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Monoclonal antibodies which recognize equatorial segment epitopes presented de novo following the A23187-induced acrosome reaction of guinea pig sperm

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SUMMARY

Acrosome-intact mammalian sperm can adhere to zona pellucida-free oocytes but are only capable of fusing if they have previously undergone the acrosome reaction. This suggests that the acrosome reaction results in presentation of at least one novel epitope which plays a role in sperm-oocyte fusion. Monoclonal antibodies were raised against unfixed acrosome-reacted guinea pig sperm and screened by indirect immunofluorescence for binding to the equatorial segment. They were back-screened against unfixed acrosome-intact sperm for absence of binding. Using this approach, two antibodies, G11 and M13, were identified which detect equatorial segment epitopes presented de novo by sperm following an A23187-induced acrosome reaction. The localization of these epitopes to the equatorial segment was confirmed at the ultrastructural level by indirect immunogold-labelling. Fluorescein isothiocyanate-labelled Fab fragments of these two antibodies also localized to the equatorial segment. Affinity chromatography and western blotting established that the two mAbs recognize the same proteins, which have M_rs of 34, 46, 48 and 51×10^3. When sperm were induced to undergo the acrosome reaction with A23187 and incubated with their discharged acrosomal contents, a further band was produced with an M_r of 30×10^3. Production of this band was inhibited in the combined presence of 100 μM phenylmethylsulphonyl fluoride and 100 μM *p*-aminobenzamidine even though these compounds do not inhibit acrosomal exocytosis. Neuraminidase and *O*-glycosidase were without effect on the proteins detected by antibodies G11 and M13. Endoglycosidase F, however, eliminated the bands of M_r 46, 48 and 51×10^3 and replaced them with a strong band of M_r 44×10^3 and two minor bands of M_r 43 and 45×10^3. Formaldehyde fixation of acrosome-intact sperm caused partial rupture of the acrosome with loss of the characteristic rouleaux (stacks) of guinea pig sperm. Indirect labelling of these formaldehyde-fixed sperm with fluorescein isothiocyanate- or gold-labelled second antibody, with or without permeabilization with 0.05% Triton X-100, showed dense labelling on the cytoplasmic face of the plasma membrane overlying the convex surface of the acrosome but little labelling elsewhere. Cryosections of acrosome-intact sperm labelled indirectly with immuno-gold showed labelling consistent with the same location, as well as sporadic labelling at other intracellular sites overlying the acrosome. Since there is no evidence that sperm can translocate intact membrane protein from the cytoplasmic face to the extracellular face of the plasma membrane during the acrosome reaction, the evidence suggests that there are two isolated antigen pools. One pool allows sperm to present epitopes de novo on the equatorial segment at the time the acrosome reaction occurs. The possible location of at least part of the precursor pool for these epitopes was established using a third monoclonal antibody, G3. This antibody binds to the equatorial segment of acrosome-reacted sperm and cross-reacts with the 34 kDa antigen recognized by antibody G11. In acrosome-intact sperm, antibody G3 binds to the extracellular face of the anterior plasma membrane of the head. It follows that the 34 kDa antigen cannot be recognized by antibodies G11 and M13 in this location, even though it is recognized by these antibodies in detergent extracts. Trypsinization of acrosome-intact sperm was without effect in generating the epitopes recognized by antibodies G11 and M13. Expression of the epitopes was not inhibited when the acrosome reaction was induced in the presence of 1 mM *p*-aminobenzamidine. The evidence suggests that the equatorial segment antigen recognized by antibodies G11 and M13 is either 34 kDa protein which has undergone a conformational rearrangement during the acrosome reaction, or a smaller protein derived from the 34 kDa protein by enzymic processing. All three antibodies (G3, G11, M13) were able to block sperm/oocyte fusion in heterologous fusion assays between guinea pig sperm and hamster oocytes. The evidence suggests that the external 34 kDa antigen (and possibly some product derived from it) may play some role in the fusion of sperm and oocyte.

Key words: mammalian sperm, equatorial segment, monoclonal antibody, membrane fusion protein

INTRODUCTION

A central event in fertilization is the fusion of sperm and egg. This, and other membrane fusions, are currently considered to be mediated by specific fusion proteins (White, 1990, 1992; Zimmerberg et al., 1993). These proteins are thought either to bring the fusing membranes in close apposition, or to destabilize the lipid at the site of contact (Burger and Verkleij, 1990; Zimmerberg et al., 1993). An important step prior to the fusion of two membranes is their adhesion.

Mammalian sperm with intact acrosomes bind intimately to zona pellucida-free oocytes but do not fuse with them (Yanagimachi and Noda, 1970a; Green, 1992), whereas sperm that have undergone the acrosome reaction both bind and fuse (Yanagimachi and Noda, 1970a). This conversion to fusion competence is inhibited by protease inhibitors (Takano et al., 1993) but treatment of acrosome-intact sperm with trypsin or acrosin fails to render them fusigenic (Takano et al., 1993). This evidence suggests that a key component of the fusion apparatus is either missing from the surface of acrosome-intact sperm, or is present but requires activation.

The precise site of fusion on the sperm surface is not known with certainty: some morphological evidence suggests that the equatorial segment is the site of fusion (Bedford et al., 1979; Koehler et al., 1982), whereas other evidence suggests that the site of fusion is wholly or partly on the post-equatorial membrane (Yanagimachi and Noda, 1970b; Talbot and Chacon, 1982). Essentially, the published data are inadequate to decide the matter. There is, however, no evidence to suggest that sperm fuse with the inner acrosomal membrane.

Functionally, fusion has been affected by a number of monoclonal antibodies, two of which are of particular note. One, M29, is against an antigen in the mouse equatorial segment (Saling et al., 1985), the other, PH-30, is against a guinea pig antigen in the post-equatorial plasma membrane (Primakoff et al., 1987). The first can block fusion completely, the second only partially. The protein recognized by PH-30 has recently been sequenced and a putative fusion sequence identified (Blobel et al., 1992). PH-30 undergoes no detectable processing, however, either positional or molecular, at the time sperm undergo the acrosome reaction and acquire fusion competence (Blobel et al., 1990). Nevertheless, recent evidence indicates that peptides derived from the fusion sequence promote fusion of phospholipid vesicles (Muga et al., 1994).

It is reasonable to assume that the change to the sperm surface which triggers fusigenicity has a molecular basis, and if that is the case, it is a reasonable working assumption that the molecular change may be detectable with monoclonal antibodies. This paper describes a set of experiments whose object was to determine whether epitopes could be found which appear de novo on the plasma membrane of the sperm head after the acrosome reaction in a region which potentially contains a component of the sperm fusion apparatus.

MATERIALS AND METHODS

Production of mAbs against acrosome-reacted guinea pig sperm

Sperm were flushed from single excised vasa deferentia and caudae epididimydes with HEPES buffer containing 145 mM NaCl, 5 mM KCl, 10 mM HEPES and 2 mM $CaCl_2$, pH 7.4, and diluted to ~2×10^7 sperm/ml. The acrosome reaction was induced with the ionophore A23187 at a final concentration of 38 μM and 10 μl/ml of dimethyl sulphoxide (10 μl of a stock solution of A23187 in dimethyl sulphoxide (2 mg/ml) per ml of sperm suspension). Half the batch of sperm were incubated at 37°C for 3-4 hours after induction of the acrosome reaction. The remainder were held at 37°C in buffer without ionophore until the end of the incubation, when ionophore was added and the two populations mixed and resuspended in phosphate-buffered saline (PBS). Balb/c mice were injected intraperitoneally with 10^7 sperm/animal without adjuvant. Following standard reimmunization procedures, spleen cells were fused with Sp2/O-Ag14 myeloma cells. Supernatants from viable hybridomas were screened for binding against unfixed, acrosome-reacted sperm by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (H + L) (Tago, Burlingame, CA, USA). mAb supernatants were isotyped, after single cell cloning, using an Amersham kit.

