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Development of the Ability to Bind to Zonae Pellucidae During Epididymal Maturation: Reversible Immobilization of Mouse Spermatozoa by Lanthanum

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ABSTRACT

Mouse spermatozoa recovered from the caput, corpus, or cauda epididymidis were examined for their ability to bind in vitro to zonae pellucidae. Since spermatozoa from the caput epididymidis do not display progressive motility as compared with more mature spermatozoa, direct comparison of the different sperm populations may not measure zona binding ability validly. To equalize the motile properties of the spermatozoa, a method was developed to immobilize vigorously motile corpus and cauda spermatozoa. Reversible immobilization was achieved by incubation in 25 μM La^{3+} which resulted in a twitching, nonprogressive type of motility. La^{3+} incubation did not appear to affect the spermatozoa adversely, since vigorous motility (equivalent to the controls) of corpus and cauda sperm was displayed upon subsequent incubation in standard La^{3+}-free culture medium. Moreover, cauda spermatozoa preincubated for 90 min in La^{3+} displayed levels of fertilization in vitro equivalent to their control counterparts.

Using this La^{3+}-immobilization technique, the zona binding ability of the different sperm population could be assayed. Gamete collision was insured under these conditions by shaking the gamete-containing dishes at 100 cycles/min. Regardless of the extent of sperm motility, a similar zona-binding pattern emerged: cauda sperm bound in high numbers, corpus sperm bound at some intermediate level (an average of 24% of cauda binding level), and caput sperm bound rarely (2% of cauda binding level). Thus it appears that, for mouse spermatozoa, the onset of fertility during epididymal transit parallels the ability to bind to zonae pellucidae.

Unlike the interaction between spermatozoa and zonae, La^{3+} was unable to support sperm binding to the egg plasma membrane, supporting the view that mouse sperm may have different sites for interaction with the zonae pellucida and the egg plasma membrane.

INTRODUCTION

A variety of alterations are known to occur in spermatozoa during transit through the epididymis (Orgebin-Crist et al., 1975; Nicolson et al., 1979; Olson and Danzo, 1981), including the acquisition of fertility (O'Rand, 1980; Bedford, 1975). But, to fertilize an egg successfully, the spermatozoon must first bind to and penetrate the zona pellucida. Unknown, however, is the temporal relationship between the acquisition of zona binding ability and fertility; the onset of fertilizing ability could be due to the appearance of zona binding ability.

One obstacle in the accurate examination of this parameter of sperm function is the vast difference in motility encountered in sperm populations isolated from various regions of the epididymis. Since sperm binding to the zona pellucida is, in part, a reflection of the collision frequency between gametes, sperm motility is a major factor to be considered. Two options are immediately available for a valid comparison of these different populations with regard to binding to zonae: to enhance the motility of nonprogressively motile sperm recovered from the proximal regions of the epididymis, or to decrease, in a nonlethal way, the motility of sperm recovered from distal, more mature, epididymal regions.

The experiments presented here utilized a method to reversibly immobilize highly motile mouse sperm. This scheme permitted a valid comparison of zona binding ability in mouse spermatozoa of varying developmental stages.

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MATERIALS AND METHODS

Media

The medium used for both capacitation and fertilization in vitro of mouse gametes was a modified Krebs-Ringer bicarbonate medium with a variety of energy sources (lactate, pyruvate, and glucose) and bovine serum albumin (20 mg/ml; Sigma, Fraction V) (Inoue and Wolf, 1975). This medium (CM), was adjusted to pH 7.4-7.5 with a 1.0 N NaOH, sterilized by Millipore filtration (0.22 μm mesh), and equilibrated with 5% CO_2 in air immediately before use. A simple medium (TN), consisting of Tris (20 mM) and NaCl (130 mM), was also used to examine sperm binding to zonae, and was sterilized similarly by filtration immediately before use. In some experiments, TN was modified by the addition of $LaCl_3$, at a concentration of 2.5×10^{-5} M (TNL).

Gametes

Tubal mouse eggs were recovered from superovulated randomly-bred Swiss mice (Taconic Farms Germantown, NY), that were approximately 6 weeks old. Cumulus cells were removed by brief (< 10 min) incubation in CM containing 0.1% hyaluronidase (Sigma, Type 1-S). Micropipetting removed stubbornly adherent cumulus cells. Zona-free eggs were used in several experiments; zonae were removed mechanically by forcing cumulus-free eggs through a very fine micropipette. All gamete manipulations occurred at 37°C under a layer of sterilized silicone oil (dimethylsiloxane, 20 cs, Contour Chemical Co., North Reading, MA), previously equilibrated with 5% CO_2 in air.

