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Early Contact Interactions between Mammalian Gametes *In Vitro*: Evidence That the Vitellus Influences Adherence between Sperm and Zona Pellucida

(fertilization/uncapacitated and capacitated sperm/hamster attachment/binding

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ABSTRACT **A study *in vitro* of interactions between gametes of the golden hamster showed that the spermatozoon associates with the zona pellucida, or outer coat of the ovum, by two successive steps termed *attachment* and *binding*. Attachment, apparently the first step in fertilization, is not species-specific; it is insensitive to temperature (2°) and is reversible. Binding, on the other hand, is species-specific, temperature-sensitive, and irreversible. Experiments with isolated zonae pellucidae indicated that the vitellus (cellular portion of the ovum) influences the interaction between the sperm and the zona pellucida by increasing the time required for the sperm to bind. This effect is exerted on the sperm while they are attached to the zona pellucida.**

The mammalian egg cell, or vitellus, is surrounded by a thick transparent envelope, the zona pellucida. This structure bears a species-specific receptor for the capacitated sperm (1). Sperm become capacitated, i.e., endowed with the ability to fertilize eggs, on contact with components of the female genital tract. Recently, capacitation was induced *in vitro* (2–4). By applying these techniques to *in vitro* fertilization of the hamster egg, we established recently that the receptor for capacitated sperm is extremely sensitive to proteolytic enzymes (1). Its destruction by a protease released from the cortical granules of the vitellus appears to be responsible for the zona reaction, a key reaction for blocking polyspermy (5). With hamster gametes *in vitro*, it was established that an uncapacitated sperm would adhere specifically to another site that was not sensitive to trypsin (1) on the zona pellucida of an egg of the same species.

We have now extended these studies, and showed that (*i*) the tight adherence of sperm to the zona pellucida is preceded by a rapid and reversible step that is not species-specific, (*ii*) capacitated sperms require a longer time to adhere to the egg than uncapacitated sperms, and (*iii*) the vitellus controls the time required for adherence through some, as yet undefined, factor acting on sperms during their attachment to the zona pellucida.

METHODS

Epididymal sperms from the golden hamster (*Mesocricetus auratus*) were collected (6) in medium 199 (7), modified to contain sodium pyruvate (30 μg/ml), crystalline bovine plasma albumin (3 mg/ml), and sodium bicarbonate (2 mg/ml). This medium was designated 199M2. Sperms were capacitated by addition of 20 μl of sperm suspension (2×10^7 motile sperm per ml) under mineral oil to equal volumes of medium 199M2 containing the contents of an oviduct (17 hr after human chorionic gonadotrophin). The culture was incubated at 37° for 5 hr. Ova introduced with the oviduct contents were removed and other ova (20–22 hr after human chorionic gonadotrophin), free of cumulus, were added to each drop of hamster sperm in medium 199M2. Cumulus was removed with hyaluronidase (450 units/ml in Dulbecco's phosphate-buffered saline pH 7.2 containing 1% polyvinylpyrrolidone). Zonae pellucidae were isolated, without damage to the vitellus, by forcing eggs through a micropipet with a 50-μm inner diameter. Experiments were conducted with replicate groups of 15–20 eggs or with zonae pellucidae.

RESULTS

Sperm attachment

Hamster ova (15 ova per drop) were placed in 40-μl drops of capacitated hamster sperm (2×10^7/ml) in medium 199M2. Observations, made within 15 sec, revealed that the eggs had become densely covered with sperm stuck to the zona pellucida by their heads (Fig. 1). All or most of these sperm were removed when the eggs were washed up to 25–30 min later. Washing is defined as the transfer of eggs and stuck sperm through three successive changes of medium 199M2 with a micropipette. We term this reversible union of egg and sperm *attachment*. After this period, sperm could not be removed by washing. We previously termed this irreversible union adherence but will now refer to it as *binding*.

When mouse or rat eggs were placed in 40-μl drops of capacitated hamster sperm in medium 199M2, or when hamster eggs were placed in drops of capacitated mouse sperm, similar numbers of sperm attached to the ova. However, washing, even after the gametes had been together for 90 min, resulted in complete removal of sperm, usually by the first transfer. Thus, heterologous sperm attach to ova but they do not bind. We also showed that attachment, but not binding, occurred when ova were previously treated with bovine pancreatic trypsin (0.1 μg/ml, Worthington Biochemicals Corp.) or when the gametes were combined at 1–2° (Table 1).

Effect of capacitation on binding

Capacitation affected profoundly the time required for sperm binding. Fig. 2 shows that uncapacitated sperm required about 3 min to bind to ova, but after their capacitation a much longer time was needed. Typically, binding began 30–35 min after combination of gametes and was complete by 40 min. Occasionally, binding began as early as 25 min and was complete at 30 min.

