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An MN9 Antigenic Molecule, Equatorin, Is Required for Successful Sperm-Oocyte Fusion in Mice[1]

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ABSTRACT

The acrosome plays an important role in fertilization. This study was designed to examine the role and behavior of a molecule, equatorin (the antigenic molecule of the monoclonal antibody mMN9), localized at the equatorial segment of the acrosome. In vitro fertilization (IVF) investigation was conducted to examine the role of this molecule, by assessing the effect of mMN9 in TYH medium (a modified Krebs Ringer bicarbonate solution) containing mMN9 at 0 (control), 25, 50, and 100 μg/ml. Under these conditions, the IVF investigation was divided into two experiments: 1) the zona pellucida (zona)-intact experiment, in which capacitated sperm inseminated cumulus- and zona-intact oocytes; and 2) the zona-free experiment, in which acrosome-reacted sperm inseminated zona-free oocytes. It was found that mMN9 did not affect sperm motility, zona binding, or zona penetration, but it significantly inhibited fertilization, reducing the rates of pronucleus and two-cell embryo formation in both the zona-intact and zona-free oocyte experiments. In addition, when judged at 5 h after insemination in the zona-intact experiment, nearly half of the unfertilized oocytes had accumulated sperm in the perivitelline space (perivitelline sperm), and concurrently we confirmed by electron microscopy the presence of many unreleased cortical granules preserved beneath the oolemma, indicating no occurrence of sperm-oocyte fusion. Confocal laser scanning light microscopy with indirect immunofluorescence demonstrated that equatorin was localized at the equatorial segment in both capacitated and perivitelline sperm (acrosome-reacted sperm). These results suggest that equatorin that is preserved at the equatorial segment is involved in the process of sperm-oocyte fusion in mice.

INTRODUCTION

Mammalian sperm are highly differentiated cells consisting of heads and flagella. The acrosome is located at the rostral region of the head. Functionally, the principal region of the acrosome is involved in the acrosome reaction, while the posterior region of the acrosome, i.e., the equatorial segment, engages in membrane fusion with the oocyte [1–4]. Although the roles of various components of sperm and oocytes have been reported and reviewed [3–7], the functional molecules specifically located at the equatorial segment are only a few. Among them, a monoclonal antibody (mAb), M29, which recognizes the equatorial segment of mouse sperm, specifically blocks sperm-oocyte fusion [8]. A 33-kDa protein, oscillin, is reported to trigger oocyte development in hamsters [9]. An interesting molecule claimed to be involved sperm-oocyte fusion in guinea pigs is PH-30 or fertilin [10]. Fertilin is a heterodimeric protein with two subunits (alpha and beta) that both have a transmembrane region; it is part of the ADAM (a disintegrin and metalloproteinase) family, which functions in cell-cell and cell-matrix interactions [11].

We produced four anti-acrosome mAbs: mMN9 [12], mMC41 [13, 14], mMN7 [15], and mMC101 [16]. Of these, mMN9 (immunoglobulin [Ig] G_{2a}) specifically recognizes the molecule in the equatorial segment of sperm in various species, including humans. We call the mMN9 antigen (MN9) ''equatorin'' because of its location in the equatorial segment. In contrast, the other three mAbs recognize molecules in the principal region of the acrosome. Equatorin is a 38- to 48-kDa protein complex in mice, and a 48-kDa protein in rats [12]. During a series of studies using these mAbs, we found that the MN7 and MC41 antigenic molecules quickly disperse into the medium during the acrosome reaction [14], whereas some amount of equatorin remains at the equatorial segment after the acrosome reaction.

Based on this background, the present study was designed to examine the following two questions: 1) whether equatorin is essential for the mouse sperm-oocyte fusion process, and 2) how equatorin behaves during the fertilization process.

MATERIALS AND METHODS

Animals

ICR strain mice at 8–16 wk of age were purchased from Kyudo Company (Kumamoto, Japan) and were maintained in the Experimental Animal Center, Miyazaki Medical College, in an automatically controlled environment (12L:12D, 20°C) with free access to food and water under the guidelines for animal welfare of the College. The animals were killed by cervical dislocation just before the experiments.

Reagents, In Vitro Fertilization (IVF) Culture Medium, and Antibodies

All organic and inorganic compounds were of analytical or culture grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated. Modified Krebs Ringer bicarbonate solution (TYH medium) [17] was used throughout the study for gamete manipulation and IVF.

