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Maturation of Monkey Spermatozoa in the Epididymis With Respect to Their Ability to Undergo the Acrosome Reaction

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ABSTRACT: Spermatozoa obtained along the length of the epididymides from five macaque monkeys (*Macaca fascicularis*) were examined for their ability to undergo the acrosome reaction under basal conditions and when stimulated with the calcium ionophore A23187. Under basal conditions (2.5 hours incubation in potentially capacitating medium) few epididymal sperm cells displayed the loss of acrosome as judged by staining with peanut agglutinin lectin that binds to the outer acrosomal membrane, regardless of the epididymal region from which they were retrieved. By contrast, there was a marked difference in response to short (0.5 hour) incubation with the calcium ionophore A23187, which induced acrosome reactions in the majority of caudal sperm: caput sperm failed to respond at all and corpus sperm were about 50% as responsive as caudal spermatozoa. There is thus a development of the ability to respond to the ionophore upon maturation. Ejaculated spermatozoa from the same monkeys displayed a higher rate of basal acrosomal loss but a slightly lower stimulated response than mature epididymal spermatozoa. With these maturational aspects, monkey epididymal sperm can serve as a model for the study of human sperm maturation.

Key words: Capacitation, primate, development, epididymis, macaque.

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The maturation of spermatozoa as they migrate through the epididymis confers on them the ability to fertilize eggs (for reviews, see Amann et al, 1993; Cooper, 1995). For this to be achieved, the sperm cells must be able to move and undergo the process of capacitation and the acrosome reaction so that passage through the zona pellucida and fusion with the vitellus can occur. Work on the ability of developing epididymal sperm to undergo the acrosome reaction has produced results that are species and treatment dependent. In the mouse, the ionophore A23187 and solubilized zonae pellucidae (Biegler et al, 1994) can elicit acrosome reactions in immature (caput and corpus) spermatozoa but in the ram, immature spermatozoa are unable to respond to the ionophore in this fashion (Williams et al, 1991). In this species, however, the use of liposomes of dilauroylphosphatidylcholine does promote acrosome reactions in caput spermatozoa, but the cells are unable to fuse with the vitellus of zona-free hamster eggs (Graham et al, 1991). Work in the goat and pig has shown that incubation in simple medium lacking substrates can induce acrosome reactions in caput epididymidal (goat: Harayama et al, 1993a) and even testicular spermatozoa (pig: Harayama et al, 1993b).

The wide disparity between species in their acrosomal response to external stimuli provides little indication that these animal models may help in the evaluation of acrosomal disorders underlying infertility in man. Characterization of sperm maturation processes in monkeys may be useful in the establishment of nonhuman primate models for the study of male infertility and fertility regulation. Maturational changes in motility potential and kinematics along the epididymis are similar in human (Yeung et al, 1993) and both cynomolgus and marmoset monkeys, despite slight shifts in the maturation profiles among species (Yeung et al, 1996). Differences between the function of mature and immature sperm have been reported on the capacities to bind to zona pellucida (cynomolgus: Mahony et al 1993) and to penetrate zona-free hamster oocytes (marmoset: Moore 1981; chimpanzee: Gould and Young, 1990). It is not known whether this is due to a direct acquisition of these functions *per se* upon maturation, or to a poor ability of immature sperm to undergo capacitation, which is a prerequisite of fertilization. Acrosome reactions can be monitored as an end point of capacitation (see Yanagimachi 1994). In the present work, the ability of cynomolgus monkey epididymal sperm at various maturational states to undergo acrosome reactions was studied after *in vitro* incubation, with and without challenge with the Ca^{2+} ionophore A23187, using a protocol established for human sperm (Cummins et al, 1991; Aitken et al, 1993).

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Materials and Methods

All chemical reagents used were obtained from Sigma Chemie GmbH (Deisenhofen, Germany) unless stated otherwise.

