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Effect of Sperm-Associated Antibodies on the Acrosomal Status of Human Sperm

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The acrosomal status of human sperm was assessed by the specific binding of *Pisum sativum* lectin to the acrosomal matrix. Immunoglobulin G (IgG) fractions of plasmas that were positive for IgG antisperm antibodies inhibited acrosomal loss, initiated acrosomal loss, or had no effect on acrosomal loss. Two of five sperm samples associated *in vivo* with only IgG, zero of one sample associated with only sperm-associated immunoglobulin A (IgA), and six of eight samples associated with both IgA and IgG underwent acrosomal loss prior to exposure to calcium ionophore. Two sperm samples associated with IgG or IgA or both were inhibited from undergoing acrosome loss after exposure to calcium ionophore. None of the seven antibody-negative sperm samples underwent an increased spontaneous acrosomal loss or were inhibited from undergoing acrosomal loss after exposure to calcium ionophore.

Key words: Antisperm antibodies, acrosome reaction, infertility spermatozoa.

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Freshly ejaculated mammalian sperm do not possess the ability to fertilize ova. Definable environmental influences are required for sperm to become capable of fertilization (Austin, 1951; Chang, 1951). This maturational process is often referred to as capacitation and encompasses many of the changes that sperm undergo from ejaculation to the initiation of the acrosome reaction (Chang, 1951). The biologic influences on the acrosomal status of mammalian sperm have been a focus of study. During the acrosome reaction, the sperm's plasma and outer acrosomal membranes fuse, vesiculate, and release enzymes contained in the acrosomal matrix (Barros et al, 1967; Yudin et al, 1988). The acrosome reaction is the result of influences of the microenvironment of the male and female reproductive tracts on sperm maturation. The specific physiologic inducer of the acrosome reaction in rodents is the zona pellucida. Contact with the zona alters the sperm membrane's permeability to calcium (Florman et al, 1989).

Previous reports indicated that nonhuman sperm antibodies can increase (Trimmer et al, 1986) or decrease (Tung et al, 1980; Podell and Vacquier, 1984; Marquant-LeGuinne and De Almeida, 1986; Saling, 1986) the number of acrosome-

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reacted sperm in animals. Although studies have shown that human sperm antibodies can potentiate (Bronson et al, 1981) or inhibit (Haas et al, 1980; Haas et al, 1985) heterologous ova penetration and inhibit homologous (Clarke et al, 1985) ova penetration, the specific steps by which human sperm antibodies interact with sperm function have not been elucidated. Our objective was to determine if the attachment of antisperm antibodies to the surface of human sperm altered sperm acrosomal loss. To investigate the influence of antisperm antibodies on the acrosomal status of human sperm, we evaluated human sperm acrosomal loss using a fluorescein-labeled lectin derived from the plant *Pisum sativum* (Cross et al, 1986).

Materials and Methods

Patient Population

Plasma assessed as negative for immunoglobulin G (IgG) antisperm antibodies by a radiolabeled antiglobulin assay (Haas et al, 1980) was collected in ethylenediamine tetraacetic acid (EDTA)-coated vacuum tubes. IgG fractions from these plasma samples then were assayed for their effect upon sperm acrosomal loss. Semen samples were obtained from 14 additional men who had previously tested positive for only IgG (n = 5) or immunoglobulin A (IgA; n = 1) or for both sperm-associated IgG and IgA on the surface of their sperm (n = 8). Sperm samples were also obtained from seven men who had tested negative for both sperm-associated IgG and IgA. The plasmas from these 14 men were not assessed for antisperm antibodies. Semen samples were allowed to liquify for 30 to 60 minutes at 37°C, were divided into two aliquots, and were assayed for either sperm-associated immunoglobulin (Ig) or the ability to undergo calcium ionophore-induced acrosomal loss.

