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Monoclonal antibody that recognizes an epitope of the sperm equatorial region and specifically inhibits sperm–oolemma fusion but not binding

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Balb/c mice were immunized with purified hamster sperm heads for induction of antisera and the production of monoclonal antibodies that recognize preferentially the equatorial segment. Twenty-six hybridoma clones secreted monoclonal antibodies with strong affinity for spermatozoa. The supernatants of 16 clones contained antibodies against the equatorial segment, of which 11 were specific to this region. Five supernatants (M1–M5) containing antibodies that bind to various regions of the sperm head were selected and assessed for the ability to inhibit hamster fertilization *in vitro* using intact and zona-free oocytes. All the supernatants inhibited fertilization compared with the control. However, M1 supernatant specifically inhibited sperm–egg fusion in a concentration-dependent manner, while sperm–oolemma binding and sperm motility remained unaffected. M1 supernatant recognized an epitope that is exclusive to the equatorial segment and expression of this epitope increased after capacitation and the acrosome reaction. Preliminary immunoblot analysis indicated that M1 monoclonal antibody recognized two protein bands of 37.5 and 34.0 kDa.

Introduction

After penetrating the outer investments of the oocyte (cumulus mass and zona pellucida), the acrosome-reacted spermatozoon fuses with the egg membrane, the oolemma. This process is essential for the incorporation of the spermatozoon into the egg cytoplasm *in vivo* (see Moore and Bedford, 1983; Yanagimachi, 1988; 1994) and is crucial for the rapid delivery of a soluble sperm factor for embryo development (Parrington *et al.*, 1996). Ultrastructural studies in the golden hamster first determined that gamete fusion in mammals is initiated by sperm plasma membrane preserved after the acrosome reaction and is restricted to a region of the sperm head called the equatorial segment (Moore and Bedford, 1978; Bedford *et al.*, 1979). This mode of sperm–egg fusion is a distinct departure from that in non-mammalian vertebrates and invertebrates, but occurs in a similar manner in all mammals examined to date, including humans (Sathananthan *et al.*, 1986) and even the marsupial opossum (Taggart *et al.*, 1993).

Common themes have emerged from the study of cell fusion events in biology (White, 1992; Hughson, 1995). It is evident that viral and cellular membrane fusion processes are mediated by a complex of specific integral and peripheral membrane proteins. Many of these proteins contain specific fusion domains which, when exposed, cause fusion of lipid components from two opposing bilayers. Exactly how these fusion proteins are activated depends on the cell type. For example, haemagglutinin-mediated fusion of influenza virus is influenced by low pH within an endosome (Bullough *et al.*, 1994). In contrast, HIV-1 viral fusion with T cells is mediated by a seven-transmembrane G protein-coupled receptor named fusin (Feng *et al.*, 1996) which may orientate and expose the fusion peptide of the gp120/41 complex. In mammalian gametes, the morphology of fusion is well documented but the biochemical interactions are poorly understood.

Only acrosome-reacted spermatozoa normally reach the perivitelline space of intact oocytes to interact with the oolemma (Moore and Bedford, 1983) and spermatozoa must undergo an acrosome reaction to be capable of fusing naturally with zona-free oocytes (Yanagimachi, 1988) or artificially with erythrocytes (Holt and Dott, 1980). Thus, completion of the acrosome reaction results in a functional sperm fusion protein on the plasmalemma overlying the apical equatorial segment. Studies with liposomes support the hypothesis that the equatorial segment is the sole fusogenic domain of the spermatozoon (Arts *et al.*, 1993). Liposomes only fuse with the equatorial region (after the acrosome reaction) and fail to diffuse to other membrane domains, indicating the presence of a lipid diffusion barrier that prevents lateral movement of intramembranous molecules.

Several putative fusion proteins have been reported for mammalian spermatozoa (Saling *et al.*, 1985; Okabe, *et al.*, 1990; Allen and Green, 1995; Toshimori, *et al.*, 1998) of which

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only one, fertilin, has been well characterized (formerly PH-30; Primakoff *et al.*, 1987). This sperm antigen was identified by a monoclonal antibody, designated PH-30, that inhibits sperm–egg fusion in the guinea-pig (Primakoff *et al.*, 1987). Support for a fusogenic role for fertilin came mostly from investigations in which the gene encoding this protein was cloned (Blobel *et al.*, 1992). Fertilin shares similarity with certain viral adhesion proteins, including membrane topology, proteolytic processing from a larger molecule, a disintegrin binding domain and a putative fusogenic domain (Wolfsberg *et al.*, 1993). However, it is doubtful that fertilin is the principal fusion protein because it is not localized to the equatorial segment of spermatozoa. The original study used immunogold labelling with PH-30 monoclonal antibody. Under the electron microscope, fertilin was clearly localized to the post-acrosomal region of acrosome-reacted guinea-pig spermatozoa and there was no labelling at the equatorial segment (Primakoff *et al.*, 1987). Since guinea-pig fertilization is essentially the same as in other mammals (Yanagimachi, 1994), this localization is incompatible with a primary role for fertilin in the fusogenic process. The argument for fertilin as a fusion protein is further disputed by the finding that the human fertilin α gene is non-functional (Jury *et al.*, 1997) and that mice deficient in fertilin β produce spermatozoa that are still capable of fusion with the oocyte (Cho *et al.*, 1998). Overall, these results indicate that fertilin is more involved in sperm–egg adhesion than in fusion (see Almeida *et al.*, 1995).

