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I am submitting herewith a thesis by Mary E. Crecink entitled "Binding of Abnormal Sperm to Mouse Egg Zonae Pellucidae." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Mary Ann Handel
Mary Ann Handel, Major Professor

We have read this thesis
and recommend its acceptance:

Joseph C. Daniel
Charles A. Davies
Alan R. Kennedy

Accepted for the Council:

Carl Munkel
The Graduate School

BINDING OF ABNORMAL SPERM TO MOUSE EGG
ZONAE PELLUCIDAE

A Thesis
Presented for the
Master of Science
Degree
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ABSTRACT

The ability of abnormal sperm to bind to the zona pellucida was examined using techniques of fertilization in vitro. Sperm from the inseminates were grouped into classes according to their morphology. After fertilization in vitro cumulus-free ova were scored by phase contrast microscopy for the number and type of sperm bound to the zona. In some cases ova were also scored by scanning electron microscopy.

The percentages of abnormal sperm bound to the zona pellucida were shown to be significantly lower than the percentages of abnormal sperm in the inseminates. In addition, the percentages of abnormal sperm bound to the ova did not increase significantly over time. When the classes of abnormal sperm in the inseminates bound to the ova and were compared, it was found that Type 3 abnormal sperm were represented in the same frequency on the zona as in the inseminate; all other abnormal classes showed a binding frequency significantly lower than their frequency in the inseminate.

The scanning electron microscopy studies demonstrated that bound sperm associate in different ways with the zona pellucida. The majority of normal sperm were bound in a plane perpendicular to the zona, had vesiculated acrosomal membranes, and were secured to the ova by strands emanating from the zona. Type 2 and Type 3 abnormal sperm bound in a plane perpendicular to the ova, exhibited vesiculated acrosomal membranes and adhered tightly to the zona, while Type 5 and Type 6 sperm were bound to the ova in a plane parallel to the zona and were loosely attached.

It was concluded from these data that while abnormal sperm are able to participate in fertilization in vitro, they are less efficient than normal

sperm. Furthermore, abnormal sperm seem to be able to undergo the process to capacitation at or near the rate of normal sperm or possibly do not require capacitation at all. Type 3 sperm seem to be exceptionally well adapted to binding to the zona pellucida and beginning the process of penetration.

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I. INTRODUCTION

Mammalian fertilization occurs in the ampulla of the oviduct, requiring sperm to negotiate successfully a series of anatomical and physical barriers while undergoing the processes of capacitation and the acrosome reaction. Once at the site of fertilization, spermatozoa must penetrate the corona radiata of the ovum, bind to and penetrate the zona pellucida, and then fuse with the plasma membrane. While normal spermatozoa are able to accomplish these processes, it is not known if morphologically abnormal sperm are similarly able to do so.

Several investigators have demonstrated that sperm, both normal and abnormal, are prevented from fertilizing ova in vitro at a variety of levels. Only a small fraction of the spermatozoa deposited in the vagina enter the oviduct. The majority remain in the uterus where they are phagocytized by neutrophils (Zamboni, 1971). Thus, the uterus provides one of the first obstacles to fertilization.

The second barrier to fertilization appears to be at the uterotubal junction. Krzanowska (1974) suggested that there is a selective reduction in the number of sperm entering the oviduct. By assessing the structure of the spermatozoa in oviductal and uterine flushings, she concluded that morphologically abnormal sperm are less capable of entering the oviduct due to some mechanism at the uterotubal junction and that this region serves as a partially effective barrier to fertilization. Recent studies by Nestor and Handel (in press) have supported these findings. Utilizing a strain of mice with unusually high percentages of abnormal sperm, they showed that the

percentages of abnormal sperm significantly decreased between uterus and oviduct.

The final barriers to fertilization are two investments of the egg, the corona radiata and the zona pellucida. The corona radiata must be penetrated and ultimately dispersed by fully capacitated sperm. The process of capacitation allows the sperm to bind to the zona pellucida of the egg and undergo the acrosome reaction (Fraser, 1981; Yanagimachi, 1978). Interspecific fertilization experiments have shown that spermatozoa from foreign species were unable to bind to the zona pellucida or to penetrate it. However, sperm from the rat, mouse, guinea pig and humans were able to activate zona-free hamster eggs (Yanagimachi, 1981). Based on these results, it has been suggested that the zona pellucida does serve as a specific barrier to fertilization. Furthermore, the acrosome reaction, essential for further progress of the sperm through the zona pellucida, involves the disruption of the membranes around the acrosome and the subsequent release of the acrosomal contents (Bleil and Wassarman, 1983; Saling et al., 1979, Saling and Storey, 1979). After the acrosome reaction has been completed and the zona is penetrated, the plasma membranes of the ova and sperm fuse (Saling et al., 1979; Saling and Storey, 1979). Bleil and Wassarman (1983), Saling et al. (1979), and Saling and Storey (1979) have further demonstrated that the sequence of the events at the site of fertilization is stringent and that any variation will cause the sperm to lose their fertilizing ability.

Normal spermatozoa are generally able to overcome the barriers of the female reproductive tract and penetrate the investments of the egg. Abnormal sperm, on the other hand, may be unable to do so. By using

different genetic strains and mutants of the mouse which exhibit variable frequencies and types of sperm abnormalities, the interaction between spermatozoa and ova, and spermatogenesis in general, can be elucidated. Fertile, random-bred mice exhibit low percentages of morphologically abnormal sperm while certain inbred strains, such as PL/J, have high percentages of abnormalities yet are fertile (Burkhart and Malling, 1981). There are many sterile mutants that demonstrate even higher percentages of sperm abnormalities, such as p^{6H}, p^{25H}, p^S (alleles at the pink-eyed dilution locus) (Bryan, 1977, 1981; Hash and Wolf, 1979; Hunt and Johnson, 1971; Johnson and Hunt, 1975; Melvold, 1979; Wolf et al., 1977), pcd (Purkinje cell degeneration) (Handel and Dawson, 1981), and bs (blind sterile) (Handel and Sotomayor, submitted). These strains and mutants provide a diversity of sperm abnormalities which can be used to study the relationship between sperm morphology and ability to participate in the various events of fertilization.

Several investigators have attempted to demonstrate that morphologically abnormal sperm are capable of successfully penetrating the cumulus cells of the corona radiata and the zona pellucida. Smith and coworkers (1979) published electron micrographs of a spermatozoan with an abnormal midpiece and flagellar fibers that had penetrated an egg. They failed, however, to document whether the sperm had been abnormal prior to fertilization or was simply photographed in the process of normal deterioration. Barros (1979) showed that sperm from infertile patients with high percentages of abnormal sperm were able to bind zona-free hamster eggs. In addition, he produced electron micrographs showing sperm with

condensed chromatin in the ooplasm and at the surface of the oocyte which he interpreted as being abnormal. Again, this may simply represent a step in the normal process of decondensation.

Krzanowska and Lorenc (1983) demonstrated that abnormal sperm were able to penetrate intact, cumulus-free, and zona-free eggs in experiments of fertilization in vitro. They found the proportions of abnormal sperm that penetrated the eggs were significantly lower in intact than in cumulus-free eggs. In addition, these researchers concluded that severely misshapen sperm penetrated the egg at a lower frequency than represented in the inseminate.

McGrath and Hillman (1980), however, using techniques of fertilization in vitro, demonstrated that sperm from the sterile mutant $\frac{t6}{t6w32}$ were not able to cross the final barriers to fertilization. By sequentially removing the investments of the egg, they were able to show that sperm from the $\frac{t6}{t6w32}$ mutants were unable to interact with the egg even when devoid of any barriers. This seems to indicate that at least some abnormal sperm are not capable of successful penetration in the final events of fertilization.

