Chapter 8
Detection of Anti-sperm Antibody (ASA) in Men

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Abstract The presence of anti-sperm antibodies (ASAs) in men can reduce fecundity; however, the relationship between the two had been disputed. It should be emphasized that a diversity of ASA exists and has effects on fertility in infertile men. Although there is an argument that the routine testing for ASA in men is not always necessary, one should be aware that in some cases of failed intrauterine insemination (IUI) or in vitro fertilization (IVF), afterward intracytoplasmic sperm injection (ICSI) is selected because of the diagnosis of ASA.

For the appropriate diagnosis of immunological infertility in men, ASA testing should be carried out as a routine test in the infertility clinic. Although several assay methods that detect ASA in infertile men have been described, there is no doubt that the most useful diagnostic method for detecting ASA is one that can assess the antibodies on the surface of ejaculated sperm. The important factors to consider in selecting the appropriate ASA assay are the availability of material being tested (motile sperm or immotile sperm), the sensitivity and specificity of the test, the ability to define immunoglobulin isotypes and location of attachment to the sperm, the quantitation of the level of ASA, and the ease of performing the test. For this purpose, the World Health Organization (WHO) has recommended the two tests, such as the mixed antiglobulin reaction (MAR) test and direct immunobead test (direct IBT).

From our investigations, for example, some of the sperm-bound antibodies are associated with complement-dependent sperm-immobilizing antibodies, indicating that there exists a heterogeneity of sperm-bound antibodies. This result might be one of the reasons for the controversy about the relationship between ASA and immunological infertility in men.

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8.1 Methods for Anti-sperm Antibody Testing in Infertile Men

Although several assay methods that detect anti-sperm antibodies (ASAs) in infertile men have been described, there is no doubt that the most useful diagnostic method for detecting ASA is one that can assess the antibodies on the surface of ejaculated sperm. For this purpose, assays which directly detect ASA on sperm membrane such as the mixed antiglobulin reaction (MAR) test and direct immunobead test (direct IBT) are recommended by the World Health Organization (WHO) [1].

Direct IBT is widely used as a screening test for ASA [2–4], and the inhibitory effects by ASA on sperm motion and fertilizing ability are evaluated to make a decision for the strategy of infertility treatments [5]. Recently, the IBT was discontinued of its production. However, the ASA test results obtained by the immunospheres (IS) assay were shown to be in agreement with the results obtained with the IBT [6]. (See Chap. 2.)

The important issue of the ASA testing is that not all of the ASAs detected by the MAR test or direct IBT are associated with the cause of infertility; they can only detect ASAs that react with the surface antigens against motile sperm. Thereafter the physicians should assess the biological effects of ASA detected whether they affect sperm function such as sperm penetrating ability through cervical mucus and fertilizing ability.

In this chapter, several direct tests for the detection of ASA for infertile men are described.

8.2 Available Tests for the Direct Detection of Anti-sperm Antibodies (ASAs)

8.2.1 Immunobead Test (IBT)

One of the methods that can detect antibodies bound to motile sperm is the direct IBT [2–4]. Immunobeads (IBs; IgG, IgA, and IgM; Irvine Scientific, Santa Ana, CA, USA) are polyacrylamide spheres with covalently bound rabbit anti-human immunoglobulin (Ig). The method for the indirect IBT was shown in Chap. 2.

For the direct IBT, suspensions of IB and a drop of washed sperm are mixed on a glass slide, and the mixture is allowed to react for 10 min. The preparation is then examined at 400× magnification with a phase-contrast microscope. The IBs adhere to the motile sperm that have surface-bound antibodies (Fig. 8.1). The percentage of motile sperm with surface antibodies is determined, the pattern of binding is noted, and the Ig class of these antibodies can be identified using different sets of IB (Fig. 8.2). A test mixture in this direct test is scored positive if at least 20% of the sperm cells show IB binding [4].