Induction of ascites and purification of mAbs

Ascites were induced by priming Balb/c mice intraperitoneally, initially with pristane, then with hybridoma cells about a week later. Ascites fluid was harvested as it developed. The ascitic fluid was treated with ammonium sulphate, initially to 25% saturation, subsequently to 50%. The cut between 25% and 50% saturation was centrifuged and dialysed against 10 mM Tris buffer, pH 8.4 and chromatographed on DEAE-Sephadex G-50. The column was washed with 50 mM NaCl, 10 mM Tris, pH 8.4, and eluted with a gradient of 50-250 mM NaCl in 10 mM Tris buffer. The mAbs eluted at approximately 150 mM NaCl.

Preparation of Fab fragments

mAbs G11 and M13 (both IgG1s) were dialyzed against 100 mM sodium acetate solution (pH 5.5) at a concentration of ~5 mg/ml. Cysteine was added from a 1 M stock solution and EDTA from a 20 mM stock solution to final concentrations of 50 mM cysteine and 1 mM EDTA. Papain (Sigma, St Louis, MO, USA) was added at 10 μg/mg antibody and the solution incubated at 37°C for 6 hours. Proteolysis was arrested by addition of solid iodoacetamide to a final concentration of 75 mM followed by incubation for 30 minutes. A preliminary time course for papain digestion was established on small amounts of mAbs by arresting the reaction at various time points and running antibody fragments on SDS-polyacrylamide gel electrophoresis. Fab fragments were purified by gel filtration on Sephadex G-100 and ion exchange chromatography on DEAE-Sephadex.

Fluorescein isothiocyanate (FITC) labelling of Fab fragments

Purified Fab fragments (2 mg/ml) were dialyzed against 0.1 M sodium carbonate (pH 9.0). Fluorescein isothiocyanate (FITC) in a freshly prepared stock solution (1 mg/ml) was added at 50 μl/ml of mAb solution in 5 μl aliquots with gentle stirring. The solution was stood for 8 hours in the dark at 4°C. Solid NH_4Cl was added to a final concentration of 50 mM. After 2 hours, the solution was gel filtered through Sephadex G-25 in PBS. The FITC-labelled protein was used immediately.

Indirect antibody labelling of live sperm

For normal screening purposes, sperm were induced to undergo the acrosome reaction with 38 μM A23187 at a sperm count of ~10^7/ml. The sperm suspension was split, as for the preparation of sperm for immunization, with a gap of 2 hours between inducing the acrosome reaction in the first batch and the second batch. Fifteen minutes after inducing the acrosome reaction in the second batch, the two batches were combined and used for indirect immunofluorescent staining. Sperm were also induced to undergo the acrosome reaction with either 9.5 or 3.8 μM A23187 (incubation for a single fixed period of 1 hour

for the whole sample). In one set of experiments, sperm were induced to undergo the acrosome reaction with 38 μM A23187 in the presence of 10 mM *p*-aminobenzamidine. After gentle centrifugation, sperm (either acrosome-intact or acrosome-reacted) were suspended in PBS and incubated with hybridoma supernatant for 30 minutes on ice. Sperm were washed twice with ice-cold PBS containing 1% fetal calf serum (FCS) and incubated on ice, either with FITC-goat anti-mouse IgG (Tago, Burlingame, CA, USA) or 10-nm gold-labelled goat anti-mouse IgG (Amersham, UK or Zymed Laboratories, South San Francisco, CA, USA). After incubation (30 minutes for FITC-labelled antibody, 2 hours for gold-labelled antibody), sperm were washed twice in PBS with 1% FCS. FITC-labelled sperm were examined by fluorescence microscopy. Gold labelled sperm were fixed with 1% glutaraldehyde in 0.14 M sodium cacodylate, pH 7.4, for 30 minutes, and processed for electron microscopy as previously described (Green, 1978). Ultrathin sections were examined at 80 kV in a Philips 410 LS electron microscope.

Indirect antibody labelling of formaldehyde-fixed sperm

Acrosome-intact sperm were suspended in PBS and fixed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 minutes. Sperm which were to be labelled after cryosectioning were then centrifuged into 10% gelatin in the same buffer at 37°C. The pellet was spread to form a thin layer (0.5-1 mm thick) and cooled on ice. The solidified layer was detached under 2.3 M sucrose with a razor blade, incubated for 6 hours at room temperature in the sucrose solution, and mounted for cryosectioning. Ultrathin sections (85-90 nm) were cut with glass knives. Sections were retrieved with sucrose droplets and deposited on Formvar-coated grids. The grids were washed with PBS and placed on droplets of hybridoma supernatant for 5 minutes. They were then washed three times with PBS and placed on droplets of 10 nm gold-labelled goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA, USA), diluted 1:20. They were post-stained with 1% uranyl acetate in distilled water. Sections were viewed at 100 kV on a Philips 410 LS electron microscope. For indirect labelling of sperm suspensions, sperm were fixed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 minutes. A portion was washed three times with ice-cold PBS containing 1% FCS and processed as for the indirect labelling of live sperm described earlier. For permeabilized sperm, formaldehyde-fixed sperm were pelleted and resuspended in 0.05% Triton X-100 in PBS for 5 minutes, washed three times with ice-cold PBS and processed as for indirect labelling.

SDS gel electrophoresis of sperm extracts

Pellets of epididymal sperm (~10^8 sperm per pellet) were extracted into 2% SDS sample buffer without reduction, heated at 100°C for 5 minutes, electrophoresed with M_r standards on 10% polyacrylamide gels and blotted onto nitrocellulose (Bio-Rad, Richmond, CA, USA). Blots were probed sequentially with mAb culture supernatants at 1:100 dilution and alkaline phosphatase(AP)-goat anti-mouse IgG (Bio-Rad). Biotinylated M_r standards were probed with AP-avidin (Bio-Rad). Alkaline phosphatase was localized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Bio-Rad) in 100 mM Tris buffer containing 5 mM $MgCl_2$, pH 9.5. Samples were reduced by addition of β-mercaptoethanol to a final concentration of 5% (v/v).

The proteolytic effect of the acrosomal contents on acrosome-reacted sperm was examined by inducing the acrosome reaction with A23187 in the absence and combined presence of the protease inhibitors phenylmethylsulphonyl fluoride (PMSF) and *p*-aminobenzamidine (pAB), both 100 μM. After incubating the sperm for 1 hour in their own secretory discharge at 37°C, sperm and supernatant were spun at 100,000 $\boldsymbol{g}_{max}$ for a further 1 hour. The pellets were extracted with SDS sample buffer in non-reducing conditions, run on 10% gels and stained as before.

The effects of deglycosylation were examined with endoglycosidase F (=endo-*β*-*N*-acetylglucosaminidase, EC 3.2.1.96, from *Flavobacterium meningosepticum*), *O*-glycosidase (=endo-*α*-*N*-acetylgalactosaminidase; =*O*-glycopeptide endo-*D*-galactosyl-*N*-acetyl-*α*-galactosaminohydrolase, EC 3.2.1.97, from *Diplococcus pneumoniae*), and neuraminidase (=sialidase; =acylneuraminyl hydrolase, EC 3.2.1.18, from *Vibrio cholerae*) (Boehringer Mannheim, Germany). A pellet of acrosome-intact sperm from two guinea pigs was extracted into 1.5 ml of buffer containing 100 mM sodium acetate, 1 mM calcium acetate, 1% SDS, pH 5.5, by pipetting and heating the sample to 100°C for 3 minutes. The cooled sample was spun at 20,000 ***g*** for 10 minutes. The supernatant was assayed for protein using a BCA protein assay kit (Pierce, Rockford, IL, USA). For enzyme incubations, the extraction buffer was adjusted to contain final concentrations of 100 mM sodium acetate, 1 mM calcium acetate, 0.2% SDS, 2% *n*-octylglucoside, 0.1 mM PMSF, 10 mM pAB, and 500 μg protein per sample tube, pH 5.5. The enzymes were added in incubation buffer (neuraminidase, 200 mU; endo F, 1 U; *O*-glycosidase, 1 mU) and final volumes adjusted to 300 μl. The final EDTA concentration, where it was added, was 50 mM. Incubation times are given in the legend to Fig. 7. Reactions were halted by diluting the sample with two volumes of extraction buffer and heating to 100°C for 3 minutes.

