Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use?

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The acrosome reaction (AR), an essential step for achieving mammalian fertilization, was recently introduced as a means of clinical evaluation of male fertility. However, most of the available techniques for acrosomal status assessment (except those employing electron microscopy) do not define whether the measurements represent partial or complete AR. We, therefore, performed a crossover investigation of the types of inducers and probes required for detecting partial or complete AR in human spermatozoa. The acrosomal status before and after stimulation with four AR inducers was evaluated after incubation for 3 h in capacitating conditions. We used a fluorescence-activated cell sorter with fluorescein isothiocyanate-conjugated monoclonal antibody CD46 (FITC–CD46) targeting the inner acrosomal membrane for detecting a complete AR, and fluorescein isothiocyanate–Pisum sativum agglutinin (FITC–PSA) targeting the acrosomal content for detection of both partial and complete AR. Without stimulation or following stimulation with progesterone, follicular fluid (FF) or phorbol myristate ester (PMA), the AR could be detected with FITC–PSA but not with FITC–CD46. Following stimulation with the calcium ionophore A23187, the AR could be detected by both FITC–PSA and FITC–CD46. These results suggest that spontaneous AR as well as AR induced by progesterone, PMA and FF are partial. In contrast, the AR induced by A23187 is total, i.e. both partial and complete. These findings are valuable for both research and clinical purposes and are a step towards an international agreement on a standard test for human sperm AR, for which there is an urgent need.

Key words: A23187/acrosome reaction/capacitation/CD46/human spermatozoa

Introduction

One of the key processes in human fertilization is the acrosome reaction (AR), usually triggered in spermatozoa upon their binding to the zona pellucida of the egg. The AR involves fusion between the plasma membrane and the underlying outer acrosomal membrane, as a result of which the acrosomal contents are released, including a variety of hydrolytic enzymes that enable the penetration of the spermatozoon to the egg (Wassarman, 1987; Kopf and Gerton, 1991; Yanagimachi, 1994). A sperm population that has undergone the AR usually contains cells at different stages of AR: some cells have released only a part of their acrosomal contents (defined as partial AR), others have fully released their acrosomal contents and their inner acrosomal membrane has become exposed (defined as complete AR) (Stock and Fraser, 1987; Kohn et al., 1997).

An ejaculated sperm population or spermatozoa incubated under capacitating conditions always contains a low level of acrosome-reacted spermatozoa, defined as spontaneous acrosome-reacted cells (Stock and Fraser, 1987). Cells that have the capacity to undergo the AR (capacitated spermatozoa; Yanagimachi, 1994) can be induced to do so, not only by the zona pellucida, but also by other agents, both natural (i.e. known to be present in the body) and pharmacological. Progesterone (Osman et al., 1989; Baldi et al., 1991; Tesarik et al., 1992; Meisel, 1995) and follicular fluid (FF) (Suarez et al., 1986; De Jonge et al., 1993) belong to the first group. The pharmacological inducers involve the Ca²⁺ ionophore A23187 (Aitken et al., 1984; Cummins et al., 1991), phorbol myristate ester (PMA) (Rotem et al., 1992; Bielfeld et al., 1994; Parinaud et al., 1995), cAMP analogues (De Jonge et al., 1991), protein kinase C stimulators (Rotem et al., 1992), pentoxifylline (DasGupta et al., 1994) and platelet-activating factor (Krausz et al., 1994).