Isolation of Plasma IgG Fraction

The procedure used to isolate plasma IgG fractions has been reported previously (McKinney and Parkinson, 1987). In brief, each plasma sample was diluted with acetate buffer, and pH was adjusted to 4.5 with sodium hydroxide. Caprylic acid (Sigma Chemical Co., St Louis, MO) was added dropwise to the plasma and thoroughly mixed; insoluble material was removed by centrifugation. The supernatant was filtered through glass wool to remove fine particles and mixed with concentrated phosphate buffered saline; pH was adjusted to 7.4 with sodium hydroxide. After cooling the supernatant to 4°C, the immunoglobulin fraction was precipitated by adding solid ammonium sulfate sufficient to achieve a 45% saturation. The sample was stirred, and the precipitated IgG was collected by centrifugation. After aspirating the supernatant, the IgG pellet was resuspended in a small volume of phosphate-buffered saline (PBS) equivalent to approximately one tenth of the original volume of plasma. Resuspended IgG was dialyzed overnight against 50 to 100 volumes of PBS; the dialyzed IgG was stored at -20°C.

The purity of the IgG sample was confirmed by gel-permeation high-pressure liquid chromatography (LKB; St Louis, MO; TSK SW 3000 column; 1 ml/min flow; 500-μL sample; 250 μL aliquots collected; buffer consisted of equal volumes of 50 mmol/L $NaSO_4$ and 20 mmol/L $NaPO_4$). The final protein concentration of IgG fractions was 0.32 mg/ml (determined by the optical density at 280 nm). Retention of antisperm antibody activity was assessed by an indirect radiolabeled antiglobulin assay (Haas et al, 1980) substituting 50 μg of the purified IgG fraction for whole plasma.

Direct Radiolabeled Antiglobulin Assay for Sperm-Associated Ig

The method used for direct radiolabeled antiglobulin assay for sperm-associated Ig has been previously described (Haas et al, 1982). In brief, sperm were washed in a modified Hanks' balanced salt solution containing 10 g/L bovine serum albumin and 1 mg/L soybean trypsin inhibitor. Triplicate sperm samples were incubated with either ^{125}I-labeled goat antihuman IgG or IgA. After washing, sperm-associated radioactivity was determined in a gamma counter. Nonspecific radioactivity (which was subtracted from each test result) was determined by performing assays in tubes without sperm. Results were expressed as the percentage of total radioactivity added to each sperm sample that remained associated with sperm.

Assessment of Acrosomal Status

When the effect of plasma IgG fractions on the acrosomal status of human sperm was studied, 7.5×10^6 sperm in 500 μL Biggers, Whitten, and Whittingham (BWW) medium were incubated for 60 minutes at 37°C with 500 μL of a 1:1 dilution of a plasma IgG fraction and BWW. Sperm were then washed twice (800g for 8 minutes) with 15 ml of BWW medium containing 0.35% human serum albumin (HSA, pH 7.4; Sigma Chemical Co.). After the second centrifugation, the supernatant was carefully removed by aspiration, and the sperm pellet was overlaid with 1 ml BWW containing 3.5% HSA and incubated for 3 hours at 37°C in an atmosphere of 5% CO_2/95% air. The necessity of exposing sperm to capacitating conditions in order to induce acrosomal loss has been previously reported (Byrd and Wolf, 1986). The supernatant containing 90% motile sperm was carefully aspirated. When the acrosomal status of sperm associated with Ig *in vivo* was studied, semen samples were washed, capacitated, and enriched for motile sperm by swim-up as described above. The latter sperm samples were obtained from different patients than those from whom the antibody-positive plasma samples were obtained.

Motile sperm were washed once with BWW containing 0.35% HSA and adjusted to a concentration of 5×10^6 sperm/ml with BWW. A 500-μL aliquot of each motile sperm sample was placed in each of two test tubes. Dimethylsufoxide (DMSO; 4 μL; Sigma Chemical Co.)

was added to one sample, and 4 μL of A23187 calcium ionophore (2.62 mg/ml; Sigma Chemical Co.) in DMSO was added to the other sample. The two sperm aliquots were incubated for 2 hours at 37°C in an atmosphere of 5% CO_2/95% air. Retention of sperm motility (percentage of motile sperm) was verified by light microscopy. Sperm were washed once in BWW medium containing 0.35% HSA, and the sperm pellet was incubated in 0.5 ml of 95% ethanol for 30 minutes at 4°C to allow permeation of the sperm plasma membrane (Cross et al, 1986).

