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MICROTECHNIQUE FOR SIMULTANEOUS DETERMINATION OF IMMOBILIZING AND CYTOTOXIC SPERM ANTIBODIES

METHODOLOGICAL AND CLINICAL STUDIES

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SUMMARY

A microtechnique for simultaneous detection of immobilizing and cytotoxic sperm antibodies was elaborated according to the principles of Terasaki and McClelland. The technique was found reproducible, easy to perform and thus suitable for large-scale examinations. The test includes determination of titres for immobilizing and cytotoxic effects and provides at the same time an opportunity to observe both complement-dependent and independent activities. Furthermore, agglutination patterns can easily be recorded. The requirement of only small volumes of test samples makes the test suitable also in testing secretions from the genital tract. In these cases, inclusion of a control for anticomplementary activity of the sample is advisable.

In serum from 288 men from infertile couples simultaneous occurrence of immobilizing and cytotoxic antibodies was observed in eight cases; all of them also had sperm-agglutinins in serum. In two of these cases, immobilizing and, in one case, cytotoxic activity was revealed in seminal plasma using undiluted samples. Patients with negative findings in serum also revealed negative findings in seminal plasma.

Immobilizing and cytotoxic antibodies were not disclosed in serum from 247 women from infertile couples.

INTRODUCTION

The reaction between surface antigens in spermatozoa and antibodies may in the presence of complement cause immobilization of the spermatozoa and classical cytotoxic effects as observed by the penetration of certain dyes into the cells. Special methods have been designed to study each of these effects (Isojima, Li & Ashitaka, 1968; Hamerlynck & Rümke, 1968), but these methods are rather time-consuming, and neither of them allows of simultaneous observation of both complement-dependent effects on the spermatozoa. Furthermore, these

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techniques require relatively large volumes of test material, making them unsuitable for the study of genital tract secretions.

The aim of this study has therefore been to develop a microtechnique which makes it possible to observe both immobilization and cytotoxicity at the same time, and which requires only small volumes of test material, so that these effects can be studied in secretions from the genital tract. In addition, we preferred to determine a titre for immobilization and cytotoxicity instead of determining the extent of these processes with undiluted test material. Consequently, the technique was designed according to the principles used for tissue typing of lymphocytes in microchambers (Terasaki & McClelland, 1964; Engelfriet & Britten, 1965; Kissmeyer-Nielsen & Kjerbye, 1967).

The technique was used to study the occurrence of sperm-immobilizing and cytotoxic antibodies in sera from women and men from infertile couples, and, as regards the men, also in seminal plasma. The occurrence of these antibodies was compared with that of sperm agglutinins.

MATERIALS AND METHODS

Two unselected groups of 247 women and 288 men from couples with infertility problems for more than 2 years were included in the study.

Venous blood was drawn from the patients at the time of the clinical examination and serum was stored at −20°C until tested. Ejaculates from the men were examined as previously described (Husted, 1975), and after separation by centrifugation the seminal plasma samples were stored at −20°C until tested.

Sperm-agglutination test. The gelatin-agglutination test of Kibrick, Belding, & Merrill (1952) was carried out in a slightly modified form (Husted & Hjort, 1974). All sera and semen samples were screened in dilutions of 1:4 and 1:16, and sera with positive or doubtful reactions were retested in four-fold titrations with spermatozoa from two donors.

Microtechnique for detection of immobilizing and cytotoxic antibodies. Serum and seminal plasma. Dilutions of inactivated (30 min at 56°C) patient's serum or seminal plasma were prepared in isotonic saline. Screening was done with an undiluted test sample and dilutions of 1:3 and 1:9. Known positive and negative sera were included in each batch.