Interaction of the sperm and the egg surface can best be observed by utilizing techniques of fertilization in vitro. Although the egg-sperm interaction is a rapid process, it is easily studied in vitro by fixing the complex at a variety of times which allows each event to be individually analyzed. Using this technique, the selective barriers within the female reproductive are removed and the barriers created by the investments of the egg manipulated; the corona radiata and zona pellucida can be left intact or removed. Since the spermatozoa are placed directly on top of the eggs, each sperm, theoretically, has an equal chance of binding to the egg. In

this way abnormal sperm which normally would have been eliminated in the female reproductive tract are present at the site of fertilization in the same concentrations as found in the inseminate. Culture conditions for mouse gametes have been established which may adequately mimic conditions in vivo. The sperm from mice require only a short capacitation time and are able to fertilize ova in vitro at high percentages (Stanger and Quinn, 1982).

In the present study, techniques of fertilization in vitro have been used to determine if morphologically abnormal sperm from normal and mutant mice can successfully overcome the final barriers at the site of fertilization. Specifically, it was asked whether morphologically abnormal sperm can bind to the zona pellucida at a frequency equal to their frequency in the inseminates. The percentages of abnormal sperm bound to the zona pellucida was compared to the percentages of abnormal sperm in the inseminate to determine if there was a significant difference between the two. In addition the association of the sperm and zona pellucida has been studied by scanning electron microscopy to determine if the abnormal sperm associates normally with the egg.

This study may be particularly important since techniques of fertilization in vitro are presently used to treat infertility in humans. Such studies may help to clarify the complex role of sperm in the fertilization process.

II. METHODS AND MATERIALS

Strains of mice

Three different strains of fertile mice served as sperm donors: the HA/ICR(Cum) random-bred line and the B6CBF₁(Cum) hybrid, both characterized by low levels of abnormal sperm, and the inbred PL/J strain, characterized by high levels of abnormal sperm. All male mice used were between 3 and 7 months old at the time they were killed.

B6D2F₁(Cum) female mice served as egg donors. Two-cell embryos which were used as a control for binding to the zona pellucida, were obtained from the mating of a HA/ICR male to a B6D2F₁ female mouse. In early experiments, females were 1 to 2 months old when they were superovulated. It was found, however, that older mice released a greater number of eggs. For that reason, in later experiments female mice were between 3 and 6 months of age when used. Animals were maintained on a 14-hour light: 10-hour dark schedule with lights on at 7 a.m. Food and water was given ad libitum.

Media

Whitten medium 514 (WM) (Whitten, 1971) modified by Hoppe and Pitts (1973) was used to culture and incubate all gametes. It was prepared with freshly distilled water from a Corning Megapure MP-3A system once per week. The medium was gased for 3 to 5 minutes with 5% CO₂ in air and filtered through a Nalgene filter unit (0.20 micron pore size).

Paraffin oil was equilibrated with WM without bovine serum albumin (BSA) by stirring the oil and medium in a 10 ml to 1 ml ratio for 96 hours. The medium was pipetted off and the oil was stored in an amber-colored bottle for up to one month. All glassware was washed in 7X detergent, treated with sulfuric-chromic acid, rinsed 20 times in tap water and 20 times in glass distilled water, and autoclaved. On the day of the experiment, surgical instruments were sterilized with 70% ethanol.

Techniques of Fertilization In Vitro

The techniques of fertilization in vitro were a modification of those described by Hoppe and Pitts (1973). Female mice were superovulated by injection of 5IU Pregnant Mare's Serum Gonadotropin (PMSG, Sigma) followed by 5IU Human Chorionic Gonadotropin (HCG, Sigma) 48 to 50 hours later. Female mice that served as two-cell embryo donors were superovulated as described above. Immediately after the HCG injection, a female mouse was placed in the cage of an HA/ICR male. Mating was confirmed by the presence of a vaginal plug on the following morning.

In order to allow equilibration of the oil, medium, and temperature 0.5 ml drops of WM were added to ten 3 mm depression slides or to ten 35x10 mm Falcon petri dishes, overlaid with 2 to 3 ml of paraffin oil, and incubated at 37°C and 5% CO₂ in air in a Napco 4100 water-jacketed CO₂ incubator the day before the experiment. Aliquots of 1 ml WM were placed in 12x75 mm culture tubes, gassed with 5% CO₂ in air, and fitted with rubber stoppers. These tubes were incubated at 37°C. In addition, 1 mg/ml of hyaluronidase (Sigma) in WM was prepared.

The male mice were killed by cervical dislocation and the reproductive tracts were removed. The testes and caput epidymides were discarded. Using 2 forceps, the sperm were gently squeezed from each cauda epididymi and vas deferens in 1 ml WM. Sperm from 2 mice were pooled. An aliquot was scored to determine the sperm concentration using a hemocytometer. The remaining suspension was overlaid with paraffin oil, incubated on a slide warmer at 37°C, and gassed with 5% CO₂ in air.

Three female mice serving as two-cell embryo donors were killed by cervical dislocation. After stripping away as much fascia as possible, the oviducts with short uterine handles were removed. Then, the oviducts were flushed under an Olympus dissecting microscope with WM and a 33-gauge needle. Two-cell embryos were collected using a micropipette and 2 to 10 two-cell embryos were pipetted into each culture dish.

Five female mice serving as egg donors were killed by cervical dislocation approximately 12 hours after HCG injection. The oviduct with a short section of the uterus was removed. Eggs were collected from the oviducts under a dissection microscope by rupturing the ampullae with a 26-gauge needle in a drop of WM plus hyaluronidase. The cumulus cells were allowed to disperse. The viable eggs were collected using a micropipette and processed through 3 washes in WM. Between washes, the eggs were placed on a warming tray at 37°C and gassed with 5% CO₂ in air. Ten to 20 eggs were pipetted into each culture dish containing WM and two-cell embryos overlaid with paraffin oil. The appropriate amount of sperm suspension was added to each culture dish so that the final concentration was

approximately 10^5 sperm/ml. The culture dishes containing the cells were then placed in an incubator at 37°C and 5% CO₂ in air.

Microscopy

Eggs and two-cell embryos were processed at 2, 3, 4, 5, and 6 hours after insemination according to techniques of Toyoda and Chang (1974). Eggs and embryos were washed 2 times in fresh WM to remove unbound sperm and then directly pipetted into a drop of 2.5% glutaraldehyde on a microscope slide. A coverslip suspended with a 3:1 mixture of vaseline to paraffin was placed over the drop of glytaraldehyde and partially compressed. The cells were allowed to fix for 10 minutes at room temperature. The glutaraldehyde was drawn off as 10% neutral formalin was added. The slide was put in a Coplin jar containing 10% neutral formalin for 10 minutes to 1 week. The eggs and embryos were then washed with distilled water, dehydrated in 95% ethanol, and stained with lacmoid (0.25%, MC/B) in 45% acetic acid.

The eggs and embryos were scored for the number and type of morphology of sperm bound to the zona pellucida. Scoring was done using the method described by Wyrobeck and Bruce (1975). A Zeiss photomicroscope with phase contrast optics was used to observe fixed and stained eggs and embryos. Photographs of different focal planes through the eggs and embryos were taken.

Some eggs also were processed for scanning electron microscopy. Eggs were processed 3 hours after insemination. Eggs were first washed 2 times in fresh WM and then pipetted into a drop of 2.5% glytaraldehyde in 0.1M Na

cacodylate (pH 7.2) with 0.01M Ca^{++} and Mg^{++} added. The eggs were allowed to fix for 3 hours at room temperature, rinsed twice in Na cacodylate buffer, and transferred to either coverslip or a small piece of aluminum foil soaked overnight in poly-L-lysine (1 ml/ml) the night before they were used. Eggs were rinsed overnight in a drop of Na Cacodylate buffer at 4°C and then were dehydrated through a graded series of acetone (from 25% to 100%). The eggs were dried in a Ladd Critical Point Dryer and gold-coated in a Denton Vacuum Automatic Evaporator. The coverslips were examined using an ETEC Autoscan microscope. The aluminum foil pieces were cut into 3 mm squares and examined with a Hitachi H-600 electron microscope with a H-6010A scanning attachment.

