

Allergic infertility: laboratory techniques to detect antispermatozoal antibodies

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Summary

Twenty sera from fertile and infertile women were examined for antibodies to spermatozoa or to seminal plasma, to determine which of the five techniques tested provided reliable evidence of allergic infertility.

Ten sera agglutinated seminal plasma coated tanned red cells, six agglutinated spermatozoa, ten gave mixed cell antiglobulin agglutination and fourteen gave immunofluorescent reactions for IgG or IgM antispermatozoal antibodies, and three were cytotoxic to sperm.

The results of tests for seminal plasma agglutinins were unrelated to those of any of the other tests, and it is believed that factors other than antibody also clump these antigen-coated cells. There was good agreement in the results obtained by direct sperm agglutination, mixed cell antiglobulin agglutination and strong immunofluorescent staining of the sperm head. The antiglobulin and immunofluorescent techniques also detect nonagglutinating ('incomplete' or 'univalent') antibody.

One of the three sera cytotoxic to sperm had no antibody detected by the other tests. The sperm may have been killed by an inflammatory substance or a systemically absorbed contraceptive. This case illustrates the necessity of combining tests for cytotoxicity with at least one technique for antispermatozoal antibody in order to detect allergic infertility.

Introduction

There is evidence that infertility in some women may be due to allergy. However, techniques to detect anti-spermatozoal antibodies differ considerably in most laboratories. In the agglutination test, for example, the antigen has been diluted whole semen (Schwimmer, Ustay & Behrman, 1967a, b; Isojima, Li & Ashitaka, 1968) or a suspension of washed spermatozoa (Kibrick, Belding & Merrill, 1952; Ansbacher, Manarang-Pangan & Srivannaboorn, 1971). Spermatozoa were living or dead, and the test read macroscopically or microscopically. The results have been interpreted to

mean that anti-spermatozoal agglutinins cause (Franklin & Dukes, 1964a, b; Schwimmer *et al.*, 1967a, b) or do not cause infertility (Phadke & Padukone, 1964).

Several techniques have been used to detect anti-seminal or anti-spermatozoal antibodies in women and men, particularly precipitation in agar gels (Bandhauer, 1966), agglutination of spermatozoa (Kibrick *et al.*, 1952; Rümke, 1959, 1965), the tanned red cell agglutination test (Southam, 1963; Schwimmer *et al.*, 1967a; Isojima *et al.*, 1968), the mixed cell antiglobulin test to detect antigens (Edwards, Ferguson & Coombs, 1964) or antibody (Parish, Carron-Brown & Richards, 1967; Parish & Ward, 1968), immunofluorescence (Feltkamp *et al.*, 1965; Parish & Ward, 1968; Hjort & Hansen, 1971), immobilization tests (Parish *et al.*, 1967; Isojima *et al.*, 1968) and the binding of radio-isotope labelled globulins (Isojima *et al.*, 1968; Noyes, 1969).

Despite the many investigations reported, few attempts were made to compare more than two techniques detecting antispermatozoal antibodies, or to identify the immunoglobulin class of the antibodies. Parish *et al.* (1967) found antispermatozoal antibodies by mixed cell antiglobulin, immunofluorescence and cytotoxic (immobilization) tests. In a more comprehensive investigation, Isojima *et al.* (1968) showed that the tanned cell agglutination test was ten times more sensitive than sperm agglutination or immobilization, and that precipitation in gels was an insensitive method of detecting anti-seminal antibodies: they also used radio-isotopes to detect globulins binding to spermatozoa.

Anti-seminal antibodies have been found in three immunoglobulin classes. Antibodies cytotoxic for spermatozoa were IgG. Antibodies harmless for spermatozoa, combining with seminal plasma antigens adsorbed to the cell surface, were IgM or IgA (Parish & Ward, 1968).

This report compares the results of several techniques used to detect IgG and IgM anti-seminal antibodies in the sera of twenty women, and examines the effects of modifying some technical procedures.

Materials and methods

Sera

The twenty samples tested were taken from 151 samples obtained by Dr A. Ward from fertile and infertile women attending the gynaecology clinics at St Mary's Hospital, Paddington. All 151 samples had been tested for agglutinins to seminal plasma. Ten sera with and ten without agglutinins were selected for further tests.

Precipitation in agar gels

Sera were tested for precipitins against seminal plasma and disrupted spermatozoa in 1.5% agar gels in buffered saline pH 7.0, containing six equidistant wells 5 mm diameter and 7 mm apart round a central well, or 3 mm distant from a trough containing antigen.

Tanned cell agglutination test

Seminal plasma was adsorbed to the surface of human group O red cells by a modification of the method of Boyden (1951). Seminal plasma was obtained from blood group O men, or from nonsecretor group A and B men and demonstrated to contain no A or B antigen by its failure to reduce the titre of anti-A or anti-B red cell agglutinins. The centrifuged cell-free plasma was dialysed against 0.001 EDTA in 0.05 M

Tris buffer in 0.9% NaCl overnight at 4°C to remove the tendency of untreated seminal plasma to clump red cells. It was then diluted 1/10, and stored frozen.

A 4% suspension of washed human group O red cells was treated with an equal volume of a 1/50 dilution of 0.5% tannic acid for 30 min. The cells were washed thrice and resuspended as a 2% suspension, which was treated with an equal volume of the treated, diluted, seminal plasma for 45 min. The cells were again washed thrice and resuspended to 1% in buffered saline pH 7.0, containing 1/50 horse serum, previously inactivated and adsorbed with group A and O human red cells.

The test sera were heated at 56°C for 30 min, and diluted in doubling dilutions in saline containing 1/50 horse serum. An equal 0.1 ml volume of the antigen-coated red cell suspension was added, mixed, and the cells allowed to settle. A rabbit anti-seminal plasma antibody was titrated with each test as a positive control, and tubes of diluent as the negative control.

Direct spermatozoal agglutination

One group O donor was used, whose samples were more free of debris than found in most samples from other donors, and which usually formed a stable suspension of washed spermatozoa.

