Characterization of sperm antigens reacting with human antisperm antibodies

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SUMMARY

Antibodies reacting with human spermatozoa have been detected by various immunological techniques in the sera of subfertile men. Different patterns of sperm agglutination are observed with different sera, either head-to-head, tail-to-tail, or tail-tip-to-tail-tip. Differences have been detected between the clinically relevant antibodies in spontaneously infertile males and the less important antibodies in males who have undergone reversal of vasectomy. It has been suggested that the variations in agglutination patterns are due either to different classes of antibody or to binding of antibody to different antigens. In the present study immunoblotting techniques were used to characterize the reactivity of solubilized sperm proteins with serum samples exhibiting different modes of sperm agglutination. This involved the electrophoretic transfer of proteins from SDS gels to nitrocellulose sheets followed by overlay with serum antibody. Using these techniques we have attempted to characterize the antigens of spermatozoa which react with sera from both spontaneously infertile and vasovasostomized men. The results showed that although antisperm antibodies bind to discrete and sperm-associated antigens, there is no substantial difference between the antigenic patterns observed with antibodies producing different types of sperm agglutination. Neither the antigens detected, nor the intensity of reaction showed significant differences although there was a tendency for head-to-head agglutinating antibodies to react more strongly with the higher molecular weight antigens. Moreover, although with sequential serum samples the patterns of agglutination may change, the antigenic pattern remains unchanged.

Keywords antisperm antibodies immunoblotting sperm antigens

INTRODUCTION

Antibodies reacting with human spermatozoa have been detected by various immunological techniques in the sera of some subfertile men (Rose *et al.*, 1976). Many of these techniques used routinely for infertility investigation such as the tray agglutination test (TAT) (Friberg, 1974a) and gelatin agglutination test (GAT) (Kibrick, Belding & Merrill, 1952) are dependent upon agglutination of the sperm by antibodies. Different patterns of sperm agglutination are observed with different sera incubated with the same spermatozoa: head-to-head (H-H), tail-to-tail (T-T), tail-tip-to-tail-tip (TT-TT) and combinations of all three. Current explanations for these differences fall into two main groups. One possibility is that the antibodies are binding to several distinct

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antigens on different parts of the spermatozoa (Rose *et al.*, 1976; Hjort, Hansen & Poulsen, 1978). The other major possibility includes various ways in which different classes of antibody are responsible for different patterns of agglutination (Friberg, 1974b, c; Husted & Hjort, 1975).

Recent results have provided evidence for the existence of at least three autoantigens in the membrane. Only one of these antigens (TT-TT antigen) appeared to be associated with a particular mode of agglutination whereas the other two antigens could be involved in both H-H and T-T agglutination (Hjort & Poulsen, 1981; Poulsen & Hjort, 1981). The unexpected finding that the same antigens may be involved in different types of agglutination has not yet been confirmed by other laboratories.

A further factor in the analysis of antisperm antibodies is the different emphasis that is placed on their clinical relevance in different groups of patients. For example, the presence of antisperm antibodies in the spontaneously infertile patients is clearly related to the infertility itself (Fjallbrant, 1968; Rumke *et al.*, 1974) and this is associated with the inability of spermatozoa from these patients to penetrate cervical mucus (Morgan *et al.*, 1977; Jager, Kremer & van Slochteren-Draaisma, 1979). However, the presence of antibodies in vasovasostomized males does not appear to influence their fertility (Thomas *et al.*, 1981; Parslow *et al.*, 1983) and normally spermatozoa from these patients will penetrate cervical mucus.

In a recent study (Parslow *et al.*, 1985) we demonstrated significant differences between these two groups of patients in the classes of antibody bound to the spermatozoa, with a higher proportion of secretory IgA antibody in the spontaneously infertile group. In addition, the amount of IgA bound to the spermatozoa correlated with the inability of sperm to penetrate cervical mucus. The reason for these differences was not clear, and it remained possible that the important differences between the two groups were related to the production of antibodies, albeit with different class distributions, to completely different antigens on the spermatozoa. In that event, certain antigens would be of relevance in infertility and others would not.

