Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin

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Summary. When western blots of human sperm proteins solubilized by acid extraction (presumably mainly acrosomal proteins) or by sodium dodecyl sulfate (SDS) were probed with biotin-conjugated *Pisum sativum* agglutinin (PSA), distinct sets of proteins were labelled in both preparations. When smears of human spermatozoa were treated with methanol either for 30 s or for 15 min and then exposed to FITC-conjugated PSA, the resulting fluorescence pattern essentially depended on the time of methanol treatment. With the longer treatment, fewer spermatozoa showed selective acrosomal labelling and more were labelled uniformly throughout, without a clear predilection for a single sperm region. With the shorter time of methanol treatment, the poorly topographically differentiated, whole-cell labelling was typical of dead spermatozoa as confirmed by a close correlation between the percentages of spermatozoa showing this type of labelling and of those stained supravitaly with Hoechst 33258. The preferential whole-cell labelling of dead spermatozoa with PSA is considered to be due to increased availability of the nonacrosomal set of PSA-reactive sites in dead spermatozoa after a short treatment with methanol, whereas this treatment is probably not sufficient to expose most of these sites when applied to living spermatozoa. The simplicity of the staining protocol makes this method feasible in routine work in a number of clinical and research applications.

Keywords: acrosomal staining; acrosome reaction; sperm viability; human

Introduction

The acrosome reaction of mammalian spermatozoa is a calcium-dependent exocytotic process that is necessary for fertilization (reviewed by Yanagimachi, 1988). A better knowledge of the phenomena occurring during the human acrosome reaction is important for understanding both the mechanisms controlling normal human fertilization and the causes of its defects. Accordingly, the frequency with which spermatozoa from a given sample undergo the acrosome reaction represents an important parameter in the clinical evaluation of sperm function (Tesarik & Testart, 1989).

A major problem in the study of the acrosome reaction of human spermatozoa is that the acrosome loss cannot be observed on living spermatozoa by phase contrast or differential interference contrast microscopy as it can be in spermatozoa of some other mammalian species

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possessing large acrosomes, such as guinea-pigs or hamsters. Electron microscopy may be used to assess the acrosomal status of human spermatozoa (e.g. Tesarik, 1985; Stock & Fraser, 1987; Yudin *et al.*, 1988), but the technique is too laborious to be applied in routine examination of spermatozoa. Consequently, various light microscope techniques (bright-field or fluorescence) using acrosomal staining with histological dyes (Talbot & Chacon, 1981), lectins (Talbot & Chacon, 1980; Cross *et al.*, 1986; Mortimer *et al.*, 1987) and monoclonal antibodies (Byrd & Wolf, 1986; Kallajoki *et al.*, 1986; Moore *et al.*, 1987) have been developed. However, all these techniques are burdened by the problem of distinction between degenerative acrosome loss accompanying sperm cell death and the true acrosome reaction. This problem has been dealt with by combining the acrosome staining with other methods that claim to distinguish between living and dead spermatozoa (for recent reviews, see Cross & Meizel, 1989; Wolf, 1989). Even though such combined protocols apparently improve the correlation of estimates of acrosome reaction with those obtained by electron microscopy (Talbot & Chacon, 1981; Thomas & Meizel, 1989), the inclusion of an additional stain makes the procedure longer and more complicated. Moreover, the addition of centrifugation steps required for the supravital staining reduces the yield of spermatozoa for smear preparation, which is inconvenient in cases where only a limited number of spermatozoa are available for this kind of examination.

In this study we describe a method for distinguishing between the true acrosome reaction and a degenerative acrosome loss in human spermatozoa using a one-step staining with fluorescein-labelled *Pisum sativum* agglutinin (PSA). Sperm compounds showing an affinity for this lectin were analysed by western blotting and the ability of the PSA stain to mark selectively dead spermatozoa under certain conditions was assessed by examining the correlation between the estimates of percentages of dead cells using PSA and those obtained using supravital staining with Hoechst 33258.

