

Inhibition of mouse fertilization *in vivo* by intra-oviductal injection of an anti-equatorin monoclonal antibody

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The monoclonal antibody mMN9 recognizes an antigenic molecule, equatorin, which is localized at the equatorial segment of the mammalian sperm acrosome. Our previous results using an IVF system indicated that mMN9 blocked sperm–oocyte fusion. Antibody-containing and control solutions were injected directly into the right and left oviductal ampullae, respectively, of anaesthetized female mice to assess the effect of mMN9 on fertilization *in vivo*. After hCG treatment, the females were mated, and their oviductal eggs and implanted embryos were examined.

mMN9 was retained in the oviductal lumen at 20 h after injection. The rates of fertilization and concomitant pregnancy were significantly lower than in the control side ($P < 0.05$). In addition, histological studies showed no evidence of pathological changes in the female reproductive tract after the injections. These results indicate that mMN9 inhibits mouse fertilization significantly under *in vivo* conditions and that this injection method should be useful for studying the effects of antibodies and agents on fertilization *in vivo*.

Introduction

Mammalian fertilization takes place in the female oviduct and requires complex, mutual interactions between spermatozoa and eggs. Fertilization involves several sequential steps, including binding of spermatozoa to the zona pellucida, acrosome reaction, sperm penetration through the zona pellucida, binding of spermatozoa to and fusion with the egg, and egg activation (Yanagimachi, 1994). Although it is thought that these steps are regulated, at least in part, by molecules (proteins) located in the acrosome of the spermatozoa, the precise mechanisms are still unclear.

The acrosome is a specialized membrane-bound organelle overlying the anterior part of the sperm head that contains hydrolytic enzymes and several antigenic molecules (Eddy and O'Brien, 1994). Functionally, the anterior region of the acrosome is involved in the acrosome reaction, whereas the equatorial segment (posterior region) engages in membrane fusion with the egg (Yanagimachi, 1994). Numerous studies have been performed on the roles of various components of the acrosome (Snell and White, 1996; Wassarman, 1999) and several functional molecules located specifically at the equatorial segment have been reported. For example, a mouse sperm 40 kDa antigen (M29) (Saling and Waibel, 1985), guinea-pig sperm 34 kDa antigen (G11/M13) (Allen and Green, 1995), hamster sperm 33 kDa protein (oscillin) (Parrington *et al.*, 1996), hamster sperm 37.5 and 34.0 kDa protein (M1) (Noor and Moore, 1999), rat epididymal 37 kDa protein (DE) (Cohen *et al.*, 2000), and several members of a disintegrin and metalloprotease (ADAM) family (Blobel, 1999; Primakoff and Myles, 2000).

Equatorin, a complex 38–48 kDa protein in mice, is the antigenic molecule of the monoclonal antibody mMN9 and is localized originally in the equatorial segment of cauda epididymal spermatozoa in various species, including humans (Toshimori *et al.*, 1992a). The studies by Toshimori *et al.* (1998), using an IVF system, indicated that equatorin is preserved at the equatorial segment after the acrosome reaction and that mMN9 blocks sperm–egg fusion significantly. The aim of the present study was to investigate the effect of mMN9 on mouse fertilization *in vivo* using an intra-oviductal injection method.

Materials and Methods

Animals

Adult ICR mice aged 8–10 weeks were purchased from Japan SLC Inc. (Hamamatsu) and maintained in the Experimental Animal Center of Miyazaki Medical College under an automatic 12 h light:12 h dark schedule at 20°C with free access to food and water. Animals were acclimated at least 5 days before use. Handling and treatment of animals were conducted in accordance with the guidelines for the care and use of laboratory animals established by the Animal Research Committee of the college.