0.05 ml of an even suspension of washed spermatozoa at 2×10^8 /ml in phosphate buffered saline pH 7.0 containing 0.5% gelatin and 0.01% azide was added to 0.5 ml of test serum, undiluted or diluted 1/5, 1/10, 1/20 and 1/40 in saline. Tubes containing saline instead of serum showed that the sperm suspension did not clump nonspecifically. The tubes were incubated at 37°C, and samples removed at 30 min, 1 and 2 hr, mounted on slides and immediately examined microscopically.

Variations of the technique are described in the Results.

Mixed-cell antiglobulin agglutination

This technique is described in detail by Chalmers *et al.* (1959) and was used to detect antibodies bound to spermatozoa by Edwards *et al.* (1964) though in our tests the sensitized indicator red cells were added without washing away the excess antiglobulin serum.

Spermatozoa were examined for IgG and IgM antibodies on their surface. The rabbit anti-human IgG and anti-IgM were monospecific (Parish, 1969) and had been inactivated and adsorbed with human A and O red cells, sheep red cells and with washed spermatozoa. The anti-IgG serum was used at a 1/1000 and the anti-IgM at a 1/50 dilution. The IgG indicator red cells were prepared by sensitizing human group O rhesus D positive red cells with non-agglutinating anti-D (IgG). The IgM indicator red cells were sheep erythrocytes sensitized with non-agglutinating human anti-sheep cell antibody (IgM) which had been separated from IgG globulins on a Sephadex G-200 column. In one test only, the IgM globulin was a nonagglutinating anti-blood group A antibody which was bound to human group A red cells.

Fig. 1 shows a diagrammatic representation of the test. Suspensions of washed group O spermatozoa were incubated in the test serum as in the direct agglutination test for 1 hr, or for 2 hr if the sperm were tested for bound antibody after direct agglutination tests. They were washed three times in a diluent of saline containing 0.05% bovine plasma albumin, to remove unadsorbed globulins, and resuspended in diluent and divided between four tubes (1-4) each containing 0.05 ml of the spermatozoal suspension. To each tube was added 0.05 ml of one of the following, tubes 1 and 3

saline, tube 2 anti-IgG, tube 4 anti-IgM. They were incubated for 20 min at 37°C, then 0.05 ml of a 1% suspension of IgG sensitized red cells were added to tubes 1 and 2, and IgM sensitized cells to tubes 3 and 4, and centrifuged for 1 min at 1800 rev/min on an MSE Minor centrifuge. Drops of the spermatozoa-red cell mixture were then mounted on slides, with the coverslips sealed with paraffin wax to prevent evaporation, and examined microscopically. During examination, the coverslips were lightly tapped to see whether the red cells were firmly adherent to the spermatozoa in positive tests.

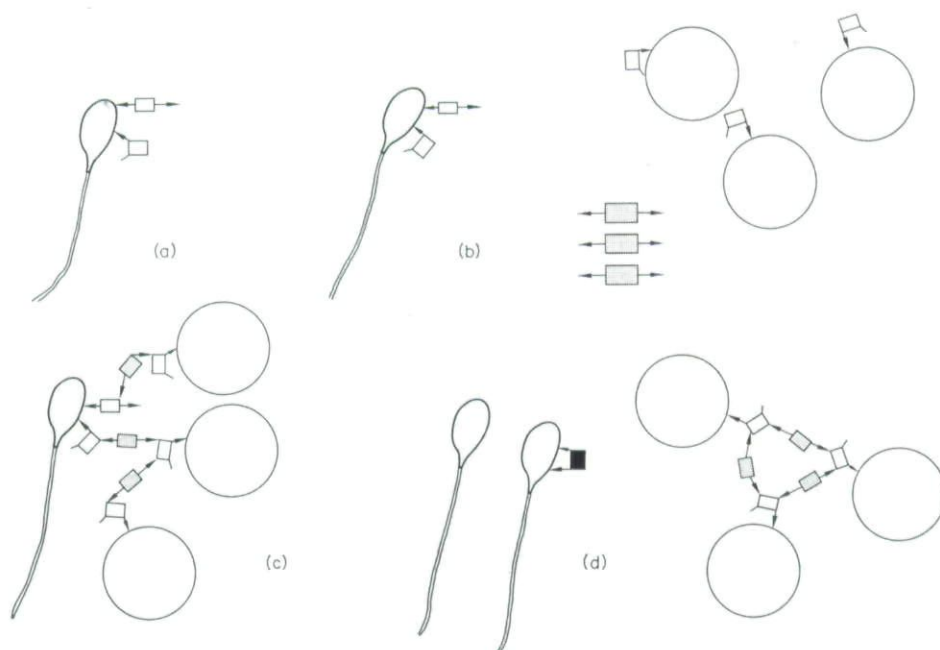


Fig. 1. Diagrammatic representation of the mixed-cell antiglobulin agglutination technique. (a) Sperm with fixed antibody, either agglutinating (divalent) or nonagglutinating. (b) Sperm treated with antiglobulin serum (speckled) corresponding to the antibody on the sperm, and to the nonagglutinating antibody on the indicator red cells. (c) Positive test in which the antiglobulin agglutinates the sperm and red cells together. (d) Negative test in which the sperm has no fixed antibody, or antibody of an immunoglobulin class (black) different to that of the antiglobulin, which, however, agglutinates the appropriately sensitized red cells.

The saline in tubes 1 and 3 controlled the specificity of the antiglobulin reaction in tubes 2 and 4. Previous tests showed no cross-reaction between the two antiglobulin sera for red cells or sperm with the alternative globulin.

Immunofluorescence

A standard technique was used. Unlike the previous tests in which a suspension of washed, unfixed, spermatozoa was used as antigen (Parish *et al.*, 1967), the spermatozoa were dried onto glass slides and fixed with absolute methanol (Hjort & Hansen, 1971).