III. RESULTS

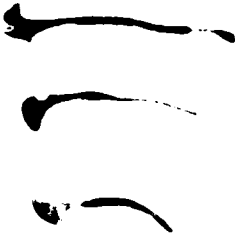
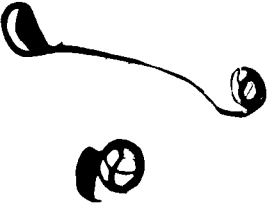
Categories of Spermatozoa

Sperm were classified into 10 categories according to their individual morphology (Figure 1). Type 1 sperm have an elongated post-acrosomal area. The heads of Type 2 sperm are also elongated but lack a rostrum. Type 3 sperm have broad post-acrosomal and rostral areas. Type 4 sperm heads are oval shaped. The heads of Type 5 sperm are amorphous in size and shape. Type 6 sperm have no nucleus but, instead, a cluster of mitochondria which appears to be an abnormal head. These sperm are similar to those described by Burkhart and Mallilng (1981). Sperm with severe tail abnormalities are categorized into 3 different classes; type 7, 8, and 9. Type 7 sperm have truncated tails with heads that are normal. Type 8 sperm have similarly truncated tails with abnormal heads. Type 9 sperm have highly coiled tails. Type 10 sperm have morphologically normal heads and tails.

Sperm Binding Assays

Sperm binding to the zona pellucida was assayed by thoroughly washing the ova, staining them with lacmoid stain and then observing them by phase contrast microscopy. To rule out the possibility of simple sperm adhesion, as opposed to true binding, two-cell embryos were used as controls. In only 2 out of 24 cases were sperm observed on two-cell embryos after washing. Scanning electron microscopy studies also demonstrated sperm binding to the

Figure 1. Classes of Spermatozoa from the Cauda Epididymes and Vasa
Deferens of Mice

1 ELONGATED POST-ACROSOMAL REGION		8 SHORTENED TAIL WITH ABNORMAL HEAD	
2 ELONGATED HEAD WITHOUT A ROSTRUM		9 HIGHLY LOOPED TAIL	
3 BROAD POST- ACROSOMAL AND ROSTRAL REGIONS			
4 OVAL HEAD			
5 AMORPHOUS		10 NORMAL	
6 MITOCHONDRIAL CLUSTER			
7 SHORTENED TAIL WITH NORMAL HEAD			

zona pellucida. It was assumed, therefore, that the sperm found on the ova were actually bound to the zona pellucida and not merely attached.

The percentages of abnormal sperm bound to the zona pellucida were compared to the percentages of abnormal sperm in the inseminates from HA/ICR, B6CBF₁, and PL/J mice. HA/ICR and B6CBF₁ mice had low percentages of abnormal sperm both in the inseminates and bound to the zona pellucida. The mean percentage of abnormal sperm in the inseminates of HA/ICR mice was 9.26 while the mean percentage of abnormal sperm bound to the ova was 3.68. The B6CBF₁ mice had 4.34 percent abnormal sperm in the inseminates but only 0.80 percent bound to the zona pellucida. PL/J mice had higher percentages of abnormal sperm. The mean percentage of abnormal sperm in these mice in the inseminates was 39.19 while 15.35 percent of the sperm bound to the zona pellucida were abnormal. The p values, determined by a Student's t Test, were 0.0001 in all cases (Table I). This indicates that the percentages of abnormal sperm bound to the zona pellucida were significantly lower than the percentages in the inseminates. Using a Pearson's Test of Correlation it was found that the number of abnormal sperm binding to the zona pellucida was not a function of time, i.e. abnormal sperm binding did not increase over time (Table II).

The difference between sperm from HA/ICR, B6CBF₁, and PL/J mice binding to the zona pellucida was studied as well. HA/ICR mice exhibited the lowest rate of sperm binding to the zona; only 59.8% of the ova scored contained bound sperm (Table III). The percentages of ova with bound sperm were much higher using B6CBF₁ and PL/J sperm. Sperm bound to 76.3% of

Table I. A Comparison of Percentages of Abnormal Sperm in the Inseminates and Bound to the Ova in Different Strains of Mice

Type of Mouse	Mean Percentages of Abnormal Sperm in the Inseminates	Mean Percentages of Abnormal Sperm Bound to the Ova	Student's t Test		
			t value	d.f.	Significance
HA/ICR	9.2577	3.6769	8.30	51	p 0.0001
B6:BF ₁	4.3375	0.8042	7.59	23	p 0.0001
PL/J	39.1935	15.3484	6.66	30	p 0.0001

Table II. A Comparison of Abnormal Sperm Binding and Hours Post HCG in Different Strains of Mice

Type of Mouse	Mean Percentage of Abnormal Sperm Bound to the ova at Hours Post HCG							Pearson's Test of Correlation		
	16	17	18	19	20	21	22	R	r ²	Significance
HA/ICR	6.67	1.45	3.03	4.03	7.58	6.63	7.69	0.125	0.016	p 0.2
B6:BF ₁	1.19	6.00	1.16	2.33	0.00	-	-	0.014	0.0002	p 0.5
PL/J	12.12	16.13	5.88	20.51	37.68	0.00	24.00	0.275	0.076	p 0.1

Table III. A Comparison of Sperm Binding in Different Strains of Mice

Males	Total Number of Ova Scored	Total Number of Ova with Sperm Bound	Percentages of Ova with Sperm Bound
HA/ICR	600	369	59.83%
B6CBF ₁	135	103	76.30%
PL/J	140	101	72.14%

the ova scored in experiments using B6CBF₁ mice (Table III). In similar experiments using PL/J mice, 72.1% of the ova scored had sperm bound (Table III).

Sperm bound to the ova were categorized by morphological type and compared by percentage to the sperm types found in the inseminates using a Student's *t* Test. In all mouse strains used it was found that the percentages of sperm types 1, 2, 5, 6, 9, and 10 bound to the ova were significantly different from those same types in the inseminates (Table IV: Figures 2-5). All of those, with the exception of Type 10 which bound in significantly higher percentages, were demonstrated to bind to the zona pellucida in significantly lower percentages than found in the inseminates (Table IV). No significant difference was found between the percentages of abnormal sperm Type 3 in the inseminates and bound to the zona pellucida (Table IV: Figures 3, 4, 6-8). The small percentages of sperm types 1, 4, 7, and 8 in the inseminates do not, however allow for a meaningful statistical analysis. For this reason,

Table IV. A Comparison of Different Types of Sperm in the Inseminates and Bound to the Zona Pellucida^a

Sperm Type	Sperm in Inseminates		Sperm Bound to Ova		Student's t Test	
	Total Number	Mean Percentage	Total Number	Mean Percentage	t value	Significance
1	46	0.163	0	0.0000	3.97	p < 0.001
2	787	2.814	20	1.165	3.58	p < 0.001
3	710	2.621	51	4.770	1.19	N.S.
4	39	0.081	2	0.041	-0.33	N.S.
5	425	1.517	7	0.694	2.29	p < 0.05
6	3246	11.593	60	4.133	2.90	p < 0.01
7	3	0.011	0	0.000	1.00	N.S.
8	2	0.007	0	0.000	1.00	N.S.
9	243	0.868	2	0.069	4.95	p < 0.0001
10	21849	80.489	1079	88.977	-3.46	p < 0.01

^aThe degree of freedom is equal to 27.

Figure 2. Photograph of a Type 6 Sperm from a HA/ICR Mouse Bound to the Zona Pellucida. The arrow indicates a type 6 sperm; all other sperm are morphologically normal. x 1024.

Figure 3. Photograph of 2 Type 6 Sperm from a PL/J Mouse Bound to the Zona Pellucida. The arrows indicate the type 6 sperm. The other sperm is morphologically normal. x 800.

Figure 4. Photograph of a type 6 sperm from a PL/J Mouse Bound to the Zona Pellucida. The arrow indicates a type 6 sperm; the asterisk indicates a type 3 sperm. x 1024.

Figure 5. Photograph of Type 2 and a Type 3 Sperm from a HA/ICR Mouse Bound to the Zona Pellucida. The arrow indicates a type 3 sperm; the asterisk indicates a type 2 sperm. All other sperm are morphologically normal. x 1024.

