20°C until use. Peritoneal fluid was collected by aspiration of fluid from the cul-de-sac during laparoscopy. Cellular material and debris were removed from the PF by centrifugation at 1,000 X g for 10 minutes and supernatant was stored as described for serum. The semen specimens were collected by masturbation from healthy donors and analyzed immediately after liquefaction according to the World Health Organization standards (9). Only specimens with counts >60 x 10^6/mL, motility rate >50%, and mixed antiglobulin reaction (MAR) percentage = 0% were used.

Mixed Antiglobulin Reaction Test

The direct MAR test (10) was performed by mixing on a microscope slide one drop (approximately 10 µL in volume) of fresh semen, one drop of latex particles coated with IgG, and one drop of antiserum from rabbit anti-human IgG (SpermMar Kit, Ortho Diagnostic Systems, Beerse, Belgium). The reactions were examined by phase-contrast microscopy at x400, and the percentage of motile spermatozoa carrying one or more latex particles was determined by the scoring of 100 motile sperm. The results were read after 2 to 3 minutes and again after 10 minutes.

Absorption of Serum and PF by Protein A and Measurement of Ig Concentration

Sixty microliters of serum or PF obtained from a fertile woman was incubated with 20 mg of insoluble protein A (minimum of 4.0 mg human IgG bound per 100 mg solid) (Sigma, St. Louis, MO) at 4°C for 12 hours. Protein A was extracted from the serum and PF by centrifugation (1,500 X g for 10 minutes). The concentration of IgG, IgA, and IgM in the serum and PF was determined by single radial immunodiffusion.

Sperm Labeling With IgG Antisperm Antibodies

Motile spermatozoa were provided by swim-up procedure using antisperm antibodies-negative semen from a fertile donor and adjusted to a concentration of 8 to 10 x 10^6/mL with Medium 199 containing 0.3% bovine serum albumin (M199-BSA-0.3%). Eight microliters of antisperm antibodies-positive male serum (whole or diluted 1:4 in M199-BSA-0.3%) was incubated with 20 µL M199-BSA-0.3% (positive control) or female serum (whole or diluted) or PF for 15 minutes at 4°C (all sera and PFs were heat inactivated at 56°C for 30 minutes) and then with 4 µL of motile sperm suspension at 4°C for 40 minutes. In competition experiments, the spermatozoa were incubated with male serum, containing PF from fertile woman. To determine antisperm antibodies in a serum or PF, 4 µL of the motile sperm suspension were added to 28 µL of the serum or PF, and the mixture was incubated at 4°C for 40 minutes. Negative controls consisting of 28 µL M199-BSA-0.3% and 4 µL of sperm suspension were included in all experiments.

Fluorescent Flow Cytometric Analysis

Second Antibody Labeling

All samples were washed twice in phosphate-buffered saline (PBS), pH = 7.4, and the cell pellet was resuspended in 25 µL of PBS. In competition experiments, various quantities (from 1 to 10 µg) of human IgG, IgA, or IgM (Sigma) were added to the spermatozoa suspension (final volume 25 µL), while the sample containing no human immunoglobulins was used as a positive control. One microliter of Fc-specific fluorescein isothiocyanate (FITC)-labeled rabbit anti-human IgG polyclonal antiserum (9512; Sigma) was added to each sample. All mixtures then were incubated at 4°C for 20 minutes, and then the cells were washed three times with PBS, resuspended in 100 µL of PBS, stained with propidium iodide (20 µg/mL) for distinguishing living spermatozoa (11), and analyzed by FCM.

Flow Cytometer

All the samples were analyzed by FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Fluorescence data of 5,000 viable cells were collected with logarithmic amplification for green fluorescence specific for antibody staining. The flow rate was 500 cells per second. Negative
controls were included in all experiments and showed a background fluorescence of essentially zero. The percentage of positive spermatozoa was determined by subtracting a background fluorescence from each histogram. Any reaction revealing a percentage of positive spermatozoa >30% was considered positive. To evaluate IgG antibody binding, the mean fluorescence intensity channel number (MFI) on a logarithmic scale was obtained. The data were analyzed by CONSORT30 and LYSIS software from Becton Dickinson.

The percentage inhibition (PI) of antisperm antibodies binding to the sperm surface after the addition of sera or PF was calculated by the formula:

\[
PI = 1 - \frac{\text{MFI of tested samples}}{\text{MFI of positive control}} \times 100\%
\]

In competition experiments, the percentage of inhibition of FITC-labeled rabbit anti-human IgG polyclonal antiserum binding to the sperm surface after the addition of IgG, IgA, and IgM was calculated using the same formula. All data are presented as the means ± SD.