In view of the conflicting evidence concerning the proposed role for fertilin and its known localization and properties after the acrosome reaction, monoclonal antibodies were used to investigate sperm antigens that may be involved in gamete fusion. Hamsters were used because their oocyte oolemma is receptive to heterologous spermatozoa and shows little species specificity. Hence, the ability of spermatozoa to fuse with the oolemma may more likely represent intrinsic sperm fusogenic capacity than cell recognition (adhesive) ability.

Materials and Methods

Reagents

All chemicals were purchased from Sigma Chemicals Ltd (Poole) unless otherwise stated.

Animals

Balb/c mice and Syrian hamsters (15–20 weeks) were maintained and housed at the University of Sheffield Field Laboratories. Females were superovulated as described by Moore and Hartman (1984). All procedures were carried out in strict accordance with the Animal (Scientific Procedures) Act 1986, and subjected to review by a local ethics committee.

Monoclonal antibody production

Monoclonal antibodies were produced as outlined by Harlow and Lane (1988). All the procedures for tissue culture were carried out in a horizontal laminar flow hood. Myeloma cells and hybridomas were cultured in 5% CO_2 in air at 37°C and 95–100% humidity (Haraeus Ltd, London). Myeloma cell lines (Sp2) were cultured in a standard medium of RPMI-1640 with L-glutamine supplemented with 10% (v/v) fetal calf serum (FCS), 1% (w/v) sodium pyruvate, and 1% (v/v) 10 000 iu benzyl penicillin and streptomycin antibiotics ml^{-1}. Hybridoma cells were maintained in complete medium containing 20% (v/v) FCS and supplemented with 1% (w/v) L-glutamine, 1% OPI (1.5% (w/v) oxaloacetate, 0.5% (w/v) sodium pyruvate), 2000 iu insulin, and 1% AH (0.01% (w/v) azaserine, 0.14% (w/v) hypoxanthine). HAT (hypoxanthine, aminopterin and thymidine) medium was used for selection of hybridomas. This was complete medium containing 2% (v/v) 50 × stock solution of hypoxanthine, aminopterin and thymidine. All media were filter sterilized using a 0.2 μm Millipore filter and stored at 37°C before use.

Preparation of sperm heads and immunization of mice

Spermatozoa from the cauda epididymidis were collected in minimum essential medium (MEM) as described by Ellis *et al.* (1985). The sperm suspension was sonicated (MSE Soniprep 150, setting 14) for 35 s to separate sperm heads and tails and also to remove the acrosomal cap and expose the inner acrosomal membrane and anterior margin of the equatorial segment. The suspension was filtered through three layers of tissue paper (Kleenex Ltd) to remove sperm tails, and the sperm heads were purified on a discontinuous Percoll density gradient; 90 and 50% Percoll in MEM for 4 h at 10 000 *g*. The sperm heads were aspirated as a discrete band close to the bottom of the gradient with the formation of a continuous gradient. An aliquot of each batch of sperm heads was prepared for light and electron microscopy and the preparation was stored at –20°C. For immunization, sperm heads were diluted to approximately 10^8 ml^{-1} and added to an equal volume of Freund's complete adjuvant. Blood samples were taken from the tail vein of female Balb/c mice (12 weeks old) for pre-immune serum. The females were then injected i.m. with approximately 10^7 spermatozoa in 0.1 ml adjuvant. After 2 weeks, the females were injected again with incomplete adjuvant. After a further 6 weeks, animals were given a booster injection of approximately 10^9 spermatozoa (0.1 ml) from the same initial preparation except without adjuvant and in PBS. Five days after the booster injection, a mouse was killed for hybridoma production by standard protocols (Harlow and Lane, 1988) and using polyethylene glycol (PEG 4000, Gibco, Paisley). Potential hybridoma cells (10^5 ml^{-1}) were pipetted into 96- and 24-well plates, and incubated in 5% CO_2 in air at 37°C. After 5 days the cells were inspected for hybridoma clones. Half of the growth medium was replenished with fresh complete medium every 4 days for 96-well plates and every 6 days for 24-well plates. Supernatant from wells containing hybridoma cells was tested for specific antibody from about 10 days after fusion.

Screening for antibodies

Sera from immunized mice and the hybridoma supernatants were screened for antibodies by indirect immuno-

fluorescence with viable and methanol-fixed hamster spermatozoa at a dilution of 1:50 as described by Ellis *et al.* (1985). Crossreactivity of positive supernatants was assessed with ejaculated rabbit and human spermatozoa and rat epididymal spermatozoa, washed by centrifugation in PBS, air dried on microscope slides and fixed in methanol. Antibodies were isotyped using a commercial kit (Sigma, Poole) according to the manufacturer's instructions.

Cloning of hybridomas by limiting dilution

Positive hybridoma cells were cloned by limited dilution in growth factor-conditioned medium in 96-well plates. This was repeated three times. The concentration of the hybrid cells was adjusted to approximately one cell per well for each clone. Supernatants were collected 10–15 days after limiting dilution. The supernatants were tested by indirect immunofluorescence to verify that a specific clone had been isolated. The cell suspensions were stored in cryotubes in a polysterene cooling container at –70°C. The following day, the cryotubes were transferred to liquid nitrogen at –196°C.