Trypsinization of acrosome-intact sperm

Sperm were flushed from excised vasa deferentia and caudae epididymides with a Ca^{2+}-free HEPES medium containing 1% BSA, 2 mM $MgCl_2$ and 100 μM EGTA. Pellets containing ~10^7 sperm were resuspended in 1 ml of this medium containing 0.5, 0.05, 0.005, and 0.0005% trypsin (w/v) and incubated at 37°C for 30 minutes. Incubations were halted by addition of 1 ml of Ca^{2+}-free medium containing 1 mM PMSF and 100 mM pAB. Sperm were washed and labelled sequentially with either mAb G11, M13 or G3 and FITC-goat anti-mouse IgG, as described earlier.

Affinity chromatography of sperm extracts

Purified mAb G11 was dialyzed against 0.1 M $NaHCO_3$ overnight and coupled to CNBr-activated Sepharose 6MB (Pharmacia). The final concentration of mAb, as assayed by loss of OD from the supernatant, was 7 mg/mL of bed. Acrosome-reacted sperm and supernatants were spun at 100,000 $\boldsymbol{g}_{max}$ for 1 hour and the pellet resuspended in 0.15 M NaCl, 10 mM Tris, 1.0% Triton X-100, 1 μM PMSF, 10 mM *p*-aminobenzamidine, pH 7.5, (~10^8 sperm/ml). The suspension was stirred for 1 hour at room temperature. The extract from 2×10^9 sperm was passed over 1 ml of G11-Sepharose bed and washed with 50 bed volumes of buffer. The bed was extracted with an equal volume of double strength SDS sample buffer, heated to 100°C for 5 minutes, and the supernatant run on an SDS gel. The nitrocellulose blot was probed with mAbs G11, M13 and G3.

Assay of fusion blocking activity

Guinea pig sperm were flushed from excised vasa deferentia and caudae epididimydes with a modified Tyrode's medium containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.09 mM pyruvic acid, 9.0 mM Na lactate, 0.5 mM hypotaurine, and 0.05 mM L(–) adrenaline bistartrate. The medium also contained 50 μg/ml pencillin G, 60 μg/ml gentamicin sulphate and 3 mg/ml bovine serum albumin (Sigma, A-7030). If necessary, the pH was titrated to 7.4 with NaOH. Sperm were incubated overnight (16 hours) at a final concentration of ~10^7 sperm/ml and then resuspended in fresh medium for 1 hour. These sperm were diluted to ~2×10^6 sperm/ml with either hybridoma culture medium or hybridoma supernatant containing antibody (G2, G3, G11 or M13) and incubated for a further hour before dilution to a concentration of ~10^5/ml with modified Tyrode's medium. Oocytes were introduced into the suspension under paraffin oil and incubated with sperm for 2 hours. Oocytes were obtained from naturally cycling hamsters on a controlled light-dark cycle (6 am to 6 pm on). Ovulating hamsters were identified by their vaginal mucus. Cumulus masses

were removed with hyaluronidase (0.01%) and zonae pellucidae with trypsin (0.01%). Oocytes from each animal (typically 10-15) were assigned to each antibody treatment group. A total of 15-17 oocytes were used in each group, and all antibodies and the culture medium were tested simultaneously against sperm from a single animal. The assay was repeated three times using sperm from three separate animals. After incubation with sperm, oocytes were pipetted vigorously to remove supernumary sperm, fixed in 1% glutaraldehyde in 0.14 M sodium cacodylate for 5 minutes, stained with Hoechst 33342 (10 μM) for a further 10 minutes, washed and examined by fluorescence light microscopy for decondensed heads.

Photography

Light micrographs were taken on a Zeiss IM35 inverted microscope with Nomarski optics and photographed using either Ilford HP5 or Kodak TMY400 film, both at 400 ASA. Fluorescent micrographs of FITC-labelled Fab fragments were taken at 1600 ASA on Ilford HP5.

RESULTS

Guinea pig sperm in which the acrosome reaction has been induced by A23187 prove effective antigens and provide straightforward access to a range of mAbs which bind to various regions of the acrosome-reacted sperm head as well as the tail. Thirty three mAbs have been obtained to date, 19 IgMs and 14 IgGs. Of these, 18 of 19 IgMs label the equatorial segment by indirect immunofluorescence and 9 of 14 IgGs. Those mAbs which label the equatorial segment were also screened against acrosome-intact sperm to determine whether they were detecting epitopes presented de novo following the acrosome reaction. Only two showed no binding. These were mAbs G11 and M13 (shown for mAb G11 in Fig. 1B-D). The remainder stained anterior plasma membrane. Care was needed, however, in staining acrosome-intact sperm with mAbs G11 and M13. When sperm were flushed from the vas deferens in Ca^{2+}-containing medium, the level of positives associated with apparently acrosome-intact sperm was ~3%. This figure rose sharply with anything other than mild pipetting to as high as 20%. When sperm were flushed from the vas deferens with Ca^{2+}-free medium (either by replacing the Ca^{2+} in HEPES medium with Mg^{2+}, or using PBS), the level of positive staining of motile, apparently acrosome-intact sperm was zero, even where the acrosome occasionally appeared swollen. Since acrosome-reacted sperm washed in Ca^{2+}-free medium undergo staining of their equatorial segments, the absence of staining in acrosome-intact sperm under the same conditions is not due to a failure of antibody to bind to presented epitope, rather the absence of the epitope itself. Labelling of the equatorial segment occurred in sperm induced to undergo the acrosome reaction at different A23187 concentrations (38, 9.5, 3.8 μM, respectively).

To check the possibility that the hybridoma supernatants themselves were causing patching of antigen into the equatorial segment from other areas of the sperm head, acrosome-reacted sperm were exposed to Fab fragments of mAbs G11 and M13. The result for G11 is shown in Fig. 1E. Direct immunofluorescent staining of the head with Fab fragments, although giving a weaker signal than by indirect immunofluorescence with whole antibody, showed staining only in the equatorial segment on the head itself, although there was now also some labelling of the tail. The same was seen for M13.

A second check was to ensure that the equatorial segment was indeed the site of mAb labelling. Indirect immunogold labelling showed this unequivocally to be the case. Fig. 2A shows an acrosome-reacted sperm en face with a glancing section through the equatorial segment. Where gold particles lie outside the equatorial segment, they are commonly associated with residual attachments of the outer acrosomal and plasma membrane vesicles formed during the acrosome reaction, although some labelling is also seen which is unassociated with vesicles. The density of labelling per unit area in the equatorial segment region is approximately 15 times higher than over the inner acrosomal membrane. Fig. 2B shows a sperm from the same pellet in sagittal section. Immunogold labelling is predominantly on the outer surface of the equato-

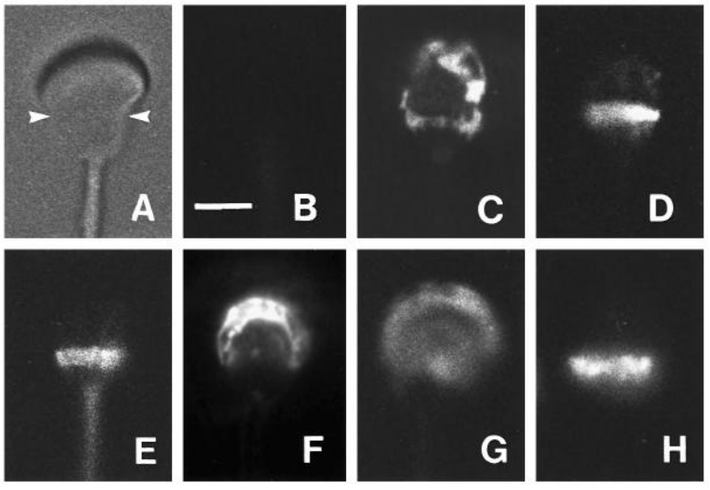

Fig. 1. Light micrographs of guinea-pig sperm en face. All micrographs are at the same magnification as B, where the bar = 5 μm. (A) Nomarski light micrograph of acrosome-intact guinea pig sperm. The arrowheads mark the position of the future equatorial segment. (B-D) Fluorescent light micrographs of guinea pig sperm labelled sequentially with mAb G11 and FITC-goat anti-mouse IgG. All three micrographs were photographed and printed under identical conditions. (B) Acrosome-intact sperm, showing no labelling of the sperm. (C) Acrosome-reacted sperm with partial retention of vesicles of plasma and outer acrosomal membrane. Fluorescence is associated with both the equatorial segment and the vestigial membrane vesicles. (D) Fully acrosome-reacted sperm in which no residual anterior plasma membrane or outer acrosomal membrane remains, showing labelling of equatorial segment only. There is no labelling of the tail. (E) Acrosome-reacted sperm labelled directly with FITC-labelled Fab fragment of mAb G11. (Photographed at 1600 ASA instead of 400 ASA to compensate for the weaker fluorescent signal from the direct label.) Some labelling of the tail is evident with this Fab fragment preparation. (F) Formaldehyde-fixed and permeabilized (0.05% Triton X-100) acrosome-intact sperm showing label in the region of the acrosomal cusp. (G,H) Sperm labelled sequentially with mAb G3 and FITC-goat anti-mouse IgG. (G) Acrosome-intact sperm showing labelling of the plasma membrane overlying the acrosome. (H) Acrosome-reacted sperm showing labelling of the equatorial segment.