Materials and Methods

Source and preparation of spermatozoa

Human semen samples were obtained from 25 healthy donors with normal parameters of sperm density, motility and morphology. The samples were left to stand for 30 min at room temperature to liquefy. Aliquots of semen samples were diluted with an equal volume of Biggers, Whitten and Whittingham (BWW) medium (Biggers *et al.*, 1971) and centrifuged at 500 *g* for 5 min. The supernatant was discarded and the sperm pellet was resuspended in 5 ml BWW and centrifuged again under the same conditions. The supernatant was discarded and the pellet of the washed spermatozoa was overlaid with 1 ml B2 medium (Api System, Montalieu-Vercieu, France). Spermatozoa were allowed to swim up to this medium during a 30 min incubation at 37°C in an atmosphere of 5% CO₂ in air. The migrated spermatozoa were then recovered from the tubes with the upper 0.5 ml of medium and incubated under the same conditions for an additional 4 h.

Induction of the acrosome reaction

The acrosome reaction was induced by ionophore A23187 (Sigma, La Verpillière, France) after incubation of spermatozoa for 4 h in B2 medium. A stock solution of 20 mmol ionophore A23187 l⁻¹ dimethylsulfoxide (Sigma) was prepared and added to sperm suspensions to give a final concentration of 10 µmol l⁻¹. Spermatozoa were then incubated with the ionophore at 37°C under 5% CO₂ in air for 30 min.

Extraction of sperm proteins

For quantitative protein extraction, pellets of washed spermatozoa were incubated for 15 min at room temperature in 300 µl of a mixture consisting of 2% (w/v) SDS (Sigma), 10% (v/v) glycerol and 0.1 mol Tris HCl buffer l⁻¹ (pH 6.8). Samples were then centrifuged at 10 000 *g* for 10 min, supernatants were decanted and used in individual experiments.

Acid extraction was used to obtain preparations enriched in acrosomal proteins. It was performed using a slight modification of the method used for the extraction of boar acrosomal proteins (Zelezna & Cechova, 1982; Cechova *et al.*, 1984). Briefly, pellets of washed spermatozoa were incubated for 16 h at 4°C in a solution containing 3% (v/v) acetic acid, 10% (v/v) glycerol and 10 mmol benzamidine l⁻¹, followed by high-speed centrifugation (4°C) and the supernatants containing solubilized acrosomal proteins were decanted.

Protein concentration in sperm extracts was determined using the method of Bradford (1976). It ranged from 0.8 mg ml⁻¹ to 2.7 mg ml⁻¹ in individual preparations.

Electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% slab gels according to the method of Laemmli (1970). Each sample was electrophoresed as duplicate aliquots on two adjacent gels. After SDS-PAGE, one of these gels was used for blotting, while the other was stained with Coomassie brilliant blue G. The apparent molecular masses of proteins from individual samples were estimated by comparing with the protein standards that were run in parallel. Western blotting was carried out as described by Towbin *et al.* (1979). Proteins were electrotransferred from polyacrylamide gels into Hybond C nitrocellulose membranes (Amersham, UK). The membrane sheets were incubated with biotin-conjugated PSA (3 µg ml⁻¹) (Sigma) in phosphate-buffered saline (PBS) followed by streptavidin-peroxidase complex (Amersham) and bound peroxidase was visualized using diaminobenzidine (Sigma) as chromogen.

Sperm staining with FITC-labelled PSA

Aliquots of sperm suspensions were used to prepare smears on microscope slides. Care was taken to remove the original culture medium from the suspensions used for smear preparation (at least two cycles of centrifugation and resuspension in protein-free PBS of BWW) because traces of proteins on smears react with fluorescein isothiocyanate (FITC) labelled PSA and produce a strongly fluorescent background which makes the evaluation of sperm staining impossible. After air-drying, sperm smears were dipped in absolute methanol for either 30 s or 15 min and allowed to dry rapidly. Such smears were either processed immediately or kept in a dry place at room temperature for up to 1 week without any disturbance of the results of subsequent staining. Methanol-treated smears were incubated for 30 min at room temperature in a moisture chamber with a solution of FITC-conjugated PSA (50 µg ml⁻¹) (Sigma) in PBS. The slides were then washed in distilled water to remove unbound probe. This washing represents another critical step in the protocol because insufficiently washed preparations are difficult to assess. For good results, slides must be rinsed in a stream of water and then dipped in excess water for at least 15 min. After drying, smears were examined immediately, without mounting, in an epifluorescence microscope. For good staining results, preparations must be examined on the day of staining, since unmounted slides rapidly lose the staining specificity.