The most frequently used techniques to measure AR are based on light microscopy, in which the acrosomal content is labelled either with a triple staining techniques (Talbot and Chacon, 1981; De Jonge et al., 1989) or with fluorescent lectins such as fluorescein isothiocyanate–Pisum sativum agglutinin (FITC–PSA) (Cross et al., 1986) or FITC–peanut agglutinin (FITC–PNA) (Kallajoki et al., 1986; Mortimer et al., 1987; Aitken and Brindle, 1993), and the AR is assessed by label disappearance (negative staining). In another method (positive staining), the inner acrosomal membrane (not the acrosomal content) is stained either by concanavalin A (Holden et al., 1990) or by specific monoclonal antibodies (Fenichel et al., 1989, 1990; D’Cruz and Hass, 1992; Dorjee et al., 1997). Consequently, the negative-staining techniques detect cells...
that have undergone both partial and complete AR, whereas positive-staining techniques identify only cells that have undergone a complete AR (Parinaud et al., 1993; Kinger and Rajalakshmi, 1995; Emiliozzi et al., 1996; Kohn et al., 1997).

Although AR assessment is widely used in research of sperm function, there appears to be no consensus with respect to a standard clinical test for measuring the AR in human spermatozoa as indicated recently by the Andrology Special Interest Group (ASIG) of the European Society of Human Reproduction and Embryology (ESHRE) (Mortimer and Fraser, 1996). The AR inducers and probes used by various groups as well as the conditions employed for achieving sperm capacitation vary to a large extent. Different inducers potentiate different levels of AR (Bielfeld et al., 1994; Parinaud et al., 1995; Emiliozzi et al., 1996) and different AR probes may give different results (Aitken and Brindle, 1993; Kinger and Rajalakshmi, 1995; Amin et al., 1996; Kohn et al., 1997), thus there are often conflicting reports from various groups. Furthermore, since electron microscopic studies have indicated that every sperm population is heterogeneous with respect to its acrosomal status (Stock and Fraser, 1987; Köhn et al., 1997), it is important to define whether an AR measurement represents spermatozoa which underwent partial or complete AR. We, therefore, initiated a crossover study to investigate the types of inducers and probes required for the detection of spermatozoa with different stages of AR status without using electron microscopy. Since ZP3 is not yet readily available in most research and clinical laboratories, we chose progesterone and FF to represent the group of natural inducers. The Ca²⁺ ionophore A23187 and PMA were selected as pharmacological inducers. As a positive-staining probe for detecting a complete AR we used the monoclonal antibody FITC–CD46 that recognizes an antigen on the inner acrosomal membrane (Anderson et al., 1989; D’Cruz and Hass, 1992; Tao et al., 1993a). As a negative-staining probe for detecting both partial and complete AR, we used FITC–PSA (Cross et al., 1986).

Materials and methods

Chemicals

A23187, progesterone, anti-mouse immunoglobulin (IgG, bovine serum albumin (BSA, fraction V powder), dimethyl sulphoxide (DMSO), pyruvic acid, propidium iodide, HEPES, and FITC–PSA were obtained from Sigma Chemicals Company (St Louis, MO, USA). PMA was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). FITC–conjugated mouse anti-human CD46 and IgG were purchased from Serotec, Oxford, UK. Percoll was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were from Merck, Germany.

Sperm preparation

Human semen samples were obtained from healthy donors with normal sperm density, motility and morphology according to World Health Organization guidelines (WHO, 1992) after 3 days of sexual abstinence. Samples were allowed to liquefy for 30–60 min at room temperature. Every sperm sample was analysed for percentage motile cells using a Makler counting chamber (Sei Medical Instruments Ltd, Haifa, Israel) and a computerized sperm analysis software program (Galal, Israel). Motile spermatozoa were then isolated using a simple discontinuous Percoll gradient of 95 and 47.5% (Draveland and Mortimer, 1985). Following centrifugation for 20 min at 300 g, the 95% percoll fraction was recovered and the spermatozoa were washed twice (300 g, 10 min) with Biggers–Whitten–Whittingham (BWW) medium (95 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate, 25 mM NaHCO₃, pH 7.4; Biggers et al., 1971), supplemented with HEPES (10 mM, pH 7.4) and 3 mg/ml BSA. The supernatants were discarded, the final pellets were resuspended in BWW medium, and the sperm concentration was adjusted to 2×10⁷ cells/ml.