**RESULTS**

Figure 1 shows results of the FCM test for a IgG antisperm antibodies-positive male serum diluted 1:16 in M199-BSA-0.3% or in sera or PF from fertile woman. Almost all spermatozoa after incubation in male serum diluted in M199-BSA-0.3% proved to be IgG antisperm antibodies-positive (MAR = 100%, positive spermatozoa = 97%; Fig. 1A). The addition of serum from a fertile woman to male serum resulted in inhibition of IgG binding to the sperm surface (positive spermatozoa = 2%; percentage inhibition = 96%; Fig. 1B). When PF was added to an antisperm antibodies-positive serum (Fig. 1C), no inhibition of IgG binding was observed (positive spermatozoa = 98%) and the amount of IgG on the sperm surface increased dramatically (percentage inhibition = −716%). Self-IgG antisperm antibodies were undetectable either in the serum or PF. Using propidium iodide, we showed that ≈80% of the cells remained alive after their incubation with the antisperm antibodies-positive serum and serum or PF from fertile woman.

A competitive inhibition of mean fluorescence intensity of spermatozoa incubated with antisperm antibodies-positive male serum diluted 1:16 in PF from fertile woman (percentage inhibition = −360%) was used to verify the specificity of the FITC-labeled anti-human IgG used in our study. Incubation of the FITC-labeled anti-human IgG with human IgG produced a dose-dependent inhibition of FITC-labeled anti-human IgG binding to spermatozoa, whereas human IgA and IgM produced no inhibition (Fig. 2).

Figure 3 shows the neutralizing effects of the same serum and PF from a fertile woman on the binding of antisperm antibodies from five different antisperm antibodies-positive male sera. The woman's serum led in three cases to inhibition of antisperm antibody binding, with the percentage inhibition ranging from 71% to 100%; in two cases (males 1 and 4), there was no inhibition, and an increase in the IgG level on
Figure 2 Dose-dependent inhibition of FITC-labeled anti-human IgG binding to the surface of antispem antibodies-positive spermatozoa after incubation with human IgG, IgA, and IgM.

The sperm surface was observed instead (percentage inhibition = -278% and -94%, respectively). No inhibition was noted in any case after the addition of the PF; rather, large increases in IgG on the sperm surface were recorded (the percentage inhibition ranged from -113% to -282%).

Table 1 presents the data on the effect of serum and PF absorption by protein A on their blocking activity. After absorption by protein A the IgG, IgA, and IgM content in serum decreased 21.4, 1.4, and 1.8 times, respectively. The IgG level in PF before absorption was 9.0 times lower than in serum, and decreased after absorption 5.0 times. The IgA level in PF decreased 2.0 times after absorption. The IgM was not detected in PF. The anti-sperm antibodies-positive male serum was diluted 1:4 in M199-BSA-0.3% (positive control) or in woman’s serum or PF. The level of blocking activity in serum before the absorption was rather high, but, after absorption, the blocking activity was not observed and IgG amount on the sperm surface was higher than that in positive control. The IgG amount on the sperm surface after the incubation with anti-sperm antibodies-positive serum containing nonabsorbed PF was higher than that in positive control, whereas, after the incubation with the absorbed PF, the IgG content on the sperm surface significantly decreased.

The effects of adding various dilutions of sera from a fertile woman to anti-sperm antibodies-positive serum on IgG binding to the sperm surface are shown in Figure 4. The decrease in IgG binding was the greatest with the undiluted serum sample. The inhibitory effect waned with an increase in serum dilution. With the serum diluted 1:4, no inhibition occurred, while the use of further dilutions (with the exception of 1:32) was found to result in elevated amounts of surface-bound IgG that exceeded several fold the total quantity of anti-sperm antibodies detected in the anti-sperm antibody-positive serum and in the female serum at a given dilution. Using the same woman’s serum absorbed by protein A, the inhibitory activity was not observed, and in all dilutions the IgG amount on the sperm surface was higher (by 34% to 70%) than that in the positive control.

The inhibition effects of sera and PFs from fertile and infertile women on binding of anti-sperm antibodies from one male serum then were compared. Almost all spermatozoa after incubation in male serum diluted in M199-BSA-0.3% proved to be IgG anti-sperm antibodies-positive (positive spermatozoa, 85% ± 8%). After sera from either fertile or infertile women with endometriosis or pelvic adhesion disease were added to the male serum, the count of IgG-positive spermatozoa decreased to 18% ± 22%.