However, based on reports that sperm penetration into the cervical mucus and in vivo fertilization (IVF) tend not to be significantly impaired unless 50% or more

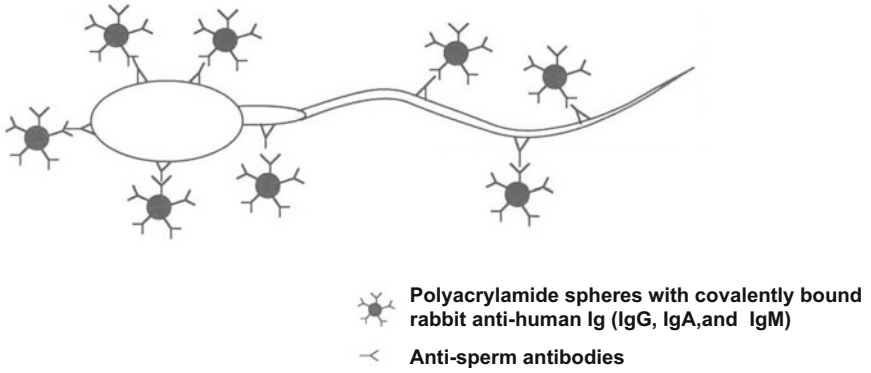

Fig. 8.1 Immunobead test (IBT). Immunobeads (IBs) are polyacrylamide spheres with covalently bound rabbit anti-human Ig. The IBs adhere to the motile sperm that have surface-bound antibodies. A test mixture in this direct test is scored positive if at least 20% of the sperm cells show IB binding [4] that is recently supported by Koriyama et al. [7]

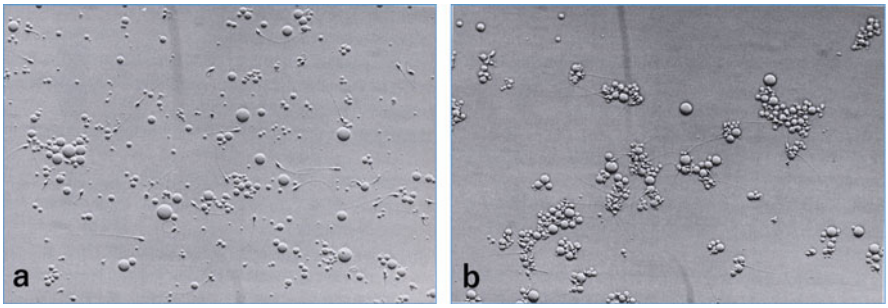

Fig. 8.2 Judgment of the immunobead test (IBT). The percentage of motile sperm with surface antibodies is determined, the pattern of binding is noted, and the Ig class of these antibodies can be identified using different sets of immunobeads (IBs). A test mixture in this direct test is scored positive if at least 20% of the sperm cells show IB binding. (**a**) A negative control. (**b**) An infertile man with ASA in sperm. The IBs are attached to the head and/or tail of the motile sperm

of the motile sperm have antibodies bound to sperm, a cutoff level of 50% was adopted in the laboratory manual published by the WHO [1]. On the contrary, Koriyama et al. [7] strongly suggested that the direct IBT is a screening test, and the value of 20% initially suggested by Bronson et al. [4] seems to be more appropriate than that of 50% in the criteria defined by the WHO [1]. The subjects they selected were 26 direct IBT-positive and 140 direct IBT-negative men. The results of post-coital tests (PCTs) for each subject were examined. A significant difference was observed in abnormal PCTs between values $<20\%$ and those $\geq 20\%$ ($P = 0.02$). However, there was no significant difference in abnormal PCTs between values $<50\%$ and those $\geq 50\%$ ($P = 0.084$). They also found that a cutoff value of

20% was correlated with the possibility of conception by the treatment with intrauterine insemination (IUI).

As for the incidence of ASA detected by the direct IBT, we have reported 18 (3.54%) out of 509 infertile men had at least 1 Ig class of ASA detected by IBT with the cutoff value of 20% [8]. Clarke et al. reported that the incidence of ASA detected by IgG and/or IgA class of IBT was 7.8% in 813 infertile men [9].