The permeated sperm were transferred to a glass microscope slide and air-dried. Sperm then were covered with a few drops of fluorescein isothiocyanate (FITC)-labeled lectin from the plant *P. sativum* (5 mg/ml in PBS; Vector Labs; Burlingame, CA) for 10 minutes at room temperature in a humidified chamber shielded from light. The slides were gently agitated in a beaker of distilled water for approximately 15 seconds. Sperm were overlaid with a solution of p-phenylenediamine (1.1 mg/ml) and sodium azide (1.1 mg/ml; Sigma Chemical Co.) in glycerol and observed by fluorescence microscopy to determine the percentage of sperm which fluoresced uniformly over their acrosomal region. One hundred sperm were evaluated in each sample. Acrosomal fluorescence was associated with acrosome-intact sperm; an absence of fluorescence or the presence of only an equatorial band of fluorescence was associated with sperm that had lost their acrosomes.

Electron Microscopy (EM)

Semen was obtained from a healthy donor and allowed to liquify at 37°C for 60 minutes. The sample was washed twice with PBS containing 0.35% HSA. After the second wash, the sperm pellet was overlaid with BWW medium containing 3.5% HSA and incubated for 3 hours. The supernatant containing motile sperm was carefully aspirated and centrifuged at 800g for 8 minutes. The supernatant was discarded, and the sperm pellet was resuspended in BWW containing 0.35% HSA. For determination of acrosomal status, the pellet was divided into four aliquots, two for electron-microscopic criteria and two for lectin assay. Each sample was adjusted to a final concentration of approximately 5×10^6 sperm/ml. Four microliters of either A23187 calcium ionophore (2.62 mg/ml) in DMSO or DMSO alone were added to two of the 500-μL aliquots of the sperm suspension, which then were incubated for 2 hours at 37°C in an atmosphere of 5% CO_2/95% air. Sperm samples were washed with 5 ml BWW containing 0.35% HSA and resuspended to approximately 25×10^6 sperm/ml. One of the ionophore-treated sperm samples and one of the DMSO-treated sperm samples were evaluated for acrosomal status with FITC-labeled lectin as described above.

The other two sperm samples (one exposed to DMSO alone and one exposed to calcium ionophore in DMSO) were processed for electron microscopy. The two sperm aliquots were placed in poly-L-lysine–coated Petri dishes and then washed three times with PBS. Five hundred microliters of sperm suspension was centrifuged for 5 minutes at 700g. Sperm adhered to the bottom of the dish, were washed twice with PBS, and were incubated for 45 minutes with 4 ml glutaraldehyde solution (2.5%; v/v). The specimen again was washed twice with PBS, overlaid with 0.1 mol/L sodium cacodylic buffer, and processed for electron microscopy.

The specimen was prepared for electron microscopy according to the method of DeBault (1973). In brief, the fixed sperm specimens were stained en bloc in uranyl acetate and dehydrated by washing nine times with increasing concentrations of ethyl alcohol; the three final washes were made with 100% ethyl alcohol. Specimens were incubated in 1 ml Epox 812 (Ernest F Fullam Co, Inc, Schenectady, NY) in ethyl alcohol (50%; v/v) for 1 hour at room temperature. Liquid was aspirated and replaced with 1 ml of 100% Epox, and specimens were incubated overnight at 4°C. Epox was aspirated, and 0.5 ml of fresh 100% Epox was added. An Epox-filled 00 Beem capsule was inverted onto the bottom of the dish for 8 to 15 hours at 60°C. Capsules were forcefully detached from the dish by twisting with a pair of pliers. This resulted in the removal of a small disc of the Epox that coated the bottom of the tissue culture dishes, similar to the effect of a cookie cutter. The Epox block containing the sperm was then microsectioned, stained for 4 minutes with 0.3% lead citrate in water at room temperature, and examined by transmission electron microscopy.