The mMN9 IgG_{2a} antibody, which was described previously [12], was purified with a protein A column kit (Ampure PA kit; Amersham International, Buckinghamshire, UK) from spent culture medium, and then the antibody was diluted (1:4) with binding buffer. The mAb concentration was estimated by a protein assay, before use of the Ampure PA kit, according to the supplier's manual for the bicinchoninic acid (BCA) method (Pierce Chemical Company, Rockford, IL). Diluted antibody was filtered through a Millipore filter (no. 22; Millipore Corp., Bedford, MA) and was applied to an Ampure PA column two times. The Ampure PA column was then washed twice with binding buffer (5

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ml each time). The purified antibody was eluted with 3 ml elution buffer, and 3 fractions of 1 ml each were collected. The pH was neutralized with 1 M Tris-HCl (pH 9.0; 0.33 ml per 1-ml fraction). The mAb concentration was also measured after the Ampure PA kit was used. The peak fraction was desalted with a Sephadex G 25M column (PD-10 column; Pharmacia Biotech, Uppsala, Sweden) that had been pre-equilibrated with TYH medium, to exchange binding buffer with TYH medium. Ten fractions of 1 ml each were collected through the PD-10 column, and the mAb concentration was again measured after use of the PD-10 column. The peak fraction (0.3–0.7 mg/ml peak yield) was stored at −4°C until use for IVF. Another mAb, mMC31 (IgG_1), which specifically recognizes the rat flagellar surface protein of 26–35 kDa [18], was prepared in the same way for the control study.

Experimental Design of the IVF Investigation

The IVF investigation was divided into two experiments: 1) the zona pellucida (zona)-intact experiment, in which capacitated sperm inseminated cumulus- and zona-intact oocytes; and 2) the zona-free experiment, in which sperm that had been acrosome-reacted using extracted zona protein (procedure described below) inseminated zona-free oocytes.

Oocytes were prepared as follows. Mature female mice were induced to ovulate by consecutive i.p. injections of 5 IU eCG followed 48 h later by 5 IU hCG. Cumulus-intact oocytes were collected 13–15 h after the injection of hCG by rupturing the oviductal ampulla region under oil in an IVF dish (Falcon Plastics, Los Angeles, CA; #3653). Before they were used in zona-intact experiments, the oocytes were transferred to another IVF dish that contained TYH medium equilibrated with 5% CO_2 in air at 37°C.

Zona-free oocytes were prepared as follows. The zona was removed according to the method of Fann and Lee [19] with slight modifications. In brief, mature oocytes collected as above were treated with 0.05% hyaluronidase in TYH medium for 5–10 min to remove cumulus cells, and then the cumulus-free oocytes were washed twice in TYH medium. The zona was removed from these oocytes by brief treatment with low-pH (2.5) TYH medium. The zona-free oocytes were transferred to an IVF dish that contained TYH medium equilibrated with 5% CO_2 in air, and they were then incubated for at least 1 h at 37°C for recovery before use.

Sperm were collected from the cauda epididymidis of mature male mice. The distal portion of each epididymis was cut using a blade, and a dense sperm mass was squeezed out of the epididymis. The sperm were allowed to disperse into 400 μl TYH medium. The sperm were diluted to a final concentration of 2×10^6 sperm/ml in TYH medium and used for capacitation and acrosome reaction.

Capacitation and Induction of the Acrosome Reaction by Zona Protein

Capacitation was achieved by the incubation of sperm prepared as above for 2 h at 37°C in a 5% CO_2 environment in TYH medium. The sperm were used for insemination.

The acrosome reaction was induced with zona protein, which was obtained essentially according to the method of Fann and Lee [19]. In brief, zonae pellucidae collected from 1000 oocytes in which the cumulus cells had been removed as described above were dissolved in 100 μl of low-pH TYH medium (pH 2.5) in a 1.5-ml tube. After centrifugation for 5 min at 150 × *g*, the supernatant containing zona protein was collected and adjusted to pH 7.2. This supernatant was used for the induction of the acrosome reaction; the reaction was induced by incubation at 37°C for 30 min after the addition of 10 μl zona protein (equivalent to 40 zona) to 100 μl of capacitated sperm suspension (equivalent to 2×10^5 sperm) in TYH medium. This procedure was basically according to the method of Lakoski et al. [20].

Zona-Intact and Zona-Free Experiments

For the zona-intact experiment, zona-intact oocytes were preincubated with mMN9 (25, 50, and 100 μg/ml) for 30–45 min in TYH medium before insemination. The capacitated sperm were then added to the oocytes in TYH medium containing mMN9 (25, 50, and 100 μg/ml).

For the zona-free experiment, acrosome-reacted sperm were preincubated with mMN9 (100 μg/ml) for 30 min in TYH medium before insemination. The sperm were then added to zona-free oocytes in TYH medium containing mMN9 (100 μg/ml).

Insemination was achieved by adding 2–10 μl of sperm suspension to 400 μl of TYH medium. The final concentration was adjusted to approximately 4×10^4 sperm/ml in all experiments.

Monitoring of Sperm Motility and IVF Events

The motility of sperm and fertilization events (sperm-binding and formation of polar bodies, pronuclei, and two-cells) were monitored under phase contrast images and were recorded with a videotape recorder system connected to an inverted light microscope (IM type; Olympus, Tokyo, Japan) through a CCD camera (Model KP-M1; Hitachi Denshi, Tokyo, Japan). The percentage of motile sperm was also estimated by the hanging drop preparation method [21]. The fertilization events were further examined with a light microscope (Optiphot; Nikon, Tokyo, Japan) equipped with differential interference contrast (Nomarski) apparatus. Formation of pronuclei and two-cells was determined at 5 h and 24 h after insemination, respectively.

