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Assessment of the Acrosomal Status of Ram Spermatozoa by RCA Lectin-Binding and Partition in an Aqueous Two-Phase System

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ABSTRACT: The acrosome reaction is an important marker for sperm function. Because different laboratory techniques may be used to detect this exocytotic process, the objective of this study was to investigate the use of fluoresceinated lectins to assess the acrosomal status of nonpermeabilized ram spermatozoa. In addition, we used centrifugal countercurrent distribution (CCCD) in an aqueous 2-phase system to assess the sperm surface modifications associated with the acrosome reaction by observing changes in their partition behavior. We analyzed the binding of 5-fluorescein isothiocyanate (FITC)–conjugated lectins to ram sperm to select a lectin that bound preferentially to the acrosomal region, which would allow differentiation of acrosome-intact from acrosome-damaged ram spermatozoa. *Ricinus communis* agglutinin (RCA) bound intensely to the anterior and weakly to the equatorial acrosomal regions. Acrosomal labeling changed when spermatozoa were induced to acrosome-react with calcium ionophore A23187. RCA acrosomal labeling significantly increased ($P < .0001$) after incubation (84% versus 28% in control samples). To determine if RCA lectin labeling could be used to assess the acrosomal status of fresh ram spermatozoa in suspension, we compared the percentage of acrosome-reacted sperm detected by the carboxyfluorescein diacetate/propidium iodide (CFDA/PI) double-fluorescent staining with the percentage detected by FITC-RCA labeling. The incidence of acrosome-reacted spermatozoa detected by CFDA/PI was not significantly different ($P = .704$; 13 comparisons in 6 different experiments) from the incidence of spermatozoa detected by FITC-RCA staining. The evaluation of the spontaneous acrosome reaction by RCA labeling (5.83%) was not significantly different ($P = .644$) from that assessed by CFDA/PI (6.88%). The percentage of induced acrosome reactions detected by CFDA/PI staining (56%) significantly correlated ($P < .0001$; $r = 0.876$) with that detected by RCA labeling (56.67%). We simultaneously carried out a comparative CCCD in an aqueous 2-phase system to analyze sperm surface changes associated with the acrosome reaction. Results revealed that sperm surface hydrophobicity decreased in samples that had been incubated with ionophore compared with the untreated-control samples. Likewise, RCA binding after CCCD showed that all acrosome-reacted cells were stained, whereas only 42% of cells were lectin-labeled in the untreated semen sample. This change in lectin reactivity of acrosome-reacted spermatozoa signals the presence of some deep membrane or intracellular residues that would affect partitioning. Therefore, the FITC-RCA–labeling procedure can be used to accurately assess the acrosomal status of ram spermatozoa in suspension.

Key words: Acrosome reaction, 2-phase partition.

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The acrosome reaction of mammalian spermatozoa is an essential contributor to fertilization because only acrosome-reacted spermatozoa can penetrate the zona pellucida and fuse with an oocyte. This event is an exocytotic reaction that involves multiple sites of fusion between the sperm plasma membrane and the outer acrosomal membrane, with subsequent vesiculation and release of the acrosomal contents (reviewed by Yanagimachi, 1994). The physiologic acrosome reaction is a well-coordinated process that can occur only in a living spermatozoan in response to natural inducers (Bedford, 1970). In contrast, loss of acrosomal content can occur with the breakdown of the membranes during cell death, and shows similar acrosomal changes (Saacke and Marshall, 1968; Bedford, 1970; Mendoza et al, 1992). The reaction can also be artificially induced by several substances; one of the most commonly used is calcium ionophore A23187 (Shams-Borhan and Harrison, 1981; Flechon et al, 1986; Pampiglione et al, 1993; Troup et al, 1994). The ionophore facilitates the passage of calcium ions across the plasma membrane (Pressman, 1976), which results in a higher-than-threshold level of calcium between the outer acrosomal membrane and the overlying plasma membrane.

