A comparison of three methods for detecting the acrosome reaction in human spermatozoa*

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This study was designed to compare three different fluorescent probes to assay the acrosome reaction in human spermatozoa: chlorotetracycline (CTC), mannosylated bovine serum albumin (BSA) labelled with fluorescein (MAF), and quinacrine (QN). Normal human sperm ejaculates were washed and allowed to swim up for 30–60 min. Samples were examined under epifluorescence for the percentage of the acrosome reacted spermatozoa, as detected by the three probes. There was no significant difference between samples of fresh, uncapacitated spermatozoa evaluated with CTC, MAF or QN; all gave <10% reacted. Following capacitation for 3 h, the percentage of spontaneously reacted spermatozoa was higher than in fresh spermatozoa; CTC and MAF gave the same percentage (12%), while QN indicated a higher percentage (18%) of reacted spermatozoa (P < 0.001). Following exposure to ionophore A23187 at 1 h, the percentage of acrosome reactions increased to a mean of 31% as detected with CTC or MAF; the mean percentage (45%) was significantly higher with QN (P < 0.0001). Further incubation up to 2 h with A23187 did not change these percentages. These results suggest that the QN probe detects the onset stage of the acrosome reaction, whereas the CTC and MAF probes detect the later stages in which the acrosomal cap is lost. Use of the two types of probe provides a means for finer resolution of the time course of the acrosome reaction in the human spermatozoon.

Key words: acrosome reaction/chlorotetracycline/human spermatozoa/mannosylated BSA/quinacrine

Introduction

The acrosome reaction is a critical physiological event permitting gamete interaction in all mammalian species, including human; indeed, it represents an absolute requirement for fertilization in vivo. Only acrosome-reacted spermatozoa have the ability to pass through the zona pellucida and subsequently fuse with the oocyte membrane to form the zygote (Yanagimachi, 1994). In order for this to occur, the acrosome reaction in vivo takes place in the sperm cell bound to the zona pellucida: the spermatozoos binds with intact plasma membrane and the acrosome reaction is induced by the zona pellucida (Saling and Storey, 1979; Florman and Storey, 1982). Spermatozoa that undergo a premature acrosome reaction are unable to penetrate the cumulus cell layer (Cherr et al., 1986; Cummins and Yanagimachi, 1986) and so lose their fertilizing potential. Spermatozoa that undergo the acrosome reaction while passing through the cumulus adhere firmly to the adjacent cumulus cells and so never reach the zona pellucida (Cummins and Yanagimachi, 1986). The acrosome reaction in vivo that leads to fertilization is best considered as a closely regulated exocytotic event (Kopf and Gerton, 1991; Storey and Kopf, 1991). A zona glycoprotein, ZP3, acts as agonist to receptors on the sperm plasma membrane (Wasserman, 1988, 1991), which in turn induces a signal transduction cascade leading to punctate membrane fusion between outer acrosomal and plasma membranes with subsequent vesiculation. The latter process leads to loss of the ‘acrosomal cap’.

The acrosome reaction can also be achieved in the absence of natural inducers using the divalent cation ionophore A23187 (Florman and Storey, 1982; Aitken et al., 1984; Byrd and Wolf, 1986). Ionophore A23187 induces acrosome reaction by mediating directly the influx of Ca2+ required for the completion of the acrosome reaction, and so bypassing the zona-activated intracellular signalling mechanisms that control this influx (Bailey and Storey, 1994). Acrosome reaction percentages induced by ionophore appear to reflect the fertilizing capacity of human spermatozoa in vitro (Byrd and Wolf, 1986; Cummins et al., 1991). Aitken et al. (1991) suggest that this reagent induces levels of sperm–oocyte fusion that are related to the potential for establishing a spontaneous pregnancy in vivo. The ionophore-induced acrosome reaction is clearly an important aspect of human sperm function to monitor for diagnostic purposes as well as for utilization in assisted reproductive technologies.