Preparation of sperm suspension. A fresh donor sperm sample of optimal quality was used, i.e. sperm count about 10×10^7 per millilitre, number of immotile spermatozoa less than 25%, morphologically abnormal forms based on the standards of MacLeod (1964) less than 30%, sperm motility characterized as 'very good'. The ejaculate should be of normal viscosity and with a very low content of leucocytes and other cellular components. The serum and seminal plasma from the donor should not contain sperm antibodies, and no signs of sperm agglutination should be seen in the ejaculate.

The fresh ejaculate was allowed to stand untouched for about 15 min at 37°C, and from the top layer, containing the majority of motile spermatozoa, portions of about 0·5 ml were transferred to each of two narrow test tubes and centrifuged at 800–1000 rev/min for 5 min. The supernatants containing seminal plasma were discarded, and the spermatozoa in one tube were resuspended in human AB Rh D negative serum-containing complement, and the spermatozoa in the other tube were similarly resuspended in the same serum, which had previously been inactivated. The suspensions were adjusted to 4×10^7 per millilitre and kept at 37°C until use, usually within half an hour. The human complement-containing serum was negative in the Kibrick test and in the slide-agglutination test. It was stored in small samples at −70°C until use.

Test procedure. Microtrays containing eighteen microchambers—six rows with three chambers in each—were used (Møller Coates A/S, Moss, Norway). The microtrays were filled with liquid paraffin. Dilutions of serum or seminal plasma, sperm suspension and Trypan Blue, respectively, were transferred to and mixed in the microchambers under the liquid paraffin by means of Hamilton microsyringes (Hamilton Microsyringe Co., Anaheim, California.

For screening, 1 μl of each of the three dilutions of a sample was deposited in two chambers, each serum thus occupying two rows with three chambers. An amount of 2 μl of the sperm suspension in human comple-

ment was added to the three dilutions in the first row, and 2 μl of the suspension in inactivated serum was similarly added to the dilutions in the second row. The mixtures were then incubated at 37°C for 2 hr.

For the demonstration of cytotoxic effect 1 μl of isotonic 0·8% Trypan Blue was added to the microchambers giving a concentration of 0·2% in the final reaction mixture. A coverslip (28 × 53 mm) was immersed into the paraffin in order to flatten the drops so that they finally filled out the microchambers. Incubation at 37°C for 1 hr followed in order to allow staining of the dead cells.

The reactions were read under an inverted microscope in phase contrast or bright field with a magnification × 320. The percentages of both immotile and stained spermatozoa in each microchamber were estimated and scored as follows. If less than 25% of the spermatozoa seemed immotile or stained, 0; if 25–50% of the cells were affected, +; 50–75%, ++; and more than 75%, +++. Differences between scores in complement-containing mixtures and mixtures with inactivated AB Rh D negative serum indicate complement-dependent effects. Only differences of at least two steps (e.g. a ++ reaction *vs* a 0 reaction) were considered of significance. Positive sera were retested in three-fold serial dilutions starting with undiluted serum, and the results for immobilizing and cytotoxic effect were expressed in titres representing the reciprocal of the highest dilution in which a two-step score difference was found.

RESULTS

(*I*) *Methodological experiments*

(*A*) *Testing of serum.* (*i*) *Antigen.* The spermatozoa were separated from the seminal plasma by centrifugation and resuspended in human AB Rh D negative serum (the source of complement) in order to reduce the final reaction volume and to avoid effects from components in seminal plasma. Washing was omitted, because this often leads to destruction of the surface membranes. Experiments with different concentrations of spermatozoa revealed that addition of a suspension with 4×10^7 spermatozoa per millilitre—giving a final concentration of 20 million per ml after addition of Trypan Blue—was most suitable for the reading of the test.

(*ii*) *Complement.* Guinea-pig and rabbit serum have in several cases been shown to exert agglutinating, immobilizing and cytotoxic effects on heterologous spermatozoa (Mancini *et al.*, 1969). The use of commercially available guinea-pig complement (Institute Pasteur) incubated with human spermatozoa at 37°C caused almost immediately complete immobilization. This effect was primarily ascribed to certain additives, as guinea-pig complement, dialysed against barbital buffer, exerted only a slight immobilizing effect during the first 4 hr of incubation. However, dialysis caused a decrease in complement activity, which rendered it less suitable for the test. On the other hand, spermatozoa suspended in normal human serum had almost unchanged motility after 8 hr, and even after 24 hr 50% of the spermatozoa were still motile. Therefore, we found human serum the most suitable source of complement for the test.