In the present study immunoblotting techniques were used to characterize the reactivity of solubilized sperm proteins with serum samples exhibiting different modes of sperm agglutination. This involved the electrophoretic transfer of sperm proteins from SDS (sodium dodecyl sulphate) gel to nitrocellulose sheets followed by overlay with serum containing antisperm antibody. Using these techniques we have attempted to characterize the antigens of spermatozoa which react with sera from both spontaneously infertile and vasovasostomized men.

MATERIALS AND METHODS

Subjects and serum samples. Serum samples obtained from 72 patients attending the Fertility Clinic at St Bartholomew's Hospital contained antisperm antibodies in titres ranging from 8 to greater than 1024, as assessed by TAT. Pure H-H type of agglutination was found in 16 sera, T-T in 14, TT-TT in two and mixed agglutination in 40 serum samples. Twenty-seven patients had reversal of vasectomy, the remaining patients were spontaneously infertile.

Serum samples were also obtained from 12 healthy men without evidence of antisperm antibodies.

Two sera containing antinuclear antibodies were received from patients with SLE (systemic lupus erythematosus).

Antisperm antibody tests. Agglutinating antisperm antibodes were measured by TAT (Friberg, 1974a) with some modifications of Hellema & Rumke (1978) and immobilizing antibodies by Micro-immobilization test (MIT) (Hellema & Rumke, 1978).

Sperm antigen preparation. Fresh semen specimens received from healthy males were treated by the 'swim up' method of Hellema & Rumke (1978) in order to obtain a suspension of only motile spermatozoa from semen by active penetration into overlying phosphate-buffered saline (PBS) layer. Pooled spermatozoa suspensions were washed three times in physiological saline and stored at $-20°C$ until used. The concentration of spermatozoa was then adjusted to 5×10^8/ml. Samples were usually made up to 50 μl and then added to double strength reducing sample buffer, giving a final concentration of 0·0625 M Tris/HCl pH 6·8, 2% SDS, 10% glycerol, 5% mercaptoethanol and

0·001% bromophenol blue as the dye. Samples were dissociated by immersion in boiling water for 30 min, centrifuged for 10 min at 10,000 *g* and the supernatant used as an antigen.

Absorption of sera with antisperm antibodies. For the absorption of sera containing antisperm antibodies with antigen preparation, packed spermatozoa were suspended in reducing buffer at a concentration of 1×10^9/ml. The preparation was boiled in a water bath for 30 min and then centrifuged for 10 min at 10,000 *g*. To 0·5 ml of the supernatant 0·5 ml of sperm agglutinating serum was added and the suspension was placed in a slowly rotating mixer for 1 h at 37°C before being used for the immunoblotting (1st antibody overlay) and for the TAT.

For the absorption of sera with whole spermatozoa, packed spermatozoa were incubated in a mixer with sperm agglutinating sera in concentration of 5×10^8/ml for 1 h at 37°C. After centrifugation the supernatant was used for the TAT and immunoblotting.

Polyacrylamide gel electrophoresis. SDS polyacrylamide gels (10%) were made by the method of Laemmli (1970) using Protean II slab cell (Bio-Rad Laboratories AG, UK). Gels were loaded with 10 µl of sperm extracts and standard solution and run overnight at a constant voltage of 25 V.

Electrophoretic blotting. After gel electrophoresis, the gel slab was removed and cut up into strips which were placed onto 1×9 cm strips of nitrocellulose (NC) sheets (45 µm pore size) obtained from Millipore. Electrophoretic blotting of the separated proteins was carried out as described by Kyhse-Andersen (1984) using a semi-dry electroblotter (Dako Ltd, UK). A 15 V potential was applied for 1 h at room temperature. The strip with the antigen extract and mol. wt markers was stained with 0·5% amido black in 50% methanol, 7% acetic acid for 2 min, blotted and dried. The remaining strips were placed into 0·06% gelatin/PBS overnight.