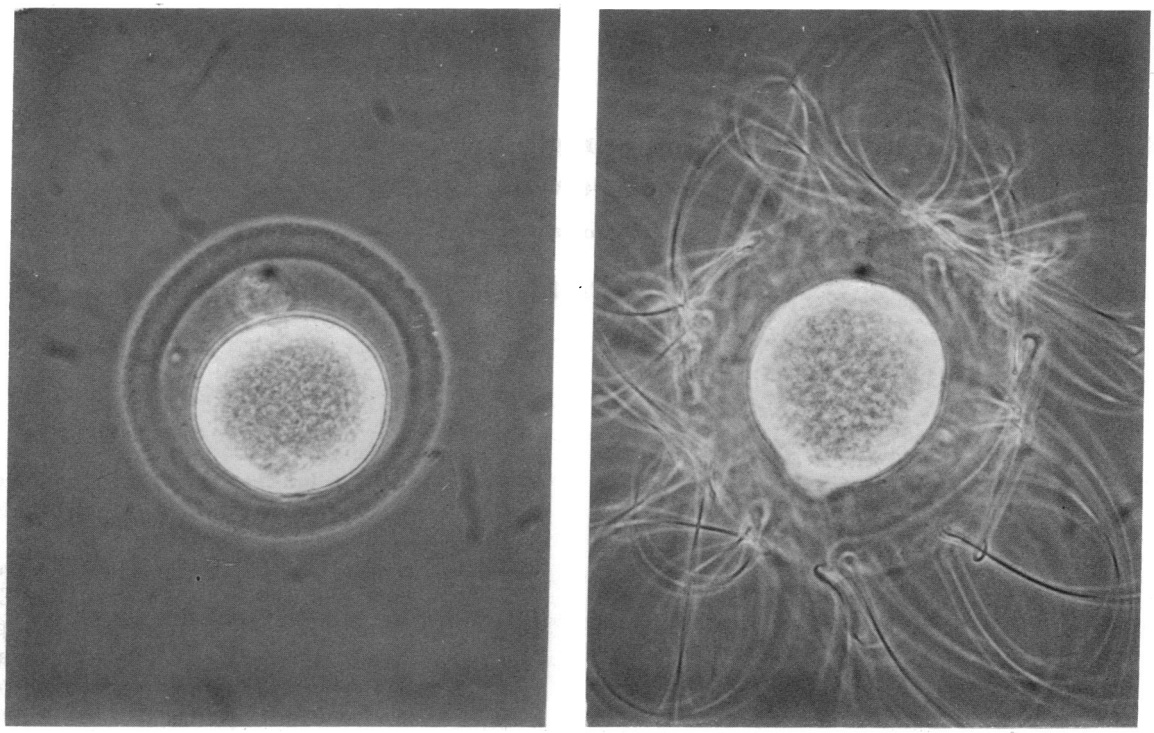

FIG. 1. Hamster eggs, exposed to capacitated sperm, then thoroughly washed 15 min later (*left*) and 40 min later (*right*) (see *Results*). Note that sperm are at first removed but later remain bound (×380).

Role of the vitellus

Comparative time-course studies were performed with intact eggs and isolated zonae pellucidae to determine whether the vitellus would influence the time required for binding of capacitated sperm to the zona pellucida. The results are shown in Fig. 3. Binding of sperm to intact ova began after 20 min and was complete by 30 min, but binding to isolated zonae pellucidae had already begun by less than 5 min and was complete by 10 min. This decrease in the time required for binding was unaffected by incubation of the eggs *in vitro* for 1 hr before isolation of the zonae pellucidae. Attachment, however, occurred almost immediately after the addition of either zonae pellucidae or whole eggs.

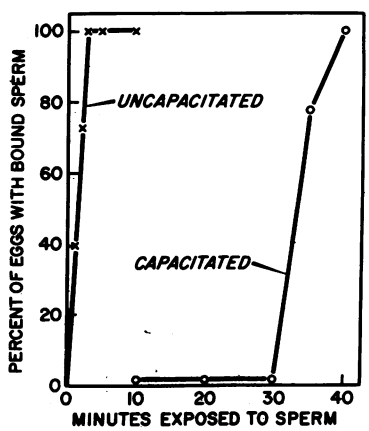

FIG. 2. Eggs were added to 40-μl drops containing uncapacitated or capacitated sperm (2×10^7/ml). After being left in the drop for the indicated period of time, the eggs were washed three times with medium 199M2, and sperm binding to the eggs was scored. Each drop contained 15 eggs.