Activity of the human complement was determined in the microsystem by testing known positive and negative sera with increasing dilutions of the complement, so that a titre was achieved. These tests showed that with the batch of serum used throughout these experiments maximal reaction was still obtained with a dilution of 1:4 in the test mixture. As the usual dilution in the test mixture was 2:3 (2 volumes C′ to 1 volume of test serum), this means that a surplus of nearly three times as much complement as necessary for maximal immobilizing and cytotoxic effect was present.

At the same time the titre of the complement giving 100% haemolysis was determined in a haemolytic system with a 3% suspension of sensitized sheep red cells. This titration was

repeated at regular intervals, offering an easy and sensitive way of checking the complement activity. The titre in this system did not decrease during storage of serum for several months.

(*iii*) *Reaction period.* The Trypan Blue solution inhibited the complement activity very effectively. This was seen in the haemolytic system with sensitized sheep red cells, in which the Trypan Blue solution was first tested for anticomplementary activity, and it was confirmed in the microsystem, in which immobilization and cytotoxicity failed to develop further after addition of the dye solution. Trypan Blue could thus be used to block further complement-dependent reactions after different periods of incubation, thereby making it possible to observe how the reactions proceeded.

Fig. 1 shows the course of the reactions with a strong and a weak antiserum, both used undiluted. Trypan Blue was added to various test mixtures at 15-min intervals, and the reactions were then read after incubation with the dye for 1 hr. It appears that the reactions

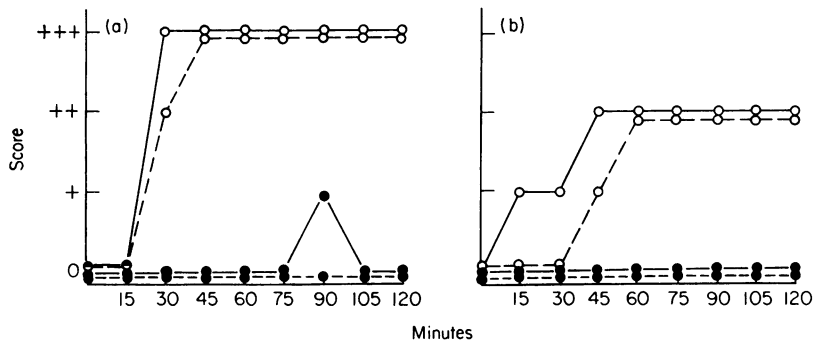

FIG. 1. Time course of immobilization and cytotoxicity. Reactions stopped at 15-min intervals by addition of Trypan Blue. (a) Strong antiserum tested undiluted (titre 9). (b) Weak antiserum tested undiluted (titre 1). (——) Immobilization; (– – –) cytotoxicity; (○) complement present; (●) complement absent.

occurred within the first hour, and an incubation period of 2 hr should therefore ensure that all antibody activities have been displayed. The curves further indicate that immobilization appeared shortly before the cells became stainable with Trypan Blue. Even after 2 hr of incubation, immotile but unstained cells could often be seen, whereas motile stained cells were never observed. This means that the scores for immobilization would sometimes be higher than those for cytotoxicity, whereas the opposite was never the case.

Sera with immobilizing and cytotoxic effects simultaneously caused agglutination. Usually, agglutinates were most prominent in the control row with inactivated complement.