In vitro *fertilization*

Investigation of the effects of monoclonal antibodies (hybridoma supernatants) on *in vitro* fertilization using intact and zona-free oocytes was conducted according to Moore and Hartman (1984) with minor modifications. Fertilization was carried out in 0.1 ml drops of Biggers, Whitten and Whittingham (BWW) medium under mineral oil (Biggers *et al.*, 1971). The medium was supplemented with 0.04% (w/v) BSA and filter sterilized using a 0.2 μm filter. The osmolarity of the medium was 308 mosmol, pH 7.2, after equilibration overnight in a humidified incubator in 5% CO_2 in air at 37°C. Sperm suspensions (5×10^6 spermatozoa ml^{-1}) were incubated in 5% CO_2 in air at 37°C for 3 h to promote capacitation as described by Bavister (1989). After 3 h, hybridoma culture supernatant containing sperm-specific monoclonal antibody or myeloma culture supernatant (0.75–1.0 μg ml^{-1}) was added to the individual drops at a 1:10 dilution or as specified and the incubation was continued for 30 min. Washed oocytes from superovulated females (Moore and Hartman, 1984) were transferred directly to the pre-incubated sperm preparation (containing antibody) or were treated with 0.1% (w/v) trypsin to dissolve the zona pellucida and washed four times in fresh medium before being placed in the drops. Approximately 15–20 oocytes were placed into 0.1 ml pretreated spermatozoa at a concentration of 10^6 spermatozoa ml^{-1}. Oocytes were assessed after 16 h for signs of fertilization as described by Aitken (1986).

Immunofluorescent localization of M1 antigen on spermatozoa bound to zona-free oocytes in the presence of M1 monoclonal antibody was achieved by gently washing oocytes by three transfers in drops of BWW medium and incubation for 1 h in medium containing fluorescein-conjugated goat anti-mouse IgG (1:100 dilution). Oocytes were washed and then examined on paraffin wax spot slides by epifluorescent microscopy.

Electron microscopic localization of M1 antigen

Preliminary ultrastructural localization of M1 antigen was performed on capacitated hamster spermatozoa after 3 h incubation as described above. Sperm suspensions were incubated with monoclonal antibody M1 hybridoma medium at 37°C in 5% CO_2 in air for 1 h. The spermatozoa were washed twice by centrifugation at 1000 *g* for 5 min and resuspended in goat anti-mouse IgG gold conjugate (10 nm) diluted 1:10 in medium and incubated for 1 h. The spermatozoa were washed by centrifugation and fixed in 2.5% (v/v) glutaraldehyde in 0.2 mol cacodylate buffer l^{-1} for 2 h at 4°C before preparation for transmission electron microscopy. Ultrathin sections were examined in a Philips CM10 electron microscope at an accelerated voltage of 80 kV.

Immunoblotting

Preliminary characterization of M1 antigen was achieved by solubilization of spermatozoa and sperm head samples (10^8 spermatozoa ml^{-1}) with 1% (w/v) sodium lauryl sulphate for 15 min, centrifugation at 5000 *g* for 5 min and recovery of supernatant by aspiration. Protease inhibitors were not added as it was assumed enzyme activity would be minimal in detergent. Extracts were then subjected to SDS-PAGE and transferred to nitrocellulose membrane for protein staining or immunoblotting with specific monoclonal antibody as described by Ellis *et al.* (1985).

Immunodot blotting was performed using hamster testis, epididymis, lung, kidney, spleen, brain and liver to determine tissue distribution of M1 antigen. Tissues were removed immediately after death and snap frozen in liquid nitrogen. The tissues were thawed and homogenized in PBS containing 2 mmol phenylmethanesulphonyl-fluoride (PMSF) l^{-1} and solubilized in SDS reducing buffer. The amount of tissue extract was determined using a bicinchoninic acid protein assay kit (Pierce Chemicals, Rockford, IL). Ten micrograms of each protein sample was blotted onto nitrocellulose membrane and processed as described above.

Results

Immunization and production of monoclonal antibodies

The procedure for isolation of hamster sperm heads was very effective. Examination of the preparation with phase-contrast microscopy revealed that the amount of non-head organelles was less than 0.1% (Fig. 1). Electron microscopy confirmed that sperm heads were free of their acrosomal caps and flagella and that little extraneous sperm material was present except for some membrane material. The equatorial segment remained intact in most sperm heads, although the plasma membrane was missing in some cases. Small particles of Percoll were bound to the sperm heads (Fig. 2).

Sera from immunized mice showed reactivity to methanol-fixed hamster spermatozoa and crossreactivity to

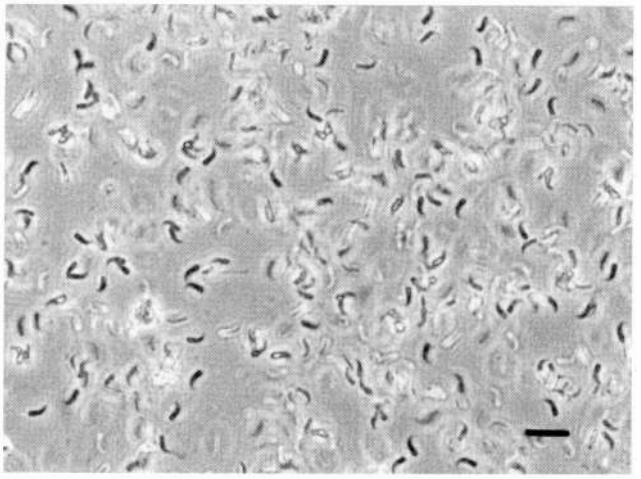

Fig. 1. Phase-contrast light micrograph of purified hamster sperm heads recovered from a Percoll density gradient. Scale bar represents 50 μm.