Supravital staining with Hoechst 33258

Suspensions of washed spermatozoa were incubated for 5 min at room temperature with the Hoechst 33258 dye (bis benzimide; Sigma) diluted in PBS at a concentration of 1 µg ml⁻¹. The free dye was washed from sperm suspensions by two cycles of centrifugation (500 g for 10 min) and the pellet resuspended in fresh PBS. These spermatozoa were then used immediately for smear preparation.

Quantitative analysis

The proportions of spermatozoa showing different topographical patterns of fluorescent staining (see below) were determined in smears. Each slide was examined by two independent observers and 200 spermatozoa were counted by each observer. The mean value from the two examinations was calculated.

Statistical analysis

Percentages of spermatozoa showing different staining patterns in individual experiments were compared, after arcsine transformation, by *t* test. Correlation between staining patterns was evaluated by linear regression analysis.

Results

Reactivity of sperm proteins with PSA in western blots

When western blots of sperm proteins were probed with PSA, the reactivity pattern strongly depended on the method of protein extraction used. In blots of acid-extracted sperm proteins (preferentially acrosomal proteins), most proteins reactive with the probe had an apparent molecular mass greater than 50 kDa, with the strongest labelling in a protein of 53–55 kDa (Fig. 1, lane a). When spermatozoa previously subjected to acid extraction were re-extracted with SDS, a

quite different set of proteins was labelled, with the exception of the 53–55 kDa protein that also appeared in this type of extract (Fig. 1, lane b). A similar PSA-binding pattern was observed when washed spermatozoa were subjected directly to SDS extraction without previous acid extraction (data not shown). The difference in the PSA-binding pattern of the two types of sperm extract shows that the lectin can bind to many proteins other than acrosomal proteins of human spermatozoa provided that these proteins are rendered accessible. The poor discrimination of individual bands in blots incubated with PSA is in accordance with the presumed glycoprotein nature of the labelled proteins whose migration in the gel might be influenced by a variable degree of glycosylation.

Fig. 1. Reactivity of *Pisum sativum* agglutinin (PSA) with human sperm proteins solubilized by different extraction procedures. Washed spermatozoa were subjected to acid extraction and the remaining pellet was re-extracted with sodium dodecyl sulfate (SDS). The amount of protein corresponding to 1×10^6 extracted spermatozoa (about 5 μ g protein in 8 μ l of extract) was loaded per lane and run on SDS-PAGE. After transblotting to nitrocellulose, proteins of both the acid extract (a) and those re-extracted with SDS (b) were probed with biotin-conjugated PSA. A major PSA-reactive protein (53–55 kDa) appearing in both types of extract is indicated by an arrow. Molecular mass standards (kDa) are indicated in (a).

Fluorescent staining patterns of permeabilized spermatozoa incubated with FITC-labelled PSA

There were four basic staining patterns after the exposure of smears of permeabilized spermatozoa to FITC-labelled PSA. These patterns were characterized by differential binding of the probe (i) to the sperm acrosomal region, and (ii) to the rest of the spermatozoa (Fig. 2). The strongly labelled acrosomal region revealed the presence of the acrosome, whereas the absence of acrosomal staining or its confinement to the equatorial segment were signs of acrosome loss, a relationship that has been demonstrated previously (Cross *et al.*, 1986). In both categories, however, some spermatozoa showed a considerable staining intensity in the rest of the sperm cell, whereas others did not (Fig. 2). Accordingly, the four staining patterns were defined as follows: pattern 1, selective staining of the whole acrosome (Fig. 2a, b); pattern 2, no staining at all or staining limited to the equatorial acrosomal segment (Fig. 2c); pattern 3, an almost uniform staining of the whole sperm cell (Fig. 2a); and pattern 4, an almost uniform staining of the whole spermatozoa but for the acrosome (Fig. 2b).

Fig. 2. Fluorescent patterns of spermatozoa stained with fluorescein isothiocyanate (FITC) conjugated *Pisum sativum* agglutinin. (a) Patterns 1 (arrowhead) and 3 (arrow); (b) patterns 1 (arrowhead) and 4 (arrow); (c) pattern 2 represented by a spermatozoon with residual equatorial staining (arrowhead) and an unstained spermatozoon (arrow). ($\times 900$).