Statistics

Fisher's exact test was used to determine an association between the presence or absence of spontaneous acrosomal loss and positive or negative results from the direct radiolabeled antiglobulin assay. Student's *t* test for the comparison of the means of two samples was used to determine significant differences in acrosomal loss.

Results

Validation of Acrosomal Status

We confirmed that acrosomal loss as assessed by electron microscopy correlated with loss assessed by the FITC-labeled lectin assay using sperm from the same semen pool that were preexposed to DMSO or calcium ionophore (Table 1). Electron microscopy of sperm exposed only to DMSO revealed 81 of 100 sperm with intact acrosomes. Transmission electron microscopy of sperm exposed to calcium ionophore revealed 26 of 100 sperm with intact acrosomes. Sperm with intact acrosomes (DMSO-exposed) were associated with a uniform fluorescence over the acrosomal region (75 ± 3.5% [mean ± SD] of four 100-sperm samples examined), whereas sperm exposed to calcium ionophore displayed only a thin "belt" of fluorescence over the equatorial segment. (Of four

Table 1. *Assessment of sperm acrosomal loss*

Method of assessment	Sperm exposed to medium (%)	Sperm exposed to Ca ionophore (%)
Transmission electron microscopy†	81*	26
Lectin binding assay‡	75 ± 3.5 (x ± SD)	28 ± 4.8 (x ± SD)

* Results are expressed as percentage of sperm with intact acrosomes (mean ± SD in lectin binding assay).
† One hundred sperm were examined.
‡ Four samples, each composed of 100 sperm, were examined.

100-sperm samples examined, 72 ± 4.8% demonstrated reacted acrosomes.)

Effect of In Vitro Antisperm Antibody Binding and Acrosomal Status

The results from the IgG fractions from six representative sera are displayed in Figure 1 and have been arbitrarily labeled A through F. Incubation of sperm with IgG from patient B caused a statistically significant increase in the percentage of sperm that had undergone acrosome loss compared to sperm exposed to medium ($P < 0.001$), compared to sperm exposed to IgG from a sperm antibody-negative plasma (patient A; $P < 0.01$), or compared to the IgG fractions from patients C ($P < 0.002$), D ($P < 0.001$), E ($P < 0.001$), and F ($P < 0.001$; Fig. 1). None of the swim-up sperm samples demonstrated an alteration in the percentage of motile sperm (approximately 90%) after exposure to any IgG fraction from an antibody-positive plasma. These sperm were exposed to the DMSO diluent only and not to calcium ionophore. It noteworthy that even when sperm had been exposed to DMSO alone, the only statistically significant decrease ($P < 0.02$) in acrosomal loss occurred when sperm were exposed to the IgG fraction from patient E. This IgG fraction was found to inhibit calcium ionophore-induced acrosomal loss.

When donor sperm were exposed to calcium ionophore after incubation with IgG fractions (Fig 1), a high and uniform percentage of sperm exhibited acrosomal loss; the sperm that did not were those exposed to IgG from patient E. IgG from patient E caused a significant decrease in sperm exhibiting acrosomal loss compared to medium ($P < 0.001$), compared to IgG from a sperm antibody negative plasma ($P < 0.01$), or compared to the IgG fractions from patients B, C, D, and F ($P < 0.01$).

