Anti-Mouse Sperm Antiserum

Fertility Inhibition *In Vitro* and Preliminary Antigen Identification

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Rabbit anti-mouse sperm (αMS) Fab fragments have been prepared, which are capable of inhibiting sperm-zona interaction (both binding and penetration) and sperm-egg fusion *in vitro*. In cumulus-intact eggs, αMS Fab did not inhibit zona penetration, but did inhibit sperm-egg fusion, implying that separate and distinguishable antigens are involved in these two fertilization events. Preliminary identification of the antigens recognized by αMS IgG, using a gel overlay technique, revealed four major antigens of 200 Kd, 55–63 Kd, 37 Kd, and 33 Kd, and three minor antigens of 110 Kd, 48 Kd, and 40 Kd.

Key words: *in vitro* fertilization, anti-sperm antibodies.

Fertilization consists of a carefully organized series of membrane-related events. Crucial to our complete understanding of fertilization is the identity of the gamete surface molecules that participate in each of these events. One route to this goal is *via* an immunologic approach combined with the use of fertilization *in vitro*, which would allow examination of the individual steps in the fertilization process.

Fertility reduction by iso-immunization with spermatozoa is a well-established phenomenon (Metz, 1979 and O'Rand, 1980), although definitive identification of the target antigen(s) has been elusive. The blockage of events both prior to sperm-egg fusion and prior to implantation may be responsible for reduced fertility (Menge et al, 1979; O'Rand, 1981; Tung et al, 1979); however, it is necessary that these events be examined individually. A variety of studies have demonstrated that anti-sperm antisera are capable of interfering with pre-fertilization events *in vitro* (For example, Menge, 1971a, 1971b; Metz, 1972; Tzartos, 1979; Tung et al, 1980; Huang et al, 1981; O'Rand, 1981; Yanagimachi et al, 1981). Thus it appears not only that molecules that are intrinsic to the sperm cell are auto- and alloantigens, but that at least some of them function during different stages of the fertilization process.

In the experiments presented, we provide the first description of a polyclonal antiserum, for mouse gametes, capable of inhibiting gamete interaction at various levels together with a preliminary identification of the sperm antigens that may be involved in these interactions. Mouse sperm surface antigens recognized by heterologous rabbit antiserum have been described (Herr and Eddy, 1980), and numerous studies on fertility reduction of mice *in vivo* have also been described (For example, Tung et al, 1979; Tsunoda and Chang, 1976; McLaren, 1966). However, the use of heterologous antiserum, coupled with fertilization *in vitro*, in the present study provides a starting point for target antigen identification and eventual isolation.

Methods

Antiserum Preparation

Female New Zealand white rabbits were immunized with epididymal mouse spermatozoa that had been washed (three times) by centrifugation and suspended finally in PBS. The rabbits received three injections (subcutaneous) over a two-month period, each injection consisting of about $0.5-1.0 \times 10^8$ spermatozoa. The rabbit was bled 10 days after the final injection. The IgG fraction of the anti-sperm serum was prepared by DEAE chromatography (O'Rand and Porter, 1979). Fab frag-

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ments of the IgG fraction were produced by digestion with papain (Porter, 1959), and were chromatographed on a Sephadex® S-200 column (2.5 × 100 cm in PBS buffer, *p*H 7.0). The Fab preparation was tested for its loss of sperm agglutinating abiltiy and complement-dependent cytotoxicity. Fab fragments of two different control antisera (anti-adjuvant and anti-BSA) were produced in a similar manner.

To determine the specificity of the anti-sperm Fab preparation, this fraction was absorbed either with homogenized mouse testis or with homogenized mouse spermatozoa recovered from the cauda epididymis. An aliquot of the Fab preparation was incubated with an equivalent volume of absorbant in a shaking water bath at 37 C. After 30 minutes, the suspension was centrifuged, and the supernatant (1×, Table 4) was assayed for removal of antibody activity. This absorption procedure was repeated for two more cycles using fresh absorbant for each cycle; the supernatant which resulted after the third absorption cycle (3×, Table 4) was also assayed for removal of antibody activity.

Cytotoxicity

Sperm immobilization (cytotoxic) activity of the IgG fraction of the anti-mouse sperm antiserum was assayed by the procedure of O'Rand and Metz (1976). Briefly, this involved mixing 10 μl of spermatozoa ($\sim 10^7$ cells/ml) with 10 μl of antibody. After 5 minutes at 37 C, 10 μl of a 1:2 dilution of guinea pig complement was added. After 20 minutes of incubation in a shaking water bath at 37 C, the number of immobilized sperm was determined by computing the average percentage of immobilized sperm from 6 different 16× objective microscope fields.