The most accurate method of assessing acrosomal status is transmission electron microscopy; however, because of the time and expense required to conduct this assay, other methods such as the use of specific acrosomal stains

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and light microscopy have been used in different species (Cross and Meizel, 1989). Specific labeling techniques have been used to enhance visualization of the acrosome with fluorescence microscopy. Lectins that bind to glycoconjugates of the acrosomal matrix or outer acrosomal membrane have been used to visualize the acrosome of human spermatozoa (Cross and Meizel, 1989) and that of bulls (Graham et al, 1990) and rams (Magargee et al, 1988). Fluorescein-conjugated plant lectins such as fluorescein isothiocyanate–conjugated *Pissum sativum* agglutinin (FITC-PSA) have been used to selectively stain the acrosome of human spermatozoa (Cross et al, 1986; Mendoza et al, 1992; Tesarik et al, 1993; Kóhn et al, 1997) and that of stallions (Farlin et al, 1992; Casey et al, 1993), monkeys (Cross et al, 1989), bulls (Cross and Watson, 1994), and rams (Graham et al, 1990; Gillan et al, 1997; Sukardi et al, 1997). Whereas spermatozoa with damaged plasma and acrosomal membranes have been reported to become fluorescently labeled over the anterior sperm head and equatorial regions, spermatozoa with intact membranes that were impermeable to these lectins did not fluoresce after exposure (Cross and Overstreet, 1987).

Because the induction of the acrosome reaction must be reflected in changes in surface properties of reacted spermatozoa, partition in aqueous 2-phase systems can be a useful way to analyze the surface modifications that result in the acrosome reaction. This technique is an efficient method for analyzing external factors that act through cell surface and plasma membrane components (Walter et al, 1985). Moreover, this procedure is based on different cell surface affinities for inmiscible aqueous solutions of polymers such as dextran and polyethylene glycol (PEG; Albertsson, 1986; Fisher and Sutherland, 1989). The upper phase is rich in PEG and is relatively more hydrophobic than the lower phase, which is rich in dextran. When cells are added to the system they partition between the interface and the PEG-rich upper phase. The extent of cell partitioning is dependent on the cell surface properties. Thus, in the same 2-phase system, cells that have different surface properties will partition to different degrees. Moreover, the properties of any given 2-phase system can be modified in several ways to effect partition of cells brought on by different cell surface molecules. Charge-insensitive phase systems partition cells on the basis of noncharge-related differences in surface properties, which is often referred to as surface hydrophobicity. A high correlation between red blood cell partition and the cell membrane ratio of polyunsaturated/monounsaturated fatty acids has been found in such a charge-insensitive system (Walter et al, 1976).

The selectivity and separation resolution can be improved severalfold by using multistep partition procedures. Countercurrent distribution (CCD) is a chromatographic process with 1 stationary (lower) phase and 1 mobile (upper) phase. The cell sample is partitioned in 1 system and the 2 phases are then, in a systematic way, brought into contact with fresh, opposite phases. Because the separation time of the rather viscous phases can be very long, special procedures have been adopted to minimize this drawback. One method is to allow phase separation to take place in thin layers of liquid, with a large area of bulk interface relative to total phase volume (thin layer countercurrent distribution (TLCCD; Treffrey et al, 1985). This separation method has already been used to reveal the heterogeneity of bovine (Cartwright et al, 1991) and rat (Cartwright et al, 1992) spermatozoa in a dextran/PEG 2–phase system. Furthermore, it has also been used for assessing subtle surface changes of bull, boar, and ram spermatozoa related to the interactions of ejaculate plasma proteins (Harrison et al, 1992).

However, the loss of viability that results from dilution and washing of sperm cells during the separation procedure presents a major technical problem (Harrison et al, 1982). Because of the long period of time necessary for phase separation at unit gravity (Albertsson, 1986), TLCCD may increase the cell death level during the separation process. Shorter separation procedures can be performed in which centrifugation speeds up the phase separation process (ie, the centrifugal countercurrent distribution [CCCD]; Akerlund, 1984). Thus, we have shown that CCCD in an aqueous 2-phase system is a resolutive technique that reveals sperm heterogeneity (Pascual et al, 1993; García-López et al, 1996; Ollero et al, 1996; Ollero et al, 1997b), the functional variability in the response of spermatozoa, which is known to be an inherent characteristic of sperm cells (Ollero et al, 1994; Watson, 1995), and to be highly involved in successful fertilization (Amann et al, 1993). This heterogeneity appears to be associated with different viability states of fractionated cells; nonviable spermatozoa have an enhanced affinity for the dextran-rich phase (ie, they are preferentially located in the left-hand fractions of the CCCD profile). Thus, we have already shown that loss of viability results in a decreased surface hydrophobicity of dead or moribund cells (Pascual et al, 1993; García-López et al, 1996; Ollero et al, 1997a; Ollero et al, 1998). Moreover, we recently reported that sperm–lectin agglutination combined with a swim-up technique leads to the selection of a very high heterogeneous and viable ram sperm population, as analyzed by CCCD (Pérez-Pé et al, 1999).