Purification of monoclonal antibody

All chemicals used in this study were purchased from Nakalai Tesque Inc. (Kyoto) unless otherwise stated. The mouse anti-equatorin monoclonal antibody mMN9 (IgG2a) was characterized previously by Toshimori *et al.* (1992a) and was purified as described by Toshimori *et al.* (1998). In

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brief, the antibody was purified from spent culture medium using an Ampure PA kit (Amersham, Little Chalfont) and eluted with elution buffer. The protein concentration was determined by the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL); the peak fraction was desalted with a PBS (pH 7.4) pre-equilibrated Sephadex G 25M column (Pharmacia, Uppsala) and the protein concentration was measured again. Only the fraction that showed the highest protein concentration (0.6 mg ml^{-1}) was used. Purified anti-rat sperm flagellar surface antibody mMC31 (IgG1) (Toshimori *et al.*, 1992b) was used as a control monoclonal antibody, as it does not bind to mouse spermatozoa or eggs.

Injection of antibody into the oviductal ampulla

A technique was developed to inject antibodies directly into the proximal region (ampulla) of the oviduct to assess the effect of mMN9 on fertilization *in vivo*. Ovulation was induced in female mice ($n = 25$) by an i.p. injection of 5 iu equine chorionic gonadotrophin (eCG) followed by 5 iu hCG 48 h later. Approximately 2 h before hCG administration, the females were anaesthetized with sodium pentobarbital (0.075 mg g^{-1} body weight). A surgical incision was made on the dorsal mid-line through the skin and each oviduct with fat pad, ovary and uterus was pulled gently out of the body cavity using forceps (Fig. 1). A fine glass micropipette with a tip diameter of approximately $30 \mu\text{m}$ was prepared using a Sutter micropipette puller (model P-97). The pipette containing solutions was attached by tubing to an Eppendorf microinjector (model 5246) so that the speed of the injection and the volume injected could be controlled. Under a dissecting microscope, $3\text{--}5 \mu\text{l}$ purified mMN9 (0.6 mg ml^{-1} in PBS) was injected slowly into the lumen of the proximal region (ampulla) of the right oviduct through the oviductal wall (Fig. 1). For controls, $3\text{--}5 \mu\text{l}$ purified mMC31 (0.6 mg ml^{-1} in PBS) was microinjected into the left ampulla. The oviducts were replaced gently and the body wall and skin were closed with wound clips. Females were caged overnight with male mice of proven fertility and were checked for the presence of vaginal plugs the next morning. Female mice with obvious plugs ($n = 13$) were included in the study. The day of appearance of a vaginal plug was designated as day 1 after mating. The numbers of female mice used in each experiment are shown (Table 1).

Egg collection and morphological analysis

Seven female mice were killed by cervical dislocation on the afternoon of day 2 after mating (44–46 h after injection; that is, 42–44 h after hCG administration). Eggs were flushed from each oviduct using a 28-gauge needle attached to a syringe under oil (Sigma Chemical Co., St Louis, MO) in plastic dishes, examined under an inverted phase-contrast microscope and photographed with a Nikon Diaphoto-300 inverted microscope equipped with a Nomarski apparatus. Fertilization was determined by

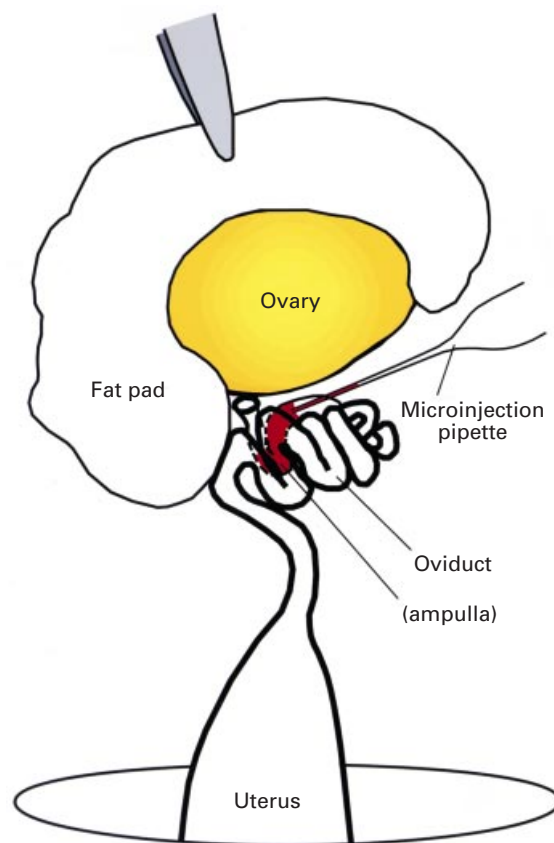


Fig. 1. Schematic representation of injection of antibody solution (red) into the oviductal ampulla region of a mouse. The ampulla was penetrated with a microinjection pipette with 1 mm outside diameter and a sharp $30 \mu\text{m}$ tip. The solution was introduced into the pipette with an Eppendorf microloader tip.