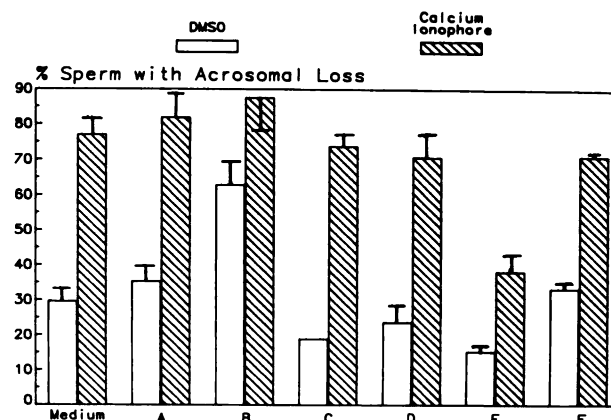

Fig 1.—The percentage (mean ± SD) of motile sperm preexposed to BWW medium or IgG fractions of plasmas negative (A [n = 4; n = number of times the IgG fraction was assessed], Neg) or positive B [n = 3], Pos Acr = positive for sperm acrosome, equatorial, and postequatorial regions; C [n = 2], Pos Tail = positive for sperm tail and equatorial regions; D [n = 3], Pos Tail; E [n = 2] Pos Equa = positive for equatorial and postequatorial regions; or F [n = 2) Pos Tail) for IgG antisperm antibodies undergoing spontaneous acrosomal loss following exposure to the DMSO diluent (open bars) or calcium ionophore-induced acrosomal loss (hatched bars) as assessed by FITC-labeled lectin binding. The alphabetical designations correspond to the patient designations in the text.

Effect of IG Associated In Vivo with Human Sperm on Acrosomal Reactivity

Based on our *in vitro* experiments, we expected three possible consequences of an Ig molecule to become attached to the human sperm surface: antibodies could induce, inhibit, or have no effect on acrosomal loss. Men without sperm-associated antibodies had less than 40% of their sperm undergoing spontaneous acrosomal loss. Using the 40% value as the lower limit, elevated levels of spontaneous acrosomal loss were demonstrated in two of five men who tested positive for sperm-associated IgG only, not in the one man positive for sperm-associated IgA only, and in six of eight men positive for both sperm-associated IgG and IgA (Fig 2). There was a statistically significant correlation ($P = 0.002$) between a positive assay result for sperm-associated Ig and premature acrosomal loss ($\geq 40\%$ acrosomal loss). Thus, in this subgroup of men, antibody binding was associated with acrosomal loss.

To assess the possibility that acrosomal loss was inhibited by the sperm-associated antibody in the remaining antibody-positive men (three with sperm-associated IgG only, one of three with sperm-associated IgA only, and two with both IgG

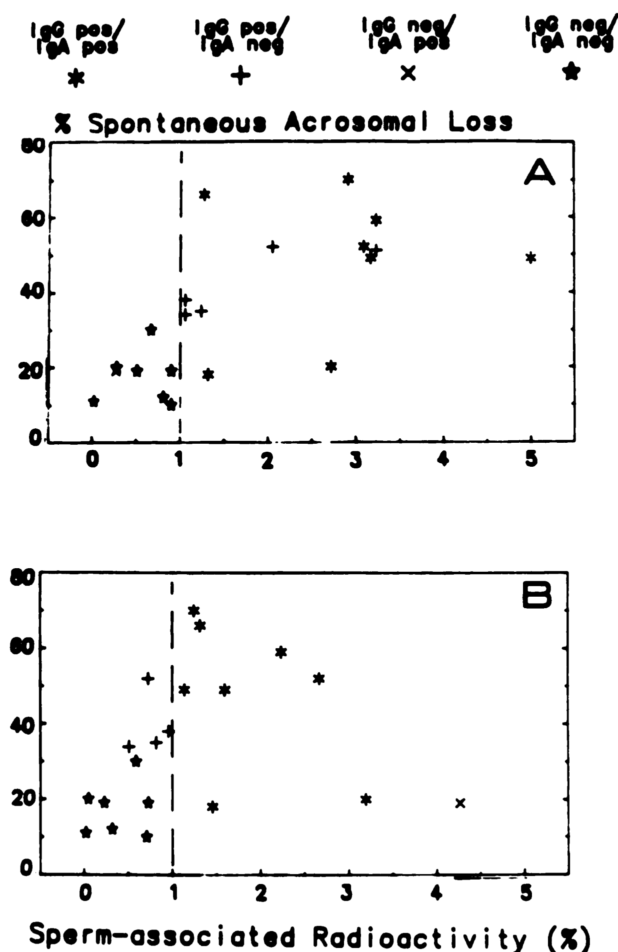

Fig 2.—The percentage of sperm undergoing spontaneous acrosomal loss from men who tested negative (<1% sperm-associated radioactivity) or positive (≥1% sperm-associated radioactivity) for *in vivo* sperm-associated IgG or IgA. The results of testing for sperm-associated IgG are shown on the abscissa in (A); results of testing sperm-associated IgA are shown on the abscissa in (B). The sperm were exposed only to DMSO, so the measured acrosomal loss was considered spontaneous.