Mouse spermatozoa were recovered from the 3 major regions of the epididymis: caput (including initial segment), corpus, and cauda (Pavlok, 1974). At least 2 males (> 10 weeks old) were used per experiment. Similar segments from different animals were pooled and placed under oil in CM, TN, or TNL, depending upon the experiment. The epididymal tubules were disrupted by puncture with 27 gauge needles. After a 10 min cell dispersion period, debris-free aliquots of the suspensions were withdrawn and maintained under oil. The sperm concentration of each suspension was estimated with a hemocytometer; a minimum of 2 determinations was performed for each suspension. The concentration of all suspensions was adjusted to 10^6 cells/ml with medium appropriate to the experiment. Throughout this study, sperm motility was evaluated subjectively with an inverted microscope using phase-contrast or interference optics (Nikon). A minimum of 5 microscope fields was examined (200X) for each sperm preparation; 20 sperm were scored for motility in each field. The percentage of motile sperm was calculated from the average of the percentage motile in the 5 fields.

To elicit capacitation in vitro, spermatozoa recovered from the cauda epididymidis were incubated in CM for 90 min at 37°C at a density of 10^6 cells/ml.

Sperm Binding Assay

Freshly recovered or incubated spermatozoa were added to cumulus-free mouse eggs at a final concentration of 10^5 cells/ml. When TNL was the medium employed during examination of gamete interaction, dishes containing the gametes were placed on a shaker device and rocked at a frequency of approximately 100 cycles/min. Shaking was not employed when the other media were used, since sperm motility appeared to be unaffected in TN or CM.

Quantitative assessment of sperm bound to eggs was accomplished by centrifugation of the gametes through a discontinuous dextran gradient, during which sperm not specifically bound to eggs were removed, while bound sperm were fixed to the egg due to glutaraldehyde in the lower layer of the gradient (Saling et al., 1978). Using this technique, eggs were recovered at 30 min after sperm addition, mounted on slides, stained with aceto-lacmoid (Toyoda and Chang, 1974), and examined with phase-contrast optics. The average number of sperm bound per egg was calculated from the total number of sperm and the total number of eggs in the sample. Variability between individual experiments was insignificant, due presumably to the use of: *a*) 2 males/experiment; *b*) the same inseminating sperm concentration throughout the study; and *c*) the density gradient centrifugation technique for gamete recovery.

Fertilization in Vitro

After incubation for 90 min, mouse spermatozoa were added to cumulus-free, zona-intact mouse eggs in 200 μl of CM. Upon addition to eggs, the sperm suspension underwent a 10- to 20-fold dilution. The gametes were recovered after 4 h at 37°C in an atmosphere of 5% CO_2 in air. The eggs were fixed in 2.5% glutaraldehyde, mounted on slides, stained and examined as above. Eggs were scored as penetrated if sperm were found within the perivitelline space and/or vitellus, and as fertilized only if both the sperm head (or pronucleus) and sperm tail were identified in the vitellus.

RESULTS

Fresh Sperm Binding

Upon release into isosmotic medium, mouse spermatozoa from either the corpus or cauda epididymidis were vigorously motile and remained so for several hours when suspended in CM or other appropriate medium. Spermatozoa recovered from more proximal regions displayed markedly different behavior. After liberation of caput epididymal sperm into CM, a characteristic pattern of motility, unlike that displayed by more mature cells, was expressed briefly. The midpiece as well as the connection between the head and the tail were rigid in caput spermatozoa, resulting in nonprogressive, circular motion. After approximately 20 min in CM, this pattern of motility degenerated into twitching of the principal piece of the sperm tail.

