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Movement Characteristics of Hamster and Guinea Pig Spermatozoa upon Attachment to the Zona Pellucida[1]

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ABSTRACT

Movement characteristics of hamster and guinea pig spermatozoa were studied by high-speed cinemicrography before and after initial binding to the zona pellucida. Hamster spermatozoa that had previously undergone motility activation in vivo or in vitro maintained vigorous behavior upon zona attachment. They continued to propagate flagellar waves of large amplitude and curvature, although these waves were much more symmetrical than when prior to zona attachment. The hamster sperm activated in vitro appeared slightly stimulated upon zona attachment. Such spermatozoa also bound to the zonae pellucidae of mouse eggs, and they exhibited similar movements. Preactivated hamster spermatozoa, fresh from the epididymis, did attach to the hamster zona, but exhibited relatively weak flagellar beats of low amplitude and frequency. Guinea pig spermatozoa, activated in vitro, also continued such movement and appeared somewhat stimulated upon attachment to the zona pellucida of their species.

INTRODUCTION

The penultimate event in the transport of spermatozoa from the male to the plasma membrane of the ovum in the female is the penetration through the zona pellucida. The mechanisms responsible for zona penetration are not yet fully understood. The role of acrosomal enzymes in this process has been a subject of interest and debate (Stambaugh, 1972, 1978; Hartree, 1971; Zaneveld, 1975; Bedford, 1974; Yanagimachi, 1981a). Regardless of the importance of such enzymatic activity in reducing the physical resistance of zona material to perforation by the spermatozoon, an active mechanism of forcing the sperm body through the zona must exist. The undulations of the sperm flagellum are believed to constitute this mechanism (Yanagimachi and Noda, 1970; Noda and Yanagimachi, 1976; Bedford, 1974; Yanagimachi, 1981a). However, relatively little is known about sperm motility during zona contact and penetration. A very few direct observations of sperm passage through the zona have been made, both visually (Pincus, 1930; Shettles, 1953; Yamagimachi, 1966) and using normal-speed cinemicrography (Yang et al., 1972; Sato and Blandau, 1979). These studies have subjectively characterized sperm flagellar movement as "vigorous," but have provided no other information on any details of that movement.

The forward thrust produced by a sperm flagellum depends upon the frequency and shape of its beats, the morphology of the sperm body, and the physical properties of the environment of the sperm, e.g., the viscosity of the environment. The nearby presence of external solid surfaces also significantly influences

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flagellar thrust (Katz and Blake, 1975; Katz et al., 1975). For spermatozoa swimming freely in fluid, symmetrical flagellar beats of moderate amplitude are the most efficient means of maximizing swimming velocity (Pironneau and Katz, 1974). It is generally believed that the spermatozoa of most mammals exhibit such movement during much of their journey from the site of insemination to the site of fertilization (Katz and Overstreet, 1980). However, the spermatozoa of a number of mammals exhibit a different form of motility during the final phases of transport prior to fertilization. Termed "activation" (Yanagimachi, 1970) or "hyperactivation" (Yanagimachi, 1981a), this movement is distinguished by episodes of whiplash-like flagellar bending that has large amplitude and curvature. These undulations are often asymmetrical, and free-swimming spermatozoa exhibiting them translate along very erratic trajectories (Katz and Overstreet, 1980). The functional significance of activated movement has not been established. It must be appreciated, however, that if a spermatozoon is physically constrained by the nearby presence of a solid surface or surfaces, then the hydrodynamically optimum beat pattern is not necessarily identical to that in free fluid. In a situation such as zona contact, in which the sperm head is anchored against a surface, large amplitude flagellar beats, rather than moderate amplitude ones, would maximize the thrust directed against the surface (Katz and Blake, 1975; Lighthill, 1976). Thus, if spermatozoa that have undergone activation retain the ability to propagate large amplitude beats during zona contact, then at least part of the biological significance of this phenomenon must be hydrodynamic. That is, it would be more efficient than preactivated movement in facilitating zona penetration.

The present study was designed to begin to elucidate the movement characteristics of spermatozoa during zona contact, and their role in the mechanics of zona penetration. The initial responses to the zona pellucida by spermatozoa that had achieved activation in vitro and in vivo, and also spermatozoa that had not yet undergone activation, were investigated. Sperm movement characteristics were recorded for analysis using high-speed cinemicrography.