Immunodiffusion

Immunodiffusion was carried out on a 2 × 2" plate in 1.5% agarose with 0.05 M barbital buffer, *p*H 8.6, in a humid chamber for 24 hours.

Fertility Studies

The procedures and materials used for the fertilization of mouse gametes *in vitro* are detailed in Saling (1981, 1982). An abbreviated account is given here.

The medium used was a modified Krebs-Ringer bicarbonate medium supplemented with energy substrates (pyruvate, lactate, glucose) and BSA (20 mg/ml; Fraction V, Sigma, St. Louis, MO), *p*H 7.4, equilibrated with 5% CO_2/95% air. This medium, designated CM, supports both capacitation and fertilization *in vitro*. Tubal mouse eggs were recovered from superovulated mature mice (randomly-bred Swiss Webster) at 13–15 hours after the hCG injection. Gametes were maintained throughout the experiment at 37 C under a layer of sterile silicone oil (dimethylsiloxane), previously equilibrated with 5% CO_2/95% air. When required, cumulus cells were removed by brief (5–10 minutes) incubation in hyaluronidase (0.1% in CM, Sigma, St. Louis, MO, Type 1-S). Zonae pellucidae were removed mechanically by forcing cumulus-free eggs through narrow-bore micropipettes. Mouse sperm suspensions were prepared by rupturing the excised caudae epididymides of mature (>12 weeks) mice in CM; 0.2 ml CM was used for each epididymis. After a 10 minute period for sperm dispersal, an aliquot of the sperm suspension was diluted 1:5 in CM. Sperm were incubated for 90–120 minutes at 37 C to permit the occurrence of capacitation.

Following the incubation interval for capacitation *in vitro*, spermatozoa were added to 200 μl of CM to achieve a final concentration of $\sim 10^5$ cells/ml. Twenty microliters of the Fab preparation under examination were then added to the capacitated sperm. After 10 minutes, mouse eggs of the type required (cumulus-intact, cumulus-free and zona-intact, or zona-free) were added to the sperm plus Fab suspensions. The gametes were recovered after 4 hours at 37 C in an atmosphere of 5% CO_2/95% air. Glutaraldehyde-fixed eggs were mounted on slides, stained with aceto-lacmoid (Toyoda and Chang, 1974), and examined with phase-contrast optics. Eggs were considered to be penetrated by the presence of spermatozoa within the perivitelline space or egg. The occurrence of fertilization was determined by identification of both the fertilizing sperm tail and the sperm head (or pronucleus), in addition to the maternal pronucleus within the egg.

Electrophoretic Techniques

Washed epididymal mouse spermatozoa were solubilized in gel sample buffer (Laemmli, 1970) and electrophoresed in a 10% polyacrylamide-SDS gel. After electrophoresis, the fixed gels were overlaid with αMS IgG, followed by affinity purified ^{125}I-goat-anti-rabbit immunoglobulin, according to the procedure of Burridge (1976, 1978).

Results

Cytotoxicity

When motile epididymal mouse sperm were incubated with the anti-mouse sperm (αMS) IgG, the suspension became agglutinated rapidly in a head-head, head-tail, and tail-tail manner, indicating that the antiserum contained antibodies that were generated toward antigens located in the sperm surface. Control IgG did not exhibit this pattern. To examine this interaction in a more defined manner, the cytotoxic titer of αMS IgG was measured. In the presence of guinea pig complement, the cytotoxic titer of αMS IgG was 1/1536 (Fig. 1).

Indirect immunofluorescence was used to ensure that papain digestion of the antiserum IgG did not alter interaction with the binding sites on the sperm surface. Comparison of the fluorescence pattern, generated by the staining of motile mouse spermatozoa with αMS IgG and αMS Fab, indi-