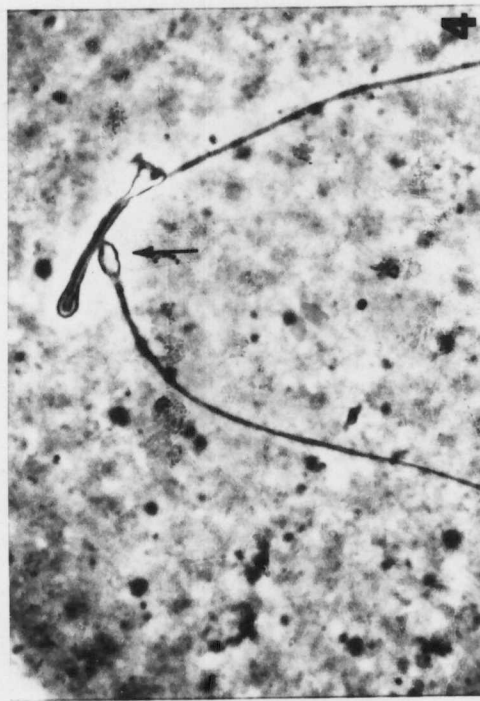
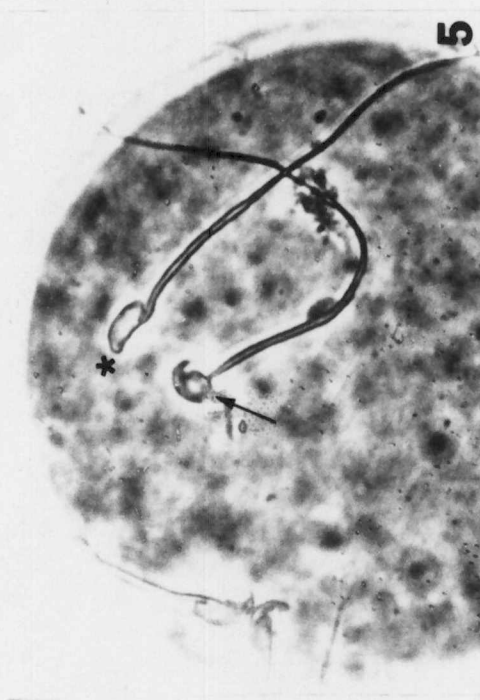
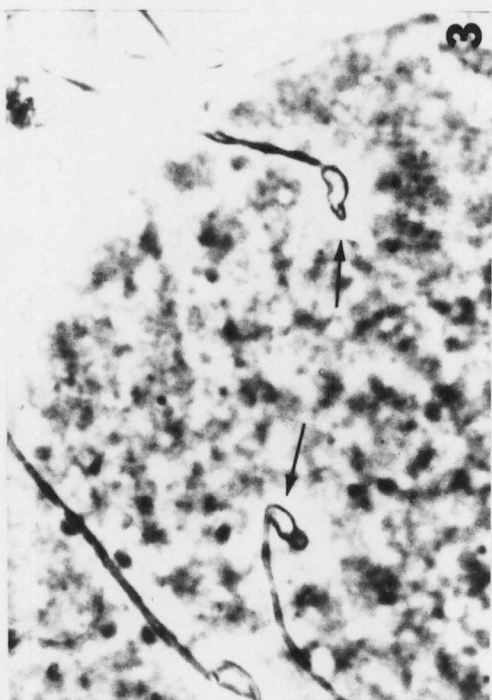
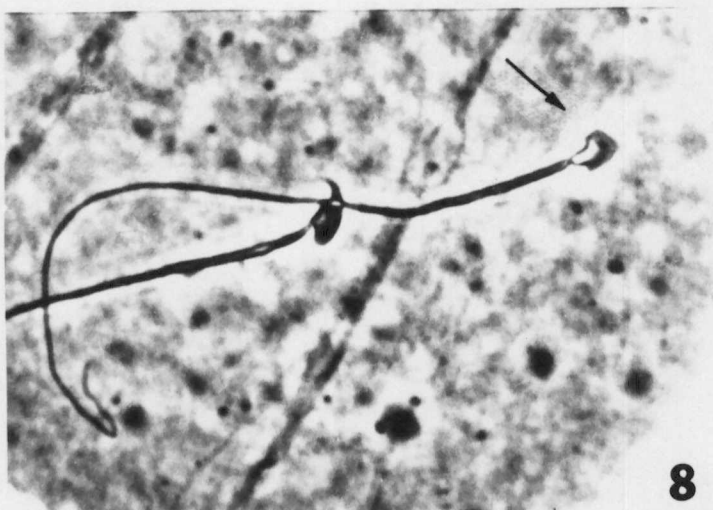
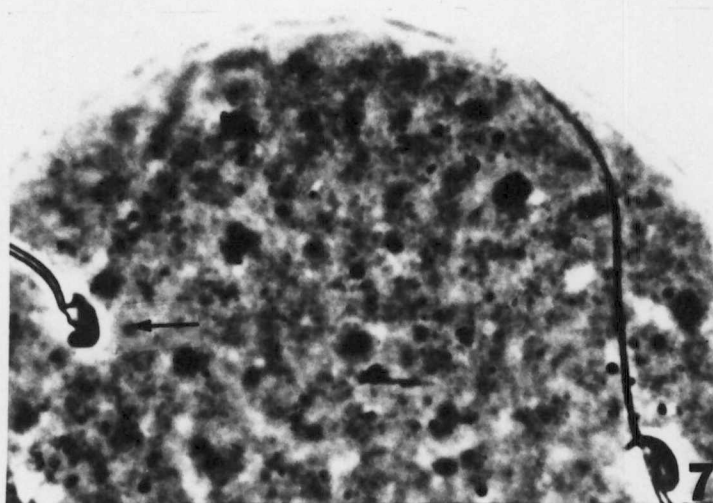
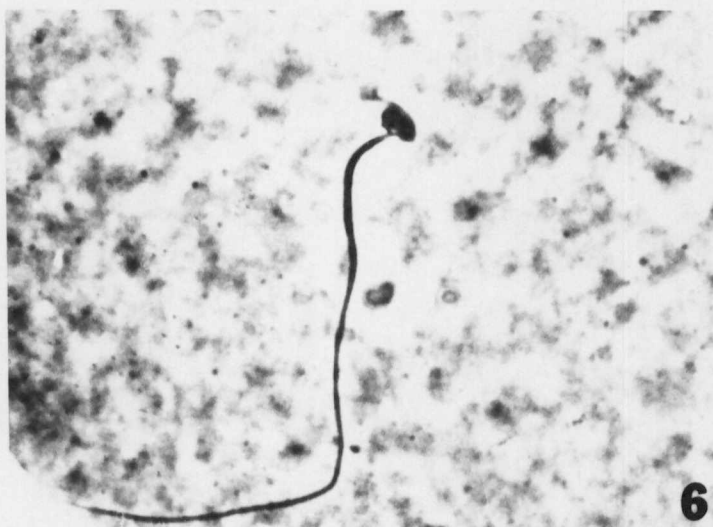


Figure 6. Photograph of a Type 3 Sperm from a HA/ICR Mouse Bound to the Zona Pellucida. x 800.

Figure 7. Photograph of a Type 3 Sperm from a PL/J Mouse Bound to the Zona Pellucida. The arrow indicates a type 3 Sperm; all other sperm are morphologically normal. x 800.

Figure 8. Photograph of a Type 3 sperm and a Type 10 Sperm from a PL/J Mouse Bound to the Zona Pellucida. The arrow indicates a type 3 sperm; all others are morphologically normal, type 10 sperem. x 1024.



the results for these groups will not be considered. Sperm types 2, 3, 5, 6, 9, and 10 were present in greater numbers in the inseminates and, therefore, lend themselves to a more reliable interpretation of the results. Similar results were found in the comparison of morphological types of sperm bound to the zona pellucida and in the inseminates from the individual strains (Table V, VI, and VII).

Electron Microscopy Studies

A total of 38 sperm from PL/J mice bound to the zona pellucida were studied by scanning electron microscopy: sixteen were normal and 22 abnormal. Various patterns of association of the sperm with the zona pellucida were observed. Three positions of the sperm on the zona pellucida were detected. Some sperm were found to lie flat on the surface of the ova so that the rostrum and the post-acrosomal area of the head and the tail were in a plane parallel to the zona pellucida. Other sperm made contact with the zona pellucida in a plane perpendicular to the ova. These sperm could further be divided into 2 groups: those sperm that contacted the zona with only the tip of the rostrum and those that laid with the convex curve of the head on the zona. Bound sperm also differed in the structure of the acrosomal membrane. In some cases the surface of the acrosomal membrane was smooth. In others the acrosomal membrane was vesiculated.