The sperm were treated with the test serum in a moist chamber for 30 min at room temperature. The slides were then washed in three changes of buffered saline for

30 min, placed in moist chambers and treated for 30 min with monospecific fluorescein-labelled rabbit anti-human IgG or anti-IgM, both previously adsorbed with powdered human skin, washed group AB red cells and also by washed spermatozoa (Parish *et al.*, 1967). They were then washed again for 30 min in three changes of saline, and coverslips were mounted with 90% glycerol and 10% phosphate buffer pH 7.2. They were examined under ultraviolet light with a $\times 40$ objective of a Zeiss microscope with a dark ground condenser, using a BG 12 excitor filter, and 53/44 Zeiss barrier filters. Spermatozoa untreated, or treated with serum not containing anti-sperm antibody and with the fluorescein labelled antiglobulin sera were dull brown or red brown, forming an excellent background for fluorescein emission in positive tests.

Cytotoxicity tests

The test cells were washed group O spermatozoa at 2×10^8 /ml in buffered saline, of which 95% were motile.

In the tube tests, 0.05 ml volumes of spermatozoal suspension, fresh human complement in serum harmless to spermatozoa, and the test serum centrifuged free of debris were mixed in narrow non-wettable plastic tubes which were firmly sealed leaving not more than a 1 cm column of air above the surface. They were incubated at 37°C and samples examined microscopically at intervals for 2 hr.

Tests were also made on slides by mixing drops of sperm suspension, complement and serum, and sealing the coverslip with nontoxic paraffin wax. The slides were incubated at 37°C and examined at intervals microscopically.

Results and technical comments

The results of the tests obtained by the different techniques on the twenty sera are summarized in Table 1. The sera were tested without knowledge of the clinical history, which was obtained afterwards.

Precipitation in agar gels

No precipitins to spermatozoal extracts or seminal plasma were found after 1–6 days incubation.

This agrees with reports by Bandhauer (1966) and Cruickshank & Stuart-Smith (1959) who found no anti-seminal precipitins in sera containing agglutinins from men with orchitis. Moreover, Isojima *et al.* (1968) showed that precipitation was very much less sensitive than agglutination in detecting antispermatozoal antibodies in a rabbit antiserum.

Tanned cell agglutination test

In order to remove a red cell clumping factor the seminal plasma has to be dialysed against an EDTA buffer before it can be used as antigen. The effect of this treatment is not known, but we have no evidence that it changes the antigens. Tanned red cells with adsorbed treated antigen prepared as described in Methods, form stable suspensions giving consistent results with control rabbit anti-seminal plasma antisera.

Sera agglutinating or clumping plasma antigen-coated tanned cells do not always contain antibodies to semen when examined by other techniques, as in samples 87, 21 and 235 (Table 1). There are two explanations of this discrepancy. Firstly, the seminal plasma antigens adsorbed by the tanned cells are not those naturally adsorbed onto the spermatozoa, thus the tanned red cell agglutination test may detect antibodies

Table 1. Summary of results of five techniques detecting serum antibodies in women to seminal plasma and to spermatozoa, related to their fertility

Serum No.	Seminal plasma	Direct aggl.		Mixed cell aggl.		Immunofluorescence		Antibody globulin class	Cytotoxicity	Fertility
		at 1/5 diln	aggl.	IgG	IgM	IgG	IgM			
146	32	—	—	—	—	+ hMt	—	IgG	—	Infertile
87	32	—	—	—	—	—	—	—	—	Fertile
82	32	—	—	—	—	± hmt	—	IgG	—	No children; exposed (pill)
27	16	—	—	—	+ + H	—	+ + HMT	IgM	—	Infertile
25	8	+	+	—	+ + H	—	+ HmT	IgM	—	Infertile
13	16	± H	± H	+ + H	—	+ + H	+ H	IgG and IgM	—	Pregnant (13 weeks)
21	8	—	—	—	—	—	—	—	—	Infertile (6 years)—probably husband
236	8	±	±	—	+	—	± H	IgM	—	Infertile (10 years)
241	1000	+	+	+	+	+ + HM	+ + Hm	IgG and IgM	+	Infertile (10 years)
235	4	—	—	—	—	—	—	—	—	Prostitute with gonococcal peritonitis
74	0	—	—	—	—	—	—	—	—	Fertile
1	0	+	+	+	—	+ HMT	—	IgG	+	Fertile (normally practise coitus interruptus)
2	0	—	—	—	—	—	+ hMT	—	+	Prostitute; positive cancer smear cervix
3	0	—	—	—	—	—	—	—	—	Infertile (2 years)
4	0	—	—	±	—	—	—	IgG	—	Infertile
230	0	—	—	±	—	+ H	—	IgG	—	
239	0	±	±	±	—	—	+ HM	IgM	—	
240	0	—	—	—	—	+ M	—	IgG	—	
237	0	—	—	—	—	—	—	—	—	
228	0	—	—	±	+ + T	+ M	+ hMT	IgG and IgM	—	
					+ + T	+ M	+ + hMT	IgG and IgM	—	

Results of agglutination tests for antibodies to seminal plasma are expressed as titres. Results of other tests are expressed as —, or ±, +, + + according to the intensity of the reaction. HMT signify strong and hmt weak reactions of head, midpiece and tail respectively of spermatozoa.

that do not react with the washed sperm used in other tests. Secondly, the agglutination or clumping induced by some sera probably results from nonantibody substances. Boettcher & Kay (1969) isolated a β -lipoprotein from one serum which agglutinated spermatozoa, and lipids in tissue extracts and sera may interact to form lines resembling specific antibody-antigen precipitates in double diffusion gel plate tests. In theory such lipid or lipid protein aggregates could clump suitable particles.

Table 2. Results of spermagglutination tests using group O sperm from the same donor, and sera diluted 1/5 and incubated for 2 hr

Serum No.	Dates, antigenic state and results of tests							
	27.5.71 Dead sperm	28.5.71 Dead sperm	1.6.71 Dead sperm	17.6.71* Dead sperm	18.10.71 Living sperm	3.11.71 Dead sperm	8.11.71 Dead sperm	13.1.72 Living semen
146				—			—	
87				—			—	—
82	— ?						—	—
27	(+)						—	
25†	(++)				+	+	+	+
13‡	(+)				(++)	+	+	—
21	—				—		—	—
236		—		+	+		+	+
241		+		+		+	++	+
235			—		—		—	—
74				—	—		—	—
1	+	(++)	+			(++)	++	—
2	—				—		—	—
3	—						—	—
4					—		—	—
230		+		(++)		—	—	—
239§			+		+	—	—	—
240			—				—	—
237			—				—	—
228			—				—	—

* Reading after 1 hr, because saline control clumped at 2 hr.