Vitellus action is on attached sperm

To determine whether the vitellus was delaying sperm binding by acting on sperm that were attached to the zona pellucida, the following experiment was performed. Sperm and eggs were mixed, and after 25 min the eggs, with sperm attached, were pipetted vigorously into a second drop of medium 199M2, a procedure that resulted in the detachment of most of the sperm. The eggs were taken out, care being taken to remove as few sperm as possible, and a fresh group of eggs was then added to each drop. After 15 min, the eggs were washed and binding was scored. The results, presented in Table 2, show clearly that sperm that had attached previously to eggs bound to 67–80% of the fresh eggs within 15 min, a period that we have shown is too short for the binding of untreated sperm. Thus, during the first exposure, the vitellus must have

TABLE 1. *Surface interactions between eggs and capacitated sperm*

Gametes		Presence (+) or absence (−) of surface interactions	
Sperm	Eggs	Attachment	Binding
Hamster	Hamster	+	+
Hamster	Hamster (trypsin treated)	+	−
Hamster (2°)	Hamster (2°)	+	−
Hamster	Mouse	+	−
Hamster	Rat	+	−
Mouse	Hamster	+	−

TABLE 2. *Time required for sperm detached from eggs to bind to fresh eggs*

Type of sperm	Minutes of exposure to second group of eggs	No. of sperm per drop ($\times 10^{-5}$)	Percent of eggs with sperm bound	
			Exp. 1	Exp. 2
Detached from eggs after 25 min	15	<0.4	67	80
Unattached remainder* after 25 min	15	>3.6	0	0
	40	>3.6	100	100
Untreated	15	4.0	0	0
	25	4.0	0	0
	40	4.0	100	100

* See *text*.

exerted an effect on the attached sperm that allowed them to bind to the second group of eggs. Binding occurred despite the relatively low sperm concentration. However, sperm that had remained unattached to the original eggs and that, except for exposure to eggs, were treated like the detached sperm, although present in much greater concentration and capable of binding to eggs eventually (i.e., after 40 min), failed to bind to the fresh eggs in the 15-min period. From these experiments it is clear that capacitated sperm, under the influence of the vitellus, undergo a change during attachment to the zona pellucida of the egg that then enables them to pass into the binding phase.

DISCUSSION

These *in vitro* experiments suggest that mammalian capacitated sperm interact with eggs as follows: First, sperm attach loosely and nonspecifically, as to species, to the zona pellucida. After 30–40 min, sperm bind firmly, and this time species-specifically to the zona pellucida. Such a two-step reaction resembles the adsorption of bacteriophage to *Escherichia coli*, which proceeds by an initial temperature-insensitive, reversible step followed by a temperature-sensitive one (8, 9).

The second, or binding, phase of the interaction between mammalian sperm and egg was shown to be under vitelline control. This is the first demonstration in mammals of a vitelline effect on the behavior of the male gamete before its penetration of this outer egg envelope. It might be argued that the effect of the vitellus is an artifact resulting from damage to the eggs. If this were so, one would then expect that sperm binding to ova damaged by treatment with such agents as cyanide or dinitrophenol would also be delayed, which was not the case (unpublished observations). Furthermore, prior incubation of eggs in medium 199M2 for 1 hr before isolation of zonae pellucidae failed to affect the time required for the binding of sperm to egg. This shows that delayed binding is not the result of damage to the zona pellucida during incubation of the eggs *in vitro* before exposure to sperm.

The nature and origin of the control emanating from the vitellus are unknown. Presumably, such material is not released by rupture of the cortical granules, since this occurs apparently only after the sperm have penetrated the zona pellucida (10).

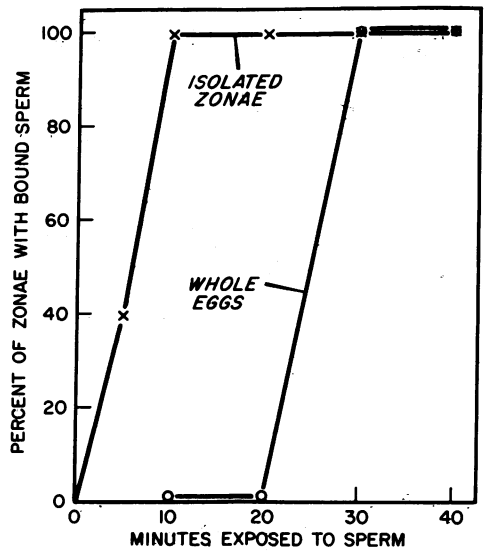

FIG. 3. Eggs or zonae pellucidae were added to 40-μl drops containing capacitated sperm (2×10^7/ml). After the indicated period of time, ova and zonae pellucidae were washed three times with medium 199M2 and sperm binding to the eggs was scored. Each drop contained 15 eggs or 15 zonae pellucidae.

It is apparent from these studies, as well as from those reported (1), that sperm do not require the presence of the vitellus in order to bind, since they bind to isolated zonae pellucidae. Such sperm are capacitated as shown by the fact that prior treatment with trypsin of isolated zonae pellucidae, as well as of whole eggs, blocks the binding of capacitated sperm (1). Binding of uncapacitated sperm, however, to isolated zonae pellucidae is unaffected by prior treatment with trypsin (unpublished observations). Whether binding to isolated zonae pellucidae and whole eggs involves similar-but-modified or different molecular groups remains to be determined.

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