A final observation was made with respect to the degree of association of the sperm with the zona pellucida. Sperm were observed to be partially embedded in the zona, held down to the egg surface by what appears to be strands of material emanating from the zona pellucida, or loosely associated

Table V. A Comparison of Sperm from HA/ICR Mice in the Inseminates and Bound to the Zona Pellucida^a

Sperm Type	Sperm in Inseminates		Sperm Bound to Ova		Student's t Test	
	Total Number	Mean Percentage	Total Number	Mean Percentage	t value	Significance
1	34	0.243	0	0.000	3.59	$p \leq 0.005$
2	461	3.300	14	1.277	3.78	$p \leq 0.005$
3	492	3.514	30	3.6546	-0.22	N.S.
4	14	0.100	1	0.081	0.22	N.S.
5	275	1.964	4	0.422	5.79	$p \leq 0.001$
6	68	0.486	2	0.113	1.67	N.S.
7	0	0.000	0	0.000	0.00	N.S.
8	2	0.014	0	0.000	1.00	N.S.
9	133	0.950	2	0.139	2.88	$p \leq 0.05$
10	12558	89.707	948	94.461	-4.61	$p \leq 0.001$

^aThe degree of freedom is equal to 13. Three hundred sixty nine ova were used in this analysis.

Table VI. A Comparison of Sperm from B6CBF₁ Mice in the Inseminates and Bound to the Zona Pellucida^a

Sperm Type	Sperm in Inseminates		Sperm Bound to Ova		Student's t Test	
	Total Number	Mean Percentage	Total Number	Mean Percentage	t Value	Significance
1	0	0.000	0	0.000	0.00	N.S.
2	91	2.275	1	0.263	5.60	p ≤ 0.05
3	10	0.250	1	0.568	-0.81	N.S.
4	1	0.025	1	0.000	1.00	N.S.
5	19	0.475	0	0.000	1.73	N.S.
6	19	0.475	2	0.798	-0.67	N.S.
7	3	0.075	0	0.000	1.00	N.S.
8	0	0.000	0	0.000	0.00	N.S.
9	30	0.750	0	0.000	4.82	p ≤ 0.05
10	3827	95.675	298	98.370	-4.82	p ≤ 0.05

^aThe degree of freedom is equal to 0.3. One hundred three ova were used in this analysis.

Table VII. A Comparison of Sperm from PL/J in the Inseminates and Bound to the Zona Pellucida^a

Sperm Type	Sperm in Inseminates		Sperm Bound to Ova		Student's t Test	
	Total Number	Mean Percentage	Total Number	Mean Percentage	t Value	Significance
1	12	0.097	0	0.000	1.89	N.S.
2	235	2.289	5	1.511	0.66	N.S.
3	208	2.311	20	3.347	-0.57	N.S.
4	24	0.087	0	0.000	1.55	N.S.
5	131	1.320	3	1.503	-0.17	N.S.
6	3159	32.433	56	12.319	3.54	$p \leq 0.01$
7	0	0.000	0	0.000	0.00	N.S.
8	0	0.000	0	0.000	0.00	N.S.
9	80	0.833	0	0.000	3.24	$p \leq 0.05$
10	5464	60.711	320	60.602	-3.68	$p \leq 0.01$

^aThe degree of freedom is equal to 8. One hundred one ova were used in this analysis.

with the zona pellucida. Sperm embedded in the zona also were observed to be secured by strands from the zona pellucida.

The majority of the normal sperm examined were found to be in a perpendicular plane to the ova: Six were bound only at the tip of the rostrum and 6 were bound at the convex curve of the sperm head (Table VIII: Figures 9 and 10). Only a small number of the normal sperm were found to bind in a plane parallel to the egg surface. Most of these sperm were found to have a relatively smooth acrosomal membrane and loose associations with the zona pellucida (Table VII: Figure 11).

The sperm that were bound in plane perpendicular to and attached at the rostral tip of the zona pellucida were found to be secured to the egg by strands emanating from the zona pellucida. Also these sperm had vesiculated acrosomal membranes (Table VIII: Figure 9). Sperm bound in a perpendicular plane to the zona and having vesiculated acrosomal membranes were shown to be secured by strands in over half of the cases when they were bound to the zona pellucida at the convex curve of the sperm head (Table VIII: Figure 9). Abnormal sperm were classified into groups and analyzed. Four classes of abnormal sperm were observed bound to the ova: types 2, 3, 5, and 6. Type 2 sperm were all bound in a plane perpendicular to the plane of the zona pellucida, had vesiculated acrosomal membranes, and were partially embedded in the zona pellucida, with or without strands from the zona (Table IX: Figures 12 and 13). Six out of seven Type 3 sperm were observed to have vesiculated acrosomal membranes: one half were parallel to the zona pellucida and one half were bound in a plane perpendicular to the plane of the zona pellucida (Table IX: Figure 14). Four out of seven of the Type 3 sperm with

Table VIII. An Analysis of Normal Sperm from PL/J Mice Bound to the Zona Pellucida Using Scanning Electron Microscopy

Vesiculated Acrosomal Membrane				
Position of the Sperm on the Zona Pellucida	Smooth Acrosomal Membrane and Loose Attachment of Sperm on the Zona Pellucida	Sperm Secured to the Ova by Strands from the Zona Pellucida	Sperm Partially Embedded in the Zona Pellucida	Sperm Secured by Strands and Embedded in the Zona Pellucida
Sperm Bound Parallel to the Zona Pellucida Surface n=4	3	1	--	
Sperm Bound Perpendicular to the Zona Pellucida by the Tip of the Rostrum n=6	-	4	2	-
Sperm Bound Perpendicular to the Zona Pellucida at the Convex Curve of the Sperm Head n=6	-	3	2	1

Figure 9. Electron Micrograph of a Morphologically Normal PL/J Sperm Bound at the Rostrum to the Zona Pellucida. Note that the sperm is bound in a plane perpendicular to the ova and has a vesiculated acrosomal cap with strands emanating from the zona onto the rostral tip (arrow). Bar = $.5\ \mu$.

Figure 10. Electron Micrograph of a Morphologically Normal PL/J Sperm Bound at the Convex Curve of the Sperm Head to the Zona Pellucida. Note that the sperm is bound in a plane perpendicular to the ova and has vesicles at the border of the acrosomal cape. Bar = $.5\ \mu$.

Figure 11. Electron Micrograph of a Morphologically Normal PL/J Sperm Loosely Bound to the Zona Pellucida. Note the smooth sperm surface. Bar = $.5\ \mu$.