Determination of the Rate of Sperm in the Perivitelline Space (Perivitelline Sperm)

This experiment was conducted in the presence of mMN9 (100 μg/ml), as was the zona-intact experiment. The occurrence of perivitelline sperm was checked at 5 h after insemination. The following criteria were used to define the oocytes with perivitelline sperm: for the experiment in the presence of mMN9, at least 2 sperm were required in the perivitelline space, whereas for the control experiment in the absence of mMN9, at least 1 sperm in the perivitelline space plus the sperm flagellar component in the ooplasm or pronucleus formation was required.

Control Experiment for IVF Investigation

The control experiment was conducted under the same insemination conditions in the absence of mMN9 (TYH medium only) or in the presence of control mAb mMC31 (100 μg/ml) in TYH.

Statistical Analyses for IVF Experiments

Each experiment was repeated 3–8 times depending on the purpose. The data were analyzed with chi-square tests.

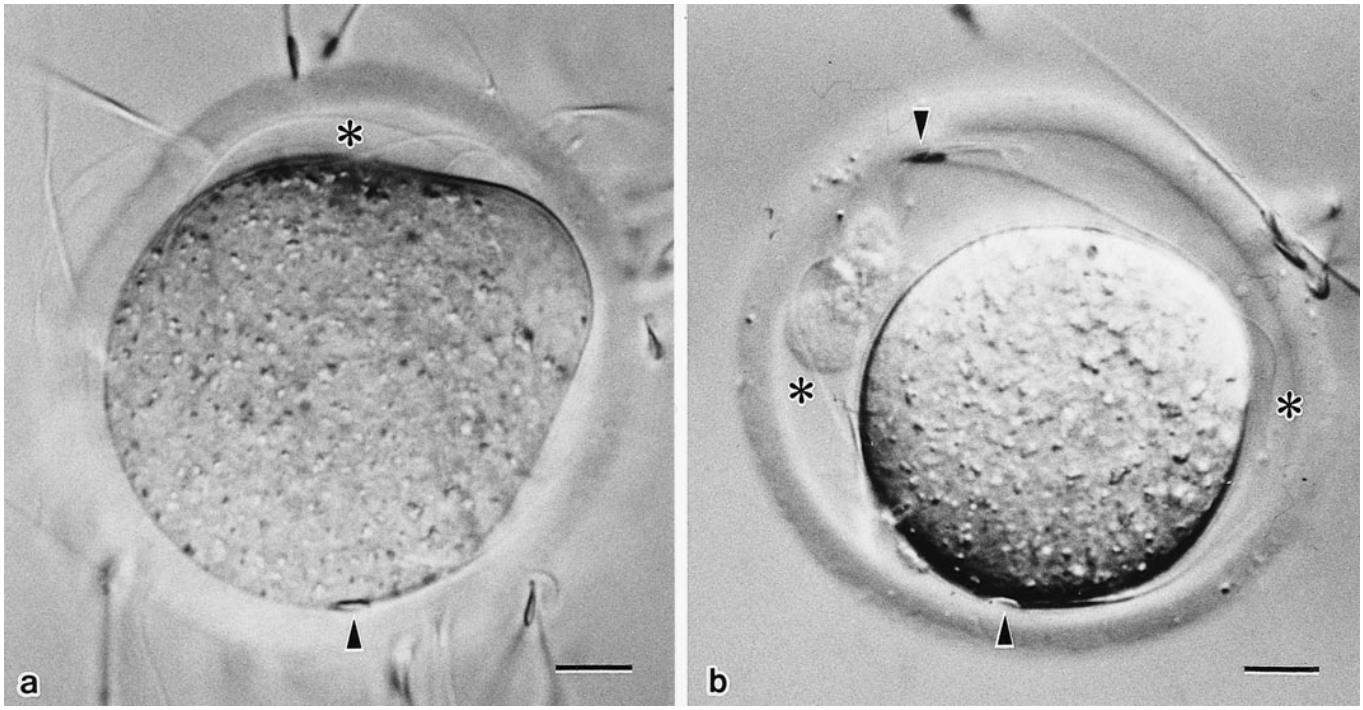

FIG. 1. Light micrographs showing gamete interaction in the zona-intact experiment, in which capacitated sperm were inseminated in the presence of mMN9 in TYH medium. Not fixed. Nomarski images. **a**) Sperm-binding after sperm were washed at 1 h after insemination; many sperm still bind to the zona pellucida. Additionally, one sperm head (arrowhead) and several flagella (asterisk) are found in the perivitelline space (perivitelline sperm). **b**) The occurrence of perivitelline sperm at 5 h after insemination. At this focus plane, at least 2 sperm heads (arrowheads) and several flagella (asterisks) are found in the perivitelline space. Bar = 14 μm.

Probability values less than 1% were accepted as significant.

Indirect Immunofluorescence (IIF) by Confocal Laser Scanning (CLS) Light Microscope

This study was performed to examine the immunoreactivity of equatorin in sperm after capacitation but before the acrosome reaction, and in perivitelline sperm recovered in the zona-intact experiment in the presence of mMN9. The perivitelline sperm were not fixed. After being washed 3 times, all the samples were incubated with the second antibody, goat fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG + IgM for 30 min. The samples were washed 3 times and then examined by a CLS light microscope (Leica, model TCS 4D; Leitz Wetzlar GBH, Wetzlar, Germany). Cross-reactivity of mMN9 to the zona pellucida and to the oolemma was also examined.