Capacitation and induction of acrosome reaction

Sperm suspensions were incubated in BWW supplemented with HEPES (10 mM, pH 7.4) and 3 mg/ml BSA under an atmosphere of 5% CO₂ at 37°C for 3 h for capacitation. Our earlier results have indicated that a period of 3 h of incubation under capacitating conditions is sufficient for achieving a maximal steady-state level of capacitated spermatozoa (Cohen-Dayag et al., 1995; Jaiswal et al., 1998). For AR induction, A23187, PMA, or progesterone (each in a stock solution of 5 mM in DMSO) was added to the capacitated sperm suspensions to give a final concentration of 10 µM (and 0.2% DMSO). Human FF (1%) was used as an inducer after acetone precipitation (Ralt et al., 1994). DMSO (0.2%) was added to sperm suspensions in control incubations. Tubes were loosely capped and incubated at 37°C and 5% CO₂ for 30 min. After incubation with the AR inducer, the spermatozoa were washed with phosphate-buffered saline (PBS) by centrifugation at 300 g for 10 min. The supernatants were aspirated and the pellets were resuspended in PBS. These sperm suspensions were then assessed for percentage motility and processed for measuring AR either by FITC–CD46 with a fluorescence-activated cell sorter (FACS) or by FITC–PSA with a fluorescence microscope.

Evaluation of the acrosomal status with FITC–PSA

Acrosome-reacted spermatozoa were identified by the acrosomal marker FITC–PSA essentially as described by Cross et al. (1986), with the modifications introduced by Tesarik et al. (1993). Briefly, an aliquot (30 µl) of spermatozoa (2×10⁷ cells/ml) was smeared onto the centre of a glass slide and allowed to air-dry. The spermatozoa on the slide were then permeabilized by methanol for 30 s at room temperature, washed with double-distilled water, dried at room temperature, incubated for 10 min with 50 µl of FITC–PSA (100 µg/ml) at room temperature in the dark, and washed with double-distilled water. The slide was then fixed with 2% formaldehyde for 15 min at room temperature in the dark, washed with double-distilled water, and then covered with a mounting fluid (Elivonol) and a cover glass. Each determination was carried out in duplicate. The slides were inspected using Zeiss Axiosvert 35 microscope equipped with a ×100 oil objective. As many as 200 cells were counted on each slide in a blind fashion. Sperm motility was used as a marker for cell viability. Because the percentage of motile spermatozoa was high (>75%) and similar before and after incubation with the AR inducers, there was no need to use supravital staining (Mortimer and Fraser, 1996).

Evaluation of acrosomal status by FACS using FITC–CD46

Spermatozoa were first incubated for 30 min at room temperature with anti-mouse IgG (0.22 mg protein/ml) in order to block non-specific sites, followed by washing the blocker off with PBS at 300 g for 10 min. The pellet was resuspended in PBS and divided into two aliquots. To one aliquot 1:50 diluted FITC–CD46 was added, and to the other aliquot 1:50 diluted FITC–conjugated mouse anti-human IgG was added as a negative control. Following 30 min incubation at room temperature in the dark, the spermatozoa were
washed with PBS by centrifugation for 10 min at 300 g, and resuspended in 1 ml PBS containing 1% formaldehyde. Immediately prior to FACS analysis, the supravital probe propidium iodide (Tao et al., 1993a) (2.5 µg/ml final concentration) was added. Samples of 10 000 cells (considered as 100%) were then analysed by a FACSScan (Beckton Dickenson).