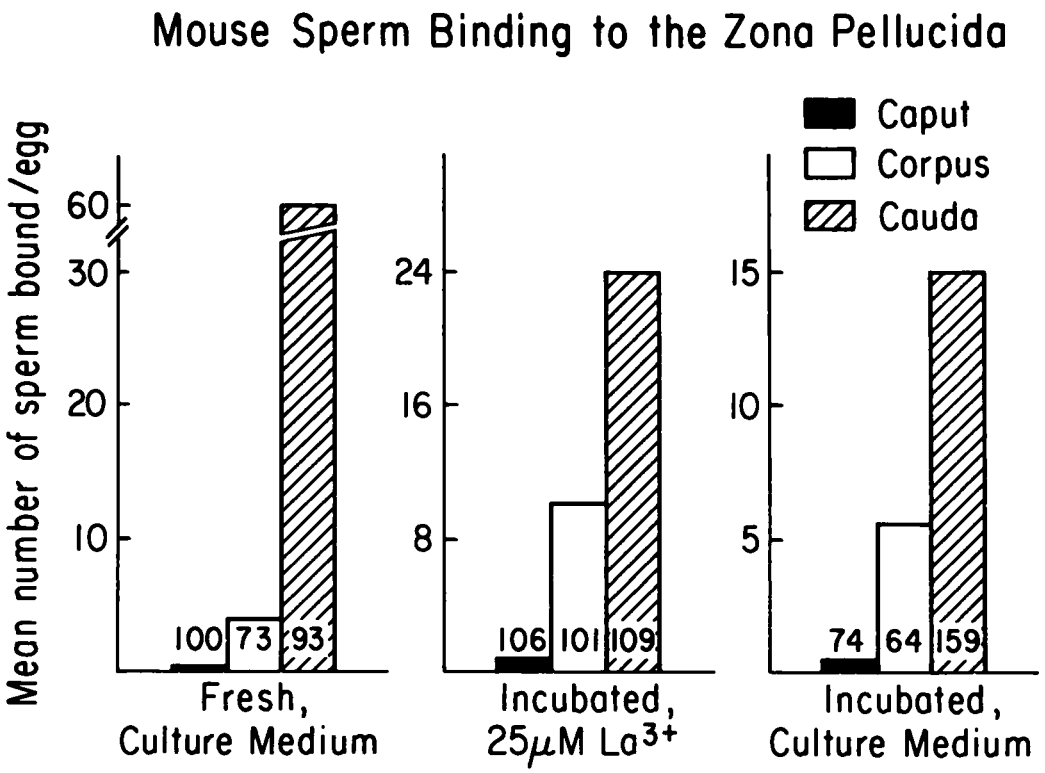

FIG. 1. Mouse sperm binding to zonae pellucidae. Mouse spermatozoa were recovered from the caput, corpus, or cauda epididymidis and maintained separately. For the histogram on the *left*, the spermatozoa were suspended in CM, the complete culture medium in which all sperm are motile for ⩾20 min (see text), and challenged with cumulus-free, zona-intact mouse eggs within 10 min of recovery. For the remaining 2 histograms the spermatozoa were suspended in either TNL (*center*) containing 25 μM La^{3+}, or in CM (*right*), and incubated at 37°C. After 90 min, the sperm were used to challenge zona-intact eggs, as above. The final concentration of all sperm suspensions was approximately equivalent (10^6 cells/ml). In all cases gametes were recovered 30 min after mixing by the density gradient centrifugation technique. The height of each column indicates the average number of sperm bound per egg; the number at the base of each column indicates the number of eggs examined. The results were compiled from 5 separate experiments.

Since caput spermatozoa were motile for a short period, their zona binding ability could be assessed if they were mixed with cumulus-free, zona-intact eggs immediately after recovery from the epididymis. In practice, 10 min was the minimum amount of time required for preparation and determination of the concentration of the sperm suspensions. The results of such an experiment (Fig. 1, *left histogram*) demonstrated that caput sperm express less than 1% of the zona binding level found in cauda sperm. Corpus sperm, though highly motile, also had low binding ability in this situation (~6% of the cauda binding level), suggesting that motility is not the sole factor necessary to achieve a firm bond between sperm and the zona pellucida.

Effect of Lanthanum on Sperm Motility and Fertility

Since the sperm's capacity to bind to zonae in vitro reaches a maximum plateau only after 15 min of incubation in the appropriate medium (Saling et al., 1978), it could be argued that determination of fresh sperm binding to zonae did not measure zona binding ability validly. Moreover, at the termination of the binding assay, after the gametes had been incubated for 30 min, few of the caput sperm were still motile. It was therefore necessary to develop a method that allowed more accurate comparison of zona binding ability among the different sperm populations.