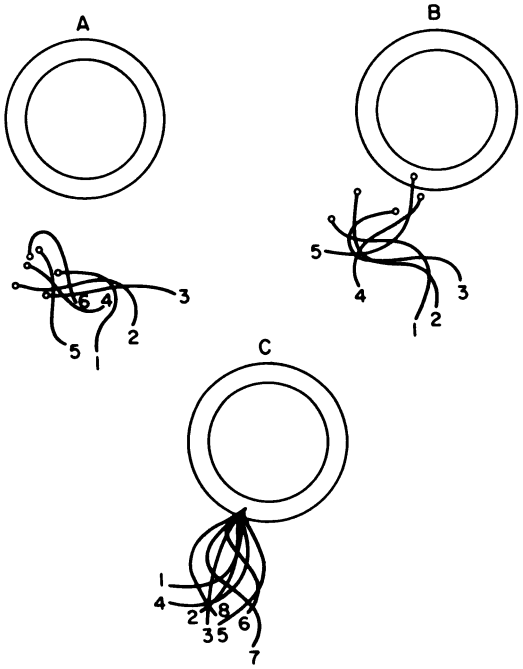

FIG. 1. Sequence of tracings from a high-speed cine film of an activated hamster spermatozoon immediately before (A), during (B), and after (C) attachment to the zona pellucida of a hamster egg. The tracings are numbered consecutively, and the interval between them is 0.02 sec. The head of the spermatozoon has been depicted as a small circle to clarify its position.

MATERIALS AND METHODS

Media Used

The medium mT-I (modified Tyrode I) was used for hamster gametes, and the medium mT-II for guinea pig gametes. The compositions of the media were as follows:

Medium mT-I. 124.80 mM NaCl, 2.68 mM KCl, 1.80 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.90 mM $NaHCO_3$, 4.50 mM D-glucose, 0.09 mM Na-pyruvate, 9.0 mM Na-lactate, 0.5 mM taurine, 0.05 mM L-epinephrine, 100 IU/ml penicillin G, and 15 mg/ml bovine serum albumin.

Medium mT-II. 108.76 mM NaCl, 2.70 mM KCl, 2.00 mM $CaCl_2$, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 5.56 mM D-glucose, 1.00 mM Na-pyruvate, 10.00 mM Na-lactate, 0.10 mM oxaloacetic acid, 100 IU/ml penicillin G, and 3 mg/ml bovine serum albumin.

Both mT-I and mT-II were handled under an atmosphere of pure air, and their pH values were 7.3–7.4 and 7.8–8.0, respectively.

Preparation of Spermatozoa

Golden hamster spermatozoa from the distal cauda epididymis were induced to undergo capacitation and acrosome reactions in vitro according to the procedure of Lui et al. (1979), with slight modifications by Yanagimachi (1981b). When incubated in the mT-I medium for 3.5 to 5 h at 37°C, the majority of

TABLE 1. Flagellar beat frequencies (Hz) of hamster and guinea pig spermatozoa before and immediately after binding to the zona pellucida of their respective species. Values are expressed as mean ± SEM. The symbols <, ≅ between adjacent values in rows and columns denote significantly less than ($P<10^{-4}$) and not significantly different from ($P>0.05$), respectively, where the order of meaning is left-to-right and top-to-bottom.

Species	Type of sperm	Free swimming		On zona pellucida
Hamster	Preactivated	6.13 ± 0.46	≅	6.02 ± 0.57
		≅		<
	Activated – in vitro	6.75 ± 0.15	<	8.12 ± 0.21
		<		≅
	Activated – in vivo	8.05 ± 0.24	≅	7.75 ± 0.37
Guinea pig	Activated – in vitro	9.48 ± 0.40	<	11.37 ± 0.89

spermatozoa were displaying "activated" motility, and 30–90% of these activated spermatozoa had no acrosomal caps (i.e., the acrosome reaction was completed). In some experiments, spermatozoa were incubated very briefly (less than 15 min) and were designated as "preactivated" spermatozoa. All these motile spermatozoa had intact acrosomes.

