A COMPARISON OF METHODS OF SCREENING FOR SPERM ANTIBODIES IN THE SERUM OF WOMEN WITH OTHERWISE UNEXPLAINED INFERTILITY*


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Sera from 102 women with infertility due to a variety of causes and from 40 pregnant women were studied for the presence of antispem factors. Three techniques were used: sperm microagglutination, sperm immobilization, and an indirect immunofluorescent technique for detection of sperm-bound immunoglobulins. There was no correlation between the results obtained using these three different techniques. Of the three, only the results of sperm immobilization tests correlated with primary unexplained infertility. The sperm microagglutination test appeared to measure nonspecific factors. Methanol fixation of spermatozoa used in the indirect immunofluorescent technique apparently resulted in nonspecific binding of immunoglobulins. When fresh spermatozoa were used no binding of immunoglobulin to spermatozoa could be demonstrated. The nature and location of the antigen(s) involved remain to be determined.

Infertility in women as a consequence of circulating antibodies directed against spermatozoa still remains an ill-defined entity. Much of the confusion concerning the clinical significance of antispematozoal antibodies stems from considerable variation in techniques used to demonstrate the presence of antibodies directed against spermatozoa.

Clinical interest in sperm isoimmunization as a possible cause of infertility was stimulated primarily by results of the microagglutination tests of Franklin and Dukes,1, 2 who reported the presence of a spermagglutinating antibody in the serum of 78% of 89 women with otherwise unexplained infertility. Extension of this study in include 487 patients showed an incidence of spermagglutinating antibody in 67.5% of women with unexplained infertility as opposed to 9.2% in patients with proven fertility.3 Subsequent studies reported by Schwimmer et al.4, 5 demonstrated spermagglutination in 73% of prostitutes and 37.5% of women of couples with primary unexplained infertility, compared with 20% in control groups. No relationship was found between the results of postcoital testing of cervical mucus and either positive circulating sperm isoagglutinins or ABO(H) incompatibility.4, 6 More recent investigations have found the incidence of positive sperm microagglutination tests to range between 7 and 29% in women with unexplained infertility and between 0 and 25% in women of known fertility.6-10 As more investigators have used the microagglutination technique it has become apparent that spontaneous agglutination is common and that serum

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factors other than immunoglobulins can agglutinate spermatozoa. Use of the gelatin agglutination test described by Kibrick and co-workers has only rarely demonstrated the presence of spermatozoal antibodies in the sera of women of fertile couples and has failed to provide correlation with results of the microagglutination test.

In 1968 Isojima and co-workers described a complement-dependent sperm immobilization test as the most reliable method for detecting antisperm antibody related to infertility. In the initial description of this method they reported the presence of a sperm-immobilizing antibody in the serum of 12% of women with primary unexplained infertility, but not in the serum of women known to be fertile. Subsequent investigators using the sperm immobilization technique confirmed these findings and reported that 4.7 to 17% of women with primary unexplained infertility possessed a sperm-immobilizing antibody.

Immunofluorescent antibody techniques have also been used to study antispermatozoal antibodies, but conflicting results have been obtained. Early attempts to demonstrate antibodies to testicular spermatozoa in sera from prostitutes failed when an immunofluorescent antibody directed against human immunoglobulins was used. However, Schwimmer and co-workers obtained uniform fluorescence of ejaculated spermatozoa when using sera demonstrating sperm microagglutination. The studies of Hjort and Hansen, who used an indirect immunofluorescent technique with methanol-fixed spermatozoa, demonstrated positive reactions in 76% of women of infertile couples. However, fluorescent patterns were also found in the sera of 58% of children, 79% of female blood donors, and 62% of pregnant women tested.

It was the purpose of this study, therefore, to compare the results of sperm microagglutination and sperm immobilization tests of infertile women with those obtained by an indirect immunofluorescent technique using both fresh and methanol-fixed spermatozoa.

MATERIALS AND METHODS

Sera from 102 infertile women were tested for the presence of antibodies directed against spermatozoa by using tests for sperm microagglutination and sperm immobilization and an indirect immunofluorescent technique. Each patient was categorized into one of four groups according to the results of infertility investigations. Patients in whom tests for tubal patency, endometrial biopsy, laparoscopy, basal body temperature curves, and husbands' seminal analyses were within normal limits were classified as having unexplained infertility. Patients in this category were further subdivided into groups with primary and secondary infertility depending upon whether or not they had conceived previously. The term organic infertility was used to include those patients who presented with anovulatory states or in whom organic lesions related to infertility, such as tubal occlusion or endometriosis, could be detected. Patients whose husbands had had at least two abnormal semen analyses were classified as having a male factor contributing to infertility.