Conventional Electron Microscopy (EM)

Conventional EM was done to see the effect of mMN9 on sperm plasmalemma, especially over the equatorial segment, and to examine the features of the cortical granule and the second meiotic chromosome in the ooplasm. For these purposes, we randomly recovered 5 oocytes at 5 h after insemination in the zona-intact experiment in the presence of mMN9, i.e., under the condition of frequent occurrence of perivitelline sperm. The samples were washed two times and then routinely processed for transmission EM analysis after fixation with 2.0% glutaraldehyde and 1% osmium tetroxide as reported previously [22]. Thin sections were made on an ultramicrotome (LKB-Produkter AB, Bromma, Sweden) and stained with both uranyl acetate and lead citrate in an LKB 2168 ultrostainer. The EM observations were made with a JEOL 200CX (Tokyo, Japan) or with a Hitachi 7100 (Tokyo) transmission electron mi-

TABLE 1. Effect of anti-equatorin monoclonal antibody mMN9 on fertilization of zona-intact oocytes.

Treatment	Antibody concentration (μg/ml)	Pronucleus formation* (n = 4)		Two-cell formation* (n = 4)	
		Total number of oocytes observed	% (mean ± SEM)†	Total number of oocytes observed	% (mean ± SEM)‡
TYH (control)	—	164	70.7 ± 4.0^{a1}	145	81.3 ± 11.7^{b1}
mMC31 (control)	100	121	70.4 ± 5.5^{a2}	64	79.3 ± 1.1^{b2}
mMN9	25	55	38.4 ± 4.9^{a3}	55	40.0 ± 5.2^{b3}
	50	54	26.5 ± 2.1^{a4}	54	28.2 ± 4.5^{b4}
	100	105	16.2 ± 8.7^{a5}	197	18.9 ± 6.3^{b5}

* Pronucleus formation was checked at 5 h after insemination; two-cell formation was checked at 24 h after insemination.
† a1, a2 vs. a3, a4, a5: $p < 0.001$.
‡ b1, b2 vs. b3, b4, b5: $p < 0.001$.

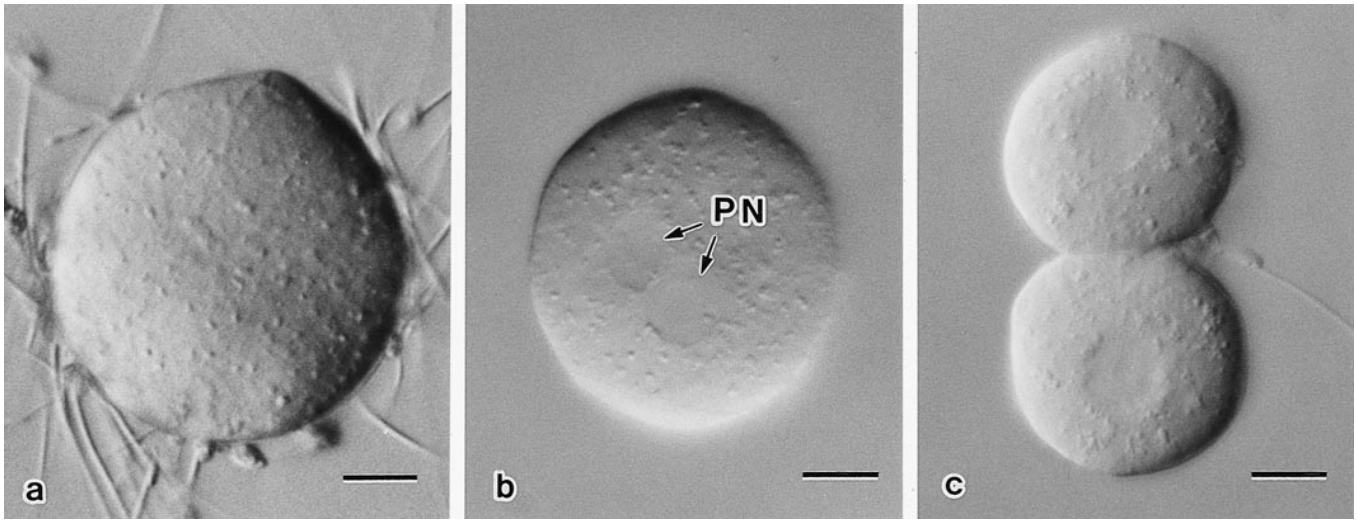

FIG. 2. Light micrographs of gamete interaction in the zona-free experiment, in which acrosome-reacted sperm inseminated zona-free oocytes. Nomarski images. **a**) Paraformaldehyde-fixed oocyte. Many sperm are attached to the oocyte surface at 2 h after insemination in the presence of mMN9. Bar = 20 μm. **b** and **c**) Control oocytes, which were inseminated in the absence of mMN9 in TYH medium. Not fixed. The oocytes have developed to the pronuclear stage (**b**) and 2-cell stage (**c**) at 5 h and 24 h after insemination, respectively. PN, pronucleus. Bar = 17 μm.

croscope at an accelerating voltage of 80 kV or 75 kV, respectively.