Hamster spermatozoa activated in vivo were collected as follows. Adult females in estrus were allowed to mate between 2100 and 2200 h of the day of estrus (i.e., Day 4 of the estrous cycle; Greenwald, 1961) and were sacrificed at ∿1000 h the next morning (i.e., Day 1 of pregnancy). The ampullary portion of the oviduct was dissected and flushed with 0.2–0.4 ml of mT-I medium. Five to 50 spermatozoa were generally collected from oviducts of one female. Some of these spermatozoa were motionless, but others were quite vigorous and were displaying "activated" motility (Katz and Yanagimachi, 1980). Since all the eggs in the ampullae were already fertilized and were in the pronuclear stage, these free spermatozoa were obviously "surplus" spermatozoa. Such surplus spermatozoa, however, have full capacity to fertilize eggs both in vitro and in vivo (Cummins and Yanagimachi, unpublished data).

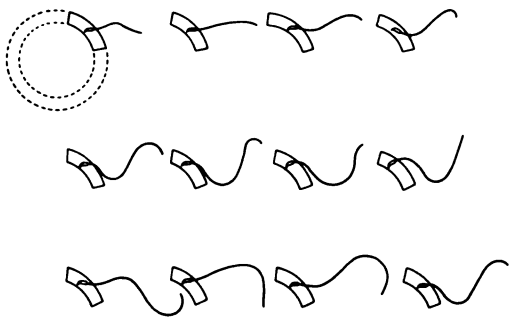

FIG. 2. Sequence of high-speed cinemicrographic tracings of an activated hamster spermatozoon immediately after binding to the zona pellucida of a hamster egg. The interval between tracings is 0.02 sec. This spermatozoon is not yet within the zona, but is positioned obliquely with respect to it.

Guinea pig spermatozoa were induced to undergo acrosome reactions according to the procedure of Fleming and Yanagimachi (1981). Briefly, spermatozoa from the distal cauda epididymis of a mature male were incubated for 1 h at 37°C in Ca^{2+}-free mT-II medium supplemented with 85 μg/ml lysophosphatidyl choline. An acute addition of Ca^{2+} (2 mM) to the medium induced synchronous acrosome reactions in the vast majority of spermatozoa within 10–15 min, and these spermatozoa displayed "activated" motility.

Preparation of Eggs

Superovulated eggs of the hamster and mouse were collected from oviducts 15–17 h after hCG injection (Nicholson et al., 1975). They were treated for 10–15 min with 0.1% bovine testicular hyaluronidase (300 USP units/mg; ICN Pharmaceutical Co., Cleveland, OH) in mT-I to remove cumulus cells, rinsed, and kept in fresh mT-I. Mature guinea pig eggs were obtained by incubating ovarian oocytes in vitro (Yanagimachi, 1978). The eggs were freed from cumulus cells by pipetting them in and out of a small-bore pipette in mT-II medium containing 0.2% hyaluronidase. They were then rinsed and kept in fresh mT-II.

Observations of Spermatozoa Approaching and Attached to Zona

An aliquot of the medium containing 4–8 eggs was placed on a slide with four pillars of a Vaseline-paraffin (10:1) mixture. After the eggs were clustered using a needle, a coverslip was placed on the pillars and compressed until it gently contacted and held the eggs (zonae) in place. The space between the slide and coverslip was filled with the minimum amount of the medium required (e.g., mT-I for the hamster). After three edges of the coverslip were sealed with a melted Vaseline-paraffin mixture, a bolus of sperm suspension was applied to the open end of the coverslip opposite that where the eggs were positioned. The open end of the coverslip was then sealed with liquid paraffin. This procedure allowed us to observe spermatozoa

during their migration toward the egg cluster, and usually it took a few minutes before the first spermatozoon reached the egg. The microscope stage was maintained at 37°C by a forced air curtain.

Since the number of surplus spermatozoa obtained from one animal is very small, the above procedure was impractical in most experiments involving such sperm. We therefore directly mixed eggs and the suspension of surplus spermatozoa and mounted them between a slide and coverslip. Sometimes spermatozoa were already on the zonae when we first examined the eggs, but we could regularly observe spermatozoa during their first approach and subsequent contact with zona surfaces.

