Effect of a Monoclonal Anti-sperm Antibody (A-1) on *in Vitro* Fertilization in the Mouse

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We prepared four monoclonal antibodies raised against mouse spermatozoa. The ability of the hybridoma culture supernatants to inhibit *in vitro* mouse fertilization was determined. One of these monoclonal antibodies, termed A-1, strongly inhibits sperm penetration into zona pellucida of egg in a concentration-dependent fashion. On the other hand, even in the presence of the same concentration of A-1 antibody, sperm binding to cumulus-free eggs was slightly inhibited and sperm-egg fusion was not inhibited at all. Immuno-fluorescence studies with A-1 showed that it recognizes an antigen(s) localized in the acrosome of mouse spermatozoa. In the case of unfixed sperm, the acrosome-intact sperm (*i.e.*, sperm bound to egg zona pellucida) and unbound sperm were slightly immunostained with A-1 antibody (1.6% and 9.0%, respectively). All the full acrosome reacted sperm (*i.e.*, sperm penetrating into the perivitelline space) were not immunoreacted. On the other hand, when sperm were fixed with methanol, approximately 80% of unbound sperm were immunoreacted with this antibody. Furthermore, almost all of zona-binding sperm were immunostained, whereas acrosome reacted, penetrating sperm were not. These data suggested that the antigen recognized by A-1 was located inside the plasma membrane of spermatozoa, and appeared on the surface of the spermatozoa during the period of penetration into egg zona pellucida. After sperm penetration into the perivitelline space this antigen must be released from the sperm.

Keywords acrosome reaction; sperm penetration inhibition; zona pellucida

Introduction

During the process of fertilization different sperm components are involved in gamete recognition.^{1–4)} The antigenic properties of the macromolecules involved in these interactions make possible the use of monoclonal antibodies for their identification and detailed characterization of their biological properties and activities. Therefore, the attempts to identify and characterize such molecules in mammalian spermatozoa using monoclonal antibodies have been carried out.^{5–11)} Lakoski *et al.* reported that the M 42 antigens participate in the zona-induced acrosome reaction.¹²⁾ Okabe *et al.* have previously reported that a monoclonal anti-sperm antibody (OBF13) blocks sperm-egg fusion.¹⁰⁾ By flow cytometric analysis, we showed that the OBF13 antigen migrates from inner acrosomal membrane to plasma membrane during “true” acrosome reaction.¹³⁾

In this paper, we newly prepared four monoclonal antibodies raised against mouse sperm and attempted to investigate what kinds of molecule would be concerned with sperm-egg interaction(s) by means of these monoclonal antibodies.

Materials and Methods

Preparation of Antigen and Immunization of Mice BALB/c and ICR mice were from Shizuoka Laboratory Animal Center, Japan. Sperm were collected as previously described.¹⁴⁾ Sperm (10^6 cells/ml) were capacitated by preincubation in modified Krebs–Ringer bicarbonate medium (m-KRB) containing 0.4% bovine serum albumine (BSA) (Fraction V, Sigma) for 60 min at 37°C in a CO₂-incubator and were washed with m-KRB by centrifugation ($600g \times 10$ min). The capacitated sperm were incubated in m-KRB containing 5 μ M A23187 (Calbiochem), 1 mM benzamidine (Aldrich) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) for 10 min at 37°C. After incubation, the sperm suspension was centrifuged at 1500 g for 15 min. The supernatant was carefully separated and centrifuged at 105000 g for 60 min at 4°C. The resulting particulate pellet was resuspended with the same volume of phosphate buffered saline (PBS). Five female BALB/c mice (7 weeks old) were immunized with pellet fraction in Freund's complete adjuvant (DIFCO). Every 14 d for 2 months the immunization was repeated with the same pellet fraction in incomplete

adjuvant (DIFCO).