A solution to this dilemma was offered by

the finding that mouse spermatozoa contain at least 2 Ca^{2+} -sensitive sites: the first site is responsible for zona binding, whereas the second site is concerned with the maintenance of motility (Heffner et al., 1980). La^{3+} can replace Ca^{2+} in the former site, but not in the latter (Saling et al., 1978; Heffner et al., 1980). When cauda epididymal mouse spermatozoa were incubated in TNL their motility declined within 10-20 min to a twitching motion that resembled caput sperm motility. This La^{3+} -dependent immobilization was apparently reversible (Fig. 2). When initial incubation of cauda epididymal spermatozoa in TNL, which led to immobilization, was followed after varying intervals with resuspension in CM, the sperm displayed a reactivation of motility that was similar in pattern and number to the motility exhibited by their nonimmobilized counterparts (i.e., cauda sperm preincubated for a similar interval in either CM or TN).

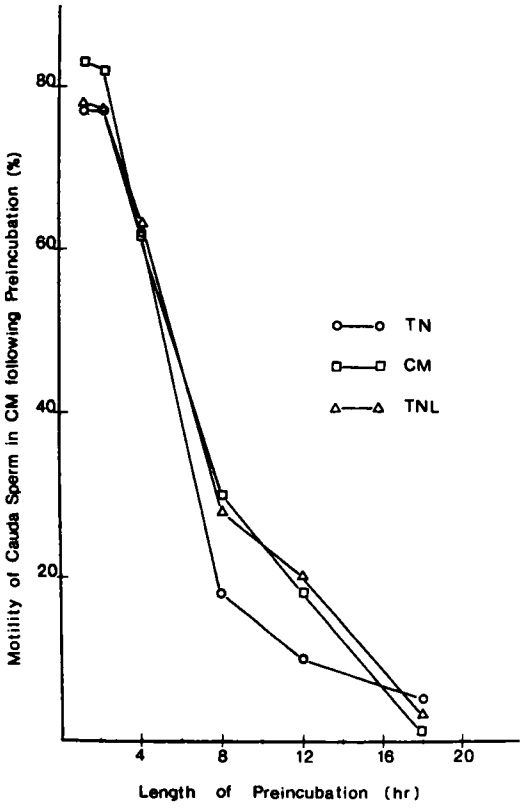

FIG. 2. Effect of preincubation in La^{3+} on subsequent sperm motility. Mouse spermatozoa from the cauda epididymidis were princubated for the times indicated in either TN, CM or TNL at a concentration of $\sim 10^6$ cells/ml. An aliquot of the preincubation suspension was diluted 1:10 in CM and assessed immediately for motility. The results represent the percentage of spermatozoa displaying vigorous, progressive motility, averaged from 3 separate trials. *TN* sperm, motile during preincubation, o—o; *CM* sperm, motile during preincubation, □—□; and *TNL* sperm, immotile during preincubation, Δ—Δ.

The period of immobilization produced by La^{3+} did not appear to have a deleterious effect upon the spermatozoa, which was ascertained by examining the fertilizing ability of sperm following La^{3+} incubation. For these experiments cauda spermatozoa were incubated in either CM or TNL for 90 min. An aliquot of the preincubated sperm was then mixed with cumulus-free, zona-intact mouse eggs. Immediately upon introduction into the CM-containing insemination vessel, both the CM- and the TNL-preincubated sperm displayed vigorous whiplash motility characteristic of capacitated (*activated*) mouse sperm (Fraser, 1977). Examination of the eggs 4 h after insemination revealed that the TNL-preincubated cauda sperm were equivalent to CM-preincubated sperm in terms of fertilizing ability: 124 of 142 eggs (87%) were fertilized by TNL-preincubated sperm, whereas 108 of 122 eggs (89%) were fertilized by CM-preincubated sperm.

Effect of Lanthanum on Zona Binding

The zona-binding ability of immotile cauda sperm in TNL could be compared with that of motile sperm in CM if the former were placed on a shaking device to insure sperm-egg collisions. When sperm were incubated in TNL for 90 min and then mixed with zona-intact eggs in TNL and shaken at 100 cycles/min, the binding levels achieved were nearly identical to those of nonshaken, but motile, gametes in CM (Table 1). It therefore appeared that the La^{3+} -dependent immobilization did not affect the sperm adversely, and permitted the occurrence of normal levels of zona binding in the absence of sperm motility.

Comparison of Binding Ability in Sperm from Different Epididymal Regions

Sperm were recovered from the caput, corpus and cauda regions of the epididymis and incubated in either TNL or CM for 90 min. When exposed to cumulus-free, zona-intact eggs for 30 min, the binding levels shown in Fig 1 (*center* and *right histograms*) were obtained.