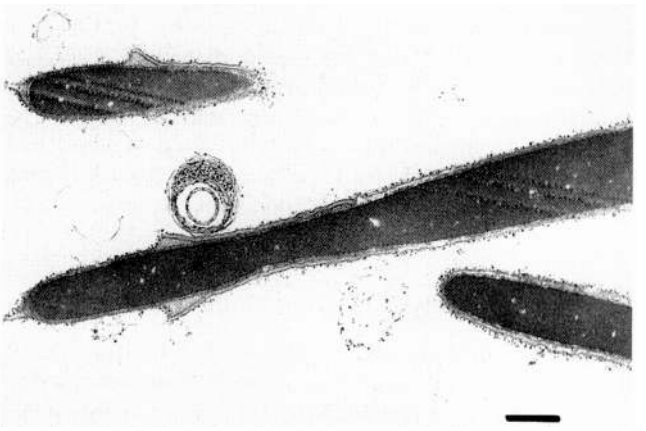

Fig. 2. Transmission electron micrograph of purified hamster sperm heads recovered from a Percoll density gradient. The plasma and acrosomal membranes have been separated from the sperm heads although some membranous vesicles remain. Percoll particles are bound to the spermatozoa. Scale bar represents 2 μm.

methanol-fixed spermatozoa from human, rat and rabbit, as detected by immunofluorescence. In each case, antibodies were localized specifically to the head region of fixed spermatozoa and always to the equatorial segment. There was strong immunofluorescence to the acrosomal and post-acrosomal region with intact hamster spermatozoa. In cases in which the acrosome cap had been lost, antibody was detected on the equatorial segment and post-acrosomal region. In rat, rabbit and human spermatozoa, immunofluorescence was restricted to the entire acrosome of intact cells and to the equatorial segment when the anterior acrosome was missing.

After initial screening protocols, two splenocyte–myeloma fusions yielded 56 hybridoma clones secreting anti-sperm antibody. Of these, 26 supernatants had high affinity for spermatozoa, as assessed by immunofluorescent localization, and showed five different patterns of staining (see Table 1). Sixteen supernatants contained antibodies that recognized the equatorial segment, of which 11 were specific to this region. One hybridoma that secreted antibody for each localization pattern was selected and cloned fully for monoclonal antibody production. Immunofluorescent localization of the binding of these monoclonal antibodies (designated M1–M5) with viable spermatozoa from the cauda epididymidis incubated in BWW medium is shown (Fig. 3). M1 localized to the equatorial segment, M2 to the neck region, M3 to the post-acrosomal region, M4 to the anterior acrosome and equatorial segment, and M5 to the equatorial segment and the 'zipper' region (see Eddy and O'Brien, 1994). Fluorescence was not observed in the absence of primary antibody, or in the presence of pre-immune serum or supernatant of non-immunoglobulin secreting cell lines. Isotyping testing indicated that all the monoclonal antibodies were IgG1 subclass, except for M1 which was IgG2a.

Effect of monoclonal antibody on in vitro *fertilization*

During incubation *in vitro*, hamster spermatozoa agglutinated spontaneously, but subsequently dispersed and became freely motile as capacitation proceeded. After 3 h, a proportion of spermatozoa showed hyperactivated motility. The addition of M3 supernatant resulted in sperm agglutination, but the other preparations had no detectable effect on sperm motility. The effect of monoclonal antibody (in culture supernatant) on fertilization *in vitro* with intact hamster oocytes is summarized (Table 2). The results indicate that all the hybridoma supernatants (M1–M5) inhibited fertilization compared with the control myeloma supernatant, but this was most significant ($P \leq 0.005$) for M1, M3 and M5. The supernatants did not have any obvious effect on sperm motility at the dilutions used, with the exception of M3 supernatant which caused sperm agglutination. Spermatozoa were frequently observed in the perivitelline space of oocytes incubated with M1 and M5

Table 1. Hybridoma clones generating sperm-specific antibodies after two myeloma–spleenocyte fusions

Fusion	Positive clones	Hybridoma producing antibodies to various regions of the sperm head				
A	22	7	5	3	7	–
B	4	3	–	–	–	1