**Evaluation of acrosomal status with FITC–CD46 using FACS in parallel with fluorescence microscopy**

We verified that the two methods, described above, yield comparable results by running four experiments in which the levels of AR were determined simultaneously by FACS and fluorescence microscopy. Using FITC–CD46 with FACS of 10 000 cells, we found spontaneous AR of 0.47 ± 0.29% (mean ± SEM) and A23187-induced AR of 11.6 ± 2.1%. Similar levels of acrosome-reacted spermatozoa were detected using the same probe with fluorescence microscopic examination of 200 sperm cells (0.12 ± 0.07 and 9.9 ± 1.4% respectively) (not significant). These results are in agreement with earlier studies, which demonstrated that the level of AR detected by FACS is similar to that found by fluorescence microscopy with FITC–CD46 (Tao et al., 1993b) or with FITC–PSA (Miyazaki et al., 1990; Uhler et al., 1993).

**Statistical analysis**

InStat 2.01 software package (Graph Pad software, San Diego, CA, USA) was used for statistical calculations. The significance of the difference between the treatments was calculated by Friedman non-parametric repeated measures test followed by Dunn’s multiple comparisons test, or by Student’s t-test, as indicated.

**Results**

We compared progesterone and the Ca²⁺ ionophore A23187 as AR inducers by measuring the level of complete AR that they induce in live cells. As shown in Figure 1 which contains typical FACS results of a given human sperm sample with FITC–CD46, only A23187 caused a remarkable increase in the level of completely acrosome-reacted cells (compare the lower right quadrants in panels A–C). Although the AR levels varied within and between donors, similar results were obtained in all the samples tested, summarized in Table I. As shown in the table, A23187 significantly increased the fraction of completely acrosome-reacted cells over the control level of spontaneous AR ($P < 0.001$ according to Dunn’s multiple comparisons test). The increase caused by progesterone was much smaller ($P \approx 0.06$).

In addition to progesterone and the calcium ionophore, we compared another natural inducer, FF (Suarez et al., 1986; De Jonge et al., 1993), and another known pharmacological inducer, PMA (Rotem et al., 1992; Bielfeld et al., 1994; Parinaud et al., 1995). This was done by simultaneously comparing the AR levels in sperm samples from five donors using four inducers and two detection probes to distinguish between complete and both partial and complete AR (Figure 2). Although the results varied from donor to donor, in all the sperm samples, a complete AR (measured with FITC–CD46) was induced only by A23187 (Figure 2A). The levels of AR induced by progesterone, FF, and PMA (0.07–1.95%) were similar to the control level of spontaneous AR (0.06–0.90%). The AR level measured with FITC–PSA in the same sperm samples (Figure 2B) was significantly higher than that measured with FITC–CD46 (Figure 2A; $P < 0.0001$ for the spontaneous AR and for progesterone induction, and $P \approx 0.003$ for induction by any one of the other inducers, according to Student’s t-test). The level of spontaneous AR as measured by FITC–PSA was 6.2–8.2%. As before, of the four tested AR inducers, A23187 was the most effective. However, unlike in the case of FITC–CD46, with FITC–PSA as a probe, the other inducers also induced the AR, though at a lower level. Since the FITC–CD46 probe that detects complete AR failed to detect acrosome-reacted cells with progesterone, PMA and FF, we conclude that incubation with these inducers results in incomplete AR. Since the level of AR detected by PSA after
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Table I. Induction of complete acrosome reaction in human spermatozoa by A23187 or progesterone, measured by FACS with FITC–CD46

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Exp. no.</th>
<th>Control</th>
<th>A23187</th>
<th>Progesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0.00</td>
<td>8.2</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.05</td>
<td>11.8</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.06</td>
<td>7.6</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.20</td>
<td>19.7</td>
<td>0.81</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>0.25</td>
<td>11.6</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.00</td>
<td>16.9</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.28</td>
<td>12.3</td>
<td>0.23</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>0.00</td>
<td>12.0</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.11</td>
<td>11.9</td>
<td>0.59</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>0.08</td>
<td>43.9</td>
<td>0.23</td>
</tr>
<tr>
<td>V</td>
<td>11</td>
<td>0.51</td>
<td>25.5</td>
<td>1.10</td>
</tr>
</tbody>
</table>

*Each experiment was carried out with a different semen sample.