† Sperm closely bound together indicating presence of IgM.

‡ Tendency for head-to-head agglutination.

§ Agglutination occurred with living sperm 18.10 but not with killed sperm on 18.10, 3.11 and 8.11.

Direct agglutination of spermatozoa

Six of the twenty sera agglutinated suspensions of washed spermatozoa from a blood group O donor (Table 1). Agglutination was manifested by random contact between different parts of the cells, but in sample 13 there was selective head-to-head agglutination which agreed with the results of other tests on this sample which also detected antibodies to the head (Table 1). The agglutination was almost certainly induced by specific antibody, because in all six, IgG or IgM antispermatozoal antibodies were detected by mixed cell antiglobulin agglutination and by immunofluorescence. Moreover, the mixed cell antiglobulin tests were usually made on the sperm used in,

and examined after, the agglutination tests. The results of the spermatozoal agglutination tests were unrelated to those of the tests on seminal plasma coated red cells which are probably not due to antibodies.

Tests using washed sperm suspended in gelatin saline were reproducible (Table 2) provided that the sample was free of debris and did not clump spontaneously. In these conditions, dead sperm (killed by sodium azide) were suitable, but living sperm (18.10) were more sensitive in detecting antibody. Sample 239 on 18.10 clearly agglutinated living sperm, but failed to agglutinate the dead sperm from the same sample.

Living spermatozoa in whole semen, diluted if necessary to obtain the concentration of 2×10^8 , provided a stable suspension but detected antibody less frequently than washed sperm as occurred in samples 13, 1 and 239 (Table 2) and discussed below.

In further tests on human sera and rabbit antisera to human spermatozoa, higher titres were obtained with washed spermatozoa than whole semen, and with motile than nonmotile sperm. Thus a suspension of washed motile sperm proved to be the best antigen. In tests on whole semen, some human sera caused clumping of fine particles, either sperm debris or aggregated plasma, onto the surface of sperm.

Spermatozoa have seminal plasma substance firmly adsorbed onto their surface, but this surface covering appears to be incomplete, leaving some of the cell membrane exposed. Therefore suspensions of washed spermatozoa can be agglutinated by antibodies to the adsorbed seminal plasma antigens or to the cell membrane.

The incidence of positive tests is lower with whole semen instead of suspensions of washed sperm in gelatin-saline diluent. This sometimes occurs when washed sperm are agglutinated by antibody combining with seminal plasma antigens adsorbed on the sperm, but when tested with whole semen the antibodies are neutralized by the free antigen in the seminal plasma, preventing agglutination. This could have occurred in the tests on sample 13. Another possibility is that soluble spermatozoal antigen is released from degenerate sperm into the plasma which neutralizes the appropriate antibodies. Moreover, it is evident that a serum may contain antibodies with several different specificities, e.g. sample 1 (Table 1) which agglutinated washed sperm but not those in whole semen (Table 2) yet were cytotoxic to sperm in both. The cytotoxic antibody may have been nonagglutinating because of its molecular form, or because it was present in small amounts and fewer molecules are required for cytotoxicity than for agglutination, or because they combined with a part of the cell not readily forming links with other cells, e.g. the midpiece.

Bacterial contamination of the sperm suspension could theoretically result in false positive tests. Mycoplasmas (Taylor-Robinson & Manchee, 1967) and bacteria (Schwimmer *et al.*, 1967b; Teague, Boyarsky & Glenn, 1971) are reported to clump sperm. In one test of this phenomenon we added living *Streptococcus viridans* (NTCC 3165) organisms to washed spermatozoal suspensions in the proportion 100 organisms per spermatozoon. The streptococci stuck to the surface of the cells within 30 min and loosely clumped them in 1 hr. Heat-killed streptococci stuck weakly to the cells without clumping, and *Bacillus subtilis* (NTCC 3610) and *Neisseria catarrhalis* (NTCC 3622) neither stuck to nor clumped the sperm. This is unlikely to be a source of confusion in agglutination tests examined microscopically, because the adherence of the bacteria bringing about clumping is readily observed.

Mixed cell antiglobulin agglutination

The principle of the technique (Fig. 1) is that spermatozoa incubated in sera containing

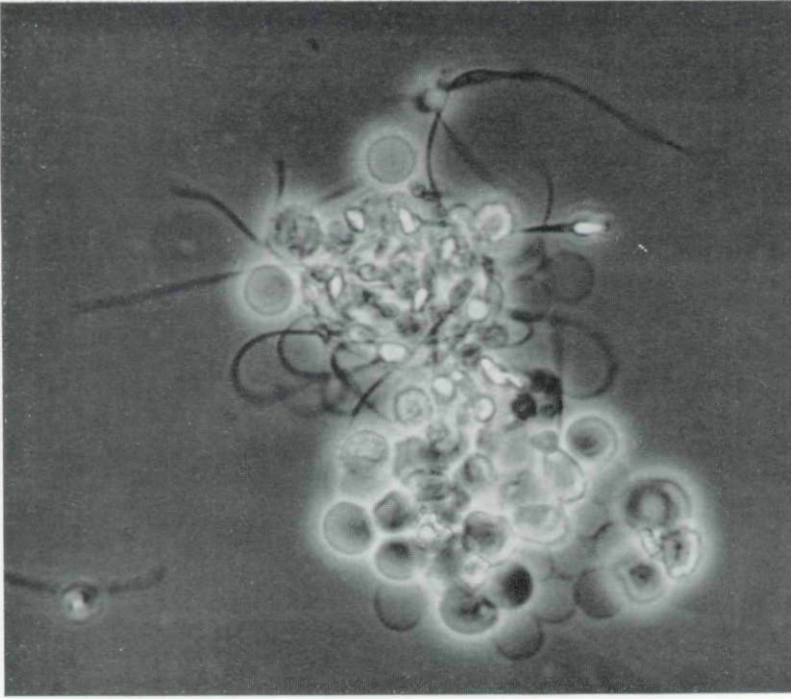


Fig. 2. Mixed cell antiglobulin positive test in which red cells and sperm are agglutinated together to detect IgG antibody on the sperm. Phase contrast.

