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Mouse Sperm Antigens That Participate in Fertilization. I. Inhibition of Sperm Fusion with the Egg Plasma Membrane Using Monoclonal Antibodies[1]

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ABSTRACT

Monoclonal antibodies (mAbs) have been generated to determine the sperm components responsible for interaction with an egg that results in fertilization. Here, we report upon a group of six different mAbs, all of which localize to a restricted region of the sperm head, the equatorial segment. Several of these mAbs demonstrated cross-reactivity with sperm from the other species tested (human, hamster, rabbit); when cross-reaction occurred, the mAb distribution was restricted to the equatorial segment despite the various configurations that this homologous region assumes in different species. When tested for an effect upon the fertilization process in vitro, ascites fluids containing two of the six mAbs, M29 and M37, displayed significant inhibition. The concentration dependency of this inhibition was observed using purified M29 immunoglobulin M, over a range of 0 to 0.2 mg/ml. The mAb inhibition of fertilization was independent of the presence of either the cellular (the cumulus) or acellular (the zona pellucida) layers surrounding the egg, indicating that the specific locus of inhibition for both of these antisperm mAbs was the egg plasma membrane. Immunologic detection of sperm components separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels followed by transfer to nitrocellulose sheets was used to identify the sperm components recognized by two of the mAbs in this group: M29, which inhibited fertilization, and M2, which did not inhibit fertilization. Using M29 mAb, a single sperm component with an apparent subunit molecular weight of approximately 40,000 was detected, whereas in the nitrocellulose strips incubated with M2 mAb two components displayed reactivity, a very prominent band at approximately 44,000 and a tight cluster of bands at approximately 36,000. Parallel nitrocellulose strips of mouse liver did not display these reactivities, consistent with indirect immunofluorescence data in which only testis and sperm, and not liver, kidney, ovary, and epididymal epithelium, demonstrated positive reactivity. These results indicate that the use of mAbs permits identification of sperm components that participate, putatively, in individual events of the fertilization process. Furthermore, using this strategy, we have identified a specific sperm component that appears to be a candidate for a role in sperm fusion with the egg plasma membrane.

INTRODUCTION

Fertilization is achieved following the successful completion of a complex sequence of events by a spermatozoon and an egg. Although these gamete interactions have been studied extensively, the molecular details of these events, particularly those concerning the sperm cell, have remained elusive. This has been due, at least in part, to a lack of probes specific for individual sperm components. Hybridoma technology offers a solution to this problem. Monospecific probes, monoclonal antibodies (mAbs), can now be generated to address this topic.

We have produced a family of monoclonal antimouse sperm antibodies using syngeneic mouse testis as the immunogen, and have demonstrated the ability of one member of this family to block mouse fertilization in vitro (Saling et al., 1983). In this report, we focus on six different mAbs that have the common property of localization at the sperm head's equatorial segment, as assessed by indirect immunofluorescence. Only two of these six mAbs inhibited fertilization in vitro; in both cases the level of the block was specifically at the egg plasma membrane, where the inhibitory

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mAbs appear to interfere with fusion between the gamete plasma membranes. This paper describes a variety of the characteristics of these six mAbs and their respective antigens.

MATERIALS AND METHODS

Monoclonal Antibody Production

Male Balb/c mice were immunized with Balb/c testis, which had been homogenized in phosphate-buffered saline (PBS) (NaCl, 137 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM; KCl, 2.5 mM; pH 7.4). A total of three immunizations were given: on Day 1 (0.5 ml of testis homogenate containing approximately 15 mg protein/ml mixed 1:1 with Complete Freund's Adjuvant), on Day 15 (the same preparation mixed 1:1 with Freund's Incomplete Adjuvant), and on Day 24 (the same preparation mixed 1:1 with PBS). The mouse was killed on Day 28 and the spleen removed for fusion with myeloma cells (X63 Ag8). The fusion procedure followed was that outlined by Kennett (1980) and modified by Lin (1981), i.e., fusion via centrifugation in the presence of polyethylene glycol 1000 (J. T. Baker Co., Phillipsburg, NJ) at a concentration of 40%. The fused cell suspensions were grown in hypoxanthine aminopterin/thymidine selection medium (Littlefield, 1964). Macroscopic evidence of growth was detected within 2 wk in a large proportion of the wells, and primary screening with indirect immunofluorescence (IIF; see below) was initiated. Those hybridoma cell lines that demonstrated potentially useful patterns were expanded and frozen with 95% fetal bovine serum/5% dimethyl sulfoxide, in addition to being cloned twice in semisolid agarose. Positive clones from each cell line were used for the production of ascites fluid in Pristane-primed Balb/c mice. The immunoglobulin subclass of the monoclonal antibodies was determined using subclass-specific rabbit antimouse immunoglobulin antibodies (Zymed Labs, Burlingame, CA) with a horseradish peroxidase-based detection scheme (Bio-Rad, Richmond, CA).