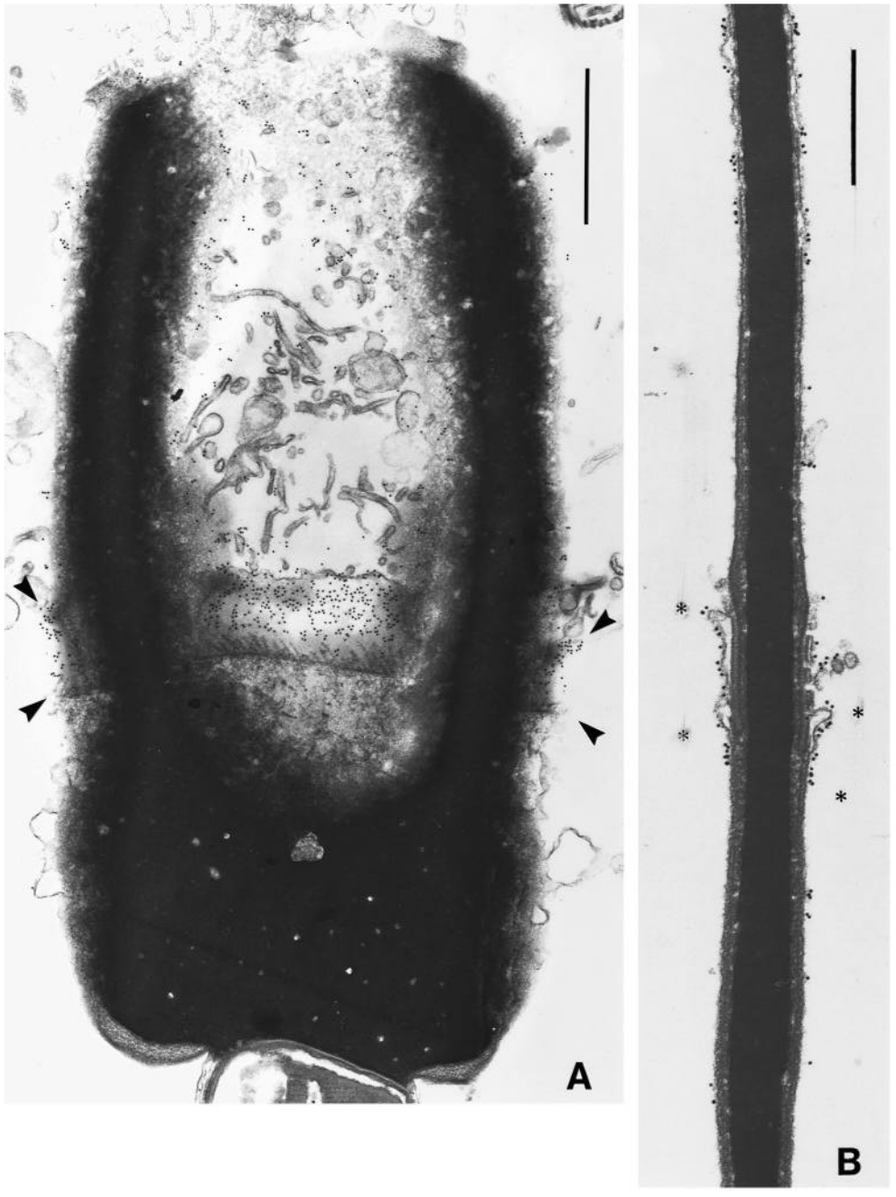

Fig. 2. (A) Electron micrograph of an acrosome-reacted guinea pig sperm labelled sequentially with mAb G11 and 10 nm gold-labelled goat anti-mouse IgG antibody, viewed en face. The equatorial segment lies between the arrowheads and traverses the sperm. There is no direct evidence that the gold labelling in the region of the equatorial segment is associated with the outer face of the equatorial segment but such a location would be consistent with the other ultrastructural evidence (see Fig. 2B). Bar, 1 μm.
(B) Sagittal section of guinea-pig sperm from the same pellet as (A). Label is predominantly associated with the external face of the equatorial segment (between asterisks). Bar, 0.5 μm.

rial segment. Low levels of labelling are also seen on the inner acrosomal membrane and the post-equatorial plasma membrane.

The epitope or epitopes which are presented de novo in the equatorial segment following the acrosome reaction potentially have two origins. They might arise by processing of an antigen which is already present in the equatorial segment plasma membrane of acrosome-intact sperm, or the epitope might arise from the diffusion of a previously sequestered antigen into the plasma membrane of the equatorial segment following the acrosome reaction. The obvious mechanism for such an introduction is for the antigen to be a constituent of the granule face of the outer acrosomal membrane and to diffuse from there to the equatorial segment once the membrane fusion associated with exocytosis has begun. An antigen with this behaviour might or might not require activation to present the epitope which is finally detected in the equatorial segment.

Efforts were made to gain evidence for one or more of these alternatives by antibody labelling following either permeabilization or cryosectioning of formaldehyde-fixed, acrosome-intact sperm. An unexpectedly complex picture emerged. Formaldehyde fixation by itself produces partial disintegration of the acrosome of acrosome-intact sperm, with significant rupture of the anterior plasma membrane and outer acrosomal membrane (Fig. 3). Stereo pairs of thick sections of formaldehyde-fixed, immunogold-labelled sperm (examined by electron microscopy at 400 kV) suggested that antibody access to the membrane surfaces of interest should be unrestricted in many sperm without the need for membrane permeabilization. Nevertheless, formaldehyde-fixed sperm were also permeabilized