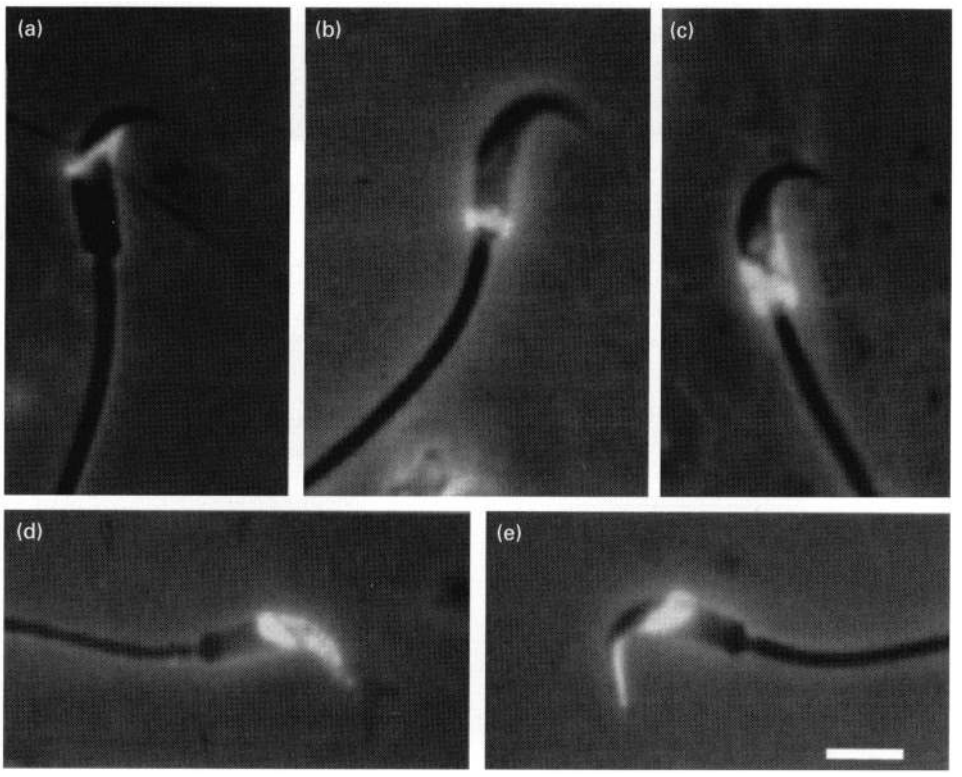

Fig. 3. Indirect immunofluorescent staining patterns for hybridoma supernatants on viable hamster spermatozoa using phase-contrast and UV epifluorescent microscopy. (a) M1, (b) M2, (c) M3, (d) M4 and (e) M5. Scale bar represents 5 μm.

antibodies, but these were not fertilized. The effect of hybridoma supernatant on sperm penetration of zona-free oocytes is summarized (Table 3). M1, M3 and M5 supernatants significantly inhibited ($P \leq 0.005$) sperm–egg fusion. The mean number of spermatozoa bound (but not fused) to zona-free oocytes when spermatozoa were preincubated with control supernatant was 9.1 ± 7.2. When spermatozoa were preincubated with M1 supernatant, a similar mean number of spermatozoa bound to oocytes (8.8 ± 6.1). In contrast, the mean number of spermatozoa bound to oocytes in the presence of M2 and M5 supernatants was 3.0 ± 22 and for M3 was 0.4 ± 0.3. These observations indicate that the inhibition of sperm–egg fusion in the presence of M1 antibody supernatant was due to inhibition of sperm–oolemma fusion rather than sperm–oolemma binding. Therefore M1 monoclonal antibody was investigated further. A titration curve for inhibition of sperm–egg fusion by M1 supernatant was determined (Fig. 4). At a sperm concentration of 10^6 ml^{-1}, maximal inhibition of sperm–egg fusion was achieved with 1:5 and 1:10 dilution ($P < 0.005$). However, there was significant inhibition ($P < 0.05$) of sperm–egg fusion at a dilution of 1:40 compared with control supernatant (86% sperm–egg fusion at the same dilution of supernatant). At each dilution of hybridoma supernatant, there was no difference in the number of spermatozoa initially bound to oocytes (12.8 ± 6.1).

Characterization of immunofluorescent localization of M1 monoclonal antibody during capacitation and sperm–egg interaction

In non-capacitated viable spermatozoa, a faint narrow band was visualized at the apical margin of the equatorial region (Fig. 5a). When spermatozoa were examined after capacitation *in vitro*, there was an increasing proportion of viable (motile) spermatozoa exhibiting greater localization of antibody over the equatorial segment, as assessed by fluorescent intensity and area (Fig. 5b). At high magnification under phase-contrast microscopy, these motile spermatozoa were also observed to have undergone the acrosome reaction. In three experiments, the development of antigen expression on spermatozoa under *in vitro* fertilization capacitating conditions was quantified. Assessment of immunolocalization of M1 in 200 spermatozoa after 1 h of incubation revealed that 88 ± 9% of cells had fluorescent staining on only the apical margin of the equatorial segment. After 3 h, this proportion decreased to 32 ± 15% and the remaining cells showed fluorescence all over the equatorial segment, although often the distribution was patchy. In methanol-fixed spematozoa, M1 monoclonal antibody localized intensely throughout the equatorial segment by indirect immunofluorescence (Fig. 5c).

Spermatozoa bound to zona-free hamster oocytes in the presence of M1 monoclonal antibody showed M1 antigen at the equatorial region of the sperm head, as detected by

Table 2. Effect of hybridoma supernatants (M1–M5) on hamster fertilization *in vitro*

Supernatant	Oocytes examined	Oocytes fertilized (%)
Myeloma control	90	52 (87)
M1	26	2 (8)*
M2	26	13 (52)
M3†	29	3 (10)*
M4	24	5 (21)
M5	25	4 (16)*

*Significantly different from control ($P \leq 0.005$).
†Sperm agglutination.

Table 3. Effect of hybridoma supernatants (M1–M5) on hamster sperm penetration of zona-free hamster oocytes *in vitro*

Supernatant	Oocytes examined	Oocytes fertilized (%)
Myeloma control	75	70 (93)
M1	70	8 (11)*
M2	58	33 (57)
M3†	11	0 (0)*
M4	38	26 (68)
M5	62	19 (31)*

*Significantly different from control ($P \leq 0.005$).
†Sperm agglutination.

immunofluorescence (Fig. 6a,b). The intensity of staining varied, although most spermatozoa showed some immunofluorescence when examined across the focal plane.

Electron microscopic localization of M1 antigen

Immunogold label was not present on intact spermatozoa. On spermatozoa that had undergone the acrosome reaction, immunogold particles were localized predominantly on the plasma membrane overlying the equatorial region (Fig. 7a,b). A few particles were located on vesiculated membranes of anterior acrosome (Fig. 7a). Immunogold label was not present elsewhere on the spermatozoa and was absent from control samples. Analysis of a random sample of 100 sections of acrosome-reacted spermatozoa for gold particle localization showed that there were 26 ± 12 particles on the equatorial segment, 6 ± 4 particles on associated membrane vesicles of the anterior acrosome, and 0.5 ± 2.0 particles at other sites, or on control samples.