Preparation of Ejaculated Sperm

Five healthy adult cynomolgus monkeys (*Macaca fascicularis*) with body weights ranging from 4.7 to 7.2 kg (mean 5.8 kg) were used for the study. They were caged individually in a temperature- and humidity-controlled room with a 12:12 hour light: dark cycle. Electroejaculation was carried out as described by Wickings and Nieschlag (1980) weekly for 2 weeks before the hemicastration. Spermatozoa were obtained from the exudate after 20–30 minutes of liquefaction at 37°C and processed for the staining of the acrosome as described below.

Preparation of Epididymal Sperm

One epididymis was obtained from each of the five monkeys during hemicastration. The animals were anesthetized initially with ketamine HCl (8–10 mg/kg; Parke-Davis, München, Germany) and anesthesia was maintained during the operation by intravenous injection of sodium pentobarbital (6 mg/kg Nembutal, CEVA, Paris, France). Loops of epididymal tubules were dissected free of capsule and blood vessels and excised from four regions corresponding to regions 2–5 in Yeung et al (1996): (region 2) the caput region where the tubule was more darkly pigmented than the adjacent regions; (3) the proximal corpus, (4) the mid-corpus, and (5) the proximal cauda (Fig. 1). These tubule fragments were washed in phosphate-buffered saline (PBS: Dulbecco's, without bicarbonate, Gibco BRL Eggenstein, Germany) and cut in several places such that spermatozoa could be gently extruded and processed for the examination of morphology and acrosomal staining outlined below.

Sperm Incubation and Staining of the Acrosome

Exudates of the ejaculate and the luminal contents released from epididymal regions 2–5 were washed through a discontinuous Percoll gradient of 35 and 70% made up in Ham's-F10 medium by centrifugation at 500 × *g* for 10 minutes. The 70% Percoll solution was prepared by mixing a 3.33×-strength Ham's-F10 solution with neat Percoll (3:7, v/v) and the 35% Percoll by mixing the 70% Percoll 1:1, v/v, with normal strength Ham's-F10 solution. Each sperm pellet was washed with 2 ml Ham's-F10 medium containing bovine serum albumin at 4 mg/ml by centrifugation at 400 × *g* for 5 minutes. Spermatozoa were resuspended in fresh medium to a cell concentration of about 20 × 10^6 ml and incubated under 5% CO_2 in air for 2 hours. The Ca^{2+} ionophore A23187 dissolved in dimethyl sulfoxide (DMSO) was added to a 100-µl aliquot of sperm suspension to give a final concentration of 5 µM, with DMSO alone (0.3%, v/v) being added to another sperm aliquot as control. Incubation was continued for 0.5 hour and the sperm were pelleted by centrifuging for 30 seconds at 3,000 × *g* (Ole Dich, Hvidovre, Denmark) and resuspending in 100 µl of hypo-osmotic swelling medium (Jeyendran et al, 1984). After a 10-minute incubation at 37°C, the sperm were recovered with the above 30-second centrifugation and fixed with 100 µl ice-cold methanol. After repelleting the sperm by centrifuging at 2,000 × *g* for 30 seconds, they were stained by 20 µl fluorescein isothiocyanate (FITC)-conjugated lectin from peanut (*Arachis hypogaea* [AH], 0.67 mg/ml PBS) for 15 minutes in the dark at room temperature. The sperm were then washed twice with 0.5 ml PBS, taken up in about 5 µl PBS, and smeared onto slides. Air-dried slides were mounted with Sigma mounting medium and examined using an Olympus BX40 fluorescence microscope fitted with a dichroic mirror (DM500), an excitation filter (BP450-480), and a barrier filter (BA515).