In all mammals including the human, spermatozoa must undergo capacitation, a biochemical change in the plasma membrane that renders the spermatozoa capable of undergoing the acrosome reaction (Austin, 1967; Yanagimachi, 1981; Florman and Babcock, 1991). Although capacitation occurs physiologically in the female reproductive tract, it can be induced in vitro using defined media based on variations of Krebs–Ringer bicarbonate (Rogers, 1978) and albumin (Go...
and Wold, 1985). Mammalian spermatozoa must be capacitated in order to undergo the acrosome reaction, whether induced by zona pellucida protein or by ionophore (Yanagimachi, 1994).

In capacitated spermatozoa, the acrosome reaction results in loss of the acrosomal cap. This can be visualized readily by light microscopy in those species that possess large acrosomes, e.g. the guinea pig and hamster. However, in most mammals, including the human, the acrosome is small and difficult to visualize. Consequently a number of different staining techniques have been developed for visualization of the human sperm acrosome, including the use of multiple histochemical stains, labelled lectins, and labelled antibodies (Koehler, 1978; Talbot and Chacon, 1980; Wolf et al., 1985; Cross et al., 1986). In this laboratory we found the chlorotetracycline (CTC) fluorescent assay, originally developed for mouse spermatozoa (Saling and Storey, 1979; Ward and Storey, 1984), to be remarkably convenient for human spermatozoa (Lee et al., 1987). Recently, Benoff et al. (1993) have shown striking changes in the binding pattern of mannosylated bovine serum albumin labelled with fluorescein (MAF) as the acrosome reaction proceeds in human spermatozoa, which provides an easily scored assay for percentage acrosome-reacted cells. All these assays depend on those membrane changes that lead to loss of the acrosomal cap. For this study, we have developed an assay based on quinacrine fluorescence (QN) for detection of the onset of the acrosome reaction, based on a previous study with N-(n-dodecyl)-9-amino acridine (NDAA) by Lee and Storey (1989). The onset is defined as the point at which pores form subsequent to punctate fusion of outer acrosomal and plasma membranes, leading to loss of the pH gradient between the normal acidic acrosomal lumen and the suspending medium. The goal of this study was to compare the MAF and QN assays with each other and with the CTC assay which had been previously validated with the triple stain assay of Talbot and Chacon (1981) and with the indirect immunofluorescent (IIF-HS19) assay of Wolf et al. (1985).

Materials and methods

Semen collection and preparation

Fresh semen samples were obtained from healthy adult donors by masturbation after 48–72 h abstinence, and were allowed to liquefy at 37°C for 15–30 min. Sperm samples (n = 44) were assessed for volume, concentration and percentage motility, using standard methods (WHO, 1992), and only samples with normal parameters (WHO, 1992) were included in the study. Liquefied ejaculates were mixed with three parts of HEPES-buffered, modified human tubal fluid medium (Quinn et al., 1985; Irvine Scientific, Santa Ana, CA, Cat # 9962) containing 5 mg/ml bovine serum albumin (mHTF/BSA). The spermatozoa were washed by centrifugation at 400 g for 10 min. The sperm pellets were resuspended in two parts of mHTF/BSA and washed again by centrifugation at 400 g for 8 min. The sperm pellet was gently overlaid with 0.5 ml mHTF/BSA, the centrifuge tubes were inclined at 45° and the motile spermatozoa were allowed to swim up out of the pellets for 30–45 min at 37°C. Sperm samples obtained by this swim-up method consistently showed forward motility >90%. Spermatozoa remained viable throughout the time-course of each experiment as shown by retention of bright fluorescence of the midpiece with all three dyes; dead spermatozoa do not retain the midpiece fluorescence (Lee and Storey, 1989).