two-cell formation at this time. The eggs were examined further with a phase-contrast microscope for evidence of spermatozoa in the perivitelline space.

Collection of ampullary fluid and immunodetection of injected mMN9

An immunofluorescence microscopy technique was used to examine the presence of mMN9 injected into the ampulla. If inhibition of fertilization had been caused by administration of antibodies, the antibody should have been detected at the time of mating in the ampullary lumen. Thus, three female mice were not mated and were killed at 20 h after injection (18 h after hCG administration), a time just after fertilization under laboratory conditions. The ampullary fluids of mMN9-injected and control mMC31-injected mice were recovered by puncturing the ampullae and were used as primary antibodies. Paraformaldehyde-fixed mouse cauda epididymal spermatozoa that had been air-dried onto slides were incubated with the primary antibodies at approximately 1:10 dilution in PBS for 1 h. After washing, the slides were incubated with fluorescein

Table 1. Numbers of female mice used in experiments

Purpose	Time after hCG administration	Total number of females	Number of females killed	
			Plug formation	No plug formation
Egg collection and morphological analysis	42–44 h (day 2)	12	7	5
Morphological analysis of implanted embryo	Day 8	10	6	4
Collection of ampullary fluid	18 h	3		Not mated (3)

isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100 in PBS; Biosource International Inc., Camarillo, CA) for 30 min and examined with an Olympus BX-50 microscope equipped with epifluorescent optics.

Immunofluorescent specificity of purified mMn9

Paraformaldehyde-fixed mouse cauda spermatozoa mounted onto slides and paraformaldehyde-fixed or unfixed mouse oviductal eggs were incubated with purified mMn9 for 1 h to confirm the immunohistochemical specificity of purified mMn9. After washing, the spermatozoa and eggs were incubated with FITC-conjugated goat anti-mouse IgG for 30 min and examined as described above.

Morphological and histological analyses of implanted embryos

Six female mice were killed on day 7 after mating (day 8 after microinjection) and the numbers of implanted embryos in the right (experimental) and left (control) uterine horns were counted under a dissecting microscope. Implanted embryos were identified by the presence of a decidual capsule. The histology of some uteri with decidual capsules was examined for evidence of embryological development, pathological or artificial changes. Tissues were fixed in Bouin's solution and embedded in paraffin wax and cut into sections by routine methods. Sections (5 μ m thickness) were stained with haematoxylin and eosin and examined by light microscopy.

Statistical analysis

Data on the fertilization rate and the number of implantation sites are expressed as mean \pm SEM and were analysed using Wilcoxon paired test. Differences between values were considered significant at $P < 0.05$.

Results

Effects of mMn9 injection on fertilization in mice

In a preliminary experiment, injection of large volume (10 μ l) of sterile PBS into the ampullae caused leakage of

the solution from the infundibulum, resulting in lower recovery of ovulated eggs in the ampullae on day 2 after mating, whereas smaller volume (3–5 μ l) injections resulted in no significant differences in the total numbers of ampullary eggs or subsequent fertilization between untreated ampullae and PBS- or control mMn31-injected ampullae (data not shown). Therefore, small volume injections of antibodies into the ampullae were used for subsequent experiments.