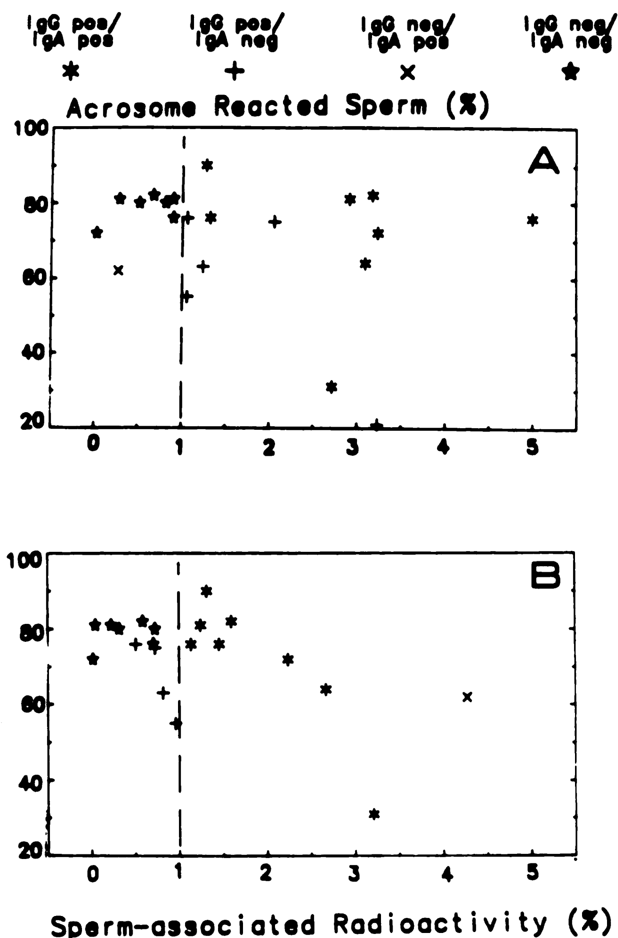

Fig 3.—The percentage of sperm with spontaneous plus ionophore-induced acrosomal loss from men who tested negative (see fig 2) or positive for *in vivo* sperm-associated IgG or IgA. The sperm were exposed to calcium ionophore in DMSO. The bars represent one SD.

and IgA), their sperm were exposed to calcium ionophore. Sperm from two antibody-positive men demonstrated impaired acrosomal loss after exposure to calcium ionophore (Fig 3). One of these men was positive for both sperm-associated IgG and IgA, whereas the other was tested only for sperm-associated IgG and found to be positive. Because of oligospermia, this man was tested for only one class of sperm-associated Ig.

Sperm from men without sperm-associated Ig did not exhibit spontaneous acrosomal loss (Fig 2) and underwent acrosomal loss when exposed to calcium ionophore (Fig 3).

Discussion

In order for fertilization to occur, human sperm must undergo the acrosome reaction shortly before penetration of the zona pellucida (Yanagimachi and Chang, 1964). Therefore, the acrosome reaction is a crucial step in human fertilization. It is assumed that the exact timing of acrosomal loss is critical to successful fertilization; if the acrosome reaction occurs too early, too late, or not at all, significant alterations in fertility might occur.