Cell Fusion and Cloning Spleen cells (4×10^8) from five immunized mice were fused with myeloma (P3U1) cells (4×10^7) using 50% w/v polyethylene glycol 4000 (Sigma) in serum-free Dulbecco's modified Eagle medium (D'MEM: Nissui Seiyaku, Japan). Following fusion, the cells were seeded (5×10^5 myeloma cells per well) in 96-well tissue culture plate (Corning, New York) and cultured at 37°C in the HAT (100 μ M hypoxanthine, 400 nM aminopterin and 16 μ M thymidine) in D'MEM containing 15% fetal calf serum (Flow, CA). After 16 d, the cultures were screened for antibody activity by enzyme-linked immuno sorbent assay (ELISA) and indirect immunofluorescence (IIF) assay as described below. Four hybridoma cell lines were cloned twice by limiting dilution. In order to prepare the conditioned medium the established hybridomas and P3U1 cells were cultured for 48 h in serum-free D'MEM. The culture supernatants were collected and concentrated to one-20th of their original volume with Centricon (Amicon, Type CF25). The immunoglobulin subclasses of the monoclonal antibodies were determined using a mouse monoclonal typing kit (The Binding Site Ltd., Birmingham, UK).

Solid Phase Assay (ELISA) of Anti-mouse Sperm Antibody Antibodies in hybridoma supernatants were measured by ELISA assay. Antigen was prepared as follows. Washed-capacitated sperm (10^5 cells) in 100 μ l of PBS were placed in wells (Sumitomo 96-well S-Plate). The cells were allowed to air-dry at room temperature onto the bottom of the plate. After drying, 3% skim milk (Morinaga) in PBS (300 μ l) was added to each well. After 1 h at room temperature the plates were used for ELISA assays or stored at 4°C until use.

ELISA assays were performed with Hybri-Clonal EIA mouse Ig(G + M) screening kit (KPL). Briefly, 50 μ l of hybridoma supernatant added to each well which was prepared as described above. After 1 h at 37°C, excess antibody was removed by washing three times with TTBS (0.05% Tween 20 in 20 mM Tris buffered saline). Fifty μ l of second antibody, peroxidase-conjugated goat anti-mouse Ig(G + M), were then added (1:500 dilution). After 1 h at 37°C the excess second antibody was removed and the wells were washed three times with TTBS. In the last step, 100 μ l of substrate solution (4 mM ABTS (2,2'-azinobis(3-ethyl-benzthiazoline-sulfonate) and 3% hydrogen peroxide) was added and incubated for 1 h at room temperature. The reaction was stopped by adding 20 μ l of 10% sodium dodecyl sulfate (SDS). The absorbance was determined at 415 nm using a microplate reader (Bio-RAD, model 3550). The background was measured as follows. The culture supernatant of myeloma (P3U1) cells was added instead of hybridoma culture supernatant.

Indirect Immunofluorescence (IIF) Staining of Fixed or Fresh Sperm To determine whether antigens are surface sperm proteins the antibody binding assays were performed with the use of the following sperm preparations

as antigen: (1) fresh (uncapacitated) sperm (2) preincubated (capacitated) sperm (3) sperm bound to zona pellucida (4) sperm penetrating into the perivitelline space. Various sperm preparations were fixed in 70% alcohol (methanol or ethanol) or 2% formaldehyde in PBS for 15 min at room temperature and washed 3 times with PBS. Living sperm and fixed sperm (10^5 cells) were incubated with hybridoma culture supernatant for 15 min at 37°C. Excess first antibody was removed by washing with PBS three times. Twenty-five μ l of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (G+M) (Antibodies Inc.) 1:500 in 5% FCS-PBS were added and incubated for 10 min at 37°C in the dark. After washing, the reactivity with the antibody was assessed under a fluorescent microscopy (VANOX-T, Olympus).

Sperm-Egg Binding Assay Sperm-egg binding assay was performed as previously described.¹⁵ Briefly, eggs were freed from their cumulus cells by hyaluronidase treatment and washed in m-KRB. Capacitated sperm (4×10^4 cells) were preincubated with various concentrations of culture supernatant for 30 min in 0.4 ml drops of m-KRB and were incubated with eggs in 5%-CO₂/95%-air for 30 min at 37°C. At the end of the culture periods, the gametes were centrifuged through a discontinuous dextran gradient. The number of bound sperm per egg was determined under phase contrast microscopy (Type IMT, Olympus).