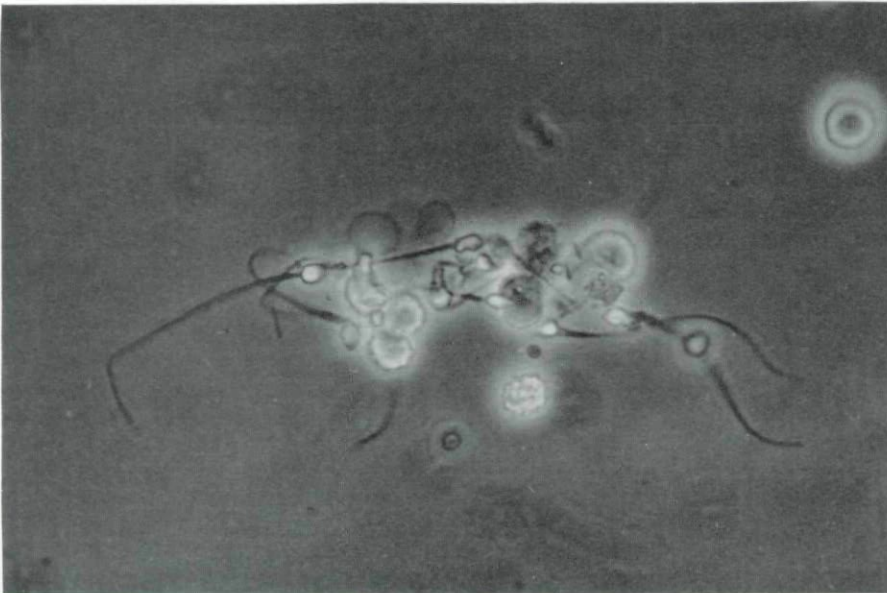


Fig. 3. Mixed cell antiglobulin positive test in which red cells are agglutinated onto the spermatozoal heads to detect IgM antibody fixed there. Phase contrast.

antispermatozoal antibodies have antibody globulin fixed to their surface. The sperm are then washed to remove all other globulins. When these sperm are treated with the appropriate antiglobulin serum it combines with the fixed antibody. If to this suspension red cells are added with the same class of immunoglobulin on their surface as that on the spermatozoa, the antiglobulin serum agglutinates the red cells to each other and to the spermatozoa (Fig. 2). Agglutinating and nonagglutinating antibodies on the sperm are detected, and by using monospecific antiglobulin sera their immunoglobulin class is identified. The technique also detects antibodies that combine selectively with the head (Fig. 3) or the tail (Fig. 4). If the red cells do not agglutinate

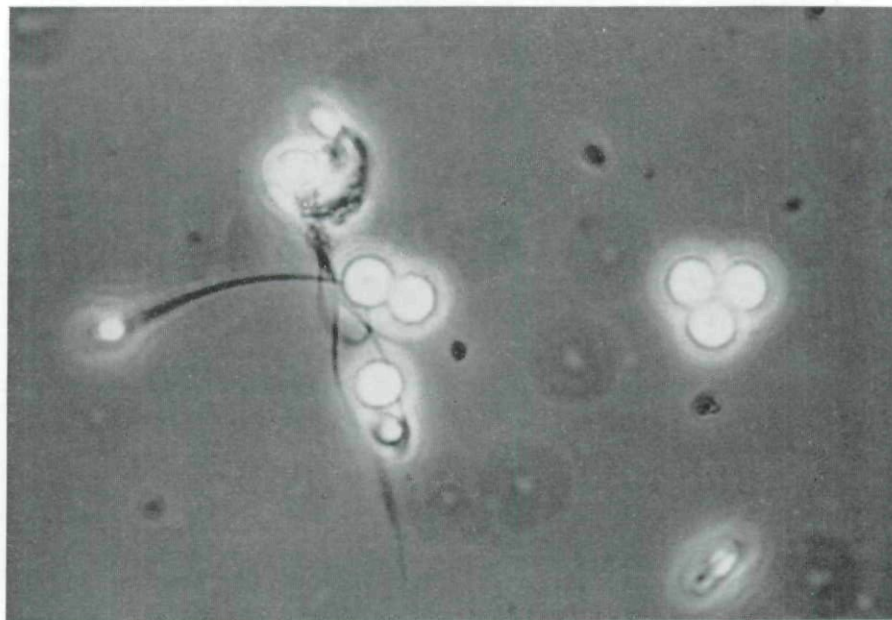


Fig. 4. Mixed cell antiglobulin positive test in which red cells are agglutinated onto the tails of the sperm to detect IgG fixed there. Phase contrast.

with the sperm it indicates either there is no antibody on the sperm, or the antibody is of a different class from the antiglobulin used to detect it. In a negative test, therefore, the red cells agglutinate together but not with the sperm (Fig. 5).

Six sera had IgG and seven IgM antibodies; of these, three contained antibodies of both classes (Table 1). Three sera had antibodies to the head only and two to the tail only. All sera that directly agglutinated the spermatozoa also had positive mixed cell antiglobulin tests, which was indirect evidence that direct agglutination was due to antibody of the particular class shown by the antiglobulin test.

The usefulness of the technique in examinations of spermatozoa is restricted by the size of the red cell indicator particle. There are several antigens in sperm, some confined to only parts of the cell as shown by immunofluorescence. The spermatozoal head measures about $5 \times 2.5 \mu\text{m}$. Red cells are about $8 \mu\text{m}$ in diameter. Therefore red cells cannot combine with sites on the head containing only small amounts of antigen, or with the midpiece, though they will distinguish between antibody on the head or tail (Fig. 6).



Fig. 5. Mixed cell antiglobulin negative test in which the red cells are agglutinated, but there is no corresponding antibody on the sperm. Phase contrast.