Hamster spermatozoa were observed with dry phase contrast optics, using 6.3×, 10×, 16×, and 40× objective lenses. Guinea pig spermatozoa were observed using 16× and 40× dry phase-contrast objective lenses. Sperm movement was recorded on Kodak 4× negative film using a high-speed Redlake Locam cine camera. Framing rates of 50 and 100 frames per second were employed for hamster spermatozoa, and 100 frames per second for guinea pig spermatozoa. Four experiments each were performed in which the interaction with the hamster zona pellucida of preactivated, in vitro-activated, and in vivo-activated hamster spermatozoa was observed and recorded. In three experiments, the interaction of in vitro-activated hamster spermatozoa with the zona pellucida of the mouse was observed. Guinea pig spermatozoa were observed and recorded during interaction with conspecific zona pellucida in four experiments. In each experiment a sample of 5–10 free swimming spermatozoa was filmed in the immediate vicinity of the eggs. We were successful in "tracking" some individual spermatozoa both before, during, and after attachment to the zona. A sample of 5–10 additional spermatozoa was filmed within ∿30 sec of zona attachment, and these sperm were subsequently observed for up to 30 min. Cine films were analyzed frame-by-frame to measure sperm flagellar beat frequencies. Statistical comparisons of the data utilized Student's t test. Sequential tracings of beat shapes were also made in many cases.

RESULTS

The acrosomes of motile hamster and guinea pig spermatozoa are readily identifiable by direct visual observation. We found that spermatozoa activated in vitro and in vivo had either apparently "intact" acrosomes (the acrosome reaction had not started), swollen acrosomes (the reaction was in progress), or no acrosomal caps (the reaction was completed). All of these spermatozoa behaved in a similar manner, when they were freely swimming or when they initially made contact with the zona pelluicida. Both in vitro- and in vivo-activated spermatozoa exhibited the typical activated patterns of motility while swimming freely (Fig. 1A). The flagellar beat frequencies of in vivo-activated spermatozoa were, however, 19% higher on the average than those for the in vitro-activated spermatozoa (Table 1), and this difference was statistically significant ($P < 10^{-4}$). Upon attachment to the hamster zonae, both classes of spermatozoa continued to beat their flagella with waves of large amplitude and curvature (Figs. 1B,C, 2). In vitro-activated hamster spermatozoa did attach to the zona pellucida of the mouse, and upon attachment they also exhibited this beat pattern. Such beats tended to be symmetrical, compared with the irregular, asymmetrical bending prior to the attachment to the zona. The beat of the flagellum was nearly planar and was in the plane of the "hook" of the sperm head. The angle of attachment of the spermatozoa to the zona was such that this plane was nearly parallel to the local tangent plane of the surface. The initial orientation of the plane of beat on the zona was otherwise random. However, many spermatozoa would pivot and swivel such that the final orientation of this plane was nearly perpendicular to the plane of the slide and coverglass (Fig. 3B).

The beat frequencies of in vitro- and in vivo-activated spermatozoa were statistically indistinguishable after attachment of the sperm heads to the hamster zona, and equaled those values of the freely swimming in vivo-activated spermatozoa. In other words, the beat frequencies of in vivo-activated spermatozoa did not tend to change, whereas those of in vitro-activated spermatozoa increased by 20% after contact with the zona (Table 1). This latter difference was significant ($P < 10^{-6}$). Both classes of spermatozoa remained vigorously

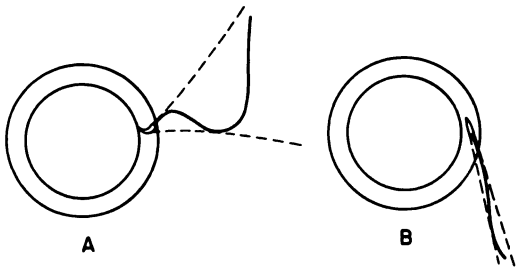

FIG. 3. Two different views of the inclination of activated hamster spermatozoa with respect to the zona pellucida of a hamster egg. In view A, the plane of the flagellar beat is parallel to the plane of microscopic focus. In view B, the plane of beat is almost perpendicular to the plane of focus. The broken lines depict the apparent envelope of the flagella beat.

motile on the zona during the subsequent 30 min of observation.

The behavior of preactivated spermatozoa fresh from the epididymis contrasted with that of the activated spermatozoa. The preactivated spermatozoa exhibited beats of low to moderate amplitude both before and after contact with the zona (Fig. 4A,B). The beats did appear more symmetrical after attachment to the zona. Flagellar beat frequencies did not change following the attachment (Table 1). Prior to attachment, the preactivated spermatozoa beat 31% more slowly than in vivo-activated spermatozoa ($P<10^{-4}$), and at frequencies not different from those in vitro-activated spermatozoa. Once attached to the zona, the preactivated spermatozoa beat 39% to 35% more slowly, on the average, than the in vivo- and in vitro-activated spermatozoa. Preactivated spermatozoa on the zona steadily reduced both beat frequencies and amplitudes during the subsequent 30 min of observation.