Indirect Immunofluorescence Assay

Cauda epididymal sperm, collected from either mature mice or golden hamsters, were fixed in 2% formaldehyde in PBS (pH 7.4) for 10 min, then were washed and suspended in PBS containing 10 mM glycine (pH 7.4). Ejaculated human and rabbit sperm were washed twice via centrifugation at approximately 300 × *g* for 10 min and then fixed and prepared as described above. Approximately 50 μl of the dilute sperm suspension were placed onto coverslips (12 mm diameter; No. 1, Carolina Biological, Burlington, NC), and allowed to settle onto the surface without letting the preparation dry. Forty microliters of supernatant from each of the wells to be tested for mAb production were added to the sperm on the coverslips and they were incubated at 37°C in a humid atmosphere of 5% CO_2/95% air for 2 h. After thorough washing in PBS, fluorescein isothiocyanate (FITC)-conjugated or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat antimouse immunoglobulins G, M, and A (IgG, IgM, and IgA) (40 μl of a 1:100 to 1:400 dilution, centrifuged before use to remove particulate material; Zymed Laboratories) was added to each coverslip as before and incubated for 45 min. After thorough washing in PBS, the coverslips were finally rinsed in water and mounted in Gelvatol (Monsanto Chemical Co., St. Louis, MO) on microscope slides.

This standard single-label IIF procedure was modified for double-label IIF using two mouse mAbs according to the method of Wessel and McClay (manuscript in preparation). In the particular experiment described, this was accomplished by incubating fresh spermatozoa with culture supernatant containing the first mAb, as described above for single-label IIF. After thorough washing in PBS, the sperm were fixed with formaldehyde as described above, and washed in PBS, followed by PBS plus 10 mM glycine. After allowing the cells to settle onto coverslips, the Fab portion of FITC-conjugated rabbit antimouse IgG, IgM, and IgA (Cappel Laboratories, West Chester, PA) was applied at saturating concentrations (for these studies, 30 μl of a 1:10 to 1:20 dilution was used). After thorough PBS washing, sperm were incubated with culture supernatant containing the second mAb under investigation as above, washed thoroughly, and incubated finally with TRITC-conjugated goat antimouse IgG, IgM, and IgA as above. Final slide preparation was achieved as described before. A Zeiss standard microscope equipped with phase contrast, Nomarski, and epifluorescence optics was used to examine the slides. Kodak Tri-X film was used for photography.

Tissue specificity of the mAbs was determined by examination of paraffin-embedded sections of mouse kidney, liver, ovary, testis, and cauda epididymidis that had been fixed in Bouin's fixative. The sections were processed for single-label IIF similarly to that described above after paraffin removal and standard dehydration.

Fertilization in Vitro

Methodology used for fertilization in vitro has been described in detail elsewhere (Saling et al., 1978; Saling and Storey, 1979; Saling, 1981). Briefly, a Krebs-Ringer bicarbonate medium, supplemented with pyruvate, lactate, glucose, and bovine serum albumin (CM), was used both for mouse sperm capacitation and fertilization in vitro. Gamete manipulations for the fertilization experiments were conducted at 37°C under a layer of sterile silicone oil (dimethylsiloxane, 20 cs; Dow Corning, Contour Chemical Co., North Reading, MA). All solutions in contact with the gametes were equilibrated with 5% CO_2/95% air. Tubal eggs were recovered from superovulated randomly bred CD-1 mice (Charles River Breeding Co., Wilmington, MA). Three types of egg preparations were used: those in which 1) the cumulus layer was left intact; 2) the cumulus layer was removed by incubation for <10 min in 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) in CM, but the zonae pellucidae were left intact; and 3) the cumulus layer was removed as above, and zonae pellucidae were removed manually by forcing the eggs through narrow-bore micropipettes.

Mouse sperm suspensions were prepared by rupturing the excised caudae epididymides of mature (≥10 wk) mice of proven fertility in CM. After dilution to approximately 10^6 cells/ml in CM, the sperm were

incubated for 90 min at 37°C in 5% CO_2/95% air to allow for capacitation. Following this interval, 20 μl of sperm were deposited into 160 μl of CM plus 20 μl of either ascites fluid, purified monoclonal antibody, or CM, to achieve a final sperm concentration of approximately 10^5 sperm/ml. Since the immunoglobulin concentration of the ascites fluids was 3–5 mg/ml generally, each mAb in ascites fluid was tested at a concentration of approximately 0.4 mg•10^5 sperm^{-1}•ml^{-1}. In the fertilization experiments employing ascites fluids (see Tables 1 and 2), multiple replicate experiments were performed using at least 3 separate lots of ascites fluid/mAb examined. Both the ascites fluids and the purified mAbs were prescreened using IIF to ensure the presence of the appropriate activity. Positive staining could be observed at ≥1:20,000 dilution generally. Purified mAb was dissolved in CM and tested at final concentrations of 0.05, 0.1, and 0.2 mg/ml. After incubating capacitated sperm with ascites fluid or mAb for 10–15 min at 37°C, the eggs were added.

Eggs were recovered after 4–5 h at 37°C in 5% CO_2/95% air, mounted on slides, and stained with acetolacmoid (Toyoda and Chang, 1974). Eggs were scored as penetrated if sperm were found within the perivitelline space and/or vitellus and as fertilized only if both sperm head (or pronucleus) and sperm tail were identified within the vitellus.

The statistical significance of the fertilization experiments was evaluated using chi-square analysis. Pertinent results of this analysis are included in the appropriate figure legend.