The effect of mMn9 on fertilization was examined at 44–46 h after injection (42–44 h after hCG administration). If fertilization had taken place normally, eggs at this time should have developed to at least the two-cell stage. Two-cell formation was suppressed significantly in eggs recovered from mMn9-injected oviducts, whereas almost all the eggs from control-injected oviducts developed normally to the two-cell stage (Fig. 2). Significantly more ovulated eggs ($48.7 \pm 9.8\%$ of 63 eggs) were fertilized in mMn9-injected oviducts compared with eggs in control oviducts ($96.7 \pm 2.5\%$ of 75 eggs) ($P < 0.05$; Fig. 3). Furthermore, approximately 20% (6 of 29) unfertilized eggs recovered from mMn9-injected oviducts had one or more spermatozoa in the perivitelline space (Fig. 4) compared with 0% (none of six) in controls. The percentage of parthenogenetic, fragmented or degenerated eggs was $< 5\%$ in both mMn9- and control-injected oviducts.

Detection of injected mMn9 in the ampulla

The presence of intraluminal mMn9 was verified by immunofluorescence microscopy. Intense immunolabelling of the equatorial segment of spermatozoa was observed in fluids recovered from ampullae injected with mMn9 (Fig. 5). In contrast, no immunoreaction was observed in fluids recovered from control-injected ampullae (not shown).

Immunostaining of spermatozoa using purified mMn9

The purified mMn9 used in this study showed strong immunoreactivity with the equatorial segment of the spermatozoa and did not crossreact with egg components, including the zona pellucida and oolemma (not shown).

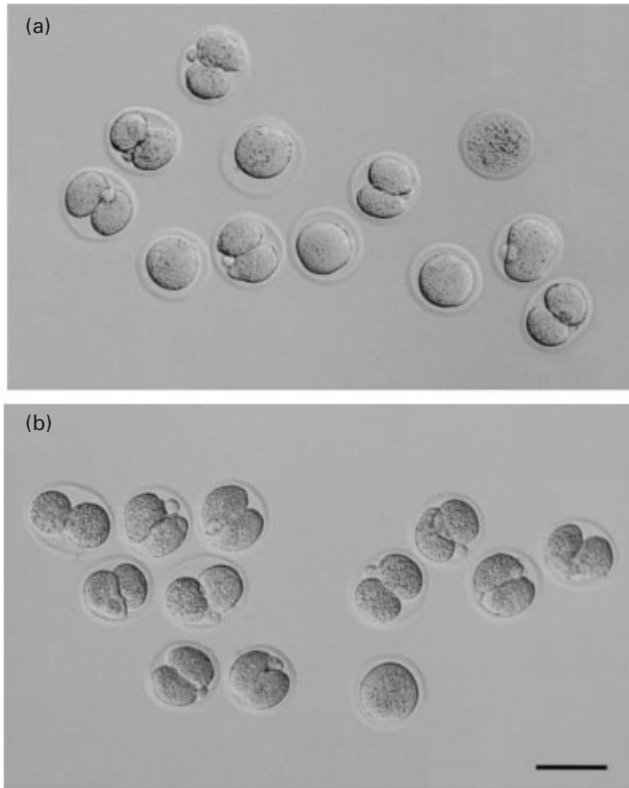


Fig. 2. Light micrographs of eggs collected from oviducts of a mouse 44 h after intra-oviductal injection (42 h after hCG administration). (a) Only 50% of eggs had developed to the two-cell stage in the mMN9-injected oviduct, whereas almost all had developed normally to the two-cell stage in the control-injected contralateral oviduct (b). Scale bar represents 100 μm .

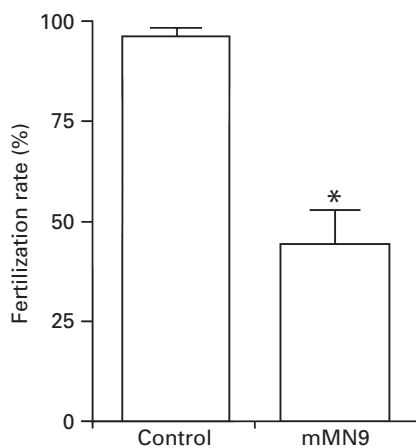


Fig. 3. Effect of intra-oviductal injection of mMN9 on fertilization in mice. Results are the percentage (mean \pm SEM, $n = 7$) of two-cell formation examined on day 2 after mating (42 h after hCG administration). *Value is significantly different from control ($P < 0.05$).