TABLE 1. Cauda epididymal mouse spermatozoa binding to zonae pellucidae following incubation.[a]

Incubation medium	Mean number of sperm/egg	Total no. sperm bound/ total no. eggs examined
CM: Culture Medium	16.0	1108/69
TNL: Tris, 20 mM NaCl, 130 mM $LaCl_3$, 25 μM	16.1	1901/118

[a]Following a 90 min incubation at 37°C in the media indicated, cauda spermatozoa were mixed with cumulus-free, zona-intact mouse eggs, that were suspended in medium homologous to that used for the spermatozoa. Microdishes containing gametes suspended in TNL were placed on a shaker and rocked at a frequency of 100 min^{-1}, while dishes with gametes in CM remained stationary. At 30 min after gamete mixing, the eggs were recovered by density gradient centrifugation. The results were compiled from 4 replicate experiments.

Regardless of the state of motility of the spermatozoa, the same zona binding pattern emerged. Whether immotile (and shaken) or freely motile, cauda sperm bound to zonae in high numbers, and corpus sperm bound at some intermediate level. Sperm recovered from the caput epididymidis bound very poorly to the zona surface under all the conditions employed.

Sperm from the 3 epididymal regions were also tested for their ability to bind to the egg plasma membrane using similar techniques. When sperm were preincubated in CM for 90 min and then mixed with zona-free eggs, a pattern similar to that found above for zona-binding ability was observed (Fig. 3). Of interest, however, is the notable difference in the effect of La^{3+}. Whereas La^{3+} was sufficient for sperm binding to zonae pellucidae, the cation was insufficient for sperm binding to the egg plasma membrane, suggesting that mouse spermatozoa have different requirements and/or binding sites for the 2 different egg surfaces.

DISCUSSION

The plasma membrane of the maturing spermatozoon appears to be the target for a variety of post-testicular modifications. These include changes in surface charge (Bedford, 1963; Cooper and Bedford, 1971; Courtens and Fournier-Delpech, 1979; Moore, 1979; Hammerstedt et al., 1979), in antigenicity (Hunter, 1969; Killian and Amann, 1973; Rodman et al., 1979; Feuchter et al., 1981), in ultrastructure (Bedford and Nicander, 1971; Olson and Hamilton, 1976), in lectin receptor distribution (Gordon et al., 1975; Nicolson et al., 1977; Olson and Danzo, 1981), in protein and glycoprotein composition (Olson and Hamilton, 1978; Nicolson et al., 1979; Bostwick et al., 1980; Kohane et al., 1980; Voglmayr et al., 1980; Olson and Danzo, 1981), and in enzymatic activity (Chulavatnatol and Yindepit, 1976; Bernal et al., 1980; Casillas et al., 1980). Interaction with zona pellucida also involves the sperm's plasma membrane (Saling et al., 1979; Saling and Storey, 1979; Phillips and Shalgi, 1980; Peterson et al., 1980). Since fertilizing ability first appears in the corpus epididymidis (Pavlok, 1974; Bedford, 1966; Orgebin-Crist, 1967; Horan and Bedford,

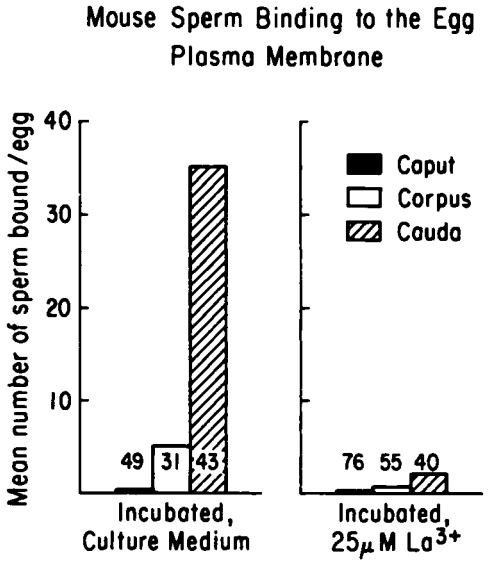

FIG. 3 Mouse sperm binding to the egg plasma membrane. Spermatozoa were prepared as described in Fig. 1, *center* and *right* histograms. After 90 min of incubation at 37°C, the sperm were mixed with zona-free mouse eggs. The final sperm concentration was $\sim 10^6$ cells/ml in all cases. Gametes were recovered at 30 min after mixing by the density gradient centrifugation technique. The height of each column represents the average number of sperm bound per egg; the number at the base of each column represents the number of eggs examined. The results were compiled from 3 separate experiments.