Eight women who became pregnant during the period of investigation were included among 40 pregnant women in the second and third trimesters who served as a fertile control group.

Blood samples obtained by venipuncture were allowed to clot and the serum was separated by centrifugation and frozen at -20° C until tested. All serum samples were stored frozen and analyzed by one person who was unaware of the patient investigations. Semen specimens were obtained from donors. Each speci-
men, obtained by masturbation, was used within 1 hour in the test procedures. Prior to use, seminal fluid volume, viscosity, and sperm morphology and motility were recorded at 30 minutes, 1 hour, and 2 hours after the specimen was obtained. Seminal fluid specimens with many inflammatory cells, bacterial contamination, spontaneous agglutination, counts less than 40 million spermatozoa/ml, less than 70% motility, or greater than 30% abnormal forms were discarded. The data were subjected to statistical analysis, using the $\chi^2$ test.

**Sperm Microagglutination Test.** The method employed was described by Franklin and Dukes; donor semen was used. Serum was inactivated at 50° C for 30 minutes. Whole serum (0.2 ml) and a 1:4 serum dilution (0.2 ml) were incubated in serologic tubes with 0.02 ml of semen at 37° C. After 1 or 2 hours of incubation a drop from each tube was examined under a microscope at x240 magnification, and the degree (1+ to 5+) and type (head-to-head, tail-to-tail, or mixed) of agglutination were recorded as described by Jones et al. Only agglutination of motile spermatozoa was included in results recorded. Agglutination of nonmotile spermatozoa or adhesion of spermatozoa to other cells was not included. Agglutination of 2+ was considered a positive test result. Sera positive in a dilution of 1:4 were also tested in a dilution of 1:16.

**Sperm Immobilization Test.** The method used was that of Isojima et al. Serum samples were inactivated by heating at 56° C for 30 minutes. Guinea pig complement was commercially obtained (Hyland, Division of Travenol Laboratories, Los Angeles, Calif.) and dissolved in distilled water. Inactivated serum (0.25 ml) was then added to fresh semen (0.025 ml) and complement solution (0.05 ml) and incubated at 37° C for 1 hour. Tubes containing saline and normal human serum and tubes containing test serum without complement were also incubated with spermatozoa to serve as controls. Following the incubation period, samples from each tube were examined under the microscope and the percentage of motile spermatozoa was recorded. The ratio of percentage of motile sperm in control samples to percentage in test samples was termed the sperm immobilization value (SIV). An SIV of 2.0 or greater was considered a positive result.

**Indirect Immunofluorescent Test.** The technique used was that described by Hjort and Hansen. Spermatozoa washed twice in saline and then resuspended were placed in drops on slides and dried. Slides were then placed into two groups; one set of slides was incubated in absolute methanol for 30 minutes for fixation of spermatozoa. Whole serum diluted 4-fold was placed on the slide for 1 hour at room temperature. Slides were washed twice with phosphate-buffered saline (pH 7.2) and then incubated with fluorescein-tagged goat anti-human immunoglobulin G, M, or A (IgG, IgM, or IgA) for 30 minutes. Slides were washed again, mounted in a glycerol-buffer mixture, and examined under a microscope equipped with an HBO-200 lamp and interference filter. Phosphate buffer and positive and negative sera found on initial testing were used in subsequent tests as positive and negative controls. Results were recorded to indicate the pattern of fluorescence and the percentage of sperm fluorescing.

**RESULTS**

**Sperm Microagglutination Tests.** Results of sperm microagglutination tests are shown in Table 1. Twenty-four of the 142 sera agglutinated donor sperm, predominantly in a head-to-head manner. Of the women with primary unexplained infertility, 24.2% showed spermagglutination, but sera from 10.4% of pregnant
TABLE 1. Results of Sperm Microagglutination Tests

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Positive sperm microagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole serum</td>
<td>1:4 dilution</td>
</tr>
<tr>
<td>Primary unexplained infertility</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>Secondary unexplained infertility</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Organic infertility</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>Male factor</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Pregnant</td>
<td>48</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>142</td>
<td>23</td>
</tr>
</tbody>
</table>

women also caused agglutination. In the other three control groups (secondary unexplained, organic, and male factor infertility, respectively) spermagglutination occurred in 16.2 to 33.3% of sera tested. Statistical analysis using the $\chi^2$ test showed no significant difference in the incidence of spermagglutination between groups. In addition, serum titers were similar in the five groups.