RESULTS

Effect of mMN9 on Sperm Motility, and Binding to the Zona and Oocyte Surfaces

All of the cellular events described above were monitored by a videotape system and analyzed. Sperm motility remained fair (70–90%) in all experimental and the control groups.

Soon after insemination, both in the presence and absence of mMN9, many sperm started to bind to the zona surface in the zona-intact experiment (Fig. 1) and to the oocyte surface in the zona-free experiment (Fig. 2). Even after brief washing, sperm remained bound to the zona surface (Fig. 1a) and to the oocyte surface (Fig. 2a).

Effect of mMN9 on Fertilization

As shown in Table 1, in the zona-intact experiment the rates of both pronucleus and two-cell formation decreased in a dose-dependent manner. The difference between the experimental and control groups was significant ($p < 0.001$). On the basis of this result, we chose 100 μg/ml of mMN9 for further experiments unless otherwise stated.

As shown in Table 2, in the zona-free experiment the rate of both pronucleus and two-cell formation also significantly decreased in the presence of mMN9. The difference between the experimental and control groups was significant ($p < 0.001$).

Occurrence of Perivitelline Sperm

This experiment was conducted in the presence of mMN9 in TYH medium, as was the zona-intact experiment, in triplicate. The control experiment was conducted in the absence of mMN9 (TYH medium only). The number of perivitelline sperm found in the presence of mMN9 was 3.6 times higher than in the control. Specifically, perivitelline sperm were found in 47 of 111 oocytes (39.6 ± 6.1; mean% ± SEM) in the presence of mMN9 (Fig. 1b), whereas they were found in only 7 of 63 control oocytes (11.0 ± 1,7; mean% ± SEM; not shown). The difference between the experimental and control groups was significant ($p < 0.001$).

Indirect Immunofluorescence by CLS

IIF-CLS light microscopy revealed that sperm recovered after capacitation but before the acrosome reaction showed an intense immunostaining pattern of ''hooked demilunes'' at the equatorial segment (Fig. 3, a and b). Acrosome-reacted sperm (perivitelline sperm) also showed an immunostaining pattern of hooked demilunes at the equatorial segment (Fig. 3, c–f). The immunostaining intensity appeared to be higher in sperm before the acrosome reaction than in perivitelline sperm (acrosome-reacted sperm). The mMN9 did not cross-react with the components of the zona

TABLE 2. Effect of anti-equatorin monoclonal antibody mMN9 on fertilization of zona-free oocytes.

	Pronucleus formation* (n = 4)		Two-cell formation* (n = 8)	
Treatment	Total number of oocytes observed	% (mean ± SEM)†	Total number of oocytes observed	% (mean ± SEM)‡
TYH (control)	81	83.9 ± 7.0^{a1}	99	83.3 ± 9.9^{b1}
mMN9 (100 μg/ml)	60	11.6 ± 9.0^{a2}	108	5.8 ± 11.0^{b2}

* Pronucleus formation was checked at 5 h after insemination; two-cell formation was checked at 24 h after insemination.
† a1 vs. a2: $p < 0.001$.
‡ b1 vs. b2: $p < 0.001$.