Capacitation and induction of the acrosome reaction

Sperm suspensions obtained by swim-up were incubated in Ham's F-10 medium containing 5 mg/ml fatty acid-free human serum albumin (HSA, Sigma Chemical Co., St Louis, MO, Cat #A-3782) for 3 h at 37°C to induce capacitation. After capacitation, the sperm samples were incubated with Ca2+ ionophore A23187 (Sigma, Cat #C-7522) at a concentration of 10 μM (Byrd and Wolf, 1986), prepared from a 10 mM stock solution in dimethyl-formamide (DMF), at 37°C to induce the acrosome reaction. Zero time was taken as the point of addition of A23187. Samples were taken at zero time and after 1 and 2 h incubations. Fresh spermatozoa prior to capacitation, capacitated spermatozoa, and A23187-treated sperm samples were evaluated for the percentage of the acrosome-reacted spermatozoa using the CTC, MAF, and QN fluorescence assays, as described below. The spermatozoa were examined at ×400 with a Zeiss microscope equipped for epifluorescence. A total of 100 spermatozoa in duplicate was scored for each determination of the percentage of the acrosome-reacted spermatozoa in each sample.

Chlorotetracycline fluorescence assay (CTC)

The assay was carried out exactly as described by Lee et al. (1987). The CTC stock solution (500 μM) was made by dissolving CTC-HCl (Sigma, Cat. # C-4881) in a buffer containing 20 mM Tris, 130 mM NaCl, 5 mM cysteine, pH 7.80 ± 0.05, stored in a light-shielded container at 4°C. Fresh CTC solutions were made up daily. A 5 μl aliquot of sperm suspension was placed on a slide at 37°C; 5 μl of CTC stock solution was rapidly added, followed within 10 s by 0.05 μl of 12.5% glutaraldehyde in 1 M Tris buffer, pH 7.8, delivered with a 1.0 μl microsyringe calibrated to 0.01 μl (Hamilton Co., Reno, NV) to give a final concentration of 0.06% glutaraldehyde. The needle tip of the microsyringe was used as a stirrer to ensure uniform mixing in the 10 ml drop. The glutaraldehyde treatment froze the fluorescent pattern such that the pattern remained stable for at least 12 h (Ward and Storey, 1984). Preparations were examined after 5 min at ×400 with a Zeiss microscope equipped for epifluorescence with the fluorescein filter module. A total of 100 spermatozoa in duplicate were scored for each determination of the percentage of the acrosome-reacted spermatozoa in each sample. Acrosome-reacted spermatozoa show lack of fluorescence on the anterior part of the head (pattern AR), while the acrosome intact spermatozoa have previously been shown to have three distinct patterns as described by Lee et al. (1987): (i) EF pattern, in which the spermatozoa show the bright band of fluorescence in the post-acrosomal region and bright fluorescence on the midpiece; (ii) DF pattern, in which the spermatozoa show the bright fluorescence on the anterior portion of the head and the dark band in the post acrosomal region as well as the bright midpiece fluorescence; (iii) CP pattern, in which the spermatozoa show fluorescence over the entire head with a bright perimeter and bright midpiece. The CP pattern is maximal after incubation of the spermatozoa to induce capacitation.

Mannosylated BSA-FITC fluorescence assay (MAF)

The method described by Benoff et al. (1993) was used. After sperm capacitation, the sperm samples were washed three times, 10 min each with core buffer (30 mM HEPES, pH 7.0, 0.5 mM MgCl2, 150 mM NaCl, 10 mg/ml BSA containing 20 mM CeCl3 and reacted with 100 μg/ml fluorescein-conjugated α-D mannosylated BSA (Man-FITC-neoglycoprotein ligand; Sigma Cat #A 7790) in calcium-supplemented core buffer for 15 min at 37°C. After
labelling, motile spermatozoa were pelleted by centrifugation, washed twice with core buffer (no calcium), and examined by epifluorescence using the fluorescein filter module. Acrosome-reacted spermatozoa showed clustering of Man-FTTC-neoglycoprotein ligand in the equatorial segment and post-acrosomal regions of the sperm head with concomitant neck midpiece labelling. The acrosome-intact spermatozoa have brightly fluorescent acrosomes. A drop of the cell suspension was examined immediately with epifluorescence, using the fluorescein filter module. Acrosome-reacted spermatozoa show a dark anterior portion of the head, while the acrosome-intact spermatozoa have brightly fluorescent acrosomes.