Antibody overlay technique. NC strips were lined onto a side of plastic Petri dishes (9 cm) and washed three times with PBS using a rotator. Each strip was incubated with 2 ml of patients' sera (first antibody) diluted 1:10 for 1 h. All incubation steps were followed by six washes in PBS containing 0·025% Tween over a 30 min period. Following washing the strips were overlaid with biotinylated sheep anti-human Ig (Amersham International, UK) (second antibody) diluted 1:50 for 1 h. The strips were overlaid with streptavidin-biotinylated peroxidase complex (Amersham International, UK) diluted 1:50 and incubated for 1 h. To reveal the peroxidase conjugate, strips were incubated in Tris-buffered saline pH 7·4 containing 20% methanol, 0·054% 4-chloro-1-naphthol and 0·03% hydrogen peroxide. The enzyme substrate reaction was stopped after 5 min with tap water.

For the detection of immunoglobulin classes, after the incubation with first antibody, the strips were overlaid with rabbit anti-human IgG, IgM or IgA (Dako Ltd, UK) diluted 1:100 and incubated for 1 hour. The strips were then overlaid with biotinylated donkey anti-rabbit Ig (Amersham) diluted 1:50 for 1 h. The incubation with peroxidase complex was carried out as above.

RESULTS

Serum antibodies were bound specifically to the chromatographed sperm proteins in 74% of a total 72 serum samples tested. The types of agglutination detected in various sera and relative molecular mass (M_r) expressed in kD are presented in Table 1. It can be seen that up to nine different sperm antigens (antigenic determinants) were detected. Examples of the antigenic patterns are presented in Fig. 1. Typically various combinations of five major bands were detected, at 120, 84, 76, 68 and 62 kD. Comparisons with other pathological sera suggested that these antigens were recognized specifically by antisperm antibodies. There was no clear relationship between the types of agglutination and the pattern of antigens bound and no correlation between the titres of antisperm antibodies as assessed by TAT and the intensity of bands on the blots. Similarly, the presence or absence of immobilisins in the sera did not show any effect on the antigenic patterns.

Serum samples which were obtained from 12 fertile men or patients without antisperm antibodies (detectable by TAT) did not reveal any specific bands.

To determine if the antigens seen on the blots were either specific to spermatozoa or due to common nuclear proteins, two sera from patients with SLE were also examined. Different patterns
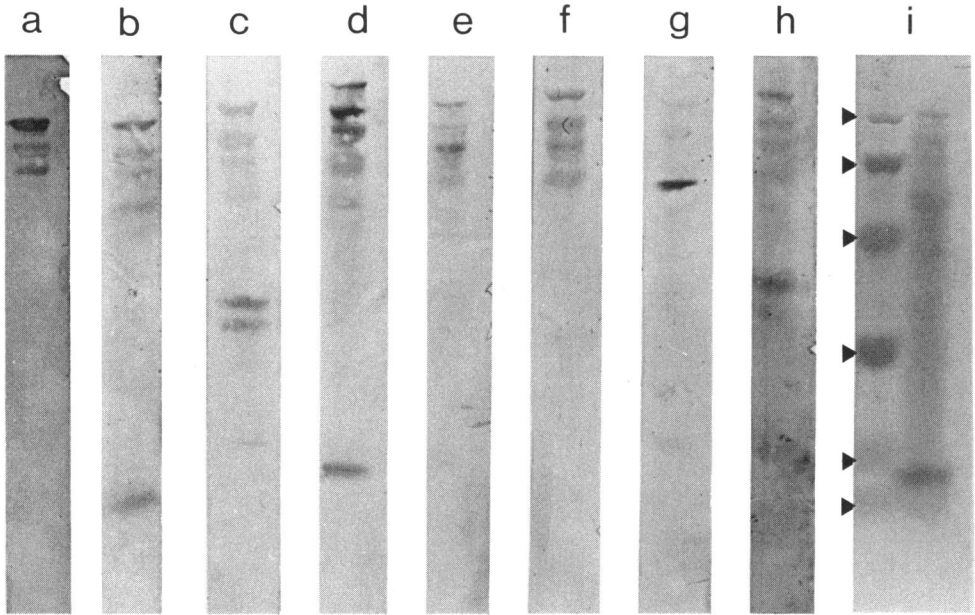
Table 1. The relationship between serum antisperm antibody titre, mode of agglutination and antigenic determinants of human spermatozoa reacting with the serum