The in vitro induction of the acrosome reaction has been demonstrated to exhibit a highly significant correlation with fertility in different species (Graham et al, 1987; Lassalle and Testart, 1992; Whitfield and Parkinson, 1992; Whitfield and Parkinson, 1995), and has thus been suggested as a potentially useful method for predicting fertility. The use of lectins to assess the acrosomal status of nonpermeabilized ram spermatozoa has not been

previously reported. The objective of this study was to investigate the use of fluorescein isothiocyanate-labeled *Ricinus communis* agglutinin (FITC-RCA_{120}) for evaluating the acrosomal status of both intact and induced-to-react ram spermatozoa. This lectin displays a primary specificity for galactose and cross-reacts to a varying degree with *N*-acetylgalactosamine (Nicolson et al, 1974). To optimize conditions, several parameters of the reaction-induction medium, including the concentration of $NaHCO_3$ and HEPES were evaluated. The FITC-RCA stain was then validated by comparing the acrosomal status of control and acrosome-reacted sperm as determined by the carboxyfluorescein diacetate and propidium iodide (CFDA/PI) stain with the FITC-RCA stain. CFDA/PI staining has already been validated as an acceptable method for assessing bovine acrosomes (Whitfield and Parkinson, 1995) and has recently been reported to have a high degree of correlation with some widely used techniques for evaluating ovine and bovine semen quality (Pérez et al, 1997a).

In addition, this paper reports the use of CCCD in an aqueous 2-phase system to assess the sperm surface changes associated with the acrosome reaction in order to further characterize the biological basis that underlies the sperm cell heterogeneity revealed by CCCD.

Materials and Methods

Semen Collection

All the experiments were carried out with fresh ram spermatozoa. Semen was collected from 3 mature *Rasa aragonesa* rams by an artificial vagina. The rams, which belonged to the National Association of Rasa Aragones Breeding (ANGRA) and ranged from 2 to 4 years of age, were kept at the School of Veterinary Medicine under uniform nutritional conditions. Second ejaculates from all 3 rams were pooled together and used for each assay in order to eliminate individual differences, as described by Ollero et al (1996).

Induction of the Acrosome Reaction

To optimize conditions of the ionophore-induced acrosome reaction, we re-evaluated several parameters of the medium described for ram spermatozoa that had been freed from seminal plasma (Shams-Borhan and Harrison, 1981) in order to use whole ram semen by measuring the CFDA/PI fluorescent staining. This process involved first establishing the concentration at which HEPES and HCO_3^- should be used to provide a good induction of the acrosome reaction and, second, assessing the effect of dimethyl sulfoxide (DMSO) on sperm viability in order to have an accurate evaluation of the control cell population. The maximum acrosome-reacted spermatozoa were obtained in the absence of HCO_3^- and with 20 mM of HEPES. Addition of DMSO to control samples did not affect cell viability (data not shown).

Calcium ionophore A23187 was dissolved in DMSO and added to 20 μL of raw semen diluted 1:200 (about 2×10^7 cells/mL) with HEPES glucose buffer (149 mM NaCl, 2.5 mM KCl, 10 mM glucose, 20 mM HEPES, and 3 mM $CaCl_2$; Shams-Borhan and Harrison, 1981). The final concentration of A23187 was 1 μM and 0.3% DMSO. Control tubes had DMSO added but no ionophore, which was shown to have no effect (data not shown). The samples were incubated at 39°C for 1 hour, after which we assessed the acrosomal status and cell viability.