(*iv*) *Staining of the dead spermatozoa.* The final concentration of 0·2% Trypan Blue in the test mixture revealed an optimal contrast between stained and unstained cells and yet a not too dark background. The staining did not occur immediately. At first the blue colour was visible only in the posterior part of the spermatozoal heads, but after 1 hr at 37°C the heads of most of the affected cells had become completely stained, which made the distinction between stained and unstained heads easy (Fig. 2). However, a small number of spermatozoa remained only partially stained, and extension of the staining period beyond 1 hr did not reduce this phenomenon significantly.

(*B*) *Testing of seminal plasma.* Inactivated seminal plasma could be tested in the microtechnique under the same conditions as serum. Experiments in which a known positive

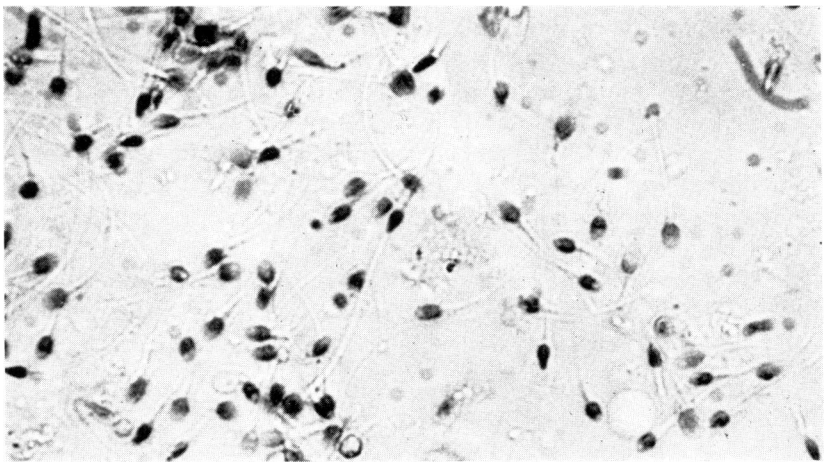

FIG. 2. A +++ reaction for immobilization and cytotoxicity. All sperm heads appear coloured, some uniformly, some mainly in the posterior part of the head. (Due to the high degree of motility of the spermatozoa in negative reactions, proper illustrations of these could not be obtained). (Magnification ×950.)

serum was diluted with inactivated normal seminal plasma and then tested showed that the complement-dependent antibody activities could be displayed just as well and with the same titres in the dilutions with seminal plasma as in control dilutions in saline.

To evaluate the anticomplementary effect of seminal plasma, the human AB Rh D negative serum used as the complement source was titrated in the presence of dilutions of inactivated seminal plasma, and after incubation for 30 min a haemolytic system consisting of 3% sensitized sheep red cells was used to determine the complement activity in the mixtures of serum and seminal plasma. In various samples of seminal plasma from healthy donors a slight anticomplementary effect was observed. A typical titration experiment is shown in Table 1.

TABLE 1. Anticomplementary effect of seminal plasma

Dilution of C′	Dilution of seminal plasma						Control PBS
	Undil.	1:1·5	1:3	1:6	1:12	1:24	
Undil.	3	3	3	3	3	3	3
1:2	3	3	3	3	3	3	3
1:4	3	3	3	3	3	3	3
1:8	1	1	2	2	2	3	3
1:16	0	0	0	0	2	2	2
1:32	0	0	0	0	0	0	0

3 = Complete haemolysis; 0 = no haemolysis.

The anticomplementary effect of seminal plasma—although weak—made it necessary to include extra controls in the testing of seminal plasma to make sure that a surplus of complement was actually present in the test mixtures. For this purpose two rows of the complement-containing mixtures were set up, and to one of them 1 μl of a 3% suspension of sensitized sheep red cells was added instead of Trypan Blue. Complete haemolysis occurred in all cases, thereby proving that a sufficient surplus of complement was present. The anticomplementary effect of seminal plasma thus seemed more a theoretical than a practical problem.

(II) Application of the test

(i) Occurrence of agglutinating, immobilizing and cytotoxic antibodies in sera from patients from infertile couples. Table 2 shows the antibody findings among the 247 women and 288 men from infertile couples.