The in vitro-activated guinea pig spermatozoa behaved analogously to their counterparts in the hamster. That is, they exhibited typical activated motility while swimming freely, and upon attachment to the zona they continued to propagate large amplitude, now symmetrical flagellar waves. Following the attachment, their average beat frequencies were 20% higher on the average than while swimming freely, and this difference was significant ($P<10^{-4}$). These activated spermatozoa on the zona remained vigorously motile during the 30 min of observation.

DISCUSSION

The biological significance of activated sperm motility has not been established (Yanagimachi, 1981a). In the rabbit, activated movement is associated with the migration of spermatozoa beyond the lower isthmus of the oviduct during the periovulatory period (Cooper et al., 1979; Overstreet and Cooper, 1979). Since rabbit spermatozoa in the lower isthmus are quiescent but can be subsequently activated both in vivo and in vitro (Cooper et al., 1979; Overstreet et al., 1980), it is possible that the assumption of activated movement plays a mechanical and/or hydrodynamic role in the release of the spermatozoa from the lower isthmus and their subsequent transport to the site of fertilization. The increased bending ability of the flagellum during activation may aid a spermatozoon in thrusting against the matrix and follicular cells of the cumulus oophorus during penetration of the structure. The results of the present study have demonstrated that the transition from the preactivated to the activated state of flagellar bending is maintained after attachment of spermatozoa to the zona pellucida. Therefore, the significance of this form of movement may indeed by hydrodynamic, i.e., in maximizing the forcing of the sperm head against the zona material. The symmetrical, large-amplitude waveforms exhibited are hydrodynamically well suited for this purpose. We noted that activated hamster spermatozoa behave similarly upon attachment to the zonae pelluicidae of mouse eggs. The reorganization of flagellar beat kinematics to a symmetrical pattern upon zona attachment may therefore be a physically mediated phenomenon, resulting from the anchoring of the sperm head to the zona.

The present study has shown that preactivated spermatozoa that have attached to the zona exhibit nonvigorous low amplitude flagellar beats. This is in marked contrast to activated spermatozoa. Since zona penetration does obviously require vigorous flagellar motion of the spermatozoon, this observation provides evidence for the lack of preparedness of the preactivated spermatozoa for fertilization. The motile machinery of the preactivated spermatozoon seems to be not yet suited to the rigors of zona penetration.

It was our impression that the final orientation of the spermatozoa with respect to the zona pellucida was not random. With time,

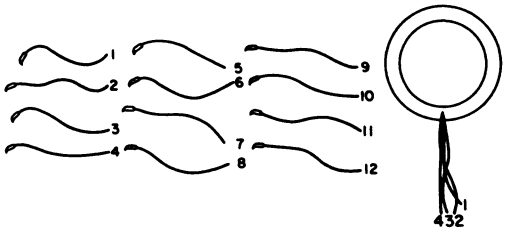

FIG. 4. Sequence of high-speed cinemicrographic tracings, numbered consecutively, of a preactivated hamster spermatozoon fresh from the epididymis. The tracings on the left depict the beat shapes while the spermatozoon was swimming freely. The tracings on the right depict the beat immediately after contact with the zona pellucida of a hamster egg; for optical clarity, the head of the spermatozoon has not been drawn. The interval between tracings is 0.02 sec.

many of the most vigorous spermatozoa rolled on the zona surface such that the planes of their beats were close to being perpendicular to the plane of the slide and coverglass. It should be emphasized that our slide preparations, as those of Sato and Blandau (1979), were sufficiently shallow that the presence of the slide and coverglass significantly influenced the hydrodynamics of sperm-zona interactions. The final orientation of spermatozoa on the zona may therefore have been a consequence of this.

The difference between the beat frequencies of in vivo- and in vitro-activated spermatozoa is interesting. This distinction could simply have reflected differences in the natural history of the two groups of spermatozoa upon release from the male and before resuspension into identical final media. It is also possible that the higher beat frequencies of those spermatozoa that had actually attained the site of fertilization in vivo reflected the greater vigor of a highly "selected" population of spermatozoa.

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