Preliminary biochemical characterization of M1 antigen

Protein from intact spermatozoa, Percoll-purified sperm heads, or 1% (w/v) sodium lauryl sulphate extraction of sperm heads was subjected to SDS-PAGE and immobilized on nitrocellulose membrane. A number of protein bands were revealed by Coomassie blue staining, including major bands at 37.5 and 26.0 kDa (Fig. 8). Immunoblotting with M1 antibody recognized two bands at 37.5 and 34.0 kDa in the purified preparations (Fig. 8). Immunodot blots of various tissue samples indicated that M1 antigen was only present in the epididymis and testis.

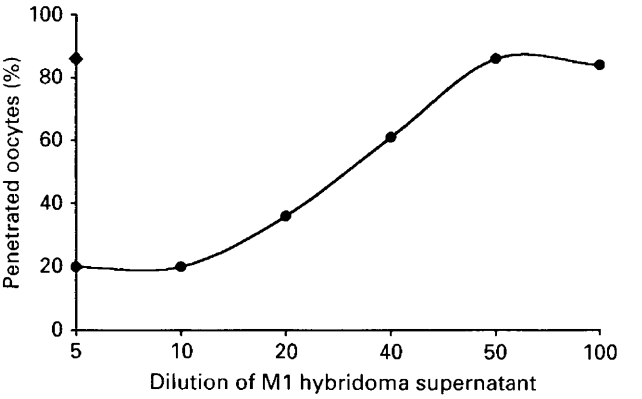

Fig. 4. Titration curve for M1 supernatant inhibition of hamster sperm–egg fusion *in vitro*. ♦, mean control value.

Discussion

The initial aim of this study was to derive monoclonal antibodies that recognized antigens of the equatorial segment. Intact spermatozoa have been used to immunize mice, but this has often led to monoclonal antibodies that recognize the apical region of the sperm acrosome and the flagellum, rather than the equatorial segment (see Moore and Hartman, 1984; Eddy and O'Brien, 1994). This may be because acrosome and tail components are more antigenic than other sperm components. The flagellum represents over 90% (v/v) of the hamster spermatozoon (Yanagimachi, 1994). In the present study, brief sonication of spermatozoa resulted in dissociation of the acrosomal cap and flagellum from the sperm head, which could then be purified by Percoll separation. In this preparation, the equatorial segment was more exposed. The anterior acrosome was absent and plasma membrane was missing from some cells, thereby increasing the possibility of obtaining antibodies to this region. Each antiserum recognized the hamster sperm equatorial segment, but also bound to the anterior acrosome. Since the purpose of the immunization was to enhance the production of antibodies to the equatorial segment, the specificities of the antisera were considered satisfactory, particularly as antibodies to the flagellum had not been generated preferentially. Immunofluorescent localization indicated that antisera crossreacted with spermatozoa from several mammalian species, indicating that some sperm epitopes may be conserved between species (Eddy and O'Brien, 1994).

The selection of hybridomas for final cloning was based on the pattern of immunofluorescence. It was decided that a variety of monoclonal antibodies to the sperm head should be produced so that valid comparisons could be made, but

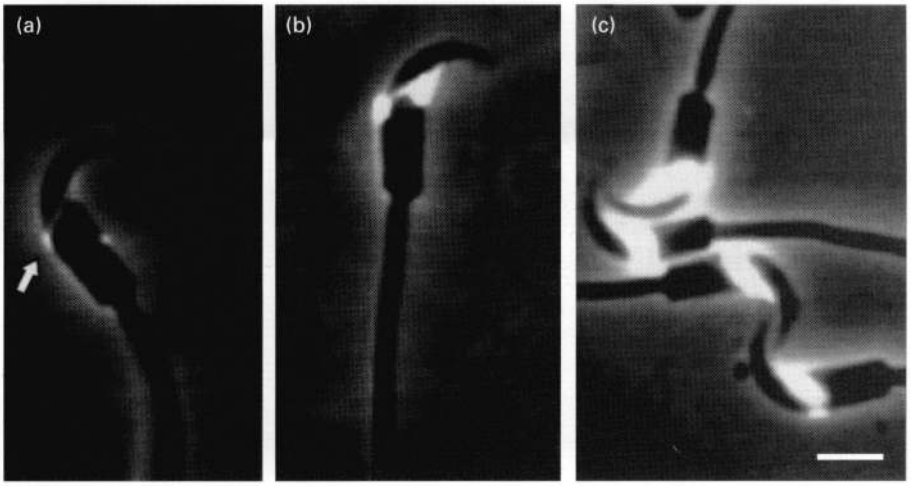

Fig. 5. Indirect immunofluorescent localization with M1 hybridoma supernatant on hamster spermatozoa using phase-contrast and UV epifluorescent microscopy. (a) Non-capacitated viable spermatozoa show staining at the apical margin of the equatorial region. (b) Fully capacitated viable spermatozoa show intense fluorescence over the entire equatorial region. (c) Methanol-fixed spermatozoa show bright fluorescence over the equatorial region. Scale bar represents 5 μm.