Antibody Purification

The immunoglobulins described here, all of the IgM class, were purified according to the method of Parham (1983). Briefly, this was accomplished by applying the 30–55% ammonium sulfate fraction of the appropriate ascites fluid to a Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) column in PBS plus 0.5 M NaCl. The first peak was pooled and dialyzed against 5 mM Tris (pH 7.0) to precipitate the IgM, which was collected by centrifugation at approximately 45,000 × *g* for 30 min at 4°C.

Antigen Characterization

Solubilization. For IIF analysis, unfixed epididymal mouse sperm in PBS were treated for 10 min at room temperature with one of four different detergents and then examined using indirect immunofluorescence, as described above. The detergents tested were: Triton X-100 (0.5%; Sigma); SDS (0.2%; Accurate Chemical and Scientific Corp., Westbury, NY); 3 - [(3 - cholamidopropyl)dimethylammonio] - 1 -propanesulfonate (CHAPS, 0.1%; Sigma); or octyl-β-glucoside (0.1%; Pierce Chemical Co., Rockford, IL).

For immunologic detection on nitrocellulose, cauda epididymal sperm were collected from 4 mature CD-1 mice of proven fertility and washed once in PBS via centrifugation at approximately 300 × *g* for 10 min. The resulting pellet (approximately 10 mg protein/8 epididymides) was resuspended in 0.2 ml treatment buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM iodoacetamide]. After thorough mixing on a vortex, the suspension was sonicated (Microultrasonic cell disruptor with 4.5" titanium probe; Kontes Corp., Vineland, NJ) at the maximum power setting for 15 s and then centrifuged (Microfuge Model 11, Beckman Instruments, Palo Alto, CA) at approximately 12,000 × *g* for 5 min. The supernatant was diluted 1:1 with water and β-mercaptoethanol was added to a final concentration of 5%. The samples were divided into aliquots and frozen (−20°C) until use, at which time they were boiled for 5 min before application to a preparative polyacrylamide gel for electrophoresis. To serve as a control for nonspecific reactivity, mouse liver extracts were prepared in the same manner, and used in parallel with the sperm extracts.

Immunologic detection of antigens transferred from SDS gels to nitrocellulose. Electrophoresis was accomplished on 12% preparative SDS-polyacrylamide gels using the buffer system of O'Farrell (1974), applying 4 mg protein of the sperm extract or 12 mg protein of the liver extract. Proteins were transferred electrophoretically onto nitrocellulose (Bio-Rad Trans-Blot) according to the method of Towbin et al. (1979) using a Hoeffer Transphor Electrophoresis Cell at 65 volts for 1 h. The blotted nicrocellulose sheet was quenched by incubation (three times for 10 min each) in Tris-buffered saline (TBS; 25 mM Tris/0.5 M NaCl, pH 7.4) containing 5% nonfat dry milk (NDM; Johnson et al., 1984), 0.05% Tween 20, and 0.02% NaN_3. The sheet was then cut into strips that were incubated individually. Each pair of strips (sperm + liver) was incubated for 16 h at room temperature with continuous rotation in the quenching buffer containing 1) no antibody; 2) concentrated M29 culture supernatant diluted 1:1 with quenching buffer; or 3) concentrated M2 culture supernatant diluted 1:1 as in (2). After washing (three times for 10 min each) in TBS with 5% NDM and 0.05% Tween 20, the strips were incubated with affinity-purified peroxidase-conjugated goat antimouse IgM (μm) (Kierkegaard and Perry, Gaithersburg, MD) diluted 1:1000 in TBS with 5% NDM and 0.05% Tween 20 for 2 h at room temperature with continuous rotation. After washing (twice with TBS with 5% NDM and 0.05% Tween 20; then once with TBS with 0.05% Tween 20), peroxidase activity was demonstrated on the strips using a HRP color development reagent (Bio-Rad) containing 4-chloro-1-naphthol. After rinsing with water, the strips were photographed (Kodak Technical Pan) and then counterstained according to the method described by Towbin et al. (1979).

RESULTS

Cellular Localization

The localization patterns of the six monoclonal antibodies under consideration on formaldehyde-fixed mature mouse sperm are presented in Fig. 1. The immunogen used for these mAbs was syngeneic mouse testis; the mAbs were screened initially on mature epididymal mouse sperm. Thus, selection has been directed toward those sperm components that

