ANTIBODIES TO SPERMATOZOA II. SPERMAGGLUTINATION TECHNIQUES FOR GUINEA-PIG AND HUMAN CELLS

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Summary. An agglutination procedure, useful for guinea-pig and human spermatozoa, has been developed. The preferred conditions are as follows: epididymal spermatozoa of guinea-pig in an immotile condition, either unwashed or diluted and centrifuged once, and at a concentration of $10 \times 10^6/\text{ml}$, are incubated with the antiserum samples at 4°C for 1 or for 2 hr of observation. Human spermatozoa (ejaculated), washed well and immotile, at a concentration of $40 \times 10^6/\text{ml}$, are incubated with the antiserum samples at room temperature (22° to 25°C) for 1 or for 2 hr of observation. The mixtures are observed in capillary tubes of about 1 to 1.5 mm in diameter and 75 to 100 mm in length. This procedure has readily allowed us to detect the presence and titre of antibodies against spermatozoa. The test has given results that are in general parallel to those obtained by the conventional Kibrick gelatin method.

INTRODUCTION

The evaluation of the presence and titre of antibodies to spermatozoa can be accomplished by means of spermagglutination, by several alternative procedures. Two general ways of performing and evaluating the agglutination are either by means of direct observation under the microscope, or by examination of a suspension in a test-tube. The microscope procedure is cumbersome and difficult to quantify; the test-tube method has been most commonly used according to Kibrick, Belding & Merrill (1952b). Both of these methods suffer from the obligatory requirement that the sperm cells retain their motility. While both these approaches will continue to be useful, it was felt that a new procedure might be developed that would be both simple and convenient, and yet be applicable to the use of immotile spermatozoa, thus making available a much greater supply of antigen. The work to be described has been done largely with guinea-pig spermatozoa, although a number of findings can also be reported for human spermatozoa.

The new method can be termed the capillary method. It involves the formation of visible agglutinates inside capillary tubing, under experimental conditions wherein sperm suspensions remain quite stable when suspended in

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normal serum. A preliminary description has appeared (Shulman & Hekman, 1971).

MATERIALS AND METHODS

Collection of spermatozoa

Guinea-pig spermatozoa were obtained in two forms. Most of the work was done with epididymal spermatozoa, although some studies were also made with ejaculated spermatozoa. For the purpose of obtaining epididymal spermatozoa, the guinea-pig was anaesthetized with ether and killed by exsanguination. The testis was removed along with the epididymis and part of the vas deferens. The epididymis was carefully separated from other structures and placed in a small Petri dish. To this tissue was added 1.0 to 2.0 ml of phosphate-buffered saline (0.12 M-NaCl+0.01 M-phosphates, pH 7.2) or plain saline (0.15 M-NaCl), and the tissue was minced gently with scissors, releasing spermatozoa into the surrounding fluid. In an alternative procedure, the spermatozoa were flushed out by forcing the saline through the epididymal channels. This was done by inserting a syringe with a 25- or 26-gauge needle into the vas deferens portion. The spermatozoa could then flow out through a small cut that had been made in the distal end of the epididymis. Ejaculated semen was obtained by electroejaculation, according to the method of Freund (1969), using an output of 40 volts, in an on-off sequence. Other details have been described (Shulman & Hekman, 1971). Human (ejaculated) spermatozoa were obtained as semen samples by masturbation from apparently healthy donors.

The concentration of each sperm suspension was measured by means of counting in a haemocytometer.

Preparation of antisera

Epididymal guinea-pig spermatozoa were washed several times with phosphate-buffered saline, and were then used as immunizing antigen. Several New Zealand White male rabbits and several female virgin guinea-pigs were used as experimental animals. In each case, a volume of 0.5 ml of the sperm suspension, generally containing 30 to 40×10^6 cells, was emulsified with an equal volume of complete Freund's adjuvant. This preparation was injected into a number of intradermal sites in the shaved back of the animal. Injections were given at intervals of 3 weeks, and bleedings were taken either every week for the rabbits or every 3 weeks for the guinea-pigs, for testing purposes. The last injection given to the guinea-pigs contained 100×10^6 cells. Final bleedings were obtained in all cases by exsanguination.