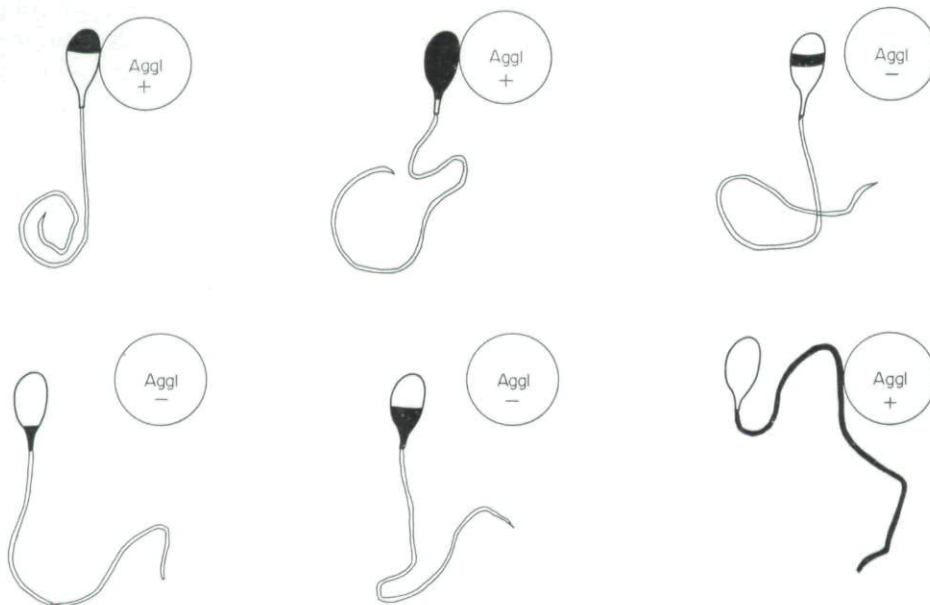


Fig. 6. Relation between immunofluorescence and mixed-cell antiglobulin agglutination. When antibody fixes to sufficient areas of the sperm detected by immunofluorescence (black areas) it can also be detected by the antiglobulin test. When the area is small, or inaccessible to the red cell (equator, midpiece or post nuclear region) no agglutination occurs. (Immunofluorescence representation is modified from a figure by Hjort & Hansen (1971) by permission of the Editor of *Clinical and Experimental Immunology*.)

In this technique it is essential to determine the correct amount of antibody to sensitize the red cells and the amount of antiglobulin to agglutinate the cells. Too much globulin on the red cells makes them clump nonspecifically, too little may result in false negative tests, which also occur if the antiglobulin serum is too strong.

Immunofluorescence

Antibodies to washed spermatozoa were detected by immunofluorescence in fourteen of the twenty sera. In five samples the antibody was IgG, in five IgM, and in four antibodies of both classes were present (Table 1). Immunofluorescence detected antibody not found by other techniques as in samples 146, 82, 2 and 239. These were either weak reactions, or strong reactions confined to the midpiece as in samples 146 and 239.

The antibodies were found to react with the head, midpiece or tail (Table 3), as described previously (Parish *et al.*, 1967; Parish & Ward, 1968) though sample 241 was once found to have antibody reacting with the equator of the head. Hjort & Hansen (1971), improving the sensitivity of the technique with an interference filter, described antibodies reacting with the acrosome, equator, postnuclear region and tail. These specificities, together with those described by us, are illustrated in Fig. 6, and are consistent with other reports of reactions with the acrosome (Feltkamp *et al.*, 1965), the whole head (Feltkamp *et al.*, 1965; Rümke, 1968; Haensch, 1969), postnuclear zone (Feltkamp *et al.*, 1965), midpiece (Feltkamp *et al.*, 1965; Rümke, 1968) and tail (Feltkamp *et al.*, 1965; Haensch, 1969). Staining of the whole spermatozoon was reported by Schwimmer *et al.* (1967b), but this results from antibodies combining with seminal plasma antigens including blood group substances adsorbed onto the spermatozoa (Parish & Ward, 1968).

Hjort & Hansen (1971) determined the titres of the sera, but we find that it is difficult to observe the end point with our filter systems because weak reactions may sometimes be observed over a range of three dilutions. The results are therefore expressed as presence or absence of antibody with a subjective assessment of intensity of staining (Table 1). When the same sera are examined on spermatozoa from one donor on several occasions the results are usually consistent (Table 3), samples 82 and 236 being the exceptions. Not all the spermatozoa in any one sample react, or react with equal intensity in immunofluorescent tests, which may be due either to cells having different amounts of seminal plasma on their surface preventing access of the antibodies to the cell membrane, or to spermatozoa varying in their antigens.

These tests were made on dried, fixed smears of spermatozoa. This technique has the advantages of being easily carried out, and reduces nonspecific staining. It has the disadvantages that the sperm are much reduced in size, and that fixation could theoretically extract methanol-soluble antigens. Tests on suspensions of washed, unfixed, spermatozoa (Parish *et al.*, 1967) have the advantages of the larger size of the cells, and the preservation of all antigens, but repeated washing of the cells in small tubes takes longer and some are inevitably lost, and there is a greater tendency for nonspecific staining, especially of debris which may adhere to the cells.

Immunofluorescence detects nonagglutinating as readily as agglutinating antibody, and also antibody to sites which have small amounts of antigen or are inaccessible. Thus, as depicted in Fig. 6, immunofluorescence detects antibody to the equator or to the midpiece which is not found by direct or by mixed cell antiglobulin agglutination.

Cytotoxic tests

Three of the sera were toxic for washed suspensions of group O spermatozoa (Table 1). Sample 1, the only one tested, was also toxic for sperm in whole semen. In samples 241 and 1 there was good though indirect evidence that the cytotoxic effect was mediated by antibody because both sera had strong agglutinins, mixed cell antiglobulin reactions and strong immunofluorescent staining of the head and midpiece. No antibody was found by other tests in sample 3 in which the cytotoxic effect may be an inflammatory substance.