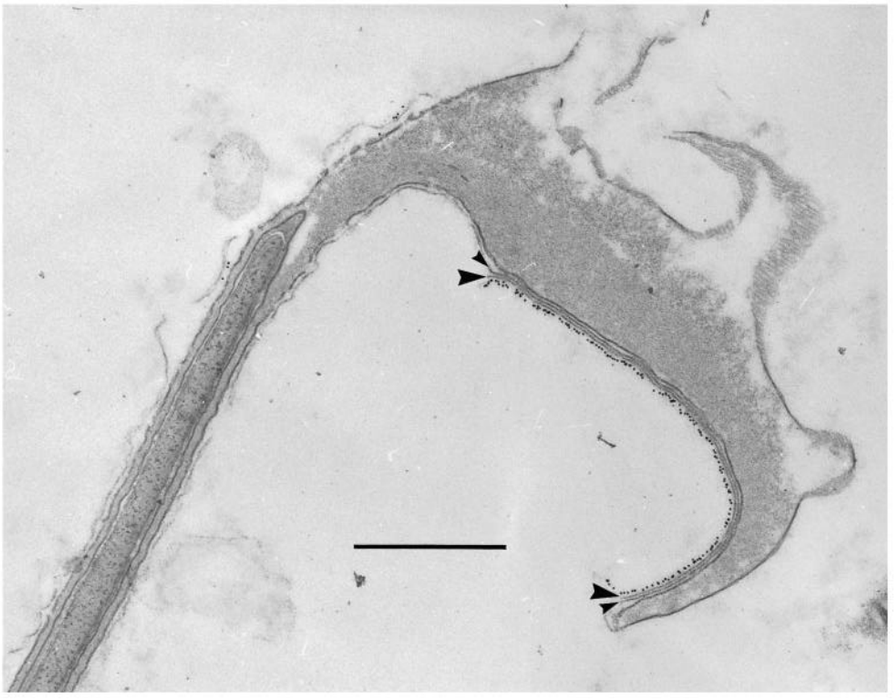

Fig. 3. Electron micrograph of the acrosome of a guinea pig sperm fixed in 4% formaldehyde in phosphate-buffered saline, permeabilized with 0.05% Triton X-100 and labelled sequentially with mAb G11 and 10 nm gold-labelled goat anti-mouse IgG antibody. Formaldehyde fixation destabilizes the acrosome but arrests its disintegration. In this micrograph, the plasma membrane overlying the convex region of the acrosome of a previously adjacent sperm remains attached to the concave face of the sperm in the micrograph. The edges of the fragment are marked by large arrowheads. Adjacent to this membrane is the plasma membrane of the sperm shown in the micrograph (marked by small arrowheads). Adjacent to that membrane is the outer acrosomal membrane. There are therefore three membranes in the concavity. The cytoplasmic face of the plasma membrane fragment from the adjacent sperm is essentially the only site of gold labelling. The sperm shown has lost the plasma membrane from its own convex region, presumably to the next adjacent sperm in the rouleaux.

with Triton X-100. Concentrations of Triton X-100 above 0.05%, or more extended exposure than 5 minutes produced significant solubilization of membranes. Indirect immunofluorescent and immunogold labelling of fixed, permeabilized, acrosome-intact sperm is shown in Figs 1F and 3. Fluorescent labelling (Fig. 1F) shows label in a crescent associated with the acrosomal cusp. Labelling is completely absent from the region of the future equatorial segment. Fig. 3 shows the site of the labelling to be the *cytoplasmic* face of the plasma membrane which overlies the convex region of the acrosome in acrosome-intact sperm. This membrane is normally strongly attached to the plasma membrane of the concave face of the acrosome in an adjacent sperm, and provides the basis for the mechanical integrity of the characteristic rouleaux (stacks) which guinea pig sperm form. Formaldehyde fixation destabilizes the acrosome, almost certainly osmotically, and the rouleaux disintegrate during subsequent washing. Part of the anterior plasma membrane is retained by the adjacent sperm. Similar micrographs are obtained with formaldehyde-fixed but unpermeabilized sperm. There is no significant gold labelling elsewhere within the head.

Similar data were obtained by immunogold labelling of cryosections (Fig. 4A-C), although in these there appeared to be patches of label as far back as the posterior tip of the acrosome, possibly associated with the cytoplasmic face of the plasma membrane (Fig. 4C). This additional label may reflect an absence of extraction by detergent in these sperm. No immunogold labelling of the outer surface of the anterior plasma membrane was seen in unfixed sperm (not shown). Where more glancing cryosections through the acrosome of acrosome-intact sperm were obtained, it was possible to identify what appears to be the boundary between antigen-containing and antigen-free regions in the membrane sheets themselves. No obvious structure or additional organization has been identified in the regions of mAb localization.

No evidence was obtained, using mAbs G11 and M13, for the presence of the precursor to the equatorial segment epitope on either the granule face of the outer acrosomal membrane or the extracellular face of the anterior plasma membrane. As a control, formaldehyde fixation of acrosome-reacted sperm did not prevent subsequent indirect mAb labelling of the equatorial segment, suggesting that the failure to detect any precursor is due to the absence of epitope, not the effect of formaldehyde fixation.

The relative molecular masses of antigens recognized by mAbs G11 and M13 were established by western blotting. Bands were obtained with M_rs of 30, 34, 46, 48 and 51×10^3 under non-reducing conditions (Fig. 5A); those with M_rs of 46, 48 and 51×10^3 survive reducing conditions (not shown). A further mAb, G3, was identified by western blotting, which recognized solely the 34 kDa protein (Fig. 6). Cross-reaction was established rigorously by isolating the antigens recognized by G11 using affinity chromatography, with subsequent western blotting of SDS gels of this material using mAbs G3 and M13 (Fig. 6).

mAb G3 was localized on sperm both by immunofluorescence and electron microscopy, in exactly the same way as for mAbs G11 and M13. By indirect immunofluorescent labelling, mAb G3 labelled the anterior plasma membrane of acrosome-intact sperm and the equatorial segment of acrosome-reacted sperm (Fig. 1G,H). Immunogold labelling of cryosectioned, formaldehyde-fixed, acrosome-intact sperm showed no

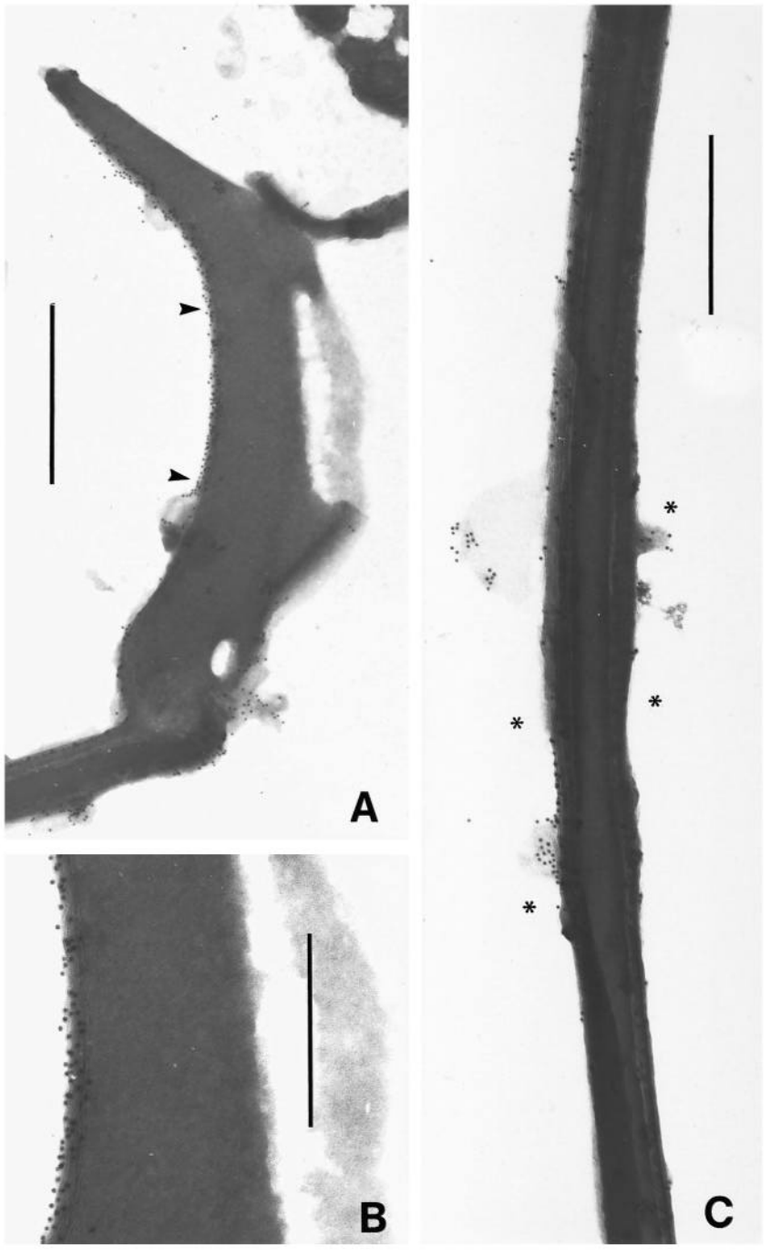

Fig. 4. Ultrathin cryo-sections of acrosome-intact guinea pig sperm labelled sequentially with mAb G11 and 10 nm gold-labelled anti-mouse IgG antibody. (A) Section through the acrosomal cusp showing label predominantly associated with the concave face of the acrosome, although with occasional localization elsewhere. The region between the arrowheads is shown at higher magnification in (B). Bar, 1 μm. (B) Higher power view of part of (A). It is not possible to establish in these micrographs the precise membrane face which is labelled. The evidence from sperm such as that in Fig. 3 indicates that it is the cytoplasmic face of an adherent plasma membrane fragment from an adjacent sperm. Bar, 0.2 μm. (C) View of the central region of the sperm head with the approximate position of the future equatorial segment identified between the asterisks. Occasional gold labelling is seen as far back as the posterior tip of the acrosome. Bar, 0.65 μm.

labelling at all, whereas immunogold labelling of formaldehyde-fixed, acrosome-intact sperm showed labelling of the external face of the anterior plasma membrane. This labelling was consistent with that seen by the indirect immunofluorescent labelling. From this evidence, it was concluded that the 34 kDa antigen recognized by all three mAbs on western blots is present on the external face of the anterior plasma membrane of sperm, but that mAbs G11 and M13 cannot detect the antigen prior to the acrosome reaction. This suggests that some form of processing of the antigen occurs during the reaction.