Several New Zealand White male rabbits were used for the production of antibodies to human spermatozoa. They were injected five times, at intervals of 2 weeks, using about 1×10^7 washed spermatozoa in complete Freund's adjuvant each time, and were bled out 1 week after the last injection.

Spermagglutination procedures

The gelatin agglutination procedure of Kibrick *et al.* (1952b) and the capillary procedure were used. For the Kibrick procedure, fresh unwashed

spermatozoa were used, which had retained their motility. The sperm cell suspension was adjusted to a concentration of 10×10^6 sperm cells/ml with saline for guinea-pig, and to 40×10^6 /ml with Baker's buffer (Kibrick *et al.*, 1952b) for human samples. This suspension was mixed at 37° C with an equal volume of 10 % gelatin in the corresponding solvent to provide the test antigen. This test antigen was then mixed with an equal volume of the antiserum or an appropriate dilution of the serum; generally, four drops of each were used. Along with the serum samples being evaluated, a known negative and a known positive serum were included in each test. The samples were mixed gently and transferred with a Pasteur pipette to a small tube of 3.0 mm inner diameter, taking care to avoid the trapping of air bubbles. The tubes were incubated at 37° C, and they were examined for agglutination after 1 hr. They were again observed after 2 hr for the purpose of obtaining clear differentiation of any earlier doubtful results.

The capillary method will be described in detail below.

Gel diffusion procedures

Gel diffusion was carried out according to the principles described in our previous publications (Shulman & Bronson, 1969; Shulman, 1971b), except that microscope slides were used, rather than dishes or plates. A 1 % solution of Noble agar (Difco labs) in saline was utilized. Sodium azide at 0.01 % was used as a preservative. Usually, 2 ml of melted agar was poured on to the slide. Reagent wells were then cut through the agar in a hexagonal pattern of six wells surrounding a central well. The wells were usually 3 mm in diameter and separated by 5 mm edge-to-edge, as produced by an LKB gel-cutter punch. In most experiments, an antiserum was placed in the central wells and the peripheral wells contained preparations of epididymal spermatozoa or related materials.

RESULTS

The new technique that has been developed for agglutination testing was stimulated by previous studies that dealt with the evaluation of specific precipitation of antibody-containing serum such as that from patients with myasthenia gravis with extracts of muscle (Lang, Shulman, Beutner & Witebsky, 1966; Shulman, Lang, Beutner & Witebsky, 1966). It had been necessary to obtain conditions under which the antigen extract would remain clear and unprecipitated for a minimal time, and during which the mixture with antiserum would show definitive positive reaction. It was eventually found that, in the geometry provided by capillary tubing and using a proper temperature, these requirements could well be satisfied. For the current studies of agglutination therefore, glass capillaries were employed which had dimensions of the order of 1 or 2 mm in diameter. Two specific kinds of tubes were used: the Kimax capillary melting point tubes, with diameter of 1.5 to 2.0 mm and length of 100 mm, and the Blu-tip capillary tubes (1.1 to 1.2 mm and 7.5 mm). The Blu-tip kind of tubes were eventually preferred, because they were shorter and easier to fill completely, eliminating any problem of air bubbles. Equal volumes of the sperm suspension and of a suitable dilution of the appropriate antiserum

were mixed in a small tube, and this mixture was aspirated into the capillary. Generally, volumes of one or two drops from a Pasteur pipette were used. Similar mixtures were always prepared with a negative control serum in place of the antiserum. Each capillary was quickly sealed at the lower end with a small amount of soft putty. The capillaries were arranged to stand up in rows on a putty plate. The assembled arrangement (with empty tubes) is shown in Pl. 1, Fig. 1.