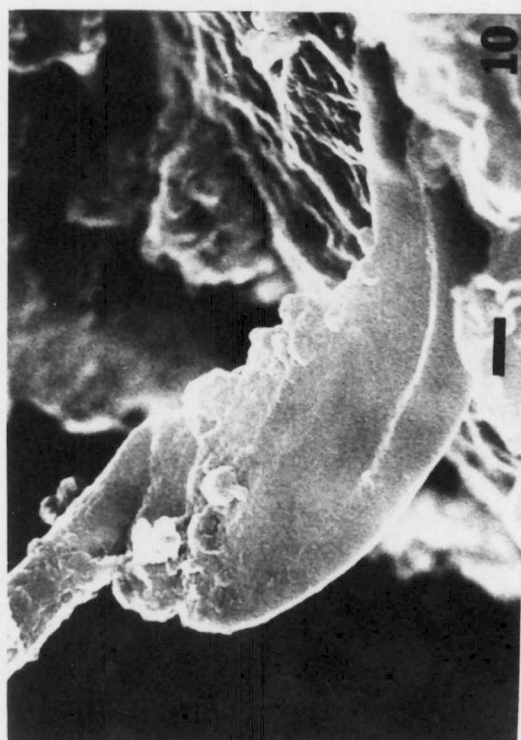
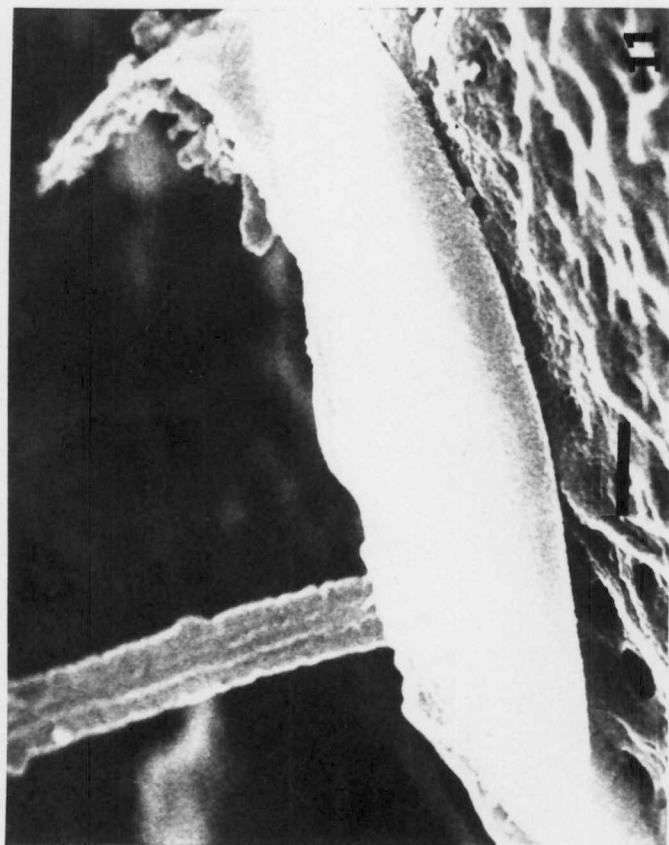


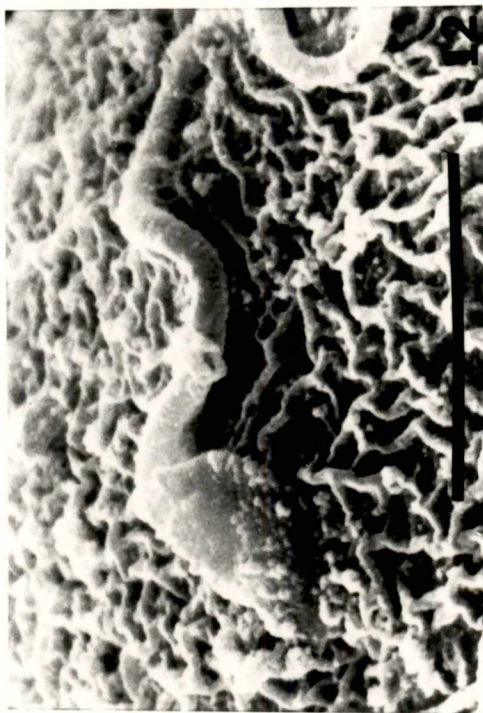
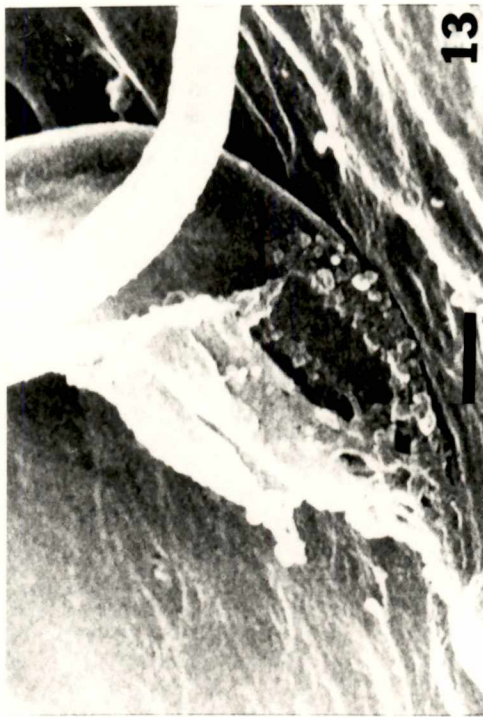
Table IX. An Analysis of Abnormal Sperm from PL/J Mice Bound to the Zona Pellucida Using Scanning Electron Microscopy

Sperm Type	Position of Sperm on the Zona Pellucida	Vesiculated Acrosomal Membrane			
		Smooth Acrosomal Membrane and a Loose Attachment to the Zona Pellucida	Sperm Secured to the Ova by Strands from the Zona Pellucida	Sperm Partially Embedded in the Zona Pellucida	Sperm Secured by Strands and Embedded in the Zona Pellucida
Type 2 n=3	Sperm Bound Perpendicular to the Zona Pellucida at the Convex Curve of the Head	-	-	2	1
Type 3 n=7	Sperm Bound Parallel to the Zona Pellucida	1	1	2	-
	Sperm Bound Perpendicular to the Zona Pellucida at the Convex Curve of the Head	-	3	-	-
Type 5	Sperm Bound Parallel to the Zona Pellucida	2	-	-	-
Type 6	Sperm Bound Parallel to the Zona Pellucida	10	-	-	-

Figure 12. Electron Micrograph of a Type 3 PL/J Sperm Bound to the Zona Pellucida at the Convex Curve of the Sperm Head. Note the Vesiculated Membrane. Bar = 5 μ .

Figure 13. Electron Micrograph of a Type 2 PL/J Sperm Bound to the Zona Pellucida at the Convex Curve of the Sperm Head. Note that the sperm is bound in a plane perpendicular to the ova and that the acrosomal membrane is vesiculated. Bar = 5 μ .

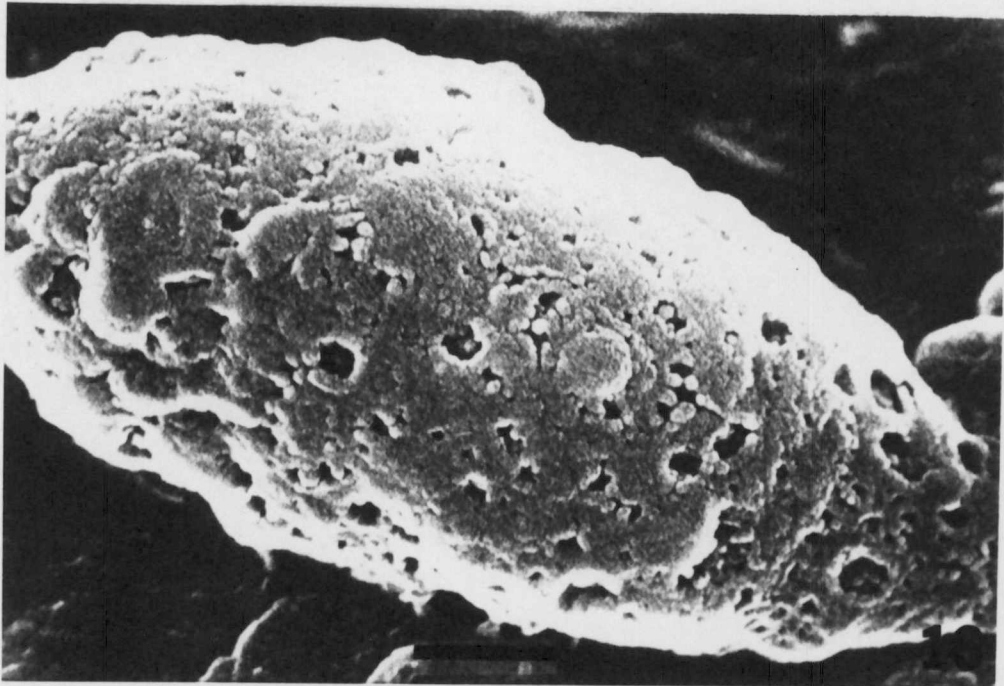
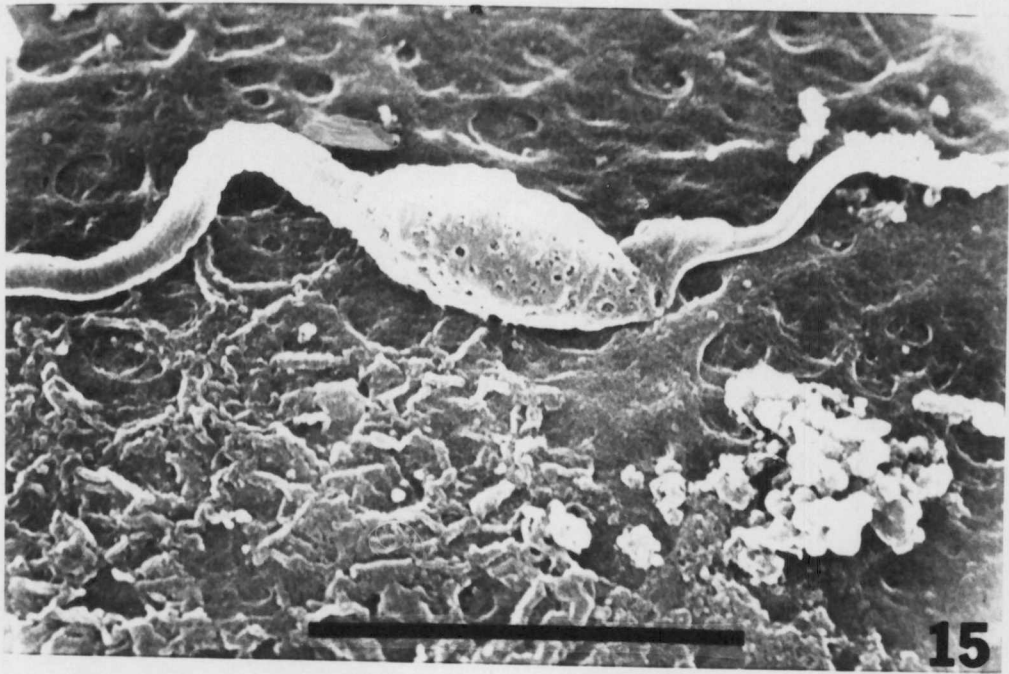
Figure 14. Electron Micrograph of the Vesiculated Membrane of a Type 2 PL/J Sperm. This is the same sperm as in Figure 12. Note the vesicles on the acrosomal membrane. (a). The zona pellucida (z) is at the bottom of the picture. Bar = .5 μ .