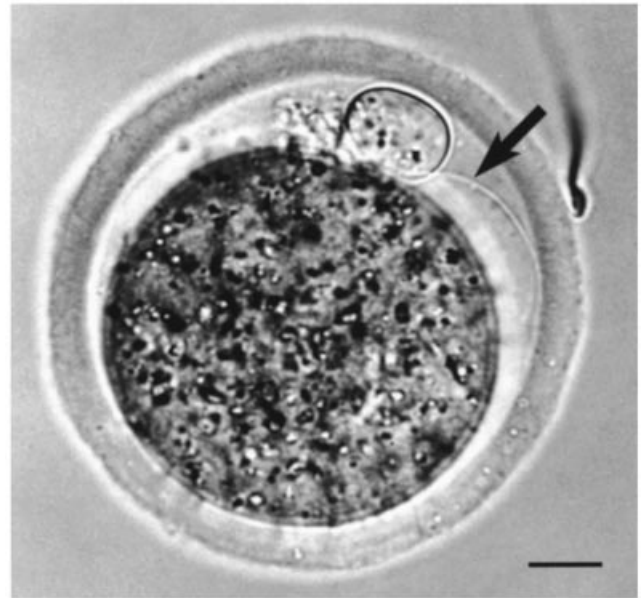


Fig. 4. Light micrograph of an egg collected from the oviduct of a mouse 44 h after intra-oviductal injection of mMN9 (42 h after hCG administration). At this focus plane, at least one sperm tail (arrow) is found in the perivitelline space. Scale bar represents 17 μm .

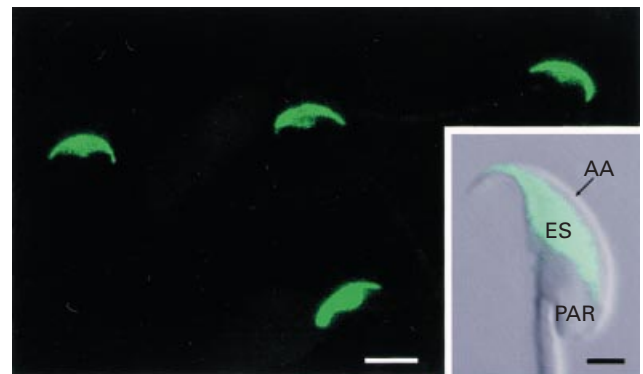


Fig. 5. Immunofluorescent detection of intraluminal mMN9 at 20 h after intra-oviductal injection. Paraformaldehyde-fixed mouse spermatozoa from the cauda epididymidis were incubated with the ampullary fluid as primary antibody, followed by FITC-conjugated secondary antibody. Note the fluorescent reaction (green) in the equatorial segment of the sperm head, which shows typical 'hooked-demilune' patterns. Scale bar represents 5 μm . Inset shows a distinct immunoreaction at the equatorial segment (ES) but not at the anterior acrosome (AA) or post-acrosomal region (PAR). Scale bar represents 1 μm .

Effects of mMN9 injection on pregnancy in mice

The effect of injection of mMN9 on subsequent pregnancy was examined on day 7 after mating (day 8 after intra-oviductal injection). All of the pregnant mice examined appeared to be healthy. The number of implantation sites