Table 1 Immunoglobulin Concentration and Blocking Activity of Serum and PF from Fertile Women Before and After Absorption With Protein A

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Preabsorption</th>
<th>Postabsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>PF</td>
</tr>
<tr>
<td>IgG (mg/mL)</td>
<td>1.10</td>
<td>0.41</td>
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<td>IgA</td>
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<td>1.00</td>
</tr>
<tr>
<td>IgM</td>
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<td>0.00</td>
</tr>
<tr>
<td>Percentage inhibition (%)</td>
<td>76</td>
<td>-79</td>
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</tbody>
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Figure 3 Inhibition of IgG anti-spem antibodies binding to the sperm surface by serum (open bars) and PF (filled bars) of fertile woman. The results are presented for anti-spem antibodies-positive sera from five infertile males.
Correlation between the inhibition of IgG antisperm antibodies binding to the sperm surface and the dilution of female serum before (filled circles) and after absorption by protein A (open circles) added to an antisperm antibodies-positive male serum.

Figure 4

When sera from pregnant women or PFs from fertile or infertile women with endometriosis or pelvic adhesion disease were added to the antisperm antibodies-positive serum, almost no change in the number of IgG-positive spermatozoa was observed.

The addition of sera from either fertile or infertile women with endometriosis or pelvic adhesion disease to an antisperm antibodies-positive male serum was followed by a reduction in the amount of IgG on the sperm surface (Fig. 5). When spermatozoa were incubated in PFs from fertile, endometriosis-, or pelvic adhesion disease-affected infertile women, a decrease in antisperm antibodies was observed only for one fertile woman and one infertile woman with endometriosis; in all other cases, the amount of IgG on the sperm surface greatly increased to exceed severalfold the quantity of antisperm antibodies detected in an antisperm antibodies-positive serum.

The addition of sera from pregnant women resulted in decreased antisperm antibodies binding to the sperm surface in one half of the patients and in increased IgG binding in the other half. In general, the inhibitory activity of sera samples from fertile or infertile women with endometriosis or pelvic adhesion disease (percentage inhibition = 87% ± 16%, 94% ± 7%, and 93% ± 10%, respectively) significantly differed from that of sera from pregnant women (percentage inhibition = −44% ± 76%; $P < 0.001$). Antisperm antibodies were detected in the sera samples obtained from fertile and infertile women with endometriosis or pelvic adhesion disease, in 30%, 15%, and 10% of the samples, respectively, as well as in 40% of the sera from pregnant women. In the PFs from fertile and endometriosis- or pelvic adhesion disease-affected women, antisperm antibodies were detected in 20%, 30%, and 33% of the samples, respectively. No correlation was found between the antisperm antibodies level and the neutralizing activity of sera or PFs.

**DISCUSSION**

This study has demonstrated that the addition of sera from fertile and infertile women with endometriosis or pelvic adhesion disease to an IgG antisperm antibodies-positive male serum resulted in significant inhibition of the antisperm antibodies binding to the sperm surface. Evidently, the decrease in proportion of live antisperm antibodies-positive spermatozoa might have resulted from their selective death. In our study, we used the fraction of motile spermatozoa obtained by swim-up procedure. All the sera and PFs we used had been preinactivated by heating and lacked spermotoxic activity. During their incubation, the spermatozoa lost much of their motility, yet >80% of the cells remained
alive and no selective death of antisperm antibodies-positive spermatozoa has been observed.

The amount of IgG on sperm surface after their incubation in an antisperm antibodies-positive serum containing PF or diluted serum from fertile women exceeded the total quantity of antisperm antibodies detected in the antisperm antibodies-positive serum and in the PF or diluted serum. When whole serum and PF are mixed with the male serum, the IgG data may be confused if there is cross-reactivity of the anti-human IgG antisemum with IgA and/or IgM. The data on competitive reduction of the FITC-labeled anti-human IgG binding to an excess of human IgG, IgA, and IgM confirm the specificity of the antibodies used in our study. In addition, we used human polyclonal antisera to IgG, IgA, or IgM along with monoclonal antibodies for the detection of antisperm IgG, IgA, and IgM antibodies in semen (5), and the results obtained with monoclonal and polyclonal antibodies are almost identical. Thus, the occurrence of “excess” Igs can not be related to the binding of IgA or IgM from serum or PF of fertile women to the sperm surface.

It may be admitted that excess Igs are antisperm antibodies from the male serum and their binding is due to the expression of new antigens in the process of incubation. However, the amount of excess IgG on the sperm surface significantly decreased after absorption of PF with protein A. We also found (data not shown) that the levels of Igs on the surface of spermatozoa preincubated in an antisperm antibodies-positive male serum appeared two to nine times higher after their incubation in antisperm antibodies-negative PF.

It is our speculation the Igs from serum or PF may bind to the antisperm antibodies and form antisperm antibodies-IgG complexes on the sperm surface. The quantity of sperm-bound IgG after incubation of spermatozoa in male serum containing absorbed and unabsorbed female serum at high dilutions was higher than after their incubation in male serum alone. These data indicate that the titer of IgG binding to the surface of antisperm antibodies-positive spermatozoa is rather high.