Evaluation of Semen Samples

We defined cell viability as both intact plasma and acrosomal membranes (Harrison and Vickers, 1990) and assessed it by fluorescent staining with CFDA/PI (Sigma Chemical Company, St Louis, Mo). The cells were then examined under a Nikon Labophot-2 fluorescence microscope with a B-2A filter at 400× magnification. The numbers of fluorescein-positive (plasma membrane-intact) spermatozoa, propidium iodide-positive (plasma membrane-damaged) spermatozoa, and fluorescein-positive acrosome (acrosomal membrane-intact) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicate for each sample.

FITC-lectin staining was conducted in suspension by mixing 7 μL of a sperm sample (about 1×10^5 cells) with 5 μL of a 12-μg/mL lectin solution in the incubation medium (5 μg/mL final concentration in HEPES glucose buffer [149 mM NaCl, 2.5 mM KCl, 10 mM glucose, 20 mM HEPES, and 3 mM $CaCl_2$]; Shams-Borhan and Harrison, 1981), followed by an incubation for 3 minutes in the dark at room temperature. A 5-μL sample was placed on the slide and covered with a coverslip. We determined the total number of cells by bright field with a Nikon Labophot-2 microscope at 400× magnification. In the same field, we assessed FITC fluorescence under epifluorescence illumination with a B2A filter at the same magnification. The acrosome appeared to have reacted by the presence of bright fluorescence from the tip of the head to the equatorial region of the spermatozoa. Acrosome-intact cells lacked such fluorescence. We counted at least 200 cells in duplicates for each sample.

Centrifugal Countercurrent Distribution

We constructed a countercurrent distribution machine in our laboratory according to a design invented by Akerlund (1984). The apparatus contains 60 chambers arranged in a circle, which allows transfers of the upper (mobile) phases relative to the lower (stationary) phases. In this system, countercurrent distribution is performed during centrifugation by keeping the denser (bottom) phases in the outer half of each chamber and the lighter (upper) phases in the inner half. Because no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 upper-batch over 60 bottom-batch phases. Each transfer in this centrifugal-enhanced countercurrent distribution includes first shaking the phases at unit gravity to thoroughly mix them and then centrifugation to separate them. After the phases have separated and while they are still rotating at full speed ($1000 \times g$), the upper (inner) phases are transferred to the next chambers. A new cycle can be performed after deceleration.

To carry out CCCD experiments, the 2-phase system we used consisted of 5.5% (wt/wt) dextran T500, 2% (wt/wt) PEG 6000, 10.5% (wt/wt) Ficoll 400, 0.25 M sucrose, 0.1 mM EGTA, 4

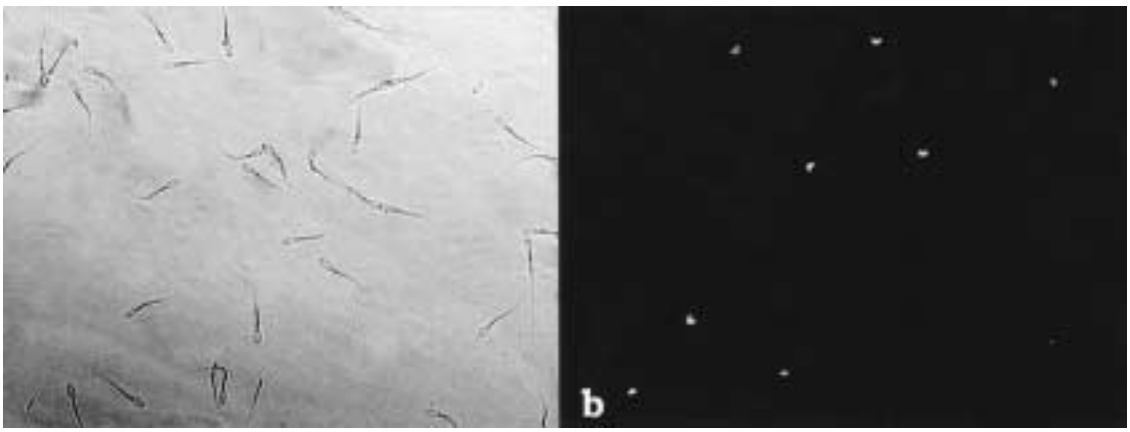

Figure 1. Paired bright-field **(a)** and epifluorescent **(b)** photomicrographs of fresh ram sperm stained with FITC-RCA (200× magnification before printing).

mM sodium phosphate (pH 7.5), and 10% (vol/vol) of 10× buffer stock HEPES (50 mM glucose, 100 mM HEPES, 20 mM KOH). We usually assembled 400-g batches by weighing stock solutions. After being thoroughly mixed, we calculated the volume ratio of the 2-phase system by sampling batches of 5 grams, equilibrated at 20°C. The volume of the system loaded in all chambers was then the estimated amount to maintain the desired volume of the bottom phase (0.7 mL).