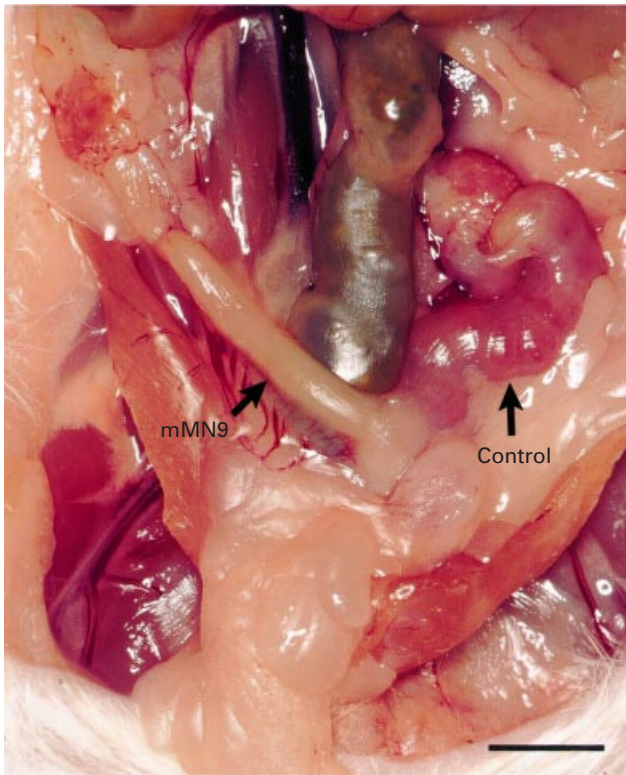


Fig. 6. Ventral view of the abdominal cavity of a mouse examined on day 7 after mating (8 days after intra-oviductal injection). No implantation site is visible in the right uterine horn of the mMN9 injection side (arrow with mMN9), whereas more than five implantation sites are visible in the left control side (arrow with control). Scale bar represents 40 mm.

was decreased markedly in the uterine horn of the mMN9-injected side, whereas the contralateral uterine horns of the control-injected side achieved normal pregnancy rates (Fig. 6). The mean number of implanted embryos in the uteri of mMN9-injected sides was 1.0 ± 0.5 compared with 5.5 ± 0.9 in the uteri of control-injected sides ($P < 0.05$; Fig. 7). Histological examination revealed that embryos recovered from both sides showed normally developed features of the advanced egg cylinder stage or early primitive streak stage, which were identified by the morphological criteria of Kaufman (1992). In addition, tissue damage such as artefactual or pathological changes was not observed in any of the embryos or uteri examined (not shown).

Discussion

An intra-oviductal injection technique has been used to demonstrate that local administration of the anti-equatorin monoclonal antibody mMN9 reduces fertilization and subsequent pregnancy in mice significantly. This is the first report demonstrating the local effect of monoclonal antibodies on fertilization *in vivo*. A passive immunization

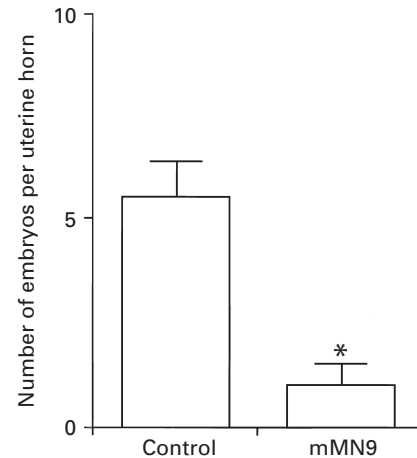


Fig. 7. Effect of intra-oviductal injection of mMN9 on pregnancy in mice. Results are expressed as the number (mean \pm SEM, $n = 6$) of implanted embryos on day 7 after mating. *Significantly different from control ($P < 0.05$).

technique has been used previously to assess the effects of specific antibodies against sperm components on fertilization *in vivo*; Saling and Waibel (1985) reported that the M29 antibody, which recognizes the equatorial segment of mouse spermatozoa, blocked fertilization in mice. Although the immunization technique (Primakoff *et al.*, 1988) would be useful for development of contraceptive vaccines (Naz *et al.*, 1995; Diekman and Herr, 1997), the intra-oviductal injection method used in the present study has several advantages for research purposes, including requiring a much smaller amount of antibody and avoiding generalized effects on the immune system. Moreover, the effects of two different solutions can be compared simultaneously under identical physiological conditions in the two oviducts of one animal. The effects of many antibodies on fertilization *in vivo* remain unknown. As all the pregnant animals examined in the present study appeared to be healthy and did not show any mechanical disturbances or pathological changes in the reproductive tract, the intra-oviductal injection does not appear to be harmful. Thus, this method provides a useful, effective and safe tool for examining the effects of antibodies on fertilization. Intra-oviductal injection has been used successfully for transfection of exogenous DNA (Relloso and Esponda, 1998), transportation of microspheres (Moore and Croxatto, 1988) and embryo transfer (Hogan *et al.*, 1994).