The relationship we detected between the concentration of female serum and the inhibition activity indicates that inhibition of antisperm antibodies binding was observed only in whole sera and those diluted twofold and 1:32. It should be noted that nonlinear data for high serum dilutions were repeated for different male sera and semen samples. Mechanisms underlying this phenomenon may involve the complex processes of interaction between the antisperm antibodies, blocking factors, and the IgG binding to the surface of antisperm antibodies-positive spermatozoa.

The blocking was specific and no inhibition of antisperm antibody binding from some male serum was observed. The increased amount of IgG detected on spermatozoa after their incubation in some antisperm antibodies-positive serum from fertile woman also may be taken as an indication that titer of blocking factors in woman’s serum was lower than titer of antisperm antibodies in the male serum.

In the present study, most of the sera from fertile as well as infertile women blocked IgG binding to spermatozoa almost completely. No differences in neutralizing activity between sera from fertile and infertile women were detectable. Antisperm antibodies-positive and antisperm antibodies-negative sera inhibited antibody binding with equal efficiencies. It should be noted, though, that we used the antisperm antibodies-positive serum in a 1:16 dilution, and it cannot be ruled out that intergroup differences could be found if antisperm antibodies were used in higher concentrations.

Peritoneal fluids, unlike sera, were found incapable of blocking antisperm antibodies binding. The absence of inhibition of antisperm antibody binding by PF or sera from some pregnant women also may be taken as an indication that titer of blocking factors in those sera or PFs was lower than titer of antisperm antibodies in the male serum.

After absorption by protein A, the IgG content in serum decreased >20 times and the inhibiting activity disappeared completely. It is probable that the Igs of the serum (mainly IgG) can block the binding of antisperm antibodies to the sperm surface.

The blocking activity may exist partly due to the presence of Igs in the female sera that are antisperm antibodies. According to Jerne’s hypothesis (12), the immune system responds to foreign substances as a regulatory network composed of idiotypes (Ab1s) and anti-idiotypes (Ab2s). The antibody Ab1 made in response to the original antigen becomes itself an antigen and elicits the synthesis of a secondary antibody, Ab2. The appearance of Ab2 in the sera of animals in response to monoclonal antisperm antibodies had been demonstrated previously (13, 14), whereas anti-antisperm antibodies in the sera of women were first identified by Naz et al. (1). These authors showed that sera from fertile women, unlike those from girls and infertile women with antisperm antibodies in their serum, contain antibodies to the anti-Fa-1 monoclonal antibody Fab. Affinity-purified Fabs from fertile women were able to neutralize up to 100% of the sperm-binding activity of Fabs from infertile women. Ab2-mediated blocking of antisperm antibodies binding cannot occur unless the antisperm antibodies-positive serum and female se-
rum contain antisperm antibodies of the same specificity and the Ab2 is present in the female serum in a sufficient amount. The source of antisperm antibodies in our study was a serum containing antibodies to surface antigens of spermatozoa from a fertile donor. Sera blocking the binding of these antibodies were obtained from fertile women and from infertile women for whom male factor infertility had been excluded. The sera of such women are therefore likely to contain antisperm antibodies specific for antigens of fertile spermatozoa, and the high neutralizing activity of these sera may be attributed to the presence of anti-idiotypic antibodies. The antisperm antibodies detectable with our method are heterogeneous and will interact with a multiplicity of sperm surface antigens, possibly with different affinities. Clearly, if the mechanisms of blocking and the role of anti-idiotypic antibodies are to be studied in depth, the binding of antisperm antibodies to individual antigens must be evaluated.

It also has been shown that the IgG4 isolated from sperm-immobilizing antibodies (SI-Abs) block an SI-Ab-positive patient's SI-Abs activity (2). One possible explanation for the blocking effect in this case may be the masking of the antigen epitopes corresponding to the IgG1 of SI-Abs by the IgG4 antibody lacking complement-activating activity.

Blood sera also may contain other factors affecting antibody-antigen interactions. Such factors may be considered to include polyanions (phospholipids, proteoglycans, heparin) that nonspecifically bind to antibodies (15) or serum proteases that were destroyed of antibodies. It should be admitted that the blocking activity of various sera may be due to the combined action of specific and nonspecific factors they contain.

In the present study we described a convenient model allowing the study the mechanisms of antisperm antibody interaction with the sperm surface using FCM. This model may be used as a basis for further examination of the mechanisms underlying the blocking of antisperm antibodies binding and for the identification of blocking factors in various biologic fluids.

REFERENCES