Assays for Sperm Penetration into Zona and Sperm Egg Fusion Capacitated sperm (4×10^4) were preincubated in 0.4 ml of m-KRB with various concentrations of hybridoma culture supernatant for 30 min and were incubated with eggs for 90 min at 37°C. Sperm penetration into zona pellucida was determined under phase contrast microscopy. For sperm-egg fusion assay, zona-free eggs were prepared as follows. Cumulus free eggs were treated in 0.1 M glycine-HCl buffered saline (pH 3.0) at room temperature under stereoscopic microscopy (Type TZ-R, Olympus). The zona-free eggs were washed three times with m-KRB. Capacitated sperm and zona-free eggs were incubated in 0.4 ml of m-KRB in the presence or absence of hybridoma culture supernatant for 4.5 h. The formation of enlarged sperm heads and/or of male and female pronuclei was observed under phase contrast microscopy or microscopy equipped with Nomarski apparatus (Type IMT, Olympus).

Results

We prepared four hybridoma cell lines (28A, A-1, 108F, 410A) producing antibodies raised against mouse sperm acrosome. The effects of these monoclonal antibodies on (1) sperm-egg binding, (2) sperm penetration into zona pellucida and (3) sperm-egg fusion were studied. Among four monoclonal antibodies, one of the monoclonal antibodies (termed as A-1) gave strong inhibition of sperm penetration into egg zona pellucida. Other antibodies didn't affect sperm penetration rate (Fig. 1). In both cases of capacitated and noncapacitated sperm, the extent of inhibition wasn't altered. The extent of inhibition by A-1 antibody was dependent on its concentration. Pretreatment of capacitated or fresh sperm with this antibody did not alter the fertilization rate. The immunoglobulin subclass of A-1 was Ig-M (data not shown).

The effect of the monoclonal antibody on sperm motility and sperm binding to the zona pellucida of mouse cumulus-free eggs was also assessed. After capacitation *in vitro*, sperm were incubated with or without A-1 antibody and then added to cumulus-free eggs. The number of sperm bound to egg was partially decreased by a monoclonal antibody A-1 compared with control (P3U1) culture supernatant (20% inhibition), but had little, if any, effect of sperm motility and sperm agglutination even at the same concentration of antibody that had completely blocked sperm penetration into zona pellucida. All other monoclonal antibodies showed no inhibition on sperm binding to egg zona pellucida (data not shown).

The effect of the monoclonal antibodies on sperm fusion into the zona-free eggs was also assessed. About forty zona-free eggs were incubated with capacitated sperm in

Fig. 1. Effects of Monoclonal Antibodies on Sperm Penetration into Zona Pellucida

Sperm were preincubated for 30 min with various antibodies (two fold dilution of culture medium) and incubated with eggs for 90 min at 37°C in the presence of the monoclonal antibodies. Sperm penetration into zona pellucida was determined by phase contrast microscopy as described in Materials and Methods. The values represent the percentages of sperm penetration rate in the presence of P3U1 culture supernatant.

Fig. 2. Indirect Immunofluorescence Staining of Mouse Sperm

Methanol-fixed sperm were incubated with A-1 monoclonal antibody and stained as described in Materials and Methods. The fluorescence was seen only in the acrosome region of almost all of the sperm ($\times 400$).

the presence of these monoclonal antibodies and pronuclear formation was observed at 4.5 h after the addition of sperm. Sperm-egg fusion was not inhibited at all even at the same concentration of A-1 antibody that had completely blocked sperm penetration into zona pellucida.

Besides, all other monoclonal antibodies showed no inhibition on sperm-egg fusion (data not shown).

In immunofluorescence experiments, the binding sites of A-1 antibody revealed acrosomal region of the sperm head only (Fig. 2). This immunofluorescence pattern was obtained in either unfixed- or fixed-sperm. Several percentages of unfixed (capacitated or uncapacitated) sperm and approximately 80% of methanol-fixed sperm were immuno-stained by A-1 antibody. Approximately half of ethanol- or formaldehyde-fixed sperm were also immunoreacted with this antibody (Fig. 3).