Attempts were made to generate the equatorial segment epitope recognized by mAbs G11 and M13 on acrosome-intact sperm by trypsinizing them at a range of trypsin concentrations but this treatment was without effect. The acrosome reaction was also induced by ionophore in the presence of 10 mM *p*-aminobenzamidine to determine whether expression of the epitope was affected by efforts to inhibit acrosomal proteases. Loss of the acrosomal matrix was slowed but presentation of the equatorial segment epitope occured normally, as assessed by indirect immunofluorescence.

Evidence was obtained, however, that the acrosome reaction may be accompanied by some proteolytic processing of the 34 kDa protein. When sperm were induced to undergo an acrosome reaction with A23187 and incubated in their own secretory supernatant for 1 hour in the absence of protease inhibitors, a protein of M_r 30×10^3 appeared (Fig. 5B), which

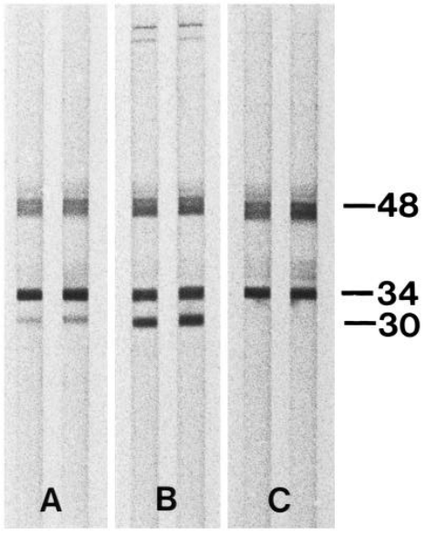

Fig. 5. Western blots of sperm extracts run on 10% polyacrylamide gels and probed with mAbs G11 and M13 (left and right of each pair of lanes, respectively). (A) Acrosome-intact sperm suspended in normal Ca^{2+}-containing medium for 1 hour without A23187, showing a faint band with an M_r of 30 kDa in addition to the bands with M_rs of 34, 46, 48 and 51×10^3. (B) Acrosome-reacted sperm incubated with their own activated acrosomal material for 1 hour at 37°C. Note the emergence of a strong band with an M_r of 30 kDa. (C) The same experiment as in (B) but with the addition of 100 μM phenylmethylsulphonyl fluoride (PMSF) and 100 μM *p*-aminobenzamidine (*p*-AB). These two protease inhibitors completely inhibit the generation of the 30 kDa band.

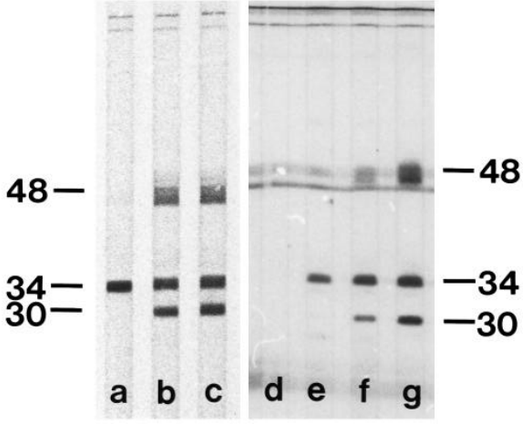

Fig. 6. Western blots of sperm extracts (lanes a-c) and material purified from sperm extracts by affinity chromatography on a mAb G11-Sepharose column (lanes d-g) probed with mAbs G3, G11 and M13. Lanes a and e, mAb G3; lanes b and f, mAb M13; lanes c and g, mAb G11. Lane d represents affinity-purified material in which the first antibody was omitted. The gel of affinity-purified material shows background elution of antibody from the affinity column (notably at about 45 kDa). mAb G3 only binds to the 34 kDa protein and cross-reacts with material immuno-absorbed by mAb G11 (lane e). mAb M13 also cross-reacts with this immuno-absorbed material (lane f).

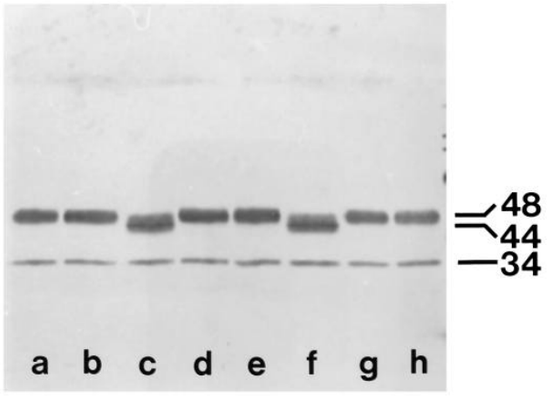

Fig. 7. Western blots of sperm extracts run on 12% polyacrylamide gels following incubation with endoglycosidase F (endo F), *O*-glycosidase, and neuraminidase and probed with mAb G11. Lane a, incubation buffer alone; reaction stopped at 0 hours. Lane b, incubation buffer alone; reaction stopped after 2 hours at 37°C. Lane c, incubation buffer, 1 U endo F, 50 mM EDTA; reaction stopped after 30 hours at 20°C. Lane d, incubation buffer, 1 mU *O*-glycosidase, 50 mM EDTA; reaction stopped after 30 hours at 20°C. Lane e, incubation buffer, 200 mU neuraminidase; reaction stopped after 2 hours at 37°C. Lane f, incubation buffer, 200 mU neuraminidase, 1 U endo F, 50 mM EDTA; sample incubated for 2 hours at 37°C with neuraminidase alone, then endo F and EDTA added; reaction stopped after a further 30 hours at 20°C. Lane g, incubation buffer, 200 mU neuraminidase, 1 mU *O*-glycosidase, 50 mM EDTA; sample incubated for 2 hours at 37°C with neuraminidase alone, then *O*-glycosidase and EDTA added; reaction stopped after a further 30 hours at 20°C. Lane h, incubation buffer alone; reaction stopped after 30 hours at 20°C. The bands have M_rs of 43, 44, and 45×10^3. A similar banding pattern was obtained with mAb M13.

Table 1.

mAb	Number of oocytes	Number of oocytes fertilized	Number of sperm fused
G2	46	41	119
G3	43	0	0
G11	48	0	0
M13	49	0	0
Hybridoma culture medium	47	43	131

was absent when the experiment was performed in the presence of PMSF and p-aminobenzamidine (Fig. 5C). Acrosome-intact sperm incubated for 1 hour without A23187 show a faint band at M_r 30×10^3 consistent with a small percentage of sperm undergoing a spontaneous acrosome reaction during isolation (Fig. 5A).

Solubilized extracts of whole sperm were also treated with endoglycosidase F, *O*-glycosidase and neuraminidase (Fig. 7). Only endoglycosidase F had any effect, lowering the M_r of the upper three bands to a triplet of bands at 43, 44 and 45×10^3. All three enzymes were without effect on the band of 34 kDa.

All three mAbs (G3, G11 and M13) were screened for their ability to block heterologous fusion between hamster oocytes and guinea pig sperm. Hybridoma supernatants were used without purification at titres of approximately 10 μg

antibody/ml. All three mAbs blocked sperm-oocyte fusion at the single concentration at which they were tested (Table 1), whereas another mAb, G2, which also labels the equatorial segment was was without effect in blocking fusion, as was hybridoma culture medium itself.