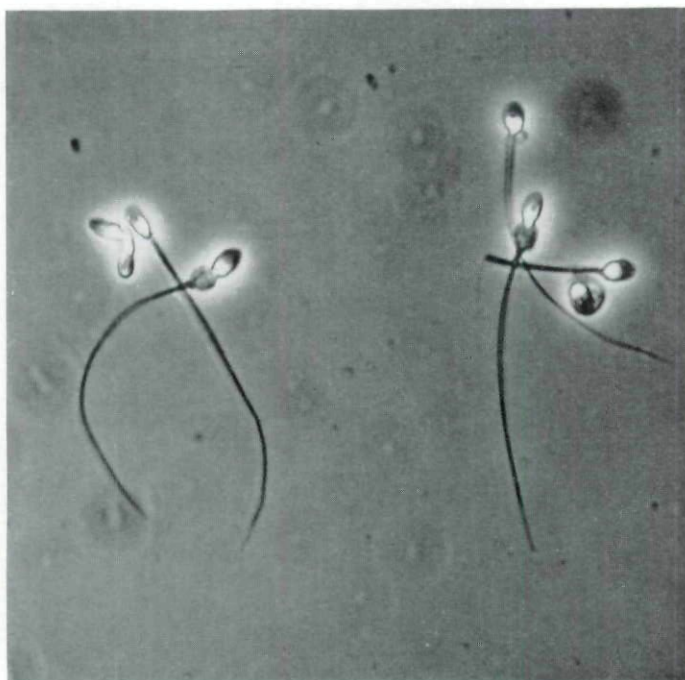


Fig. 7. Cytotoxic immobilization of spermatozoa with enlargement of midpiece, induced by IgG antibody to midpiece, and complement. Phase contrast.

Serum samples 1 and 3 immobilized and lysed the spermatozoa; sample 241 immobilized without lysis. The cytotoxic activity of the sera heated at 56°C was dependent upon the presence of fresh-complement containing serum, except for sample 1 which in the absence of complement immobilized or reduced motility of the sperm without lysis. It should not be assumed that the cytotoxicity of sample 3, in which no antibody was found, was complement-dependent. It is possible that fresh serum contains substances which could be activated by an inflammatory agent in the sample, which was obtained from a person with carcinoma of the cervix.

The results of cytotoxic tests are clearly observed. The spermatozoa either continue to move and live, or stop moving and probably die. In the three sera of this series and in other tests we have seen three types of cytotoxic change.

1. The commonest is immobilization without morphological change.
2. Immobilization with enlargement of the midpiece (Fig. 7).
3. The least frequent: lysis and complete disruption of the spermatozoa (Fig. 8).

These responses are usually complement-dependent. In the third type of response in the absence of complement there is no disruption but the spermatozoa may be immobilized, as seen in sample 1 of this series, and previously (Parish *et al.*, 1967).

It is essential to ascertain that any toxic changes are due to factors in the serum or the mucus, and are not due to chemicals or detergents on the glassware, or to dehydration following faulty sealing of tubes or slides. Immobilization with or without enlargement of the midpiece can be induced by poisons or adverse physical conditions.

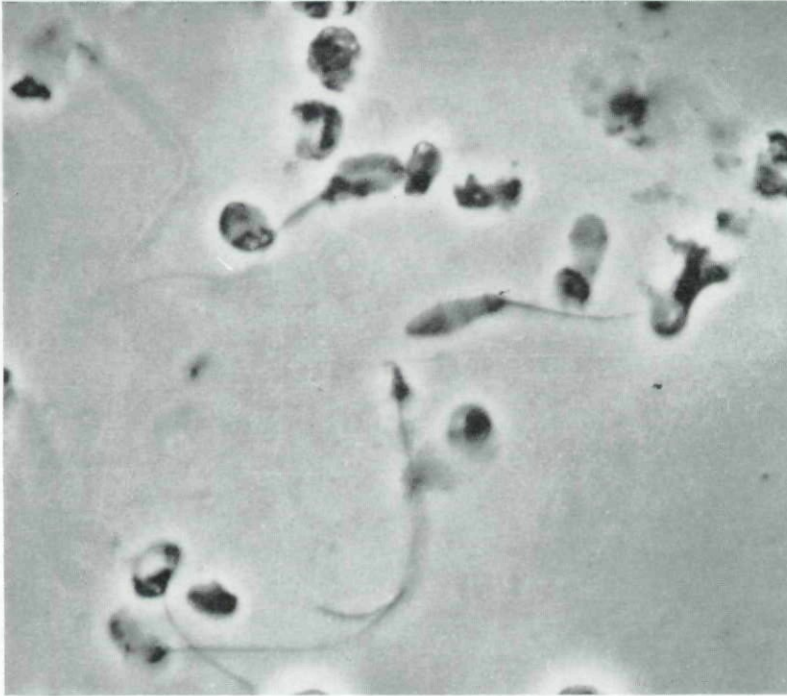


Fig. 8. Cytotoxic disruption of spermatozoa induced by IgG antibody to head and midpiece, and complement (from an earlier study). Phase contrast.

Moreover, some semen samples contain many abnormal spermatozoa, and even normal spermatozoa when immobilized or dead tend to present the flat face of the head instead of the thin biconcave edge, which may be misconstrued as cytotoxic morphological changes.

Tests for cytotoxicity are usually made to observe immobilization or disruption that could cause infertility. We see no advantage in the use of nigrosin, trypan blue, acridine orange or other dyes to observe cytotoxic changes, particularly as acridine orange itself can be harmful. If an agent immobilizes the sperm *in vivo*, the woman will be infertile even if the spermatozoal enzymes still function.

Discussion

The tests were made without knowledge of the clinical history of the serum donors, and most tests were read without knowledge of the results of the other techniques. The results show good agreement between techniques but no exact relation to the clinical history (Table 1). Antispermatozoal antibodies were found in five of six women with

infertility of unknown cause; in a seventh infertile woman (sample 21), who had no antispermatozoal antibodies, the infertility is believed to be due to the husband.