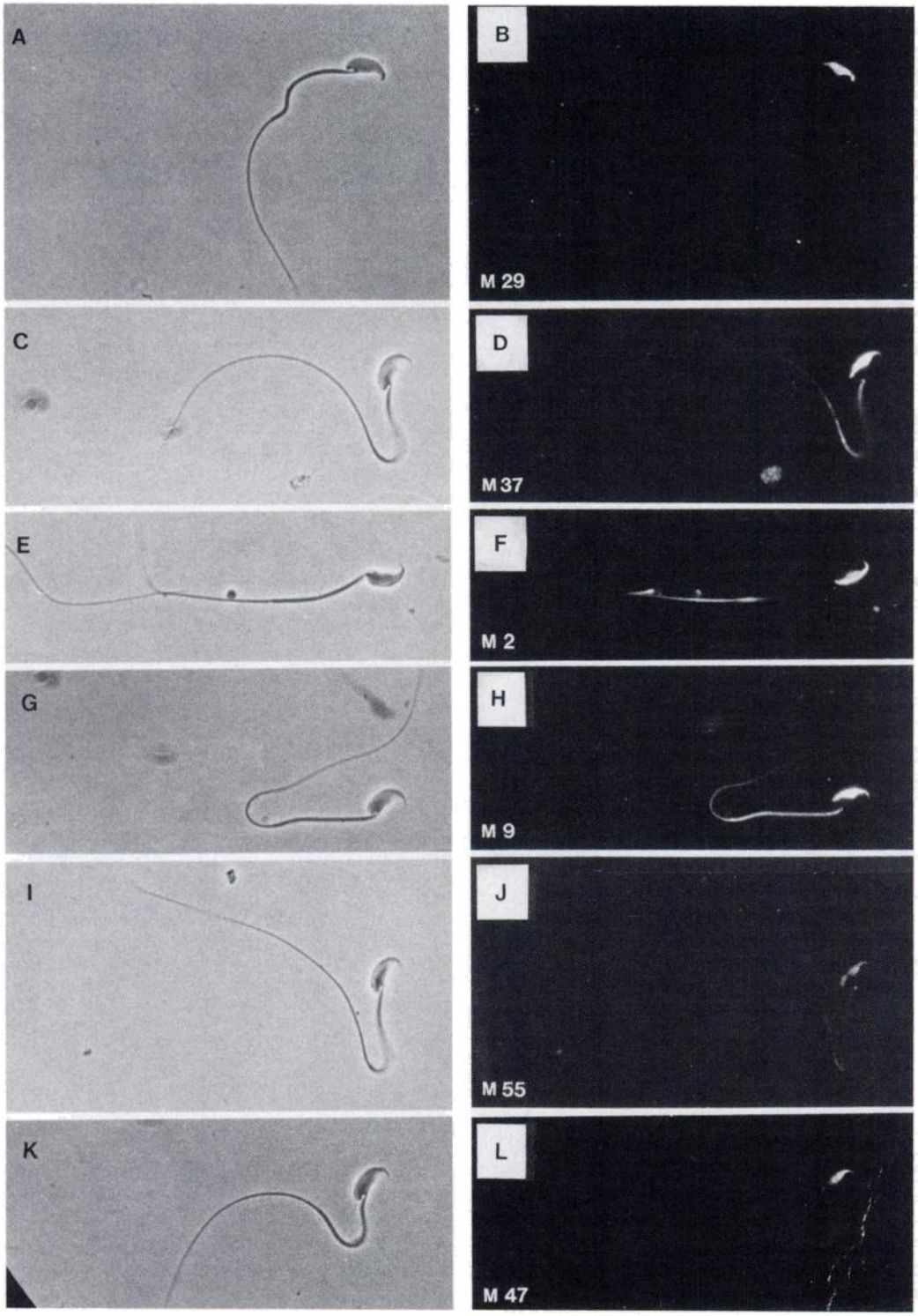

FIG. 1. Paired phase-contrast (*a,c,e,g,i,k*) and epifluorescent (*b,d,f,h,j,l*) photomicrographs demonstrating the indirect immunofluorescent localization patterns of the 6 mAbs indicated on formaldehyde-fixed cauda epididymal mouse sperm. Two of the mAbs (M29 and M55) are distributed on the equatorial segment alone, whereas the other 4 mAbs (M37, M2, M9, and M47) display additional variable staining on the sperm tail. Control specimens (parental myeloma cell line culture supernatant or PBS) display very faint, if any, fluorescence over the entire sperm cell, which appears totally dark in the epifluorescent micrographs under the photographic conditions used.

arise in the testis and persist throughout spermatogenesis. The common region of distribution was the equatorial segment of the mouse sperm head, although several of the mAbs were also found in a patchy, variable distribution along the length of the sperm tail. Undiluted culture supernatants were used to produce the micrographs shown in Fig. 1, but ascites fluids containing each of these mAbs could be diluted 1:20,000 to produce a similar level of intensity.

When formaldehyde-fixed mouse sperm were stained with these mAbs, all of the sperm in the population displayed the equatorial segment fluorescence depicted in Fig. 1. In contrast, if a freshly acquired, unfixed population was examined, very few sperm displayed fluorescence of any kind; such a sperm preparation will consist of approximately 70–80% acrosome-intact sperm (Saling and Storey, 1979). However, since the acrosome of the mouse spermatozoon is impossible to distinguish with phase-contrast optics, the relationship of the sperm's acrosome to mAb staining could not be determined using single-label IIF. Two independent methods have been used to resolve this issue. Hamster sperm provided an indirect measure of mAb localization on the mouse sperm, since the acrosome in that species can be identified readily with light optics. Unfixed hamster sperm displayed mAb staining only in the absence of the acrosome, suggesting that it was only after loss of the acrosome from the cell that the mAbs had access to the appropriate cellular area: the crypt exposed to the external environment following the acrosome reaction, comprised of the inner and outer acrosomal membrane surfaces. Direct confirmation of this suggestion was achieved using a double-label IIF technique developed for use with monoclonal antibodies (Wessel and McClay, manuscript in preparation). Fresh, unfixed mouse sperm were exposed to culture supernatant containing either M29, M37, M2, M9, M47, or M55 mAb, washed in PBS, fixed in 2% formaldehyde, and then incubated with saturating concentrations of the Fab portion of FITC-conjugated rabbit antimouse IgG, IgM, and IgA. After thorough washing in PBS, the sperm were incubated in culture supernatant containing M42 mAb, an antibody that localizes to the plasma membrane overlying the acrosome (Saling and Lakoski, this issue) and can be used as a diagnostic tool for the presence of the acrosome in mouse sperm. Following exposure to the second mAb and thorough washing, sperm were incubated in TRITC-conjugated goat antimouse IgG, IgM, and IgA.