Sperm agglutination pattern	Patient	MIT titre	TAT titre	Approximate M_r expressed in kD								
				120	84	76	68	62	53	38	35	18
T-T	R.H.*	16	>1024	+++	++	+	+	+				++
	G.C.	Neg	1024	++	++	++	+	++				
	M.D.	Neg	32	+++	++	++		++				++
	R.F.*	N	256	+++	++	+	++	+			++	
	M.M.*	2	128	+			++				+	
	P.S.*	Neg	32	++	++	+		+				
	M.E.	Neg	64	+	+	++						
	I.B.*	32	1024	+	++			+				
	B.R.	N	32	+	+		+			+		
	J.H.	64	>1024	+			+				+	
	L.S.	8	256									
	A.S.	4	1024									
	C.B.	Neg	256									
	W.W.	2	512									+++
H-H	M.S.*	Neg	512	+++	++	++		+				
	L.L.*	Neg	32	+++	++	++		++				
	M.S.	Neg	64	++	++	+++		+				
	H.M.	Neg	16	+++	++		++	++				
	V.M.	Neg	128	+++	++	++						
	A.C.	Neg	128	+	+	++						
	M.F.	Neg	>1024	+				++				
	J.S.	Neg	64	++	+			+				
	K.G.	Neg	16	++		+		+				
	J.C.	Neg	128	++	++							
	D.E.	Neg	8	++			+					
	A.H.	Neg	512	++	+							
	G.G.	Neg	32									
	S.K.*	4	512					+				
	N.H.	Neg	256									
	P.P.	N	>1024									
TT-TT	B.O.	Neg	32	++	+				+++	+		
	J.B.	Neg	64						+++	+++		
T-T/H-H	L.K.*	2	64	+			+					
	P.H.*	32	1024	++	+			+				

Group		Serum titre	Sperm agglutination
H-H/T-T	J.S.*	2	256
	L.M.	Neg	64
	M.G.	Neg	64
	R.B.	Neg	>1024
	R.M.	32	512
	M.S.*	Neg	>1024
	J.S.	Neg	64
	R.R.*	16	>1024
	R.G.*	2	256
	D.E.	Neg	32
	B.M.	4	256
	P.P.	Neg	>1024
	M.H.	N	256
	A.S.	4	>1024
	P.H.	Neg	128
	K.T.*	8	1024
	R.S.*	Neg	32
	G.P.*	4	>1024
	J.G.*	4	>1024
	R.L.*	Neg	512
T-T/TT-TT	H.M.	Neg	32
	C.C.*	Neg	128
	R.B.	N	>1024
	R.D.	Neg	>1024
	B.C.*	4	1024
	L.S.	2	256
	T.M.	16	256
	R.C.	Neg	>1024
	D.K.	8	512
	T.H.*	N	512
H-H/TT-TT **T-T/H-H/TT-TT** **T-T/TT-TT/H-H**	R.T.*	Neg	512
	W.B.*	2	512
	A.H.	32	>1024
	M.P.	8	512
	C.C.*	Neg	512
	F.M.*	Neg	>1024
	B.B.	Neg	32
	J.W.*	16	>1024

* Vasovasostomized males.   N, Neat (result positive in undiluted serum).

Fig. 1. Sperm antigens recognized by antisperm antibodies present in various serum samples (Lanes a–h). Left side of the stained blot (Lane i) shows standards of following M_r in kD: 94, 67, 43, 30, 20·1 and 14·4.
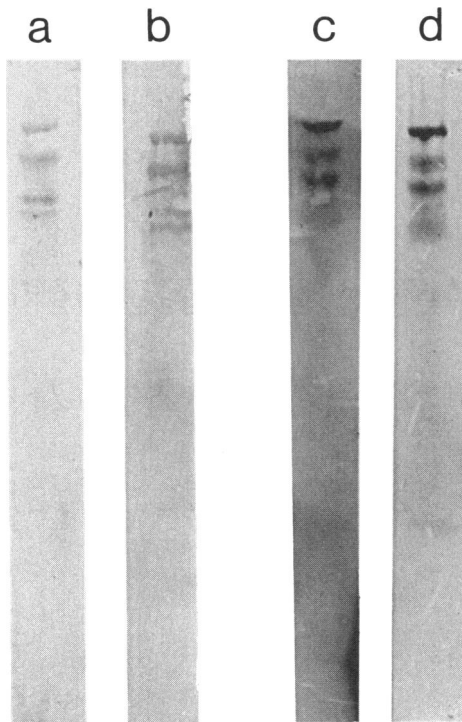
Fig. 2. Sperm antigens observed with serum samples of same patients taken at different times. The antigenic pattern is identical despite a change in the agglutination mode (a, b) and disappearance of the sperm agglutinating activity (as determined by TAT) after a treatment (c, d).