8.2.2 Mixed Antiglobulin Reaction (MAR) Test

In 1978, Jager et al. reported a simple and rapid test, named the mixed antiglobulin reaction (MAR) test, for the detection of ASA of the IgG class on freely swimming sperm in fresh human semen [10]. The test is based on a modification of the famed Coombs test [11]. Motile mixed agglutinates between erythrocytes sensitized with incomplete anti-Rh antibodies and freely swimming sperm with surface ASA are formed after mixing both cell types together with anti-IgG antiserum (Fig. 8.3). Agglutination of the red blood cells serves as an internal control.

Said et al. [12] described the simple initial version of the assay entailed mixing of three ingredients as single drop and covering them with a cover slip. The semen sample is mixed with a suspension of Group O, Rh-positive, human red cells of R_1 R_2 type, sensitized with human IgG in addition to rabbit or goat, undiluted, monospecific anti-IgG antiserum. The reaction is then observed after 10 min of incubation. Since the red cells are coated with IgG as well as the sperm cells if they have antibodies on them, the added anti-IgG antiserum will then link together the two kinds of cells. Agglutination can be seen under a light microscope as mixed clumps

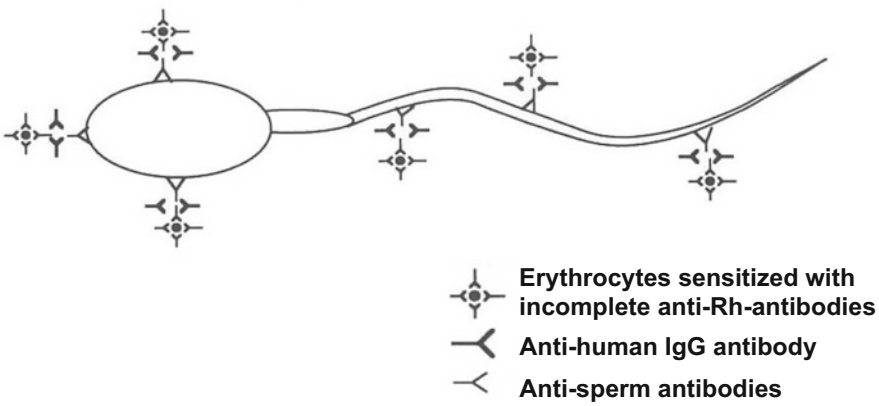

Fig. 8.3 Mixed antiglobulin reaction (MAR) test. The formation of motile mixed agglutinates between erythrocytes sensitized with incomplete anti-Rh antibodies and freely swimming sperm with surface ASA, after mixing both cell types together with anti-IgG antiserum. Agglutination of the red blood cells serves as an internal control

of sperm and red blood cells with a slow "shaky" movement. Results of the MAR test are indicated as percentages of motile sperm incorporated into the mixed agglutinates. The site of attachment could be also noted. No interpretation of the test was given unless agglutination of red blood cells and the presence of sufficient motile spermatozoa are observed. A MAR test was considered positive and of clinical significance when >50% agglutination is seen [13].

The test can be applied on ejaculates with sperm concentrations down to one million per ml, provided the motility is sufficient. The percentage of motile sperm found to be coated with ASA of the IgG class, and the extent of the coating, proved to be correlated with the agglutination titer of circulating ASA and with the inhibition of sperm penetration into cervical mucus. The test can be used as a screening for the presence of anti-sperm autoantibodies in serum and semen.

The MAR test correlates with most other ASA tests, such as SIT and IBT [14]. Although MAR test is considered an ideal method for screening of ASA, it is not without limitations [15]. The assay cannot be used in patients with oligozoospermia, asthenozoospermia, and azoospermia. Also it must be performed on a fresh sample and can be difficult to quantitate due to the presence of debris, semen viscosity, mucus, and microbial factors. Commercially available SpermMAR kit uses an antiserum against human IgG to induce mixed agglutination between antibody-coated latex beads conjugated with human IgG [16]. The SpermMAR kit can be considered a superior alternative to erythrocyte MAR since it is time- and cost-effective. One formulation of the kit contemplates the assessment of IgA as well as IgG classes. The assay can be successfully used for the evaluation of male partners of infertile couples if included routinely in semen analysis [17]. An indirect MAR using serum or seminal plasma samples and donor sperm can be considered in cases with azoospermia. However, it has been reported as difficult to interpret. Therefore, ASA should be rather detected using another approach in these cases.