Sperm Immobilization Tests. Results of sperm immobilization tests are shown in Table 2. Seven sera caused greater than 50% inhibition of sperm motility; five of these were from women with primary unexplained infertility (SIV values of these sera ranged from 2.1 to 4.7). The other two sera were from patients with polycystic ovarian disease. The SIV values for these latter two patients were 2.5 and 2.9. When analyzed statistically by the $\chi^2$ test, the incidence of sperm immobilization was significantly greater with sera from patients with primary unexplained infertility than with sera from patients with other causes of infertility or from pregnant patients.

Indirect Immunofluorescent Tests. Results of indirect immunofluorescent tests using methanol-fixed spermatozoa are shown in Table 3. Binding of fluorescein-conjugated antisera to human immunoglobulins was demonstrated in 67 of 142 sera tested. When present, fluorescence was usually observed in less than 60% of spermatozoa. No significant difference in the incidence of positive reactions was found between any of the groups tested.

Binding of individual immunoglobulins was evaluated by the use of heavy chain-specific, fluorescein-labeled, anti-human immunoglobulin antisera. As is shown in Table 4, binding of specific immunoglobulins did not vary significantly among the five groups tested. Anti-human IgM was bound more frequently than antisera to the other immunoglobulins and was bound predominantly to the acrosomal segment of the fixed spermatozoa. In contrast, anti-human IgG bound to any part of the spermatozoa but most often bound to the sperm tail. Binding of IgA antibody was demonstrated only infrequently.

To evaluate indirect immunofluorescence further, the 67 sera that had shown binding of anti-immunoglobulin antibodies when methanol-fixed spermatozoa were used as substrate were then evaluated for binding capabilities using fresh, washed and unwashed spermatozoa as substrate. Under these conditions, binding of the fluorescein-conjugated immunoglobulins could not be demonstrated.

TABLE 2. Results of Sperm Immobilization Tests

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Positive sperm immobilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Primary unexplained infertility</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td>Secondary unexplained infertility</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Organic infertility</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Male factor</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>142</td>
<td>7</td>
</tr>
</tbody>
</table>
with any of the sera, even after prolonged incubation periods.

Comparison of Results Obtained with the Three Procedures. Of the 24 sera causing sperm microagglutination, 2 were also capable of causing sperm immobilization. One of the two patients had primary unexplained infertility and the other had polycystic ovarian disease (organic infertility). When tested by indirect immunofluorescence using fixed spermatozoa, 10 of the 24 sera that produced sperm microagglutination were positive. Two of the ten patients had primary unexplained infertility, and demonstrated binding of anti-human IgM and IgG antibody to the acrosome and tail of the spermatozoa, respectively. The remaining eight sera were from one patient with secondary unexplained infertility, four patients with organic infertility, and three pregnant women.

Three of seven sera producing sperm immobilization also reacted by indirect immunofluorescence; the majority showed binding of anti-IgM. Results obtained with indirect immunofluorescence were also compared with results obtained with the first two techniques. Of the 25 sera binding IgG antibody, 4 also caused sperm-agglutination, and 2 sera demonstrated sperm immobilization. Only two sera, one from a patient with organic infertility and the other from a pregnant control, caused sperm microagglutination and also showed binding of both anti-human IgG and IgM antibodies by immunofluorescence.

Only one serum, from a patient with primary unexplained infertility, reacted positively when tested by all three techniques.

DISCUSSION

The results of sperm microagglutination studies demonstrated no difference in the incidence of head-to-head agglutination between patients with primary unexplained infertility and patients with other causes of infertility or pregnant women. These findings differ markedly from the initial reports of Franklin and Dukes, but are in agreement with more recent studies. Much of the difficulty encountered with sperm microagglutination techniques is related to the poor reproducibility of the method, as was illustrated by Mettler and Gradl.