We analyzed three different sperm trials (each about 2.5×10^7cells): the acrosome-reacted sample, the DMSO-control sample, and an untreated-control sample. The acrosome-reacted and DMSO-control samples were prepared as described earlier. For the untreated semen sample, aliquots of 0.5 mL of fresh semen were diluted 1:20 with the 2-phase system medium (polymer-free medium) and filtered twice through Millipore disks with pore sizes of 5 μm. Aliquots of 0.5 mL of the samples with about 2.5×10^7 cells were loaded into chambers zero and 30, and 29 transfers were carried out. Thus, we analyzed 2 different cell samples at the same time to allow direct comparison between them. Shaking and centrifugation were each performed for 60 seconds. After the run, the solutions were transformed into a 1-phase system by the addition of 1 volume of the dilution medium (polymer-free medium). The fractions were then collected and the cells counted under a light microscope. All operations were carried out at 20°C.

Partition results are expressed as the number of cells recovered in each fraction. Total viable cells in each fraction were assessed by percent viability × recovered cells in each fraction/100. As a consequence of the separation procedure, populations of sperm cells with a marked affinity for the lower dextran-rich phase (mainly because of low hydrophobicity) partition themselves in the left part of the profile, those that partition themselves almost equally in both phases congregate in the central sector, and those that have a high affinity for the upper PEG-rich phase (mainly because of high hydrophobicity) partition themselves in the right sector of the profile.

Reagents and Media

FITC-lectins were obtained from Vector Laboratories, Burlingame, Calif. Calcium ionophore A23187 (free acid) and DMSO were obtained from Sigma Chemical Company, St Louis, Mo. Dextran T-500 (Mr 500 000) and Ficoll 400 (Mr 400 000) were obtained from Pharmacia, Uppsala, Sweden. Polyethylene glycol (Mr 6 000) was purchased from Serva Feinbiochemica, Heidelberg, Germany. All other chemicals used were of analytical reagent grade.

Statistics

Obtained results are shown as means ± the standard deviation of the number of samples indicated in each case. Statistical analysis of data was carried out by the Mann-Whitney test using InStat software (Macintosh version 2.01) to determine significant differences between samples before and after the acrosome reaction. The correlation of acrosome integrity between RCA labeling and CFDA/PI staining was analyzed by linear correlation.

Results

The ability of 5 lectins (ECL, Jacalin, PNA, PSA, and RCA) to label the surface of the acrosomal region of fresh ram spermatozoa was screened. RCA_{120} displayed the most specific labeling of the acrosomal region of nonpermeabilized sperm stained in suspension. RCA bound intensely to the anterior portion of the acrosomal region (Figure 1b). Figure 1a reveals that not all spermatozoa in the sample were labeled with RCA. In order to prove whether this different labeling corresponded to different acrosomal status, we analyzed the RCA-labeling pattern before and after induction of the acrosome reaction by A23187 and compared it with that detected by the CFDA/PI double-fluorescent staining.

Medium values of 30 different sperm samples (Table) showed that the proportion of acrosome-reacted spermatozoa was slightly increased during the incubation in the absence of ionophore (DMSO-control samples), which accounted for a spontaneous acrosome reaction of 6.85% as assessed by CFDA/PI. The increase in the percentage

*CFDA/PI and RCA staining of fresh spermatozoa and after 1 hour's incubation with DMSO or A23187**

	CFDA/PI			RCA		
Medium	Reacted acrosomes	Spontaneous AR	Induced AR	Labeled acrosomes	Spontaneous AR	Induced AR
Fresh	22.22 ± 5.29		…	22.88 ± 7.08	…	…
DMSO	28.70 ± 6.04	6.85 ± 5.54	…	29.20 ± 7.77	6.95 ± 5.57	…
A23187	84.40 ± 7.51	…	57.7 ± 9.16	87.83 ± 7.41	…	58.63 ± 9.08