Quinacrine fluorescence assay (QN)

A 1 µl aliquot of 0.5 mM quinacrine reagent (quinacrine dihydrochloride, Sigma, Cat # 3251) prepared from a 10 mM stock solution in H2O, was added to a 20 µl sperm suspension and mixed gently. A drop of the cell suspension was examined immediately with epifluorescence, using the fluorescein filter module. Acrosome-reacted spermatozoa show a dark anterior portion of the head, while the acrosome-intact spermatozoa have brightly fluorescent acrosomes.

Statistical analysis

Statistical significance of the data was determined by paired Student's t-test and analysis of variance (ANOVA), using the StatView statistics program. A value of P < 0.05 was taken as statistically significant.

Results

The results with each probe, comparing the different percentages of acrosome-reacted spermatozoa under different conditions, are shown in Table I. The sperm samples (n = 44) obtained by the swim-up technique had a forward progressive motility >90%, a concentration range of 40–110×10^6 cells/ml, and viability >95%. In these samples, >90% of spermatozoa as freshly obtained by swim-up were acrosome intact; there was no significant difference in the percentage acrosome-reacted cells as determined by the three different probes (Table I). Capacitation of the sperm samples for 3 h in Ham F-10 medium containing 5 mg/ml HSA resulted in a significant increase in the number of spontaneously acrosome-reacted spermatozoa compared with zero time (P < 0.0001). Evaluation of the acrosomal status showed that whereas there was no significant difference between the percentage of acrosome-reacted spermatozoa as detected by the CTC and MAF assays, the QN assay gave a significantly higher percentage of acrosome-reacted spermatozoa (P < 0.0001) compared to the other two assays (Table I).

Addition of the ionophore A23187 at a concentration of 10 µM to the sperm samples resulted in induction of the acrosome reaction. Within 5 min after addition of ionophore, there was no significant increase in percentage acrosome-reacted spermatozoa compared to capacitated spermatozoa prior to ionophore addition (Table I). This determination ruled out a possible 'jump' in acrosome reactions caused by ionophore addition and also rules out an effect of increase in intracellular Ca^{2+} on the fluorescence emission of QN in the acrosomal lumen. After 1 h exposure to ionophore A23187, there was a highly significant increase in the percentage acrosome-reacted spermatozoa in comparison with the results after 1 h (Table I).

These data were also analysed by ANOVA to show the range of variation within each group of samples. The box plots are shown in Figure 1 for the QN assay, Figure 2 for the MAF assay, and Figure 3 for the CTC assay.

Table I. Percentage of acrosome-reacted spermatozoa as detected by the chlortetracycline fluorescence assay (CTC), mannosylated bovine serum albumin fluorescence assay (MAF) and quinacrine fluorescence assay (QN) respectively. Values are shown at zero time, after 3 h under capacitation conditions, and 1 and 2 h following incubation with ionophore A23187. Values are means ± SD

<table>
<thead>
<tr>
<th></th>
<th>CTC (n = 44)</th>
<th>MAF (n = 44)</th>
<th>QN (n = 44)</th>
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</thead>
<tbody>
<tr>
<td>Fresh (zero time)</td>
<td>7.11 ± 2.1a</td>
<td>6.34 ± 2.2a</td>
<td>8.2 ± 2.3a</td>
</tr>
<tr>
<td>Capacitation (3 h)</td>
<td>12.8 ± 3.1b</td>
<td>11.9 ± 3.8b</td>
<td>17.5 ± 3.5c</td>
</tr>
<tr>
<td>Ionophore A23187 (1 h)</td>
<td>32.5 ± 4.9d</td>
<td>30.9 ± 6.2d</td>
<td>45.3 ± 5.3e</td>
</tr>
<tr>
<td>Ionophore A23187 (2 h)</td>
<td>34.1 ± 3.8d</td>
<td>32.5 ± 5.6</td>
<td>46.9 ± 5.6e</td>
</tr>
</tbody>
</table>

Values bearing the same superscript letter are not significantly different (P > 0.05). Values bearing different superscript letters are significantly different (P < 0.0001).