1972), it becomes important to determine whether the appearance of fertility reflects modification of the sperm plasma membrane sufficiently to permit sperm-zona pellucida interaction. The results of the experiments presented here are consistent with this possibility. In all of the experimental situations examined, sperm recovered from the caput epididymidis failed to bind to zonae pellucidae, whereas those recovered from the corpus epididymidis were the least mature cells to display zona binding ability. No attempt was made in the present study to differentiate the regions of the epididymis further than caput, corpus, cauda.

Like many physiological processes, the binding of mouse spermatozoa to zonae pellucidae depends upon Ca^{2+} (Saling et al., 1978). Earlier studies showed that La^{3+}, at a concentration of 5 μM, would substitute for Ca^{2+} in permitting this reaction; the latter cation is used standardly at a concentration of 1.7 mM. The significance of that finding was the indication that the transport of Ca^{2+} across the sperm membrane is not necessary for zona binding, since lanthanide cations are potent inhibitors of transmembrane movement of Ca^{2+} (Mela, 1968). Mouse spermatozoa have an additional Ca^{2+} sensitive site; the initiation of sperm motility is Ca^{2+} independent, but its maintenance appears to be Ca^{2+} dependent (Heffner, et al., 1980). This site can be distinguished from the Ca^{2+} dependent zona binding site by its ionic specificity. Sr^{2+}, but not La^{3+}, can replace Ca^{2+} in the motility maintenance reaction (Heffner, et al., 1980). With regard to motility, the results of the present study demonstrated that sperm immotility in the presence of La^{3+} can be reversed immediately by incubation with Ca^{2+}. Furthermore, the state of immobilization produced by La^{3+} did not affect mouse sperm adversely, since cauda epididymal sperm, incubated with La^{3+} initially, displayed high levels of fertility when mixed with eggs in the standard fertilization in vitro medium, CM. Prudent use of this technique to immobilize sperm nonlethally may allow examination of the role of motility in sperm function.

The inability of TNL-preincubated mouse sperm to bind to the egg plasma membrane is intriguing. This result suggests that the spermatozoon's binding site for the zona pellucida is distinct from that for the egg plasma membrane. When the morphology of the sperm cell during its encounter with these 2 egg surfaces under conditions that may lead to fertilization is considered, the result is less surprising. In the mouse, the fertilizing spermatozoon binds to the zona pellucida *via* its plasma membrane (Saling and Storey, 1979; Saling et al., 1979). Sperm that have penetrated the zona exhibit reacted acrosomes exclusively (Bedford, 1967; Piko, 1969; Austin, 1975; Chang and Hunter, 1975; Gwatkin, 1976; Yanagimachi, 1977; Bedford and Cooper, 1978). Thus interaction between the plasma membrane overlying the sperm's acrosome (preferential region of Ca^{2+}, and thus La^{3+}, binding; see Saling and Storey, 1979) and the plasma membrane of the egg would not occur normally. Alternatively, La^{3+} may affect the egg plasma membrane directly in an adverse manner. Although unintended, a small amount of La^{3+} could be present during the binding experiments due to carry-over during sperm transfer.

The onset of zona binding ability in epididymal mouse spermatozoa could result from several mechanisms: an unmasking or transformation of a zona binding component(s) that is already present in the spermatozoon, or the addition of a zona binding component(s), possibly epididymal secretory protein, to the sperm surface during transit through the epididymis. Evidence that both of these processes can occur has been obtained in various species; in fact, Olson and Danzo (1981) have demonstrated that, within the class of Con A receptors on the surface of epididymal rat spermatozoa, different individual components may either increase or decrease during epididymal transit. The nature of the alterations on the mouse sperm surface that allow zona binding are unresolved. Yet 2 distinct populations of epididymal spermatozoa may now be defined in terms of their zona binding ability. Identification of the molecular changes responsible for this functional distinction is both necessary and accessible to study.

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REFERENCES

Austin, C. R. (1975). Membrane fusion events in fertilization. J. Reprod. Fertil. 44:155–166.

Bedford, J. M. (1963). Changes in the electrophoretic properties of rabbit spermatozoa during passage through the epididymis. Nature (Lond) 200: 1178–1180.