TABLE 2. Occurrence of agglutinating, immobilizing and cytotoxic activity in sera from women and men from infertile couples

	Number of cases	Immobilizing antibodies	Cytotoxic antibodies
Women			
Sperm agglutinins present	2	0	0
Sperm agglutinins absent	245	0	0
Total	247	0	0
Men			
Sperm agglutinins present	19	8	8
Sperm agglutinins absent	269	0	0
Total	288	8	8

Only in two cases (0·9%) were agglutinins demonstrated in the female sera and only in low titres (4 and 4). Immobilizing or cytotoxic antibodies were not found at all.

In the male sera sperm agglutinins were present in nineteen cases (6·6%), and in eight of these the sera were found also to exert immobilizing and cytotoxic effects in the presence of complement. None of the sera without sperm agglutinins showed any immobilizing or cytotoxic activity. Complement-independent reactions were not observed.

Table 3 shows a comparison between the findings in the male patients with both agglutinins and immobilizing and cytotoxic antibodies and those with only agglutinins in serum. Immobilization and cytotoxicity occurred in seven of the nine sera with agglutinin titres of $\geqslant 64$, whereas these activities were observed only in one serum with an agglutinin titre lower than 64. The titres for immobilization and cytotoxicity were identical in the six sera, but in two cases the titre for immobilization was one step higher than that for cytotoxicity.

The sperm counts in the patients in the two groups did not show any difference.

TABLE 3. Sperm counts in patients with sperm agglutinins in serum, with and without immobilizing and cytotoxic antibodies, respectively

	Sperm agglutinins		Titre for immobilization				Titre for cytotoxicity				Sperm count (millions per ml)		
	Titre < 64	Titre ⩾64	1	3	9	27	1	3	9	27	5<	5–20	>20
Agglutinating sera with C′-dependent activity	1	7	2	4	2	0	3	4	1	0	2	1	5
Agglutinating sera without C′-dependent activity	9	2			0				0		4	2	5

(ii) Occurrence of agglutinating, immobilizing and cytotoxic antibodies in seminal plasma from men from infertile couples. Testing of seminal plasma from 117 men without sperm agglutinins in serum revealed entirely negative results, both in the agglutination test and in the microtechnique for immobilization and cytotoxicity.

In contrast, sperm agglutinins were found in seminal plasma in twelve of the nineteen patients with agglutinins in serum. The agglutinin titres in seminal plasma were to some degree correlated with the findings in serum. Thus eight of the nine men with titres in serum of 64 or more had agglutinating activity in seminal plasma, whereas this was the case with only four of the ten men with lower titres in serum.

Two cases showed identical agglutinin titres in serum and seminal plasma. In the remaining ten cases, the titres in seminal plasma were from one to three four-fold titre steps lower in seminal plasma than in serum.

Immobilizing effect was observed in two cases with undiluted seminal plasma, but only in one of them was a simultaneous cytotoxic effect observed. The agglutinin titres in these two patients were 1024 and 256, respectively, in serum and 256 in seminal plasma.

These results lead to the conclusion that although immobilizing and cytotoxic antibodies may be found in seminal plasma, it seems to be a very rare phenomenon.

DISCUSSION

The microtechnique used for demonstration of sperm-immobilizing and cytotoxic activity in human serum and seminal plasma is easy to perform and read, thus being suitable for large-scale examinations. The reading was not done by exact counting, but the reactions were scored into one of four classes—each with a range of 25%—and in each case compared with the result in an inactivated but otherwise identical test mixture. The results were given as titres, the titre being the highest dilution at which a score difference of at least two steps was recorded between the complement-containing test mixture and the inactivated control mixture (i.e. a difference in the percentages of affected spermatozoa greater than 25%). If desired, exact counting could be done without modification of the test.