Examination of the fluorescence pattern of sperm prepared in this way (Fig. 2) indicated that this panel of mAbs bound to the equatorial segment of live sperm only after the acrosome was lost, since those sperm that have acrosomes (as indicated by positive M42 fluorescence; Fig. 2c,i) did not demonstrate equatorial segment staining (Fig. 2b,h) with any of the six mAbs examined. Conversely, those sperm that had lost their acrosomes (indicated by the lack of M42 staining; Fig. 2f,l) displayed equatorial segment staining (Fig. 2e,k); all of the equatorial segment-staining mAbs under consideration conformed to this same pattern with regard to equatorial segment localization. Sperm tails stained quite variably when unfixed sperm were exposed to culture supernatant containing M2, M9, M37, or M55 mAb, and could not be correlated with presence of the acrosome. The spermatozoon shown in panels (m), (n), and (o) in Fig. 2 serves as a control to indicate the separation of the signals produced by the two fluorophores employed.

Several of the mAbs displayed cross-reactivity with sperm of other species. M29 demonstrated the widest range, staining the equatorial segment of fixed hamster, rabbit, and human sperm, as reported prevously (Saling et al., 1983). M2, M9, and M47 reacted similarly with hamster and human sperm, whereas M37 and M55 did not appear to cross-react with sperm from any of the three species examined. When cross-reaction occurred, fluorescence was routinely observed at the

FIG. 2. Double-label indirect immunofluorescence micrographs showing individual cells viewed with optics appropriate for phase-contrast (*a,d,g,j,m*), fluorescein isothiocyanate (*b,e,h,k,n*), or tetramethyl rhodamine isothiocyanate (*c,f,i,l,o*). Cells shown in the fluorescein column (*b,e,h,k,n*) were exposed to the mAb indicated while unfixed. Following extensive PBS washing, subsequent manipulations involved fixed cells.

TABLE 1. Effect of monoclonal antibodies localized to the equatorial segment on fertilization of zona pellucida-intact mouse eggs.[a]

Ascites fluid	No. of eggs examined	Zonae penetrated, % (range)	Eggs fertilized, % (range)
Control (parent myeloma cell line)	322	86 (67–96)	80 (61–85)
M2	78	60[b] (25–83)	54[c] (25–83)
M9	185	76[c] (50–87)	68[c] (43–79)
M47	144	74[c] (60–100)	62[c] (48–83)
M55	152	73[c] (64–83)	60[c] (47–72)
M29	265	35[d] (14–61)	2[e] (0–14)
M37	151	26[e] (17–43)	9[e] (0–29)

[a]Refer to *Materials and Methods* for experimental details. Data were compiled by averaging the results of 8 replicate experiments; 5–9 female mice were used per experiment, totaling 57 female mice. The range of the fertilization and zona penetration results is shown in parentheses.

[b]Differs from control ($P<0.025$).

[c]Not significantly different from control ($P>0.20$).

[d]Differs from control and from M9, M47, and M55 with $P<0.001$, and differs from M2 with $P<0.01$.

[e]Differs from control and from M2, M9, M47, and M55 ($P<0.001$).

region homologous to that found with mouse sperm, despite the varied configurations that the equatorial segment assumes in different species (see, for example, Yanagimachi, 1981). Examination of various mouse tissues (kidney, liver, ovary, testis, and cauda epididymidis) for cross-reactivity revealed antibody binding in the case of the testis only. Spermatozoa were not retained within the lumen of the epididymis under these conditions, and thus are not available for examination of mAb localization pattern. It is noteworthy, however, that epididymal tissue itself did not cross-react with these mAbs. To eliminate the possibility that Bouin's fixation altered or abolished mAb distribution, a suspension of cauda epididymal mouse sperm was fixed in Bouin's, and then prepared for IIF as described for our standard procedure. The immunofluorescent patterns generated were identical to those shown in Fig. 1, lending support to the idea that Bouin's fixation did not distort the tissue specificity results and, moreover, that these mAbs appear to react only with testicular tissue and with spermatozoa.

Fertilization in Vitro

To test whether these mAbs affect the fertilization process, ascites fluids containing each of the 6 mAbs were generated. Two of the six equatorial segment-staining mAbs, M29 and M37, were effective at blocking the fertilization of mouse eggs that either had their cumulus layer intact (data not shown) or their cumulus cells removed with hyaluronidase treatment (Table 1). Although fertilization was reduced significantly, sperm penetration through the zonae pellucidae was not reduced as substantially. A major difference between the eggs recovered after either M29 or M37 treatment and those recovered after treatment with any of the other 4 mAbs was the observation that multiple (4 to >10) sperm were often found within the perivitelline space in eggs inseminated with the M29- and M37-treated sperm. This was an unusual finding for the experimental conditions used; in the latter treatment group, perivitelline sperm were rare.