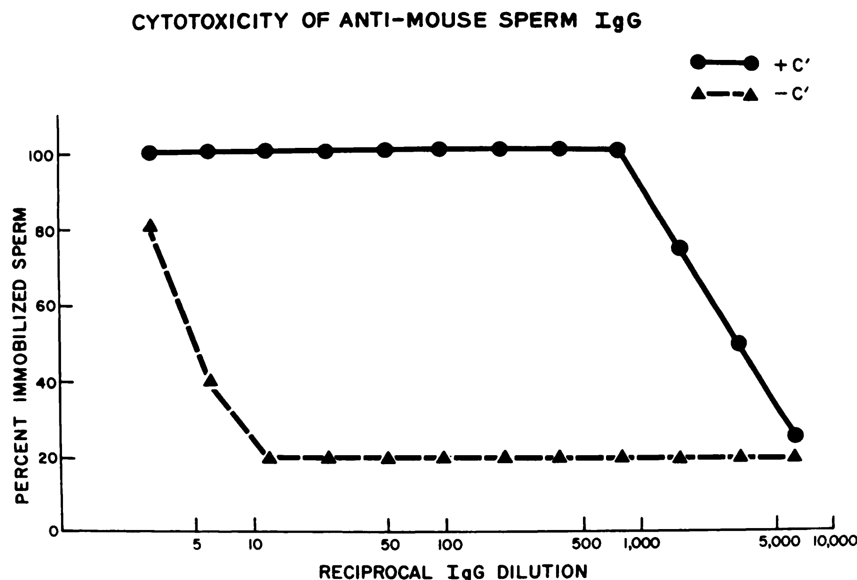

Fig. 1. Complement-dependent cytotoxic activity of rabbit anti-mouse sperm IgG on freshly isolated mouse epididymal sperm. +C': guinea pig complement present; −C': guinea pig complement absent.

cated that in both cases the entire sperm cell was uniformly and intensely fluorescent (data not shown). Control Fab did not label.

Cross Reactivity

The reactivity of αMS IgG toward various mouse tissues was tested by immunodiffusion (Fig. 2). Both reproductive (testis and cauda epididymal sperm) and non-reproductive (brain, spleen, liver and kidney) tissues were tested; only testis and spermatozoa formed precipitin bands with αMS IgG.

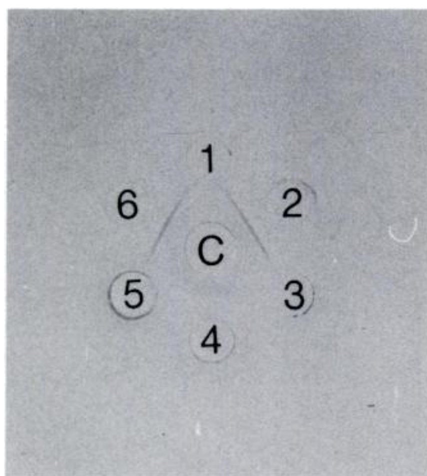

Fig. 2. Immunodiffusion plate using 1.5% agarose in 0.05 M barbital buffer, pH 8.6. Center well (C) contains 20μl of rabbit anti-mouse sperm IgG (3.4 mg/ml). Well #1: mouse brain; well #2: mouse cauda epididymal sperm; well #3: mouse kidney; well #4: mouse liver; well #5: mouse spleen; well #6: mouse testis. Each well received 10 μl of tissue extract.

Fertilization Studies

Incubation of either capacitated or freshly recovered non-capacitated epididymal mouse spermatozoa in αMS Fab for long periods (≤6 hours) permitted levels of motility (high) and agglutination (low) that were indistinguishable from that exhibited by control sperm suspensions that either contained no Fab preparation or contained anti-adjuvant or anti-BSA Fab fragments at protein concentrations, comparable to that in the immune Fab preparation.

Brief preincubation of capacitated mouse spermatozoa with αMS Fab, at a concentration of 3.4mg protein/ml, resulted in a complete loss of their ability to fertilize cumulus-intact mouse eggs, despite the finding that sperm penetration through the zonae pellucidae of these eggs was not abolished entirely, but decreased by 63% (Table 1). When the average number of sperm that had penetrated through the zona pellucida in this case is considered (Table 1), it appears that this capability was only slightly impaired in αMS Fab-treated spermatozoa. Inhibition of cumulus matrix dispersion was not observed in the presence of any of the Fab-treated sperm.