Previously published reports conclude that some antisperm antibodies interfere with or promote ovum penetration (Bronson et al, 1981; Haas et al, 1980; Haas et al, 1985; Clarke et al, 1985), although the precise mechanism of the antibody's influence is unclear. Sperm antibodies can inhibit

the acrosome reaction of the sea urchin (Podell and Vacquier, 1984), the guinea pig (Tung et al, 1980; Marquant-LeGuinne and De Almeida, 1986), and the mouse (Saling, 1986). In the mouse, a specific monoclonal antibody that associates with the murine sperm acrosome has been isolated. This antibody significantly inhibits both mouse fertilization and the acrosome reaction (Saling and Lakoski, 1985). Similar results have been found with certain monoclonal antibodies against sea urchin sperm (Trimmer et al, 1985). It is not known whether antibodies which inhibit acrosomal loss attach to a receptorlike molecule or whether the antibody's position is inhibitive because of its close proximity to the receptor (Tung et al, 1980).

The inhibitory activities of antisperm antibodies may also be due to a stabilization of antigens on the plasma membrane. Some seminal plasma components stabilize the sperm acrosome, thereby inhibiting a premature acrosome reaction by inhibiting capacitation (Eng and Oliphant, 1978). Capacitated washed sperm, when reexposed to these inhibitory substances, regress to their former noncapacitated state (Chang, 1957). It is not known whether antisperm antibodies which inhibit acrosomal loss do so in a manner similar to the action of seminal plasma components.

The plasma membrane overlying the acrosome (Saling et al, 1979), the inner acrosomal membrane (Yanagimachi, 1977), and the plasma membrane (Yanagimachi, 1981) over the equatorial and postacrosomal regions all have been reported to be the site of binding to the zona pellucida. It is logical to assume that sperm antibodies attached to these locations could either mimic or disturb sperm–zona pellucida binding and possibly alter the sperm's acrosomal status. The latter possibility is plausible since zona pellucida binding appears to be integral to initiating the acrosome reaction in some species (Florman and Storey, 1982). The limited number of plasma IgG fractions assessed in this study did not allow a precise correlation of an antibody's site of attachment to the sperm surface and the effect of the antibody on the sperm's acrosomal status.

Monoclonal antibodies against the plasma membrane of sea urchin sperm have been reported to increase calcium influx, although the acrosome reaction is not induced unless subtle increases in pH are also present (Trimmer et al, 1986). Many antisperm antibodies in our study stimulated a spontaneous acrosomal loss similar to the action of surface components of the zona pellucida. Either close proximity or actual attachment to the zona pellucida is necessary to initiate the acrosome reaction in mice (Saling et al, 1979), limiting the number of capacitated sperm which have undergone acrosomal loss at any one time. This is important, since acrosome reacted sperm have only a few hours during which they are capable of fertilization, even though their motility is maintained for a longer interval (Barros et al, 1973).

A shortened interval may exist for acrosome-reacted sperm to reach the ampulla of the Fallopian tube and affect fertilization. Acrosome-reacted human sperm may be capable of fertilization if they contact the ova during their shortened reproductive lifespan (Morales et al, 1988), possibly accounting for pregnancies that occur after insemination or *in vitro* fertilization with sperm from antisperm antibody-positive men.

Our results imply that sperm from a majority of men with sperm-associated Ig may undergo premature acrosomal loss shortly after ejaculation. The antibody-bound sperm from these men may have a decreased lifespan, not because of a direct cytolytic effect of the antibody, but because of a prematurely triggered acrosomal loss mediated by the antibody. Sperm from this subpopulation of antibody-positive men should be incubated with ova after a shorter postejaculation interval than is usual in most *in vitro* fertilization protocols. In addition, with sperm from these men, intrauterine insemination after ovulation may have to be performed to enhance the achievement of pregnancy.

In this study, antisperm antibodies affected acrosomal loss in different ways: 1) inhibition of acrosomal loss; 2) premature initiation of acrosomal loss; or 3) no effect on acrosomal loss. The majority of sperm associated with Ig *in vivo* underwent spontaneous acrosomal loss prior to the addition of calcium ionophore. Our experiments did not examine whether men whose sperm-associated Ig triggered spontaneous acrosome loss also had plasma Ig associated with a parallel affect on sperm acrosomal status. Our data imply that a crucial step in human sperm function, the sperm acrosome reaction, can be altered by antisperm antibodies, an alteration that may result in infertility.

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