*CFDA/PI indicates carboxyfluorescein diacetate/propidium iodide; RCA, *Ricinus communis* agglutinin; AR, acrosome reaction; and DMSO, dimethyl sulfoxide.

of acrosome-reacted spermatozoa was significantly higher ($P < .0001$) after incubation with ionophore (84.4% vs 28.7% in control samples). This value of acrosome-reacted spermatozoa detected by CFDA/PI was not significantly different ($P = .711$; 30 comparisons in 15 different experiments) from the incidence of spermatozoa detected by FITC-RCA labeling. Likewise, the evaluation of the spontaneous acrosome reaction by RCA labeling (6.95%) was not significantly different ($P = .758$) from that assessed by CFDA/PI (6.85%, Table). Therefore, a positive correlation ($r = 0.776$, $P < .0001$) was determined between the percentage of induced acrosome reactions detected by CFDA/PI staining (57.7%) and that detected by RCA labeling (58.6%). The regression line determined by the method of least squares was $y = 0.762x + 14.64$ (Figure 2).

In order to determine whether the status of the acrosome could significantly modify the CCCD behavior of ram spermatozoa, the untreated sperm–control sample, the DMSO–control sample, and that obtained after induction of the acrosome reaction were analyzed by CCCD. Under identical conditions CCCD yields highly reproducible results, as reported here and elsewhere (Albertsson, 1986; Ollero et al, 1994, 1996, 1997b). The distribution profiles presented in this study are representative of 4 different experiments and show important differences in the surface characteristics of spermatozoa before and after the induction of the acrosome reaction by A23187 (Figure 3). A main viable cell-enriched population and some other smaller populations with lower viable cell contents were found in the untreated-control sample (Figure 3a). The profile of acrosome-reacted spermatozoa (Figure 3c) was strongly displaced to the left and showed an important decrease in cell surface heterogeneity because only 1

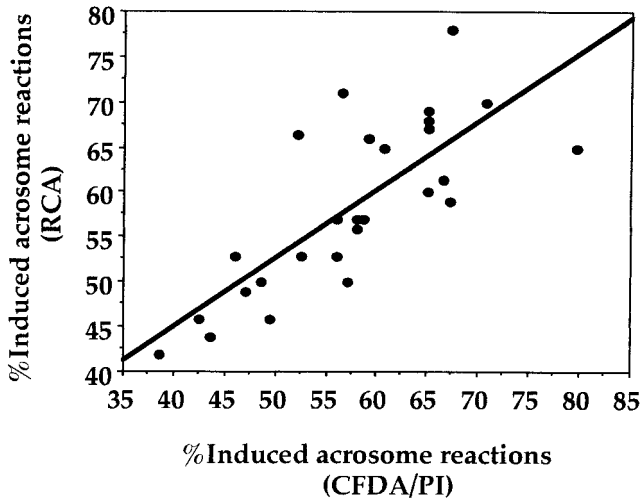

Figure 2. Correlation of induced acrosome reaction as determined by CFDA/PI dyes (abscissa) and FITC-RCA labeling (ordinate).