Bedford, J. M. (1966). Development of the fertilizing ability of spermatozoa in the epididymis of the rabbit. J. Exp. Zool. 163:319–330.

Bedford, J. M. (1967). Experimental requirements for capacitation and observations on ultrastructural changes in rabbit spermatozoa during fertilization. J. Reprod. Fertil. (Suppl 2):35–48.

Bedford, J. M. (1975). Maturation, transport and fate of spermatozoa in the epididymis. Handbook of Physiology, sect. 7, Endocrinology, vol. 5, Male Reproductive System (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Bethesda, MD, pp. 303–317.

Bedford, J. M. and Cooper, G. W. (1978). Membrane fusion events in the fertilization of vertebrate eggs. Cell Surf. Rev. 5:65–125.

Bedford, J. M. and Nicander, L. (1971). Ultrastructural changes in the acrosome and sperm membranes during maturation of spermatozoa in the testis and epididymis of the rabbit and monkey. J. Anat. 108:527–543.

Bernal, A., Torres, J., Reyes, A. and Rosado, A. (1980). Presence and regional distribution of sialyl transferase in the epididymis of the rat. Biol. Reprod. 23:290–293.

Bostwock, E. F., Bentley, M. D., Hunter, A. G. and Hammer, R. (1980). Identification of a surface glycoprotein on procine spermatozoa and its alteration during epididymal maturation. Biol. Reprod. 23:161–169.

Casillas, E. R., Elder, C. M. and Hoskins, D. D. (1980). Adenylate cyclase activity of bovine spermatozoa during maturation in the epididymis and the activation of sperm particulate adenylate cyclase by GTP and polyamines. J. Reprod. Fertil. 59:297–302.

Chang, M. C. and Hunter, R.H.F. (1975). Capacitation of mammalian sperm: Biological and experimental aspects. Handbook of Physiology, sect. 7, Endocrinology, vol. 5, Male Reproductive System (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Bethesda, MD, pp. 339–351.

Chulavatnatol, M. and Yindepit, S. (1976). Changes in surface ATPase of rat spermatozoa in transit from the caput to corpus epididymis. J. Reprod. Fertil. 48:91–97.

Cooper, G. W. and Bedford, J. M. (1971). Acquisition of surface change by the plasma membrane of mammalian spermatozoa during epididymal maturation. Anat. Rec. 169:300–301.

Courtens, J. L. and Fournier-Delpech, S. (1979). Modifications in the plasma membranes of epididymal ram spermatozoa during maturation and incubation *in utero*. J. Ultrastruct. Res. 68:136–148.

Feuchter, F. A., Vernon, R. B. and Eddy, E. M. (1981). Analysis of the sperm surface with monoclonal antibodies: topographically restricted antigens appearing in the epididymis. Biol. Reprod. 24:1099–1110.

Fraser, L. (1977). Motility patterns in mouse spermatozoa before and after capacitation. J. Exp. Zool. 202:439–444.

Gordon, M., Dandekar, P. V. and Bartoszewicz, W. (1975). The surface coat of epididymal, ejaculated and capacitated sperm. J. Ultrastruct. Res. 50:199–207.

Gwatkin, R.B.L. (1976). Fertilization. Cell Surf. Rev. 1:1–54.

Hammerstedt, R. H., Keith, A. D., Hay, S., Deluca, N. and Amann, R. P. (1979). Changes in ram sperm membranes during epididymal transit. Arch. Biochem. Biophys. 196:7–12.

Heffner, L. J., Saling, P. M. and Storey, B. T. (1980). Separation of calcium effects on motility and zona binding ability in mouse spermatozoa. J. Exp. Zool. 212:53–59.

Horan, A. H. and Bedford, J. M. (1972). Development of the fertilizing ability of spermatozoa in the epididymis of the Syrian hamster. J. Reprod. Fertil. 30:417–423.

Hunter, A. C. (1969). Differentiation of rabbit sperm antigens from those of the seminal plasma. J. Reprod. Fertil. 20:413–418.

Inoue, M. and Wolf, D. P. (1975). Sperm binding characteristics of the murine zona pellucida. Biol. Reprod. 13:340–346.

Killian, G. J. and Amann, R. P. (1973). Immunoelectrophoretic characterization of fluid and sperm entering and leaving the bovine epididymis. Biol. Reprod. 9:489–499.