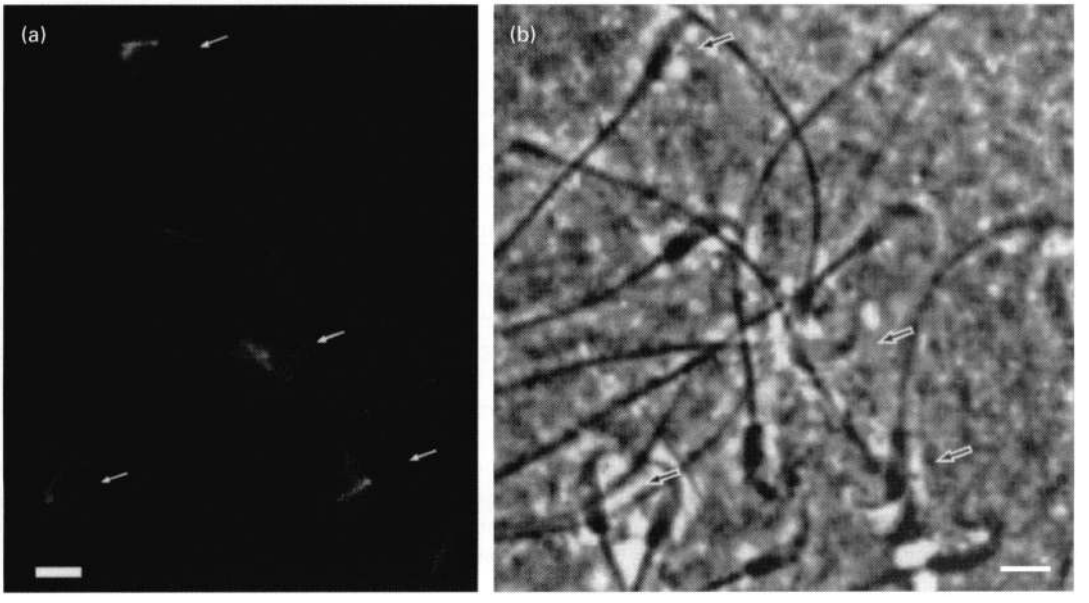

Fig. 6. Indirect immunofluorescent localization of M1 antigen on hamster spermatozoa bound to zona-free oocytes *in vitro*. Corresponding (a) epifluorescent and (b) phase-contrast micrographs. M1 antigen localized to the equatorial region (arrows) of spermatozoa bound to the surface of the oocyte. Scale bars represent 8 μm.

with a principal objective of identifying antigens involved in sperm–egg fusion. Hybridoma supernatant was added to sperm suspensions at the end of a 3 h incubation period when capacitation was considered complete and a high proportion of spermatozoa had hyperactivated motility. On the basis of studies indicating that sperm–egg fusion can only occur after sperm capacitation and the acrosome reaction (see Yanagimachi, 1994), it was surmised that at this stage pertinent antigens would be exposed (or modified). A primary screen with zona-intact oocytes revealed that M1, M3 and M5 antibody supernatants markedly inhibited fertilization. M1 and M5 supernatants had no effect on sperm motility, but M3 caused considerable sperm agglutination.

M3 and M5 supernatant caused a reduction in sperm binding to the oolemma compared with the control; this was not observed with M1 supernatant. A dose-dependent curve of M1 antibody inhibition of sperm–egg fusion was obtained (with no change in sperm–egg binding) that was consistent

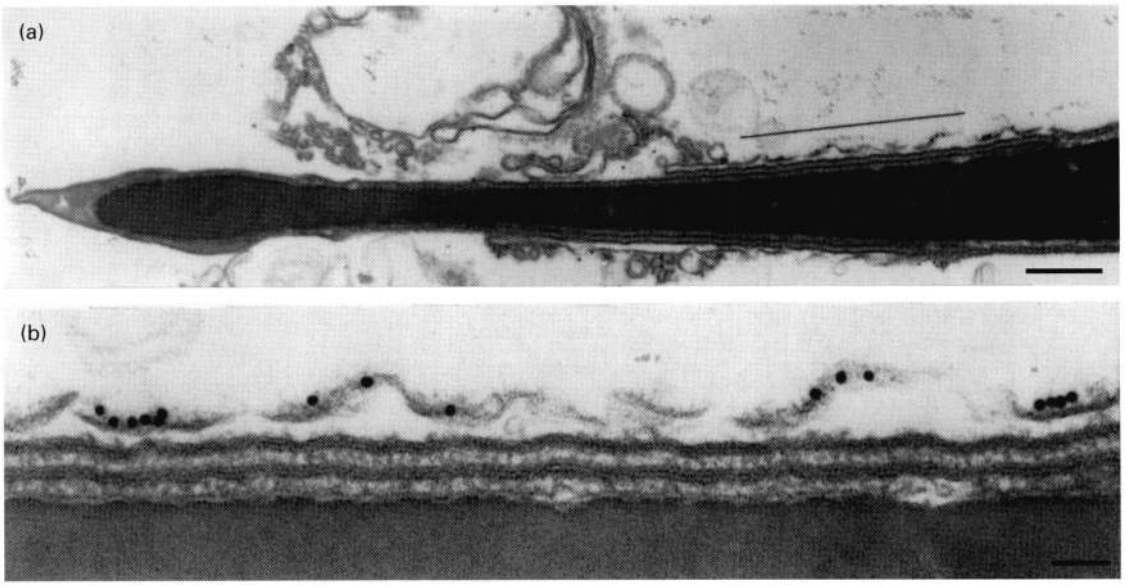

Fig. 7. Immunogold localization of M1 antigen on hamster spermatozoa. (a) An acrosome-reacted spermatozoon with associated membranes of the anterior acrosome. The membranes over the equatorial region are intact. (b) The region below the line in (a) at higher magnification. Particles were localized predominantly on the plasma membrane overlying the equatorial segment. No particles were present on intact spermatozoa. Scale bars represent (a) 0.5 μm and (b) 0.1 μm.