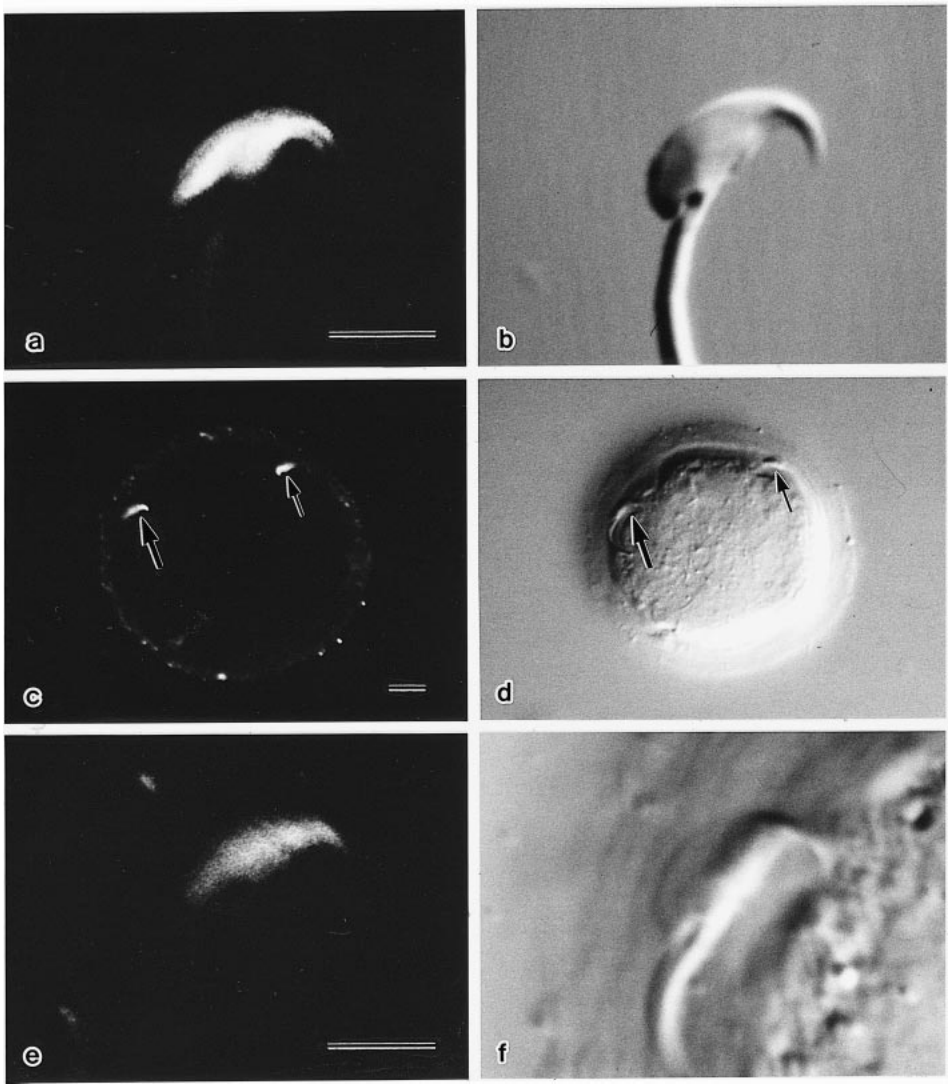

FIG. 3. Paired immunofluorescence (**a**, **c**, and **e**) and Nomarski (**b**, **d**, and **f**) micrographs taken by confocal laser scanning light microscopy. Not fixed. **a** and **b**) A spermatozoon after capacitation but before acrosome reaction. The equatorial segment is intensely immunostained, showing typical hooked demilune pattern (lateral view). Bar = 5 μm. **c–f**) An oocyte and perivitelline sperm, recovered at 1 h after insemination in the zona-intact experiment in the presence of mMN9. **c** and **d**) Low magnification showing two sperm heads immunostained in the perivitelline space (arrows). Several fluorescence spots distributed sporadically near the zona surface are contaminations. Bar = 10 μm. **e** and **f**) Higher magnification of a spermatozoan head indicated by large arrows in **c** and **d**. The equatorial segment (lateral view) shows typical hooked demilune immunostaining pattern, but the immunostaining intensity appears to be somewhat reduced. Bar = 5 μm.

pellucida and oolemma (Fig. 3, c and e). In addition, the sperm that bound to the zona surface showed a strong immunostaining intensity after capacitation but before the acrosome reaction (Fig. 3a).

Conventional EM

The plasmalemma over the equatorial segment was intact in many sperm that were recovered in the zona-intact experiment in the presence of mMN9; a representative spermatozoon is shown (Fig. 4a). We also incubated the acrosome-reacted sperm (reaction induced by zona protein) with mMN9 (100 μg/ml) for 2 h and then observed the status of the plasmalemma. Most of the sperm observed had intact plasmalemmas (not shown).

To examine the features of the cortical granule and the second meiotic chromosome in the ooplasm, we thoroughly checked the features of the ooplasm of 5 oocytes by the serial sectioning method. One oocyte was fertilized, showing pronucleus formation. In the remaining 4 oocytes, a number of unreleased cortical granules were apparently preserved in the ooplasm with the second meiotic metaphase chromosome (Fig. 4, b–e). In these 4 oocytes, we found a total of 10 perivitelline sperm (1–4 sperm per oocyte). No sperm penetrated into the ooplasm. The perivitelline sperm had intact plasmalemmas not only on the head (Fig. 4d) but also on the flagellum (Fig. 4e).

DISCUSSION

Antibodies directed against sperm components are reported to block fertilization both in vitro and in vivo [8, 23–26]. However, the mechanism of this blocking and the precise role of many antigenic molecules are as yet largely unknown.

In the present study, the anti-acrosomal mAb mMN9 significantly reduced the fertilization rate in both the zona-intact and zona-free experiments. This effect was most prominently demonstrated in the zona-free experiment in the presence of mMN9; the rates of both pronucleus and two-cell formation were greatly reduced (both to the range of 5–12%). However, even in this situation, many sperm could bind to the oocyte surface. On the other hand, mMN9 had no effect on sperm motility. Sperm-binding to the zona surface was not affected by mMN9, nor was sperm penetration through the zona pellucida inhibited. These findings imply that equatorin is not involved in the processes that occur up to binding to the oocyte surface but that it is involved in the process of sperm-oocyte fusion.