Relationship between the occurrence of different PSA staining patterns and the degree of sperm permeabilization

When permeabilization of spermatozoa was carried out by exposure of dried sperm smears to methanol for 30 s, most spermatozoa showed patterns 1 or 2 of PSA staining and only a few showed patterns 3 or 4; however, the percentage of spermatozoa showing patterns 3 and 4 of PSA staining increased considerably when the methanol treatment was prolonged to 15 min (Fig. 3).

Fig. 3. Representation of individual fluorescent staining patterns in the same sperm populations incubated for *in vitro* capacitation and permeabilized with methanol for either (▨) 30 s or (■) 15 min. Data are means $\pm$ SEM for five sperm samples.

Relationship between whole-cell staining with PSA and cell death as monitored with Hoechst 33258

When spermatozoa were exposed supravitaly to Hoechst 33258, smeared on slides, permeabilized with methanol for 30 s and stained with FITC-labelled PSA (Fig. 4), virtually all those accepting the supravital nuclear stain showed patterns 3 or 4 of staining with the lectin. In the evaluation of the relationship between the type of PSA staining and the quantitative representation of Hoechst-positive spermatozoa, percentages of spermatozoa showing patterns 3 and 4 of PSA staining were thus pooled and the resulting sperm subpopulation is referred to as whole-cell staining with PSA in this part of the analysis. When the percentage of spermatozoa showing this whole-cell staining with PSA was plotted against the percentage of spermatozoa in the same samples stained supravitaly with Hoechst 33258, similar values on both axes were read and a strong positive correlation ($r = 0.98$) was obtained (Fig. 5). By contrast, percentages of spermatozoa showing the whole-cell staining with PSA exceeded considerably those of spermatozoa stained supravitaly with Hoechst 33258 when the time of methanol treatment was prolonged to 15 min (Fig. 5), and the correlation between the two variables was weak ($r = 0.50$).

Fig. 4. Double-fluorescence analysis of spermatozoa incubated with (a) fluorescein isothiocyanate (FITC) conjugated PSA and (b) Hoechst 33258. Two spermatozoa showing pattern 3 of PSA staining (arrows) are also supravitaly stained with Hoechst 33258. Two other spermatozoa showing pattern 1 of PSA binding (arrowheads in (a)) were not labelled with the Hoechst dye (b). ($\times 1000$).

Discussion

Pisum sativum agglutinin has been proposed as an acrosomal stain based on the empirical finding that, when applied on permeabilized spermatozoa, it gives essentially the same staining patterns as an antiserum to the acrosomal enzyme acrosin (Cross *et al.*, 1986). This lectin from edible pea shows an affinity for terminal α -D-glucosyl and α -D-mannosyl residues of glycoproteins (Trowbridge, 1974). Accordingly, PSA would be expected to bind all glycoproteins carrying the corresponding saccharide groups provided that they are available for the reaction with the lectin. Such glycoproteins are probably quite abundant in any type of animal cell and, in spermatozoa, they are not necessarily confined to the acrosome. In fact, a great number of proteins from SDS extracts of human spermatozoa were shown in this study to bind PSA in western blots. However, a

Fig. 5. Correlation between the percentages of spermatozoa stained supravitaly with Hoechst 33258 and those showing a whole-cell staining with FITC-labelled PSA evaluated in aliquots of the same sperm samples. Spermatozoa were permeabilized with methanol for either 30 s (■) or 15 min (□) before exposure to the lectin. The curve fit is displayed only for the 30 s permeabilization time.

much more selective binding of the lectin was observed in blots of proteins from sperm acid extracts. Acid extraction is known to preferentially release acrosomal proteins from mammalian spermatozoa and it is currently used as part of the procedure for acrosin isolation from spermatozoa of different mammalian species (e.g. Meizel & Mukerji, 1975; Cechova *et al.*, 1984) including man (Drahorad *et al.*, 1988; Tesarik *et al.*, 1990). A major PSA-reactive protein in blots of acid extracts of spermatozoa observed in this study had an apparent molecular mass of 53–55 kDa, which correspond to that reported for human proacrosin (Siegel *et al.*, 1987). Proacrosin is known to be a glycoprotein, and binding of PSA to its saccharide groups may thus be a molecular mechanism underlying the staining of sperm acrosomes with this lectin. Further focused studies will be needed to confirm this.