Next, the reactivity of A-1 against sperm bound to egg zona pellucida was studied. Capacitated sperm and cumulus-free eggs were incubated for 30 min at 37°C. The gametes were washed 3 times by pipetting with m-KRB. Free sperm (not bound to zona pellucida) and sperm bound to zona pellucida were immediately incubated with A-1 monoclonal antibody for 15 min at 37°C, and stained with FITC-conjugated second antibody as described in Materials and Methods. The sperm bound to zona pellucida were rarely reacted to A-1 antibody (1.6% of sperm were stained)

Fig. 3. Indirect Immunofluorescence of Mouse Sperm before or after Fixation

Capacitated sperm were fixed with 70% alcohol or 2% formaldehyde in PBS for 15 min at room temperature. After washing with PBS, fixed sperm and unfixed sperm were immunostained with A-1 monoclonal antibody as described in Materials and Methods. Data represent mean $\pm$ S.D. a) $p < 0.05$; b) $p < 0.01$ compared with unfixed sperm.

while 9.0% of free sperm were immunostained (data not shown).

Furthermore, the reactivity of A-1 against sperm penetrating into the perivitelline space was studied. Capacitated sperm and cumulus-free eggs were incubated for 90 min at 37°C. The gametes were washed 3 times by pipetting with m-KRB. About half of the gametes were fixed with 70% methanol before immunostaining as described previously. The penetrating sperm into the perivitelline space showed no immunofluorescence (Fig. 4). The sperm showed no immunofluorescence after methanol-fixation, too.

Discussion

It has been generally shown that most monoclonal sperm antibodies showing inhibition of fertilization react with sperm acrosomal antigens.^{6,10} In the mouse, it is well established that spermatozoa located within the perivitelline space have undergone the acrosome reaction.^{16,17} In addition, only acrosome-intact sperm can bind to the zona pellucida.¹⁸ And the acrosome reaction occurs after binding to egg.¹⁹

In this study, we prepared anti-mouse sperm monoclonal antibody (A-1) which reacts with the acrosomal region of sperm. This antibody rarely reacts with fresh sperm but reacts with most methanol-fixed sperm. In addition, a sperm-egg binding was slightly prevented by this antibody. These results suggested that A-1 antigen(s) are located inside the plasma membrane of acrosome-intact spermatozoa. Furthermore, sperm failed to penetrate into egg zona pellucida in the presence of A-1 antibody, and no sperm penetrating in the perivitelline space were immunostained with this antibody before or after fixation. These results suggested that A-1 antigen(s) should appear on the sperm surface just when sperm are penetrating into zona pellucida, and that, after the completion of sperm penetration through zona pellucida, these antigen(s) must be freed from sperm. In other words, A-1 antibody appears to react with just acrosome-reacting sperm. Although, several percentages of unfixed sperm were immunoreacted with A-1 antibody, this phenomenon may be accountable for either the rupture of plasma membrane of sperm during the immunostaining procedure or partial degradation of plasma membrane by cell death.

The cross reactivity of A-1 monoclonal antibody to

Fig. 4. Indirect Immunofluorescence Staining of Mouse Sperm Bound to Egg Zona Pellucida or Penetrating into Perivitelline Space

The sperm observed under phase contrast (A) and fluorescence (B) microscopy. No fluorescence were observed in any sperm.

unfixed-sperm from hamster, rat and rabbit cauda epididymis and human ejaculated sperm was preliminarily assayed. Only hamster sperm were reacted (ca. 30% of sperm) with this antibody. In addition, the acrosomal crest region released from hamster sperm was immunoreacted as well as the sperm head.

Several monoclonal antibodies against mouse sperm have been reported to be able to inhibit the sperm penetration. Saling and Lakoski reported a monoclonal antibody (M42) which inhibits sperm penetration into egg zona pellucida,⁶ and that M42 antibody inhibits sperm acrosome reaction induced by zona pellucida.^{7,20} Lee *et al.* reported that monoclonal antibodies (termed MS204 and MS207) inhibit sperm penetration through the egg plasma membrane only when zona pellucida is present.²¹

In this study, we prepared a monoclonal antibody (A-1) that inhibits sperm penetration into egg zona pellucida but the inhibition mechanism is still not clear. Further experiments are needed to analyze the mechanism.

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