vesiculated acrosomal membranes were scored secured to the ova by strands emanating from the zona pellucida (Table IX). Type 5 and Type 6 sperm were all found to bind in a plane parallel to the zona pellucida. They were loosely attached to the zona pellucida and had smooth acrosomal membranes (Table IX: Figures 15 and 16).

Figure 15. Electron Micrograph of a Type 6 PL/J Sperm Bound to the Zona Pellucida. Note that the binding is loose and no vesicles are seen on the acrosomal membrane. Bar = 5 μ .

Figure 16. Electron Micrograph of a Type 6 PL/J Sperm Bound Loosely to the Zona Pellucida. Note that there are no vesicles on the acrosomal membrane. Bar = .5 μ .



IV. DISCUSSION

An Overview

The purpose of this investigation was to determine if structurally abnormal spermatozoa from different strains of mice are capable of binding to the zona pellucida of ova in significant numbers using techniques of fertilization in vitro. It was hoped that such an investigation also would help to clarify the structural associations between sperm and egg during fertilization.

The percentages of abnormal sperm bound to the zona pellucida were shown to be significantly lower than the percentages of abnormal sperm in the inseminates using a Student's *t* Test. In addition, the percentages of abnormal sperm bound to the ova did not increase significantly with increased time of incubation as demonstrated by a Pearson's Test of Correlation. When the types of abnormal sperm bound to the ova and in the inseminates were compared, it was found that the sperm with broad acrosomal areas in the inseminates did not differ significantly from that type bound to the ova. All other abnormal classes that were discussed showed a significantly lower binding frequency.

The scanning electron microscopy studies demonstrated that bound sperm lie in different orientations to the zona pellucida, exhibit different conditions of the acrosomal membrane, and show differences in the degree of adhesion. The majority of the normal sperm were bound in a plane perpendicular to the zona pellucida, had vesiculated acrosomal membranes, and were secured to the ova by strands emanating from the zona. The orientation, condition

of the acrosomal membrane and the degree of adhesion varied according to the morphology of the abnormal sperm. Generally, Type 2 and Type 3 sperm bound in a plane perpendicular to the ova, exhibited vesiculated acrosomal membranes, and adhered tightly to the zona pellucida. Type 5 and Type 6 sperm which have severely misshapen heads or no nucleus bind in a plane parallel to the zona and are loosely attached.

Krzanowska and Lorenc (1983) have studied the ability of morphologically abnormal sperm to penetrate the various investments surrounding the egg. By comparing the percentages of abnormal sperm surrounding the egg to the percentages of abnormal sperm penetrating either the cumulus oophorus, zona pellucida, or plasma membrane, they were able to demonstrate that the percentages of abnormal sperm surrounding the egg were significantly different from the corresponding percentages of abnormal sperm penetrating the investments of the egg. This suggested that morphologically abnormal sperm are capable of participating in fertilization in vitro but are less efficient than normal sperm in the penetration of the egg investments. Additionally, the authors concluded that the investments surrounding the egg do serve as a partially effective barrier to abnormal sperm in fertilization in vitro.

Krzanowska and Lorenc (1983) also have demonstrated in zona-free and cumulus-free ova that severely misshapened sperm heads, classified by them as Class 4 (Krzanowska, 1976), penetrate the zona at significantly lower percentages than those surrounding the ova. Abnormal sperm classes 3a and 3b, which have a flattened appearance and correspond to the Class 3 abnormal sperm described in the present investigation, were found to penetrate the ova at percentages similar to those same classes surrounding the ova.

The present investigation supports the findings of Krzanowska and Lorenc (1983) in that the percentages of abnormal sperm bound to the zona pellucida were significantly lower than the corresponding percentages of abnormal sperm in the inseminates. Additionally, these data form a basis for the suggestion that abnormal spermatozoa are less efficient in binding to the zona pellucida and thus are less able to participate in fertilization in vitro.

The present investigation does, however, differ from that of Krzanowska and Lorenc (1983) in several ways. Different statistical analyses were performed. Krzanowska and Lorenc (1983) used a Chi-squared Test of Independence which was adjusted for a large sample size. Several issues may be raised about the inappropriateness of this test with these data. First, the percentages of abnormal sperm surrounding the egg served as the expected value. It is, however, questionable whether this can legitimately be used since the number of abnormal sperm in the inseminate of any given mouse differs, as does the actual number of sperm used to inseminate the egg. From experiment to experiment, then, the "expected" values may differ significantly and may not represent the theoretical value. Furthermore, Snedecor (1956) states that percentages can be used with Chi-squared Test only if the sample size is actually 100 or if some other method is employed that would give weight to the original sample size. Krzanowska and Lorenc (1983) attempted to fulfill this criterion but were able to do so in only 2 of 6 cases presented. Their total numbers of sperm scored ranged from a low of 73 to a high of 124.

In the present study, a Student's *t* Test was used to analyze the data rather than a Chi-squared Test. This seemed a more appropriate test for

the analysis of the difference between the percentages of abnormal sperm bound to the zona pellucida and the percentages of abnormal sperm in the inseminates. The Student's *t* Test is routinely used to determine if 2 means are significantly higher or lower than each other. In addition, the Student's *t* Test is relatively unaffected by 2 common violations; sampling from non-normal distributions and from different populations having unequal variations (Liebermann, 1971). The Student's *t* Test, then, seems to better accommodate the use of percentages and give more reliable interpretation of the results.