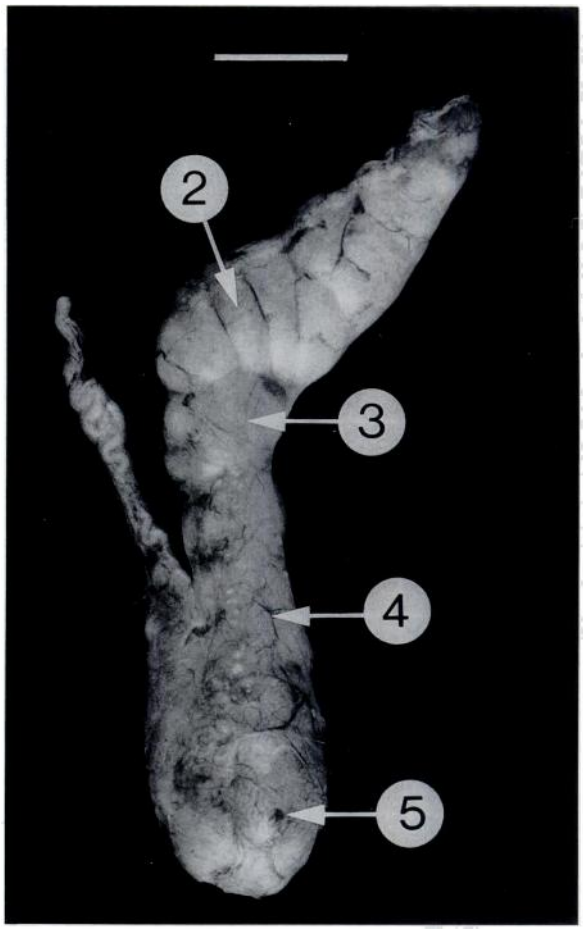

FIG. 1. Photomicrograph of the epididymis of *Macaca fascicularis* showing the four regions from which spermatozoa were sampled in this study. These regions correspond to regions 2–5 in Yeung et al (1996): region 2, mid-caput; region 3, proximal corpus; region 4, distal corpus; region 5, proximal cauda.

The staining patterns of the monkey sperm head by the lectin AH were classified into 4 categories (A, B, C, and D, see definition in Results), based on the description for human sperm by Mortimer (1994). Only the staining patterns of sperm with swollen tails were scored (100 from each sample), by one or two observers, and the number of nonswollen sperm (as described by Jeyendran et al, 1984) was also noted. Coefficients of variation between the 2 observers were 4.5% for acrosome-intact sperm (pattern A) and 12.8% for the patterns of acrosomal loss (B + C + D).

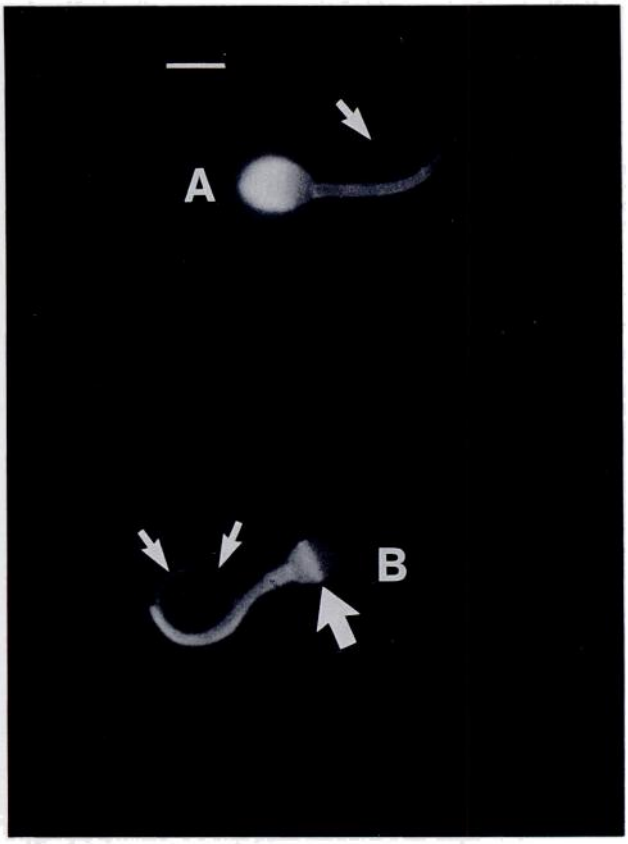

FIG. 2. Immunofluorescence micrographs of acrosome-intact (sperm A; whole acrosomal region stained by the lectin) and acrosome-reacted spermatozoa indicated by the acrosomal loss (sperm B; large arrow indicates lectin-stained equatorial region, classified as staining pattern C). Small arrows indicate coiled sperm tails. Bar = 5 μm.