Table 2. Distribution of IgG and IgA antibody in relation to antigenic determinants of human spermatozoa reacting with sera containing antisperm antibodies

Sperm agglut. pattern	TAT titre	Approximate M_r expressed in kD																	
		120		84		76		68		62		53		38		35		18	
		G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A
T	>1024	+++		++		+	+	++	+	+	+							+	+
	1024	+++	+					++	+					+		+			
	32	++	+	++	+	++	+											+	+
H	512	++		++		++				++									
	16	++	+	++	+			++	+	++	+								
TT	32	++	+	+	+									+++		+++			
T/TT	256													++					
H/T	256																	+	+
T/H/TT	512	++	+	++	+	+				++	+	++	+			++			

T, T-T; H, H-H; TT, TT-TT.

of bands were detected (M_r 72 and 66 kD). This observation strongly suggests that the antigens recognized with sera from patients with antisperm antibodies were specific to infertility.

In order to find out whether the sperm agglutinating activity in serum could be removed, three serum samples (Patients R.H., R.T., B.O.) were incubated with reduced sperm antigen preparation. Reduced intensity of all bands was noted in two sera (R.H. and R.T.) and complete disappearance in one serum (B.O.). The TAT results showed a decrease of sperm agglutinating activity in one serum (R.H.) and complete disappearance in two sera (R.T. and B.O.). The same serum samples were incubated with the reducing buffer free of spermatozoa. These control sera reacted like the intact sera and did not show any reduction in activity in either the blotting technique or TAT. Following absorption of these three sera with whole packed spermatozoa, all antigenic bands with the exception of M_r 84 and 62 kD were removed. The TAT showed a decrease in sperm agglutinating titres (from one to five titre steps) in all samples.

The pattern of antigens bound by antisperm antibody were analysed in serum samples obtained sequentially from seven patients (four spontaneously infertile and three vasectomy reversals) in whom change of agglutination mode occurred either spontaneously or after steroid treatment. The pattern of antigens remained unchanged despite the changes in the patterns of agglutination, e.g. from T-T/TT-TT to H-H (Fig. 2 a, b). Three serum samples were examined which were positive by TAT with different titres and type of agglutination and which became negative following prednisolone treatment. The blotting technique demonstrated that the antigenic pattern remained the same; bands of identical molecular weights and intensity were found (Fig. 2 c, d). The same was also true for one serum sample which was negative before the patient's vasectomy reversal but became positive after the operation.

Antigens of M_r 120, 84, 76, 68, 62, 53, 38, 35, 18 kD were recognized by IgG antibody and those of M_r 120, 84, 76, 68, 62, 53, 18 kD were also seen to some extent by IgA antibody. IgM was not detected (Table 2).

Four seminal plasma (SP) samples containing high titres of antisperm antibodies were examined in the same manner as the serum samples but revealed no bands.

DISCUSSION

This analysis of sperm antigens has shown that antisperm antibodies are binding to discrete and specific sperm-associated antigens, but there is no substantial difference between the antigenic patterns observed with antibodies causing sperm immobilization or producing different types of sperm agglutination. This is in agreement with absorption studies of Poulsen & Hjort (1981) which showed that agglutinating sera reacted with the same antigens independently of their mode of

agglutination. The polypeptide chains of 32, 41, 77 and 120 kD revealed by their sera are comparable to those of 35, 38, 76 and 120 kD in our findings. In our studies neither the antigens detected, nor the intensity of reaction, show significant differences. There is a tendency for H-H agglutinating sera to react more strongly with antigens of high molecular weight while other agglutination modes react more strongly with bands of lower molecular weight. The number and intensity of the bands was not related to antisperm antibody titre. Sera with relatively low titres of antibody (< 32) reacted with as many as five different antigens while many sera with very high titres (> 512) did not react at all.