8.2.3 Panning Test

In 1984, Hancock RJ et al. [18] described the use of "panning" procedures as shown in Fig. 8.4 to detect immunoglobulin on the sperm surface. Wells in leukocyte migration trays were coated with anti-immunoglobulin by leaving the appropriate antibody diluted in phosphate-buffered saline (PBS) in the wells at room temperature for 1–2 h and then washing the trays five times in PBS. Sperm were centrifuged at 550 × *g* for 5 min, resuspended in ASA diluted in PBS plus 5% fetal calf serum (FCS) or control media, and incubated for 1 h at 37 °C. Sperm were then diluted in PBS and centrifuged, and the cells were resuspended in PBS plus 5% FCS to a concentration of approximately five million/mL. Following this, 0.25 mL volumes were incubated in the antibody-coated wells for 1 h at room temperature. The contents of the wells were then shaken out and the wells gently washed by rocking trays in a bath of PBS. The degree to which sperm remained attached to the wells was determined by microscopy using an inverted microscope.

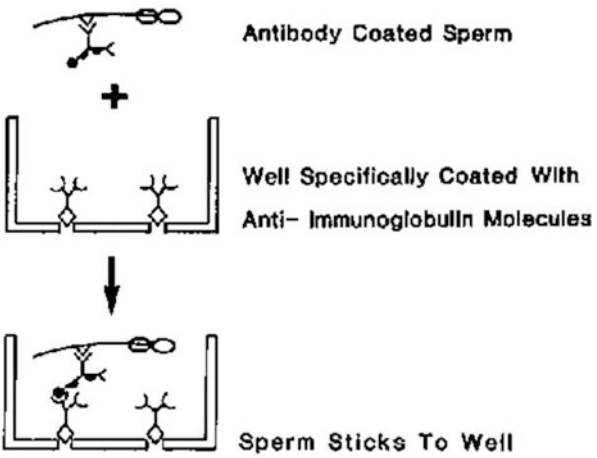

Fig. 8.4 Panning test. Diagram summarizes rationale of the panning test. Sperm with ASA at surface will bind to anti-immunoglobulin-coated wells and remain attached when the wells are washed, while sperm without ASA will not

Later, Hancock RJ [19] developed monospecific and cross-reactive human monoclonal antibodies to HLA antigens. He used the panning assays, and the results correlated with those of agglutination assays, but not immunofluorescence or enzyme-linked immunosorbent assays (ELISA). He concluded that panning assays can be used for the detection of antibodies to sperm and can be useful where target sperm are of poor motility. The assays may be more sensitive for antibodies to superficial antigens.

8.2.4 Direct Sperm-Immobilization Test (Direct SIT)

As there is a diversity of ASA themselves, their biological activities are varied. We developed a direct sperm-immobilization test (SIT) to detect sperm-immobilizing antibodies on the sperm surface [20] and demonstrated that sperm-immobilizing antibody bound to the sperm surface is one of the causes of asthenozoospermia [21].

For the direct SIT, 12 μL of a suspension of the patient's sperm (40×10^6 sperm/mL) and 1 μL of active or heat-inactivated guinea pig serum (Sigma, Tokyo, Japan) as complement source are mixed in a Terasaki plate (Greiner, Frickenhausen, Germany) and incubated at 32 °C for 60 min. Heat-inactivated guinea pig serum is used as a control. The sperm-immobilization value (SIV) is calculated by dividing the sperm motility in the control containing heat-inactivated complement by that containing active complement. The SIV of two or more is considered positive. In our study, 9 out of 13 males with ASA tested positive in D-SIT, giving a positive rate of 69% [8]. All males with sperm-immobilizing antibodies were diagnosed as having asthenozoospermia by computer-aided sperm analysis (CASA) (Table 8.1).

Table 8.1 Direct tests for the detection of anti-sperm antibodies (ASA) bound to sperm surface in infertile men

1.	Immunobead test (IBT)
2.	Mixed antiglobulin reaction (MAR) test
3.	Panning test
4.	Direct sperm-immobilization test (direct SIT)

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