Kohane, A. C., Gonzalez Echeverria, F.M.C., Pineiro, L. and Blaquier, J. A. (1980). Interaction of proteins of epididymal origin with spermatozoa. Biol. Reprod. 23:737–742.

Mela, L. (1968). Interactions of La^{3+} and local anesthetic drugs with mitochondrial Ca^{2+} and Mn^{2+} uptake. Arch. Biochem. Biophys. 123:286–293.

Moore, H.D.M. (1979). The net surface charge of mammalian spermatozoa as determined by isoelectric focusing. Changes following sperm maturation, ejaculation, incubation in the female tract and after enzyme treatment. Int. J. Androl. 2:449–462.

Nicolson, G. L., Usui, N., Yanagimachi, R., Yanagimachi, H. and Smith, J. R. (1977). Lectin-binding sites on the plasma membranes of rabbit spermatozoa. Changes in surface receptors during epididymal maturation and after ejaculation. J. Cell Biol. 74:950–962.

Nicolson, G. L., Brodginski, A. B., Beattie, G. and Yanagimachi, R. (1979). Cell surface changes in the proteins of rabbit spermatozoa during epididymal passage. Gamete Res. 2:153–162.

Olson, G. E. and Danzo, B. J. (1981). Surface changes in rat spermatozoa during epididymal transit. Biol. Reprod. 24:431–443.

Olson, G. E. and Hamilton, D. W. (1976). Morphological changes in the midpiece of wooly opossum spermatozoa during epididymal transit. Anat. Rec. 186:387–404.

Olson, G. E. and Hamilton, D. W. (1978). Characterization of the surface glycoproteins of rat spermatozoa. Biol. Reprod. 19:26–35.

O'Rand, M. G. (1980). Antigens of spermatozoa and their environment. Immunological Aspects of Infertility and Fertility Regulation (D. S. Dhindsa and G.F.B. Schumacher, eds.). Elsevier/North Holland, New York, pp. 155–171.

Orgebin-Crist, M.-C. (1967). Maturation of spermatozoa in the rabbit epididymis: Fertilizing ability and embryonic mortality in does inseminated with epididymal spermatozoa. Ann. Biol. Anim. Biochim. Biophys. 7:373–389.

Orgebin-Crist, M.-C., Danzo, B. J. and Davies, J. (1975). Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. Handbook of Physiology, sect. 7, Endocrinology, vol. 5, Male Reproductive System (D. W. Hamilton and R. O. Greep, eds.). American Physiological Society, Bethesda, MD, pp. 319–338.

Pavlok, A. (1974). Development of the penetration activity of mouse epididymal spermatozoa *in vivo* and *in vitro*. J. Reprod. Fertil. 36:203–205.

Peterson, R. N., Russell, L., Bundman, D. and Freund, M. (1980). Sperm-egg interaction: direct evidence for boar plasma membrane receptors for porcine zona pellucida. Science 207:73–74.

Phillips, D. M. and Shalgi, R. M. (1980). Surface properties of the zona pellucida. J. Exp. Zool. 213:1–8.

Piko, L. (1969). Gamete structure and sperm entry in mammals. Fertilization, vol. 2 (C. B. Metz and A. Monroy, eds.). Academic Press, New York, pp. 325–403.

Rodman, T. C., Litwin, S. D., Romani, M. and Vidali, G. (1979). Life history of mouse sperm protein. J. Cell Biol. 80:605–620.

Saling, P. M., Storey, B. T. and Wolf, D. P. (1978). Calcium-dependent binding of mouse epididymal spermatozoa to the zona pellucida. Dev. Biol. 65:515–525.

Saling, P. M. and Storey, B. T. (1979). Mouse gamete interactions during fertilization *in vitro*. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. J. Cell Biol. 83:544–555.

Saling, P. M., Sowinski, J. and Storey, B. T. (1979). An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae *in vitro*: sequential relationship to the acrosome reaction. J. Exp. Zool. 209:229–238.

Toyoda, U. and Chang, M. C. (1974). Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fertil. 36:9–22.

Volgmayr, J. K., Fairbanks, G., Jackowitz, M. A. and Colella, J. (1980). Post-testicular developmental changes in the ram sperm cell surface and their relationship to luminal fluid proteins of the reproductive tract. Biol. Reprod. 22:655–667.

Yanagimachi, R. (1977). Specificity of sperm-egg interactions. Immunobiology of Gametes (M. Edidin and M. H. Johnson, eds.). Cambridge University Press, Cambridge, pp. 255–295.