The zona reaction (hardening), which is mediated by

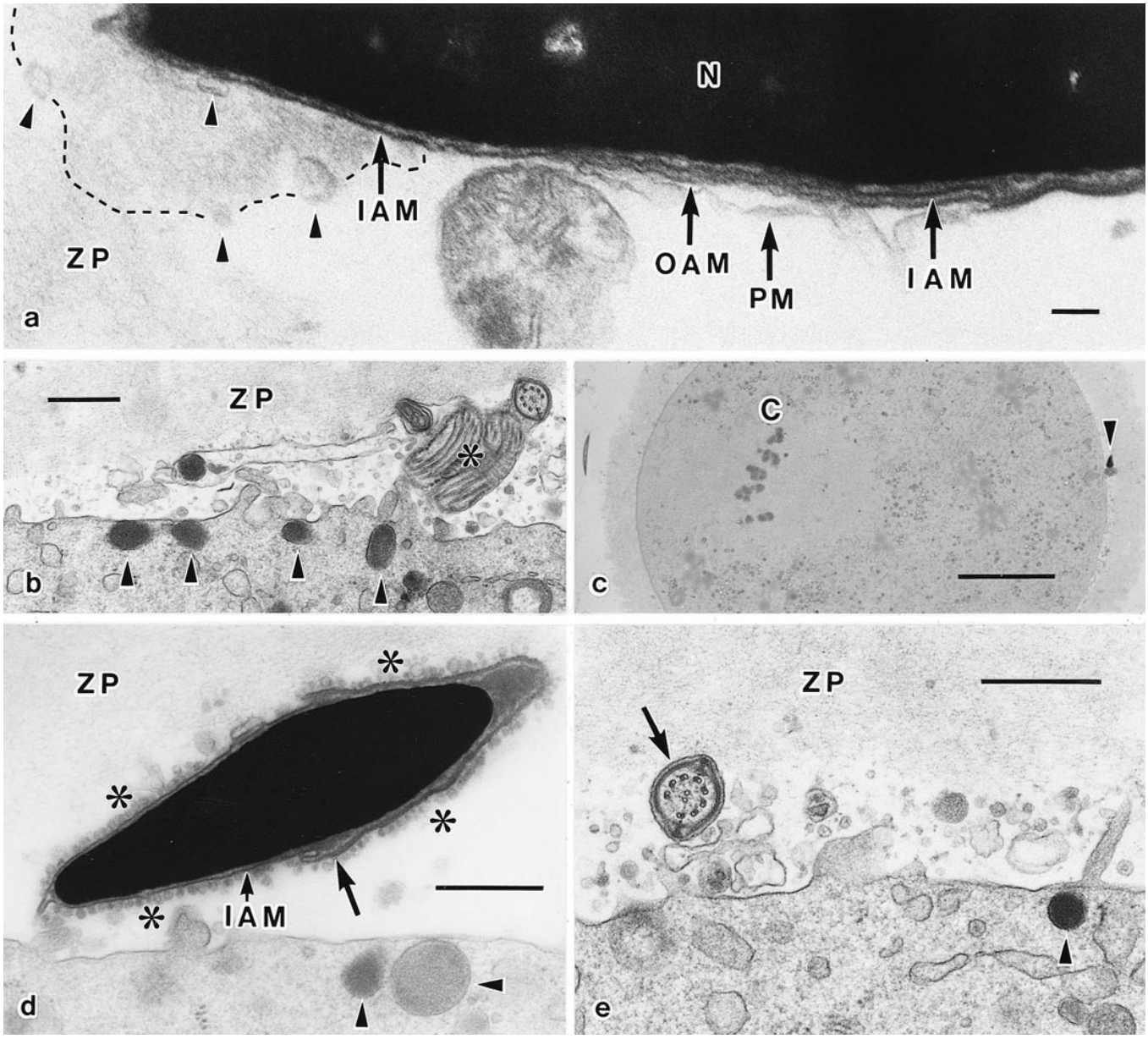

FIG. 4. Conventional electron micrographs showing various features of gamete interaction in the zona-intact experiment in the presence of mMN9 at 5 h after insemination. All these micrographs were taken from the same oocyte after serial sectioning. **a**) A spermatozoon during the acrosome reaction (dotted line shows the approximate border of the dispersing acrosomal content). Note the intact, but undulating, plasmalemma (PM) over the equatorial segment. Several acrosome-reacted vesicles (arrowheads) are seen between the sperm head and zona pellucida (ZP). N, nucleus. IAM, inner acrosomal membrane. OAM, outer acrosomal membrane. Bar = 0.1 μm. **b**) Many cortical granules (arrowheads) are found beneath the oolemma. The principal piece region of the flagellum (asterisk) is found in the perivitelline space. ZP, zona pellucida. Bar = 0.5 μm. **c**) The chromosome (C) of this oocyte shows the second meiotic metaphase. A perivitelline sperm head (arrowhead) is also found in the perivitelline space. Bar = 1 μm. **d**) A perivitelline sperm head is partially covered with the plasmalemma (arrow), but the anterior part of the head has already lost the acrosome contents and the associated membranes, exposing the inner acrosomal membrane (IAM). Many vesicles (asterisks) are found around the head, but the origin is unclear at present. Cortical granules (arrowheads) are also seen. ZP, zona pellucida. Bar = 0.5 μm. **e**) A cortical granule (arrowhead) and the principal piece region covered with plasmalemma (arrow) in the perivitelline space. ZP, zona pellucida. Bar = 0.5 μm.

cortical granule exocytosis, is a major component of the polyspermy block mechanism in vertebrates [4]. In this study, the incidence of perivitelline sperm was significantly higher in the presence of mMN9 than in its absence. This was further supported by the conventional EM result that many unreleased cortical granules were preserved in the unfertilized ooplasm with the second meiotic metaphase, even at 5 h after insemination. In mice, the period of 5 h after insemination is generally long enough for fertilized oocytes to develop to the pronuclear stage, as shown in the control experiment (Fig. 2b). These findings suggest that the zona reaction was not initiated in the presence of mMN9, indicating that cortical granule exocytosis did not occur, or at least did not occur efficiently. This means that sperm-oolemma fusion could not take place, or at least could not take place efficiently. Thus, oocyte activation could not be induced.