If eggs, which have had their cumulus cells removed by brief hyaluronidase treatment, were challenged with αMS Fab-preincubated sperm as above, both the ability to penetrate the zona pellucida as well as the ability to fuse with the egg plasma membrane were lost entirely (Table 2). Although sperm binding to zonae pellucidae was

TABLE 1. Effect of Fab Fragments upon Fertilization Using Cumulus-intact Mouse Eggs

Papain-Digested Antiserum	% Eggs Penetrated (Number of Eggs Penetrated/ Total Number of Eggs)	% Eggs Fertilized (Number of Eggs Fertilized/ Total Number of Eggs)	Mean Sperm/Egg (Number of Penetrated Sperm/ Total Number of Penetrated Eggs)
None	85% (62/73)	78% (57/73)	4.2 (258/62)
Anti-Adjuvant	77% (52/68)	75% (51/68)	5.7 (297/52)
Anti-BSA	76% (50/66)	70% (46/66)	4.7 (233/50)
Anti-Mouse Sperm	37% (40/109)	0 (0/109)	3.1 (122/40)

not investigated quantitatively, observation of the interacting gametes indicated that this pre-fertilization step was curtailed severely when spermatozoa were pretreated with αMS Fab, despite the presence of highly motile "activated" mouse sperm in the preparation. As was also the case with the cumulus-intact eggs, high levels of both penetration and fertilization, as well as sperm binding to the zona pellucida, were observed in the cumulus-free eggs that were inseminated with control Fab pre-treated spermatozoa (Table 2).

Similarly, when zonae pellucidae were removed mechanically from mouse eggs, fertilization was not achieved if the eggs were challenged with capacitated sperm preincubated in αMS Fab (Table 3). As seen previously, high levels of fertilization were observed using the control Fab preparations (Table 3).

In the experiments described above, αMS Fab was used at a concentration of 3.4 mg protein/ml. To examine the effect of varying concentrations of αMS Fab on fertilization, cumulus-free eggs were challenged with mouse sperm that had been pretreated with three different concentrations of αMS Fab. As was found in the earlier studies, none of 71 eggs were penetrated or fertilized when sperm were pre-incubated with 3.4 mg αMS Fab/ml. When 1.3 mg/ml were used for sperm preincubation, one of 34 eggs (3%) were fertilized; when 0.5 mg/ml was used, six of 47 eggs (13%) were fertilized. This approximate titration suggests that the inhibitory activity of the αMS Fab is concentration-dependent.

The inhibitory activity of αMS Fab was removed by absorption of the Fab preparation with either mouse testis or mouse spermatozoa (Table 4). When the αMS Fab was absorbed once with homogenates of these tissues, a fairly low level of fertilization was achieved, whereas when the Fab preparation was absorbed three times, significantly higher levels of both penetration and fertilization were observed (Table 4).

Antigen Identification

Identification of the mouse epididymal sperm proteins recognized by the αMS IgG using the gel

TABLE 2. Effect of Fab Fragments upon Fertilization *In Vitro* Using Cumulus-free, Zona-intact Mouse Eggs

Papain-Digested Antiserum	% Eggs Penetrated (Number of Eggs Penetrated/ Total Number of Eggs)	% Eggs Fertilized (Number of Eggs Fertilized/ Total Number of Eggs)	Mean Sperm/Egg (Number of Penetrated Sperm/ Total Number of Penetrated Eggs)
None	94% (30/32)	94% (30/32)	6.1 (183/30)
Anti-Adjuvant	94% (32/34)	82% (28/34)	7.9 (252/32)
Anti-BSA	100% (32/32)	81% (26/32)	3.2 (102/32)
Anti-Mouse Sperm	0 (0/33)	0 (0/33)	0

TABLE 3. Effect of Fab Fragments upon Fertilization *In Vitro* Using Zona-free Mouse Eggs

Papain-Digested Antiserum	% Eggs Fertilized (Number of Eggs Fertilized/ Total Number of Eggs)	Mean Sperm/Egg (Number of Penetrated Sperm/ Total Number of Penetrated Eggs)
None	100% (52/52)	3.6 (187/52)
Anti-Adjuvant	100% (48/48)	4.3 (208/48)
Anti-BSA	98% (41/42)	4.5 (183/41)
Anti-Mouse Sperm	1% (1/85)	1.0 (1/1)