The observed difference between the pattern of PSA-binding proteins in western blots prepared from SDS- and acid extracts of human spermatozoa indicates that different compartments of the sperm cell differ substantially in glycoprotein composition. When FITC-labelled PSA is used as a fluorescent stain for human spermatozoa, the resulting staining pattern will thus depend on the ease with which the probe enters these different compartments. Probe entry into a cell and its reaction with intracellular affinity sites depends on multiple factors, such as membrane permeability, the presence of competitive binding sites interposed between the cell surface and the affinity sites of interest, and the presence of competitive intracellular ligands reacting with the affinity sites of interest. The relative contribution of each of these factors to the resulting binding pattern is difficult to assess and, consequently, most of the existing sperm staining protocols are based on empirical tuning of the working conditions to obtain the result required.

The type of membrane permeabilizing agent, the conditions of its application and the time of treatment are important for staining of the sperm acrosome as are the type and conditions of use of the stain or probe. The use of PSA as a selective acrosomal stain was pioneered by Cross *et al.* (1986) who permeabilized spermatozoa by resuspension of centrifuged sperm pellets in 95% (v/v) ethanol followed by incubation at 4°C for at least 30 min. Spermatozoa were then dried onto a microscope slide and incubated with FITC-conjugated PSA ($100 \mu\text{g ml}^{-1}$) for 5–10 min. We have made several modifications to this protocol. Living spermatozoa were allowed to air-dry on slides

and only then exposed to the permeabilizing agent. Methanol was used as the permeabilizing agent instead of ethanol, and the time was either 30 s or 15 min. The incubation of slides with the FITC-labelled lectin was increased to 30 min. With a time of methanol treatment of 30 s, we have confirmed a strong correlation of values for acrosome reaction frequency with those obtained by electron microscopy (Tesarik *et al.*, 1990). In this study we have shown that prolonging the methanol fixation has a considerable effect on the pattern of staining with PSA, mainly by augmentation of the percentage of spermatozoa that show a poorly differentiated, whole-cell staining with the lectin. However, slight variation of the fixation time (20 s to 1 min) did not influence the staining results. This is important for the feasibility of the proposed staining method because variations occur, owing to the effect of atmospheric conditions on the rate of methanol evaporation, which are difficult to avoid.

The change in the staining pattern after prolonging the methanol treatment may be due to a higher degree of membrane permeabilization, removal of competitive ligands occupying the binding sites for PSA, or both. The short methanol treatment (30 s) appears to be just sufficient to allow a limited interaction of PSA with its intracellular binding sites, with a clear predilection for acrosomal components. However, many new sites become reactive with the lectin after the prolonged methanol treatment, which leads to the loss of selectivity and to the appearance of a whole-cell staining in many spermatozoa.

With the short permeabilization time, spermatozoa showing any of the whole-cell staining patterns with PSA were essentially those that were stained supravitaly with Hoechst 33258, and the percentages of spermatozoa showing the whole-cell staining with PSA and those accepting the Hoechst nuclear stain were both strongly correlated and similar in all sperm samples tested. In view of the above reasoning, this finding can be explained by the breakdown of the barrier function of sperm membranes in dead cells (stained supravitaly with Hoechst 33258) leading to exposure of lectin-binding sites after a short methanol treatment that is not efficient enough to make these sites available when the spermatozoon has been living at the time of smear preparation. Alternatively, dead cells may lack ligands that, in living cells, competitively block the lectin-binding sites.

Whatever the exact mechanism underlying the preferential whole-cell staining of dead spermatozoa with PSA, this relationship has a great practical significance because it enables considerable simplification of the evaluation of acrosome reaction of human spermatozoa. This can now be done after a one-step staining with a commercially available FITC-labelled PSA conjugate and scoring both the true acrosome reaction (pattern 1 versus pattern 2 of PSA staining) and sperm cell death (patterns 3 & 4) by fluorescent microscopy using the same filter adjustment. Alternatively, staining with PSA (FITC or TRITC conjugate) can be used as part of a double-labelling protocol with which the acrosome reaction is evaluated conjointly with other sperm properties, reflected by the reactivity with a second stain or probe. Finally, the use of peroxidase-labelled PSA will bring this technique within the reach of those who prefer to use bright-field light microscopy rather than fluorescence microscopy for this type of examination. The simplicity of the staining protocol, the relatively low cost of reagents and the wide range of applications warrant the use of this method for both clinical and research purposes.

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