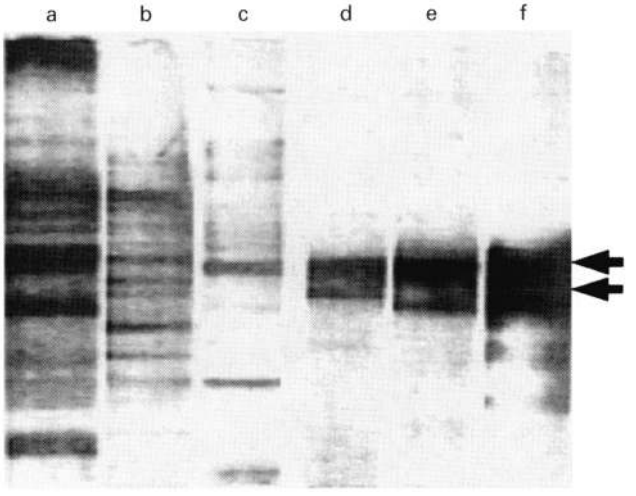

Fig. 8. Detection of M1 antigen on hamster spermatozoa after SDS-PAGE and immobilization of protein on nitrocellulose membrane. Lanes a–c are stained with Coomassie blue: a, intact spermatozoa; b, Percoll-purified sperm head; c, SDS extract of Percoll-purified sperm heads. Lanes d–e are the corresponding preparations to those in lanes a–c, immunoblotted with M1 hybridoma supernatant. Main protein bands (arrows) are at 37.5 and 34.0 kDa.

with interference with a receptor-mediated process (see Wassarman, 1990). M1 antigen clearly localized to the equatorial segment of spermatozoa bound to the oolemma. Thus, the antibody may inhibit sperm–oolemma fusion rather than sperm–oolemma binding. Alternatively, M1 antibody may prevent sperm head decondensation after gamete fusion. The zona-free egg penetration test relies on detecting swollen sperm heads in the vitellus (Aitken, 1986). Therefore, using standard protocols it is not possible to distinguish between antibody inhibition of sperm–egg fusion or antibody inhibition of sperm head decondensation after fusion. Further investigations using Hoescht 33342-stained spermatozoa (Green, 1993) may resolve this problem.

Preliminary ultrastructural localization of M1 using a pre-embedding immunogold protocol indicated that M1 antigen was localized to plasma membrane overlying the equatorial segment after the acrosome reaction, but was absent from the intact cells. Immunofluorescent staining of live intact and acrosome-reacted spermatozoa produced similar results, with the exception that on intact spermatozoa, immunogold particles were not detected at the apical margin of the equatorial segment, while immunofluorescent localization showed faint staining at this site. Although immunogold binding was not intense, it was highly specific and very few particles were observed away from the equatorial segment plasma membrane or on associated acrosomal membrane vesicles. The intensity of gold particle binding probably reflects the stringency of the washing procedures rather than the amount of antigen present. Gold particles on the equatorial segment (but not elsewhere) were often in small clusters, indicating aggregation of antigen in the membrane.

Exactly how M1 is presented on the plasma membrane overlying the equatorial segment remains to be determined. When sperm membranes were permeabilized by methanol fixation, M1 antigen was localized to the entire equatorial segment, indicating that the primary location of the antigen is intracellular. Ultrastructural observations with disrupted cells also indicate antigen localization to a site between the outer acrosomal and plasma membrane (M. Mat Noor and H. D. M. Moore, unpublished). Allen and Green (1996)

proposed that novel antigens or epitopes are expressed on the surface of the equatorial segment after the acrosome reaction through the modification of existing antigens by released acrosomal enzymes or by translocation of antigens from an intra-acrosomal site. Neither of these proposals is supported by the preliminary observations of the present study. M1 epitope is not located in the anterior acrosome before the acrosome reaction and does not obviously emanate from acrosomal matrix material after the acrosome reaction. Therefore, a direct translocation from the anterior acrosome is unlikely. The principal location of M1 before the acrosome reaction appears to be between the outer acrosomal and plasma membrane of the equatorial segment. During the acrosome reaction, membrane fusion occurs between the plasma membrane and the outer acrosomal membrane at the anterior margin of the equatorial segment. It is possible that M1 diffuses from its intracellular site during membrane fusion. Since substantial membrane remodelling may occur with the onset of membrane fusion, an alternative proposal is that the antigen is translocated directly across the plasma membrane overlying the equatorial segment after the acrosome reaction.

The characteristics of M1 antigen are similar to those reported for several other putative fusion proteins. Saling *et al.* (1985) reported that in mouse spermatozoa, M29 monoclonal antibody inhibited sperm–egg fusion, as detected by sperm head decondensation, but not sperm–egg adhesion, and recognized a determinant of 40 kDa. Antigen was localized to the equatorial segment of mouse acrosome-reacted spermatozoa. Toshimori *et al.* (1998) reported that MN9 monoclonal antibody inhibited mouse sperm–oocyte fusion and bound to an antigenic complex (designated 'equatorin') in the equatorial segment with a molecular mass of 38–48 kDa. In guinea-pig spermatozoa, G11 and M13 monoclonal antibodies inhibited sperm–egg fusion in a heterologous assay with zona-free hamster oocytes and recognized a 34 kDa protein present on the plasmalemma of the equatorial segment after the acrosome reaction (Allen and Green, 1995). Further ultrastructural investigations are in progress to investigate the precise expression of M1 in relation to the acrosome reaction and sperm–egg fusion.

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