TABLE 4. Effect of Absorption upon Levels of Fertilization *In Vitro*

Papain-Digested Antiserum		% Eggs Penetrated (Number Eggs Penetrated/Total Number of Eggs)	% Eggs Fertilized (Number of Eggs Fertilized/Total Number of Eggs)	Mean Sperm/Egg (Number of Penetrated Sperm/Total Number of Penetrated Eggs)
None		76% (64/84)	64% (54/84)	3.2 (204/64)
Anti-Mouse Sperm		>1% (1/105)	>1% (1/105)	1.0 (1/1)
Absorbed with Mouse Testis	(1×)	4% (2/57)	0% (0/57)	2.0 (4/2)
	(3×)	67% (24/36)	17% (6/36)	6.8 (162/24)
Absorbed with Mouse Sperm	(1×)	37% (15/41)	27% (11/41)	1.8 (27/15)
	(3×)	86% (36/42)	57% (24/42)	3.4 (122/36)

overly technique is shown in figure 3. At least four major bands can be seen; these have apparent molecular weights of 200 Kd (A), 55–63 Kd (C), 37 Kd (F), and 33 Kd (G). Minor bands are shown at 110 Kd (B), 48 Kd (D), and 40 Kd (E). The most prominent band, at (C), was resolved on 7–15% gradient gels into two bands, one of 63 Kd and a second of 59 Kd. Several minor bands of lower molecular weight, between 12 Kd and 32 Kd, were also resolved on gradient levels (data not shown).

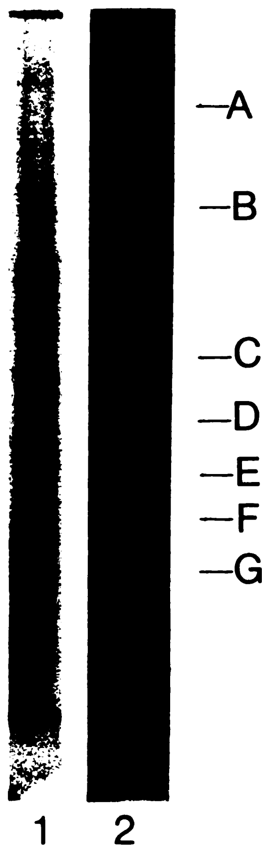

Fig. 3. Lane 1: SDS-PAGE on a 10% slab gel of mouse epididymal sperm. Coomassie blue stained, approximately 5 μg protein loaded. Lane 2: autoradiograph of a parallel 10% gel which was reacted with RαMSIg and ^{125}I-goat anti-rabbit IgG. The molecular weights of the labeled bands are: A, 200 Kd; B, 110 Kd; C, 55-63 Kd; D, 48 Kd; E, 40 Kd; F, 37 Kd; and G, 33 Kd.

Discussion

This study has taken the first step in identifying, by heterologous antiserum, a group of mouse sperm antigens that have an essential function in fertilization. Use of a fertilization *in vitro* system indicated that αMS Fab contained activity capable of inhibiting sperm-zona binding, sperm-zona penetration, and sperm-egg fusion. Unaffected by this αMS Fab preparation were cumulus cell dispersion and the display of "activated" sperm motility. Information concerning an effect of αMS Fab upon the induction of the acrosome reaction is not yet available. Absorption of the antiserum with mouse testis indicated that the essential antigen(s) is testicular in origin. Electrophoretic analysis of mouse sperm, coupled with the gel overlay technique, pointed to at least eight polypeptides that may be considered candidates for specific functions during the fertilization process.

The observation that αMS Fab-treated sperm penetrated the zona pellucida only when cumulus cells were present was a surprising finding which could be explained in several ways. It is possible that there are common molecular configurations on cumulus and sperm cell surfaces which bind specifically the particular antibody that functions to inhibit zona penetration. Alternatively, the cumulus cells or the extra-cellular matrix could adsorb the Fab fragments non-specifically, effectively removing them from the sperm surface. Further experimentation will be needed to distinguish between these possibilities. However, these results do emphasize the contention that interactions between spermatozoa and egg plasma membrane are separate and distinguishable from interactions between spermatozoa and zonae pellucidae.

Comparison of the antigens recognized by our

antiserum with those recognized by the rabbit anti-mouse sperm serum of Herr and Eddy (1980) indicates that the 59 Kd (C), 40 Kd (E), and 33 Kd (G) bands (Fig. 3) are most likely identical to antigens which they determined to be on the surface of mouse sperm by ^{125}I-labeling. Isolation of individual antigens and absorption of the antiserum with them could determine whether each is functional in fertilization. Alternatively, monoclonal antibodies to isolated protein bands might also facilitate elucidation of their function in fertilization.

Finally, we would like to stress that fertilization *in vitro* is a powerful tool with which the fertilization process may be dissected into its component parts and, combined with immunologic procedures, it will undoubtedly lead to the unraveling of many of the subtleties of fertilization.

Acknowledgments

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