Statistics

Percentages were transformed by substituting the arcsin of the square-root of the proportion before statistical analysis using one-way analysis of variance followed by the Tukey multiple range test. Differences among sperm from different epididymal regions or ejaculates were considered significant with $P < 0.05$. Mean values given in the text were retransformed into percentages and upward standard errors (sem) calculated as the differences between the retransformed mean value and retransformed mean + SEM value.

Results

Spontaneous Acrosomal Loss

Viable spermatozoa, with tails that swelled in the hypo-osmotic swelling test, could be clearly distinguished and categorized by their labeling with lectin-FITC into one of the four patterns: (A) brightly stained acrosomal region, (B) dotted or patchy staining covering <50% of the acrosomal region, (C) equatorial band as the only stained acrosomal area, and (D) complete loss of acrosomal staining. Pattern A indicated acrosome-intact cells, whereas the other three patterns demonstrated various extents of acrosomal loss, representing sperm undergoing (pattern B) or having completed (patterns C and D) the acrosome reaction (Cross et al, 1986; Mortimer et al, 1987). In all cases, the majority staining pattern was either A or C (Fig. 2), with the minority pattern B or D in <5% of sperm (Table 1).

Induction of Acrosomal Loss by the Ca^{2+} Ionophore A23187

After an incubation of 2.5 hours, spontaneous acrosomal loss rarely occurred in sperm from any epididymal region (≤5%, patterns B + C + D). When the ionophore was

Table 1. *Distribution of the different staining patterns of the sperm head by the lectin AH-FITC, as percentages of viable sperm, with or without the presence of the ionophore A23187, and the percentage of viable sperm (with swollen tails in the hypo-osmotic swelling test), in sperm samples obtained from various regions of the epididymis and the ejaculates of five cynomolgus monkeys**

Sperm sample	Pattern A	Pattern B	Pattern C	Pattern D	% Viable
Without A23187					
Caput	98.9 + 0.5	0.2 + 0.4	0.1 + 0.2	0.04 + 0.12	93.8 + 1.8
Proximal corpus	96.7 + 0.9	0.8 + 1.0	1.7 + 0.4	0.04 + 0.12	92.1 + 1.6
Mid corpus	94.9 + 3.2	0.7 + 0.7	4.1 + 4.0	0.00 + 0.00	90.8 + 1.7
Proximal cauda	94.9 + 2.8	0.4 + 0.6	3.7 + 3.0	0.16 + 0.26	91.8 + 1.8
Ejaculate	79.1 + 7.1	3.1 + 0.9	6.5 + 2.6	1.63 + 0.66	87.2 + 2.7
With 5 μM A23187					
Caput	98.3 + 0.3[a]	0.5 + 0.5	0.2 + 0.2[a]	0.16 + 0.26[a]	94.4 + 1.4
Proximal corpus	67.7 + 8.7[b]	3.0 + 2.5	27.0 + 7.3[b]	0.12 + 0.36[a]	90.7 + 1.0
Mid corpus	53.6 + 8.6[b]	5.3 + 1.8	39.8 + 7.0[b]	0.12 + 0.36[a]	90.7 + 1.0
Proximal cauda	33.6 + 7.1[c]	1.6 + 1.1	62.1 + 7.0[c]	1.28 + 0.95[a]	90.1 + 1.0
Ejaculate	49.4 + 2.2[b,c]	3.9 + 0.7	42.5 + 2.7[b]	3.88 + 0.73[b]	88.3 + 0.9

* Values are percentages (mean + SEM, retransformed data; values in the same column with different superscripts (a, b, c) are significantly different ($P < 0.05$).