To examine whether the zona pellucida was involved in the apparent block to fertilization achieved with M29 and M37 mAbs, mouse eggs from which zonae had been removed manually were challenged with sperm in the presence of these mAbs. The results of those experiments (shown in Table 2) indicated that both M29 and M37 remained effective at preventing fertilization in the absence of the zona pellucida. These results indicate that these two mAbs exerted their inhibitory effect specifically at the level of sperm interaction with the egg plasma membrane. Although it was not investigated quantitatively, sperm binding to the egg

plasma membrane was unaffected qualitatively in the presence of either M29 or M37, suggesting that these mAbs interfere specifically with fusion of the gamete membranes.

To ensure that the inhibitory effect observed using the ascites fluid preparations was due to the particular antibody under examination, and not to a peculiar contaminating component, purified M29 IgM and M2 IgM were compared for their effect upon fertilization inhibition (Fig. 3). Increasing concentrations of M29 IgM, but not M2 IgM, reduced fertilization in vitro in a dose-dependent manner. Reduced zona penetration was also dose dependent, but was affected to a much smaller extent (e.g., 0.2 mg M29/ml reduced fertilization by 97%, yet decreased zona penetration by only 53%), consistent with the ascites fluid results shown in Table 1.

Antigen Characterization

Indirect immunofluorescence (to identify cellular location) followed by fertilization in vitro (to assess inhibitory activity) was the typical sequence used in this study to identify mAbs of interest. M29 was actually one of the first mAbs tested in this scheme; the antibody was found subsequently to be of the IgM class. Thereafter, to control for possible nonspecific effects due to the presence of a molecule as large as an IgM, other mAbs with the same cellular distribution as well as of the same immunoglobulin class were chosen for comparison. Thus, all six of the mAbs described here were of the IgM class.

TABLE 2. Effect of monoclonal antibodies localized to the equatorial segment on fertilization of zona pellucida-free mouse eggs.[a]

Ascites fluid	No. of eggs examined	Eggs fertilized, % (range)
Control (parent myeloma cell line)	178	96 (92–100)
M2	85	97[b] (89–100)
M29	244	5[c] (0–12)
M37	116	14[c] (0–29)

[a]Refer to *Materials and Methods* for experimental details. Data were compiled by averaging the results of 5 replicate experiments; 9–12 female mice were used per experiment, totaling 54 female mice. The range of the fertilization levels observed is shown in parentheses.

[b]No significant difference from control level ($P>0.75$).

[c]Differs from control level and from M2 level ($P<0.001$).

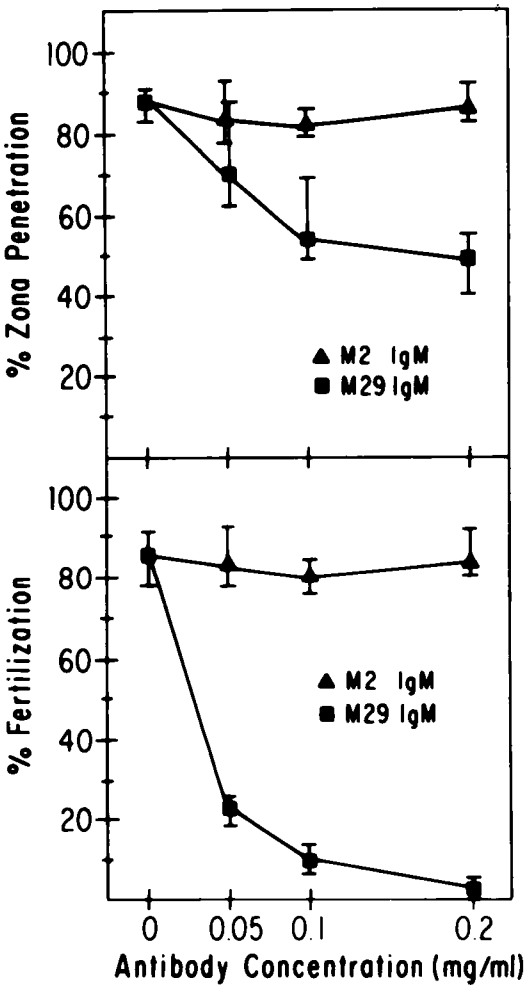

FIG. 3. Effect of purified monoclonal IgM (M29 vs. M2) on mouse fertilization and zona penetration in vitro (refer to *Materials and Methods* for experimental details). Results represent the average of two experiments (with the range indicated by the *bars* at each point), using 58–69 cumulus-free, zona-intact mouse eggs per point. Chi-square analysis revealed that M29 at 0.05 mg/ml did not affect zona penetration significantly ($P<0.5$), whereas at higher doses it decreased zona penetration significantly (at 0.1 mg/ml, $P<0.025$; at 0.2 mg/ml, $P<0.01$) compared to either the control or to the M2 values. With respect to fertilization inhibition, M29 IgM differed significantly ($P<0.001$) from the control and M2 values at all concentrations examined.

Since this set of mAbs shared a common cellular location, yet differed with respect to their ability to block fertilization, determination of whether the antigen(s) recognized by these mAbs were the same or different became a relevant question. Our initial experiments in this direction were performed with two aims: to determine possible differences in the antigens recognized by these 6 mAbs based upon their differential detergent solubilization, and to establish appropriate extraction procedures for eventual use in identifying and isolating these antigens. Prior to fixation, mouse sperm were incubated with detergent (Triton X-100, 0.5%; SDS, 0.2%; CHAPS, 0.1%; or octyl-β-glucoside, 0.1%) and then prepared for indirect immunofluorescence (Table 3). Although the results were varied, some common patterns emerged. Fluorescence from the sperm head could be eliminated for all of the mAbs except M9 and M47 by each of these detergents. Tail fluorescence was more inconsistent in its response to detergent treatment, its appearance being characteristic for the particular mAb under examination rather than the detergent used.