Negative results are indicated by the maintenance of a homogeneous turbid appearance throughout the liquid while positive results are shown by the development of discrete opaque clumps, separated by clear regions of intervening liquid. Occasionally, in the less diluted serum samples, these reactions involve a fairly rapid settling out of the turbid material, rather than an aggregation into clumped regions. Weaker positive results can be distinguished as mixtures with tiny clumps, between which there is not a completely clear liquid. Several capillary tubes, containing diverse results are shown in Pl. 1, Fig. 2.

Effects of experimental conditions: guinea-pig spermatozoa

Most of these studies have been conducted with epididymal spermatozoa of the guinea-pig, that is, the same type of material which had been used for the stimulation of antibody production in the rabbits and guinea-pigs. A number of parameters have been explored, including the effects of motility of the spermatozoa, washing of the spermatozoa, concentration of the cell suspension, time and temperature of incubation, nature of diluent, and titration of the antisera. A few studies were also made with ejaculated spermatozoa.

Sperm motility. It was discovered that immotile spermatozoa, produced by adding an equal volume of 1 % sodium bicarbonate solution to the suspension or by overnight refrigeration, can give positive results. After such treatment, any remaining motility consists of a sluggish motion of the tails, without forward progression of the heads. In fact, cell suspensions that had been refrigerated for as long as 1 week were often still found to be effective in this test, although the titre of the antiserum might be somewhat less in tests with sperm cells that are 3 or more days old. By contrast, the use of fresh motile spermatozoa has invariably led to negative results or to lower titres. For example, two antiserum samples gave titres of 1:128 with immotile spermatozoa, whereas with a motile sample, their titres were 1:32 and 1:16.

Sperm concentration. The test has been attempted at several initial concentrations of the cellular suspension. A concentration of 10×10^6 /ml was found to be optimal, and this level was selected for the standard procedure with guinea-pig spermatozoa.

Effects of washing. It has been found that agglutination can be obtained with unwashed spermatozoa. If necessary, some degree of gentle washing can be attempted, although this may cause an increasing degree of non-specific agglutination.

Nature of diluent. It has been found that the result is the same whether the sperm cells are diluted with saline, phosphate-buffered saline or with Baker's buffered glucose solution (Kibrick, Belding & Merrill, 1952a; Kibrick *et al.*, 1952b). Dilution with saline is now our preferred procedure.

PLATE 1

FIG. 1. Arrangement of a typical set of empty capillary tubes, as displayed on a putty plate for the capillary method of spermagglutination.

FIG. 2. Selected capillary tubes, containing anti-serum-sperm mixtures, after a 2-hr incubation. The four tubes on left show positive results in a dilution series of six steps; the two on right show negative results with negative serum.

(Facing p. 34)

Temperature of incubation. The test mixtures were incubated at room temperature (approximately 20° C) and at 4° C in several preliminary studies. At room temperature, the cells and clumps settled out too rapidly but at 4° C the test works well.

Time of incubation. The capillaries are generally examined after 1 hr, and again after 2 hr, of incubation. The control mixtures are usually found to remain clear for 2 hr, and thus the maximal titre is obtained. If, however, the control mixtures are no longer clear at 2 hr, this observation should be discarded, and the 1-hr reading should be used, assuming there was no ambiguity at this point.

TABLE 1
COMPARISON OF TITRES IN CAPILLARY AGGLUTINATION (WITH IMMOTILE SPERMATOCYTES), AND IN KIBRICK AGGLUTINATION (WITH MOTILE SPERMATOCYTES), USING GUINEA-PIG EPIDIDYMAL SPERMATOCYTES AND RABBIT ANTISERA

Rabbit antiserum	Titre with Kibrick method	Titre with capillary method	Gel diffusion
R43, pre	1 : 4	< 1 : 4	n.d.
a	1 : 32	1 : 8	—
b	1 : 32	1 : 8	n.d.
c	1 : 64	1 : 64	+
d	1 : 64	1 : 64	+
R44, pre	1 : 8	< 1 : 4	n.d.
a	1 : 8	1 : 4	—
b	1 : 256	1 : 16	n.d.
c	1 : 128	1 : 64	+
d	1 : 128	1 : 16	+
R45, pre	1 : 8	< 1 : 4	n.d.
a	1 : 8	1 : 4	—
b	1 : 32	1 : 8	n.d.
c	1 : 128	1 : 128	+
d	1 : 128	1 : 32	+
NRS	1 : 4	< 1 : 4	

pre = preimmunization bleeding. a,b,c,d = successive trial bleedings. NRS = normal rabbit serum. + = precipitation between wells containing sperm suspension and serum; — = no precipitation. n.d. = not done.