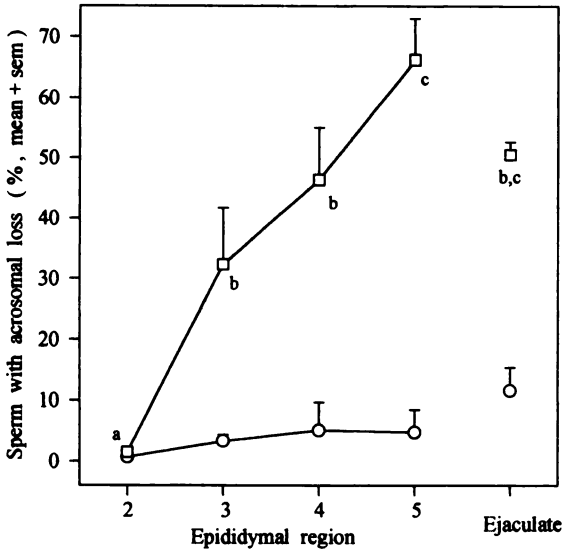

FIG. 3. The percentage of monkey spermatozoa from different regions (see Fig. 1) of the epididymis and the ejaculate (abscissa) displaying the loss of acrosome (ordinate) when incubated under basal conditions (○) or after incubation with the ionophore A23187 (□). Values with different letters (a, b, c) are significantly different ($P < 0.05$). There is no statistical difference among basal values.

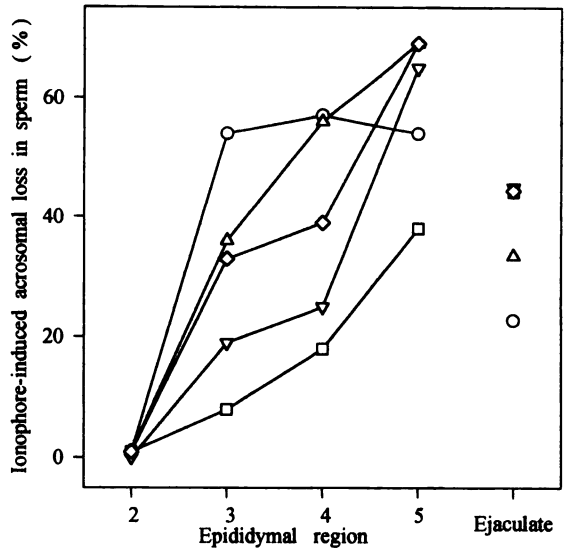

FIG. 4. The net percentage of cells displaying the ionophore-induced acrosomal loss (ordinate) of sperm cells from different regions of the epididymis (see Fig. 1) and the ejaculate (abscissa) from individual monkeys, each animal being represented by a different symbol.

present during the last 0.5 hour, acrosomal loss was detected with increasing percentages in epididymal sperm from more distal regions (Fig. 3). Almost all of these increases took the form of staining pattern C (Fig. 2; Table 1). There was no stimulation of acrosomal loss by A23187 in the immature sperm from the caput epididymidis from any of the five monkeys (Fig. 3). In spite of large inter-animal variations, the trend of increase along the epididymis in the response to ionophore was consistent. In four of five monkeys, net induction of acrosomal loss by ionophore increased stepwise from the proximal corpus to the proximal cauda. In the fifth animal, maximum stimulation was already achieved in sperm obtained from the proximal corpus epididymidis (Fig. 4). Compared to cauda epididymidal spermatozoa from the same monkey, the response of ejaculated sperm to ionophore was decreased in all five animals (Figs. 3, 4) such that the net induction of acrosomal loss was significantly lower in the ejaculated sperm (37.7 + 4.6%) than the cauda sperm (59.2 + 5.9%).

Sperm Vitality Revealed by Hypo-osmotic Swelling

Epididymal sperm from all regions exhibited >90% swollen tails upon hypo-osmotic challenge, and this was not affected by the presence of A23187 (Table 1). There was no significant difference among epididymal or ejaculated sperm with respect to membrane integrity.