Concerning the localization of equatorin, previously we reported that equatorin is originally localized in the narrow gap between the outer and inner acrosomal membranes at the equatorial segment in cauda epididymal sperm [12]. The present IIF results indicate that equatorin is localized at the

equatorial segment in both capacitated and acrosome-reacted (perivitelline) sperm. Additionally, the IIF intensity appeared to be higher in the capacitated sperm than in the perivitelline sperm. This result suggests that the IIF intensity of the capacitated sperm reflects the net amount of equatorin that is originally localized within the equatorial segment before the acrosome reaction, whereas the reduced equatorial segment staining observed in the perivitelline sperm reflects the total amount of equatorin localized at the equatorial segment (equatorin that remains within the equatorial segment plus equatorin that is exocytosed and reaches the outer surface of the plasmalemma over the equatorial segment). It is probable that equatorin is exocytosed during penetration into the perivitelline space and reaches the plasmalemma over the equatorial segment or oolemma under in vitro and in vivo conditions, because previous EM data showed that the dense materials lodged in the equatorial segment gradually diminished during gamete interaction, especially during the approach to the oolemma and penetration into the ooplasm [1, 3, 4]. Therefore, there is a possibility that the exocytosed equatorin mediates an inevitable molecular event of sperm-oocyte fusion. Alternatively, the exocytosed equatorin may biophysically and biochemically modify the molecule(s) on the plasmalemma over the equatorial segment or oolemma, and such a modification may be involved in the sperm-oolemma fusion. In fact, alteration of intra-acrosomal molecules leading to gamete interaction is found in acrosin; the hamster acrosin, which is a major component of the acrosome, is reported to alter biophysical and biochemical characteristics of the plasmalemma over the equatorial segment during the acrosome reaction, and the modified plasmalemma is supposedly involved in the sperm-oocyte interaction [27]. Another example is oscillin, a 33-kDa protein localized in the equatorial segment in hamsters, which is involved in triggering oocyte development by inducing Ca^{2+} oscillation activity in the oocyte [9]. However, the precise behavior of oscillin during gamete interaction is unclear.

As to the sperm-oocyte fusion-related molecule, an interesting molecule is fertilin (PH-30). Mature fertilin is thought to play dual functions in sperm-oolemma binding and fusion via integrin/disintegrin-like interactions [28], since fertilin is a heterodimeric protein with two subunits (alpha and beta) that both have a transmembrane region of disintegrin and metalloproteinase [10]. However, it is reported that fertilin is initially localized to the whole head in testicular sperm and is redistributed to the postacrosomal region during epididymal maturation [29–31]. If so, a question is why the fertilin is not localized at the equatorial segment, where gamete membrane fusion starts [1, 3, 4].

mMN9 appears not to directly affect the zona pellucida and oolemma, since it did not cross-react with the zona pellucida and oolemma. However, the rat epididymal glycoprotein DE (37 kDa) is reported to be redistributed from the dorsal region to the equatorial segment during capacitation, and then it is involved in sperm-oocyte fusion [32, 33]. In this case, the glycoprotein DE interacts with complementary sites on oolemma. Therefore, we cannot totally exclude the possibility that equatorin may bind to the oolemma. To determine this, a more sophisticated approach is required.

The present conventional EM results indicate that mMN9 itself does not have a harmful effect on the sperm plasmalemma. This is inferred not only because the sperm both on the zona surface and after incubation with mMN9 had an intact plasmalemma over the equatorial segment, but also because the plasmalemma of perivitelline sperm was intact on both the head and flagellum, even at 5 h after insemination. It is known that sperm plasmalemma remains intact during penetration into the perivitelline space under in vivo condition in mice [34].

The findings reported here lead to the conclusion that equatorin preserved at the equatorial segment is involved in an inevitable process of sperm-oocyte fusion, and also suggest several possibilities for the role(s) of equatorin, such as mediator, modulator, or activator. Presumably, mMN9 antibody interferes with such a function(s) of equatorin, which eventually results in inhibition of oocyte activation and development. However, many questions remain such as how, precisely, equatorin acts during penetration into the perivitelline space; a satisfying explanation for the mechanism is not yet apparent. Further biochemical and immunoelectron microscopic investigations are surely required to address the questions raised in this study, and eventually to determine precisely the physiological role and behavior of equatorin.

Finally, it is noteworthy that equatorin may be one of the candidate molecules for contraception in many mammalian species, including humans, since mMN9 significantly inhibits fertilization, as shown in this study, and since the antigenic molecule equatorin is widely distributed in many mammalian species such as mice (38–48 kDa), rats (48 kDa), hamsters, guinea pigs, rabbits, monkeys, and humans [12].

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