Titration results on guinea-pig spermatozoa

It has been found with a group of rabbit antisera that titres as high as 1 : 128 can be obtained. The most concentrated solution of antiserum that has been used has been a 1 : 4 dilution. By contrast, normal sera usually did not show agglutination at dilutions of 1 : 4 or higher, although they often showed agglutination if used undiluted or at a 1 : 2 dilution. If the normal serum gives a titre higher than 1 : 8, the data and sperm sample are discarded.

Antibody levels obtained against spermatozoa in rabbits and guinea-pigs immunized with suspensions of guinea-pig epididymal spermatozoa have also

been evaluated, comparing several different methods. These included the Kibrick procedure and the capillary procedure. The results in general showed that when one of these methods gave a negative result, the other also gave a negative result, and that the titres for positive results were in satisfactory agreement. As another part of this study, gel diffusion precipitation has been attempted in an effort to study the antigens of spermatozoa. As will be described more fully in another report (Hekman & Shulman, 1971), guinea-pig spermatozoa, despite vigorous washing, show the presence of sufficient freely diffusing

TABLE 2

COMPARISON OF TITRES IN CAPILLARY AGGLUTINATION (WITH IMMOTILE SPERMATOZOA), AND IN KIBRICK AGGLUTINATION (WITH MOTILE SPERMATOZOA), USING GUINEA-PIG EPIDIDYMAL SPERMATOZOA AND GUINEA-PIG ANTISERA

<i>Guinea-pig antiserum</i>	<i>Titre with Kibrick method</i>	<i>Titre with capillary method</i>
GP40, pre	<1 : 8	<1 : 8
a	<1 : 8	<1 : 8
b	1 : 32	1 : 16
c	1 : 64	1 : 32
d	1 : 256	1 : 128
e	1 : 128	1 : 64
GP41, pre	<1 : 8	<1 : 8
a	<1 : 8	<1 : 8
b	1 : 16	1 : 8
c	1 : 16	1 : 64
d	1 : 256	1 : 128
e	1 : 256	1 : 128
GP42, pre	1 : 8	<1 : 8
a	1 : 16	<1 : 8
b	1 : 64	1 : 32
c	1 : 128	1 : 64
d	1 : 512	1 : 128
e	1 : 256	1 : 128
NGPS	<1 : 8	<1 : 8

pre = preimmunization bleeding. a,b,c,d,e = successive trial bleedings. NGPS = normal guinea-pig serum.

antigen for lines of precipitation to be formed in gel diffusion reactions with antisera against the spermatozoa. Making use of these reactions as another form of confirmation that antibody activity has been indicated in these sera, it has been possible to show that these precipitation results also parallel the negative or positive results that are obtained for the same antisera in capillary agglutination. Several sets of comparisons of this type are illustrated in Table 1, citing the tests with rabbit antisera. The three guinea-pig antisera were also evaluated by comparison of a series of different bleedings by the two methods of agglutination testing, with the results seen in Table 2. Concordant positive results were clearly seen.

The studies described above have been made with epididymal sperm cells. A few studies were also carried out with ejaculated spermatozoa. After they became immotile on refrigerated storage, specific agglutination could be well achieved with rabbit antiserum, and with similar titres compared to the use of epididymal spermatozoa.

Studies on human spermatozoa

A few studies have been made on human spermatozoa. Rabbit antisera, containing heteroantibodies, were used in these studies, from three animals that had been injected with washed human spermatozoa from normal donors.