Discussion

Whereas sperm from various epididymal regions exhibited similarly low percentages of spontaneous acrosomal loss, they responded differently to the challenge with Ca^{2+} ionophore. In the presence of A23187, there was a clear profile of maturational increase in the ability of epididymal spermatozoa to undergo the acrosome reaction, with a nondetectable incidence of induced acrosomal loss in the caput sperm and a maximum induction of about 60% in the mature spermatozoa from the proximal cauda epididymidis. This profile was consistent among different monkeys despite the variability in the extent of increase.

In contrast to those of epididymal origin, ejaculated sperm differed by a slightly higher rate of spontaneous acrosomal loss and a lower response to ionophore stimulation. These observations may be explained if spontaneous acrosomal loss is considered nonphysiological and reflects the instability of the sperm membranes and that aged cells respond less well to ionophore. There is evidence for this in the hamster, where sperm in the cauda region of a surgically created abdominal epididymis, in which storage is impaired, exhibit an increase in spontaneous acrosomal loss (Bedford and Yanagimachi, 1991). Sperm aged in the hamster cauda after ligation of the efferent ducts also display a lower acrosome response to A23187 challenge within the first 3 hours of incubation than controls (Cuasnicú and Bedford, 1989). With these observations in mind, the ejaculated spermatozoa in this study were obtained from the second of two weekly ejaculation sessions and might have contained a large proportion of cells from the old sperm reserve left after the

first weekly ejaculation. On the other hand, the proximal cauda epididymidal spermatozoa were obtained after both of these ejaculations, so the epididymal spermatozoa were probably considerably younger mature cells than the ejaculated spermatozoa in this study. Superiority in the kinematic qualities of cauda epididymidal sperm over ejaculated sperm under these circumstances has also been shown (Yeung et al, 1996). The lower stimulation of acrosomal loss in ejaculated sperm might reflect the higher resistance to capacitation conditions because of the additional coating of decapacitation factor(s) originating from accessory glands (see Yanagimachi, 1994).

Acrosomal loss of ejaculated sperm from cynomolgus monkeys occurring on the zona pellucida requires an elevation of intracellular cyclic AMP (Vandevoort et al, 1994) and is stimulated by the protein kinase C pathway (Tollner et al, 1995). These inductions require Ca^{2+} in the medium. Although the use of the ionophore in the study of sperm function is to induce Ca^{2+} influx, bypassing some of the physiological sequence of events leading to acrosomal exocytosis, it has been demonstrated that the response of human sperm to A23187, with respect to oocyte penetration, requires a sufficient incubation period (Aitken, 1994). It is not clear whether the failure of immature sperm to respond to Ca^{2+}-ionophore challenge by exhibiting acrosomal loss to the same extent as mature sperm indicates their incompetence in capacitation or their failure to respond to the anticipated induced Ca^{2+} influx.

The ability of sperm to undergo the acrosome reaction *in vitro,* as indicated by acrosomal loss, is often employed to assess sperm function, although it is generally accepted now that the acrosome reaction of physiological importance is that occurring on the surface of the zona pellucida (Cross et al, 1988). The extent of spontaneous acrosomal loss by epididymal or ejaculated spermatozoa obtained in this study was low compared to values published for the mouse (Lakoski et al, 1988; Biegler et al, 1994), sheep (Graham et al, 1991), and boar (Harayama et al, 1993a) but in the range of that displayed by goat epididymal spermatozoa (Harayama et al, 1993b). After incubation with A23187 the acrosomal loss was induced only in sperm from the proximal corpus epididymidis (region 3) or more distally. There clearly is a maturational component to the ability of the cells to respond that occurs in the epididymis, as found for the ram (Williams et al 1991) but not for the mouse (Lakoski et al, 1988; Biegler et al, 1994).

The present findings in the monkey differ partially from one report in man in which maturing spermatozoa obtained from epididymides of men undergoing castration for prostatic carcinoma displayed increasing rates of spontaneous acrosomal loss and increasing responses to long-term incubation at 4°C, as detected by the triple stain technique (Haidl et al, 1994).

In conclusion, the present findings indicate maturational changes in the function of epididymal spermatozoa from the cynomolgus monkey, which may serve as a model for the study of the development of human spermatozoa.

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