Figure 1. Box plots showing the percentage of acrosome-reacted human spermatozoa as assayed by the quinacrine fluorescence assay (QN). Data show fresh sample at time zero, following incubation for 3 h under capacitation conditions in Ham's F-10 medium containing 5 mg/ml human serum albumin and after treatment with ionophore A23187 (10 µM) for 1 and 2 h respectively. Circles represent points outside the 10th and 90th percentiles.
A.-H Amin et al.

Figure 2. Box plots showing the percentage of acrosome-reacted human spermatozoa as assayed by the mannosylated bovine serum albumin fluorescence assay (MAF). Other details are as for Figure 1.

Figure 3. Box plots showing the percentage of acrosome-reacted human spermatozoa as assayed by the chlortetracycline fluorescence assay (CTC). Other details are as for Figure 1.

Discussion

The QN assay consistently resulted in a higher value for the percentage acrosome-reacted spermatozoa, once this percentage exceeded 10%, than did the CTC or MAF assays. This result is consistent with the QN probe detecting the onset stage of the acrosome reaction, defined as the point at which the pH gradient between the originally acidic acrosomal lumen and the suspending medium is collapsed. Quinacrine is a weak base and accumulates in the acidic acrosomal compartment in acrosome-intact spermatozoa as determined by the pH gradient between the compartment’s lumen and the suspending medium. This accumulation of the fluorescent probe results in bright fluorescence emission from the anterior portion of the sperm head encompassing the acrosome. The onset of the acrosome reaction involves punctate fusion of the outer acrosomal and plasma membranes followed by formation of pores at the points of fusion (Bedford and Cooper, 1978). These pores allow the ionic composition of the acrosomal compartment and the suspending medium to equilibrate (Lee and Storey, 1989), resulting in loss of anterior head fluorescence. The interpretation that quinacrine is detecting the early stage of the acrosome reaction is based on the results of the detailed study of Lee and Storey (1989) using pH probes to assess the acrosome reaction. The CTC assay monitors the loss of the outer acrosomal/plasma membrane (Saling and Storey, 1979) as the pores grow to the point of hybrid membrane vesicle formation (Bedford and Cooper, 1978). This set of membrane reactions necessarily follows in time the initial pore formation. Interestingly, the MAF assay parallels the CTC assay, presumably because both probes monitor the loss of the hybrid membrane vesicles (Benoff et al., 1993).

The observations that all the acrosome reactions that can be induced by ionophore have occurred within the first hour of incubation and that the difference in percentage of acrosome reactions detected by the QN assay compared to the CTC and MAF assays persists as a constant percentage over the 2 h incubation suggests that only a subset of those spermatozoa that undergo onset pore formation continue on to complete the acrosome reaction. The reactions controlling the progress from onset to completion of the acrosome reaction are at present obscure. One consistent feature of the results obtained with normal donors by induction of the acrosome reaction by ionophore A23187, as diagnosed by the three different probes in this study, is that the mean percentage of completely acrosome-reacted spermatozoa did not exceed 50% (Table I). Similar results were reported by Wolf et al. (1985), Byrd and Wolf (1986) and Lee et al. (1987) for induction by ionophore A23187, and by Suarez et al. (1986) for induction by human follicular fluid. The percentage registered by QN assay did exceed this figure on occasion. The most reasonable explanation for this result is that those spermatozoa which did not reach the onset of the acrosome reaction remained uncapacitated or imperfectly capacitated. Those that did reach onset but did not complete the acrosome reaction may be capacitated but, as pointed out above, may lack certain reaction pathways needed for this completion. The combined use of this set of fluorescent probes for detection of the acrosome reaction in human spermatozoa provides one experimental approach to the differentiation of these various physiological states of the human sperm cell, and so helps to elucidate the unknown reaction pathways mediating the various later stages of the acrosome reaction in human spermatozoa. This in turn could be of assistance in diagnosing male infertility arising from the incompetence of spermatozoa in undergoing the acrosome reaction (Calvo et al., 1994a,b).

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Acrosome reaction in human spermatozoa

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References