In contrast, the results of sperm immobilization tests showed a significant difference between patients with primary unexplained infertility and other groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>No. showing binding of anti-human IgG</th>
<th>No. showing binding of anti-human IgM</th>
<th>No. showing binding of anti-human IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary unexplained infertility</td>
<td>33</td>
<td>7&quot;</td>
<td>10&quot;</td>
<td>1</td>
</tr>
<tr>
<td>Secondary unexplained infertility</td>
<td>18</td>
<td>2</td>
<td>4</td>
<td>2&quot;</td>
</tr>
<tr>
<td>Organic infertility</td>
<td>37</td>
<td>5</td>
<td>9&quot;</td>
<td>2&quot;</td>
</tr>
<tr>
<td>Male factor</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Pregnant</td>
<td>48</td>
<td>9&quot;</td>
<td>17&quot;</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>142</td>
<td>25</td>
<td>40</td>
<td>4</td>
</tr>
</tbody>
</table>

*Some sera reacted with two types of immunoglobulins.
tested. Our results showed an incidence of 15.2% of sperm-immobilizing factor in sera of patients with primary unexplained infertility, whereas sera of only two patients with organic infertility, and no pregnant women, were positive. These data confirm those of earlier studies, which have reported an incidence of 4.7 to 17% of sperm-immobilizing factors in patients with primary unexplained infertility, with none being detected in pregnant women. Our results, as have earlier studies, suggest that the sperm immobilization technique is a method of detecting antisperm activity in serum that may be of clinical significance.

The use of an indirect immunofluorescent technique using methanol-fixed spermatozoa demonstrated binding of human immunoglobulin to spermatozoa in 57.5% of women with primary unexplained infertility. However, positive reactions were also observed in a large proportion of patients with organic infertility, and even pregnant women. These data are similar to those of Hjort and Hansen. They reported immunoglobulin binding to sperm in 76% of the female partners of infertile couples, but also noted positive results in 68% of children, 75% of female blood donors, and 62% of pregnant women. These findings suggest either that binding of immunoglobulin to sperm under these conditions is nonspecific or that binding of immunoglobulin to sperm does not interfere with sperm function. To investigate these possibilities, fresh spermatozoa were used instead of fixed spermatozoa, in view of the known protein-denaturing effects of methanol. The failure to demonstrate immunoglobulin binding with fresh spermatozoa suggests, in fact, that methanol fixation resulted in alteration of the cell surface membrane, leading to nonspecific binding of immunoglobulin and/or fluorescein-labeled anti-human immunoglobulin antibodies.

Comparison of results obtained using the sperm microagglutination and sperm immobilization techniques have shown little or no correlation between results, since only 2 of 24 sera causing sperm-agglutination were also capable of causing sperm immobilization. Poor correlation between spermagglutination and immobilization techniques has been demonstrated previously, and it has been suggested that the two methods may detect different immunologic reactions. Previous studies have shown the sperm microagglutination factor to be a β-globulin, whereas the sperm-immobilizing factor has been shown by Isojima and co-workers to be a globulin absorbed by spermatozoa and dependent upon complement for its activity.

In the present study only 13 of 67 sera reacting in the indirect immunofluorescent procedure also demonstrated antispermatozoal activity in one or both of the other methods used. Staining patterns observed failed to demonstrate a relationship between either the type of immunoglobulin or region of spermatozoa involved and the results of other tests of antispermatozoal activity. Previous reports have shown that binding of immunoglobulin specifically to the acrosome, equatorial segment, neck, or tail of fixed spermatozoa may often be found in normal human sera. Binding of IgG to the tail of spermatozoa has been reported to occur in a high proportion of sera positive in the sperm immobilization method, but it was observed in only 29% of sera causing sperm immobilization in the present study. Sera causing binding of anti-human IgM immunoglobulin were largely from patients who had not demonstrated antispermatozoal activity with other testing procedures.

With respect to the lack of correlation between sperm immobilization and indirect immunofluorescent techniques, nonspecific binding has already been discussed. Other possibilities exist. It was suggested by Hjort and Hansen that
the high incidence and ubiquitous nature of the weakly reactive antibodies may reflect cross-reactivity between antibodies directed at both sperm-coating antigen (scaferin) and lactoferrin. More recently, Hansen\(^5\) proposed that the indirect immunofluorescent technique with fixed spermatozoa involves antigens in structures beneath the cell membrane of the spermatozoon, whereas other methods reflect reactions with antigens on the cell membrane. If this latter possibility is considered, inability to demonstrate antibody binding to fresh spermatozoa by sera causing sperm immobilization suggests antigen unavailability in fresh sperm or very loose binding of immunoglobulin which is removed by saline washings.

REFERENCES

2. Franklin RR, Dukes CD: Further studies on sperm-agglutinating antibody and unexplained infertility. JAMA 190:682, 1964