DISCUSSION

The experiments described in this paper were designed to identify sperm membrane proteins which might play a role in membrane fusion with the oocyte. To this end, a panel of monoclonal antibodies (mAbs) was developed against the surface of the head of acrosome-reacted guinea pig sperm. The major criterion employed in screening the mAbs was that of de novo expression of an epitope in the equatorial segment following the acrosome reaction. This criterion was intended to parallel the conversion of sperm to fusion competence when they undergo the acrosome reaction, and was based on the assumption that acquisition of fusion competence reflects a molecular change on the sperm surface. It was implicit in the experimental design that this change would be detectable by mAbs.

For ease of experimental manipulation, the acrosome reaction was induced by the ionophore A23187 at a concentration of 38 μM for a sperm count of ~10^7sperm/ml (Green, 1978). These conditions induce the acrosome reaction in the sperm population within minutes and allow a synchronous start for subsequent manipulations. A23187 is hydrophobic and partitions rapidly into cell membranes, lowering the concentration in bulk solution as it does so. Since the cells act as a large sink for the ionophore, the effect that a given concentration of A23187 produces on cells is determined both by the cell concentration and their size. The concentration of A23187 used in these experiments is the same as that used previously to generate membrane fragments of guinea pig sperm for immunization (Myles et al., 1981; Primakoff and Myles, 1983). In those experiments, however, the sperm concentration was 100-fold lower, entailing an A23187 to sperm ratio 100-fold higher than ours. A23187 at concentrations of ~4 μM has been used to induce the acrosome reaction and fusion competence in human sperm at sperm counts of ~10^7/ml (Aitken et al., 1993; McLaughlin et al., 1993), that is, a ten-fold lower concentration than ours. However, human sperm have a considerably smaller surface area than guinea pig sperm and the difference in membrane concentration of ionophore in the two sperm is likely to be considerably less than ten-fold. The question that arises from these considerations is whether the concentration of A23187 in any way influences the outcome of the acrosome reaction, and hence the conclusions that can be drawn from these artificially acrosome-reacted sperm. The distorting effect, if it exists, is likely to be crucial to the screening rather than the immunization. The evidence suggests, however, that the ionophore concentration relative to sperm numbers is not a variable in determining the pattern of indirect immunofluorescence in acrosome-reacted sperm since a 10-fold lower ionophore concentration (~4 μM) lowers the rate at which the population of sperm undergoes the acrosome reaction but does not affect the pattern of immunofluorescent staining in acrosome-reacted sperm. The conclusions from the functional tests of antibody blockade of fusion using in vitro fertilization are unaffected by the A23187 concentration since A23187 is not used for the capacitation of sperm in these experiments.

Two monoclonal antibodies, mAbs G11 and M13, satisfy the principal screening criterion: they recognize an antigen in the equatorial segment of acrosome-reacted guinea pig sperm but show no recognition of acrosome-intact sperm when applied extracellularly. Unexpectedly, however, they also localize to an intracellular site which is on the cytoplasmic face of part of the anterior plasma membrane. On the face of it, there must be two antigen pools, one on the cytoplasmic face of the anterior plasma membrane, the other on the extracellular face. If flip-flop of membrane protein during the acrosome reaction is discounted as a serious possibility, the evidence points inevitably to the conclusion that the pools are independent. Armed with this assumption, it is possible to make some provisional assignment of antigens to the two pools. mAb G3 recognizes solely the 34 kDa antigen on western blots and binds solely to the external surface of acrosome-intact sperm, evidenced by the absence of intracellular mAb G3 binding in either permeabilized or cryo-sectioned sperm. Although this evidence suggests that the 34 kDa plasma membrane antigen faces extracellularly, it does not, by itself, preclude the possibility that some of the higher molecular mass antigens, those with M_rs of 46, 48 and 51×10^3, respectively, might also face extracellularly. By contrast, mAbs G11 and M13 recognize only internal antigens in acrosome-intact sperm, indicating that the internal antigens are experimentally distinguishable from the extracellular antigen(s) which both mAbs can recognize after the acrosome reaction. It follows from these observations that the 34 kDa protein cannot be recognized by mAbs G11 and M13 on the extracellular face of the plasma membrane of acrosome-intact sperm even though the evidence indicates that it is there, but it can be recognized by mAbs G11 and M13 when the antigen is solubilized in detergent. This indicates that detergent solubilization of the 34 kDa protein makes an epitope accessible to the two mAbs, possibly because it frees antibody-epitope binding from some steric constraint which is present in the membrane. The evidence also suggests that the antigens which can be recognized by mAbs G11 and M13 on the cytoplasmic face of the plasma membrane are distinct from the 34 kDa antigen. They may be the 46, 48, and 51 kDa antigens which are unrecognized by mAb G3. If that is the case, then they appear to be absent from the external face of the plasma membrane, since they can be recognized in undischarged sperm by mAbs G11 and M13 which show no binding to the external face of the anterior plasma membrane.

The 34 kDa antigen also appears to be present in the equatorial segment once sperm have undergone the acrosome reaction since mAb G3 binds there, but whether this 34 kDa protein is now capable of being recognized by mAbs G11 and M13 is unclear. It may be that the 34 kDa protein undergoes some conformational rearrangement during the acrosome reaction which allows it to retain its molecular mass while exposing a novel epitope for mAbs G11 and M13 to recognize. Alternatively, it is possible that proteolytic cleavage of the 34 kDa protein or some other precursor presents novel epitope(s) recognized by mAbs G11 and M13. Having said that, however, it should be recalled that trypsinization of acrosome-intact sperm does not produce the epitope(s), arguing against proteolytic cleavage of the 34 kDa protein as their source. Moreover, inducing the acrosome reaction in the presence of

a trypsin inhibitor fails to prevent their appearance, again arguing that proteolytic cleavage is not the formative step. It is worth noting that trypsinization of acrosome-intact sperm also fails to make them fusigenic (Takano et al., 1993).

Western blotting provided evidence that the 34 kDa protein recognized by mAb G3 co-migrates with an antigen recognized by mAbs G11 and M13. Complete identity of the two proteins was confirmed by affinity purification of the mAb G11 antigens and subsequent western blotting with mAbs G3 and M13. Evidence was also obtained from western blots that sperm generate a 30 kDa antigen by proteolysis following the acrosome reaction (Fig. 5) but this protein is only detected by mAbs G11 and M13 and not mAb G3, possibly because proteolysis cleaves the epitope recognized by mAb G3. The dependence on the acrosome reaction for production of the 30 kDa protein suggests that its precursor has to be accessible to acrosomal proteases, since inhibition of its formation by PMSF suggests that it arises as a consequence of acrosin cleavage. This suggests, in turn, that the precursor protein is either on the extracellular face of the anterior plasma membrane of acrosome-intact sperm or the granule face of the outer acrosomal membrane. The obvious precursor is the 34 kDa protein, and it may be that the 30 kDa protein is the form of the 34 kDa antigen which is recognized by mAbs G11 and M13 in the equatorial segment. However, the evidence, already discussed, that serine proteases are not involved in epitope presentation for these mAbs argues against that, and there is no direct evidence, at this stage, that the 30 kDa protein can be recognized by mAbs G11 and M13 in the equatorial segment. Although the discussion has proceeded so far as if the antigens were integral membrane proteins, there is no direct evidence at this stage that that is the case. The antigens may be surface-bound proteins. This does not in itself undermine the potential for distinct antigens in different pools.

The diffuse nature of the bands of 46, 48, 51 kDa suggests post-translational modification of a single protein, but no firm indication has been obtained as to the precise nature of the modification. Although *N*-glycosidase treatment reduced the M_rs of these bands in tandem, the persistence of multiple bands after *N*-glycosidase treatment suggests that some of the post-translational modification which gave rise to the original heterogeneity is still present. The 34 kDa band is completely unaffected. It is possible that mAbs G11 and M13 both recognize a post-translational modification shared by all five bands (e.g. GPI-linkage, tyrosine sulphation, etc.) although clearly there would have to be a mechanism for concealing this modification when it was on the surface of acrosome-intact sperm and allowing its expression when the acrosome reaction occurred. A summary of the behaviour of the three antigens is shown in Fig. 8.