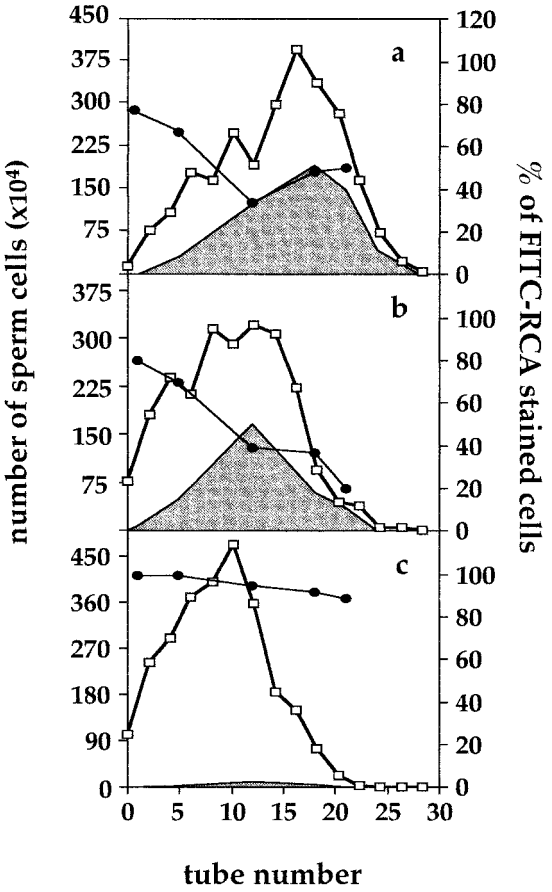

Figure 3. CCCD profile of untreated **(a)**, DMSO-control **(b)**, and induced to acrosome react **(c)** sample. Dark area indicates the total number of viable cells; □, number of cells in each chamber; ●, percentage of FITC-RCA stained cells.

main population was found. Cell viability was almost zero along all profiles whereas a very important viable cell population still remained in the DMSO-control sample (Figure 3b). RCA-lectin binding after CCCD showed that all acrosome-reacted cells were stained (Figure 3c), whereas the total percentage of stained cells in the DMSO-control sample was 46% (Figure 3b) and 42% in the untreated-semen sample (Figure 3a).

Discussion

Although correlations between simple assessment of semen and fertility have been low, promising results have been derived from the assessment of the acrosome reaction in vitro. The relationship between in vitro induction of the acrosome reaction and fertility in bovine semen is sufficiently close (Lenz et al, 1983) for the predictive use of the acrosome reaction to have been suggested for determining the fertility of individual bulls in commercial artificial insemination (Whitfield and Parkinson, 1992).

FITC-PSA has been used to stain ram sperm populations before and after induction of the acrosome reaction (Graham et al, 1990; Cross and Watson, 1994; Gillan et al, 1997; Pérez et al, 1997b; Sukardi et al, 1997). The technique used, ethanol fixation and air drying, likely renders these cells permeable to the lectin conjugates so that both intracellular- and membrane-associated binding must be considered. However, the precise changes after induction of the acrosome reaction in viable (unfixed) sperm has received little attention. The results of this study are, therefore, the first on the relationship between induction of the acrosome reaction in unfixed sperm and the staining in suspension with FITC-RCA on nonpermeabilized ram spermatozoa.

An important aspect of lectin use is the potential problem of lectin toxicity; such toxicity could lead to artifactual cell death as has already been shown (Ashworth et al, 1995). In this study we minimized toxic effects by incubating samples with 5 μg/mL of fluorescein-labeled lectin for 3 minutes. No toxic effects were found in these conditions (data not shown). In fresh semen samples, only some spermatozoa were fluorescent after incubation with FITC-RCA, thus demonstrating clear discrimination between negatively and positively stained spermatozoa (Figure 1). Because spermatozoa have not been ethanol-fixed, this pattern unequivocally corresponds to the surface-labeling pattern of living sperm cells and some dead sperm that may have exposed internal receptors caused by a disruption of the plasma membranes, acrosomal membranes, or both. In this study, sperm viability was examined with 2 supravital stains, an inclusion and an exclusion stain. This use of combination staining allows an accurate definition of a viable sperm, considered here as both intact plasma and acrosomal membranes.

Highly consistent results were obtained after induction of the acrosome reaction by A23187. The double assessment by using the CFDA/PI fluorescent staining and RCA labeling showed a significant correlation between the proportion of spermatozoa that were labeled by FITC-RCA and that of the acrosome-reacted spermatozoa detected by CFDA/PI (Figure 2). These data indicate that galactose groups in ram spermatozoa are intracellular and became exposed to the surface of the cell during the acrosome reaction.

However, even if sperm viability is determined after induction of the acrosome reaction, it does not give an indication of whether the acrosome reaction is physiological (ie, a true acrosome reaction; Didion et al, 1989), or a degenerative loss of the acrosome that occurs after death. These dead spermatozoa can obscure the physiologic acrosome reaction and could lead to an erroneous interpretation of experimental results. Incubation of control samples containing DMSO instead of ionophore allows determination of the degenerative acrosomal loss, which would be beneficial in determining the percentage of spermatozoa undergoing the acrosome reaction. Our results confirmed that some fresh ram spermatozoa spontaneously underwent the acrosome reaction when incubated at 39°C for 1 hour in buffer (Table). The percentage of lost acrosomes was about 7% assessed by either CFDA/PI fluorescent staining or FITC-RCA labeling. The incidence of acrosome-reacted cells significantly increased after incubation with ionophore. Assessment of induced acrosome reactions by CFDA/PI staining very significantly correlated with assessment by FITC-RCA labeling.