Because it was the detergent most generally effective in removing sperm head fluorescence, SDS was selected as the agent for membrane extraction in the antigen identification experiments. At present, we have succeeded in identifying the antigens recognized by two of the mAbs discussed here. Fig. 4 demonstrates that when a SDS extract of cauda epididymal mouse sperm was separated on a 12% SDS-polyacrylamide gel and then transferred to nitrocellulose, specific sperm components were identified depending upon the mAb used. M29 mAb recognized a single sperm component with subunit M_r of approximately 40,000 (lane 4, (arrowhead); in contrast, M2 mAb recognized a prominent band at approximately 44,000 as well as a cluster of bands centered at approximately 36,000 (lane 6, arrowheads). On parallel nitrocellulose strips containing similarly prepared extracts of mouse liver, reaction of these mAbs with the same antigens was not detected. Instead, the weak bank at approximately 100,000 was observed in the nitrocellulose strips of mouse liver incubated with either M29 (lane 3) or M2 (lane 5). Nitrocellulose strips of either mouse sperm or mouse liver in which mAb was not included in the first incubation (lanes 1 and 2), serving as controls for the specificity of the secondary antibody and subsequent color development reagents, demonstrated no reaction.

DISCUSSION

Fertilization is an intricate process that has resisted molecular description, particularly with regard to the spermatozoon; hybridoma technology has now provided a powerful means of approaching this topic. Monoclonal antibodies, generated against mouse testis, have been used here to identify and isolate a sperm component that appears to participate in a specific event of

TABLE 3. Effect of detergents on the immunofluorescent distribution of monoclonal antibodies localized to the equatorial segment.[a]

	Sperm treatment[b]				
mAb	Fixed (control)	Triton X-100 (0.5%)	SDS (0.2%)	CHAPS (0.1%)	Octyl-β-glucoside (0.1%)
M29	EqSeg	–	PPsp	–	–
M37	EqSeg, PPsp	–	–	–	–
M2	EqSeg, PPsp	PP	PP	PPsp	PPsp
M9	EqSeg, PPsp	EqSeg, Neck, MP, PP	PP	N.D.	N.D.
M47	EqSeg, PPsp	–	–	EqSeg, PPsp	EqSeg, PPsp
M55	EqSeg	–	–	–	–

[a]Refer to *Materials and Methods* for experimental detail.

[b]Key: –, no fluorescence visible; *EqSeg*, equatorial segment; *MP*, midpiece; *Neck*, small dot of fluorescence at junction of head and midpiece; *PP*, principal piece of tail; *PPsp*, spotty and heterogeneous fluorescence distribution along length of principal piece; and *N.D.*, not determined.

the fertilization process. In the case of the mAbs described here, it is most likely that this event is fusion between sperm and egg plasma membranes.

Previous ultrastructural studies (Yanagimachi and Noda, 1970; Noda and Yanagimachi, 1976; Bedford and Cooper, 1978; Moore and Bedford, 1978) show that the equatorial segment is an area of the sperm head critical for fertilization, since it is the region of the cell that is involved initially in fusion with the egg plasma membrane. For this reason, we have concentrated upon 6 different mAbs all of which share the property of localization to the equatorial segment. Moreover, two of these six mAbs were found to inhibit fertilization in vitro.

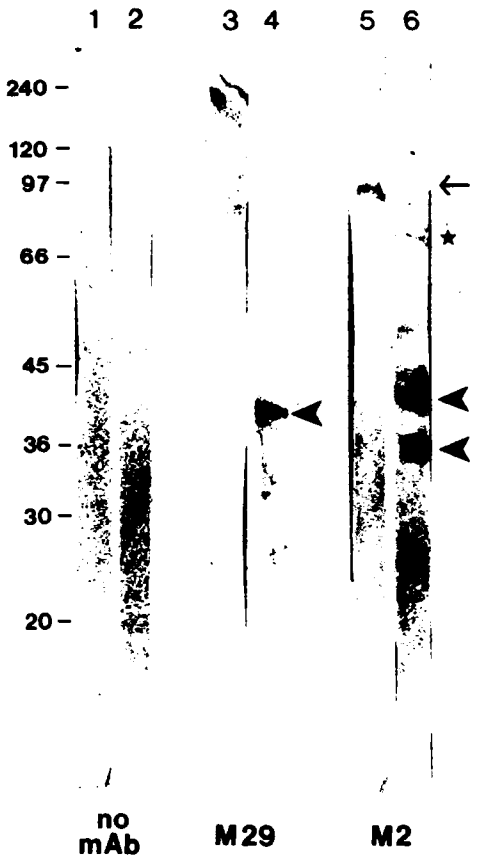

FIG. 4. Immunologic detection of the antigens recognized by M29 mAb and M2 mAb. Sodium dodecyl sulfate extracts of either mouse liver (*lanes 1, 3, and 5*) or mouse sperm (*lanes 2, 4, and 6*) were electrophoresed in a 12% polyacrylamide gel, followed by transfer of the proteins to nitrocellulose. *Lanes 1 and 2*: nitrocellulose strips incubated in buffer containing no primary (i.e., monoclonal) antibody. *Lanes 3 and 4*: nitrocellulose strips incubated in M29. *Lanes 5 and 6*: nitrocellulose strips incubated in M2. The *arrowheads* indicate the sperm components recognized specifically by the respective mAbs (*lanes 4 and 6*); the *arrow* indicates a liver component recognized mutually by both mAbs, but not by sperm; and the *star* indicates a tear in the nitrocellulose strip due to serrated forceps. Molecular weight standards ($\times 10^{-3}$) are indicated to the left of lane 1.