The results discussed so far were all derived from a primary screen of mAbs based on antibody location on the sperm head. It is also possible to screen mAbs for their ability to block function, notably fusion of sperm with the oocyte. From an experimental point of view, it is technically much easier to undertake these screens of fusion-blocking activity with hamster oocytes because of their availability and ease of handling. There is, however, some doubt about the direct relevance of data acquired by heterologous fertilization. In general terms, it is unlikely that each mammalian species has evolved a fundamentally different molecular mechanism for sperm fusion with oocytes. Undoubtedly some divergent evolution has occurred at the molecular level between species and this would serve to account for the failure of in vitro fertilization to occur universally between different mammalian species. In vitro fertilization is possible between some mammalian species, however, notably between hamster oocytes and sperm from a number of other mammalian species (including guinea pig). When it can occur, it seems likely that it simply reflects an absence of substantial molecular diver-

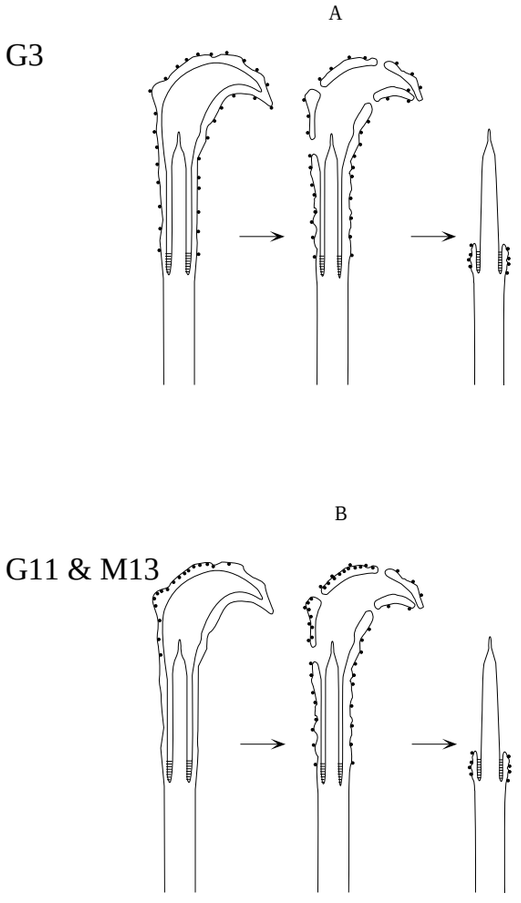

Fig. 8. Diagrammatic representation of the proposed behaviour of the three mAbs described in this paper. (A) mAb G3 recognizes an antigen on the extracellular surface of the anterior plasma membrane of acrosome-intact sperm. Its distribution is approximately co-extensive with the acrosome. When the bulk of this anterior plasma membrane is lost during the acrosome reaction, the remnant in the equatorial segment retains the antigen. There is no evidence for any other pool of mAb G3 antigen which western blotting suggests is a 34 kDa protein. (B) In acrosome-intact sperm, mAbs G11 and M13 recognize antigen(s) only on the cytoplasmic face of the anterior plasma membrane. When the acrosome reaction takes place, the two mAbs recognize an additional pool of antigen on the extracellular face of the anterior plasma membrane which, like the antigen recognized by mAb G3, is partly retained in the equatorial segment. This antigen may be the 34 kDa protein which has undergone conformational change. However, it may also be an antigen derived from the 34 kDa protein or some other precursor by enzymic processing.

gence in the mechanism of membrane fusion between the two species involved, not the substitution of an entirely novel, non-physiological alternative mechanism of fusion. Even so, results obtained from heterologous fertilization should clearly be treated with caution. The tentative conclusion from the data in this paper is that the equatorial segment may contain proteins which play a role in membrane fusion. This conclusion is identical to that obtained from mouse where M29, a monoclonal antibody against mouse testis, labels the mouse equatorial segment specifically, fails to bind to acrosome-intact mouse sperm, and blocks homologous fertilization completely (Saling et al., 1985). Any role which the equatorial segment plays in sperm/egg fusion is presumably in addition to the role implicated for the post-equatorial region by PH-30 (Primakoff et al., 1987).

As to why mAbs G11 and M13 should recognize two distinct pools of antigen, it should be noted that both pools are in regions of the sperm (the cytoplasmic face of the anterior plasma membrane and the extracellular face of the equatorial segment) which are candidate membrane fusion sites (the equatorial segment for fusion to oocytes, the cytoplasmic face of the acrosomal membrane for acrosomal exocytosis). It may be, therefore, that the two regions share molecular features which play a role in membrane fusion.

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REFERENCES

Aitken, R. J., Buckingham, D. W. and Fang, H. G. (1993). Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J. Androl.* **14,** 132-141.

Bedford, J. M., Moore, H. D. M. and Franklin, L. E. (1979). Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. *Exp. Cell Res.* **119,** 119-126.

Blobel, C. P., Myles, D. G., Primakoff, P. and White, J. M. (1990). Proteolytic processing of a protein involved in sperm-egg fusion correlates with acquisition of fertilization competence. *J. Cell Biol.* **111,** 69-78.

Blobel, C. P., Wolfsberg, T. G., Turck, C. W., Myles, D. G., Primakoff, P. and White, J. M. (1992). A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. *Nature* **356,** 248-251.

Burger, K. N. J. and Verkleij, A. J. (1990). Membrane fusion. *Experientia* **46,** 631-644.

Green, D. P. L. (1978). The induction of the acrosome reaction in guinea-pig sperm by the divalent metal cation ionophore A23187. *J. Cell Sci.* **32,** 137-151.

Green, D. P. L. (1992). Comparison of H33342 and propidium iodide as fluorescent markers for sperm fusion with hamster oocytes. *J. Reprod. Fertil.* **96,** 581-591.

Koehler, J. K., de Curtis, I., Stenchever, M. A. and Smith, D. (1982). Interaction of human sperm with zona-free hamster eggs: a freeze-fracture study. *Gamete Res.* **6,** 371-386.

McLaughlin, E. A., Ford, W. C. and Hull, M. G. (1993). Effects of cryopreservation on the human sperm acrosome and its response to A23187. *J. Reprod. Fert.* **99,** 71-76.

Muga, A., Neugebauer, W., Hirama, T. and Surewicz, W. K. (1994). Membrane interaction and conformational properties of the putative fusion peptide of PH-30, a protein active in sperm-egg fusion. *Biochemistry* **33,** 4444-4448.

Myles, D. G., Primakoff, P. and Bellve, A. R. (1981). Surface domains of the guinea pig sperm defined with monoclonal antibodies. *Cell* **23,** 433-439.

Primakoff, P. and Myles, D.G. (1983). A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* **98,** 417-428.

Primakoff, P., Hyatt, H. and Tredick-Kline, J. (1987). Identification and purification of a sperm surface protein with a potential role in sperm-egg fusion. *J. Cell Biol.* **104,** 141-149.

Saling, P. M., Irons, G. and Waibel, R. (1985). Mouse sperm antigens that participate in fertilization. I. Inhibition of sperm fusion with the egg plasma membrane using monoclonal antibodies. *Biol. Reprod.* **33,** 515-526.

Takano, H., Yanagimachi, R. and Urch, U. A. (1993). Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with the oolemma: inhibitor studies using the golden hamster. *Zygote* **1,** 79-91.

Talbot, P. and Chacon, R. S. (1982). Ultrastructural observations on binding and membrane fusion between human sperm and zona pellucida-free hamster oocytes. *Fertil. Steril.* **37,** 240-248.

White, J. M. (1990). Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* **52,** 675-697.

White, J. M. (1992). Membrane fusion. *Science* **258,** 917-924.

Yanagimachi, R. and Noda, Y. D. (1970a). Physiological changes in the post-nuclear cap region of mammalian spermatozoa: a necessary preliminary to the membrane fusion between sperm and egg cells. *J. Ultrastruct. Res.* **31,** 486-493.

Yanagimachi, R. and Noda, Y. D. (1970b). Electron microscope studies of sperm incorporation into hamster egg. *Am. J. Anat.* **128,** 429-462.

Zimmerberg, J., Vogel, S. S. and Chernomordik, L. V. (1993). Mechanisms of membrane fusion. *Annu. Rev. Biophys. Biomol. Struct.* **22,** 433-466.

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