The various technical parameters were all re-explored, because the first experiments showed that only very low titres could be obtained if the conditions were identical to those used for guinea-pig spermatozoa. The best results were obtained with spermatozoa that had been washed and resuspended in either saline or Baker's buffer, as contrasted with unwashed preparations. As a result of the washing, the spermatozoa were invariably immotile; however, experiments with both motile and immotile unwashed spermatozoa, showed that the results were independent of motility. Various concentrations of spermatozoa were used and the highest titres were obtained with a level of $40 \times 10^6/\text{ml}$, although 20×10^6 or 80×10^6 also gave similar results. Mixtures were incubated at several temperatures, and room temperature (22° to 25°C) was found to be best. Utilizing the combination of optimal parameters, a rabbit antiserum gave a maximum titre of 1:256. Titres of 1:128 or 1:256 could be repeatedly achieved with a number of different semen specimens. These results may be compared with a titre of 1:512, which was obtained in a separate experiment, using motile spermatozoa in a Kibrick procedure. Further details on this form of the capillary method are being published elsewhere (Shulman & Shulman, 1971).

DISCUSSION

Antibodies against spermatozoa can show a number of properties, and they can be detected by several procedures. These various procedures and properties have been recently reviewed (Shulman, 1971a, 1971b; Shulman & Hekman, 1971). Although such antibodies have been studied since 1899, agglutination methods were rarely used during the early years. The first clearly described microscope method seems to be that of Henle, Henle & Chambers (1938), who termed it the 'slide agglutination reaction'. Essentially similar procedures were used by Snell (1944), who studied mouse spermagglutinins after immunization between inbred strains, and by Flocks, Bandhaur, Patel & Begley (1962) who studied rabbit antisera to human sperm cells. More recently, a rather different procedure was introduced by Franklin & Dukes (1964a, b) and then adopted by Schwimmer, Ustay & Behrman (1967), for study of antibodies in women's sera. In this test, the sperm concentration is quite low, being diluted 1/11 (of $50 \times 10^6/\text{ml}$), and the observations are made between $\frac{1}{2}$ and 4 hr after mixing. Agglutinating activity was thus said to be present in a very high incidence among the infertile women tested, and even in 20% in the control sera. It is

not clear that this test is highly specific for an antibody activity, nor have reasons been given for the choices of experimental conditions. The possibility that the agglutinating activity in some of the serum samples of this type is not in fact due to antibody has been claimed in a study by Boettcher & Kay (1969).

An alternative method has been based on direct (unmagnified) observation of agglutinating mixtures. Early efforts had encountered the difficulty that suspensions of spermatozoa in normal serum or even in saline solution sometimes produced spontaneous agglutination that was difficult to distinguish from specific agglutination. This problem was first considered by Mudd (1927), who proposed that the tubes be centrifuged after incubation and then shaken to resuspend the sediments until an even suspension was reached in the control tubes. Building on these ideas, Kibrick *et al.* (1952a, b) developed successive improvements in procedure, suggesting first the use of narrow bore tubes (5 × 65 mm) in order to permit resuspending by inversion (three times gently) instead of by shaking. They also suggested that semen samples be selected with a high initial sperm count, permitting appreciable dilution to $40 \times 10^6/\text{ml}$, in order to remove much of the seminal plasma. Further, they emphasized that the sperm cells must be motile, or much lower titres will be seen; and finally, incubation should be carried out at 37° C for 2 hr, or less if the controls begin to show granular sedimentation (Kibrick *et al.*, 1952a). Even with these refinements, the test was still not sufficiently sensitive. By means of incorporating some gelatin in the sperm suspension the disruption of weak unions could be sufficiently minimized, while allowing the sperm motility to favour clump formation (Kibrick *et al.*, 1952b).