It appears that both M29 and M37 mAbs blocked fertilization by interfering with fusion between sperm and egg membranes, since fertilization was prevented by each of these two mAbs independently of the presence of the cumulus layer or the zona pellucida. Purified M29 IgM blocked fertilization in vitro in a concentration-dependent manner as well, supporting the suggestion that it is the antibody itself that exerts the inhibitory effect and not a contaminant of the ascites fluid. A partial reduction in the ability of M29- or M37-treated sperm to penetrate the zona was also found (Table 1). An entirely satisfying explanation for this observation is not yet apparent. However, in a companion paper (Saling and Lakoski, this issue), we describe completely different mAbs that specifically inhibited sperm penetration through the zona pellucida; the antigens recognized by those mAbs were entirely distinct from the antigens presented here.

At the light microscopic level, it was difficult to determine the precise event blocked by M29 and M37. Sperm binding to the egg plasma membrane did not appear to be affected by these mAbs. Ultrastructural examination of gametes treated with the inhibitory mAbs will be necessary to determine whether membrane fusion had occurred and it was another, immediately subsequent, event that was prevented. However, it is noteworthy that multiple sperm were found within the perivitelline space routinely. This observation suggests that the zona reaction, which renders the zona impenetrable to subsequent sperm entry and is thought to result from cortical granule dehiescence (Barros and Yanagimachi, 1971; Wolf and Hamada, 1977), did not occur in eggs in the M29 or M37 mAb-treated gamete preparations. These findings argue that gamete membrane fusion was not initiated, nor have the cortical granules fused with the egg plasma membrane in response to the sperm's attachment.

The equatorial segment is a particularly complex region. Since the mAbs described here did not bind to live acrosome-intact sperm, but did bind to live acrosome-reacted sperm, two

possibilities for antigen localization on the sperm head exist. It seems most likely that the antigens are located in the crypt of the equatorial segment exposed to the external environment after the acrosome reaction has occurred, but it is also possible that a consequence of the acrosome reaction is alteration of the sperm surface in the region of the equatorial segment, whereby the appropriate antigenic determinants are exposed. Immunoelectron microscopic studies in progress should resolve this issue.

Our mAbs have been generated for use as monospecific probes to dissect the fertilization process; the methodology that we have used has exploited known properties of the system. Since the testis contains highly immunogenic tissue, production of mAbs was not a particularly difficult task. Selection of individual mAbs upon which to focus is more problematic. Our strategy has been to screen large numbers of supernatants rapidly with IIF using fixed, mature mouse sperm, which directed our attention to those mAbs with localization patterns of potential interest. Thereafter, a more difficult functional assay, the ability to inhibit fertilization in vitro, was performed using the prescreened mAbs. The use of IIF as a primary screen permitted the detection and maintenance of cell lines producing mAbs of very restricted distribution, such as the six mAbs described here. Radioligand or enzyme-linked binding assays, when used as the primary screening assay, tend to select mAbs directed toward antigens distributed on the sperm tail or over the entire cell, since this type of localization will provide the largest signal generally (Saling, unpublished observations; Moore and Hartman, 1984).

The M29 mAb displayed a very restricted localization pattern on the sperm cell, being confined to the equatorial segment. The estimated subunit molecular weight of the antigen recognized by this mAb was 40,000. In contrast, M2 mAb displayed a wider cellular distribution, seen at the equatorial segment as well as variably along the length of the tail. The antigens recognized in this case had estimated molecular weights of 44,000 and a cluster centered at 36,000. Whether either or both of these M2 antigens are found on the head as well as the tail remains to be determined. It will also be of interest to identify the components recognized by the four other mAbs described here that localize to the equatorial segment. Unique components, not identified by either M29 or M2, may be found. Alternatively, some of these uncharacterized mAbs may recognize the same components as those described here, but bind at perhaps a different antigenic determinant of the molecule. The possibility of probing the "active" site(s) of the moiety will then be created if this occurs for a pair of mAbs, one of which is inhibitory toward fertilization while the other had no effect. A mouse sperm component with a molecular weight close to one of the M2 components (28,000) has been reported recently by Gaunt (1982). Some of the characteristics of that moiety appear similar to those that we have reported here. However, since the localization of that antigen is described as the sperm surface at the anterior tip of the acrosome, it appears unlikely that the antigens are the same.

The findings reported here support the observations of Primakoff and Myles (1983) that sperm membranes are comprised of multiple domains, each of which may have quite sharp demarcations and a unique composition. Here, however, we have also begun to define specific components within a particular domain that are candidates for involvement in that domain's physiologic role.

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