As well as antigens which are inherent to spermatozoa, some antigens may be derived from seminal plasma (sperm coating antigens (SCA)). Among these SCA are the ABO blood group antigens. We think, however, that it is very unlikely that we are detecting ABO blood group antigens, as spermatozoa from several donors with different ABO status were used and the same blotting patterns were obtained with various preparations. It would still be interesting in future investigations to determine if any of the antigens we have detected are derived from SP and further whether all these antigens are tissue-specific. It would be unlikely that the antibodies are cross-reacting with other tissues as no association has been found between antisperm antibodies and antibodies directed against other tissues, e.g. gastric parietal cells, pancreatic islet cells, adrenal cortex, thyroglobulin and thyroid microsomes (Parslow, 1985).

The absorption experiments with whole spermatozoa suggest that the majority of the antigens detected are of membrane origin. Only 84 and 62 kD bands were not removed suggesting that these were probably of cytoplasmic or nuclear origin. These internal antigens could be protamines, as antibodies to protamines of similar molecular weights have been identified in infertile and vasectomized men (Samuel, Kolk & Rumke, 1978). It is possible that some antigens are destroyed and lost in the reduction and boiling process, which would explain the lack of reaction with sera containing high levels of antisperm antibodies but the complete absorption of activity obtained by incubation with the reduced sperm preparation suggests that the antigens were intact.

We have tried using two different methods of cell membrane preparation to obtain an antigenic extract, without success; no binding was observed in the antibody overlay experiments.

The observation that, with sequential serum samples the antigenic pattern remained unchanged although the patterns of agglutination changed, only leaves variations in the antibody class as the reason for the different types of agglutination. In this event changes in the agglutination pattern seen in the follow-up studies would be little more than changes in the predominant isotype. Our experiments, however, showed no specificity related to the antibody class although IgM antibody was not seen in these experiments. Indeed, it would be difficult to see what factors are likely to have provoked such a change in patients whose possession of antisperm antibodies is effectively a chronic condition. There is a possibility that other factors rather than antigen specificity are involved. The different agglutination types could be reflecting the affinity or extention of antigen-binding site of the antibody molecules. The effect of an electric charge could also be relevant since the sperm heads carry higher positive charge than the sperm tails (Lindahl, 1968). Alternatively, the agglutination patterns can change with time (Linnet & Hjort, 1977) and could be therefore due to mobility of the antigens on the surface of spermatozoa after reacting with antibody.

The absence of binding to separated sperm proteins using seminal plasma samples may perhaps be easily explained by the different assay systems used for detection of the antibody. It is possible that in seminal plasma the antisperm antibodies are complexed with soluble sperm antigens or antigenic components of the SP itself. During TAT testing the sperm surface may successfully compete for the binding of this complexed antigen. However the avidity of such a reaction is likely to be much higher than the binding of antibody to the individual denatured sperm proteins on the NC blots. Fractionation of seminal plasma to separate the antibody from the antigen should answer this question.

The preliminary characterization of the antigens has prepared the way to investigate which antigens are significant in infertility. It is likely that antigens of high molecular weights play the important role in infertility since these were most frequently recognized by antibodies from sera of infertile patients. In support Lee *et al.* (1983) reported an association of high molecular weight antigen (90 kD) on human sperm with autoimmune infertility. Lehmann *et al.* (1985), on the other

hand, correlated an antigen of 14 kD with infertility, but their patients were selected on the basis of 'unexplained infertility' and the results of serum antisperm antibody tests were unknown.

Clearly, there is no simple answer to the question of which antigens play the major role in immunological infertility. It is now essential to obtain the antibodies binding to the specific antigens in a purified form. This will enable a full characterization of the effect that individual antibody–antigen reactions have upon the behaviour of the spermatozoa.

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