The scoring system and the definition of a positive reaction demand the use of optimal sperm samples, in particular as regards motility and percentage of immotile spermatozoa. It is of advantage to use the same selected donors for large test series. Known positive and negative control sera tested in each batch should guarantee a constant sensitivity in the system. Occurrence of complement-independent immobilization—if such exists—would easily be detected by comparison with the reaction obtained with the negative control serum.

The use of human complement in the test system was preferred, principally because the test should give the most realistic characterization of the possible effects of the sperm antibodies in the human organism, but also because heterologous complement often exerts a toxic action on human spermatozoa. Even with complement from carefully selected guinea-pigs, it was difficult to avoid a slight immobilizing effect on the spermatozoa, so that the score in the negative control sera increased from 0 to +.

In order to ensure that optimal conditions exist in the system the complement-containing serum should be titrated in the microsystem, particularly when a new portion of serum is taken into use. Titration in the system—or more easily in the haemolytic system—should be done at regular intervals to check that the serum stored at −70°C still has the same complement activity.

The microtechnique was found suitable for the testing of seminal plasma, and it can probably also be used for testing of other secretions of the genital tract. In order to ensure that such specimens do not have an anticomplementary effect it is advisable to set up, parallel with the test, a third row with test mixtures of spermatozoa, test sample and complement to which sheep red cells are added instead of Trypan Blue. The occurrence of complete haemolysis ensures complement surplus in the mixture.

The complement-free control mixture is nearly the same as used in slide-agglutination tests (Wilson, 1954; Friberg, 1974), the main difference being that we prepared the sperm suspension in inactivated AB Rh D negative serum instead of in buffer. Consequently, it is not surprising that agglutination patterns—including the Franklin–Dukes phenomenon (Franklin & Dukes, 1964) with small head-to-head agglutinates—could also be studied. Agglutination was also seen in the complement-containing test mixtures, but in positive tests usually not to the same degree as in the control mixtures. This could possibly be explained by the rapid immobilization of the spermatozoa, which would decrease the tendency to form agglutinates.

Immobilizing and cytotoxic effects were not observed in sera from women, and only in two cases did a weak reaction in the Kibrick test occur. This is in contrast to the findings of Isojima *et al.* (1972), who found immobilizing antibodies in 17·2% of women with unexplained infertility. This discrepancy may be explained by a greater sensitivity in the immobilization test used by Isojima *et al.*, but it should be realized that the two series of patients are not comparable because our study includes unselected women from infertile couples.

Even if the titre of immobilization was in some cases higher than that of cytotoxicity the two phenomena usually occurred together, and in no case was an isolated strong reaction of either immobilization or cytotoxicity observed. This would suggest that these phenomena are the effect of only one antibody which at the same time causes agglutination. The titres of immobilization and cytotoxicity were low compared with the titre of agglutinating antibodies. The studies of Rümke & Hellinga (1959), Fjällbrant (1969), Friberg (1974) and

Husted & Hjort (1975) have shown that agglutinins with a high titre in serum from men were mostly IgG antibodies. In order to activate complement in a type-II hypersensibility reaction at least two IgG antibody molecules should be bound very near to each other on the antigen, and this would mainly be possible in the lower dilutions of serum.

In seminal plasma which had sperm-agglutinating activity, sperm immobilization occurred in two cases and cytotoxicity in one. As sperm agglutinins in seminal plasma are mostly IgA, and the IgG concentration is only about 1% of that in serum, the reaction may have been caused by activation of complement via the alternate pathway by aggregated IgA molecules, but the possibility that IgG antibodies were also present cannot be ruled out.

As far as *in vivo* reactions are concerned, the anticomplementary effect of seminal plasma indicates that immobilization and cytotoxicity can hardly be displayed in the ejaculate. This is confirmed by the observation that spermatozoa in seminal plasma containing immobilizing and cytotoxic antibodies are not rapidly immobilized. Attention should therefore be directed to the fate of the antibody-coated spermatozoa in the female organism.

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