The two studies also differ in the various lines and strains of mice used. Krzanowska and Lorenc (1983) used sperm from the C57Bl/Kw inbred strain. This strain is characterized by about a 30% level of morphologically abnormal sperm present within the inseminates and by low percentages of penetration in cumulus free eggs (15%; Krzanowska and Lorenc, 1983). In the present study sperm from random-bred HA/ICR mice, hybrid B6CBF₁ mice, and inbred PL/J mice were used. HA/ICR and B6CBF₁ mice exhibit low percentages of abnormal sperm, 9.3% and 4.3%, respectively, and bind to the zona pellucida in vitro 44 to 65% higher than sperm from C57Bl/Kw strain (HA/ICR were 44% higher and B6CBF₁ were 65% higher) (Table III, page 15). The PL/J strain was used because they are characterized by high percentages of abnormal sperm (39.2%) and also bind in vitro at levels which are approximately 57% higher than penetration in the C57Bl/Kw strain (Table VII, page 24).

Capacitation

The process of capacitation and its role in the fertilization process has been debated for many years. Capacitation is generally considered to be the acquisition of binding and fertilizing capabilities by the sperm. Even so, our understanding of what exactly constitutes capacitation is not clear. Yanagamachi (1981), Fraser (1977, 1981), Olds-Clarke (1983), Tessler et al. (1981), and Bedford (1983) believe that capacitation not only preconditions the spermatozoa for penetration through the zona pellucida but involves two essential events as well: a hyperactivated modification of the flagellar beating and the ability to undergo the acrosome reaction. The hyperactivation described by these researchers involves an accelerated whiplash flagellar beating and may cause the initiation of the acrosome reaction and, in addition, may add needed thrust to propel the sperm through the semi-solid zona pellucida.

The acrosome reaction, however, can occur in vitro without prior capacitation. Guinea pig sperm have been shown to undergo an almost immediate acrosome reaction when exposed for 15 minutes to the calcium ionophore, A23187 (Green, 1978), or when placed on a slide and pressed under a glass coverslip (Barros et al., 1973). Spermatozoa can also undergo the acrosome reaction without the accompanying hyperactivated step if the zona pellucida is removed from the egg (Fraser, 1981). Finally, it is known that chickens (Howarth, 1970) and invertebrates, such as the sea urchin, do not require sperm capacitation to initiate the acrosome reaction. These data, then, lead one to question the necessity of the process of capacitation for the penetration of the zona pellucida.

Fraser (1983) found that mouse sperm require approximately 120 minutes to become fully capacitated. After that time, binding and penetration of the zona pellucida was rapid and synchronous. In the present study, binding to the zona pellucida was analyzed at 2 to 6 hours after insemination. Sperm were not preincubated. Therefore, if abnormal sperm require a longer incubation time than normal sperm, their binding would be expected to increase significantly with time after 2 hours. However, this was not the case. Abnormal sperm binding was not found to be a function of time, i.e. the number of abnormal sperm bound to the zona pellucida did not significantly increase with time. This seems to indicate that abnormal sperm capable of binding to the zona pellucida are able to undergo the process of capacitation at or near the rate of normal sperm or that their capacitation is not essential for binding.

Sperm Zona Binding Related to Morphology

Zamboni (1971) has shown that the post-acrosomal cap is the region where the sperm initially interacts with the egg. This seems to involve a loose attachment of the sperm and then irreversible binding to the zona pellucida (Bleil and Wassarman, 1983). Saling et al. (1979) found that this initial interaction required an intact but capacitated acrosomal membrane. Contrary to this Toshimori (1982) contended that it is the naked inner acrosomal membrane of the rostrum that first contacts the zona pellucida during penetration. In addition protease sensitive binding sites on the zona pellucida of the egg have been implicated in the recognition of the sperm (Hartmann and Gwatkin, 1971; Bleil and Wassarman, 1980, 1981, and 1983). These zona

pellucida surface receptors have been postulated to react or bind to some surface component of the sperm membrane.

Percentages of Type 3 sperm bound to ova were not found to differ significantly from percentages of Type 3 sperm in the inseminates in the present study. Similar findings, however, were also reported by Krzanowska and Lorenc (1983). From these data, it was concluded that Type 3 sperm have an advantage over other abnormal sperm types in binding to the zona pellucida. Type 3 sperm, because of their broad post-acrosomal and rostral areas, may have additional binding sites for the zona pellucida surface receptors to contact. This may account for increased ability to recognize the zona pellucida and bind in higher numbers than would otherwise be expected.

Sperm which are elongated or have narrower post-acrosomal caps than morphologically normal sperm, for example Type 1 or Type 2 sperm, would be expected to bind to the zona pellucida at significantly lower levels than in the inseminates. A significantly lower level of Type 2 sperm binding to the zona pellucida was found.

Yanagimachi (1981) and others, as discussed earlier, have described a hyperactive motility once the sperm is capacitated and within the oviduct that is required for penetration of the zona pellucida. They concluded that motility is an important factor in fertilization. Consequently, since Type 9 sperm have highly coiled tails and are relatively immotile, they may lack the necessary thrust needed for penetration of the zona pellucida. This may account for the rarity of Type 9 sperm bound to the zona pellucida. Type

10 sperm are normal, and as would be expected, bind at significantly higher percentages than they are represented in the inseminates.

Electron Microscopy Studies

Barros et al. (1984) have studied the penetration of the zona pellucida by golden hamster sperm. Using scanning and transmission electron microscopy, they were able to show that sperm bound to the zona pellucida had a corrugated appearance on the outer surface of the acrosome. This appearance was correlated to the presence of vesicles on the plasma and outer acrosomal membranes. To them, these vesicles represented a part of the acrosome reaction. Therefore, sperm having these vesicles were considered to have begun this process.

In the present scanning electron microscopy studies, both normal and abnormal sperm from PL/J mice were able to successfully bind to the zona pellucida in vitro. It seems that each sperm must contact the zona pellucida in a plane perpendicular, or nearly so, to the zona pellucida before it can securely bind and eventually penetrate the zona. Sperm that lie in a plane parallel to the zona pellucida do not seem to attach as tightly to the zona and can maintain only a loose association with it. Yanagimachi (1966), using the golden hamster, and Toshimori (1982), using the mouse, have proposed that sperm approach and bind to the zona pellucida at an oblique angle. These data are supported by the findings of this study.

In addition, it seems that sperm must be tightly attached to the ova; either by being partially embedded in the zona pellucida or by being secured by strands emanating from the zona pellucida. This must occur before the acrosome reaction can take place since membrane vesicles were found only

on sperm that are tightly associated with the zona pellucida. Sperm that are loosely attached do not exhibit vesiculated sperm surface membranes. These results correspond to the results reported by Saling et al. (1979) and Saling and Storey (1979). They proposed that sperm from mice first bind to the zona pellucida and then undergo the acrosome reaction.

Abnormal sperm were also observed using scanning electron microscopy. Type 2 and Type 3 sperm were able to bind tightly to the zona pellucida in a plane perpendicular to the ova. Vesiculated acrosomal membranes were also observed. Again, these data seem to indicate that binding is a prerequisite to the acrosomal reaction. Type 5 and Type 6 sperm were not found to bind tightly to the zona pellucida or to undergo the acrosome reaction. It is questionable, then, whether these types of sperm are capable of penetration of the zona pellucida and ultimately of completing the fertilization process.

Conclusion

From these studies it appears that abnormal sperm, while capable of binding to the zona pellucida, are less effective and may be incapable of progressing further. However, certain types of abnormal sperm, specifically Type 3 sperm, seem to be exceptionally well adapted to binding to the zona pellucida and beginning the process of penetration. These findings may be important considerations when using techniques of fertilization in vitro and embryo transfer in humans since abnormal sperm can successfully participate in fertilization in vitro. It would be useful to know if these sperm activate a normal developmental process.

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VITA

Mary E. Crecink was born in Oakland, California, on April 11, 1955. She attended elementary schools in Starkville, Mississippi and graduated from Starkville High School in May 1973. The following August she entered Mississippi University for Women, and in May 1977 she received a Bachelor of Science degree in Biology. After four years of teaching biology in Forest, Mississippi, she received a Master of Science Education in August 1982. In the winter of 1982 she accepted a teaching assistantship at The University of Tennessee, Knoxville, and began study toward a Master of Science degree with a major in Zoology. She received that degree in December 1984.

Ms. Crecink will remain at the University to begin study toward a Doctor of Philosophy degree with a major in Zoology.