A23187 induction was ~2-fold higher than the level measured by FITC–CD46 ($P < 0.005$), it appears that, after A23187 induction, ~50% of the cells are partially acrosome-reacted and ~50% are completely acrosome-reacted.

Discussion

Correct assessment of the level of acrosome-reacted cells in a sperm population is of great importance for both basic research of mammalian reproduction and for clinical evaluation of male fertility (Cummins *et al.*, 1991; Fenichel *et al.*, 1991; Ohashi *et al.*, 1992; Pilikian *et al.*, 1992; Yovich *et al.*, 1994; Parinaud *et al.*, 1995). However, most studies, excluding those employing transmission electron microscopy, have not distinguished between complete and partial AR. Here we demonstrated that, after 3 h of capacitation, (i) a spontaneous AR results in partially acrosome-reacted cells only (Table I and Figures 1 and 2); (ii) progesterone, PMA and FF induce only partial AR; and (iii) A23187 induces both partial and complete AR at a ratio of ~1:1.

Our study, which is the first crossover investigation carried out with two different probes and four different inducers (representing both natural and pharmacological inducers) (Table I and Figure 2), endorsed previous suggestions that probes targeting the inner acrosomal membrane, like FITC–CD46, identify only spermatozoa that have undergone a complete AR (Parinaud *et al.*, 1993; Emiliozzi *et al.*, 1996; Köhn *et al.*, 1997), whereas probes targeting the acrosomal content, such as FITC–PSA, detect both completely and partially acrosome-reacted spermatozoa (Aitken and Brindle, 1993; Köhn *et al.*, 1997). Our study also confirmed earlier observations that different inducers potentiate different levels of AR (Bielfeld *et al.*, 1994; Parinaud *et al.*, 1995; Emiliozzi *et al.*, 1996). Furthermore, it provided an explanation for these differences, demonstrating that some inducers (e.g., progesterone, PMA and FF) trigger only partial AR, whereas others (e.g., A23187) induce both complete and partial AR (Table I; and Figures 1 and 2). This appears to be the reason why, in earlier studies, A23187 always yielded higher AR levels than progesterone, FF or PMA (Parinaud *et al.*, 1995; Emiliozzi *et al.*, 1996). This also explains why, when the AR level was measured with CD46, progesterone was wrongly concluded to have no measurable effect on the AR level (Carver-Ward *et al.*, 1996).

The findings of this study may be exploited for defining...
which probes and inducers should be used for determining partial or complete AR without a need for electron microscopy. Thus, for potentiating partial AR, inducers such as progesterone, PMA or FF should be used, and the measurement should be carried out with probes that target the acrosomal content. e.g. FITC–PSA. For inducing a complete AR, A23187 should be used. With this inducer, using probes that target the inner acrosomal membrane, e.g. FITC–CD46, will detect only the completely acrosome-reacted cells; while probes such as FITC–PSA will detect both partially and completely acrosome-reacted cells. The results obtained with each of these two probes, PSA and CD46, were demonstrated to be independent of the measuring technique, FACS or a fluorescence microscope [(Miyazaki et al., 1990; Tao et al., 1993b; Uhler et al., 1993) and our own results with CD46 (see Materials and methods)]. It should be emphasized that the conclusions made herein with respect to the various inducers apply to spermatozoa preincubated for 3 h under capacitating conditions. It was reported that progesterone added after longer periods of capacitation (20–24 h) (Meizel et al., 1997) or at concentrations as high as 1 mM (Parinaud et al., 1997) induced some spermatozoa to undergo AR, as detected by positive-staining probes that measure complete AR.

The availability of convenient probes and inducers for complete and partial AR, as revealed in this study, makes it possible to address questions such as whether sperm penetration into the egg requires a complete or partial AR. This is also a step towards an international agreement on a standard test for human sperm AR, for which there is an urgent need, especially for diagnostic purposes.

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References
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