This gelatin agglutination method has been put to use very extensively by Rümke and co-workers (Rümke, 1954, 1965; Rümke & Hellinga, 1959), who have described and illustrated the method in detail. The procedure has also been used by Flocks *et al.* (1962) and by Fjällbrant (1965). Apparently, the only earlier application of the Kibrick method to guinea-pig spermatozoa was that of Otani & Behrman (1963), who described this method for guinea-pig epididymal spermatozoa and guinea-pig antibodies. They took readings rather quickly, since agglutination occurred in 10 to 20 min in normal guinea-pig serum, for which no explanation was offered. In our studies, the mixtures with normal serum always remained clear for at least 2 hr. Otani & Behrman isolated the sperm suspension by flushing the epididymis though, in our experience, a procedure of this type requires more time for the manipulations, and this delay can lead to a heightened tendency toward spontaneous agglutination. We, therefore, preferred the procedure of mincing the tissue and thus obtaining the cells quickly. We would emphasize the use of minimal time, temperature and manipulation to obtain sperm suspensions which will then remain unclumped for some hours, as we have described (Shulman & Hekman, 1971). Quite recently, Rümke & Titus (1970) described the application of the Kibrick procedure to rat spermatozoa. They emphasized the preferred use of 30° C for the incubation conditions with this species.

It would still be desirable to have an alternative procedure that is based on the use of immotile spermatozoa, and this has been the goal of these present studies on the use of capillary containers. The avoidance of non-specificity of

agglutination in this method is dependent on the dimensions of the tubing and the low temperature conditions of incubation; no addition of gelatin is necessary.

The preferred requirement for the use of immotile spermatozoa in this test is not at all clear; nor is it clear why motile spermatozoa must be used in the Kibrick test. Tyler (1945) and Kibrick *et al.* (1952a, p. 422, p. 431) have made speculations in this regard, but these suggestions may not be fully convincing for the motility can be expected to disrupt weak unions, as well as to help form them. It seems that the best basis for speculating on this problem has been elusive.

In the capillary procedure, nonetheless, this preference for the use of immotile spermatozoa causes no technical problem since a supply of motile spermatozoa can be readily immobilized when desired, as described above. For human spermatozoa, centrifuging causes a decrease in motility so that a washing procedure is necessarily associated with some loss of motility. The capillary procedure can still be used and satisfactory results obtained. The capillary method would therefore seem to have a greater versatility than the other agglutination procedures.

Some studies have also begun on human spermatozoa. Positive results could be obtained under somewhat different conditions than those used with guinea-pig spermatozoa. The most striking point, perhaps, is the much better activity that is obtained by using well-washed spermatozoa. This is presumed to be due to the removal of the large quantity of seminal plasma antigens, some of which may inhibit the reaction with spermatozoa. The guinea-pig epididymal sperm suspensions would not be expected to show a similar reaction. The preferred use of room temperature for incubation was also in contrast to the preferred 4° C condition for the guinea-pig spermatozoa. Although the earlier experiments with human spermatozoa had suggested the need for concentrations considerably above 100×10^6 cells/ml, the changes in other parameters made it possible to get satisfactory results with only 40×10^6 cells/ml. The capillary method under these conditions gave titration results that were within a half or a quarter of the titres in comparable Kibrick tests. It may yet be possible to develop a sensitive procedure for human spermatozoa that can then also be performed by the capillary method.

It was of some interest that antibodies against guinea-pig spermatozoa could be elicited in guinea-pigs. Our attempts to do the same in rabbits had been unsuccessful, although rabbit autoantibodies against seminal plasma antigens could be readily produced (Shulman, Riera & Yantorno, 1968; Shulman, 1969). The production of rabbit antibodies against rabbit spermatozoa has been reported by some workers (Mudd & Mudd, 1929; Henle, 1938) though these activities were not studied by agglutination procedures. Since there are many seminal plasma antigens, as illustrated in man (Herrmann & Schirren, 1961; Shulman & Bronson, 1968, 1969; Hekman & Rümke, 1969; Herrmann, 1969; Shulman, 1969, 1971a, b) and guinea-pig (Maruta & Moyer, 1967), the possibilities of interaction between these substances and the spermatozoa, already partially understood (Shulman, 1971b), should be better clarified in regard to possible effects of spermagglutination testing.

The studies of the production and properties of anti-guinea-pig epididymal

sperm antibodies, as elicited in rabbits and guinea-pigs, is being published elsewhere (Hekman & Shulman, 1971).

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