A cytotoxic factor was present in three samples. Two of these had strong agglutinins, and antibodies to the head and midpiece detected by other techniques. One (241) was infertile for 10 years; the other was fertile, but the cytotoxic factor, presumably antibody, may have been formed after the last child was conceived, or appeared only intermittently in the serum, or was present in the serum but not the cervical mucus or uterine fluids and therefore did not prevent conception. The third sample (3) which was cytotoxic to spermatozoa in the absence of antibody detected by any test illustrates the importance of combining tests for cytotoxicity with at least one other for antibody, preferably immunofluorescence, before attributing the toxicity to allergic infertility. Samples of cervical mucus not infrequently, and sera occasionally, contain substances harmful for spermatozoa in the absence of detectable antibody. The toxic substance in sample 3, obtained from a prostitute, may have been an inflammatory substance associated with the cervical cancer. As it was inactivated by heating it was unlikely to have been a contraceptive agent which had been absorbed systemically, though such agents in serum could immobilize spermatozoa, and in the absence of other tests for antispermatozoal antibodies, the immobilization might be attributed erroneously to allergy.

There is no doubt that some cases of infertility result from women forming antibodies cytotoxic to spermatozoa. Isojima *et al.* (1968) found such antibodies in the sera of three of twenty-five infertile women, and Ansbacher *et al.* (1971) found agglutinating and cytotoxic antibodies in 11.4% infertile women, but did not differentiate between the two tests. Cytotoxic antibodies were found in the cervical mucus of three women by Parish *et al.* (1967), and in the mucus of two other women and in the serum of one by Parish & Ward (1968). The cytotoxic antibodies examined by Parish and colleagues were IgG, complement-dependent, specific for the spermatozoal head and midpiece which enlarged and disrupted, and were harmless for human macrophages which ingested the dead sperm in the presence of the antibody. Isojima *et al.* (1968) found the serum antibody immobilizing spermatozoa to be a γ -globulin, complement-dependent, absorbed from the serum only by spermatozoa, and when eluted and iodinated reacted strongly with spermatozoa. The participation of complement was reported earlier by Rümke & Hellinga (1959).

This technique is commonly termed an immobilization test. There is, however, no necessity to distinguish it from other antibody-mediated cytotoxic tests just because the target cell is motile. As had already been described, occasionally antispermatozoal antibodies with complement disrupt the sperm in a manner similar to antibody and complement-mediated lysis of erythrocytes or tissue cells. Cytotoxic is a better designation than immobilization for these tests.

The results obtained by direct agglutination of spermatozoa, mixed cell antiglobulin and strong immunofluorescent staining of the head agree well, with two exceptions in samples 4 and 27 which caused no direct agglutination and in which the antibodies were 'incomplete' or non-agglutinating. These consistent results support the contention that in this investigation direct spermagglutination is due to specific antibody, probably against cell membrane antigens.

The results of the tanned cell agglutination tests for anti-seminal plasma antibodies were unrelated to those of the other tests for anti-spermatozoal antibodies. Clumping of seminal plasma coated cells occurring in some sera is thought to be due to nonanti-

body substances, possibly lipoproteins, as reported above. In other sera, however, e.g. sample 241, agglutination of the seminal plasma coated cells is probably due, at least in part, to antibody. Of the four to seven antigens observed in prostatic secretions or seminal plasma, some are specific for semen and are therefore antigenically foreign to women (Rao & Sadri, 1959; Quinlivan, 1969). Tanned cells do not adsorb antigens equally from a mixture, resulting in some selection. Spermatozoa firmly adsorb seminal plasma antigens (Weil, 1961; Weil & Rodenburg, 1962), including the blood group substances A or B if the man secretes them (Edwards *et al.*, 1964; Boettcher, 1965; Parish *et al.*, 1967), and another for which the name scaferrin is proposed (Roberts & Boettcher, 1969). Though the use of sperm from persons of blood group O as antigen eliminates complications due to blood group antibodies, it remains unlikely that sperm and tanned red cells adsorb the same antigens from the seminal plasma. Thus these tests may detect antibodies to different seminal plasma antigens. It is also theoretically possible for a woman to form antibodies to the semen of one man and not of another, because small antigenic differences exist in the seminal plasma (De Fazio, Lewis & Ketchel, 1969) and spermatozoa (Fellows & Dausset, 1970) of different men. They may even form anti-seminal plasma antibodies without ever being exposed to semen, because scaferrin in seminal plasma has some determinants in common with lactoferrin (Roberts & Boettcher, 1969).

The influence on fertility of antibodies to seminal plasma antigens or to spermatozoa is still to be assessed, though there is a tendency for antibodies to seminal plasma to be found more frequently and in higher titre in infertile than fertile women, particularly women in whom no cause for infertility can be found.

There is another possibility that has not been fully investigated, that antibodies reacting with seminal plasma antigens firmly adsorbed by the spermatozoa are cytotoxic. Spermatozoa which had adsorbed A or B blood group substance were unharmed by subsequent treatment with human immune-type anti-A or anti-B antibodies with complement (Parish *et al.*, 1967; Isojima & Tsuzuku, 1968), just as spermatozoa were unharmed when treated with IgA and IgM antibodies to seminal plasma antigens other than blood group substances, adsorbed onto the spermatozoa (Parish & Ward, 1968). However, it has been claimed that sperm with adsorbed blood group substance A have reduced anaerobic glycolysis when treated with anti-A (Ackerman, 1967).

It is concluded that women exposed to semen which contains organ-specific antigens may respond by forming anti-seminal antibodies, just as they might respond to multiple blood transfusions by forming anti-leucocyte antibodies. In most women these antibodies do not hinder fertilization. In some women, however, the antibodies are cytotoxic, immobilizing or even disrupting spermatozoa. The techniques recommended for investigations of allergic infertility are cytotoxic tests, together with immunofluorescence or mixed cell antiglobulin agglutination as supporting evidence that the factor harmful to the spermatozoa is antibody. As antibody can be formed locally in the cervix (Parish *et al.*, 1967) it is advisable to examine the cervical mucus as well as the serum.

Acknowledgments

We thank Dr Audrey Ward and Dr Kay Capner of St Mary's Hospital, Paddington, London, for the twenty sera which were obtained from patients examined by them as part of their investigation on serum substances agglutinating seminal plasma coated particles.

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