Concerning the specificity of immunoreactivity of the antibody mMN9, the results of the present *in vivo* study indicate that mMN9 reacts with the equatorial segment of the spermatozoa and does not react with egg components. Furthermore, our previous results using an IVF system indicated that mMN9 does not crossreact with components of zona-free eggs and also revealed that acrosome-reacted perivitelline spermatozoa showed a positive

immunoreaction at the equatorial segment (Toshimori *et al.*, 1998). These findings indicate that mMN9 may be able to penetrate the zona pellucida.

A significant reduction of fertilization rate was observed in the present study. This result, combined with our previous observations indicating that mMN9 blocks sperm–egg fusion *in vitro* (Toshimori *et al.*, 1998), indicates strongly that equatorin plays a functional role in sperm–egg fusion. Importantly, the immunofluorescence analysis revealed that intraluminal fluids could bind specifically to the equatorial segment of the sperm at 20 h after injection of mMN9, which is the specific immunostaining pattern of mMN9 (Toshimori *et al.*, 1992a; present study). This finding indicates clearly that mMN9 can be retained and is active in the ampullary lumen for at least 20 h after injection, a period that is of sufficient duration for mouse spermatozoa to complete fusion with eggs. Similarly, Saling and Waibel (1985) reported that M29 antibody (IgM class) was detected in the oviduct at 14–16 h after i.p. administration, although its access route was not investigated.

Consistent with the *in vitro* findings of Toshimori *et al.* (1998), it was found that spermatozoa bind to the zona pellucida and remain in the perivitelline space of eggs from mice that received mMN9 *in vivo*. These results indicate that mMN9 does not affect sperm binding to or penetration through the zona pellucida, but does affect sperm–egg fusion. Perivitelline spermatozoa were observed more frequently in eggs treated with mMN9 during IVF, probably as a result of high sperm concentrations (4×10^4 spermatozoa ml⁻¹) in the culture medium (Toshimori *et al.*, 1998), although fewer than 100 spermatozoa actually reach the mouse ampulla during natural fertilization (Harper, 1994). Recent studies using knockout mice also reported perivitelline spermatozoa in eggs from mated female mice lacking egg surface protein CD9, which has an essential role in sperm–egg fusion (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miller *et al.*, 2000; Miyado *et al.*, 2000). These results provide further support for the involvement of equatorin in sperm–egg fusion in mice.

A high rate of failure of pregnancy was observed in the uterine horns of the mMN9-injected side. This failure is probably the result of inhibition of fertilization rather than of subsequent events such as embryonic development or implantation, as mMN9 binds specifically to spermatozoa and does not bind to the egg, oviduct or uterus (Toshimori *et al.*, 1992a). However, the possibility of a suppressive effect of mMN9 incorporated into fertilized eggs or later stage embryos cannot be excluded. The fate of the equatorin molecule within the eggs and embryos remains to be studied.

In addition to equatorin, several sperm molecules are involved in mammalian sperm–egg fusion (Myles *et al.*, 1993; Evans, 1999). Fertilin α/β and cyritestin, members of the ADAM family of transmembrane proteins on spermatozoa, are thought to interact with integrin receptors on the egg plasma membrane through their disintegrin domains (Blobel, 1999; Primakoff and Myles, 2000). mMN9 inhibits fertilization both *in vivo* and *in vitro* (Toshimori

et al., 1998), presumably by inhibiting sperm–oocyte fusion. Further molecular and ultrastructural studies will be helpful for understanding the precise mechanism by which equatorin is involved in fusion.

In conclusion, the present study is the first to demonstrate that fertilization and subsequent pregnancy can be reduced by local administration of an anti-sperm antibody by mechanisms involving inhibition of sperm–egg fusion. The injection method developed in the present study should be useful for studying the effects of antibodies and agents on fertilization *in vivo*.

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