It is worth noting that the number of sperm with negative FITC-RCA fluorescence may also include nonviable, acrosomeless sperm. Unlike intact sperm in which the acrosome reaction has been induced, nonviable, acrosomeless sperm are PI-positive. Inclusion of these sperm in the total number of FITC-RCA positive cells may result in an increase in the scatter of the correlation between FITC-RCA and CFDA/PI fluorescence. Studies are now underway to determine the percent of acrosomeless sperm in our preparation using simultaneous FITC-RCA and PI labeling.

Given the capacity of CCCD in aqueous 2-phase systems to analyze sperm surface changes, taking into account loss of viability (Pascual et al, 1993; Ollero et al, 1996, 1997a, 1997b) and different states of maturity (Ollero et al, 1994), we were able to study the induction of the acrosome reaction by ionophore A23187 by assessing changes in ram sperm partition behavior. As we have proven, ram spermatozoa incubated in the acrosome-reaction medium have different partition behaviors when

either DMSO (Figure 3b) or ionophore was added (Figure 3c). The loss of acrosome induces a significant displacement to the left of the CCCD profile with an important decrease in cell heterogeneity. During the acrosome reaction, fusion of the plasma membrane with the acrosomal vesicular membrane takes place, the intracrosomal content is released, and the inner side of the vesicular membrane is exposed to the extracellular medium. Therefore, the number of saccharide residues exposed to the phase system should change as a consequence of the loss of acrosome, thus accounting for a different CCCD distribution. Because the low phosphate concentration in the 2-phase system we used in our experiments could result in a relatively charge-insensitive partition of cells (Albertsson, 1986; Cartwright et al, 1991), the different partition behavior must be interpreted as a change in hydrophobicity of the spermatozoa surface by induction of the acrosome reaction. A higher affinity for the lower dextran-rich phase would indicate a decreased hydrophobicity of the acrosome-reacted sperm surface. The saccharide residues exposed to the medium as a consequence of the acrosome reaction would decrease the hydrophobicity of the sperm cells, as has already been described (Amann et al, 1993); however, these results are in disagreement with our previous report in which we had shown that loss of acrosome did not appear to significantly affect the partitioning of ram spermatozoa (Pascual et al, 1996). This contradiction could be the result of differences in the experimental conditions because, in that paper, not only had the spermatozoa been washed with Percoll gradient, but the phosphate concentration in the 2-phase system was also higher (7 mM), which increased the partition that occurred in the upper PEG-rich phase.

The DMSO-control sample (Figure 3b) showed different surface characteristics from the untreated sample (Figure 3a), although cell viability of both samples was closed. This observation could be interpreted as the effect of the incubation at 39°C for 1 hour on the DMSO-control sperm sample as well as the different medium in which the samples were diluted (the 2-phase system medium for the untreated–control sample and the acrosome-reaction–induction medium for that of the DMSO-control). This affects cell partitioning but to a much lesser extent than that produced by the loss of the acrosome.

In fact, confirmation of the effect of acrosomal loss within the sperm surface characteristics could be further achieved by estimating the RCA-labeling pattern along CCCD chambers. Changes in lectin reactivity of acrosome-reacted spermatozoa indicate the recognition of some deep membrane or intracellular residues that affect partitioning. From these results it can be concluded that analysis of ram sperm surface characteristics by CCCD allows assessment of semen quality on the basis of the rule that the more the cells are fractionated to the right, the more the integrity of the acrosome is protected and, hence, more capacity for fertilization could be expected.

This study also demonstrates that FITC-RCA–lectin binding can be a useful method for assessing the acrosomal status of fresh ram spermatozoa in suspension. This technique is the only described method by which the acrosomal status of unfixed ram spermatozoa can be assessed. Because it is simple and reliable, this acrosomal labeling technique should have broad applications in practical use such as the evaluation of sperm quality for cryopreservation or artificial insemination. Likewise, its application in research will enable sperm biologists to make more rapid progress in investigations of acrosomal physiology in the ram.

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